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REVIEW

Infected cell protein 0 functional domains and their coordination in herpes simplex virus replication

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Abstract

Herpes simplex virus 1 (HSV-1) is a ubiquitous human pathogen that establishes latent infection in ganglia neurons. Its unique life cycle requires a balanced "conquer and compromise" strategy to deal with the host anti-viral defenses. One of HSV-1 α (immediate early) gene products, infected cell protein 0 (ICP0), is a multifunctional protein that interacts with and modulates a wide range of cellular defensive pathways. These pathways may locate in different cell compartments, which then migrate or exchange factors upon stimulation, for the purpose of a concerted and effective defense. ICP0 is able to simultaneously attack multiple host pathways by either degrading key restrictive factors or modifying repressive complexes. This is a viral protein that contains an E3 ubiquitin ligase, translocates among different cell compartments and interacts with major defensive complexes. The multiple functional domains of ICP0 can work independently and at the same time coordinate with each other. Dissecting the functional domains of ICP0 and delineating the coordination of these domains will help us understand HSV-1 pathogenicity as well as host defense mechanisms. This article focuses on describing individual ICP0 domains, their biochemical properties and their implication in HSV-1 infection. By putting individual domain functions back into the picture of host anti-viral defense network, this review seeks to elaborate the complex interactions between HSV-1 and its host.

Key words: Subcellular translocation; Herpes simplex virus 1; Infected cell protein 0; E3 ubiquitin ligase; Protein modification; ND10 nuclear bodies; Chromatin repression

Core tip: Due to the genomic limitation, viruses often use multifunctional proteins to ensure viral replication. Coordination of the multiple viral functions is critical for a successful viral infection. Infected cell protein 0 (ICP0) is notoriously multi-functional in terms of simultaneously targeting many host machineries located in different cellular compartments. Understanding the molecular basis of ICP0 multifunctionality is important for not only the elucidation of herpes simplex virus pathogenicity but also the delineation of host defense mechanisms.

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INTRODUCTION

Herpes simplex virus 1 (HSV-1) is a ubiquitous virus that infects over 70% of the world adult population. It causes a wide range of clinical manifestations, including cold sores, genital ulceration, keratitis, and herpes encephalitis. Once infected, HSV-1 establishes a lifelong latency in human trigeminal ganglia. Its sporadic reactivation nourishes a wide spread of the virus. It is one of the most prevalent opportunistic pathogens that can cause severe diseases in newborns or immunocompromised patients. Infected cell protein 0 (ICP0), an α (immediate early, IE) gene product of HSV-1, is a key regulator that activates viral gene expression in both lytic and latent infections[1]. This multifunctional protein plays a critical role in viral counteractions against the host anti-viral defenses.

In early studies, viral proteins expressed in HSV-1 infection were classified into two groups: Virion proteins and infected cell proteins (ICPs)[2]. Both groups were numbered in the order of their descending molecular weight, with number "1" representing the largest protein on high resolution polyacrylamide gels[2]. ICP0 was named outside of the natural numbers for two reasons. First, the protein level of ICP0 was significantly lower than other ICPs. ICP0 was not detected in the initial efforts of numbering the ICPs[2]. It was only discovered after a cycloheximide treatment, which augmented mRNA accumulation and boosted a sudden protein production following the cycloheximide withdrawal[3]. The second reason why ICP0 was named differently was its anomalous mobility in denaturing polyacrylamide gel electrophoresis. The relative position of ICP0 *vs* other ICPs was not consistent on gels with different acrylamide concentrations, which made it impossible to give ICP0 a fixed position in the descending order of molecular weight.

Later on ICP0 was found to be extensively post-translationally modified[4-8] and to undergo quick turnover at early infection[9,10]. The complex biochemical properties of ICP0 likely contribute to the aforementioned low abundancy and abnormal mobility. Three decades of studies have showed that ICP0 is an important viral multifunctional protein to counteract against host anti-viral defenses. It is essential for low multiplicity infection in cultured cells and for latency reactivation in animal models. However, the complexity of how ICP0 carries out those biological functions is not well understood. Understanding the biochemical foundations of ICP0 at different infection phases will help to elucidate the molecular basis of ICP0 functionality. Individual functions of ICP0 as E3 ubiquitin ligase or chromatin remodeler have been discussed elsewhere[11-16]. This review will focus on dissecting ICP0 biochemical properties and seek to understand the profound coordination in the multiple functions of ICP0.

THE TIMELINE OF REVEALING ICP0 ACTIVITIES, A BRIEF HISTORICAL OVERVIEW

Initially, ICP0 was found to transactivate HSV-1 promoters when co-transfected in mammalian cells, similar to many other IE viral proteins such as ICP4 of HSV[17,18], T antigen of SV40[19], and E1A of adenovirus[20]. However, it was quickly realized that the mechanism of ICP0 transactivation was quite different from that of other viral gene activators. For example, ICP4 is essential for viral replication. Deletion of ICP4 led to abnormal viral expression and defective DNA replication[21,22]. In the case of ICP0, gene deletion did not affect viral expression or DNA replication at high multiplicity of infection (MOI) but it had great impact on the viral yield when MOI was lower than 0.1[23]. In experimental animals, deletion of ICP0 mildly reduced the efficiency of latency establishment but completely abolished the latency reactivation[24], whereas ICP4 or ICP27 deletion rendered the mutant virus neither able to replicate in the eyes nor to establish latent infection[24]. Moreover, many viral IE proteins contain a DNA binding domain and they work in mechanisms similar to cognate transcription activators such as GAL4, but ICP0 did not bind to the DNAs it activated[25,26]. Extensive functional analysis showed that ICP0 can transactivate a wide range of cellular promoters or promoters from other DNA or RNA viruses, with no requirement of a specific *cis*-sequence[27-29]. Therefore, ICP0 is defined as a promiscuous transactivator.

The unique functionality of ICP0 energized a great amount of interests in the virology field. In early 1990s, a series of mutagenesis analyses identified a cysteine-rich region required for the ICP0 transactivation activity[30-32], which was later determined as a C3HC4 zinc binding really interesting new gene (*RING*) finger motif[33-35]. Conserved RING finger sequences were found in a large family of E3 ubiquitin ligases[36,37]. Later on, ICP0 was also proven to be an E3 ubiquitin ligase[38,39]. The discovery that various ICP0 substrates imposed restrictions on viral expression in the absence of ICP0[40-45] eventually led to a conclusion that one major function of ICP0 is to target host defensive molecules for ubiquitin-mediated proteasomal degradation. By degrading the restrictive host factors, ICP0 alleviates host defense and promotes viral gene expression.

Starting in the late 1990s, several labs made the efforts to identify ICP0 interacting proteins. From pull-down assays, yeast-2-hybrid screenings and coimmuno-

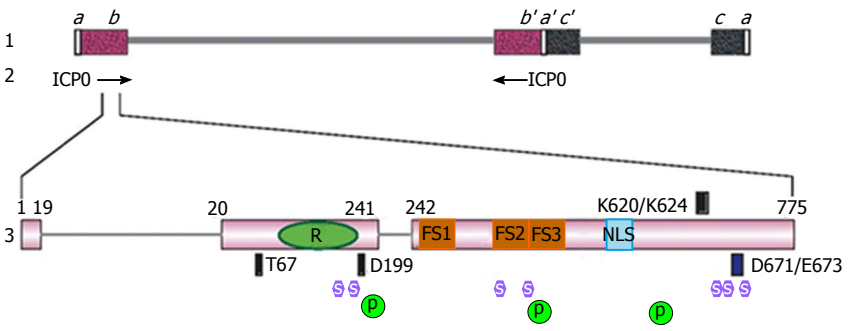

Figure 1 Schematic diagram of infected cell protein 0 gene structure and functional domains. Line 1: Genome structure of HSV-1; Line 2: Locations of the two inverted copies of ICP0 gene in the HSV-1 genome; Line 3: *ICP0* gene structure and domain properties. The amino acid numbers are labeled above the illustration of *ICP0* gene. RING finger domain, Proline-rich ND10-FSs and nuclear localization signal are represented by a green oval with "R", brown squares with "FS" and a blue rectangle with "NLS", respectively. The binding sites for RNF8 (T67), Cyclin D3 (D199), USP7 (K620/K624), and CoREST (D671/E673) are represented by the dark blue boxes above or beneath the *ICP0* gene. The positions of the seven SLSs are represented by lavender hexagons with "S" in the center. The positions of the three phosphorylation clusters are represented by dark green circles with "P" in the center. ICP0: Infected cell protein 0; HSV-1: Herpes simplex virus 1; RING: Really interesting new gene; ND10: Nuclear domains 10; NLS: Nuclear localization signal; USP7: Ubiquitin-specific protease 7; SLSs: SIM-like sequences.

precipitations, a wide range of cellular proteins were found to interact with ICP0[46-49]. Therefore ICP0 carries out viral counteractions by degrading restrictive factors and modulating repressive complexes, and consequently ICP0 enhances viral expression and replication. To better understand the coordination of ICP0 functional domains in counteracting host defenses, this review summarizes the current knowledge of ICP0 domains and ICP0 binding partners, and discusses their implications in HSV-1 infection.

ICP0 GENE STRUCTURE

The gene that encodes for ICP0 protein, also called *α0* gene, is located within the inverted sequences *ab* and *b′a′* that flank the unique long (U_L) region[50] (Figure 1). Therefore, the *ICP0* gene is one of the few HSV-1 genes that are diploid in the genome. The *ICP0* gene is also among the few HSV-1 genes that contain introns[51]. There are two introns of 765 and 136 nucleotides, respectively, intervening the three exons that encode for ICP0 amino acids 1-19, 20-241 and 242-775[51]. It is quite curious why the *ICP0* gene would evolve to bear introns because these introns do not seem to have significant functions in viral replication and alternative splicing of ICP0 has not been observed in infected cells. In one report, the ICP0 cDNA virus had a slight delay of gene expression depending on the cell-type used[52], whereas in another report differences between wild type virus and ICP0 cDNA viruses were not observed[53]. In animal models, recombinant viruses containing ICP0 deleted of introns showed no obvious defects in latency establishment and reactivation[54].

There is an in-frame stop codon located inside intron 2, which predicts a truncated form of ICP0 (ICP0R) if alternative splicing occurs. Overexpression of ICP0R inhibited the transactivation activity of the co-transfected wild type ICP0[55,56], suggesting ICP0R can work as a dominant negative to repress ICP0 activity. Although a band at the size of ICP0R was detected at low level in some cell lines[57], it remains unclear whether this is a product from alternative splicing or a product of proteolytic cleavage of ICP0. The function of ICP0R in the infection context is unknown.

One important fact about the *ICP0* gene is that the coding strand of ICP0 is anti-sense to the latency-associated transcript (LAT), the only transcript that is abundantly expressed in latently infected ganglia neurons[58,59]. The concept of LAT functioning as the anti-sense RNA to ICP0 mRNA has been explored and microRNAs identified in the LAT region have been shown to regulate ICP0 expression. Likely these actions fine-tune the basal level expression in latency maintenance and reactivation[60-62].

ICP0 PROTEIN: DOMAINS AND FUNCTIONS

RING finger domain and E3 ubiquitin ligase activity

The three exons of *ICP0* gene encodes for a 775-amino acid protein. It contains many functional domains and interacts with multiple binding partners (Table 1). The most important functional domain of ICP0 is the aforementioned C3HC4 zinc containing RING finger, which is located within exon 2 and spans through residues 116-156[63] (Figure 1). The promiscuous transactivator ability of ICP0 relies on a functional RING finger domain. Deletions or mutations of the consensus cysteine or histidine in the RING finger domain completely abolish the transactivation activity[63,64]. Recombinant viruses containing such deletions or mutations replicate at a rate similar to that of the ICP0-null virus[53,63,65]. This region is highly conserved among α-herpesviruses[34,63]. The structure of ICP0 RING finger has been solved by nuclear magnetic resonance (NMR)[35].

The RING finger domain of ICP0, like many RING superfamily members[36,66,67], works as an E3 ubiquitin ligase. Mediated by the E2 conjugating enzyme UbcH5a[68,69], ICP0 uses this domain to ubiquitinate its

Table 1 Infected cell protein 0 functional domains

Domain	Location	Function in HSV-1 replication	Section	Ref.
ICP0 cis-elements				
RING finger	aa 116-156	E3 ubiquitin ligase, degrading PML, Sp100, *etc.*	RING finger domain and E3 ubiquitin ligase activity	[63-65]
Proline-rich region	aa 241-553	Containing redundant ND10-fusion segments	Proline-rich region and ND10-fusion	[105]
NLS	aa 500-506	Nuclear localization	Nuclear localization domain and ICP0 nuclear/cytoplasmic translocation	[90]
Dimerization domain	aa 617-711	ICP0 self-dimerization, *in vivo* functions unclear	Dimerization	[115-117]
ND10-retention domain	aa 669-775	Retaining ICP0 at ND10	ND10-retention	[53]
SLSs			SUMO interaction motif and ICP0 substrate recognition	[113]
SLS-4	aa 361-367	Binding to SUMO-1/2/3, stimulating *in vitro* polyubiquitination		
SLS-5, SLS-7	aa 651-655, 681-685	Binding to SUMO-1, cooperating with SLS-4		
ICP0 binding partners				
RNF8	T67	Degrading RNF8 to regulate DNA damage responses	RNF8	[42,43]
Cyclin D3	D199	Involved in nuclear-to-cytoplasmic translocation of ICP0	Cyclin D3	[46,133-135]
BMAL1	aa 20-241	Activating viral transcription *via* BMAL1/CLOCK	BMAL1	[48,140]
EF-1δ	aa 543-768	Inhibiting translation *in vitro*, *in vivo* functions unclear	EF-1δ interaction	[96]
USP7	K620/K624	USP7 degradation, Cell-dependent ICP0 stabilization,	USP7 interaction	[47,85,88,123]
CoREST	D671/E673	Dislodging HDAC from REST/CoREST/HDAC repressor	CoREST interaction	[49,124]
WDR11	N/A	Regulating virion assembly and egress	WD repeat protein 11	[143]

ICP0: Infected cell protein 0; HSV-1: Herpes simplex virus 1; RING: Really interesting new gene; ND10: Nuclear domains 10; PML: Promyelocytic leukemia; Sp100: Speckled 100 kDa; NLS: Nuclear localization signal; SLS: SIM-like sequence; BMAL1: Brain and muscle ARNT-like protein 1; EF-1δ: Elongation factor 1δ; USP7: Ubiquitin-specific protease 7; SUMO: Small ubiquitin-like modifier; CLOCK: Circadian locomotor output cycles kaput.

substrate proteins and targets them for proteasomal degradation. The first two ICP0 substrates, promyelocytic leukemia (PML) and Sp100 (speckled 100 kDa) were identified by Chelbi-Alix and de Thé[40]. PML and Sp100 are the major organizer proteins for the dynamic nuclear bodies called nuclear domains 10 (ND10s) or PML nuclear bodies (for reviews, see references[70,71]). ND10s are nuclear structures that are composed of over 150 constituents[72]. They are involved in many cellular functions including gene regulation[73,74], cell cycle arrest[75], apoptosis[76], DNA repair[77] and anti-viral defense[78]. Degradation of PML and Sp100 by ICP0 leads to the dispersal of ND10 bodies[79]. In ICP0-null virus infection, depletion of PML and Sp100 was shown to compensate for the loss of ICP0 and to increase viral replication[41,80]. In PML$^{-/-}$ mouse embryotic fibroblasts (MEF), interferon (IFN) caused minimal effects on low multiplicity HSV-1 infection, whereas IFN treatment of PML$^{+/+}$ MEF reduced viral growth at least 1000 folds[81], suggesting that PML can mediate the IFN inhibition on viral replication. Taken together, PML is an important factor in host defense pathways and ICP0 targets PML, and maybe also Sp100, to alleviate anti-viral repressions.

Additional ICP0 substrates identified up to date include DNA-dependent protein kinase K (DNAPK)[82], centromeric proteins C and A (CENP-C and CENP-A)[83,84], ubiquitin-specific protease 7 (USP7)[85], RNF8[43], the 111-kDa isoform of poly (ADP-Ribose) glycohydrolase[86], interferon inducible protein 16 (IFI16)[44], and tripartite motif (TRIM) protein TRIM27[87]. Among these substrates, siRNA knock-down of RNF8 or IFI16 promoted the replication of ICP0-null virus[43,45], suggesting the involvement of these two proteins in host anti-viral defenses. However, depletion of TRIM27 reduced the viral yield in the absence of ICP0[87], and overexpression of USP7 accelerated gene expression in wild type HSV-1 infection[88]. These results indicate that not all ICP0 substrates place simple direct repressions on viral gene expression. Some of the substrate proteins may be degraded to regulate a more complicated cell network in order to benefit the overall viral outcome, especially the balanced actions in latent infection.

The E3 ubiquitin ligase activity of ICP0 RING finger is highly regulated by multiple factors, including its subcellular location, its phosphorylation status, and its other functional domains. For example, a failure of ICP0 to completely merge with ND10 bodies blocked substrate access and abolished PML degradation[53], and two amino acid substitutions in the C-terminal CoREST binding site (D671A/E673A) also negatively affected PML degradation[89]. The regulatory mechanisms of ICP0 E3 are not completely understood. Some of the known regulations will be discussed more in detail as we describe other important ICP0 properties in this review.

Nuclear localization domain and ICP0 nuclear/cytoplasmic translocation

ICP0 contains a nuclear localization signal (NLS) mapped to the short stretch of basic amino acids VRPRKRR located at residues 500-506[90] (Figure 1). This arginine-rich NLS is sufficient and necessary for the nuclear localization of transiently transfected ICP0[90]. However, in infected cells, ICP0 is not an exclusively nuclear protein. Its subcellular distribution is regulated by many other factors in addition to the NLS.

First of all, ICP0 undergoes localization changes during the infection process. Early in infection, newly synthesized ICP0 is immediately transported into the nucleus in the presence of the NLS. Once inside the nucleus, ICP0 is immediately localized to the dynamic nuclear structure ND10[91]. This leads to the aforementioned degradation of ND10 organizers, PML and Sp100[40], and the subsequent disruption of ND10 nuclear bodies[79]. The dynamic interaction between ICP0 and ND10 is critical for the efficient access of ICP0 to its substrates, PML and Sp100, and their subsequent degradation[53], which will be discussed in depth in section "Proline-rich region and ND10-fusion".

After the dispersal of ND10 bodies, ICP0 diffuses throughout the nucleus. Once its nuclear functions are completed, ICP0 is translocated into the cytoplasm[92,93]. Many important ICP0 functions are carried out in the nucleus, where ICP0 degrades PML and interacts with REST/CoREST chromatin repressor (see section "CoREST interaction") early in infection. Pre-transfection of irrelevant DNA before infection can prolong ICP0 nuclear localization and delay the cytoplasmic translocation, especially in cell lines that poorly express transgenes[93]. These results suggest that ICP0 is kept within the nucleus until its nuclear functions are completed[93].

It is not yet clear how the NLS containing ICP0 protein is translocated into the cytoplasm at late infection. Either the NLS is modified late in infection so that newly translated ICP0 cannot enter the nucleus, or a nuclear export signal (NES) is unmasked late in infection so that nuclear ICP0 is exported. So far, a functional NES has not been identified.

Multiple viral factors have been found to participate in regulating the nuclear-to-cytoplasmic translocation of ICP0. For example, deletion of ICP4 caused ICP0 to lose its nuclear localization. Even at early infection, ICP0 expressed in the ICP4-null virus infected cell was solely found in the cytoplasm[94]. On the other hand, deletion of ICP27 retained ICP0 within nucleus throughout the infection and overexpression of ICP27 facilitated ICP0 export into the cytoplasm[94]. Since ICP27 is highly expressed in ICP4-null virus infected cells, ICP27 is likely the factor promoting ICP0 export. Another viral protein, VP22, has also been reported to play a role in the ICP0 cytoplasmic translocation. Deletion or mutation in VP22 restricted a series of viral proteins, including ICP0, inside the nucleus[95]. Whether or not VP22 affects a general nuclear export pathway and therefore indirectly delays ICP0 translocation remains unclear.

Functions of cytoplasmic ICP0 are not understood either. Kawaguchi *et al*[96] reported an interaction between ICP0 and translation elongation factor 1δ (EF-1δ) (also see in section "EF1δ interaction") and showed that ICP0 inhibited *in vitro* translation *via* this interaction. However, regulation of cellular translation by ICP0 is yet to be seen *in vivo*. Paladino *et al*[97] showed that ICP0 lacking NLS stayed in the cytoplasm and blocked IRF3 activation in infected cells. It remains unknown whether ICP0 directly interacts with IRF3 or secondary mediators are involved in this inhibition. Small amount of ICP0 has also been found in the tegument of purified virions[98,99]. Although the function of virion-associated ICP0 is not clear, it has been reported that ICP27 dependent cytoplasmic translocation of ICP0 is required for the incorporation of ICP0 into virions[100]. Delboy *et al*[101,102] also showed that an active ubiquitination was important for ICP0 to be incorporated into virions. Both RING finger mutation and proteasome inhibition precluded ICP0 from associating with virions. Since defective ubiquitination sequesters ICP0 within the ND10 bodies and prevents the cytoplasmic translocation of ICP0[89,92], Nicola's results are consistent with the observation that cytoplasmic localization of ICP0 in late infection is a prerequisite for the incorporation of ICP0 into virions. Since up to 49 cellular proteins have also been found in purified virions[99], the selection mechanism of low copy tegument proteins and their biological significance are not clear.

Proline-rich region and ND10-fusion

In the center of ICP0 protein, there is a long stretch of proline-rich region spanning residues 241 to 553. Initial deletion mapping found that serial deletions from the carboxyl-end of this region resulted in a progressive loss of the ICP0 transactivator activity[55], indicating the importance of this region in ICP0 functions. Multiple repeats of the PxxP motif in this region can interact with the Src homology 3 (SH3) domain in Cbl-interacting protein 85 kDa (CIN85), and a few other Src kinase family members[103,104]. Recently, Zheng *et al*[105] demonstrated that the proline-rich sequences were important to direct the fusion of ICP0 with ND10 nuclear bodies. As discussed above, ICP0 is localized to ND10 at early infection. This colocalization process is composed of three sequential dynamic steps: ND10-adhesion, ND10-fusion and ND10-retention[53]. Among these steps, a successful ICP0-ND10 fusion is essential for the ICP0 E3 ligase to access and degrade its substrate PML[53]. The proline-rich region of ICP0 is critical for the ND10-fusion step[105]. Zheng *et al*[105] showed that three proline-rich segments located at residues 242-291, 343-391, and 393-441, termed ND10-FS1, ND10-FS2 and ND10-FS3, respectively (Figure 1), redundantly facilitated the ND10-fusion of ICP0. Deletion of one or two ND10-FSs did not substantially affect the fusion process. However when all three ND10-FSs were deleted, ICP0 was blocked from entering the ND10 bodies[105]. Since most

of the cellular PML is located at ND10, the ICP0-ND10 fusion ensures a quick access of ICP0 to large amount of substrate and leads to an effective PML degradation. This likely increases the efficiency of ICP0 destroying the host restrictive factor PML and therefore enhances gene expression. The redundancy in proline-rich segments indicates the importance of ND10-fusion process in HSV-1 infection. Whether the redundant ND10-FSs synergistically improve the speed of ND10 fusion is a very important question waiting to be answered. It is also unknown whether ND10-FSs work *via* interacting with a SH3 domain or other proline-interacting motifs.

Small ubiquitin-like modifier interaction motif and ICP0 substrate recognition

Small ubiquitin-like modifier (SUMO) is a unique type of post-translational modification found on a variety of proteins. Protein SUMOylation functions in almost every aspect of a cell's life, including cell cycle, genome integrity, subcellular transport, and host immune defenses (for reviews, see references[15,106-108]). The SUMO moiety is recognized by hydrophobic sequences called the SUMO-interaction motif (SIM)[109,110]. RING-type E3 ubiquitin ligases that contain a SIM and specifically recognize SUMOylated substrates are classified as SUMO-targeted ubiquitin ligases (STUBL)[111,112]. Boutell *et al*[113] identified seven putative SIM-like sequences (SLSs) scattering throughout the ICP0 open reading frame (Figure 1). In yeast-2-hybrid assays, mutations in SLS-4 abolished the interaction between ICP0 and SUMO-2/3, whereas mutations in SLS-5 and SLS-7 did not affect such binding. SLS-4 was also found to be necessary for the *in vitro* ubiquitination of a SUMO-2 chain, indicating that ICP0 can work as a STUbL to preferentially recognize SUMOylated proteins for ubiquitination[113]. However, a recombinant virus containing mutant SLS-4 did not affect the degradation of endogenous PML in infected cells, while PML with all SUMOylation sites mutated were still degraded by ICP0[113], suggesting a more complex regulation on ICP0 substrate recognition in addition to the SUMO-SIM interaction. Moreover, although mutations in SLS-5 and SLS-7 did not interfere with the binding between ICP0 and SUMO-2/3, a recombinant virus carrying triple mutations in SLS-4/5/7 greatly demolished the ability of ICP0 to degrade PML[114]. This suggests there may be differences in the SLS affinities and multiple SLSs may work synergistically in PML degradation.

The C-terminus of ICP0 and a diverse array of functions

The C-terminus of ICP0, broadly defined for the region from downstream of NLS to the carboxyl-end, may be the most active but also the least understood region of ICP0. At least five major functions or interactions have been described in this region.

Dimerization: First, ICP0 is a protein known to aggregate and dimerize *in vitro* and *in vivo*[115-117]. In chromatography purification, ICP0 was fractionated at a much bigger molecular weight[117]. When wild type and mutant ICP0 were co-transfected into the same cell, the wild type ICP0 was able to correct the subcellular distribution of a mislocated mutant ICP0. The dimerization domain has been mapped to C-terminal residues 617-711[115]. The biological function of ICP0 dimerization is not yet clear.

ND10-retention: The second function of ICP0 C-terminus is related to the ND10 localization property of ICP0. Initial data showed that ICP0 lacking the C-terminus was evenly dispersed throughout the nucleus, compared to the full-length ICP0 that was colocalized to the ND10 bodies[117]. This led to an assumption that the C-terminus of ICP0 is responsible for ND10 localization[117,118]. However, recent results from Gu *et al*[53] showed that the C-terminus of ICP0 was not involved in the recruitment of ICP0 to ND10. In the absence of C-terminus, ICP0 did not aggregate at ND10 but had the ability to degrade PML. When a double mutant of both C-terminal truncation and RING finger mutation was introduced, ICP0 was found to localize at ND10. These results suggest that the C-truncated ICP0 undergoes adhesion and fusion steps to enter ND10, but it cycles in and out of ND10 in a more accelerated mode. Only when the inactive RING blocks the enzymatic reaction into a transition state, can the ICP0-ND10 colocalization be observed in a steady-state immunofluorescence staining. Therefore the C-terminus of ICP0 is responsible for the retention, but not the recruitment, of ICP0 to ND10.

USP7 interaction: The C-terminus of ICP0 also interacts with various proteins, such as USP7[47], CoREST[49] and EF-1δ[96], which are from proteasome pathway, chromatin repressor complex and translational machinery, respectively.

USP7 is the first ICP0 interacting protein identified *via* a GST pull-down/protein sequencing assay[47,119]. This is a deubiquitinase that regulates the ubiquitination status of many important cell check point proteins, such as p53[120], RE1-silencing transcription factor (REST)[121], and phosphatase and tensin homolog (PTEN)[122]. The minimum sequences required for the strong binding between the two are amino acids 615-633 of ICP0 and amino acids 535-889 of USP7[123]. The crystal structure of USP7 C-terminal ubiquitin-like domains bound with ICP0 peptide has been solved. Salt bridges between K620/K624 of ICP0 and D762/D764 of USP7 are critical for the interaction, while the peripheral residues form a binding pocket to support the strong ICP0-USP7 interaction[123]. Consistent structural data have also been obtained from NMR assays[124].

Initial *in vitro* ubiquitination assays showed that ICP0-USP7 interaction inhibited ICP0 autoubiquitination but promoted USP7 polyubiquitination[8,85]. Consistent with these observations, the ICP0-USP7 interaction was found essential for the degradation of USP7 by ICP0 in infected cells[85,88]. However, regarding to ICP0 autoubiquitination,

different groups have reported contradictory results[85,88]. Boutell *et al*[85] used HSV-1 (strain 17+) and reported that wild type ICP0 stayed at a steady level after cycloheximide treatment, whereas an R623L/K624I mutant virus, of which ICP0 was incapable of binding to USP7 and was quickly degraded in the presence of cycloheximide. On the other hand, Roizman and colleagues demonstrated that wild type ICP0 of HSV-1 (strain F) underwent rapid degradation at early infection and was only stabilized late in infection[9,10]. Furthermore, they found that a K620I mutant virus that abolished ICP0-USP7 interaction had enhanced, not reduced, viral gene expression but showed defects in plaque formation[88]. Therefore, ICP0-USP7 interaction may have profound biological significances, depending upon the virus strains and cell lines. Since both ICP0 and USP7 have a wide range of different substrates that are involved in critical cellular pathways, the interaction between ICP0 and USP7 may be more important in fine-tuning the ubiquitin status of these check point proteins than simply regulating ICP0 self-stability. A complex balance of these proteins may in return affect ICP0 stability in a cell type dependent manner.

CoREST interaction: CoREST binding to ICP0 was discovered by co-immunoprecipitation[125]. CoREST is the corepressor partner for REST[126]. REST/CoREST are the key components of a chromatin regulatory complex that determines neural cell fate during development[127]. The CoREST binding of ICP0 is mapped to the amino acids D671/E673[89]. Gu *et al*[125] showed that ICP0-CoREST interaction depended on the presence of viral kinases U_S3 and U_L13, and a prolonged infection resulted in less binding, suggesting that ICP0-CoREST interaction is a regulated transient process. This interaction was found essential for the dissociation of HDAC1 from REST/CoREST complex in HSV-1 infection[89,125]. A recombinant virus carrying a dominant negative CoREST incapable of HDAC1-binding showed a higher viral productivity in the absence of ICP0, which means the disruption of HDAC1-CoREST interaction is beneficial for viral replication[49]. Furthermore, on the molecular level, a recombinant virus containing D671A/E673A mutations had less acetylated histone H3 compared with the wild type virus or a mutant virus that kept the effective ICP0-CoREST interaction[128]. In contrast to these results, Everett showed that depletion of CoREST did not improve the yield of ICP0-null virus[129]. The seemingly contradictory results are reconciled from the fact that lysine-specific demethylase-1 (LSD1), another important component in the REST/CoREST complex, is required in HSV-1 replication[130]. Therefore the stoichiometry of REST/CoREST/LSD1/HDAC components[127] may play a role in determining the interaction to different viral proteins at different infection phases.

EF1δ interaction: Interaction between ICP0 and EF-1δ was identified through a yeast-2-hybrid screening[96]. The binding has been mapped to the C-terminal residues 543-768 and found to inhibit *in vitro* translation[96]. However, *in vivo* function of this interaction is not clear.

For all these different C-terminal functions it is not clear how these seemingly unrelated activities coordinate in this region. Are there different subsets of ICP0 distributed in distinct subcellular compartments? Or some of the components from different pathways converge at certain cellular hubs, such as ND10? Answers to these questions will be the key to understanding the complex functions of ICP0 in both lytic and latent infections.

Other ICP0 interaction partners

Cyclin D3: Cyclin D3 is identified as an ICP0-interacting protein by a yeast-2-hybrid screening[46]. D-type cyclins form complexes with cyclin-dependent kinases to regulate G1 to S phase transition[131,132], which can be manipulated by many DNA viruses for the purpose of promoting DNA synthesis in infected cells[133]. ICP0 interacts with Cyclin D3 through its amino acid D199 located in exon 2, downstream to the RING finger domain (Figure 1). The D199-Cyclin D3 interaction is important in the nuclear-to-cytoplasmic translocation of ICP0. Mutation in cyclin D3 binding site or treatment by CDK4 inhibitor during the infection prevented ICP0 from translocating to the cytoplasm[134,135], whereas insertion of cyclin D3 gene into the HSV-1 genome to overexpress cyclin D3 led to an accelerated cytoplasmic translocation[135,136]. The regulation of the cell cycle during HSV-1 infection is a profound event involving multiple factors. For example, HSV-1 ICP22 and U_L13 are found to participate in G2/M transition[137], and CDK inhibitor roscovitine inhibits HSV-1 gene transcription without affecting PML degradation[138,139]. Moreover, the D199 dependent nuclear-to-cytoplasmic translocation of ICP0 is a process that depends on viral DNA replication and the expression of a late protein(s)[92]. Therefore different cell cycle regulatory pathways are interwoven with ICP0 phosphorylation, translocation and possibly other infection events. The concerted efforts from both viral and cellular sides determine the ultimate productivity of an HSV-1 infection.

Brain and muscle ARNT-like protein 1: Brain and muscle ARNT-like protein 1 (BMAL1) interacting with ICP0 is also identified by a yeast-2-hybrid screening[48]. The interaction site to BMAL1 is located in the exon 2 of ICP0[48]. BMAL1 and circadian locomotor output cycles kaput (CLOCK), a histone acetyltransferase, forms a heterodimer to regulate mammalian circadian oscillation[140]. During HSV-1 infection, CLOCK is stabilized and recruited to ND10, which acts as a transcription activator to stimulate viral transcription and replication[141].

RNF8: The identification of ICP0-RNF8 interaction was based on the observation that RNF8 was degraded by ICP0 in HSV-1 infection[42,43]. RNF8 is an RING type E3 ubiquitin ligase that plays a key role in histone ubiquitination and chromatin remodeling upon DNA double-

stranded break (DBS) damage[142,143]. ICP0-RNF8 binding is mapped to the phosphorylated amino acid T67 of ICP0, and amino acid R42 of RNF8[43]. A recombinant virus carrying the T67A mutation did not degrade RNF8 but had no problems in degrading DNAPK or USP7, which means ICP0-RNF8 interaction is likely important for a specific RNF8 substrate recognition[43]. Interestingly, knock-down of RNF8 only mildly delayed *ICP27* gene transcription and had no effects on viral DNA replication, suggesting that the involvement of ICP0-RNF8 interaction in responding to DBS DNA damage is, again, a complex action.

WD repeat protein 11: WD repeat protein 11 (WDR11) is a newly reported ICP0 interacting protein identified by co-immunoprecipitation[144]. Taylor *et al*[144] showed that the trans-Golgi network localized WDR11 pulled down several viral proteins including gB, VP16 and VP5 in additional to ICP0, suggesting its possible role in virion assembly and egress.

POST-TRANSLATIONAL PROCESSING OF ICP0

Modification

ICP0 protein contains 775 amino acids, but the apparent molecular weight of ICP0 is about 110 kDa[3], suggesting the presence of post-translational modifications for ICP0. First of all, ICP0 is highly phosphorylated. On two-dimensional gel electrophoreses, ICP0 phosphorylation status changes along with the progression of infection[6]. The phosphorylation sites on ICP0 has been mapped to three phosphor-clusters by tandem mass spectrometry. Cluster 1 is at residues 222-250, cluster 2 is at residues 356-386, and cluster 3 is at residues 505-528[145] (Figure 1). Davido and colleagues showed that serine/threonine mutations in these clusters demolished the transactivation activity of ICP0 and reduced the viral replication in mice[145,146]. Viral protein UL13 was found important for ICP0 phosphorylation[147]. However, how ICP0 phosphorylation coordinates with ICP0 localizations or ICP0 protein-protein interactions to affect the infection is not yet known.

Other modifications of ICP0 are understudied. ICP0 is believed to be nucleotidylated because it can be radiolabeled in infected cells cultured with [α-^{32}P]GTP or [2-^{3}H]ATP containing medium[5]. ICP0 may also be ubiquitinated because it is found to autoubiquitinate itself in *in vitro* polyubiquitination assays[8].

Proteolytic cleavage and rapid turnover

At least in the infection of HSV-1 (strain F), ICP0 undergoes a rapid degradation at early infection in both proteasome dependent and proteasome independent manners. The protein is then stabilized at late infection[9]. The proteasome independent cleavage occurs in the central region of ICP0 and the rapid turnover depends on the *cis* presence of an active RING finger as well as the phosphorylation status of ICP0[9,10].

CONCLUSION

Like all herpesvirus family members, HSV-1 establishes latent infection. The peculiar life cycle of HSV-1 necessitates a close interaction and a delicate balance between the virus and its host. ICP0 of HSV-1, a unique multifunctional protein, plays a key regulatory role to enhance gene expression in lytic infection and to reactivate virion production from latent infection. This protein is tightly regulated on transcriptional, post-transcriptional and post-translational levels. Through its intrinsic functional domains and its ability to interact with a wide range of binding partners, ICP0 can target many cellular protein for proteasomal degradation and regulate various cell pathways *via* protein-protein interactions.

To achieve its multiple functions, ICP0 undergoes modification and subcellular translocation. Early in infection, ICP0 is immediately imported into the nucleus upon synthesis. Once inside the nucleus, it is recruited to adhere at and then fuse with ND10 to co-mingle with ND10 components. The ND10-fusion process ensures ICP0 to quickly access large amounts of PML and Sp100 for degradation and to extensively interact with many of the regulatory factors located within ND10. This early step in HSV-1 infection is vital for the outcome of a productive infection, not only by destroying and dispersing the repressive factors but also by capturing favorable factors that help establishing replication compartment. Upon viral DNA entering the nucleus, host cell attempt to silence the foreign intrusion by: (1) forming ND10 bodies near viral DNA[148]; (2) recruiting chromatin repressors[149]; and (3) stimulating IFN responses[45]. In a way, HSV-1 deploys ICP0 to approach ND10 is a "smart" move because ND10 serves as a molecular hub for many cellular pathways and it is able to recruit component factors upon specific stimulations[78]. Therefore, adopting factors recruited to ND10 during infection while destroying and repelling restrictive components is an effective strategy to boost viral replication. In fact, various cellular check point proteins such as USP7, CoREST, Cyclin D3, BMAL1 and CLOCK are all recruited to ND10 upon infection and they are found in HSV-1 replication compartments[47,135,141,149]. In fact, HSV-1 replication compartments are established at the sites where ND10 loci have been located before their dispersal[149]. ICP0 interacting with the molecular hub ND10 is a major adaptation to coordinate the multitasking of ICP0 functions. Likely the sequential steps of ICP0-ND10 interaction, ND10-adhesion, ND10-fusion, ND10-retention[53], play important roles in achieving the "destroy and then take over" strategy.

Once the replication compartments are set up in the infected cells, ICP0 may have additional functions in a diffused pattern in nucleus and then in the cytoplasm. Whether the trafficking of ICP0 is regulated by post-translational modification or proteolytic processing is currently unknown. Solving the road map of ICP0 being in the right place at the right time will be a continuous

interest in the near future for herpes virology field.

ICP0 is required for latency reactivation[24]. The subtle balance of ICP0 level in latent infection may be achieved by microRNA regulation. The rapid turnover of ICP0 on the protein level may also be essential for the maintenance and reactivation of latent infection. After all, one good way to achieve massive spreading is to keep the sporadic but not severe recurrent infections.

REFERENCES

1 **Roizman B**, Knipe DM., Whitley RJ. Herpes Simplex Viruses, in Fields Virology, 6 Edition, Lippincott Williams & Wilkins, 2013: 1823-1897

2 **Honess RW**, Roizman B. Proteins specified by herpes simplex virus. XI. Identification and relative molar rates of synthesis of structural and nonstructural herpes virus polypeptides in the infected cell. *J Virol* 1973; **12**: 1347-1365 [PMID: 4357511]

3 **Honess RW**, Roizman B. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. *J Virol* 1974; **14**: 8-19 [PMID: 4365321]

4 **Ackermann M**, Braun DK, Pereira L, Roizman B. Characterization of herpes simplex virus 1 alpha proteins 0, 4, and 27 with monoclonal antibodies. *J Virol* 1984; **52**: 108-118 [PMID: 6090689]

5 **Blaho JA**, Mitchell C, Roizman B. Guanylylation and adenylylation of the alpha regulatory proteins of herpes simplex virus require a viral beta or gamma function. *J Virol* 1993; **67**: 3891-3900 [PMID: 8389911]

6 **Advani SJ**, Hagglund R, Weichselbaum RR, Roizman B. Post-translational processing of infected cell proteins 0 and 4 of herpes simplex virus 1 is sequential and reflects the subcellular compartment in which the proteins localize. *J Virol* 2001; **75**: 7904-7912 [PMID: 11483735 DOI: 10.1128/JVI.75.17.7904-7912.2001]

7 **Weber PC**, Spatz SJ, Nordby EC. Stable ubiquitination of the ICP0R protein of herpes simplex virus type 1 during productive infection. *Virology* 1999; **253**: 288-298 [PMID: 9918887 DOI: 10.1006/viro.1998.9502]

8 **Canning M**, Boutell C, Parkinson J, Everett RD. A RING finger ubiquitin ligase is protected from autocatalyzed ubiquitination and degradation by binding to ubiquitin-specific protease USP7. *J Biol Chem* 2004; **279**: 38160-38168 [PMID: 15247261 DOI: 10.1074/jbc.M402885200]

9 **Gu H**, Poon AP, Roizman B. During its nuclear phase the multifunctional regulatory protein ICP0 undergoes proteolytic cleavage characteristic of polyproteins. *Proc Natl Acad Sci USA* 2009; **106**: 19132-19137 [PMID: 19850872 DOI: 10.1073/pnas.0910920106]

10 **Zhu Z**, Du T, Zhou G, Roizman B. The stability of herpes simplex virus 1 ICP0 early after infection is defined by the RING finger and the UL13 protein kinase. *J Virol* 2014; **88**: 5437-5443 [PMID: 24574411 DOI: 10.1128/JVI.00542-14]

11 **Everett RD**. ICP0, a regulator of herpes simplex virus during lytic and latent infection. *Bioessays* 2000; **22**: 761-770 [PMID: 10918307 DOI: 10.1002/1521-1878(200008)22:8<761::AID-BIES10>3.0.CO;2-A]

12 **Hagglund R**, Roizman B. Role of ICP0 in the strategy of conquest of the host cell by herpes simplex virus 1. *J Virol* 2004; **78**: 2169-2178 [PMID: 14963113 DOI: 10.1128/JVI.78.5.2169-2178.2004]

13 **Roizman B**, Gu H, Mandel G. The first 30 minutes in the life of a virus: unREST in the nucleus. *Cell Cycle* 2005; **4**: 1019-1021 [PMID: 16082207 DOI: 10.4161/cc.4.8.1902]

14 **Boutell C**, Everett RD. Regulation of alphaherpesvirus infections by the ICP0 family of proteins. *J Gen Virol* 2013; **94**: 465-481 [PMID: 23239572 DOI: 10.1099/vir.0.048900-0]

15 **Everett RD**, Boutell C, Hale BG. Interplay between viruses and host sumoylation pathways. *Nat Rev Microbiol* 2013; **11**: 400-411 [PMID: 23624814 DOI: 10.1038/nrmicro3015]

16 **Zhou G**, Du T, Roizman B. The role of the CoREST/REST repressor complex in herpes simplex virus 1 productive infection and in latency. *Viruses* 2013; **5**: 1208-1218 [PMID: 23628827 DOI: 10.3390/v5051208]

17 **Gelman IH**, Silverstein S. Identification of immediate early genes from herpes simplex virus that transactivate the virus thymidine kinase gene. *Proc Natl Acad Sci USA* 1985; **82**: 5265-5269 [PMID: 2991915 DOI: 10.1073/pnas.82.16.5265]

18 **Quinlan MP**, Knipe DM. Stimulation of expression of a herpes simplex virus DNA-binding protein by two viral functions. *Mol Cell Biol* 1985; **5**: 957-963 [PMID: 2987684 DOI: 10.1128/MCB.5.5.957]

19 **Myers RM**, Rio DC, Robbins AK, Tjian R. SV40 gene expression is modulated by the cooperative binding of T antigen to DNA. *Cell* 1981; **25**: 373-384 [PMID: 6269743 DOI: 10.1016/0092-8674(81)90056-8]

20 **Nevins JR**. Mechanism of activation of early viral transcription by the adenovirus E1A gene product. *Cell* 1981; **26**: 213-220 [PMID: 7332929 DOI: 10.1016/0092-8674(81)90304-4]

21 **Dixon RA**, Schaffer PA. Fine-structure mapping and functional analysis of temperature-sensitive mutants in the gene encoding the herpes simplex virus type 1 immediate early protein VP175. *J Virol* 1980; **36**: 189-203 [PMID: 6255206]

22 **DeLuca NA**, McCarthy AM, Schaffer PA. Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. *J Virol* 1985; **56**: 558-570 [PMID: 2997476]

23 **Stow ND**, Stow EC. Isolation and characterization of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate early polypeptide Vmw110. *J Gen Virol* 1986; **67** (Pt 12): 2571-2585 [PMID: 3025339 DOI: 10.1099/0022-1317-67-12-2571]

24 **Leib DA**, Coen DM, Bogard CL, Hicks KA, Yager DR, Knipe DM, Tyler KL, Schaffer PA. Immediate-early regulatory gene mutants define different stages in the establishment and reactivation of herpes simplex virus latency. *J Virol* 1989; **63**: 759-768 [PMID: 2536101]

25 **Purifoy DJ**, Powell KL. DNA-binding proteins induced by herpes simplex virus type 2 in HEp-2 cells. *J Virol* 1976; **19**: 717-731 [PMID: 183021]

26 **Wilcox KW**, Kohn A, Sklyanskaya E, Roizman B. Herpes simplex virus phosphoproteins. I. Phosphate cycles on and off some viral polypeptides and can alter their affinity for DNA. *J Virol* 1980; **33**: 167-182 [PMID: 6245226]

27 **Nabel GJ**, Rice SA, Knipe DM, Baltimore D. Alternative mechanisms for activation of human immunodeficiency virus enhancer in T cells. *Science* 1988; **239**: 1299-1302 [PMID: 2830675 DOI: 10.1126/science.2830675]

28 **Gius D**, Laimins LA. Activation of human papillomavirus type 18 gene expression by herpes simplex virus type 1 viral transactivators and a phorbol ester. *J Virol* 1989; **63**: 555-563 [PMID: 2536091]

29 **Kwun HJ**, Han HJ, Lee WJ, Kim HS, Jang KL. Transactivation of the human endogenous retrovirus K long terminal repeat by herpes simplex virus type 1 immediate early protein 0. *Virus Res* 2002; **86**: 93-100 [PMID: 12076833 DOI: 10.1016/S0168-1702(02)00058-8]

30 **Chen JX**, Zhu XX, Silverstein S. Mutational analysis of the sequence encoding ICP0 from herpes simplex virus type 1. *Virology* 1991; **180**: 207-220 [PMID: 1845823 DOI: 10.1016/0042-6822(91)90025-7]

31 **Everett RD**. A detailed mutational analysis of Vmw110, a trans-acting transcriptional activator encoded by herpes simplex virus type 1. *EMBO J* 1987; **6**: 2069-2076 [PMID: 2820720]

32 **Everett R**, O'Hare P, O'Rourke D, Barlow P, Orr A. Point mutations in the herpes simplex virus type 1 Vmw110 RING finger helix affect activation of gene expression, viral growth, and interaction with PML-containing nuclear structures. *J Virol* 1995; **69**: 7339-7344 [PMID: 7474166]

33 **Freemont PS**, Hanson IM, Trowsdale J. A novel cysteine-rich sequence motif. *Cell* 1991; **64**: 483-484 [PMID: 1991318]

34 **Everett RD**, Barlow P, Milner A, Luisi B, Orr A, Hope G, Lyon D. A novel arrangement of zinc-binding residues and secondary structure in the C3HC4 motif of an alpha herpes virus protein family. *J Mol Biol* 1993; **234**: 1038-1047 [PMID: 8263911 DOI:

10.1006/jmbi.1993.1657]
35 **Barlow PN**, Luisi B, Milner A, Elliott M, Everett R. Structure of the C3HC4 domain by 1H-nuclear magnetic resonance spectroscopy. A new structural class of zinc-finger. *J Mol Biol* 1994; **237**: 201-211 [PMID: 8126734 DOI: 10.1006/jmbi.1994.1222]
36 **Freemont PS**. RING for destruction? *Curr Biol* 2000; **10**: R84-R87 [PMID: 10662664]
37 **Metzger MB**, Pruneda JN, Klevit RE, Weissman AM. RING-type E3 ligases: master manipulators of E2 ubiquitin-conjugating enzymes and ubiquitination. *Biochim Biophys Acta* 2014; **1843**: 47-60 [PMID: 23747565 DOI: 10.1016/j.bbamcr.2013.05.026]
38 **Boutell C**, Sadis S, Everett RD. Herpes simplex virus type 1 immediate-early protein ICP0 and is isolated RING finger domain act as ubiquitin E3 ligases in vitro. *J Virol* 2002; **76**: 841-850 [PMID: 11752173 DOI: 10.1128/JVI.76.2.841-850.2002]
39 **Hagglund R**, Van Sant C, Lopez P, Roizman B. Herpes simplex virus 1-infected cell protein 0 contains two E3 ubiquitin ligase sites specific for different E2 ubiquitin-conjugating enzymes. *Proc Natl Acad Sci USA* 2002; **99**: 631-636 [PMID: 11805320 DOI: 10.1073/pnas.022531599]
40 **Chelbi-Alix MK**, de Thé H. Herpes virus induced proteasome-dependent degradation of the nuclear bodies-associated PML and Sp100 proteins. *Oncogene* 1999; **18**: 935-941 [PMID: 10023669 DOI: 10.1038/sj.onc.1202366]
41 **Everett RD**, Parada C, Gripon P, Sirma H, Orr A. Replication of ICP0-null mutant herpes simplex virus type 1 is restricted by both PML and Sp100. *J Virol* 2008; **82**: 2661-2672 [PMID: 18160441 DOI: 10.1128/JVI.02308-07]
42 **Lilley CE**, Chaurushiya MS, Boutell C, Landry S, Suh J, Panier S, Everett RD, Stewart GS, Durocher D, Weitzman MD. A viral E3 ligase targets RNF8 and RNF168 to control histone ubiquitination and DNA damage responses. *EMBO J* 2010; **29**: 943-955 [PMID: 20075863 DOI: 10.1038/emboj.2009.400]
43 **Chaurushiya MS**, Lilley CE, Aslanian A, Meisenhelder J, Scott DC, Landry S, Ticau S, Boutell C, Yates JR, Schulman BA, Hunter T, Weitzman MD. Viral E3 ubiquitin ligase-mediated degradation of a cellular E3: viral mimicry of a cellular phosphorylation mark targets the RNF8 FHA domain. *Mol Cell* 2012; **46**: 79-90 [PMID: 22405594 DOI: 10.1016/j.molcel.2012.02.004]
44 **Orzalli MH**, DeLuca NA, Knipe DM. Nuclear IFI16 induction of IRF-3 signaling during herpesviral infection and degradation of IFI16 by the viral ICP0 protein. *Proc Natl Acad Sci USA* 2012; **109**: E3008-E3017 [PMID: 23027953 DOI: 10.1073/pnas.1211302109]
45 **Orzalli MH**, Conwell SE, Berrios C, DeCaprio JA, Knipe DM. Nuclear interferon-inducible protein 16 promotes silencing of herpesviral and transfected DNA. *Proc Natl Acad Sci USA* 2013; **110**: E4492-E4501 [PMID: 24198334 DOI: 10.1073/pnas.1316194110]
46 **Kawaguchi Y**, Van Sant C, Roizman B. Herpes simplex virus 1 alpha regulatory protein ICP0 interacts with and stabilizes the cell cycle regulator cyclin D3. *J Virol* 1997; **71**: 7328-7336 [PMID: 9311810]
47 **Everett RD**, Meredith M, Orr A, Cross A, Kathoria M, Parkinson J. A novel ubiquitin-specific protease is dynamically associated with the PML nuclear domain and binds to a herpesvirus regulatory protein. *EMBO J* 1997; **16**: 1519-1530 [PMID: 9130697 DOI: 10.1093/emboj/16.7.1519]
48 **Kawaguchi Y**, Tanaka M, Yokoymama A, Matsuda G, Kato K, Kagawa H, Hirai K, Roizman B. Herpes simplex virus 1 alpha regulatory protein ICP0 functionally interacts with cellular transcription factor BMAL1. *Proc Natl Acad Sci USA* 2001; **98**: 1877-1882 [PMID: 11172044 DOI: 10.1073/pnas.041592598]
49 **Gu H**, Roizman B. Herpes simplex virus-infected cell protein 0 blocks the silencing of viral DNA by dissociating histone deacetylases from the CoREST-REST complex. *Proc Natl Acad Sci USA* 2007; **104**: 17134-17139 [PMID: 17939992 DOI: 10.1073/pnas.0707266104]
50 **Wadsworth S**, Jacob RJ, Roizman B. Anatomy of herpes simplex virus DNA. II. Size, composition, and arrangement of inverted terminal repetitions. *J Virol* 1975; **15**: 1487-1497 [PMID: 167196]
51 **Perry LJ**, Rixon FJ, Everett RD, Frame MC, McGeoch DJ. Characterization of the IE110 gene of herpes simplex virus type 1. *J Gen Virol* 1986; **67** (Pt 11): 2365-2380 [PMID: 3023529 DOI: 10.1099/0022-1317-67-11-2365]
52 **Poon AP**, Silverstein SJ, Roizman B. An early regulatory function required in a cell type-dependent manner is expressed by the genomic but not the cDNA copy of the herpes simplex virus 1 gene encoding infected cell protein 0. *J Virol* 2002; **76**: 9744-9755 [PMID: 12208953 DOI: 10.1128/JVI.76.19.9744-9755.2002]
53 **Gu H**, Zheng Y, Roizman B. Interaction of herpes simplex virus ICP0 with ND10 bodies: a sequential process of adhesion, fusion, and retention. *J Virol* 2013; **87**: 10244-10254 [PMID: 23864622 DOI: 10.1128/jvi.01487-13]
54 **Natarajan R**, Deshmane S, Valyi-Nagy T, Everett R, Fraser NW. A herpes simplex virus type 1 mutant lacking the ICP0 introns reactivates with normal efficiency. *J Virol* 1991; **65**: 5569-5573 [PMID: 1654452]
55 **Weber PC**, Wigdahl B. Identification of dominant-negative mutants of the herpes simplex virus type 1 immediate-early protein ICP0. *J Virol* 1992; **66**: 2261-2267 [PMID: 1312631]
56 **Weber PC**, Kenny JJ, Wigdahl B. Antiviral properties of a dominant negative mutant of the herpes simplex virus type 1 regulatory protein ICP0. *J Gen Virol* 1992; **73** (Pt 11): 2955-2961 [PMID: 1331297 DOI: 10.1099/0022-1317-73-11-2955]
57 **Everett RD**, Cross A, Orr A. A truncated form of herpes simplex virus type 1 immediate-early protein Vmw110 is expressed in a cell type dependent manner. *Virology* 1993; **197**: 751-756 [PMID: 7504367 DOI: 10.1006/viro.1993.1651]
58 **Stevens JG**, Wagner EK, Devi-Rao GB, Cook ML, Feldman LT. RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons. *Science* 1987; **235**: 1056-1059 [PMID: 2434993 DOI: 10.1126/science.2434993]
59 **Rock DL**, Nesburn AB, Ghiasi H, Ong J, Lewis TL, Lokensgard JR, Wechsler SL. Detection of latency-related viral RNAs in trigeminal ganglia of rabbits latently infected with herpes simplex virus type 1. *J Virol* 1987; **61**: 3820-3826 [PMID: 2824816]
60 **Cui C**, Griffiths A, Li G, Silva LM, Kramer MF, Gaasterland T, Wang XJ, Coen DM. Prediction and identification of herpes simplex virus 1-encoded microRNAs. *J Virol* 2006; **80**: 5499-5508 [PMID: 16699030 DOI: 10.1128/JVI.00200-06]
61 **Umbach JL**, Kramer MF, Jurak I, Karnowski HW, Coen DM, Cullen BR. MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs. *Nature* 2008; **454**: 780-783 [PMID: 18596690 DOI: 10.1038/nature07103]
62 **Jiang X**, Brown D, Osorio N, Hsiang C, Li L, Chan L, Ben–Mohamed L, Wechsler SL. A herpes simplex virus type 1 mutant disrupted for microRNA H2 with increased neurovirulence and rate of reactivation. *J Neurovirol* 2015; **21**: 199-209 [PMID: 25645379 DOI: 10.1007/s13365-015-0319-1]
63 **Lium EK**, Silverstein S. Mutational analysis of the herpes simplex virus type 1 ICP0 C3HC4 zinc ring finger reveals a requirement for ICP0 in the expression of the essential alpha27 gene. *J Virol* 1997; **71**: 8602-8614 [PMID: 9343218]
64 **Everett RD**. Analysis of the functional domains of herpes simplex virus type 1 immediate-early polypeptide Vmw110. *J Mol Biol* 1988; **202**: 87-96 [PMID: 2845096 DOI: 10.1016/0022-2836(88)90521-9]
65 **Everett RD**. Construction and characterization of herpes simplex virus type 1 mutants with defined lesions in immediate early gene 1. *J Gen Virol* 1989; **70** (Pt 5): 1185-1202 [PMID: 2543774 DOI: 10.1099/0022-1317-70-5-1185]
66 **Tyers M**, Willems AR. One ring to rule a superfamily of E3 ubiquitin ligases. *Science* 1999; **284**: 601, 603-604 [PMID: 10328744 DOI: 10.1126/science.284.5414.601]
67 **Joazeiro CA**, Weissman AM. RING finger proteins: mediators of ubiquitin ligase activity. *Cell* 2000; **102**: 549-552 [PMID: 11007473 DOI: 10.1016/S0092-8674(00)00077-5]
68 **Gu H**, Roizman B. The degradation of promyelocytic leukemia and Sp100 proteins by herpes simplex virus 1 is mediated by the ubiquitin-conjugating enzyme UbcH5a. *Proc Natl Acad Sci USA* 2003; **100**: 8963-8968 [PMID: 12855769 DOI: 10.1073/

pnas.1533420100]
69 **Vanni E**, Gatherer D, Tong L, Everett RD, Boutell C. Functional characterization of residues required for the herpes simplex virus 1 E3 ubiquitin ligase ICP0 to interact with the cellular E2 ubiquitin-conjugating enzyme UBE2D1 (UbcH5a). *J Virol* 2012; **86**: 6323-6333 [PMID: 22438555 DOI: 10.1128/JVI.07210-11]
70 **Maul GG**, Negorev D, Bell P, Ishov AM. Review: properties and assembly mechanisms of ND10, PML bodies, or PODs. *J Struct Biol* 2000; **129**: 278-287 [PMID: 10806078 DOI: 10.1006/jsbi.2000.4239]
71 **Everett RD**, Chelbi-Alix MK. PML and PML nuclear bodies: implications in antiviral defence. *Biochimie* 2007; **89**: 819-830 [PMID: 17343971 DOI: 10.1016/j.biochi.2007.01.004]
72 **Van Damme E**, Laukens K, Dang TH, Van Ostade X. A manually curated network of the PML nuclear body interactome reveals an important role for PML-NBs in SUMOylation dynamics. *Int J Biol Sci* 2010; **6**: 51-67 [PMID: 20087442 DOI: 10.7150/ijbs.6.51]
73 **Zhong S**, Salomoni P, Pandolfi PP. The transcriptional role of PML and the nuclear body. *Nat Cell Biol* 2000; **2**: E85-E90 [PMID: 10806494 DOI: 10.1038/35010583]
74 **Cohen N**, Sharma M, Kentsis A, Perez JM, Strudwick S, Borden KL. PML RING suppresses oncogenic transformation by reducing the affinity of eIF4E for mRNA. *EMBO J* 2001; **20**: 4547-4559 [PMID: 11500381 DOI: 10.1093/emboj/20.16.4547]
75 **Wang ZG**, Delva L, Gaboli M, Rivi R, Giorgio M, Cordon-Cardo C, Grosveld F, Pandolfi PP. Role of PML in cell growth and the retinoic acid pathway. *Science* 1998; **279**: 1547-1551 [PMID: 9488655 DOI: 10.1126/science.279.5356.1547]
76 **Bernardi R**, Pandolfi PP. Role of PML and the PML-nuclear body in the control of programmed cell death. *Oncogene* 2003; **22**: 9048-9057 [PMID: 14663483 DOI: 10.1038/sj.onc.1207106]
77 **Carbone R**, Pearson M, Minucci S, Pelicci PG. PML NBs associate with the hMre11 complex and p53 at sites of irradiation induced DNA damage. *Oncogene* 2002; **21**: 1633-1640 [PMID: 11896594 DOI: 10.1038/sj.onc.1205227]
78 **Geoffroy MC**, Chelbi-Alix MK. Role of promyelocytic leukemia protein in host antiviral defense. *J Interferon Cytokine Res* 2011; **31**: 145-158 [PMID: 21198351 DOI: 10.1089/jir.2010.0111]
79 **Everett RD**, Freemont P, Saitoh H, Dasso M, Orr A, Kathoria M, Parkinson J. The disruption of ND10 during herpes simplex virus infection correlates with the Vmw110- and proteasome-dependent loss of several PML isoforms. *J Virol* 1998; **72**: 6581-6591 [PMID: 9658103]
80 **Everett RD**, Rechter S, Papior P, Tavalai N, Stamminger T, Orr A. PML contributes to a cellular mechanism of repression of herpes simplex virus type 1 infection that is inactivated by ICP0. *J Virol* 2006; **80**: 7995-8005 [PMID: 16873256 DOI: 10.1128/JVI.00734-06]
81 **Chee AV**, Lopez P, Pandolfi PP, Roizman B. Promyelocytic leukemia protein mediates interferon-based anti-herpes simplex virus 1 effects. *J Virol* 2003; **77**: 7101-7105 [PMID: 12768029 DOI: 10.1128/JVI.77.12.7101-7105.2003]
82 **Parkinson J**, Lees-Miller SP, Everett RD. Herpes simplex virus type 1 immediate-early protein vmw110 induces the proteasome-dependent degradation of the catalytic subunit of DNA-dependent protein kinase. *J Virol* 1999; **73**: 650-657 [PMID: 9847370]
83 **Everett RD**, Earnshaw WC, Findlay J, Lomonte P. Specific destruction of kinetochore protein CENP-C and disruption of cell division by herpes simplex virus immediate-early protein Vmw110. *EMBO J* 1999; **18**: 1526-1538 [PMID: 10075924 DOI: 10.1093/emboj/18.6.1526]
84 **Lomonte P**, Sullivan KF, Everett RD. Degradation of nucleosome-associated centromeric histone H3-like protein CENP-A induced by herpes simplex virus type 1 protein ICP0. *J Biol Chem* 2001; **276**: 5829-5835 [PMID: 11053442 DOI: 10.1074/jbc.M008547200]
85 **Boutell C**, Canning M, Orr A, Everett RD. Reciprocal activities between herpes simplex virus type 1 regulatory protein ICP0, a ubiquitin E3 ligase, and ubiquitin-specific protease USP7. *J Virol* 2005; **79**: 12342-12354 [PMID: 16160161 DOI: 10.1128/JVI.79.19.12342-12354.2005]
86 **Grady SL**, Hwang J, Vastag L, Rabinowitz JD, Shenk T. Herpes simplex virus 1 infection activates poly(ADP-ribose) polymerase and triggers the degradation of poly(ADP-ribose) glycohydrolase. *J Virol* 2012; **86**: 8259-8268 [PMID: 22623791 DOI: 10.1128/JVI.00495-12]
87 **Conwell SE**, White AE, Harper JW, Knipe DM. Identification of TRIM27 as a novel degradation target of herpes simplex virus 1 ICP0. *J Virol* 2015; **89**: 220-229 [PMID: 25320289 DOI: 10.1128/JVI.02635-14]
88 **Kalamvoki M**, Gu H, Roizman B. Overexpression of the ubiquitin-specific protease 7 resulting from transfection or mutations in the ICP0 binding site accelerates rather than depresses herpes simplex virus 1 gene expression. *J Virol* 2012; **86**: 12871-12878 [PMID: 22993145 DOI: 10.1128/JVI.01981-12]
89 **Gu H**, Roizman B. The two functions of herpes simplex virus 1 ICP0, inhibition of silencing by the CoREST/REST/HDAC complex and degradation of PML, are executed in tandem. *J Virol* 2009; **83**: 181-187 [PMID: 18945770 DOI: 10.1128/JVI.01940-08]
90 **Mullen MA**, Ciufo DM, Hayward GS. Mapping of intracellular localization domains and evidence for colocalization interactions between the IE110 and IE175 nuclear transactivator proteins of herpes simplex virus. *J Virol* 1994; **68**: 3250-3266 [PMID: 8151787]
91 **Everett RD**, Maul GG. HSV-1 IE protein Vmw110 causes redistribution of PML. *EMBO J* 1994; **13**: 5062-5069 [PMID: 7957072]
92 **Lopez P**, Van Sant C, Roizman B. Requirements for the nuclear-cytoplasmic translocation of infected-cell protein 0 of herpes simplex virus 1. *J Virol* 2001; **75**: 3832-3840 [PMID: 11264372 DOI: 10.1128/JVI.75.8.3832-3840.2001]
93 **Kalamvoki M**, Roizman B. Role of herpes simplex virus ICP0 in the transactivation of genes introduced by infection or transfection: a reappraisal. *J Virol* 2010; **84**: 4222-4228 [PMID: 20164233 DOI: 10.1128/JVI.02585-09]
94 **Zhu Z**, Cai W, Schaffer PA. Cooperativity among herpes simplex virus type 1 immediate-early regulatory proteins: ICP4 and ICP27 affect the intracellular localization of ICP0. *J Virol* 1994; **68**: 3027-3040 [PMID: 8151771]
95 **Tanaka M**, Kato A, Satoh Y, Ide T, Sagou K, Kimura K, Hasegawa H, Kawaguchi Y. Herpes simplex virus 1 VP22 regulates translocation of multiple viral and cellular proteins and promotes neurovirulence. *J Virol* 2012; **86**: 5264-5277 [PMID: 22357273 DOI: 10.1128/JVI.06913-11]
96 **Kawaguchi Y**, Bruni R, Roizman B. Interaction of herpes simplex virus 1 alpha regulatory protein ICP0 with elongation factor 1delta: ICP0 affects translational machinery. *J Virol* 1997; **71**: 1019-1024 [PMID: 8995621]
97 **Paladino P**, Collins SE, Mossman KL. Cellular localization of the herpes simplex virus ICP0 protein dictates its ability to block IRF3-mediated innate immune responses. *PLoS One* 2010; **5**: e10428 [PMID: 20454685 DOI: 10.1371/journal.pone.0010428]
98 **Yao F**, Courtney RJ. Association of ICP0 but not ICP27 with purified virions of herpes simplex virus type 1. *J Virol* 1992; **66**: 2709-2716 [PMID: 1313896]
99 **Loret S**, Guay G, Lippé R. Comprehensive characterization of extracellular herpes simplex virus type 1 virions. *J Virol* 2008; **82**: 8605-8618 [PMID: 18596102 DOI: 10.1128/JVI.00904-08]
100 **Sedlackova L**, Rice SA. Herpes simplex virus type 1 immediate-early protein ICP27 is required for efficient incorporation of ICP0 and ICP4 into virions. *J Virol* 2008; **82**: 268-277 [PMID: 17959681 DOI: 10.1128/JVI.01588-07]
101 **Delboy MG**, Siekavizza-Robles CR, Nicola AV. Herpes simplex virus tegument ICP0 is capsid associated, and its E3 ubiquitin ligase domain is important for incorporation into virions. *J Virol* 2010; **84**: 1637-1640 [PMID: 19906912 DOI: 10.1128/JVI.02041-09]
102 **Delboy MG**, Nicola AV. A pre-immediate-early role for tegument ICP0 in the proteasome-dependent entry of herpes simplex virus. *J Virol* 2011; **85**: 5910-5918 [PMID: 21471243 DOI: 10.1128/JVI.00267-11]
103 **Liang Y**, Kurakin A, Roizman B. Herpes simplex virus 1 infected cell protein 0 forms a complex with CIN85 and Cbl and mediates the degradation of EGF receptor from cell surfaces. *Proc Natl Acad*

Sci USA 2005; **102**: 5838-5843 [PMID: 15824310 DOI: 10.1073/pnas.0501253102]

104 **Liang Y**, Roizman B. State and role of SRC family kinases in replication of herpes simplex virus 1. *J Virol* 2006; **80**: 3349-3359 [PMID: 16537602 DOI: 10.1128/JVI.80.7.3349-3359.2006]

105 **Zheng Y**, Gu H. Identification of three redundant segments responsible for herpes simplex virus 1 ICP0 to fuse with ND10 nuclear bodies. *J Virol* 2015; **89**: 4214-4226 [PMID: 25631093 DOI: 10.1128/JVI.03658-14]

106 **Hay RT**. SUMO: a history of modification. *Mol Cell* 2005; **18**: 1-12 [PMID: 15808504 DOI: 10.1016/j.molcel.2005.03.012]

107 **Geiss-Friedlander R**, Melchior F. Concepts in sumoylation: a decade on. *Nat Rev Mol Cell Biol* 2007; **8**: 947-956 [PMID: 18000527 DOI: 10.1038/nrm2293]

108 **Wimmer P**, Schreiner S, Dobner T. Human pathogens and the host cell SUMOylation system. *J Virol* 2012; **86**: 642-654 [PMID: 22072786 DOI: 10.1128/JVI.06227-11]

109 **Song J**, Durrin LK, Wilkinson TA, Krontiris TG, Chen Y. Identification of a SUMO-binding motif that recognizes SUMO-modified proteins. *Proc Natl Acad Sci USA* 2004; **101**: 14373-14378 [PMID: 15388847 DOI: 10.1073/pnas.0403498101]

110 **Hecker CM**, Rabiller M, Haglund K, Bayer P, Dikic I. Specification of SUMO1- and SUMO2-interacting motifs. *J Biol Chem* 2006; **281**: 16117-16127 [PMID: 16524884 DOI: 10.1074/jbc.M512757200]

111 **Sun H**, Leverson JD, Hunter T. Conserved function of RNF4 family proteins in eukaryotes: targeting a ubiquitin ligase to SUMOylated proteins. *EMBO J* 2007; **26**: 4102-4112 [PMID: 17762864 DOI: 10.1038/sj.emboj.7601839]

112 **Prudden J**, Pebernard S, Raffa G, Slavin DA, Perry JJ, Tainer JA, McGowan CH, Boddy MN. SUMO-targeted ubiquitin ligases in genome stability. *EMBO J* 2007; **26**: 4089-4101 [PMID: 17762865 DOI: 10.1038/sj.emboj.7601838]

113 **Boutell C**, Cuchet-Lourenço D, Vanni E, Orr A, Glass M, McFarlane S, Everett RD. A viral ubiquitin ligase has substrate preferential SUMO targeted ubiquitin ligase activity that counteracts intrinsic antiviral defence. *PLoS Pathog* 2011; **7**: e1002245 [PMID: 21949651 DOI: 10.1371/journal.ppat.1002245]

114 **Everett RD**, Boutell C, Pheasant K, Cuchet-Lourenço D, Orr A. Sequences related to SUMO interaction motifs in herpes simplex virus 1 protein ICP0 act cooperatively to stimulate virus infection. *J Virol* 2014; **88**: 2763-2774 [PMID: 24352468 DOI: 10.1128/JVI.03417-13]

115 **Ciufo DM**, Mullen MA, Hayward GS. Identification of a dimerization domain in the C-terminal segment of the IE110 transactivator protein from herpes simplex virus. *J Virol* 1994; **68**: 3267-3282 [PMID: 8151788]

116 **Lium EK**, Panagiotidis CA, Wen X, Silverstein SJ. The NH2 terminus of the herpes simplex virus type 1 regulatory protein ICP0 contains a promoter-specific transcription activation domain. *J Virol* 1998; **72**: 7785-7795 [PMID: 9733814]

117 **Meredith M**, Orr A, Elliott M, Everett R. Separation of sequence requirements for HSV-1 Vmw110 multimerisation and interaction with a 135-kDa cellular protein. *Virology* 1995; **209**: 174-187 [PMID: 7747467 DOI: 10.1006/viro.1995.1241]

118 **Perusina Lanfranca M**, Mostafa HH, Davido DJ. Two overlapping regions within the N-terminal half of the herpes simplex virus 1 E3 ubiquitin ligase ICP0 facilitate the degradation and dissociation of PML and dissociation of Sp100 from ND10. *J Virol* 2013; **87**: 13287-13296 [PMID: 24089549 DOI: 10.1128/JVI.02304-13]

119 **Meredith M**, Orr A, Everett R. Herpes simplex virus type 1 immediate-early protein Vmw110 binds strongly and specifically to a 135-kDa cellular protein. *Virology* 1994; **200**: 457-469 [PMID: 8178435 DOI: 10.1006/viro.1994.1209]

120 **Li M**, Chen D, Shiloh A, Luo J, Nikolaev AY, Qin J, Gu W. Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. *Nature* 2002; **416**: 648-653 [PMID: 11923872 DOI: 10.1038/nature737]

121 **Huang Z**, Wu Q, Guryanova OA, Cheng L, Shou W, Rich JN, Bao S. Deubiquitylase HAUSP stabilizes REST and promotes maintenance of neural progenitor cells. *Nat Cell Biol* 2011; **13**: 142-152 [PMID: 21258371 DOI: 10.1038/ncb2153]

122 **Song MS**, Salmena L, Carracedo A, Egia A, Lo-Coco F, Teruya-Feldstein J, Pandolfi PP. The deubiquitinylation and localization of PTEN are regulated by a HAUSP-PML network. *Nature* 2008; **455**: 813-817 [PMID: 18716620 DOI: 10.1038/nature07290]

123 **Pfoh R**, Lacdao IK, Georges AA, Capar A, Zheng H, Frappier L, Saridakis V. Crystal Structure of USP7 Ubiquitin-like Domains with an ICP0 Peptide Reveals a Novel Mechanism Used by Viral and Cellular Proteins to Target USP7. *PLoS Pathog* 2015; **11**: e1004950 [PMID: 26046769 DOI: 10.1371/journal.ppat.1004950]

124 **Pozhidaeva AK**, Mohni KN, Dhe-Paganon S, Arrowsmith CH, Weller SK, Korzhnev DM, Bezsonova I. Structural Characterization of Interaction between Human Ubiquitin-specific Protease 7 and Immediate-Early Protein ICP0 of Herpes Simplex Virus-1. *J Biol Chem* 2015; **290**: 22907-22918 [PMID: 26224631 DOI: 10.1074/jbc.M115.664805]

125 **Gu H**, Liang Y, Mandel G, Roizman B. Components of the REST/CoREST/histone deacetylase repressor complex are disrupted, modified, and translocated in HSV-1-infected cells. *Proc Natl Acad Sci USA* 2005; **102**: 7571-7576 [PMID: 15897453 DOI: 10.1073/pnas.0502658102]

126 **Andrés ME**, Burger C, Peral-Rubio MJ, Battaglioli E, Anderson ME, Grimes J, Dallman J, Ballas N, Mandel G. CoREST: a functional corepressor required for regulation of neural-specific gene expression. *Proc Natl Acad Sci USA* 1999; **96**: 9873-9878 [PMID: 10449787 DOI: 10.1073/pnas.96.17.9873]

127 **Qureshi IA**, Gokhan S, Mehler MF. REST and CoREST are transcriptional and epigenetic regulators of seminal neural fate decisions. *Cell Cycle* 2010; **9**: 4477-4486 [PMID: 21088488 DOI: 10.4161/cc.9.22.13973]

128 **Ferenczy MW**, Ranayhossaini DJ, Deluca NA. Activities of ICP0 involved in the reversal of silencing of quiescent herpes simplex virus 1. *J Virol* 2011; **85**: 4993-5002 [PMID: 21411540 DOI: 10.1128/JVI.02265-10]

129 **Everett RD**. Depletion of CoREST does not improve the replication of ICP0 null mutant herpes simplex virus type 1. *J Virol* 2010; **84**: 3695-3698 [PMID: 20106915 DOI: 10.1128/JVI.00021-10]

130 **Liang Y**, Vogel JL, Narayanan A, Peng H, Kristie TM. Inhibition of the histone demethylase LSD1 blocks alpha-herpesvirus lytic replication and reactivation from latency. *Nat Med* 2009; **15**: 1312-1317 [PMID: 19855399 DOI: 10.1038/nm.2051]

131 **Sherr CJ**. D-type cyclins. *Trends Biochem Sci* 1995; **20**: 187-190 [PMID: 7610482]

132 **Malumbres M**, Barbacid M. Cell cycle, CDKs and cancer: a changing paradigm. *Nat Rev Cancer* 2009; **9**: 153-166 [PMID: 19238148 DOI: 10.1038/nrc2602]

133 **DeCaprio JA**. How the Rb tumor suppressor structure and function was revealed by the study of Adenovirus and SV40. *Virology* 2009; **384**: 274-284 [PMID: 19150725 DOI: 10.1016/j.virol.2008.12.010]

134 **Van Sant C**, Kawaguchi Y, Roizman B. A single amino acid substitution in the cyclin D binding domain of the infected cell protein no. 0 abrogates the neuroinvasiveness of herpes simplex virus without affecting its ability to replicate. *Proc Natl Acad Sci USA* 1999; **96**: 8184-8189 [PMID: 10393969]

135 **Kalamvoki M**, Roizman B. ICP0 enables and monitors the function of D cyclins in herpes simplex virus 1 infected cells. *Proc Natl Acad Sci USA* 2009; **106**: 14576-14580 [PMID: 19706544 DOI: 10.1073/pnas.0906905106]

136 **Van Sant C**, Lopez P, Advani SJ, Roizman B. Role of cyclin D3 in the biology of herpes simplex virus 1 ICPO. *J Virol* 2001; **75**: 1888-1898 [PMID: 11160688 DOI: 10.1128/JVI.75.4.1888-1898.2001]

137 **Advani SJ**, Brandimarti R, Weichselbaum RR, Roizman B. The disappearance of cyclins A and B and the increase in activity of the G(2)/M-phase cellular kinase cdc2 in herpes simplex virus 1-infected cells require expression of the alpha22/U(S)1.5 and U(L)13 viral genes. *J Virol* 2000; **74**: 8-15 [PMID: 10590085 DOI: 10.1128/JVI.74.1.8-15.2000]

138 **Schang LM**, Rosenberg A, Schaffer PA. Transcription of herpes simplex virus immediate-early and early genes is inhibited by

roscovitine, an inhibitor specific for cellular cyclin-dependent kinases. *J Virol* 1999; **73**: 2161-2172 [PMID: 9971799]

139 **Davido DJ**, Von Zagorski WF, Maul GG, Schaffer PA. The differential requirement for cyclin-dependent kinase activities distinguishes two functions of herpes simplex virus type 1 ICP0. *J Virol* 2003; **77**: 12603-12616 [PMID: 14610183 DOI: 10.1128/JVI.77.23.12603-12616.2003]

140 **Glossop NR**, Hardin PE. Central and peripheral circadian oscillator mechanisms in flies and mammals. *J Cell Sci* 2002; **115**: 3369-3377 [PMID: 12154068]

141 **Kalamvoki M**, Roizman B. Circadian CLOCK histone acetyl transferase localizes at ND10 nuclear bodies and enables herpes simplex virus gene expression. *Proc Natl Acad Sci USA* 2010; **107**: 17721-17726 [PMID: 20876123 DOI: 10.1073/pnas.1012991107]

142 **Panier S**, Durocher D. Regulatory ubiquitylation in response to DNA double-strand breaks. *DNA Repair* (Amst) 2009; **8**: 436-443 [PMID: 19230794 DOI: 10.1016/j.dnarep.2009.01.013]

143 **Luijsterburg MS**, van Attikum H. Close encounters of the RNF8th kind: when chromatin meets DNA repair. *Curr Opin Cell Biol* 2012; **24**: 439-447 [PMID: 22464734 DOI: 10.1016/j.ceb.2012.03.008]

144 **Taylor KE**, Mossman KL. Cellular Protein WDR11 Interacts with Specific Herpes Simplex Virus Proteins at the trans-Golgi Network To Promote Virus Replication. *J Virol* 2015; **89**: 9841-9852 [PMID: 26178983 DOI: 10.1128/JVI.01705-15]

145 **Davido DJ**, von Zagorski WF, Lane WS, Schaffer PA. Phosphorylation site mutations affect herpes simplex virus type 1 ICP0 function. *J Virol* 2005; **79**: 1232-1243 [PMID: 15613350 DOI: 10.1128/JVI.79.2.1232-1243.2005]

146 **Mostafa HH**, Thompson TW, Kushnir AS, Haenchen SD, Bayless AM, Hilliard JG, Link MA, Pitcher LA, Loveday E, Schaffer PA, Davido DJ. Herpes simplex virus 1 ICP0 phosphorylation site mutants are attenuated for viral replication and impaired for explant-induced reactivation. *J Virol* 2011; **85**: 12631-12637 [PMID: 21937654 DOI: 10.1128/JVI.05661-11]

147 **Ogle WO**, Ng TI, Carter KL, Roizman B. The UL13 protein kinase and the infected cell type are determinants of posttranslational modification of ICP0. *Virology* 1997; **235**: 406-413 [PMID: 9281521 DOI: 10.1006/viro.1997.8710]

148 **Maul GG**, Ishov AM, Everett RD. Nuclear domain 10 as preexisting potential replication start sites of herpes simplex virus type-1. *Virology* 1996; **217**: 67-75 [PMID: 8599237 DOI: 10.1006/viro.1996.0094]

149 **Gu H**, Roizman B. Engagement of the lysine-specific demethylase/HDAC1/CoREST/REST complex by herpes simplex virus 1. *J Virol* 2009; **83**: 4376-4385 [PMID: 19193804 DOI: 10.1128/JVI.02515-08]

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ORIGINAL ARTICLE

Basic Study

Modelling the prevalence of hepatitis C virus amongst blood donors in Libya: An investigation of providing a preventive strategy

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Author contributions: Daw MA designed the study, extracted the data, and drafted and finalized the manuscript; Shabash A and El-Bouzedi A collected the data and contributed to the drafting of the manuscript; Dau AA drafted, analyzed, and corrected the manuscript; Habas M collected the data and reviewed the clinical status of all patients; all authors read and approved the final manuscript.

Institutional review board statement: The study was reviewed by the board of Faculty of Medicine Tripoli Libya and found that the utilization and analysis of microbial epidemiological data did not require oversight by Libyan National Ethics committee. Hence no ethical approval was needed for this study.

Institutional animal care and use committee statement: We declare that no animals or human volunteers were used in the study.

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Data sharing statement: All data will be made freely available via the corresponding author (mohamedadaw@gmail.com). There are no security or licensing matters related to the study.

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Abstract

AIM: To determine hepatitis C virus (HCV) seroprevalence among the Libyan population using blood donors and applying the autoregressive integrated moving average (ARIMA) model to predict future trends and formulate plans to minimize the burden of HCV infection.

METHODS: HCV positive cases were collected from 1008214 healthy blood donors over a 6-year period from 2008 to 2013. Data were used to construct the ARIMA model to forecast HCV seroprevalence among blood donors. The validity of the model was assessed using the mean absolute percentage error between the observed and fitted seroprevalence. The fitted ARIMA model

was used to forecast the incidence of HCV beyond the observed period for the year 2014 and further to 2055.

RESULTS: The overall prevalence of HCV among blood donors was 1.8%, varying over the study period from 1.7% to 2.5%, though no significant variation was found within each calendar year. The ARIMA model showed a non-significant auto-correlation of the residuals, and the prevalence was steady within the last 3 years as expressed by the goodness-of-fit test. The forecast incidence showed an increase in HCV seropositivity in 2014, ranging from 500 to 700 per 10000 population, with an overall prevalence of 2.3%-2.7%. This may be extended to 2055 with minimal periodical variation within each 6-year period.

CONCLUSION: The applied model was found to be valuable in evaluating the seroprevalence of HCV among blood donors, and highlighted the growing burden of such infection on the Libyan health care system. The model may help in formulating national policies to prevent increases in HCV infection and plan future strategies that target the consequences of the infection.

Key words: Autoregressive integrated moving average model; Libya; Hepatitis C virus; Blood donors

Core tip: Hepatitis C virus (HCV) infection has major consequences and an overwhelming impact particularly among developing countries, hence prediction of its prevalence is important for future planning to mitigate its impact. This is an innovative study highlighting the importance of using a modified mathematical model to forecast and predict the future prevalence and consequence of HCV infection using data collected from blood donors. The results will allow strategists in health care services to plan immediate and long-term policies.

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INTRODUCTION

Hepatitis C virus (HCV) has been known to be one of the leading causes of chronic viral hepatitis with devastating consequences such as cirrhosis and hepatocellular carcinoma which are the major reasons for liver transplantation[1]. The geo-epidemiology of HCV infection varies greatly and is dynamic over time[2]. Indeed, 3% of the world's population are chronically infected with HCV and over 3 million new infection occur each year[3]. Africa and Asia represent the largest reservoir of chronic HCV infection[4,5], though prevalence varies from one country to another and among regions within each country[2,3]. The prevalence of HCV infection are highest in Africa, ranging from 1% to 26%, and Egypt, Senegal, and Cameroon have the highest rates worldwide[6].

Hepatitis C is well documented in Libya and different studies have shown the prevalence of HCV infection and genotypes among Libyans[7-9]. Recently a comprehensive study in over 1% of the Libyan population has shown that the prevalence of HCV infection is 1.2%, varying from 0.6% to 2.2% according to the region within the country[10]. The prevalence indicated an alarming increase in HCV among the younger generation, particularly within new emerging risk groups in Libyan society such as intravenous drug users (IVDUs)[10,11]. As age increases and disease progresses among infected individuals, there will be an increase in expected complications. This will place an increasing burden on the Libyan health care system which is still developing. Hence, studies should be directed to formulate policies to combat the effects of infection.

Early identification of epidemics of infectious diseases and prediction of their outcomes are an important step toward implementing effective intervention measurements and reducing mortality and morbidity[12]. Such goals are a challenge in health care surveillance studies. Mathematical dynamic modeling has contributed greatly in exploring such challenges. Surveillance data however, are usually necessary for these modeling purposes[13].

Different statistical models including linear regression and correlation coefficients have been used for prediction of viral hepatitis. Autoregressive integrated moving average (ARIMA) or Box-Jenkins has potential application in studies of disease dynamics[14]. Helfinstein was the first to show that the ARIMA model can be used successfully for forecasting and predicting the different relationships between viral infections and associated diseases[15]. Different studies applied such modeling to detect spikes, steps, and trends for hepatitis E, hepatitis B and hepatitis C infection[16,17]. A considerable effort has been undertaken to forecast the epidemiology of hepatitis C, and different models were used to estimate the burden and complications of the infection[18]. Recently, Corson *et al*[19] used a mathematical model to project the future of HCV among IVDUs and its impact on the future development of HCV-related morbidity and mortality.

Blood donors are generally considered to be a healthier cohort of any community and viral hepatitis seropositivity among them may mirror the seroprevalence in the general population[20]. In Libya, a study conducted between 1991 and 2001 indicated that the prevalence of HCV infection ranged from 1.2% to 1.6% among blood donors, similar to the prevalence among the general population reported in 2014 of 1.2%, though it was much higher (20.5%) among hospital personnel[7,10]. Therefore, modeling and forecasting using HCV data from blood donors may provide an opportunity for planning

Table 1 Number of blood donors included in the study, Tripoli, Libya, 2008-2013

Study period	No. of volunteers	No. HCV seropositive
2008	35859	937
2009	65330	1713
2010	254177	3958
2011	173873	3060
2012	260139	4480
2013	218836	3749
Total	1008214	17897

2011-2013 were post-revolution years where many Libyan were injured during the Libyan conflict (2011)[35]. HCV: Hepatitis C virus.

and intervention to control HCV infection. In this study, we aimed to forecast the prevalence of HCV trends among blood donors by analyzing HCV dynamics and highlighting the need for further intervention strategies.

MATERIALS AND METHODS

Ethical consideration

The study was reviewed by the Board of the Faculty of Medicine, Tripoli, Libya, who declared that the utilization and analysis of microbial epidemiological data did not require oversight by the Libyan National Ethics Committee. Hence, no ethical approval was needed for this study.

Study population

A total of 1008214 healthy individuals, aged 18 to 50 years, were recruited from three different main blood banks in Tripoli over 6 years from 2008-2013 (Table 1). These included Tripoli Central Hospital, Karda Teaching Hospital, and Tripoli Reference Laboratory. Each person was subjected to screening for known risk factors associated with blood donation according to the national and international standards applicable in all three hospitals. Those who failed to meet the criteria for blood donation were excluded from the study, such as those who had previous blood transfusion, jaundice, a history of illicit drug-taking, and other potential risk factors.

Laboratory diagnosis

The laboratory analysis was carried out using ELISA (Vitros EciQ, Orthodiagnostic-Switzerland), and samples were considered to be confirmed positive according to the manufacturer's instructions. HCV infection was defined as the presence of anti-HCV antibodies in the serum as detected by ELISA.

Modeling

The ARIMA model was developed to forecast the incidence of HCV infection among blood donors in Libya. This was applied using data for 72 mo between January 2008 and December 2013 to then forecast the incidence of HCV infection from January to December 2014 and predict the prevalence of HCV infection from 2008 to 2055 under nonexclusive expectation. The model was constructed

Table 2 Seroprevalence of hepatitis C virus among blood donors, Tripoli, Libya, 2008-2013

Yr	Prevalence (%)	OR	95%CI		Minimum	Maximum
			Lower	Upper		
2008	2.6	1.4	2.3	2.8	2.0	3.2
2009	2.6	1.4	1.7	3.4	1.1	4.1
2010	1.5	0.8	1.3	1.6	0.9	2.1
2011	1.7	0.7	1.3	2.0	0.8	2.8
2012	1.7	1.1	0.8	2.6	0.6	2.9
2013	1.7	1.1	0.9	2.5	0.7	2.7
Total	1.8	1.1	1.4	2.5	1.0	3.0

using the Box-Jenkins method. The identification and selection steps for ARIMA were carried *via* autocorrelation and partial autocorrelation functions. The model parameters were determined by the maximum likelihood method. Goodness-of-fit among ARIMA models was compared using diagnostic checks such as residual analysis and other relevant information. The accuracy of the model was finally subjected to critical estimation and rigorous checking to fulfill the required criteria for the model. The details of the ARIMA model were recently described by Yu *et al*[21] in 2013 and used to analyze the epidemiology of HIV infection among the Korean population.

Statistical analysis

Data were analyzed using Microsoft Excel, Minitab version 15 (State College, PA, United States), and SPSS version 16 (SPSS Inc., Chicago, IL, United States). A *P*-value < 0.05 indicated a significant difference between HCV and HBV prevalence. The Excel 2007 forecast function was used to predict the number of infected people. The following equation was used to calculate the expected number of infected persons each year, 1-Y = 613.2X + 836.5, where Y = number of expected infected persons with HCV, and X = the serial number for the year calculated from 2008; for example for 2009 and 2010 the serial numbers were 2 and 3, respectively.

RESULTS

A total of 1008214 volunteer blood donors were screened for HCV over a 6-year period from 2008 till 2013. Of these, 17897 were found to be positive for anti-HCV antibodies, with an overall prevalence of 1.8%. There was no apparent monthly difference in HCV infection among individuals screened during the same year. Based on year-to-year analysis, a substantial variation in the seroprevalence of HCV was observed as shown in Table 2. The highest prevalence of HCV infection was reported in 2008 and 2009 as (2.6%) though it was 1.5% in 2010 and 1.7% in 2011-2013. In 2008, 35869 individuals were reviewed, of whom 937 (2.6%) were positive for anti-HCV antibodies. During 2009 the number of screened people doubled, and 1713 (2.6%) were positive for anti-HCV antibodies. In 2010, the number screened was 254177, a 7-fold increase compared with 2008, and 3958 (1.5%) were positive for HCV antibodies. In

2011, 3060 (1.7%) were HCV-positive. Although 260139 people were screened during 2012, the largest number over the 5-year period, the prevalence was the same (1.7%) as to that in 2011 and 2013, when a combined total of 218836 persons were screened (Table 1).

ARIMA modeling was applied to the data for identification, estimation, and then forecasting of HCV infection. The first stage was construction of an estimation model followed by forecasting and model evaluation. The data collected from 2008-2013 was used to construct the ARIMA model as depicted by Box *et al*[14]. Figure 1 shows the sample autocorrelation and partial autocorrelation functions [autocorrelation function (ACF) and partial ACF (PACF)] for the case structure which allowed identification of an appropriate ARIMA form to model the stationary series. A small variation was noted but it was not statistically different from zero which confirms the adequacy of the ARIMA model. The model forecast a steady increase for the following 6 years.

The sample ACF and PACF in Figure 2 showed a good fit which allowed us to determine the appropriate ARIMA model for HCV seroprevalence among blood donors. The adequacy of the model was evident as the residuals of autocorrelation had little variation with no significant difference ($P > 0.05$).

The plot of observed *vs* fitted values indicated that the model provided an excellent fit of the data as shown in Figure 3. The ARIMA model was used to forecast HCV prevalence for 72 mo over the 6-year period from January 2008 to December 2013 (Figure 3). Detailed analysis of observed *vs* forecast values of HCV prevalence over the study period showed a steady increase, with a maximum value at 1.8% to 2.01%, and an increasing tendency beyond the observed period in the short-term forecast (January-December 2014), reaching a maximum of 700 per 10000 population (2.3% to 2.7%). This was then used as a basis for estimating the prevalence of HCV infection among the Libyan population up to 2055, based on 6-year periods (Figure 4). According to our model, the prevalence of HCV infection will decrease and thus all sequelae of the infection will continue to decrease steadily in the future.

DISCUSSION

HCV infection has been known to be an important cause of chronic liver diseases though accurate representative epidemiological data are difficult to obtain, particularly in developing countries, as this infection has been considered to be endemic[2]. Statistical analysis of surveillance data on the prevalence of various infections was shown to be helpful in establishing a hypotheses to highlight and anticipate the dynamics of HCV infection and subsequently implement appropriate preventive measures and allocation of required resources[22]. The ARIMA model is one of the most widely used forecasting techniques due to its structured modeling base and acceptable forecasting performance[23].

In this study, we developed a calibrated ARIMA model for HCV infection with the aim of taking full advantage of available epidemiological information from registered blood donors in Libyan blood banks. The overall prevalence of HCV among the blood donors was found to be 1.8%, ranging from 2.5% in 2008 to 1.7% in 2013. This is consistent with a recent comprehensive study published by our group who reported that the prevalence of HCV among the Libyan population varied from 0.6% to 2.2%[10]. Comparing such results with regional published data, the prevalence of HCV infection was found to be similar to that in neighboring countries, with 1.6% in Tunis and 1.8% in Algeria, though it was higher in Egypt (22%)[24,25]. However, this was higher than the prevalence among developed countries such as the United States and Germany (< 1.5%)[26,27]. Hence, further studies are needed to elucidate the different factors associated with the higher prevalence of HCV among Libyan blood donors.

In our study, the applied model showed accuracy for the prevalence and dynamics of HCV infection among blood donors over a 6-year period and the forecast after that. This is in agreement with other studies who also declared that this model provides a better forecast than traditional methods for case notification of an infectious disease[28]. Although the prevalence of HCV was steady over the last 3 years (1.7% for 2011-2013), we predicted an increase for the year after. This was consistent with other studies from China and Latin America which showed that the prevalence of HCV was steady or increasing and that the number of infected individuals will increase[29,30]. This suggested that other risk factors are set to play a major role in continued new infection. Further studies are needed to clarify such an assumption.

Despite the increase in rates of HCV seropositivity in this study, we did not predict the burden of HCV infection over the next decades, nor did we calculated the estimated number of individual morbidities associated with HCV infection. However, different studies have shown that the prevalence of HCV-related cirrhosis is expected to increase by 24% within a decade, though decompensated cirrhosis cases and hepatocellular carcinoma will increase by 50% within the same period[31-33]. Hence, further studies are needed to elucidate such consequent complications of HCV infection among Libyan populations.

Modeling studies have projected a dismal future for HCV infection and related disease burden. In general, these models make forecasts based on current conditions of low rates of screening and treatment, and thus do not include a widespread program of identifying and treating the large proportion of undiagnosed HCV-infected individuals[34]. According to the results of our model, the incidence of the more serious outcomes of HCV infection will continue to rise, at least until 2055, unless modified. In our projections of HCV infection to 2055, we did not take into account the effective HCV prevention programs and the possible impact of the use of antiviral medications. Both these developments could have a considerable impact on our future projections, and thus the prevalence of HCV infection projected to 2055 may be less than that estimated by our model. Furthermore,

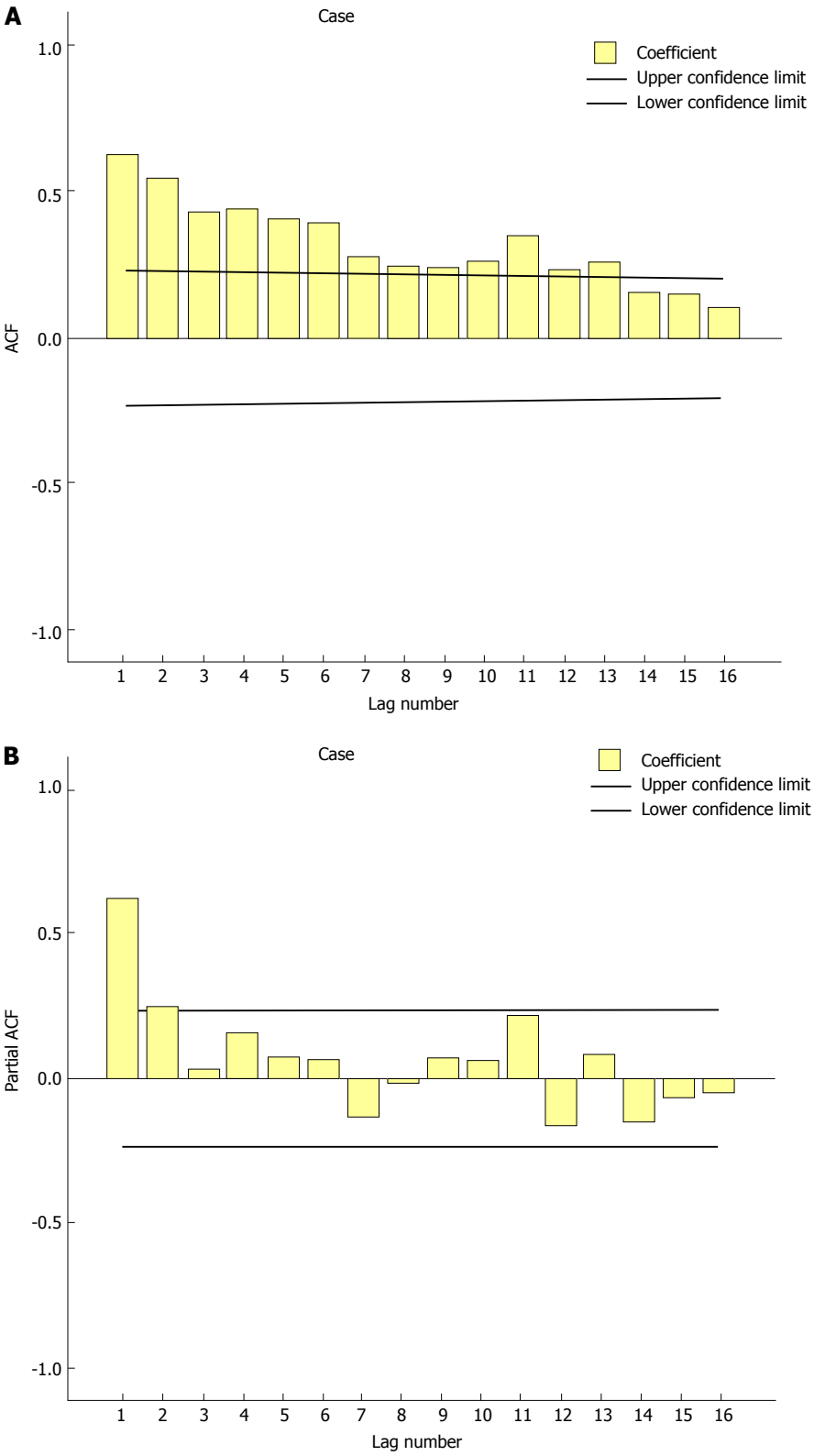

Figure 1 Correlogram and partial correlogram for a case structure control used for autoregressive integrated moving average model. A: ACF; B: Partial ACF. ACF: Autocorrelation function.

socioeconomic conditions in the country should be taken in consideration, particularly among developing countries; Libya is experiencing a major challenge regarding its geographical, political and social-ethnic identity[35,36]. Thus, future planning regarding infectious disease should be prioritized[37,38].

Monitoring HCV seropositivity among blood donors could be used to evaluate the effectiveness of the national efforts and guidelines to provide safe blood donation and good blood bank services[39]. In many

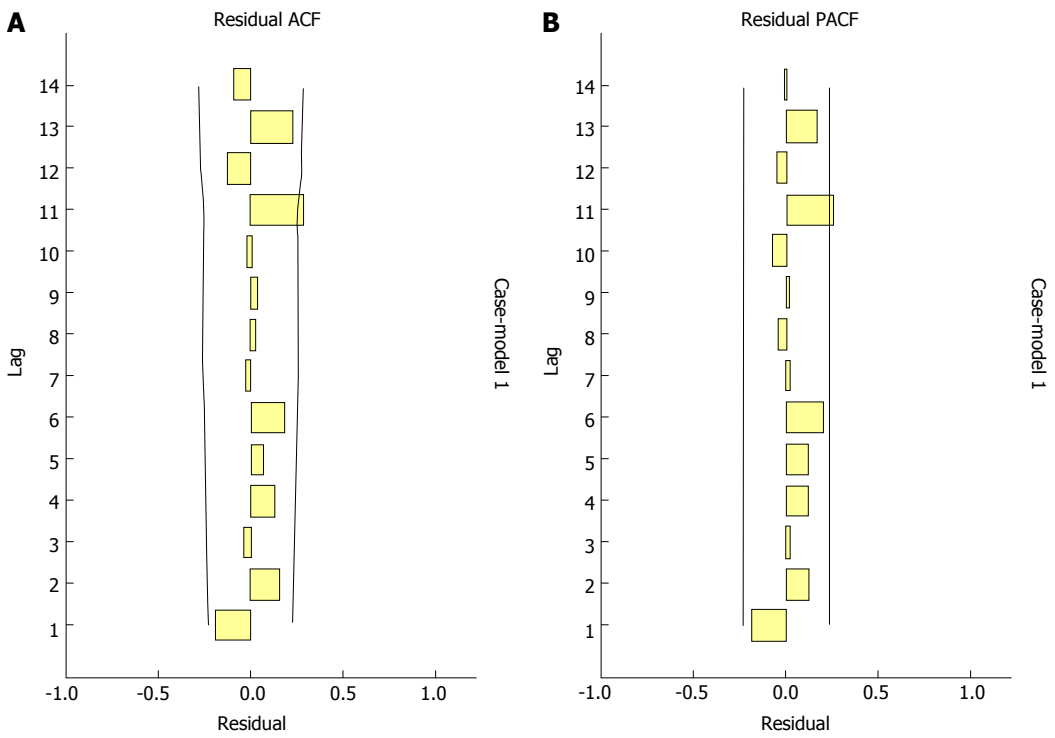

Figure 2 Residual plots for the final autoregressive integrated moving average (2, 1, 7) model of hepatitis C virus seroprevalence among volunteer blood donors in Libya, 2008-2013. A: ACF; B: Partial ACF. Lines indicate 95%CI. ACF: Autocorrelation function.

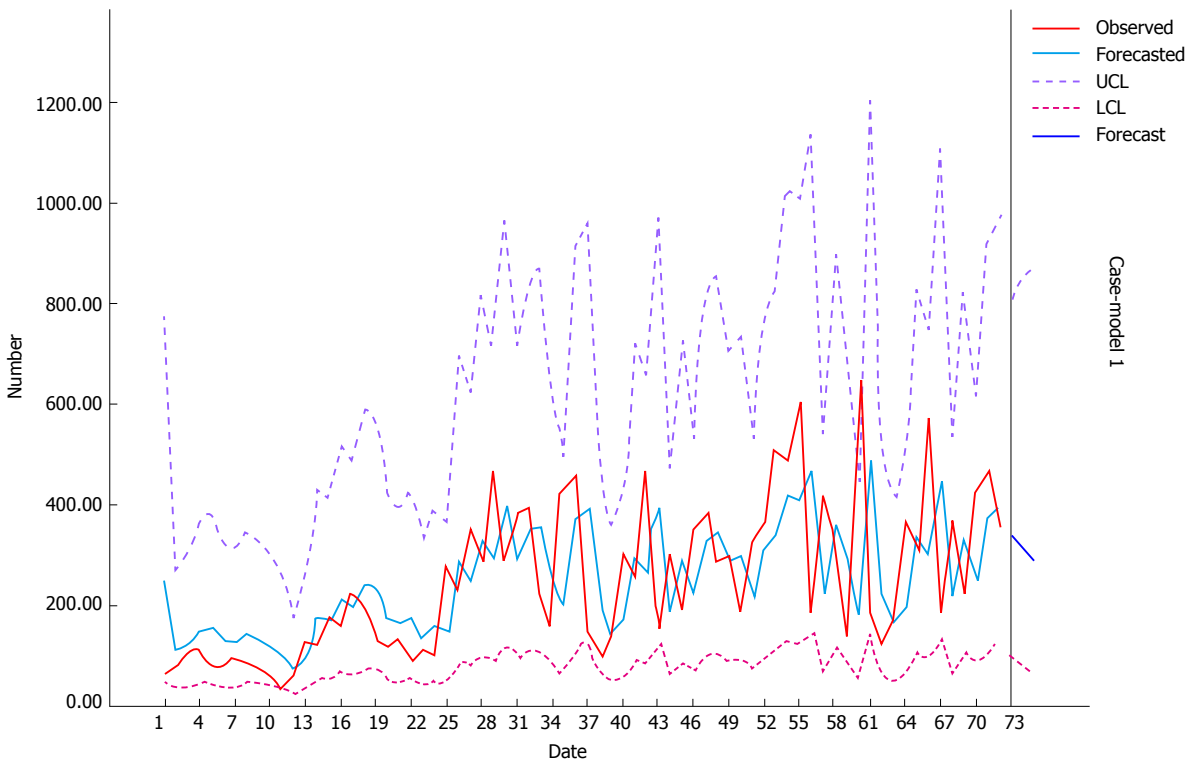

Figure 3 Number of observed and forecast hepatitis C virus seropositive volunteers among blood donors in Libya, 2008-2013. Date: Period of observation (months: 2008-2013); Number: Estimated number of HCV seropositive/month. UCL: Upper confidence limit; LCL: Lower confidence limit; HCV: Hepatitis C virus.

countries, HCV transmission rates decreased markedly with the introduction of blood screening[40]. Despite such a decline, mathematical models still predict a continuing rise in the prevalence of HCV infection within blood banks[41]. This was evident in our study where the increase in HCV seroprevalence may be attributed to the lack of quality assurance within the blood donation system. In Arab countries, blood transfusion is still a

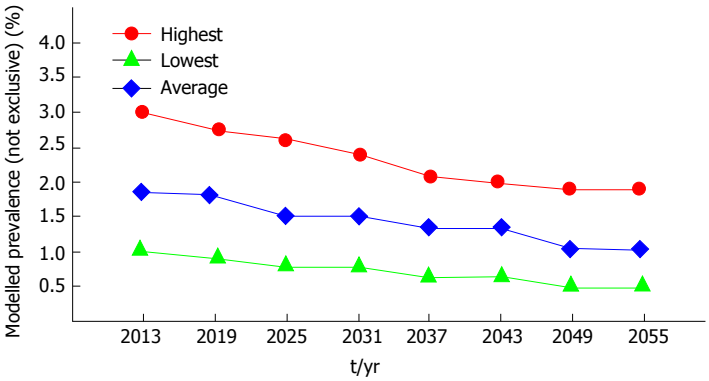

Figure 4 Modeled prevalence (not exclusive[1]) of hepatitis C virus infection by 6-year period, Libya, 2008-2055. [1]Estimates assume stable risk populations and HCV infection risks and do not adjust for treatment. HCV: Hepatitis C virus.

problem due to lack of an organized infrastructure and altruistic volunteers. The main sources of blood donation are usually relatives and friends who attend because of social pressure and in an emergency where questions regarding risk behaviors are rarely asked[2].

Many difficulties surround the determination of HCV prevalence using blood donors, since high risk groups including IVDUs are often excluded from blood donation, leading to underestimation of the true prevalence of HCV infection[42]. Nevertheless, our data do not necessarily represent the true HCV prevalence among the general population and thus are in need of further updating. The applicability and effectiveness of this monitoring system in its practical application as conducted here is able to detect the epidemic situation of HCV infection in Libya. However, such an infection is dynamic and evolves over time. Therefore, the model should be periodically reassessed and updated to maintain long-term sustainability and precision. This study highlights the need for preventive initiatives and strategies to be adapted by health care policy-makers to reduce HCV infection.

In conclusion, there is an important need for monitoring and predicting the prevalence of HCV infection to reduce the substantial consequences particularly in developing countries. The model applied was verified and could be used to monitor and predict the epidemiology of HCV infection. A better understanding of the epidemiology of HCV infection will allow health authorities to revise and plan new strategies within the health care system.

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COMMENTS

Background

Viral hepatitis, particularly hepatitis C virus (HCV) is known to be a serious problem particularly among developing countries. Hence, using a simple and reliable method for predicting the future course and consequences of this infection are a priority for researchers and health care planners. Reliable data regarding the prevalence of HCV infection among blood donors are available in all blood banks and this could be used to achieve such objectives.

Research frontiers

Studies on the prevalence of HCV infection utilizing blood bank data could be used as a basis for future planning. However, such studies are rare and few researchers have focused on using such data as a model for future planning.

Innovations and breakthroughs

This is a novel study which applied a mathematical model utilizing basic data from blood banks regarding the pathogen HCV. This is a rare study which modeled data to predict the prevalence of HCV infection among the general population over the next 50 years (2008-2055).

Applications

The practical approach of this study allows strategists and health care professionals to plan appropriate intervention and prevention methods not only to minimize the spread of HCV infection but also to reduce the associated consequences and complications, such as hepatocellular carcinoma and cirrhosis, and may be used further for other infections such as hepatitis B virus and human immunodeficiency virus.

Terminology

The ARIMA model is an autoregressive integrated moving average or Box-jenkins mathematical model which has a potential application in studying disease dynamics. The model can be used successfully for forecasting and predicting the relationships among viral infections and associated diseases.

Peer-review

This is a well conducted epidemiologic study carried out in a developing country.

REFERENCES

1 **Howell J**, Angus P, Gow P. Hepatitis C recurrence: the Achilles heel of liver transplantation. *Transpl Infect Dis* 2014; **16**: 1-16 [PMID: 24372756 DOI: 10.1111/tid.12173]

2 **Daw MA**, Dau AA. Hepatitis C virus in Arab world: a state of concern. *ScientificWorldJournal* 2012; **2012**: 719494 [PMID: 22629189 DOI: 10.1100/2012/719494]

3 **Mohd Hanafiah K**, Groeger J, Flaxman AD, Wiersma ST. Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence. *Hepatology* 2013; **57**: 1333-1342 [PMID: 23172780 DOI: 10.1002/hep.26141]

4 **Karoney MJ**, Siika AM. Hepatitis C virus (HCV) infection in Africa: a review. *Pan Afr Med J* 2013; **14**: 44 [PMID: 23560127]

5 **Zidan A**, Scheuerlein H, Schüle S, Settmacher U, Rauchfuss F. Epidemiological pattern of hepatitis B and hepatitis C as etiological agents for hepatocellular carcinoma in iran and worldwide. *Hepat Mon* 2012; **12**: e6894 [PMID: 23233864 DOI: 10.5812/hepatmon.6894]

6 **Jobarteh M**, Malfroy M, Peterson I, Jeng A, Sarge-Njie R, Alabi A, Peterson K, Cotten M, Hall A, Rowland-Jones S, Whittle H, Tedder R, Jaye A, Mendy M. Seroprevalence of hepatitis B and C virus in HIV-1 and HIV-2 infected Gambians. *Virol J* 2010; **7**: 230 [PMID: 20843322 DOI: 10.1186/1743-422X-7-230]

7 **Daw MA**, Elkaber MA, Drah AM, Werfalli MM, Mihat AA, Siala IM. Prevalence of hepatitis C virus antibodies among different populations of relative and attributable risk. *Saudi Med J* 2002; **23**: 1356-1360 [PMID: 12506296]

8 **Elasifer HA**, Agnnyia YM, Al-Alagi BA, Daw MA. Epidemiological manifestations of hepatitis C virus genotypes and its association with potential risk factors among Libyan patients. *Virol J* 2010; **7**: 317 [PMID: 21073743 DOI: 10.1186/1743-422X-7-317]

9 **Alashek WA**, McIntyre CW, Taal MW. Hepatitis B and C infection in haemodialysis patients in Libya: prevalence, incidence and risk factors. *BMC Infect Dis* 2012; **12**: 265 [PMID: 23082935 DOI: 10.1186/1471-2334-12-265]

10 **Daw MA**, El-Bouzedi A. Prevalence of hepatitis B and hepatitis C infection in Libya: results from a national population based survey. *BMC Infect Dis* 2014; **14**: 17 [PMID: 24405790 DOI: 10.1186/1471-2334-14-17]

11 **Daw MA**, Dau AA, Agnan MM. Influence of healthcare-associated factors on the efficacy of hepatitis C therapy. *ScientificWorldJournal* 2012; **2012**: 580216 [PMID: 23346018 DOI: 10.1100/2012/580216]

12 **Rong L**, Guedj J, Dahari H, Perelson AS. Treatment of hepatitis C with an interferon-based lead-in phase: a perspective from mathematical modelling. *Antivir Ther* 2014; **19**: 469-477 [PMID: 24434478 DOI: 10.3851/IMP2725]

13 **Chatterjee A**, Guedj J, Perelson AS. Mathematical modelling of HCV infection: what can it teach us in the era of direct-acting antiviral agents? *Antivir Ther* 2012; **17**: 1171-1182 [PMID: 23186606 DOI: 10.3851/IMP2428]

14 **Box GEP**, Jenkins GM. Time series analysis, control, and forecasting. San Francisco, CA: Holden Day, 1976: 10

15 **Helfenstein U**. Box-Jenkins modelling of some viral infectious diseases. *Stat Med* 1986; **5**: 37-47 [PMID: 3961314]

16 **Ren H**, Li J, Yuan ZA, Hu JY, Yu Y, Lu YH. The development of a combined mathematical model to forecast the incidence of hepatitis E in Shanghai, China. *BMC Infect Dis* 2013; **13**: 421 [PMID: 24010871 DOI: 10.1186/1471-2334-13-421]

17 **Sutton AJ**, Gay NJ, Edmunds WJ, Hope VD, Gill ON, Hickman M. Modelling the force of infection for hepatitis B and hepatitis C in injecting drug users in England and Wales. *BMC Infect Dis* 2006; **6**: 93 [PMID: 16762050]

18 **Akhtar S**, Carpenter TE. Stochastic modelling of intra-household transmission of hepatitis C virus: evidence for substantial non-sexual infection. *J Infect* 2013; **66**: 179-183 [PMID: 23103288 DOI: 10.1016/j.jinf.2012.10.020]

19 **Corson S**, Greenhalgh D, Taylor A, Palmateer N, Goldberg D, Hutchinson S. Modelling the prevalence of HCV amongst people who inject drugs: an investigation into the risks associated with injecting paraphernalia sharing. *Drug Alcohol Depend* 2013; **133**: 172-179 [PMID: 23791029 DOI: 10.1016/j.drugalcdep.2013.05.014]

20 **Khodabandehloo M**, Roshani D, Sayehmiri K. Prevalence and trend of hepatitis C virus infection among blood donors in Iran: A systematic review and meta-analysis. *J Res Med Sci* 2013; **18**: 674-682 [PMID: 24379843]

21 **Yu HK**, Kim NY, Kim SS, Chu C, Kee MK. Forecasting the number of human immunodeficiency virus infections in the korean population using the autoregressive integrated moving average model. *Osong Public Health Res Perspect* 2013; **4**: 358-362 [PMID: 24524025 DOI: 10.1016/j.phrp.2013.10.009]

22 **Firmino PR**, de Mattos Neto PS, Ferreira TA. Correcting and combining time series forecasters. *Neural Netw* 2014; **50**: 1-11 [PMID: 24239986 DOI: 10.1016/j.neunet.2013.10.008]

23 **Zhang X**, Zhang T, Young AA, Li X. Applications and comparisons of four time series models in epidemiological surveillance data. *PLoS One* 2014; **9**: e88075 [PMID: 24505382 DOI: 10.1371/journal.pone.0088075]

24 **Ezzikouri S**, Pineau P, Benjelloun S. Hepatitis B virus in the Maghreb region: from epidemiology to prospective research. *Liver Int* 2013; **33**: 811-819 [PMID: 23530901 DOI: 10.1111/liv.12135]

25 **Breban R**, Doss W, Esmat G, Elsayed M, Hellard M, Ayscue P, Albert M, Fontanet A, Mohamed MK. Towards realistic estimates of HCV incidence in Egypt. *J Viral Hepat* 2013; **20**: 294-296 [PMID: 23490375 DOI: 10.1111/j.1365-2893.2012.01650.x]

26 **Sheikh MY**, Atla PR, Ameer A, Sadiq H, Sadler PC. Seroprevalence of Hepatitis B and C Infections among Healthy Volunteer Blood Donors in the Central California Valley. *Gut Liver* 2013; **7**: 66-73 [PMID: 23423771]

27 **Offergeld R**, Ritter S, Hamouda O. [HIV, HCV, HBV and syphilis surveillance among blood donors in Germany 2008-2010]. *Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz* 2012; **55**: 907-913 [PMID: 22842883 DOI: 10.1007/s00103-012-1516-1]

28 **Liu Q**, Liu X, Jiang B, Yang W. Forecasting incidence of hemorrhagic fever with renal syndrome in China using ARIMA model. *BMC Infect Dis* 2011; **11**: 218 [PMID: 21838933 DOI: 10.1186/1471-2334-11-218]

29 **Ji ZH**, Li CY, Lv YG, Cao W, Chen YZ, Chen XP, Tian M, Li JH, An QX, Shao ZJ. The prevalence and trends of transfusion-transmissible infectious pathogens among first-time, voluntary blood donors in Xi'an, China between 1999 and 2009. *Int J Infect Dis* 2013; **17**: e259-e262 [PMID: 23195637]

30 **Kershenobich D**, Razavi HA, Sánchez-Avila JF, Bessone F, Coelho HS, Dagher L, Gonçales FL, Quiroz JF, Rodriguez-Perez F, Rosado B, Wallace C, Negro F, Silva M. Trends and projections of hepatitis C virus epidemiology in Latin America. *Liver Int* 2011; **31** Suppl 2: 18-29 [PMID: 21651701 DOI: 10.1111/j.1478-3231]

31 **Weinmann A**, Koch S, Niederle IM, Schulze-Bergkamen H, König J, Hoppe-Lotichius M, Hansen T, Pitton MB, Düber C, Otto G, Schuchmann M, Galle PR, Wörns MA. Trends in epidemiology, treatment, and survival of hepatocellular carcinoma patients between 1998 and 2009: an analysis of 1066 cases of a German HCC Registry. *J Clin Gastroenterol* 2014; **48**: 279-289 [PMID: 24045276 DOI: 10.1097/MCG.0b013e3182a8a793]

32 **Pinchoff J**, Drobnik A, Bornschlegel K, Braunstein S, Chan C, Varma JK, Fuld J. Deaths among people with hepatitis C in New York City, 2000-2011. *Clin Infect Dis* 2014; **58**: 1047-1054 [PMID: 24523215]

33 **Kershenobich D**, Razavi HA, Cooper CL, Alberti A, Dusheiko GM, Pol S, Zuckerman E, Koike K, Han KH, Wallace CM, Zeuzem S, Negro F. Applying a system approach to forecast the total hepatitis C virus-infected population size: model validation using US data. *Liver Int* 2011; **31** Suppl 2: 4-17 [PMID: 21651700 DOI: 10.1111/j.1478-3231.2011.02535.x]

34 **Edlin BR**, Eckhardt BJ, Shu MA, Holmberg SD, Swan T. Towards a more accurate estimate of the prevalence of hepatitis C in the United States. *Hepatology* 2015 Jul 14; Epub ahead of print [PMID: 26171595 DOI: 10.1002/hep.27978]

35 **Daw MA**, El-Bouzedi A, Dau AA. Libyan armed conflict 2011: Mortality, injury and population displacement. *Afr J Emerg Med* 2015; **14**:101-107 [DOI: 10.1016/j.afjem.2015.02.002]

36 **Daw MA**, El-Bouzedi A, Dau AA. The assessment of efficiency and coordination within Libyan Healthcare System during the Armed Conflict-2011. *Clinical Epidemiology & Global Health* 2015; In press [DOI: 10.1016/j.cegh.2015.07.004]

37 **Daw MA**, El-Bouzedi A, Dau AA. Geographic distribution of HCV genotypes in Libya and analysis of risk factors involved in their transmission. *BMC Res Notes* 2015; **8**: 367 [PMID: 26293137 DOI: 10.1186/s13104-015-1310-x]

38 **Daw MA**, El-Bouzedi A. Viral haemorrhagic fever in North Africa; an evolving emergency. *J Clin Exp Pathol* 2015; **5**: 215 [DOI: 10.4172/2161-0681.1000215]

39 **Watkins NA**, Dobra S, Bennett P, Cairns J, Turner ML. The

management of blood safety in the presence of uncertain risk: a United kingdom perspective. *Transfus Med Rev* 2012; **26**: 238-251 [PMID: 22126710 DOI: 10.1016/j.tmrv.2011.09.003]

40 **Kim MJ**, Park Q, Min HK, Kim HO. Residual risk of transfusion-transmitted infection with human immunodeficiency virus, hepatitis C virus, and hepatitis B virus in Korea from 2000 through 2010. *BMC Infect Dis* 2012; **12**: 160 [PMID: 22817275 DOI: 10.1186/1471-2334-12-160]

41 **Lucky TT**, Seed CR, Keller A, Lee J, McDonald A, Ismay S, Wand H, Wilson DP. Trends in transfusion-transmissible infections among Australian blood donors from 2005 to 2010. *Transfusion* 2013; **53**: 2751-2762 [PMID: 23461827 DOI: 10.1111/trf.12144]

42 **Zou S**, Dorsey KA, Notari EP, Foster GA, Krysztof DE, Musavi F, Dodd RY, Stramer SL. Prevalence, incidence, and residual risk of human immunodeficiency virus and hepatitis C virus infections among United States blood donors since the introduction of nucleic acid testing. *Transfusion* 2010; **50**: 1495-1504 [PMID: 20345570 DOI: 10.1111/j.1537-2995.2010.02622.x]

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ORIGINAL ARTICLE

Basic Study

Pathogenicity of a currently circulating Chinese variant pseudorabies virus in pigs

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Abstract

AIM: To test the pathogenicity of pseudorabies virus (PRV) variant HN1201 and compare its pathogenicity with a classical PRV Fa strain.

METHODS: The pathogenicity of the newly-emerging PRV variant HN1201 was evaluated by different inoculating routes, virus loads, and ages of pigs. The classical PRV Fa strain was then used to compare with HN1201 to determine pathogenicity. Clinical symptoms after virus infection were recorded daily and average daily body weight was used to measure the growth performance of pigs. At necropsy, gross pathology and histopathology were used to evaluate the severity of tissue damage caused by virus infection.

RESULTS: The results showed that the efficient infection method of RPV HN1201 was *via* intranasal inoculation

at 10^7 $TCID_{50}$, and that the virus has high pathogenicity to 35- to 127-d old pigs. Compared with Fa strain, pigs infected with HN1201 showed more severe clinical symptoms and pathological lesions. Immunochemistry results revealed HN1201 had more abundant antigen distribution in extensive organs.

CONCLUSION: All of the above results suggest that PRV variant HN1201 was more pathogenic to pigs than the classical Fa strain.

Key words: Pseudorabies virus; Pathogenicity; Virus variant; Gross pathology; Histopathology

Core tip: Pseudorabies virus (PRV) variant HN1201 has pathogenicity in 35 to 127-d old pigs *via* intranasal inoculation at 10^7 $TCID_{50}$. Intranasal inoculation is more efficient than intramuscular inoculation for PRV challenge. PRV variant HN1201 showed higher pathogenic ability, as shown by the more severe clinical symptoms, pathological lesions, and abundant antigen distribution in extensive organs than the classical PRV Fa strain.

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INTRODUCTION

Pseudorabies virus (PRV), also known as Aujeszky's disease virus or Suid herpesvirus type 1 (SuHV-1), is the causative agent of pseudorabies (PR). Belonging to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, and genus *Varicellovirus*, the virus causes substantial economic losses in the pig industry worldwide[1-3]. The PRV genome is a double-stranded linear DNA which is about 143 kb in size and has about 70 ORFs[4,5]. This pathogen can infect numerous mammals, including carnivores, ruminants, and rodents, yet pigs are the only natural host for PRV as the reservoir of the virus[6,7]. PRV infection is characterized by neurologic symptoms and death in newborn piglets, respiratory disorders in elder pigs, and reproductive failure like stillbirths and abortions in sows. Like other alphaherpesviruses, PRV can establish a lifelong latent infection in the peripheral nervous system of infected pigs. Latently infected pigs can be recognized as a source of reinfection when the latent viral genome reactivates spontaneously and the infectious virus is developed[8].

Attenuated live or killed PRV vaccines have played a critical role in the control and eradication of PR. Bartha-K61, a vaccine imported from Hungary, have been widely used in China since the 1970s, and was reported to provide complete protection from field virus infection[2]. Nevertheless, since October 2011, severe PRV outbreaks have occurred on pig farms and spread rapidly to the northern parts of China[9,10]. Most of the infected farms had used Bartha-K61 vaccine according to the manufacturer's instructions, and the serum samples obtained from the infected pigs had a considerable positive rate of gE Ab detected by ELISA (IDEXX Laboratories, Westbrook, United States)[10,11]. The affected pigs presented with multiple clinical signs, including high fever (usually ≥ 40.5 ℃), depression, anorexia, respiratory distress, shivering, and systemic neurological symptoms[11,12]. Pathologic examination of viscera samples collected from dead pigs from different provinces displayed consolidation, edema, and hemorrhage in the lungs, as well as necrosis in the kidneys, indicating that newly-emerging PRV variants may have higher pathogenicity than the classical strains[13]. The PRV infection in vaccinated pig herds indicates that the traditional Bartha-K61 vaccine could not provide complete protection to the current prevalent PRV variants in China[11,14]. Accordingly, it is imperative to study the pathogenicity of the currently circulating PRV variant strains and develop newly effective vaccines to tackle the problem.

In this study, we first established a PRV variant HN1201 infection model in pigs according to different inoculation routes, virus loads, and pig ages. The characterized PRV variant HN1201 was then compared with the virulent classical PRV strain Fa to determine pathogenicity.

MATERIALS AND METHODS

Viruses and cells

The PRV variant HN1201 was previously isolated from the brain of infected pigs in Henan province[12]. Briefly, the infected pig brain sample was homogenized and the supernatant of homogenization was subjected to 0.22 μm filtration. The filtrated supernatant was inoculated on a PK-15 cell monolayer until the appearance of CPE after 3 d. The virus was harvested after two cycles of freeze-thaw and store at -80 ℃ until use. The classical PRV Fa was purchased from the Institute of China Veterinary Medicine Inspection[15]. Permissive PK-15 cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 5% fetal bovine sera.

Experiment design and animals

To establish a PRV HN1201 infection model in pigs, in the first animal experiment, twenty 60-d old pigs, five 35-d old pigs, and five 127-d old pigs were used to evaluate the pathogenicity of the virus by different inoculating routes, virus loads, and ages of pigs. Twenty 60-d old pigs were randomly allocated into the first four groups

Table 1 Outcome of infections with pseudorabies virus HN1201 strain in pigs

Group	Pig age (d)	Pig No.	Virus titer	Route	Euthanized
1	60	5	10^7	im	3/5
2	60	5	10^7	in	5/5
3	60	5	10^6	in	2/5
4	60	5	10^5	in	1/5
5	35	5	10^7	in	5/5
6	127	5	10^7	in	5/5

im: Intramuscular; in: Intranasal.

(Table 1). Pigs in group 1 and group 2 were inoculated with 10^7 $TCID_{50}$ PRV HN1201 strain *via* intramuscular (im) and intranasal (in) routes, respectively. Pigs in group 3 and 4 were inoculated *via* the intranasal route with 10^6 $TCID_{50}$ and 10^5 $TCID_{50}$ of PRV HN1201 strain, respectively. To test the susceptibility of pigs to the virus at different ages, five 35-d old pigs in group 5 and five 127-d old pigs in group 6 were inoculated *via* the intranasal route with 10^7 $TCID_{50}$ PRV HN1201 (Table 1).

In the second animal study, ten 56-d old pigs were randomly divided into two groups with five pigs in each group. Pigs in group Ⅰ were inoculated with 10^7 $TCID_{50}$ PRV HN1201 *via* the intranasal route and group Ⅱ were inoculated with classical PRV Fa strain with the same dose and route.

All pigs used in the above two animal trials were free of PRV and excluded by using gB- and gE-ELISA Kits (HerdChek PRV, IDEXX, United States) and PCR method. All pigs were also free of porcine reproductive and respiratory syndrome virus, classical swine fever virus, and porcine circovirus 2. Experimental pigs in different groups were insulated in separate rooms throughout the study. After virus inoculation, rectal temperature and clinical signs were recorded on a daily basis. At 14 d post-inoculation (dpi), all surviving pigs were humanely euthanized and necropsied, and different organ samples were collected. The collected samples were subjected to pathological examination and gently inflated with 10% neutral-buffered formalin for immunohistochemistry examination. All animal trials in this study were approved by the Animal Care and Ethics Committee of the China National Research Center for Veterinary Medicine.

Histopathology and immunohistochemistry

Representative samples were cut from the fixed tissues and processed into paraffin blocks. Sections approximately 3-4 μm thick were cut into slides. Duplicates of the same sections were used for hematoxylin and eosin (H and E) staining and immunohistochemistry staining separately, as previously described[16]. The H and E staining was operated automatically by Leica fully automatic dyeing machine according to standard procedures. Immunohistochemistry staining was performed as below. The prepared paraffin sections were mounted on APES-treated slides and incubated overnight at 37 ℃. The slides were de-waxed *via* routine method by Leica automatic dyeing machine. The samples were blocked with 3% peroxide-methanol for 20 min at room temperature for endogenous peroxidase ablation and rinsed by phosphate buffer solution (PBS) twice. The following steps were carried out in a moisture chamber: (1) Samples were incubated with blocking buffer containing normal horse serum (Beijing Zhongshan Jinqiao, China) with 1:20 dilution with PBS at 37 ℃ for 20 min; (2) The horse serum was discarded and samples were incubated in PRV monoclonal antibody 3B5 solution (Beijing Tian Tech Biotechnology, China) with 1:800 dilution in PBS (pH 7.3) at 37 ℃ for half an hour and then 4 ℃ overnight; (3) After rinsing with PBS three times, HRP goat anti-mouse IgG (BTI, United States) with 1:100 dilution in PBS (pH 7.3) was added, and the slides were incubated for 1 h at 37 ℃; (4) After rinsing with PBS three times, the slides were incubated with AEC and kept at room temperature without light for 5-10 min; (5) After rinsing with PBS three times, the slides were stained with hematoxylin (freshly prepared) 1:10 dilution for 10 s; (6) The unbound hematoxylin was washed away by running water, and the slides were placed into water for 2 min; and (7) The slides were allowed to dry naturally and then mounted with water-soluble tablet seal before visualization by 200 × microscope photographs. The results were determined by negative (-) and positive (+), with positive signals interpreted as low (+), moderate (++), and intense (+++), according to the intensity of staining.

Animal care and use

The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to laboratory conditions (23 ℃, 12 h light/12 h dark, 50% humidity, and *ad libitum* access to food and water) for two weeks prior to experimentation. All animals were euthanized by barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) for tissue collection. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the National Research Center for Veterinary Medicine (IACUC protocol number: 2015010402).

Statistical analysis

Differences of body temperature and body weight between two infected groups in the second animal trial were determined by using *t*-test in GraphPad Prism 5.0 Software (San Diego, CA). Differences were considered statistically significant when $P < 0.05$.

RESULTS

Experimental infection of PRV HN1201

For routes of infection, all pigs in group 1 and group 2 inoculated with 10^7 $TCID_{50}$ PRV HN1201 strain *via* intramuscular and intranasal routes, respectively, showed PRV-specific clinical symptoms such as fever (40.0 ℃-41.5 ℃), respiratory distress, excessive

Table 2 Clinical manifestations of pseudorabies virus HN1201 and Fa infection

Groups	Respiratory symptom					Neurological symptom			
	Vomit	Respiratory distress	Cough	Sneeze	Salivation	Circling	Posterior paralysis	Muscle tremors	Lay recumbent and paddle
	+	+	-	+	-	+	-	+	-
Ⅰ (HN1201)	-	+	+	+	-	+	-	+	-
	+	+	+	+	-	-	+	+	+
	-	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+
	-	-	-	+	-	-	-	-	-
Ⅱ (Fa strain)	-	-	-	+	-	-	-	-	-
	-	-	-	+	-	-	-	-	-
	-	-	-	+	-	-	-	-	-
	+	+	-	+	+	+	-	+	+

Each row represents one pig in the corresponding group.

salvation, and neurological signs including convulsion and ataxia. All pigs in group 2 were euthanized due to moribund conditions from 5 to 7 dpi. Compared with group 2, three pigs in group 1 were euthanized from 5 to 7 dpi and the other two pigs survived until the end of the study (terminated at 14 dpi, Table 1).

All pigs in group 3 ($10^{6.0}$ $TCID_{50}$) showed severe respiratory symptoms and neurological signs, as described above, with two being euthanized 6 dpi. Compared to pigs in group 3, respiratory symptoms such as coughing and shivering were more often observed in group 4 ($10^{5.0}$ $TCID_{50}$). There was one pig out of the five in group 4 that showed neurological signs, and was euthanized by the end of study (terminated at 14 dpi).

Young piglets are more susceptible to PRV infection than elder pigs[6]. To determine the pathogenicity of PRV HN1201 in pigs of different ages, 35, 60, and 127-d old pigs were inoculated with 10^7 $TCID_{50}$ of virus. After virus inoculation, pigs of different ages showed the clinical symptoms as described above. All pigs in group 5 (35-d old pigs) were euthanized from day 4 to day 6 and all pigs in group 6 (127-d old pigs) were euthanized from day 5 to day 8 due to the moribund conditions. Therefore, unlike the classical PRV strains, this PRV variant strain showed high pathogenicity in pigs of different ages.

Comparison of pathogenicity between PRV variant HN1201 and classical Fa strain

Since the above results showed PRV variant HN1201 has high pathogenicity in pigs of different ages, a classical PRV Fa strain was used to compare pathogenicity. To exclude the bias of pathogenicity of two PRV strains due to the age of experimental pigs, ten 56-d-old healthy pigs were randomly assigned to two groups, with five pigs in each group. Pigs in groups Ⅰ and Ⅱ were inoculated with PRV HN1201 and Fa strain, respectively, *via* intranasal method at 10^7 $TCID_{50}$.

As expected, all pigs in group Ⅰ displayed high fever, anorexia, depression, respiratory symptoms, and neurological signs as described in the first animal study. In contrast, four pigs in group Ⅱ had no respiratory or neurological symptoms, aside from sneezing (Table 2), while only one pig showed the same clinical signs as pigs in group Ⅰ. Gross pathology examination at necropsy showed that PRV HN1201 infection led to severe pulmonary consolidation and necrosis in the lung (Figure 1A), encephalic hemorrhage in the brain (Figure 1B), and hemorrhage and necrosis in the tonsil (Figure 1C). By contrast, pigs infected with PRV Fa showed only slight hemorrhage in the lung tissue (Figure 1D) and had no obvious changes in the brain or tonsil (Figure 1E and F). No other obvious pathologic change was found after two virus infection in heart, liver, spleen, and kidney tissues. There was no significant difference in rectal temperature between the two groups in the first 5 d of study (Figure 2A). Pigs in group Ⅰ had significant body weight losses compared to pigs in group Ⅱ at 6 dpi (Figure 2B). At 5 dpi, two pigs were euthanized in group Ⅰ and one pig was euthanized in group Ⅱ. At 6 dpi, another three pigs were euthanized in group Ⅰ and all remaining pigs in group Ⅱ survived to the end of the study.

Organ samples of pig tonsil, lung, cerebellum, lymph nodes, kidney, and liver were collected for histological examination and immunohistochemistry staining. Typical PRV infection is characterized by necrosis in multiple organs. As shown in Figure 3, necrosis, congestion, or hemorrhage in all above organs of PRV HN1201-infected pigs were observed after H&E staining (Figures 3A-G), with neuronal intra-nuclear inclusions also being observed in the brain. Compared to the HN1201 infection, PRV Fa-infected pigs only showed neuronal degeneration, necrosis in the brain, Purkinje cell degeneration, and necrosis in the cerebellum (Figure 3H and Ⅰ). In accordance with histopathology results, immunohistochemistry staining showed significant strong positive signals in all of the above organs obtained from pigs infected with HN1201 virus, whereas only brain and cerebellum samples of one RPV Fa-infected pig revealed positive results (Table 3).

DISCUSSION

Since late 2011, outbreaks of PR-like diseases have occurred on numerous Bartha-K61-vaccinated pig farms and gradually spread in China, causing huge economic

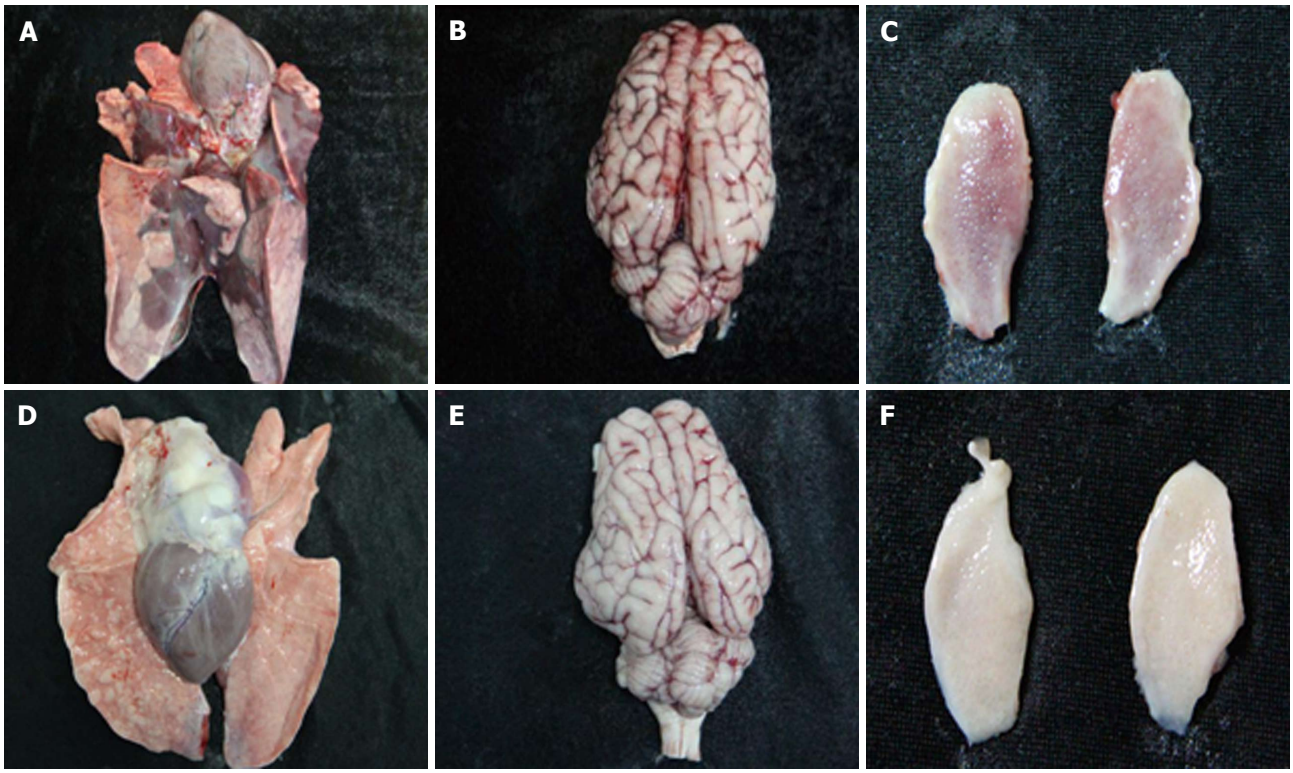

Figure 1 Gross pathology examination at necropsy. Lung, brain, and tonsil samples after PRV HN1201 (A-C) or Fa strain infection (D-F). PRV: Pseudorabies virus.

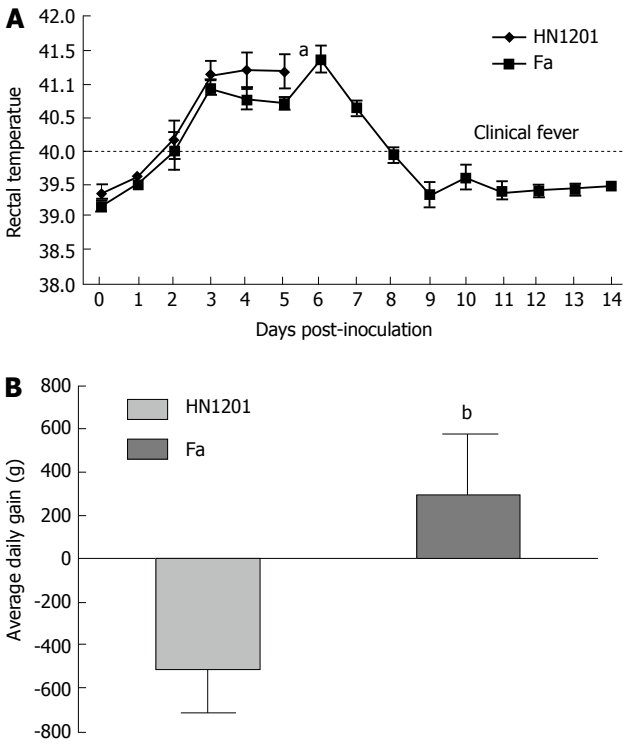

Figure 2 Rectal temperature (A) and average daily (B) body weight gain (6 d post-inoculation) of pigs after pseudorabies virus HN1201 or Fa infection. $^{a}P < 0.05$, $^{b}P < 0.001$.

losses to the Chinese swine industry[10,13]. Recent studies have shown that PRV variants contributed to the recent outbreaks of PR, and the traditional Bartha-K61 vaccine could not provide complete protection against the emerging PRV strains[11,14]. Similar to classical PR, the disease is characterized by the sudden death of new born piglets, respiratory and neurological symptoms in growing pigs, and stillbirth or the birth of weak piglets from sows. However, the pathogenicity of the new emerging PRV variant was never delineated and compared with classical PRV strains. Therefore, it is necessary to determine the pathogenicity of the current PRV variants before any control measures are implemented to control the disease.

PRV is tropic for both the respiratory and nervous systems of swine. Viral particles enter sensory nerve endings, thereby innervating the infected mucosal epithelium. Morbidity and mortality associated with PRV infection varies with host age, the animal's overall health status, and infectious dose[2]. In this study, we first tested the pathogenicity of PRV variant HN1201 by different routes of virus infection, virus loads for inoculation, and pig ages. Our results showed that intranasal infection is more effective than intramuscular infection when 10^7 $TCID_{50}$ viruses were used for inoculation. Pigs infected with PRV 1201 by the intranasal route showed more severe clinical symptoms and higher mortality rates than those with intramuscular routes, and virus loads were positively correlated with mortality rates. The pathogenicity of some other PRV variant strains have been studied recently[17]. In a study by Luo *et al*[17] (2014), pigs infected with the 10^6 $TCID_{50}$ PRV TJ strain by the intranasal route showed higher mortality than those with a lower dose or were infected by the

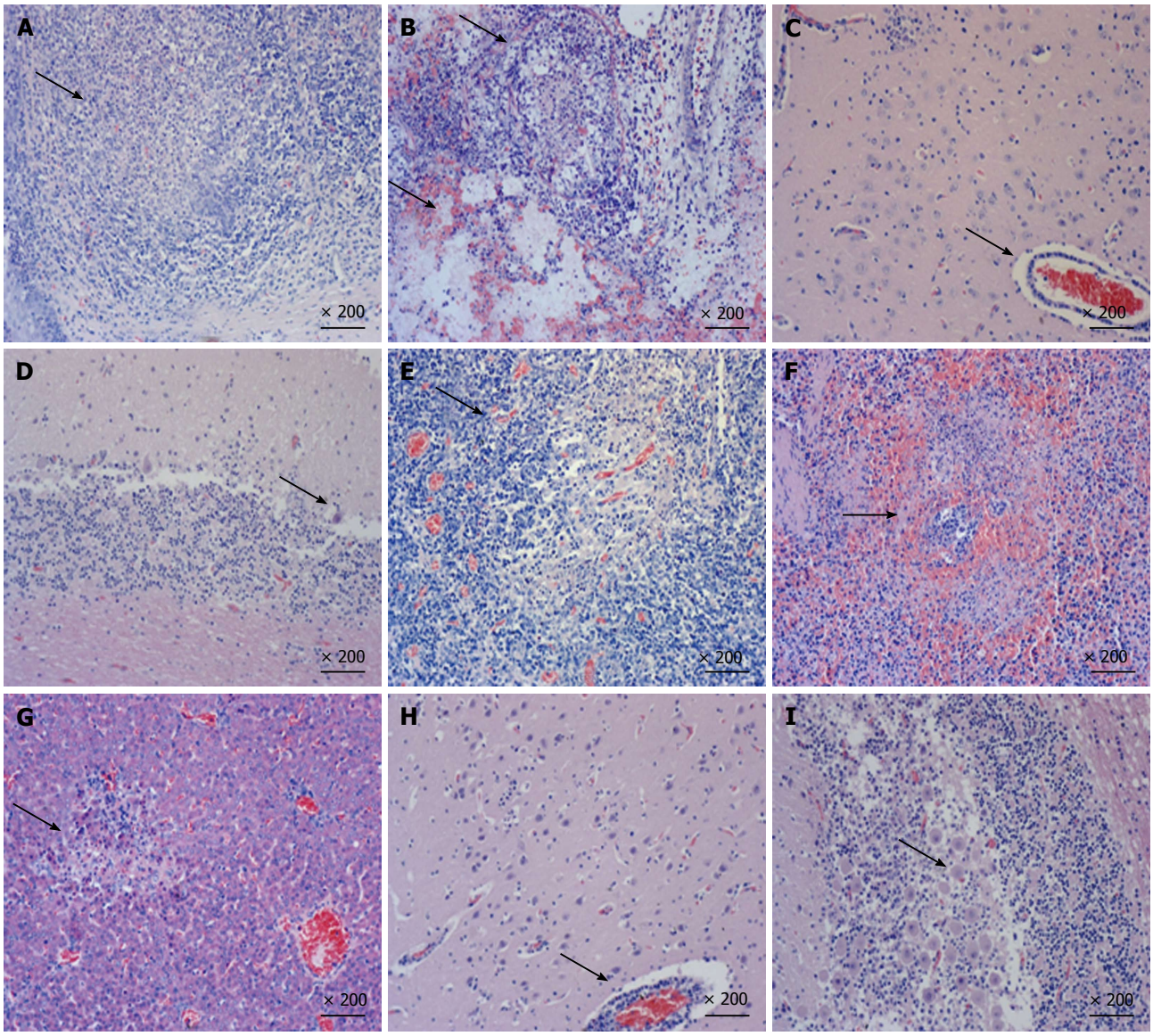

Figure 3 Hematoxylin and eosin staining of multiple tissues of pigs inoculated with HN1201 (A-G) and Fa strain (H and I). A: Tonsil - tonsillar lymphoid tissue necrosis and formation of large necrotic foci; B: Lung - vascular congestion and hemorrhage (lower arrow), with bronchial epithelial necrosis and necrotic cells within the lumen (upper arrow); C: Brain - lymphocyte infiltration around the small blood vessels in the brain cortex, non-suppurative encephalitis; D: Cerebellum - Purkinje cell degeneration and necrosis; E: Hilar lymph nodes - vascular dilatation and congestion, and lymphatic tissue necrosis; F: Spleen - white pulp structure disappeared and white necrotic marrow lymphocytes formed large necrotic foci; G: Liver - necrotic foci formation; H: Brain - coalescing non-suppurative encephalitis with neuronal degeneration and perivascular cuffing; I: Cerebellum - Purkinje cell degeneration and necrosis.

intramuscular route, which is consistent with our results. Bama miniature pigs injected intramuscularly with $10^{7.0}$ $TCID_{50}$ PRV strain HeN1, another virulent PRV variant, the animals exhibited only transient fever for 3-5 d, and no other clinical symptoms or postmortem changes were observed[10]. Differences in pathogenicity and mortality caused by different PRV viruses could be explained by the virus load for inoculation, viral strain, and breed of pigs, although these three viruses also share a more than 99.0% similarity in their whole genome sequences. Besides routes of inoculation and virus load, PRV HN1201 could infect pigs from 35 to 127 d old with PRV-specific clinical symptoms, indicating that the PRV HN1201 strain is highly pathogenic to pigs.

To compare the pathogenicity of newly-emerging PRV variants with the classical PRV strain, PRV HN1201 and Fa strains were used to infect pigs. Our results showed that HN1201-infected pigs showed more severe clinical signs and higher mortality rates than Fa-infected pigs (5/5 *vs* 1/5). Pigs in the PRV HN1201-infected group displayed high fever, anorexia, depression, respiratory symptoms, and neurological signs. In comparison, four pigs in the PRV Fa-infected group had no respiratory or neurological symptoms, aside from sneezing. Meanwhile, pigs infected with HN1201 had steady body weight loss as compared with pigs infected with the Fa strain (Figure 2B). Retarded growth was more often observed in young piglets after PRV infection. However, the loss of body weight of 56-d old pigs after PRV infection was seldom observed, which proves the high virulence of

Table 3 Virus antigen distribution and intensity in different organs of pseudorabies virus HN1201 or Fa strain by immunohistochemistry staining

Groups	Tonsil	Lung	Lymph nodes			Brain	Cerebellum	Spleen	Liver
			Mandibular	Superficial inguinal	Mesenteric				
HN1201	3+	3+	3+	2+	2+	2+	1+	2+	2+
	3+	2+	2+	2+	2+	2+	1+	3+	-
	3+	3+	3+	2+	2+	1+	2+	3+	+
	3+	3+	++	3+	2+	1+	2+	2+	2+
	3+	3+	3+	2+	3+	2+	2+	3+	3+
Fa strain	-	+	-	-	-	-	-	-	-
	-	-	-	-	-	2+	2+	-	-
	-	-	-	-	-	2+	2+	-	-
	-	-	-	-	-	2+	2+	-	-
	-	-	-	-	-	2+	2+	-	-

The positive staining signals were interpreted as negative (-), low (1+), moderate (2+), or intense (3+), according to the intensity of staining. Each row represents one pig in the corresponding group.

PRV HN1201.

Gross pathological examination at necropsy revealed more severe damage to the lung, tonsil, brain, cerebellum, and lymph nodes in pigs infected with HN1201 strain than in the Fa strain group. In line with pathological results, histopathology examination showed remarkably obvious necrosis in multiple tissues, such as the tonsil, lung, brain, spleen, and liver in HN1201-infected pigs; in contrast, necrosis caused by PRV Fa infection was only limited to the brain and cerebellum. Immunochemistry results also showed that PRV HN1201 infection lead to more extensive virus antigen distribution in different organs with more intense staining, while Fa infection only had one cerebellum sample from one pig that showed positive. Previous studies reported that inoculation of PRV through the nasal cavity resulted in virally-induced neuropathological lesions[2]. The kinetics and locations of lesion appearance were consistent with a transneuronal spread of PRV from the nasal epithelium to synaptically-connected higher-order structures in the nervous system. The intense PRV antigen location and severe lesions of the brain, tonsil, and lung coincided with the typical respiratory and neurological symptoms, and may be due to intranasal infection. Therefore, the above results further suggest the higher pathogenicity of PRV HN1201 when compared to the classical Fa strain.

In conclusion, PRV HN1201 infection is more effective through the intranasal route than the intramuscular inoculation route, and the virus is highly pathogenic to different ages of pig. Compared with classical PRV Fa strain, HN1201 causes more severe clinical symptoms and pathological lesions, with extensive antigen distribution in different organs.

COMMENTS

Background

Highly virulent pseudorabies virus (PRV) variants are circulating in most Chinese pig farms, causing huge economic losses. The pathogenicity of these PRV variants have not been previously compared with classical PRV strains.

Research frontiers

The authors aimed to test the pathogenicity of a newly-emerging PRV variant in pigs of different inoculation routes, virus loads, and ages. Differences in pathogenicity between the newly-emerging PRV variant and the classical PRV strain were also compared.

Innovations and breakthroughs

This study demonstrates that the currently-circulating PRV HN1201 variant has higher pathogenicity in pigs than the classical PRV Fa strain *via* the manifestation of more severe clinical symptoms and pathological lesions, with extensive antigen distribution in different organs.

Applications

The authors proved the PRV variant to be more pathogenic in pigs as compared to the classical Fa strain, which may partially explain the inefficacy of current commercial PRV vaccines. Thus, a better understanding of the differences of pathogenicity between variant and classical PRV may facilitate the development of more effective vaccines.

Terminology

Pathogenicity of pseudorabies virus is the potential capacity of PRV to cause PR-like syndrome in pigs. Pathogenicity of viruses may change due to virus mutation and/or recombination. Study into the pathogenesis of currently-circulating field viruses may provide first-hand data for disease control.

Peer-review

This manuscript reports the analysis of the pathogenicity of a new PRV variant that the commonly-used vaccine cannot protect against, and is therefore causing massive economic losses in China. The pathogenicity of this variant and the classical PRV Fa stain is also compared. The experiment design and results were clear and convincing. It will be interesting to see if the authors can further explore the mechanisms of the enhanced pathogenicity of the PRV variant behind these phenomena.

REFERENCES

1 **Mettenleiter TC**. Pseudorabies (Aujeszky's disease) virus: state of the art. August 1993. *Acta Vet Hung* 1994; **42**: 153-177 [PMID: 7810409]

2 **Pomeranz LE**, Reynolds AE, Hengartner CJ. Molecular biology of pseudorabies virus: impact on neurovirology and veterinary medicine. *Microbiol Mol Biol Rev* 2005; **69**: 462-500 [PMID: 16148307 DOI: 10.1128/MMBR.69.3.462-500.2005]

3 **Roizman B**, Baines J. The diversity and unity of Herpesviridae. *Comp Immunol Microbiol Infect Dis* 1991; **14**: 63-79 [PMID: 1935001 DOI: 10.1016/0147-9571(91)90122-T]

4 **Klupp BG**, Hengartner CJ, Mettenleiter TC, Enquist LW. Complete, annotated sequence of the pseudorabies virus genome. *J Virol* 2004; **78**: 424-440 [PMID: 14671123 DOI: 10.1128/JVI.78.1.424-440.2004]

5 **Szpara ML**, Tafuri YR, Parsons L, Shamim SR, Verstrepen KJ, Legendre M, Enquist LW. A wide extent of inter-strain diversity in virulent and vaccine strains of alphaherpesviruses. *PLoS Pathog* 2011; **7**: e1002282 [PMID: 22022263 DOI: 10.1371/journal.ppat.1002282]

6 **Mulder WA**, Pol JM, Gruys E, Jacobs L, De Jong MC, Peeters BP, Kimman TG. Pseudorabies virus infections in pigs. Role of viral proteins in virulence, pathogenesis and transmission. *Vet Res* 1997; **28**: 1-17 [PMID: 9172836]

7 **Müller T**, Hahn EC, Tottewitz F, Kramer M, Klupp BG, Mettenleiter TC, Freuling C. Pseudorabies virus in wild swine: a global perspective. *Arch Virol* 2011; **156**: 1691-1705 [PMID: 21837416 DOI: 10.1007/s00705-011-1080-2]

8 **Rziha HJ**, Mettenleiter TC, Ohlinger V, Wittmann G. Herpesvirus (pseudorabies virus) latency in swine: occurrence and physical state of viral DNA in neural tissues. *Virology* 1986; **155**: 600-613 [PMID: 3024403 DOI: 10.1016/0042-6822(86)90220-5]

9 **Wang TY,** Xiao Y, Yang QY, Wang YZ, Sun Z, Zhang CL, Yan SJ, Wang J, Guo LH, Yan H, Gao ZY, Wang LL, Li XD, Tan FF, Tian KG. Construction of a gE-deleted pseudorabies virus and its efficacy to the new-emerging variant PRV challenge in the form of killed vaccine. *Biomed Int Res* 2015; In press [DOI: 10.1155/2015/684945]

10 **An TQ**, Peng JM, Tian ZJ, Zhao HY, Li N, Liu YM, Chen JZ, Leng CL, Sun Y, Chang D, Tong GZ. Pseudorabies virus variant in Bartha-K61-vaccinated pigs, China, 2012. *Emerg Infect Dis* 2013; **19**: 1749-1755 [PMID: 24188614 DOI: 10.3201/eid1911.130177]

11 **Gu Z**, Dong J, Wang J, Hou C, Sun H, Yang W, Bai J, Jiang P. A novel inactivated gE/gI deleted pseudorabies virus (PRV) vaccine completely protects pigs from an emerged variant PRV challenge. *Virus Res* 2015; **195**: 57-63 [PMID: 25240533]

12 **Wu R**, Bai C, Sun J, Chang S, Zhang X. Emergence of virulent pseudorabies virus infection in northern China. *J Vet Sci* 2013; **14**: 363-365 [PMID: 23820207 DOI: 10.4142/jvs.2013.14.3.363]

13 **Yu X**, Zhou Z, Hu D, Zhang Q, Han T, Li X, Gu X, Yuan L, Zhang S, Wang B, Qu P, Liu J, Zhai X, Tian K. Pathogenic pseudorabies virus, China, 2012. *Emerg Infect Dis* 2014; **20**: 102-104 [PMID: 24377462 DOI: 10.3201/eid2001.130531]

14 **Wang CH**, Yuan J, Qin HY, Luo Y, Cong X, Li Y, Chen J, Li S, Sun Y, Qiu HJ. A novel gE-deleted pseudorabies virus (PRV) provides rapid and complete protection from lethal challenge with the PRV variant emerging in Bartha-K61-vaccinated swine population in China. *Vaccine* 2014; **32**: 3379-3385 [PMID: 24793946 DOI: 10.1016/j.vaccine.2014.04.035]

15 **Zhu L**, Yi Y, Xu Z, Cheng L, Tang S, Guo W. Growth, physicochemical properties, and morphogenesis of Chinese wild-type PRV Fa and its gene-deleted mutant strain PRV SA215. *Virol J* 2011; **8**: 272 [PMID: 21639925 DOI: 10.1186/1743-422X-8-272]

16 **Zhang C**, Guo L, Jia X, Wang T, Wang J, Sun Z, Wang L, Li X, Tan F, Tian K. Construction of a triple gene-deleted Chinese Pseudorabies virus variant and its efficacy study as a vaccine candidate on suckling piglets. *Vaccine* 2015; **33**: 2432-2437 [PMID: 25865469 DOI: 10.1016/j.vaccine.2015.03.094]

17 **Luo Y**, Li N, Cong X, Wang CH, Du M, Li L, Zhao B, Yuan J, Liu DD, Li S, Li Y, Sun Y, Qiu HJ. Pathogenicity and genomic characterization of a pseudorabies virus variant isolated from Bartha-K61-vaccinated swine population in China. *Vet Microbiol* 2014; **174**: 107-115 [PMID: 25293398 DOI: 10.1016/j.vetmic.2014.09.003]

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ORIGINAL ARTICLE

Observational Study

Neuropathology of JC virus infection in progressive multifocal leukoencephalopathy in remission

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Author contributions: SantaCruz KS is a neuropathologist who reviewed all histopathology and determined the diagnosis, captured the histological images and helped to write the MS; Roy G was a resident in training who reviewed the medical chart and drafted and edited the MS; Spigel J performed the autopsy, contributed and edited clinical data and reviewed the MS; Bearer EL is an experimental neuropathologist who reviewed the pathology, edited the MS, added the references, selected the images, prepared the figures and replied to reviewers' comments.

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Abstract

AIM: To investigate the neuropathology of the brain in a rare case of remission following diagnosis of progressive multifocal leukoencephalopathy (PML).

METHODS: Consent from the family for an autopsy was obtained, clinical records and radiograms were retrieved. A complete autopsy was performed, with brain examination after fixation and coronal sectioning at 1 cm intervals. Fourteen regions were collected for paraffin embedding and staining for microscopic analysis. Histologic sections were stained with Luxol blue, hematoxylin/eosin, and immunostained for myelin basic protein, neurofilament, SV40 T antigen and p53. The biopsy material was also retrieved and sections were stained with hematoxylin/eosin and immunostained for SV40 and p53. Sections were examined by American Board of Pathology certified pathologists and images captured digitally.

RESULTS: Review of the clinical records was notable for

a history of ulcerative colitis resulting in total colectomy in 1977 and a liver transplant in 1998 followed by immune-suppressive therapy. Neurological symptoms presented immediately, therefore a biopsy was obtained which was diagnosed as PML. Immunotherapy was adjusted and clinical improvement was noted. No subsequent progression was reported. Review of the biopsy demonstrated atypical astrocytes and enlarged hyperchromatic oligodendroglial cells consistent with JC virus infection. Strong SV40 and p53 staining was found in glial cells and regions of dense macrophage infiltration were present. On gross examination of the post-mortem brain, a lesion in the same site as the original biopsy in the cerebellum was identified but no other lesions in the brain were found. Microscopic analysis of this cerebellar lesion revealed a loss of myelin and axons, and evidence of axonal damage. This single burned-out lesion was equivocally positive for SV40 antigen with little p53 staining. Examination of thirteen other brain regions found no other occult sites.

CONCLUSION: Our study reveals residual damage, rare macrophages or other inflammation and minimal evidence of persistent virus. This case demonstrates the possibility of complete remission of PML.

Key words: Progressive multifocal leukoencephalopathy; Progressive multifocal leukoencephalopathy; JC virus; Remission; Demyelinating

Core tip: Progressive multifocal leukoencephalopathy after organ transplant is rapidly fatal in most cases, with an average time to death of 6.4 mo. We report a case with no clinical progression over 14 years despite ongoing immunosuppressive therapy. At initial diagnosis the biopsy demonstrated classic histopathological features of JC virus. At autopsy, microscopic analysis of the cerebellar lesion revealed a residual loss of myelin and evidence of axonal damage without evidence of viral activity. These results suggest that JC virus can be kept in check even in a setting of immunosuppression, and argue for more investigation into the microbiome of the brain.

SantaCruz KS, Roy G, Spigel J, Bearer EL. Neuropathology of JC virus infection in progressive multifocal leukoencephalopathy in remission. *World J Virol* 2016; 5(1): 31-37 Available from: URL: http://www.wjgnet.com/2220-3249/full/v5/i1/31.htm DOI: http://dx.doi.org/10.5501/wjv.v5.i1.31

INTRODUCTION

Progressive multifocal leukoencephalopathy (PML) is a demyelinating disease of the central nervous system caused by reactivation of latent JC virus in immunocompromised individuals. Oligodendroglial cells are preferentially infected with consequent loss of myelin and coalescing demyelination plaques, sometimes leading to mild-to-moderate axonal loss associated with axonal spheroids. Long-term survival in patients with PML is increasingly common in human immunodeficiency virus (HIV)-infected people treated with highly active antiretroviral therapy (HAART)[1-8]. In contrast, prolonged survival in patients with immunosuppression following solid organ transplant is unusual[9,10]. Here we present an unusual case in which there was a 14-year clinical remission after biopsy-proven PML, despite continued immuno-suppression after liver transplant.

PML had been universally fatal, usually within 6 mo, until the late 1990's. Although no specific treatment has proven particularly effective, enhancing natural immunity by reducing the effects of HIV virus or by altering immunosuppressant therapy has been shown to improve survival[11]. One explanation for prolonged survival is improved cellular immune responses against JC virus (JCV) in long term survivors *vs* those with poor outcomes[12]. Detectable cytotoxic T lymphocytes specific for JCV -T or VP-1 have been shown to be a prognostic indicator of long-term survival in HIV patients[12]. Although long-term survival in immunosuppressed transplant patients has been described[13,14] these cases are unusual[9] and detailed neuropathologic descriptions of residual demyelinating plaques in patients in complete remission are few, possibly because they are very rare or not frequently examined post-mortem[15]. This study therefore fills an important gap in our knowledge of pathological processes that appear in long-term survival.

MATERIALS AND METHODS

Consent for the autopsy from the family was obtained as approved by Presbyterian Hospital. According to Internal Review Board of University of New Mexico Health Sciences Center neither post-mortem material nor case reports require IRB approvals. Clinical records were retrieved, and the clinical history together with results of all brain imaging studies that had been performed at UNM (12/2006 and 4/2008) reviewed. The original imaging studies were not available, and, in the absence of neurological symptomatology, imaging and CSF sampling were not performed during the final hospitalization, nor was post-mortem brain imaging done.

A complete autopsy was performed with subsequent examination of the brain after fixation. Gross examination of the brain included coronal sectioning of the neocortex at 1 cm intervals, and sectioning of the cerebellum and brainstem at 0.5 cm intervals. The surface of each slice was examined. Thirteen brain regions were selected, slabs 1.5 cm × 1.5 cm × 0.1 cm dissected and these were submitted for paraffin embedding. Histologic sections were stained with Luxol blue, hematoxylin/eosin, and for myelin basic protein (Dako, polyclonal rabbit anti-human), neurofilament (Dako, Clone 2F11), SV40 T antigen (Calbiochem, Ab-2, PAb 416) or p53 (Dako,

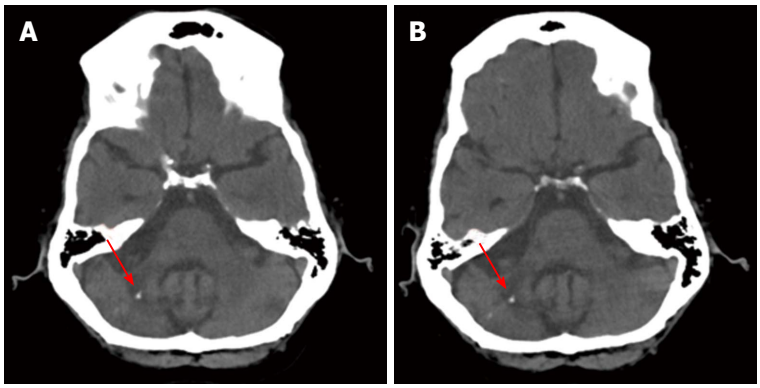

Figure 1 Computed tomography scans show no progression. A: Axial slice from the CT scan of 2006 showing the cerebellar lesion (red arrow), a small calcification, and atherosclerosis of cerebral vasculature; B: Axial slice of the same coordinates as in (A) from the CT scan of 2008 showing the cerebellar lesion (red arrow), as well as the calcification. Note the radiolucency of the cerebellar peduncle and the loss of tissue density in the cerebellum. This location corresponds to that of the 1999 biopsy and the histologic sections examined at autopsy. CT: Computed tomography.

Clone DO7) by immunohistochemistry at TriCore Reference Laboratories, Albuquerque, NM. The SV40 T-antigen (Ab-2) antibody is a mouse monoclonal antibody with specific determinants unique to the SV40 large T antigen and non-reactive with the small T antigen. The antigenic epitope is between Ile83 and Lys128 of the SV40 large T antigen, a region highly homologous to the JC virus large T antigen. For each immunostain, positive and negative controls were run in parallel. The paraffin block containing the biopsy was retrieved together with its slides from archives, new sections were made and also stained for SV40 and p53. Sections were examined on an Olympus BX40 microscope using 4 ×, 10 ×, 20 × and 40 × objectives, and digital images captured on an Olympus DP26 camera using cellSens Standard software. Images were prepared for figures using Adobe Photoshop to resize, create multi-image panels, adjust levels and add lettering.

RESULTS

Case history

A 76-year-old woman with a history of ulcerative colitis had a total colectomy in 1977 and subsequently developed sclerosing cholangitis. She received an orthotopic liver transplant in 1998. In 1999 neurological symptoms occurred, primarily consisting of ataxia in a setting of immunosuppressive therapy for the liver transplant. She was found to have a white matter lesion involving cerebellar white matter with no other sites of involvement. Brain biopsy performed in March 1999 showed classic changes of PML. The dosage of immunosuppressive therapy with Tacrolimus and Sirolimus was subsequently adjusted to minimize progression of further neurological disease and her mild cerebellar symptoms stabilized.

Her medical history was also significant for right hip fracture in 2003, status post hip replacement complicated by infection and requiring long term antibiotic therapy, chronic renal insufficiency, due to congenital hypoplastic kidney, end stage renal disease on dialysis since 2008, cardiovascular disease with episodes of atrial fibrillation and rapid ventricular response, hypothyroidism, gout and recurrent infections.

She had multiple hospital admissions from February 2012 to March 2013 due to gastroenteritis with subsequent workup for stool pathogens that was negative. She developed pancreatic insufficiency with findings on ultrasound examination that showed an atrophic right kidney, small liver and pancreatic cysts. Due to recurrence of the gastrointestinal illness, and to ultrasound findings, there was concern for an intraductal papillary mucinous neoplasm of the pancreas with associated pancreatic insufficiency. Computed tomography scan of the abdomen showed diffuse dilatation of the pancreatic duct, as well as a liver abscess. She died six days following abdominal imaging studies on March 25, 2013.

Mortality was due to complications related to remote liver transplantation for primary sclerosing cholangitis. An intraductal papillary mucinous neoplasm of the pancreatic duct was identified at autopsy with associated chronic atrophic pancreatitis. Post mortem examination determined the immediate cause of death to be due to infection from the liver abscess, cardiac arrhythmia and cardiomegaly.

Neurologic and Radiographic studies: The patient was seen by a neurologist at UNM on 3/2003, 2/2005, 12/2006, 6/2007, 4/2008, 7/2009, 4/2010 and 9/2011. During this period symptoms were stable on the reduced immunosuppressant protocol.

Imaging from 4/4/2008 was read as unchanged compared to the computed tomography (CT) done 12/12/2006 (Figure 1). Both images show diffuse cerebral as well as cerebellar atrophy. A region of greater volume loss and accompanying low attenuation appeared in the right cerebellar hemisphere and middle cerebellar peduncle. Small foci of calcification were noted. These were not significantly changed between the 2006 and 2008 images. No new areas of abnormal attenuation were identified within the brain. Vertebral and internal carotid artery calcifications were also noted.

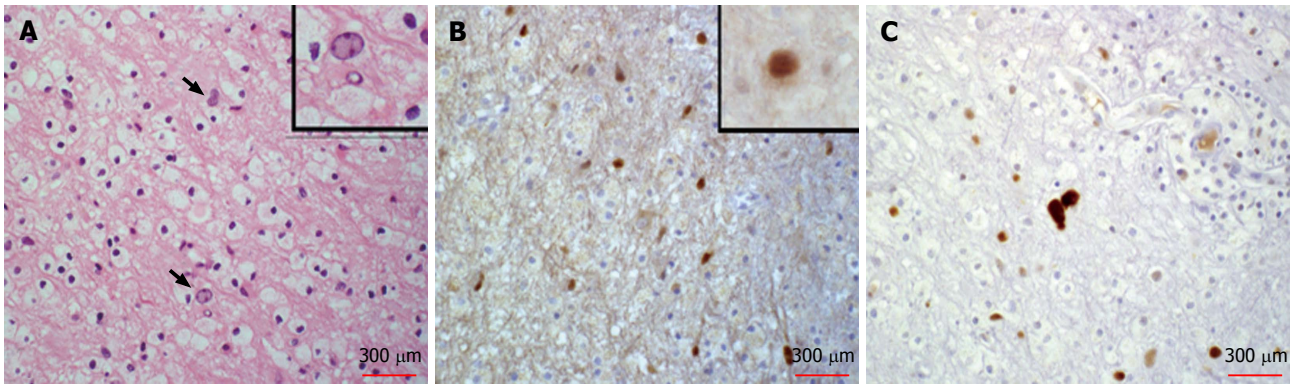

Figure 2 Histopathology of the original diagnostic biopsy. A: Histopathology of the cerebellar biopsy specimen from 1999 stained with hematoxilin and eosin. Atypical glia is indicated by arrows. Inset shows higher magnification of one example. Numerous macrophages surround the atypical cells and fill the parenchyma; B: Immunohistochemical staining for SV40 T antigen of another section from the biopsy shows numerous scattered strongly positive brown stain in nuclei of atypical glial cells, consistent with oligodendroglial infection by JC virus. Inset shows a higher magnification of another example of a positive glial cell; C: Immunohistochemistry for p53 detected strongly positive atypical glia and scattered weaker staining of macrophages consistent with inflammatory reaction to viral infection (A-C).

Pathological examination

Histopathologic review of the hematoxilin and eosin stained slides from the cerebellar stereotactic biopsy specimen of 1999 showed the classic features of PML, including demyelination with abundant macrophages, no lymphocytes or granulocytes and relative preservation of axons. Occasional enlarged oligodendroglial cells with dense chromatin and atypical astrocytes were also present (Figure 2). To detect virus, SV40 T antigen immunostaining was used. JC virus is a papovavirus in the polyoma family. SV40 monoclonal antibody was raised against a short peptide from the large T antigen of Simian Virus 40, another member of this virus family. This antibody also recognizes the large T antigen from both JC and BK viruses. It does not recognize small T antigens. SV40 immunostaining highlighted nuclei of infected oligodendroglial cells and occasional bland-appearing astrocytes, but did not highlight atypical astrocytes in the biopsy. Staining for p53 was performed to detect secondary viral effects on glia as support for the diagnosis[16]. The p53 antibody stained the nuclei of atypical glial cells. Thus these atypical astrocytes were likely reactive rather than infected. This review of the biopsy confirmed the previous diagnosis of PML.

At autopsy, gross examination of the brain surface and of coronal sections at 1 cm intervals revealed no ventricular enlargement, and no periventricular, or other white matter abnormalities. The brain was thoroughly examined by coronal sectioning from forebrain to brain stem and no areas of softening or discoloration were found. PML frequently extends initially to periventricular regions, yet no cerebral lesions were found in this case. Sagittal sectioning of the cerebellum revealed a 1.0 cm × 0.8 cm × 0.5 cm focus of tissue softening just lateral to the vermis on the left, in the region of the original biopsy. This is the area of the lesion identified in the 2006 and 2008 CT brain scans. This area of softening included cerebellar white matter and the dentate nucleus.

On histologic examination of the post-mortem brain sections from the original lesion in the cerebellar white matter were remarkable for white matter rarefaction, as evidenced by loss of myelin that was nearly proportional to axonal loss (Figure 3). Thus repair of the damage had not occurred. However, continuing damage was not detected. The heavy macrophage infiltration observed in the 1999 biopsy was absent, and macrophages were not apparent. Classical features of PML, such as enlarged oligodendroglial cells, were also mainly absent, atypical astrocytes were rare and only weak nuclear SV40 or p53 immunostaining was noted (Figure 3). We considered weak staining of the cytoplasm of glial cells to be non-specific since this low level of background staining was present in normal tissue within the section and also present in the negative control where no virus was present. A few rare cells displayed slightly more intense staining, which are shown in insets (Figure 3). Due to these rare cells, we cannot rule out residual viral antigens or continued low level of expression from latent virus in this burnt-out lesion.

There was minimal involvement of overlying cerebellar folia and minimal depletion of cerebellar granular cells, as determined by lack of detectable pathologic processes (Figure 3). Thirteen additional histologic sections from throughout the brain were dissected and processed according to the standard neuropathological brain examination procedure. Random sections of periventricular white matter, adjacent to the lateral ventricles and the aqueduct, where infection is most likely to spread, revealed no diagnostically significant abnormality. Sections from the pons were also stained for SV40 and p53 and no staining was detected.

Remarkably, fourteen years after diagnosis the lesion appeared confined to a single focus in the cerebellum, as in the original presentation. No additional foci throughout the brain, which are normally common in this multifocal disease, were found.

DISCUSSION

PML is a demyelinating disease of the brain caused by

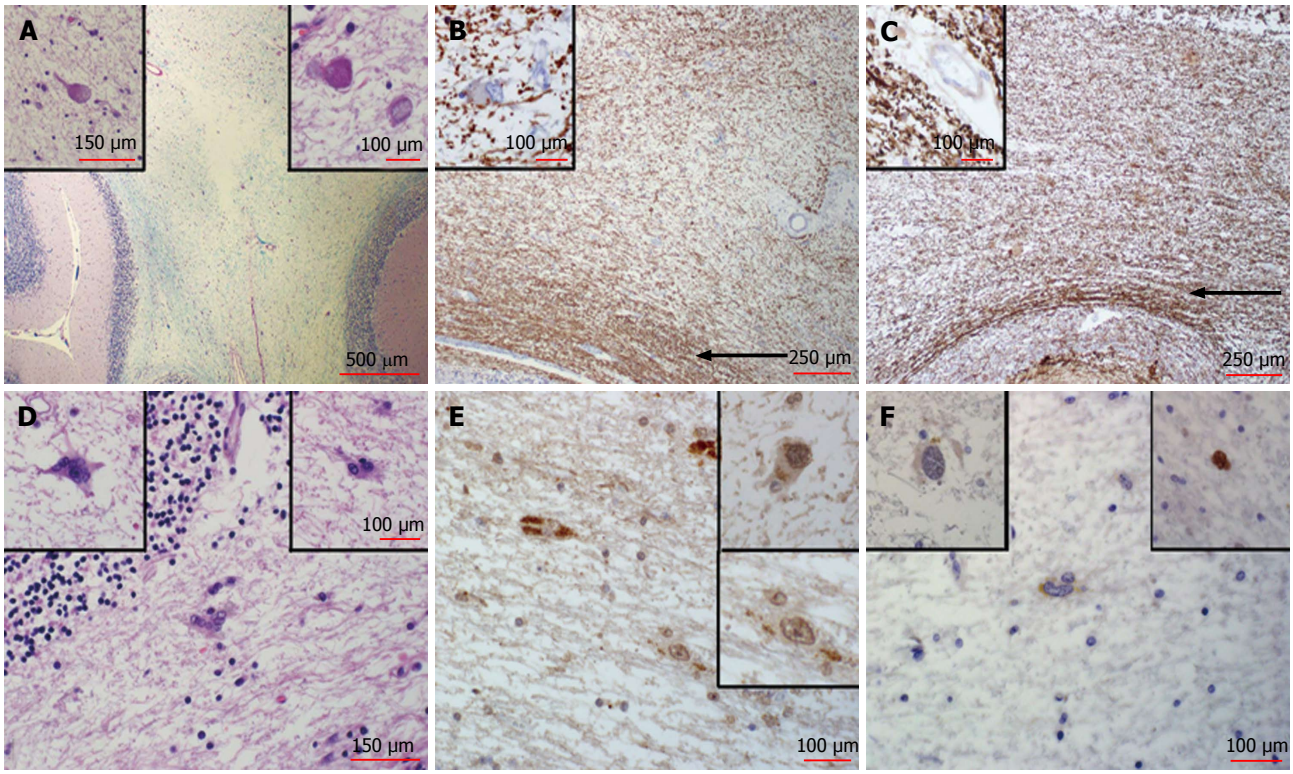

Figure 3 Post-mortem histologic analysis. A: The cerebellar lesion examined by histology shows a severe loss of myelinated fibers in the white matter, as stained by Luxol blue/Periodic Acid-Schiff. Note central pale area in the image. Also note that the surface folia, stained pink at the lower corners of the image, display no involvement. Inset (left) shows a higher magnification of an axonal spheroid from the region of the lesion, indicative of residual axonal damage, stained by H and E. Inset (right) shows another axonal spheroid from the same region at higher magnification also stained by H and E. Note that the pale lesion lacks evidence of active viral infection, with no macrophage infiltration or other inflammatory processes; B: Myelin basic protein immuno-staining reveals a severe loss of myelinated fibers stained brown in the cerebellar lesion. Note relative sparing of subcortical U-fibers (arrow). Inset (left) Higher magnification of a cell in the lesion stained for myelin basic protein that shows ragged myelin distribution. This demyelination appears to be quiescent; C: Section of the lesion in the cerebellum stained for neurofilament protein shows a loss of axons nearly proportional to the loss of myelin. Note relative sparing of subcortical U-fibers (arrow). Inset (left) shows higher magnification of neurofilament staining in the area of the lesion. Axons apparently lost in the acute phase in 1999 have not regenerated; D: Examples of rare atypical astrocytes found at the periphery of the lesion in H and E stained sections. Insets at higher magnification; E: SV40 immunostaining for T antigen shows some non-specific granular pattern within the cytoplasm of a few glial cells in the region of the lesion, some faint nuclear staining and scattered acellular granular deposits. This staining suggests latent infection with low levels of residual antigen. Insets show examples at higher magnification; F: The p53 immunostain shows that atypical astrocytes are mostly negative. Inset (left) another example of an atypical astrocyte that does not stain at the same magnification, and inset (right) a rare example of a p53 positive atypical glial cell. Macrophages were very rare and p53 staining was not observed. H and E: Hematoxilin and eosin.

the polyomavirus, JCV in immunosuppressed individuals. Although long-term survival has been reported in PML, the histological appearance of a demyelinating plaque in complete remission has not been well described. Here we show that demyelination in the original lesion was not repaired despite 14 years of remission, while evidence of continuing acute infection was absent.

The lack of defined viral particles, absence of progressive lesions, and inflammatory processes suggests that the virus had either been cleared or was latent. Since polyomaviruses persist in cells in a latent form, either episomally or when integrated into cellular DNA[17,18], surviving glia in the lesion could harbor latent virus and continue to express viral antigens at low levels without producing sufficient infective particles to spread the virus.

The risk of PML is present throughout the post transplantation period with a higher case fatality and incidence than reported in HIV patients on HAART or multiple sclerosis patients treated with natalizum[9]. There is no cure for PML, but prolonged survival rates are becoming increasingly common; although in one series, patients with cerebellar lesions tended to have a worse clinical outcome[6]. Magnetic resonance imaging brain findings typically show leukomalacia with ventricular enlargement secondary to destruction of the white matter at the site of previous PML lesions, and focal areas of subcortical atrophy with preservation of the cortical ribbon[6,8].

Although this case illustrates the classic histological features of PML at initial presentation together with neurological symptoms, imaging findings in the cerebellum and JCV confirmed biopsy, the patient's neurological symptoms were non-progressive despite continued immunosuppression. At autopsy, only residual damage in the location of the original lesion was observed, and histopathologic features of active infection in this region were absent, presumably indicating an effective cellular immune response against the virus. Serological workup of HIV cases has suggested a role for $CD8^+$ cytotoxic T-lymphocytes against JCV[12], although in this current

case no significant lymphocytic presence was detected in either the cerebellar biopsy or the post-mortem brain. Recent reports suggest findings of mutated JCV in CSF may correlate with slower or halted disease progression in HIV but no correlation was found in transplant recipients despite similarly mutated virus[2,19,20].

One of the earliest histopathological descriptions of patients with long term survival with PML revealed classical findings of progressive multifocal leukoencephalopathy, but with numerous eosinophils[21]. Viral particles were found in oligodendrocyte nuclei and cytoplasm with electron microscopy. Other cases of long term survival in non-HIV-infected patients are so rare as to be reportable, and include immunosuppressed patients for leukemia-lymphoma treatment[6,7,22] as well as solid organ transplant such as kidney[14] and liver[13]. The current case is unusual in that the neurological status was stable and at autopsy, gross evidence of multifocal pathology was absent, and histologic evidence of active viral infection was absent. Despite detection of low levels of viral antigen in the cerebellar region by immunostaining at autopsy this patient was clinically stable for fourteen years. No progression was detected symptomatically, neurologically, or radiographically. No evidence of progressive demyelination or spread of pathology beyond the original lesion was found in post-mortem evaluation of the brain.

A multicenter, retrospective cohort study of cases of PML was performed among transplant recipients at Mayo Clinic, Johns Hopkins University, Washington University, and Amsterdam Academic Medical Center[9]. The incidence of PML was calculated at 1.24 per 1000 post transplantation person-years. In this study of 69 cases of PML associated with solid organ and bone marrow transplantation, median survival following symptom onset was 6.4 mo for solid organ *vs* 19.5 mo for bone marrow recipients; with survival beyond one year of only 55.7%[9]. Anti-retroviral treatment for HIV improves the immune system and is beneficial for those with progressive multifocal leukoencephalopathy[1]; however the only effective treatment for iatrogenically immunosuppressed patients appears withdrawal or reconfiguration of life-saving immunosuppressive therapy and consequent enhancement of their natural immunity.

The mechanisms for reactivation of latent JCV in brain are poorly understood but thought to be related to immune competence. Viral and/or host genotypes may also play a role, since variation in human leukocyte antigens correlates with antibody response[23]. Other viruses latent in brain include herpes simplex virus (HSV). While HSV DNA is found in a large percentage of normal brains, little evidence exists as to whether HSV reactivates in brain[24]. Attempts to correlate HSV reactivation in the brain with the risk of neurodegenerative diseases such as Alzheimer's are on-going[25,26]. How either HSV or JCV are kept in check in the immune-competent infected person remains a mystery.

Our study reveals residual damage, rare macrophages, a few reactive astrocytes and minimal evidence of persistent viral antigen expression with no evidence of viral replication and infective particle production. This case demonstrates the possibility of complete remission of PML with long-term survival in a patient after solid organ transplant who was maintained on immunosuppressive therapy.

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COMMENTS

Background

JC virus (JCV) causes progressive multifocal leukoencephalopathy (PML), especially in immun-compromised patients. After solid organ transplant is typically rapidly progressive and fatal.

Research frontiers

Here the authors present a case of PML in a patient who received a solid organ transplant that did not progress for over 14 years despite on-going immune-suppression. The diagnosis was validated by pathological analysis of brain biopsy.

Innovations and breakthroughs

Rare insight into the histopathology of quiescent JCV infection and residual damage, not repaired after 14 years, are presented. This is the first reported histopathology of a JCV-induced lesion in remission.

Applications

This report demonstrates that PML progression may be halted but the original lesion does not repair.

Terminology

JCV is a polyoma genetically similar to SV40. "JC" stands for John Cunningham, the first patient in which the virus was discovered. It is very common in the general population but only causes overt disease in immune compromised hosts. PML thought to be caused by JCV, is a rare and usually fatal disease of the white matter in the brain.

Peer-review

JCV is a human polyomavirus that infects greater than 60% of the human population during childhood, and establishes a latent infection in healthy individuals. Replication of the neurotropic strain of JCV in glial cells causes the fatal demyelinating disease of the central nervous system, PML, which is seen in patients with underlying immunocompromised conditions. PML has also been described in patients with autoimmune diseases treated with immunomodulatory therapies. PML is a mortal disease and there is no specific therapy. Long-term survivors have been reported with no sign of viral reactivation and replication. There is little known about neuropathologic description of long-term survivals. In this manuscript, authors provided an interesting case report of a long-term PML survivor with immunohistological evaluation. These observations are interesting for the readers of the Journal.

REFERENCES

1 **Engsig FN**, Hansen AB, Omland LH, Kronborg G, Gerstoft J, Laursen AL, Pedersen C, Mogensen CB, Nielsen L, Obel N. Incidence, clinical presentation, and outcome of progressive

multifocal leukoencephalopathy in HIV-infected patients during the highly active antiretroviral therapy era: a nationwide cohort study. *J Infect Dis* 2009; **199**: 77-83 [PMID: 19007313 DOI: 10.1086/595299]

2 **Ferenczy MW**, Marshall LJ, Nelson CD, Atwood WJ, Nath A, Khalili K, Major EO. Molecular biology, epidemiology, and pathogenesis of progressive multifocal leukoencephalopathy, the JC virus-induced demyelinating disease of the human brain. *Clin Microbiol Rev* 2012; **25**: 471-506 [PMID: 22763635 DOI: 10.1128/CMR.05031-11]

3 **Focosi D**. Does contrast enhancement predict survival in progressive multifocal leukoencephalopathy? *J Infect Dis* 2009; **199**: 1410-1411; author reply 1411-1412 [PMID: 19358677 DOI: 10.1086/597622]

4 **Gasnault J**, Costagliola D, Hendel-Chavez H, Dulioust A, Pakianather S, Mazet AA, de Goer de Herve MG, Lancar R, Lascaux AS, Porte L, Delfraissy JF, Taoufik Y. Improved survival of HIV-1-infected patients with progressive multifocal leukoencephalopathy receiving early 5-drug combination antiretroviral therapy. *PLoS One* 2011; **6**: e20967 [PMID: 21738597 DOI: 10.1371/journal.pone.0020967]

5 **Gheuens S**, Pierone G, Peeters P, Koralnik IJ. Progressive multifocal leukoencephalopathy in individuals with minimal or occult immunosuppression. *J Neurol Neurosurg Psychiatry* 2010; **81**: 247-254 [PMID: 19828476 DOI: 10.1136/jnnp.2009.187666]

6 **Lima MA**, Bernal-Cano F, Clifford DB, Gandhi RT, Koralnik IJ. Clinical outcome of long-term survivors of progressive multifocal leukoencephalopathy. *J Neurol Neurosurg Psychiatry* 2010; **81**: 1288-1291 [PMID: 20710013 DOI: 10.1136/jnnp.2009.179002]

7 **Stam FC**. Multifocal leuko-encephalopathy with slow progression and very long survival. *Psychiatr Neurol Neurochir* 1966; **69**: 453-459 [PMID: 5971458]

8 **Tavazzi E**, White MK, Khalili K. Progressive multifocal leukoencephalopathy: clinical and molecular aspects. *Rev Med Virol* 2012; **22**: 18-32 [PMID: 21936015 DOI: 10.1002/rmv.710]

9 **Mateen FJ**, Muralidharan R, Carone M, van de Beek D, Harrison DM, Aksamit AJ, Gould MS, Clifford DB, Nath A. Progressive multifocal leukoencephalopathy in transplant recipients. *Ann Neurol* 2011; **70**: 305-322 [PMID: 21823157 DOI: 10.1002/ana.22408]

10 **Shitrit D**, Lev N, Bar-Gil-Shitrit A, Kramer MR. Progressive multifocal leukoencephalopathy in transplant recipients. *Transpl Int* 2005; **17**: 658-665 [PMID: 15616809 DOI: 10.1007/s00147-004-0779-3]

11 **Antinori A**, Cingolani A, Lorenzini P, Giancola ML, Uccella I, Bossolasco S, Grisetti S, Moretti F, Vigo B, Bongiovanni M, Del Grosso B, Arcidiacono MI, Fibbia GC, Mena M, Finazzi MG, Guaraldi G, Ammassari A, d'Arminio Monforte A, Cinque P, De Luca A. Clinical epidemiology and survival of progressive multifocal leukoencephalopathy in the era of highly active antiretroviral therapy: data from the Italian Registry Investigative Neuro AIDS (IRINA). *J Neurovirol* 2003; **9** Suppl 1: 47-53 [PMID: 12709872 DOI: 10.1080/13550280390195388]

12 **Du Pasquier RA**. JCV-specific cellular immune response correlates with a favorable clinical outcome in HIV-infected individuals with progressive multifocal leukoencephalopathy. *J Neurovirol* 2001; **7**: 318-322

13 **Boulton-Jones JR**, Fraser-Moodie C, Ryder SD. Long term survival from progressive multifocal leucoencephalopathy after liver transplantation. *J Hepatol* 2001; **35**: 828-829 [PMID: 11738115 DOI: 10.1016/S0168-8278(01)00202-1]

14 **Crowder CD**, Gyure KA, Drachenberg CB, Werner J, Morales RE, Hirsch HH, Ramos E. Successful outcome of progressive multifocal leukoencephalopathy in a renal transplant patient. *Am J Transplant* 2005; **5**: 1151-1158 [PMID: 15816900 DOI: 10.1111/j.1600-6143.2005.00800.x]

15 **Gheuens S**, Wüthrich C, Koralnik IJ. Progressive multifocal leukoencephalopathy: why gray and white matter. *Annu Rev Pathol* 2013; **8**: 189-215 [PMID: 23092189 DOI: 10.1146/annurev-pathol-020712-164018]

16 **Yang B**, Prayson RA. Expression of Bax, Bcl-2, and P53 in progressive multifocal leukoencephalopathy. *Mod Pathol* 2000; **13**: 1115-1120 [PMID: 11048806 DOI: 10.1038/modpathol.3880206]

17 **Coelho TR**, Almeida L, Lazo PA. JC virus in the pathogenesis of colorectal cancer, an etiological agent or another component in a multistep process? *Virol J* 2010; **7**: 42 [PMID: 20167111 DOI: 10.1186/1743-422X-7-42]

18 **Wold WS**, Green M, Mackey JK, Martin JD, Padgett BL, Walker DL. Integration pattern of human JC virus sequences in two clones of a cell line established from a JC virus-induced hamster brain tumor. *J Virol* 1980; **33**: 1225-1228 [PMID: 6245274]

19 **Jensen PN**, Major EO. A classification scheme for human polyomavirus JCV variants based on the nucleotide sequence of the noncoding regulatory region. *J Neurovirol* 2001; **7**: 280-287 [PMID: 11517403 DOI: 10.1080/13550280152537102]

20 **Delbue S**, Elia F, Carloni C, Tavazzi E, Marchioni E, Carluccio S, Signorini L, Novati S, Maserati R, Ferrante P. JC virus load in cerebrospinal fluid and transcriptional control region rearrangements may predict the clinical course of progressive multifocal leukoencephalopathy. *J Cell Physiol* 2012; **227**: 3511-3517 [PMID: 22253012 DOI: 10.1002/jcp.24051]

21 **Kepes JJ**, Chou SM, Price LW. Progressive multifocal leukoencephalopathy with 10-year survival in a patient with nontropical sprue. Report of a case with unusual light and electron microscopic features. *Neurology* 1975; **25**: 1006-1012 [PMID: 1237816 DOI: 10.1212/WNL.25.11.1007]

22 **Demir E**, Liebert UG, Söylemezoglu F, Yalaz K, Köse G, Anlar B. Childhood case of progressive multifocal leukoencephalopathy with improved clinical outcome. *J Child Neurol* 2005; **20**: 241-244 [PMID: 15832618 DOI: 10.1177/08830738050200031301]

23 **Sundqvist E**, Buck D, Warnke C, Albrecht E, Gieger C, Khademi M, Lima Bomfim I, Fogdell-Hahn A, Link J, Alfredsson L, Søndergaard HB, Hillert J, Oturai AB, Hemmer B, Kockum I, Olsson T. JC polyomavirus infection is strongly controlled by human leucocyte antigen class II variants. *PLoS Pathog* 2014; **10**: e1004084 [PMID: 24763718 DOI: 10.1371/journal.ppat.1004084]

24 **Roizman B**, Whitley RJ. An inquiry into the molecular basis of HSV latency and reactivation. *Annu Rev Microbiol* 2013; **67**: 355-374 [PMID: 24024635 DOI: 10.1146/annurev-micro-092412-155654]

25 **Bearer EL**. HSV, axonal transport and Alzheimer's disease: in vitro and in vivo evidence for causal relationships. *Future Virol* 2012; **7**: 885-899 [PMID: 23335944 DOI: 10.2217/fvl.12.81]

26 **Itzhaki RF**. Herpes simplex virus type 1 and Alzheimer's disease: increasing evidence for a major role of the virus. *Front Aging Neurosci* 2014; **6**: 202 [PMID: 25157230 DOI: 10.3389/fnagi.2014.00202]

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REVIEW

Inflammatory and oxidative stress in rotavirus infection

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Abstract

Rotaviruses are the single leading cause of life-threatening diarrhea affecting children under 5 years of age. Rotavirus entry into the host cell seems to occur by sequential interactions between virion proteins and various cell surface molecules. The entry mechanisms seem to involve the contribution of cellular molecules having binding, chaperoning and oxido-reducing activities. It appears to be that the receptor usage and tropism of rotaviruses is determined by the species, cell line and rotavirus strain. Rotaviruses have evolved functions which can antagonize the host innate immune response, whereas are able to induce endoplasmic reticulum (ER) stress, oxidative stress and inflammatory signaling. A networking between ER stress, inflammation and oxidative stress is suggested, in which release of calcium from the ER increases the generation of mitochondrial reactive oxygen species (ROS) leading to toxic accumulation of ROS within ER and mitochondria. Sustained ER stress potentially stimulates inflammatory response through unfolded protein response pathways. However, the detailed characterization of the molecular mechanisms underpinning these rotavirus-induced stressful conditions is still lacking. The signaling events triggered by host recognition of virus-associated molecular patterns offers an opportunity for the development of novel therapeutic strategies aimed at interfering with rotavirus infection. The use of N-acetylcysteine, non-steroidal anti-inflammatory drugs and PPARγ agonists to inhibit rotavirus infection opens a new way for treating the rotavirus-induced diarrhea and complementing vaccines.

Key words: Rotaviruses; Oxidative stress; Inflammatory signaling; Antioxidant treatment; Anti-inflammatory treatment

Core tip: Rotavirus entry into the host cell requires cell surface molecules providing binding, chaperoning and oxido-reducing functions. Sialic acid/integrin $\alpha 2\beta 1$, heat shock cognate protein 70 and protein disulfide isomerase (PDI) seem to perform these functions. Recently, the cell surface oxido-reduction activity based at least on PDI has been highlighted as a potential determinant of the conformational changes that are required by viral structural proteins in order to facilitate virus entry. The rotavirus-induced oxidative stress and inflammatory signaling is an attractive target for therapeutic intervention as antioxidant and anti-inflammatory treatment has

proved to efficiently inhibit rotavirus infection.

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INTRODUCTION

Rotaviruses are the major cause of severe, acute, and dehydrating diarrhea in children under 5 years of age worldwide. World Health Organization estimates that more than 25 million outpatient visits and 2 million hospitalizations attributable to rotavirus infections occurred each year[1]. Child deaths caused by rotaviruses were estimated at more than 453000 in 2008 globally[2]. Rotaviruses belong to the family Reoviridae and their 11-segmented double-stranded RNA (dsRNA) genome is encapsidated within a non-enveloped virion composed by three concentric protein layers [triple-layered particle (TLP)][3]. The outer layer is made of two structural proteins, VP4 and VP7. The middle layer is composed of VP6 that surrounds the core shell. The inner layer is composed of the core shell (VP2), which encloses VP1, VP3 and the genomic RNA[4,5].

Rotavirus entry into the host cell seems to be mediated by the sequential interaction of virions with various cell surface molecules including sialic acid (SA)[6], heat shock cognate protein 70 (Hsc70)[7,8], integrins[9-11] and protein disulfide isomerase (PDI)[12]. Virion penetration into the host cell involves the loss of VP4 and VP7 converting the TLP into a double-layered particle (DLP), which becomes transcriptionally active by generating positive-strand RNAs (mRNAs)[13]. Besides translation into viral proteins, positive-strand RNAs also serve as template for synthesizing the new dsRNA genomic segments. Electron-dense structures, named viroplasms, appear early in the cytoplasm of rotavirus-infected infected cells. The dsRNA synthesis and the initial steps of virion assembly occur in viroplasms[14]. Several structural and non-structural virus-encoded proteins accumulate in viroplasms for participating in the formation of viroplasms and contributing to dsRNA synthesis and viral replication[15,16]. The newly assembled DLPs bud into the endoplasmic reticulum (ER) lumen where a transiently acquired envelope is later replaced by an outer protein layer consisting of VP4 and VP7[17]. Releasing of mature virions from infected cells take place by either cell lysis or by a non-classical, Golgi apparatus-independent, vesicular transport pathway[16,17].

Here, we review the current knowledge on the oxidative stress and inflammation responses induced by rotavirus infection and the contribution of these responses to viral pathogenesis. The analysis of the implication of cellular proteins having oxidoreductase, thiol isomerization and chaperone activities is also emphasized in the context of rotavirus entry into the host cell.

OXIDATIVE STRESS

Balancing oxidation-reduction (redox) status in cells seems to be a crucial event for maintaining life[18,19]. Molecular oxygen has the ability to form free radicals which are highly reactive species having a single unpaired electron in their outermost shell. Reactive oxygen species (ROS) include the superoxide anion (O_2^-) that is transformed into H_2O_2 through the reaction catalyzed by superoxide dismutase (SOD). H_2O_2 may interact with transition metals such as iron and cupper to form the hydroxyl radical (OH^-). Reactive nitrogen species (RNS) are initially produced in cells by the reaction of nitric oxide (NO) and O_2^- that produces peroxynitrite ($ONOO^-$), whereas NO is biosynthesized by various nitric oxide synthases. ROS and RNS are normally generated by cellular metabolism and at low or moderate concentrations play physiological roles including cellular response to infectious agents, cellular signaling, induction of mitogenic response, neurotransmission, blood pressure regulation, smooth muscle relaxation, and immune regulation[20]. Oxidative stress occurs when the production of ROS and other reactive species overwhelm the capacity of cellular antioxidant defenses to detoxify these potentially injurious species. Redox imbalance can be produced through an increased generation of ROS, depletion of cellular antioxidant molecules and decrease in antioxidant molecules[21]. Harmful effects of ROS are represented by oxidative damage to proteins, lipids and DNA, whereas RNS can cause protein nitrosylation, lipid oxidation and DNA fragmentation[20]. On the other hand, excessive ROS and RNS have been linked to pathogenesis of cancer, cardiovascular disease, atherosclerosis, hypertension, ischemia/reperfusion injury, diabetes mellitus, neurodegenerative diseases, rheumatoid arthritis, pulmonary disease, and ageing[20,22]. Oxidative stress has been implicated in pathological conditions associated with different human inflammatory diseases[23]. Cells have developed mechanisms to deal with damaging oxidative environments. These mechanisms include intracellular redox systems such as GSH/GSSG (the glutathione system), $NADH/NAD^+$, $NADPH/NADP^+$ and Trx(SH)2/Trx(S-S) (the thioredoxin system). GSH is the most abundant and ubiquitous intracellular antioxidant in cells from higher organisms and oxidative stress is commonly associated with decreased GSH or increased GSSG levels. However, cellular oxidative stress has been defined in terms of the disruption of biological redox signaling events rather than a simple imbalance between pro- and ant-oxidant systems[24,25]. Increasing data suggest that oxidative stress is involved in the pathogenesis of many diseases and disorders, including infectious diseases caused by viruses affecting the gastrointestinal tract.

INTESTINAL REDOX BALANCE

The mammalian gastrointestinal epithelium, the largest surface area contacting the external environment, consists

of five major cell types (enterocytes, mucus-secreting goblet cells, hormone-secreting enteroendocrine cells, Paneth cells, and tuft cells)[26]. Normally, the villus tip enterocytes at 4-5 d post-differentiation spontaneously undergo anoikis (apoptosis) before being shed into the gut lumen[27]. Rotaviruses proliferate in the non-dividing mature enterocytes localized near the tips of the villi causing alterations in the small intestinal epithelium leading to diarrhea[28]. Homeostatic control of the intestinal redox environment seems to be a critical factor for maintaining intestine functions. Cells from intestinal epithelium must face the challenge not only of endogenously generated ROS but also of oxidant agents, mutagens and carcinogens accessing the luminal environment. Mucosal integrity is ensured by the luminal redox balance of the GSH/GSSG and cysteine/cystine (Cys/CySS) couples, that are also involved in maintaining luminal nutrient absorption, mucus fluidity, and microbiota[27,29]. Normal intestinal cell transition from proliferative state to non-dividing differentiated state or apoptosis has been associated with increasing oxidation of intracellular GSH/GSSG or extracellular Cys/CySS redox systems[29]. The homeostasis of the mucosal GSH is maintained through GSH uptake[30], regeneration from GSSG[31], and *de novo* synthesis[32]. Nevertheless, the extracellular/luminal redox environment is predominantly maintained by the Cys/CySS couple, with contributions from the GSH system[33]. Recent advances on intestinal redox biology suggest that the loss of intestinal homeostasis caused by oxidative stress in the mucosal and adjacent tissues can alter nutrient digestion and absorption, stem cell proliferation, enterocyte apoptosis, and immune response[27]. Understanding the mechanisms by which rotaviruses alter the intestinal homeostasis through the induction of oxidative stress open the way for designing new strategies based on the use of antioxidants as therapeutic tools for treating the severe and dehydrating rotavirus-induced diarrhea.

INNATE IMMUNE RESPONSE

Innate immunity, the first arm of the host immunity system, plays an important role in immediately controlling the pathogen invasion before induction of the mechanisms leading to an adaptive immune response. Innate immune system activation occurs through the recognition of pathogens by the germ-line-encoded pattern-recognition receptors (PRR). These receptors recognize specific structures present in pathogens, such as bacterial wall components or viral dsRNA. PRRs function by recognizing conserved pathogen-associated molecular patterns (PAMP) that are expressed by the invading pathogens. PRRs include toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and AIM2-like receptors. Ten different TLRs have been identified in humans, whereas there are 12 functional TLRs known in mice[34]. TLR9 is activated upon stimulation with viral DNA, TLR7 and TLR8 are activated by viral single-stranded RNA, while TLR3 activation is produced by viral double-stranded RNA[35]. Following receptor activation by virus associated molecular patterns and recruitment of several adaptor proteins, signaling pathways are activated resulting in the induction of cytokine production in virus-infected cells. Activation of TLRs stimulates nuclear factor-κB (NF-κB) and IRF3/7 signaling leading to the expression of type I interferons (IFNs) IFN-α and IFN-β, the production of pro-inflammatory cytokines, such as pro-interleukin (IL)-1β, and the activation of natural killer cells[36]. RLRs, including RNA helicases such as retinoic acid inducible gene I (*RIG-I*) and melanoma differentiation associated gene 5 (*MDA-5*), and double stranded RNA-dependent protein kinase (PKR) are particularly important in viral infections[37]. The NLR family consists of 22 proteins in humans and 34 in mice[38]. NLRs are involved in various innate immunity-associated functions including their assembly into multimeric protein complexes named as inflammasomes which are in charge of processing precursors of cytokines IL-1β and IL-18[39]. NLRP1, NLRP3, NLRP6, NLRP12 and NLRC4 have been found in distinct inflammasomes which participate in the recognition of different stimuli such as bacteria and viruses, among others[39].

Host cells response to viral infections through an early innate response consisting in the expression and secretion of type Ⅰ, Ⅱ and Ⅲ IFN which, in turn, stimulate the expression of numerous IFN-stimulated gene (*ISG*) products having antiviral activities[40]. The IFN-regulatory factor (IRF) family of transcription factors comprises nine members (IRF1 to IRF9) which play crucial roles in activating innate and adaptive immune responses to viral infection[41]. IRF3, IRF5, and IRF7 are particularly important for inducing the expression of type Ⅰ IFN[42]. The activation of the NF-κB by virus infection plays an important role during the induction of innate immune responses[43]. Transcription of type Ⅰ IFN is induced by activation of RLRs RIG-I and MDA-5 following recognition of cytoplasmic RNA[44]. NF-κB plays a role in the expression control of over 500 genes involved in immune inflammatory responses, acute-phase inflammatory responses, angiogenesis, oxidative stress responses, cell adhesion, differentiation, apoptosis, AIDS, atherosclerosis, asthma, arthritis and metastasis[45,46]. The central role played by NF-κB signal pathway in physiological and pathological conditions has made it a potential target for pharmacological intervention[45,47].

Rotavirus infection stimulates early antiviral gene expression and IFN-β *via* a signaling pathway that involves the participation of IFN-β promoter stimulator 1 which is recruited to signaling complexes after activation of RIG-I or MDA-5[48,49]. However, rotavirus PAMPs have not been exactly characterized and some rotavirus replication products have been suggested as activators of RIG-I and MDA-5[48,49]. The exact identification of rotavirus PAMPs that are recognized by RLRs have been judged to be critical for understanding of rotavirus-host cell interactions[50]. Endosomal and cell surface membrane-associated PRRs, including TLR3, TLR7 and TLR9, have been implicated in rotavirus recognition for stimulating innate immune response to infection[51-53]. An increased level of type Ⅰ and Ⅱ IFNs has been found in children and

animals as a consequence of rotavirus recognition by host PRRs[54,55]. However, some studies have suggested that whereas rotaviruses are able to trigger IFN production, they also can suppress the IFN effects[56]. Evidence has been provided that both IFN-α/β and IFN-γ play an important role in host response to rotavirus infection. However, their relative contribution may depend on the nature of rotavirus strain, site of replication, synergistic effects of IFN-γ, sustained replication and host age[50]. It has been shown that type Ⅱ IFNs have a relatively modest effect in restricting early replication of homologous rotavirus strains in comparison with a higher effect on heterologous strains[57]. Further studies are needed to assess the roles of TLRs and IFNs during the early infection by homologous and heterologous rotavirus strains.

CELLULAR PROTEINS CONTRIBUTING REDOX AND CHAPERONE ACTIVITIES

Cellular proteins having oxidoreduction and/or chaperone activities have been shown to be essential for successful replication of many viruses. In this context, PDI and Hsc70, and other related cellular proteins, deserve to be highlighted. The PDI family of dithiol-disulfide oxidoreductases comprises at least 17 members in mammalian cells[58] and up to 21 members including other organisms[59]. PDI is mostly present in the ER where it catalyzes the oxidative formation of disulfide bonds in nascent proteins entering the secretory pathway[60,61]. Conversely, PDI acts as a reductase on cell membrane surface, thereby reducing cell membrane-bound protein disulfide bonds[59,62]. Erp57, a protein disulfide isomerase chaperone similar to PDI, has been found to be involved in ER quality control of newly-synthesized glycoproteins[63]. Erp57 is located in the ER but it is also present on the cell surface and plasma membrane lipid microdomains (rafts) from some cells[64]. PDI family proteins catalyze the introduction, reduction and isomerization of disulfides bonds and are also enzymatic chaperones reconstructing misfolded proteins. Human PDI is a 57 kDa protein containing four characteristic thioredoxin-like domains, two of which containing the common structural motif CXXC in the active site[65].

PDI redox activity can be inhibited by cell membrane-impermeant thiol/disulfide-reactive agents such as DTNB [5, 5-dithio-bis-(2-nitrobenzoic acid)] and bacitracin[66,67]. Recent studies have shown that Bak, a pro-apoptotic Bcl-2 protein, mediates the pro-apoptotic function previously reported for several PDI members. This Bak-dependent function of PDI is performed by inducing mitochondrial outer membrane permeabilization, linking in this way ER chaperone proteins and apoptotic signalling[68]. NADPH oxidase complex (Nox) is the major contributor of ROS in cells. PDI has been shown to interact with Nox within the ER and also in the citosol[69,70]. The PDI overexpression has been shown to produce an increase in NADPH oxidase activity, leading to increased levels of cellular ROS[71].

In the context of cellular chaperone activity, Hsc70 has been shown to play an important role in the virus life cycle by modulating infectivity[72,73], serving as a receptor molecule[7,8] or participating in viral assembly and morphogenesis[74,75]. Hsc70 is a constitutively expressed molecular chaperone belonging to the Hsp70 family. Hsc70 has been reported to be involved in protection from several forms of cellular stress performing multiple cellular functions including assistance in folding of nascent polypeptides, prevention of protein aggregation, translocation of proteins across membranes, chaperone mediation of autophagy, survival of cancer cells, and disassembly of clathrin-coated vesicles[76]. Hsc70 has been reported to protect cells from oxidative stress and apoptosis[77]. Although Hsc70 has not been reported as a cell surface receptor facilitating attachment of Japanese encephalitis virus (JEV) virions, it has been found to be associated with virus penetration *via* clathrin-mediated endocitosis[78]. There is evidence showing that NF-κB p65-induced cell proliferation is dependent on a NF-κB p65-mediated decrease of Hsc70 levels[79]. The above-mentioned evidences indicate that chaperone and oxidoreduction activities are present at different subcellular locations which can be used by viruses during their life cycle stages. Further studies must be conducted in order to better understand the specific implications of chaperone and oxidoreduction activities in both physiological and pathophysiological conditions.

PDI IMPLICATION IN VIRUS ENTRY

PDI redox function has been found to be needed for entry of some viruses into the host cell. Early studies demonstrated that human immunodeficiency virus (HIV) entry was inhibited by membrane-impermeant thiol/disulfide-reactive agents through inhibiting PDI redox function[66] or other cell-surface molecules showing redox activity[80,81]. PDI and thioredoxin-1 have been shown to reduce the disulfide bonds present on HIV glycoprotein gp120 facilitating the virus entry[82]. It has been suggested that endothelial PDI reduces integrins β1 and β3 causing the internalization of dengue virus[83]. Avian leukosis virus[84] and Sindbis virus[85] entry has been found to be dependent on the generation of free thiols in their fusion protein. The conserved cysteine residues from the hepatitis B virus (HBV) envelope protein coating hepatitis delta virus particles have been shown to be essential for virus entry[86]. Generation of free thiols in Newcastle disease virus fusion (F) protein have been shown to be required for virus entry into cells and cell fusion[87,88]; it has been suggested that PDI family isomerases could be responsible for such thiol generation[89]. Cell surface PDI has been found to facilitate the infection of HeLa cells by mouse polyoma virus[90]. Studies have identified novel functions of PDI that are relevant for various diseases including virus infections[91-93].

Rotavirus infectivity inhibition has been reported to be caused by treatment of MA104 cells with DTNB, bacitracin or anti-PDI antibodies[12]. It was suggested that thiol/disulfide exchange activity on cell membrane surface was involved in rotavirus infection as DTNB can modified

thiol-containing cell surface proteins and bacitracin can react with proteins containing the tetra-peptide motif CXXC. The cell surface PDI implication in rotavirus entry was concluded from results showing a physical *in vitro* interaction between PDI and TLPs and a significant rotavirus inhibition caused by cell pre-treatment with anti-PDI monoclonal antibodies (mAbs)[12]. In the same study, it was observed that infectivity of rotavirus TLPs was reduced by pre-treating them with DTNB, whereas pre-treatment of TLPs with bacitracin or anti-PDI mAb did not affect TLP infectivity. These findings suggested that rotavirus virions contain thiol groups that are required for virus infectivity. From this study, it was concluded that membrane-impermeant thiol/disulfide-reactive agents and anti-PDI mAbs inhibit rotavirus infectivity at entry but during a post-binding step[12]. The implication of PDI during the rotavirus entry process has been further studied using synthetic peptides derived from rotavirus structural protein amino acid sequences potentially mediating cell surface PDI–substrate interactions[94]. Cysteine-containing VP4 and VP7 peptides were observed to cause a significant inhibitory effect of infectivity when added to MA104 cells by competing with infectious virions. It was also found that antibodies against these cysteine-containing VP7 or VP4 peptides significantly inhibited rotavirus infectivity suggesting that PDI can use at least these viral amino acid sequences for interacting with rotavirus structural proteins[94]. Interestingly, antibodies to VP7-derived amino acid sequences inhibited virus infectivity only after virions were attached to host cell surface membrane. These finding allowed authors to suggest that these VP7 amino acid sequences were exposed after a cell surface interaction-dependent conformational change occurred[94]. From these findings it can be summarized that a thiol/disulfide exchange is contributing to rotavirus entry to MA104 cells and that cell-surface PDI is a potential target for DTNB and bacitracin-induced infectivity inhibition as cell surface thiol/disulfide exchange blockade prevented at least viral structural proteins from being modified by cell surface proteins catalyzing thiol/disulfide exchange (*i.e.*, PDI). Incubation of TLPs, VP5, VP6 or VP7 with rPDI or PDI in membrane-enriched fractions resulted in redox changes in viral proteins as such proteins reacted with maleimide, a thiol reactive moiety (Rivera M, Guerrero CA, Acosta O. Manuscript in preparation). Taken together, the above described findings suggest that cell surface PDI reducing activity is implicated during rotavirus entry. This fact opens the way for the rational design of membrane-impermeant thiol/disulfide compounds able to specifically inhibit the virus entry into the host cell.

ROTAVIRUS ENTRY INTO HOST CELL

Several cell-surface molecules have been involved in the early interactions between rotavirus virions and host cells. Rotavirus entry seems to occur by sequential interactions between virion proteins and various cell surface molecules[95,96]. After these initial interactions, the internalization of rotaviruses into the host cell takes place through distinct endocytic pathways that are determined by the viral structural protein VP4[97-99]. The rotavirus spike protein VP4 is cleaved by trypsin into N-terminal VP8* and C-terminal VP5* fragments to prime TLPs for efficient infectivity[100]. The structural characterization of an infectious rotavirus particle has allowed authors to propose a model involving a sequence of conformational changes in VP4 leading to the distortion of host cell membrane during entry[4,101,102]. However, the complete understanding of the mechanisms by which rotavirus enter cells is still lacking. Rotavirus structural proteins VP4 (VP5* and VP8*), VP6 and VP7[96,103] have been involved in different interactions with cell surface molecules during entry. Experimental results indicated that N-acetyl neuraminic (sialic) acid (SA)-dependent/neuraminidase-sensitive strains bind first through VP8* to SA before interacting with integrin $\alpha2\beta1$ whereas this integrin is directly bound by SA-independent/neuraminidase-insensitive strains through VP5*[104-106]. Available evidence has indicated that SA is a crucial determinant for the binding of both neuraminidase-sensitive and neuraminidase-insensitive rotavirus strains[6]. Most commonly occurring human VP4 serotypes use their VP8* subunit to interact with cell surface GM1 ganglioside containing the internal N-acetylneuraminic acid, the most common SA[107,108]. This is in contrast with most animal rotaviruses that bind terminal sialic acids without using GM1 for VP4 cell binding or infection[107]. It has also been shown that VP8* of a human rotavirus strains specifically recognizes histo-blood group antigens[109-112]. After the initial binding to SA and integrin $\alpha2\beta1$, post-binding studies have led to conclude that that rotavirus interacts with cell surface Hsc70[7,8]. Similar studies have also shown that rotavirus virions interact with integrins $\alpha4\beta1$, $\alpha x\beta2$ or $\alpha v\beta3$ after their binding to $\alpha2\beta1$[9,10,113]. Recently, it has been reported that rotaviruses also interact with reducing cell surface PDI during entry[12,94], most probably through their structural proteins VP5*, VP6 and VP7 which are potential substrates of PDI (Rivera M, Guerrero CA, Acosta O, manuscript in preparation). Post-binding interactions of VP5 and VP6 with Hsc70 has been well documented[7,8,103], whereas post-binding interactions with integrins $\alpha4\beta1$, $\alpha x\beta2$ or $\alpha v\beta3$ have involved VP7[9,10,113,114]. However, the sequence in which these post-binding interactions occur has not been yet established. Interactions of cell surface molecules and rotavirus structural proteins are summarized and schematized in Figure 1.

Crystallographic studies of VP5* have suggested that the trypsin cleavage of VP4 is determinant in generating conformational changes priming the VP8* and VP5* cleavage products for interacting with their corresponding cell surface receptors[101,102]. It has been hypothesized that a conformational transition from a dimer to a folded-back trimer of VP5* would facilitate the interaction of VP5* with the lipid bilayer membrane, resembling the fusogenic conformational changes in enveloped-virus fusion proteins[101]. Regarding the functional identity of Hsc70, this protein could be a candidate contributing to

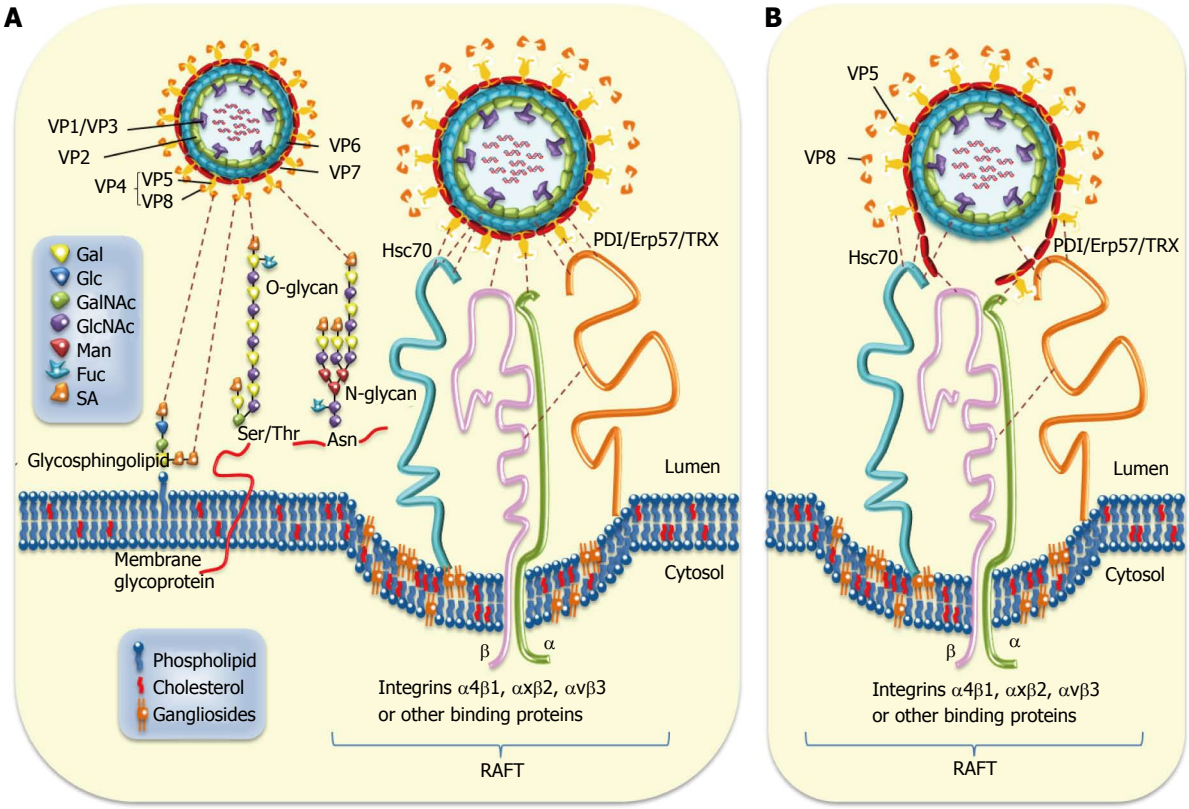

Figure 1 Rotavirus-cell surface interactions during entry. A: The rotavirus particle-associated proteins (VP1/2/3/4/5/6/7) that enclose the viral genome are represented. Cell surface molecules including sialic acid (SA), Hsc70, PDI, Erp57, thioredoxin (TRX), and integrins α4β1, αxβ2, and αvβ3 are also represented. Infection is initiated by the VP8*-mediated binding (attachment) of virion to terminal or non-terminal (neuraminidase -resistant) SAs located on cell surface glycolipids including gangliosides or to SAs located on cell surface glycoproteins. The N- and O-substituted derivatives of neuraminic acid (SAs) are indicated. Neuraminidase-resistant rotavirus strains can bind directly to integrin α2β1 through VP5* DGE sequence. SA-dependent strains bind first through VP8* to SA before interacting with integrin α2β1 through VP5*. A putative caveolae containing raft-associated cell surface receptors is depicted. The sequence of virion-cell interactions taking place after binding to α2β1 has not yet been established. However, several interactions involving rotavirus structural protein (VP5*, VP7 and VP6) and raft-associated cell surface receptors (Hsc70, PDI and integrins α4β1, αxβ2 and αvβ3) have been documented. Interactions between rotavirus structural proteins and cell surface molecules are illustrated; B: Disruption of rotavirus proteins (VP5*, VP7 and VP6) caused by cell surface-associated chaperone (Hsc70, PDI) and oxido-reductase activities (PDI, integrin αvβ3) is depicted. Hsc70: Heat shock cognate protein 70; PDI: Protein disulfide isomerase.

such conformational transition. Hsc70 has been proposed as a penetration receptor mediating JEV entry into cell by generating conformational changes in the envelope glycoprotein E of JEV, the protein responsible for receptor binding and membrane fusion[115]. Most likely, Hsc70 not only plays a role in anchoring rotavirus virions to cell membrane but also generating conformational transitions in VP5* to facilitate its transition from a dimeric to trimeric conformation. Other studies have suggested that VP5* bound to integrin α2β1 could undergo conformational changes associated to its trimerization[10,116]. Integrin α2β1 has also been shown to undergo conformational changes and activation that may facilitate binding of VP5* to cell membrane[10].

Chaperones such as Hsc70 commonly interact with hydrophobic regions of target proteins to perform ATP-dependent protein complex disassembly[117]. Although Hsc70 interacts with VP4 through the domains aa 642-658[8] and aa 531-554[103], the potentially fusogenic domain of VP5* (aa 385-404) could be a Hsc70 substrate. Hsc70-TLP interaction in solution seemed to induce conformational changes in VP5* and VP7[118]. Moreover, there are studies showing that DLPs interact physically with Hsc70 at least through the VP6 sequence aa 280-297 and that cell treatment with a synthetic peptide comprising this sequence was able to inhibit infection by animal and human rotavirus strains[103]. In the same study, the presence of antibodies to the VP6 synthetic peptide was shown to also inhibit rotavirus infectivity, suggesting that DLPs interact with Hsc70 during the entry process. Overall, it is not unlikely that post-binding interactions of rotavirus virions with Hsc70 might facilitate the generation of conformational changes in VP5* leading to the trimeric conformation able to destabilize de lipid bi-layer of cell membrane or endocytic vesicle[119,120].

Despite the identification of these potential receptors, there is no known single cell surface protein whose reaction with specific antibodies leads to an almost complete abolition of rotavirus infectivity. For instance, partial inhibition of rotavirus infectivity by anti-Hsc70 or anti-integrin antibodies might be reflecting the existence of alternative entry routes[96,99,121,122] or "dead-end" pathways[123]. Partial inhibition of rotavirus infectivity by anti-PDI mAbs might be suggesting that rotaviruses use

alternative entry paths or that the anti-PDI mAbs used partially inhibited PDI activity[12]. These mAbs have been shown to inhibit PDI activity by 49% to 90%, depending on the assay system used[66,124-126]. The finding that bacitracin greatly inhibit PDI-TLP interaction *in vitro* suggested that the CXXC motif in the PDI catalytic domain was required for this interaction rather than the presence of free thiols in virion proteins, as shown by the insensitivity of this interaction to DTNB treatment[12]. Obviously, the PDI's chaperone activity implication in PDI-TLP interaction cannot be ruled out because such activity has been reported to have become notably reduced by bacitracin treatment.

PDI, Hsc70 and integrin αvβ3 have been found to interact in lipid microdomains ("rafts")[12,127], which have been proposed as being essential platforms facilitating efficient interaction between virus particles and cellular receptors[96,128]. On the other hand, some reports have indicated that PDI forms complexes with integrins α2β1 and αvβ3[129,130] that have been identified as cell surface rotavirus receptors in MA104 cells. Since integrin β3 is known to be an endothelial cell-surface PDI substrate[129], it would be interesting to determine whether free thiol generation in this integrin is required for its activation and interaction with rotavirus during entry. Evidence has been provided that IL-1α-mediated innate response of macrophages to adenovirus implicating the interaction of virus RGD motif with integrin β3 for triggering the activation of pro-inflammatory responses to the virus[131]. Interestingly, results have been presented that a specific inhibitor of integrin β3 (a secondary adenoviral receptor) attenuated the cytokine release and the inflammatory hepatic toxicity induced by an oncolytic adenovirus without interfering with its infectivity and oncolytic properties[132]. Integrin β3 expression has been shown to be required and up-regulated by classical swine fever virus (CSFV) infection[133]. However, PDI expression has been found to be inhibited in the heart, liver, spleen, lung, kidney and mesenteric lymph node tissue from a CSFV-positive pig[134]. Evidence has been provided that dengue virus serotype 2 (DV2) induce up-regulation of integrin β3 which is also required for DV2 entry into the cell[135]. However, studies using intestinal cell lines showed that rotavirus infection up-regulated the expression of integrins α2β1 and β2, whereas down-regulated that of integrins αvβ3, αvβ5, and α5β1[136]. It would be interesting to examine whether cell surface PDI activates integrin β3 to facilitate rotavirus infection since PDI expression has been up-regulated by rotavirus infection[127]. It has been found that dengue virus infection increases cell surface PDI expression for activating integrins β1 and β3 and facilitating virus entry into epithelial cells[83]. Chaperone and thiol-disulfide exchange activities are schematized in Figure 2.

Rotavirus virion binding to the cell surface and the subsequent post-binding events seem to involve conformational changes and oxidoreduction reactions in the virus structural proteins. Regarding the properties of cell surface proteins interacting with virus structural proteins, it can be proposed that conformational changes could be produced by the chaperone activity characterizing Hsc70 and PDI, whereas redox status changes involving also conformational changes could be induced by oxidoreductase and thiol/disulfide isomerase activities present in PDI and integrin αvβ3[129,137]. On the other hand, there is evidence suggesting that thiol isomerases such as PDI and Erp57 bind to β3 subunit of integrins αⅡbβ3 and αvβ3 for regulating their function during thrombus formation and that αⅡbβ3 also has an endogenous thiol isomerase activity[138]. These results have led to propose that integrin β3 function might be regulated by both exogenous and endogenous thiol isomerase activity and that PDI inhibitors could be useful therapeutic tools for treating integrin-associated diseases[138].

Rotavirus structural proteins VP4, VP6 and VP7 have been reported to contain cysteine residues able to form intramolecular disulfide bonds[139-141]. However, crystal structure studies of VP6[142] VP8*[108] and VP5*[101,143] have shown that these proteins lack disulfide bonds. Rotavirus VP4 from many SA-dependent animal strains contains five conserved cysteines at positions 203, 216, 318, 380 and 774. It has been shown for simian RRV and SA11 that their VP4 contains two disulfide bonds residing in the VP8* (Cys-203/Cis-216) and VP5* (Cys-318/Cis-380) domains[139]. A SA-independent variant of RRV was reported to have an additional cysteine at position 267 that was able to form an alternative disulfide bond implicating Cys-318 while co-existing with the disulfide bond Cys-318/Cys-380[144]. The presence of highly conserved disulfide bonds in VP5* has been suggested to facilitate bringing together the trypsin cleavage sites, the integrin binding site and the putative fusogenic peptide into intimate proximity[121,139]. The mutant VP5* containing mixed species of disulfide bonds was supposed to have an altered conformation explaining its ability interact with the host cell surface independently from SA interaction[144]. In this context, the concept of functional disulfide bonds[145] could be extended to the interactions between disulfide bond-containing proteins of rotavirus virions and the cell surface proteins having thiol isomerase activity including PDI and integrins. Interactions of rotavirus structural proteins and cell surface molecules during entry are summarized in Table 1.

Research aims at unraveling the mechanisms involved in rotavirus entry is very critical for understanding versatility of rotaviruses in using different cell surface receptors. However, the accumulated findings on rotavirus entry mechanisms suggest that in addition to the initial attachment to SA-containing molecules, rotavirus structural proteins undergo conformational changes mediated by cell surface chaperone and thiol-disulfide activities. Clearly more research is needed to fully understand if rotavirus certainly use alternative entry pathways or at least partially shared pathways that finally lead to the conversion of TLPs into transcriptionally active

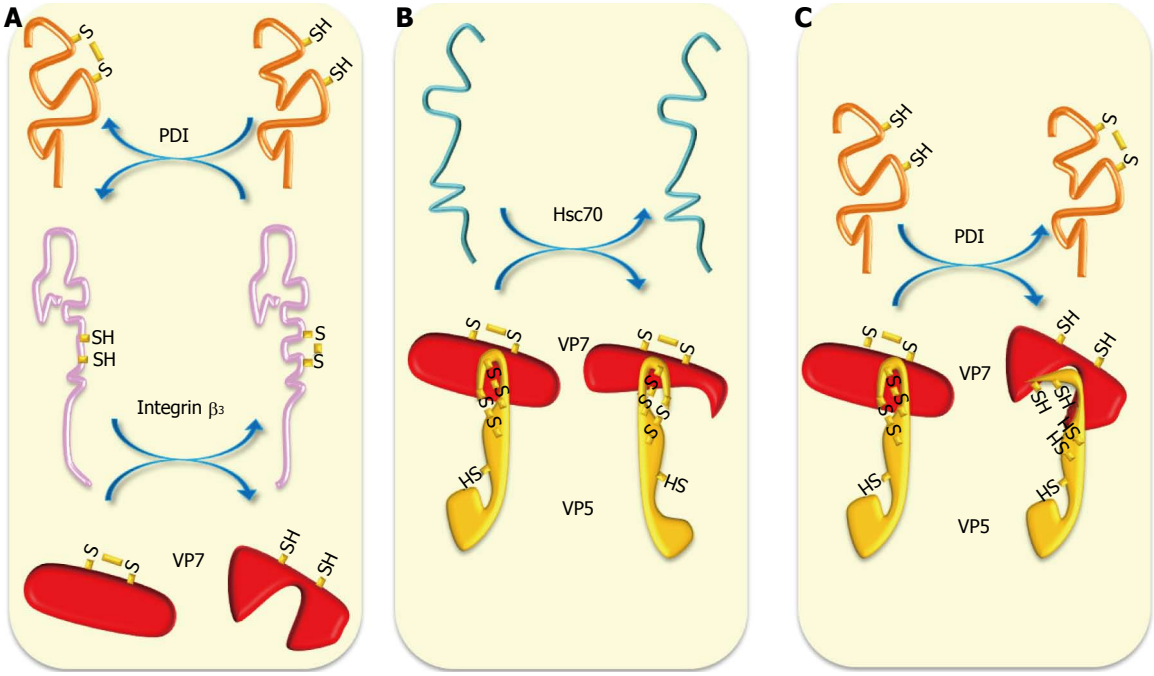

Figure 2 Schematic representation of chaperone and oxidoreduction activities during rotavirus entry. A: Cell surface reducing PDI has been shown to form complexes with integrins α2β1 and αvβ3 to generate free thiols in these integrins. Reducing integrin β3 can reduce thiol-disulfide bonds present in VP7; B: Chaperone activity of Hsc70 can induce conformational changes in VP5* and VP7 priming them for further interactions; C: Cell surface reducing PDI can reduce thiol-disulfide-containing VP5* and VP7 generating in them conformational changes needed for further interactions and entry. Hsc70: Heat shock cognate protein 70; PDI: Protein disulfide isomerase.

DLPs[146].

DLP-TLP INTER-CONVERSION

Rotavirus entry process contributes to convert TLPs into transcriptionally active DLPs, whereas newly formed DLPs are converted into new TLPs. Although the mechanisms involved in this inter-conversion are not entirely composed by the same reactions in opposite directions, it sounds illustrative to compare some of these membrane-associated reactions: Those aimed at removing VP4 and VP7 from TLPs to generate DLPs and those aimed at coating DLPs to generate TLPs. Cell membrane must possess molecular systems capable of inducing the necessary conformational changes facilitating viral proteins to disturb cell membrane for penetration[147]. In the case of rotaviruses, potential receptors having chaperone and oxidoreductase activity has been identified[7,12]. The ER has been described as complex membranous network that is used by many viruses during infection[148]. ER participates in rotavirus assembly especially during the late steps of the morphogenesis events converting DLPs into TLPs. NSP4 recruits VP4 and DLP to the ER membrane before budding into the ER lumen where a transient membrane layer is removed and replaced by VP7 to generate mature TLPs budding from the ER[16,149]. The mechanisms involved in removing the ER-derived transient membrane layer are unclear, except that unassembled VP7 has been reported to have a membrane lytic activity[150]. Although formation of virus-induced ER-derived structures is considered critical for viral replication and assembly[148], viral infections induce ER stress and interferon responses that are interfered by viruses to ensure viral replication or pathogenesis[151].

Despite the advances in structural characterization, the sequence of events occurring during uncoating for generating and releasing DLPs into cytoplasm is still unknown[4,97]. These events involve removing of structural proteins VP4 and VP7 to produce DLPs. To this step, the general event could be assumed as a set of reactions proceeding in the opposite direction to those reported for the morphogenesis of TLPS from DLPs during the ER budding. Removing VP4 and VP7 led to generating a hydrophobic surface that might facilitate the translocation of DLPs into the cytoplasm through cellular or endosomal membrane. However, the sequence of VP4 and VP7 assembly did not explain the mechanisms of entry-associated uncoating[123]. Recoating experiments in which rotavirus DLPs are recoated *in vitro* with recombinant outer proteins have been useful in approaching the sequence of virion assembly *in vivo*. These experiments allow obtaining an efficient *in vitro* coating of DLPs that favors the hypothesis that *in vivo* VP4 assembly precedes that of VP7[123]. Reversing the coating assembly during entry means that VP7 should be removed before VP4, except that VP4 underwent a previous trypsin cleavage that generates VP8* and VP5*. However, VP8* is supposed to be released from VP5* before cell membrane destabilization[101].

Studies using cysteine-containing synthetic peptides derived from VP4 and VP7 suggested that VP4 and VP7 probably are PDI substrates as pre-treatment of cells with these peptides inhibited rotavirus infection[94].

Table 1 Cell surface molecules interacting with rotavirus structural proteins during entry

Cellular molecule	Rotavirus protein	Activity	Viral protein motif involved	Ref.
Sialic acid	VP8*	Binding	Carbohydrate binding site	[108]
α2β1	VP5*	Post-binding	DGE (VP5*)	[106]
α4β1	VP7, VP5*	Post-binding	YGL (VP5*); LDV o LDI (VP7)	[11,121]
αxβ2	VP7	Post-binding	GPR (VP7)	[114,121]
αvβ3	VP7	Post-binding, oxidoreduction	161NEWLCNPMD169	[10,113]
Hsc70	VP5*, VP7, VP6	Chaperoning	aa 642-658 (VP5*); aa 280-296 (VP6); aa 531-554 (VP5*)	[8,103]
PDI	VP5*, VP7, VP6	Chaperoning, oxidoreduction	aa 200-219 (VP4); aa 189-210 and aa 243-263 (VP7)	[12,94, Rivera M, Guerrero CA, Acosta O, manuscript in preparation]
HBGAs	VP8*	Binding	Carbohydrate binding site	[111]

Hsc70: Heat shock cognate protein 70; PDI: Protein disulfide isomerase; HBGAs: Histo-blood group antigens.

Similarly, pre-treatment of TLPs with antibodies against these peptides led to decreased infectivity. These findings allow hypothesizing that the disulfide bonds present in VP8* or VP5* could be reduced by PDI facilitating the TLP uncoating. The fact that PDI can produce *in vitro* modifications in the redox status of VP4 and VP7 (Rivera M, Guerrero CA, Acosta O, manuscript in preparation) gives support to this hypothesis. It would be interesting to know whether the mechanism causing the release of VP8* from VP5* involves redox reactions. Disulfide bond reduction could be a plausible candidate mechanism for ensuring the conformational changes needed for detachment of VP5* from integrin α2β1, contributing in this way to make the entry process irreversible. VP7 maturation in the ER lumen has been reported to involve oxidation reactions caused by the oxidant PDI to generate intra-molecular disulfide bonds and a proper conformation to ensure its assembly on DLPs[140,152,153]. The contribution of disulfide bonds to VP7 conformation seems to be crucial for the structural and functional roles of this protein during assembly and interaction of virions with cell surface receptors. Since virus entry leads to uncoating of TLPs by losing VP4 and VP7 to release the transcriptionally active DLPs into cytoplasm, it is tempting to propose that the reducing cell surface PDI could contribute to destabilize VP7 by reducing its disulfide bonds beside the contributions of the decreased Ca^{2+} concentration and acidification reported to occur in the endocytic environment[154-156]. DLPs in the absence of VP4 and VP7 have been shown to be able to bud into the ER lumen. Taking into account that the reduced VP7 seem to have low affinity for DLPs, it is not unlikely that DLPs present in the endosomal vesicles can bud into the cytoplasm through the permeabilized endosomal membrane[156]. In the opposite redox reaction, oxidized VP7 has been shown to be required to remove the transient lipid envelope in a calcium-dependent process to generate mature TLPs in the ER lumen[16]. Research aims at knowing whether the reducing PDI is modifying the redox status of VP7 in the endosomal membrane would be useful to determine the potential participation of redox reactions during generation of DLPs *in vivo*. Interaction between the outer capsid proteins (VP4 and VP7) and PDI has been demonstrated *in vitro* and also the generation of free thiols in these proteins after this interaction. Similarly, thiol groups are generated in the outer capsid proteins after TLPs contact the cell surface, suggesting that PDI or other related thioredoxins are able to reduce disulfide bonds in viral proteins (Rivera M, Guerrero CA, Acosta O, manuscript in preparation). Studies characterizing potential cell surface receptors for rotavirus infection of small intestinal villus cells from mice showed that raft-associated Hsc70, PDI and integrin β3 played an important role in the rotavirus entry process as previously shown for MA104 cells[127]. It has been reported that integrin subunit β3 and integrin α2β1 are present on the cell surface of murine and human enterocytes[157,158], and that rotavirus-susceptible MA104, COS7 and Caco-2 cells also contain cell surface receptors including αvβ3 and Hsc70[113,128]. The colocalization of PDI, integrin β3, Hsc70 and rotavirus particles in lipid microdomains (rafts) from MA104 and intestinal villus cells[12,127] suggest that PDI reducing function at cell surface can activate either integrins or VP7 to interact each other during entry[94].

The role of thiol-disulfide exchange during rotavirus infection is well documented, but the detailed processes of this implication still remain incompletely elucidated. Although PDI has emerged as a significant contributor for generating thiol-disulfide-associated conformational changes in rotavirus structural proteins during uncoating and assembly of viral particles, contributions from integrins and other thioredoxins cannot be ruled out. Again, a better understanding about the involvement of thiol-disulphide exchange in the rotavirus infection process could facilitate the identification of potential targets of therapeutic strategies.

OXIDATIVE STRESS AND ROTAVIRUS INFECTION

Several studies have demonstrated the implication of redox balance disruption in the establishing of viral infection and the progression of virus-induced diseases[159]. The oxidants induced by viral infections include superoxide anion (O_2^-)[160], which can be transformed into hydroxyl

radical (OH^-), nitric oxide radical (NO), H_2O_2 or peroxynitrite ($ONOO^-$) through enzymatic and non-enzymatic reactions. The findings showing pathogenic interactions between ROS and HIV stimulated research into the role these interactions may play in the pathogenesis of many viruses, opening the way for novel antioxidant-based antiviral therapeutic strategies[161,162]. ROS may modulate the viral replication and cellular response, and also contribute to viral pathogenesis[163,164]. Virus-induced oxidative stress has been reported during HIV[165], influenza virus[166], HBV[167], hepatitis C virus[168], encephalomyocarditis virus (EMCV)[169], respiratory syncytial virus (RSV)[170], dengue virus[171,172], and JEV[173] infections.

Early studies on rotavirus infection showed decreased SOD and glutathione peroxidase activities in whole intestine homogenates from infant mice[174]. A more recent study reported that rotavirus infection was able to induce an increase in inducible nitric oxide synthase (iNOS) mRNA in murine ileum and iNOS expression also in murine ileum upon exposure to NSP4[175]. NSP4-induced release of NO metabolites was reported in cultured human intestinal epithelial cells incubated with purified NSP4[176]. Increased NO metabolites were also observed in mice infected with murine rotavirus EDIM beside upregulation of iNOS mRNA in ileum, but not in duodenum or jejunum[176]. A prospective clinical study including acutely rotavirus-infected children showed that viral infection stimulated NO production[176]. However, studies using Caco-2 cells infected with SA11 rotavirus showed that viral infection increased the expression of the mitochondrial superoxide dismutase (MnSOD) within the first 48 h.p.i. This increased SOD expression was correlated with a decrease in ROS generation during the early phase of infection (8 h.p.i.) and a lack of cellular glutathione (GSH) depletion[177]. Despite the increase in enzyme activity was not directly proportional to the rise in protein expression level in the cell lysates studied, during the later post-infection times ROS returned to the control levels even in the presence of increased MnSOD protein expression. This fact was interpreted as being due to an overproduction of mitochondrial ROS that overwhelmed the activity of the MnSOD[177]. Induction of MnSOD overexpression has been shown to occur as a consequence of increased production of ROS through a pathway involving inflammatory cytokines such as tumor necrosis factor-α (TNF-α) or IL-1[178,179] and activation of the NF-κB factor by ROS[180]. ER stress was found to increase ROS[181] and also induce MnSOD through nuclear factor NF-κB and AP-1 activation after exposure of HeLa cells to various agents interfering with ER functions[182].

Acute gastroenteritis in piglets has been associated with increased levels of high-mobility group box 1 (HMGB1) protein (a nuclear DNA-binding protein), and serum haptoglobin and ceruloplasmin which suggest an acute phase response[183]. A significant decrease of total antioxidant capacity and antioxidant enzyme activities has been found in serum from piglets affected with acute enteritis. Increased values of oxidative stress indices, including the malondialdehyde (MDA) and NO concentrations in serum have also been associated with pathological condition[183]. However, HMGB1 protein, acute phase response and oxidative stress indices were even more prominent in the cases in which porcine rotavirus infection took place.

Although there is a large body of information available about the involvement of oxidative stress in viral infection and its effects on cell functions leading to cell death, the extent to which oxidative stress is part of a natural defense response of cells to virus infection or a mechanism by which viruses induce cell injury is still unknown. Advances in the understanding of the role of oxidative stress in rotavirus infection might contribute to improved treatment strategies of rotavirus-induced diarrhea. Interestingly, rotavirus infection of cultured cell lines, and *in vivo* conditions using animals and human patients has been shown to be inhibited by anti-oxidant therapy[184-186]. These findings encourage research to clarify the role of virus induced-oxidative stress as a damaging by-product of infection or a condition required for a successful viral life cycle.

ER STRESS AND ROTAVIRUS INFECTION

Disruption of protein folding homeostasis in the ER leads to unfolded or misfolded protein accumulation in the ER lumen and alteration in the calcium homeostasis. Protein misfolding in the ER contributes to the pathogenesis of many diseases. ER stress of intestinal epithelial cells activates signaling pathways known as unfolded protein response (UPR) which have been associated with inflammatory bowel disease[187]. Alterations in ER homeostasis are normally sensed and followed by activation of the UPR pathway in order to restore homeostasis by activating genes implicated in protein folding. Failure to resolve ER stress causes activation of apoptotic pathways that lead to cell death[188]. Misfolded proteins in the ER activate UPR and induce oxidative stress and apoptosis *in vitro* and *in vivo* in mice, whereas antioxidant treatment counteracts UPR activation, oxidative stress, and apoptosis[189]. Release of calcium from the ER increases the generation of mitochondrial ROS leading to toxic accumulation of ROS within ER and mitochondria. On the other hand, sustained ER stress has been found to potentially stimulate inflammatory response through UPR pathways. Moreover, ROS produced as a consequence of inflammation or mitochondrial dysfunction could aggravate ER malfunction[190]. This picture suggests that a networking occurs between ER stress, inflammation and oxidative stress. Dysfunctional UPR pathways have been associated with numerous diseases including several neurodegenerative diseases, stroke, metabolic disorders, cancer, inflammatory disease, diabetes mellitus, cardiovascular disease, among others[190]. A crosstalk between ROS generation and ER stress response has been proposed as the ER-stress-associated redox status may be correlated with ER-stress-associated ROS[191]. Although the production of ROS has been correlated with ER stress in many pathological states, the detailed mechanisms on how changes in the protein-folding environment in the ER lumen cause oxidative stress are still unclear[192].

Proper protein folding and disulfide bond formation

that take place in the ER are critically dependent on the redox status of the ER lumen. This compartment is highly oxidizing showing a high ratio of GSSG/GSH, which contrasts with the citosol environment[193]. The oxidizing environment of ER lumen is required to ensure disulfide bond formation and avoid aggregation or unfolded protein accumulation in this compartment[194]. Resident enzymes of the ER lumen contribute to regulate redox status and facilitate disulfide bond formation and isomerization[195]. The oxidative folding of proteins is facilitated by a family of ER oxidoreductases including ERp57 and PDI among others[196]. Oxidative folding catalyzed by ER oxidoreductases leads to their reduction, whereas their reoxidation is performed by ERO-1, an enzyme that can use molecular oxygen as an electron acceptor[191,197]. Then, disulfide bond formation catalyzed by ERO-1 is a significant source of the total generation of ROS in the cell as the incomplete oxygen reduction leads to the anion superoxide formation[198].

Given that in the ER occurs the major synthesis and folding of secreted and transmembrane proteins, alterations in the protein flux into the ER have been associated ER stress. Recent studies have shown that viroporins, small hydrophobic virus-encoded proteins that oligomerize to form aqueous pores through cellular membranes, play important role in virus replication by affecting normal physiology of host cell and contributing to viral pathogenesis[199,200]. Since replication of most RNA viruses occurs in intimate interaction with the ER and causes ER stress in the infected cells, its underlying mechanisms are a central issue of the research about virus-host interactions. Many viroporins localize to the ER where alter the membrane potential of the ER and modulate the ER stress response and autophagy induction[201]. Viral infections can act as stress signals that alter ER homeostasis affecting negatively ER functions[151,202]. Many viruses have been shown to cause ER stress and induce one or more branches of the UPR in the infected cells[199,203]. Some studies have shown that several viruses induce ER stress and UPR signaling but also modulate UPR for protecting the infected cells from ER stress-mediated death to ensure virus replication[204-206]. Several properties of the viroporins suggest that they might also modulate the virus-induced ER stress response[207]. The relatively high concentration of Ca^{2+} in the ER lumen is needed for proper functioning of many calcium dependent chaperones and enzymes including PDI[208]. Many viroporins, including rotavirus NSP4, induce leakage of ER luminal calcium into the cytosol[209,210] affecting the calcium-dependent protein folding machinery and consequentially inducing ER stress. Translocation of NSP4 to mitochondria has been observed to dissipate mitochondrial membrane potential and induce apoptosis during the early infection. However, the pro-apoptotic activity of NSP4 was counteracted by NSP1, which activates PI3K/AKT[211]. In addition, autophagy could be induced by the increased Ca^{2+} concentration in the cytosol as it has been shown for foot and mouth disease virus[212]. Rotavirus NSP4, a protein inducing diarrhea in young mice, has been shown to anchor to the ER through its N-terminus, where its domain spanning amino acids 47-90 has been found to insert into ER membrane and show structural characteristics of viroporins[209]. NSP4 has also been shown to modulate autophagy induction in the virus-infected cells as cellular autophagy is required by rotaviruses to ensure their successful replication[213].

Rhesus rotavirus (RRV) has shown to induce ER stress in the rhesus monkey epithelial cell line MA104 and also activate two components of the UPR pathway[214]. However, this ER-mediated signaling was interrupted at the transcription level by the non-structural protein 3 (NSP3). Specific virus-encoded proteins have been identified as inducers of UPR during infection in the case of coronavirus[215], dengue 2 virus[216], human cytomegalovirus[217] and West Nilo virus[218]. In contrast, a single specific virus protein in RRV-infected MA104 cells did not trigger the activation of UPR. It was supposed that a multifactorial event involving either the budding of the DLPs into the ER, the formation of viroplasms, or the activation of genome replication could be the inducer of the UPR[214].

Rotavirus infection has been shown to induce ER stress leading to disturbances in the cellular calcium compartments and generation of ROS. Rotavirus-induced diarrhea involves a series of secretory and osmotic mechanisms[219] where NSP4 plays a key role by inducing release of intracellular deposits of calcium from enterocytes[220,221] and altering ion secretion[222]. It has been reported a NSP4-dependent chloride secretion in human enterocytes[223], which has also been demonstrated in Caco-2 cells infected with SA11 rotavirus[224]. In this case, NSP4-dependent chloride secretion was associated with an increase in ROS and a decreased reduced (GSH) to oxidized (GSSG) ratio. The same effects were observed when Caco-2 cells were treated with purified NSP4, whereas the increase in ROS and the GSH imbalance were strongly inhibited by N-acetylcysteine. These findings suggested an association between oxidative stress and rotavirus-induced diarrhea[224]. There are data supporting the hypothesis that ROS can induce intestinal epithelial cell apoptosis in mice through the Fas and Fas-L expression[225].

A number of stimuli and insults, including pathogen invasions such as virus infections have been found to induce ER stress affecting protein folding function and other disturbances including alterations in calcium homeostasis and increase of ROS. Then, the ER-induced UPR signaling has emerged as a central subject in the context of pathological processes including virus infections. However, the UPR-associated molecular mechanisms leading to minimize the accumulation and aggregation of misfolded proteins in response to virus infections need further investigation to be completely understood. The knowledge gained from UPR mechanisms could provide basis for antiviral development.

N-ACETYLCYSTEINE IN THE TREATMENT OF VIRAL INFECTIONS

N-acetylcysteine (NAC) is an amino acid that functions as a cysteine pro-drug and glutathione (GSH) precursor, the most powerful cellular antioxidant[226]. NAC is readily deacetylated primarily in the liver to yield L-cysteine thereby promoting intracellular GSH synthesis[227]. It has been used during several decades as mucolytic agent and also for the treatment of various disorders including paracetamol intoxication[228]. NAC has been also used for treatment of numerous disorders linked to oxidative stress including gastrointestinal[229], renal[230], cardiovascular[231], pulmonary[232], hepatic[233], psychiatric and neurological disorders. The mucolytic activity of NAC is due to its ability to break up the disulfide bonds of the high molecular weight glycoproteins present in the mucus. NAC functions in cells as a free radical scavenger antioxidant agent as it reacts with ROS such as H_2O_2 and OH^-[234].

NAC has been used in the treatment numerous infectious diseases, including virus infections. A significant reduction of the incidence of clinical symptoms and improvement of cell-mediated immunity were reported after treatment with NAC[235]. Similarly, GSH has been reported to inhibit infection by influenza virus in both cultured cells and mice[236]. High doses of NAC have proven to be synergistic with oseltamivir treatment in protecting mice from fatal influenza infection[237], whereas a synergistic combination of NAC and ribavirin was also effective in preventing mice from lethal influenza virus infection[238]. A long-term NAC administration attenuated influenza symptoms in elderly patients with chronic degenerative disease[235]. In addition, a patient infected with the A/H1N1 influenza virus improved rapidly after treatment with a high-dose NAC therapy in combination with antiviral medication[239]. NAC has been shown to reduce H5N1-induced cytopathic effects, virus-induced apoptosis and the production of some pro-inflammatory molecules whereas it inhibited the activation of oxidant sensitive pathways including NF-κB and mitogen activated protein kinase p38[240]. However, a universal inhibitory activity against influenza A viruses has not yet been demonstrated[241]. A successful outcome was reported following early administration of NAC to children affected with dengue hemorrhagic fever or dengue shock syndrome complicated by acute liver failure[242]. Before highly active antiretroviral therapy (HAART), NAC was tested to replenish GSH levels in HIV-infected patients since cysteine and GSH levels decrease as the HIV disease progresses[243,244]. However, NAC has been offered as a useful adjunct therapy to increase protection against oxidative stress, improve immune system function and increase detoxification of acetaminophen and other drugs in patients treated with HAART[245].

The sensitivity of rotavirus infection to NAC has recently been demonstrated. A study that screened for drugs with the potential ability to interfere with cellular redox reactions, found that infection of MA104 and Caco-2 cells with several rotavirus strains was significantly inhibited by NAC in both cell systems[184]. On the other hand, the rotavirus NSP4-induced chloride secretion has been shown to be inhibited by pre-treating Caco-2 cell with NAC, suggesting that the enterotoxic effect of NSP4 is stress oxidative-dependent[224]. Inhibition of rotavirus infection by NAC was further demonstrated in ICR mice infected with rotavirus ECwt as the percentage of viral antigen-positive villus cells was significantly decreased by NAC treatment[185]. The use of NAC as a therapeutic tool for treatment of rotavirus disease in children was also demonstrated. Administration of NAC after the first diarrheal episode was shown to decrease the number of diarrheal episodes, excretion of fecal rotavirus antigen, and resolution of symptoms after 2 d of treatment[186].

There is demonstration that TNF-α stimulates HIV transcription through activation of NF-κB[246] and that this stimulation is inhibited by NAC treatment[247]. It has been also found that intracellular thiols regulate NF-κB activation since low thiol levels lead to its activation whilst high thiols levels inhibit its activation[248]. NAC has been shown to be a potent inhibitor of NF-κB activation in terms of inhibiting its nuclear migration and DNA binding activity in vascular endothelial cells[249]. NAC and other antioxidants have been reported to inhibit hydrogen peroxide-induced NF-κB activation[45]. Moreover, NAC has been reported to block NF-κB activation by interfering with IκB kinase (IKK) activation and inhibitor of κB phosphorylation, which suggested that ROS could be ubiquitous mediators of NF-κB activation[250,251]. However, it has been reported that NAC inhibits NF-κB activation in Hela and L929 cells independently of its anti-oxidative function. NAC seems to block selectively TNF-induced signaling by decreasing the affinity of receptor for TNF[252]. The NAC inhibitory effect on NF-κB activation appears to be a well established fact.

It has been reported that NAC inhibits the expression of VCAM-1 by interfering with the binding of NF-κB to the VCAM-1 κB motif[253,254]. Many harmful effects of TNF-α associated to endothelial dysfunction have been partially prevented by increasing GSH through NAC treatment[255]. NAC has also been reported to completely inhibit ROS, JNK and NF-κB activation induced by leptin, suggesting that hyperleptinemia is sensitive to redox signaling[256]. Attenuation of TNF-α-induced p38 mitogen-activated protein kinase (MAPK) activity in pulmonary vascular endothelial cells was obtained by NAC treatment, suggesting that p38 MAPK pathway is regulated by redox environment[257]. Activation of NF-κB in response to various signals, including IL-1, TNF and H_2O_2 can be inhibited by NAC treatment, suggesting that ROS are common signaling modulators[258]. Moreover, NAC was found to enhance the effect of IFN-α on liver tumor cells through inhibition of NF-κB[259]. On the other hand, some studies suggested that NAC inhibits the upstream IKK activation induced by TNF-α[260].

The NAC antiviral activity has mainly been associated with inhibition of pro-inflammatory molecules including those belonging to the NF-κB pathway and its associated

generation of ROS. These findings suggest that inflammatory and oxidative stress pathways are intimately involved in the virus infection-associated pathogenesis. Nevertheless, the underlying mechanisms of NAC treatment of virus infections need further research in order to differentiate the direct and indirect effects associated with its antioxidant ability. A probable direct effect on disulfide bonds harbored in cellular and virus-encoded proteins cannot be excluded.

ROTAVIRUS INFECTION AND INFLAMMATORY SIGNALING

RNAs from rotavirus replication are sensed by RIG-I and MDA-5, which result in induction of an IFN-mediated innate immune response involving the activation of IRF3[48,49]. However, NSP1 from group A rotavirus is involved in evading innate immune response by antagonizing the induction of IFN and IFN-stimulated gene (*ISG*) products[261,262]. Rotavirus NSP1 has been shown to be involved in the evasion of innate immune response by interfering with the induction of IFN *via* induction of the degradation of IRF-3, IRF-5 and IRF-7. NSP1 from several rotavirus strains has been shown to target IRF3 for proteasome degradation during early post-infection[263-265]. Recent studies indicated that NSP1 can induce degradation of IRF proteins (IRF3 to IRF9) by targeting their IRF association domains needed for their dimerization and nuclear translocation[261]. Nevertheless, there is evidence indicating that NSP1 from some rotavirus strains such as OSU is inefficient in degrading IRF-3[266]. It has been reported that IRF3 is activated and remains stable in cells following infection with porcine rotavirus strain OSU. An alternative mechanism for blocking induction of IFN-β by rotavirus strain OSU has been recently reported[266]. Results from this work showed that NF-κB activation was blocked in cells infected with rotavirus strain OSU due in part to stabilization of phosphorylated IκBα. It was found that the $SCF^{\beta\text{-TrCP}}$ E3 ligase was targeted for proteosome degradation by NSP1, which provided an explanation for the IκBα stabilization and the consequent absence of NF-κB activation in virus-infected cells. Most human group A rotaviruses encode NSP1s that contain a C-terminal recognition motif (DSGxS) for β-transducing repeat-containing protein (β-TrCP)[267]. This feature allows NSP1 to inhibit NF-κB activation by inducing proteasome-dependent degradation of β-TrCP[266]. Many NSP1s from group A rotaviruses that lack the β-TrCP recognition motif are then able to induce the degradation of IRF3, IRF5 and IRF7[261,263].

Regarding that rotaviral RNAs have the potential of triggering activation of IFN, it has been also suggested that sequestering of viral RNAs in the viroplasms and in the progeny capsids could contribute to delay the antiviral innate response in rotavirus-infected cells[50]. Moreover, it has been shown that infection of MA104 cells with rotavirus RRV is able to block expression of NF-κB-dependent gene expression without reducing NF-κB activation. This suggested that rotavirus can efficiently activate NF-κB in MA104 cells although this activated transcription factor was not functional in enhancing gene expression[268]. On the other hand, the role of NSP1 has been studied in modulation of apoptosis and it has been found that NSP1 contributes to the establishment and replication of bovine rotavirus wild type A5-13 in MA104 cells by inhibiting apoptosis through the activation of the pro-survival pathways PI3K/Akt and NF-κB during early infection stages[269].

Studies on malnutrition and concomitant rotavirus infection in neonatal piglets have suggested an inflammatory response during rotavirus infection. It has been shown that concentrations of intestinal prostaglandin E2 (PGE2) were elevated early after rotavirus infection regardless of nutritional state[270]. However, malnutrition increased PGE2 response to rotavirus infection while prolonged diarrhea in rotavirus infected and malnourished piglets was found to be associated with more intense and sustained expression of local mediators or markers of intestinal inflammation[270]. Rotavirus pro-inflammatory actions have been suggested based on studies in which the rotavirus infection of cultured cells or mice was significantly inhibited by treatment with various peroxisome proliferator-activated receptor gamma (PPARγ) agonists and nonsteroidal antiinflammatory drugs (NSAIDs)[184,185,271]. Cyclooxygenase-2 (COX-2), which is responsible for increased synthesis of prostaglandins[272], seems to be mainly regulated by various MAPKs and transcription factors such as NF-κB[273,274]. Moreover, PKA-mediated ERK1/2 and NF-κB pathways have been shown to be involved in the COX activity induction during rotavirus infection[271]. The nonspecific COX inhibitor indomethacin has been shown to significantly reduced rotavirus Wa infection of Caco-2 cells. Similarly, inhibition of the ERK1/2 and p38 MAPK pathways resulted in a significant decrease of rotavirus infection of Caco-2 cells[271]. Antiviral effects have been obtained by treatment with COX-2 inhibitors[275,276]. PPARγ ligands have been found to downregulate the transcriptional activation of COX-2 through multiple mechanisms[277], including the inhibition of multiple steps of the NF-κB pathway[278]. Evidence has been provided that rotavirus infectivity in MA104 and Caco-2 cells and mice is significantly inhibited not only by NAC, but also by pioglitazone and rosiglitazone which are drugs affecting the NF-κB pathway involved in the COX-2 transcriptional activation[184,185]. PPAR-γ agonists have been highlighted as potential therapeutic tools due to their ability to down-regulate the inflammatory responses to respiratory virus-related pulmonary inflammation[279]. PPARs participate antagonizing oxidant and inflammatory pathways such as NF-κB, AP1, and STAT[280,281]. Down-regulation of these signaling pathways by thiazolidine-2-4-diones (TZDs), including pioglitazone and rosiglitazone, has led to reduced levels of oxidative products in monocyte/macrophages[282]. PPARγ has emerged as an anti-inflammatory and anti-oxidant gene since its encoded product may directly modulate the expression of several antioxidant and pro-oxidant genes in response to oxidative stress[283-285].

However, it should be noted that oxidants such as ROS could interact with NF-κB signaling pathways in many ways. The transcription of genes depending on NF-κB influences the ROS levels, and in turn, the ROS levels also regulate the NF-κB activity levels. It has been argued that ROS influence is context-dependent and even cell-type specific being either positive or negative for NF-κB signaling[286].

Clearly rotavirus NSP1 has been implicated in down-regulating interferon expression being a key factor in the evasion of host innate immune response. However, the NSP1 mechanism for anti-interferon activity seems to be rotavirus strain-dependent. A more comprehensive understanding of the rotavirus pro-inflammatory actions could lead to identification of potential targets of anti-inflammatory therapeutics. Cellular innate response to rotavirus infection is schematized in Figure 3.

ROTAVIRUS INFECTION AND PROTEIN SYNTHESIS

Viruses are fully dependent on the host cell translation machinery to produce their proteins needed for viral replication. Viruses take control of host ribosomes, translation factors and signaling pathways involved in protein synthesis. This control ensures the production of virus-encoded proteins and the inhibition of cellular innate defenses[287]. Most cellular mRNAs use a cap-dependent mechanism for their translation that involves the binding of a complex termed eIF4F comprised of eukaryotic initiation factors eIF4G, eIF4E and eIF4A to cap structure located at the 5′ end of the mRNA. However, some cellular and many viral mRNAs use a cap-independent mechanisms for initiating translation that involves an internal ribosome-entry site located in the 5′untranslated region of mRNAs that is use during ER stress[288]. Viruses have evolved a wide range of strategies for exploiting and controlling the cellular translation machinery. Several virus-encoded functions are dedicated to controlling the cellular translation machinery including its initiation, elongation and termination steps[289].

Early in the infection process rotaviruses takes over the host cell translation machinery, inducing a shut off of host cell-directed protein synthesis although not all cellular proteins stop being synthesized[290]. Rotavirus NSP3 has been implicated in the inhibition of cellular mRNA translation by binding to eIF4G or interfering with the shuttling of nascent cellular mRNAs[291,292]. Binding of NSP3 to eIF4G disturbs its interaction with poly(A)-binding protein which is required for the initiation of cellular mRNA translation. However, siRNA-mediated knockdown of NSP3 expression and a NSP3 defective mutant failed to interfere rotavirus-directed synthesis and its replication[293,294]. Rotavirus-induced phosphorylation of eIF2α in a double-stranded PKR-dependent manner has been reported to inhibit cellular translation[295]. However, the presence of naked RNA in rotavirus infected cells as part of the viral cycle is an unresolved question[50].

Increase of jejunal protein synthesis in rotavirus-infected piglets has been reported[296,297]. It was hypothesized that this increased protein synthesis was mainly due to actively proliferating enterocytes differentiating and migrating up the villus. However, it was suggested that rotavirus activates mTOR signaling through $p70^{S6K}$ since rotavirus-induced mobilization of calcium has been shown to be a stimulator of $p70^{S6K}$[298]. However, in these studies it was not specified whether the increased protein synthesis was either cell or virus-directed. On the other hand, expression of some specific cellular proteins is increased during viral infections[299,300]. COX-2, an enzyme induced by pro-inflammatory agents, has been reported to be increased in infections caused by RSV[301], gammaherpesvirus 68[302], influenza virus[275], herpes simplex virus[301], and EMCV[303]. Despite Hsc70 is a constitutively expressed protein, it has been shown to be increased following infection with SV40[304], Autographa californica multiple nucleopolyhedrovirus[305], JEV[78], and white spot syndrome virus[306]. Evidence has been shown that rotavirus ECwt infection of mice induce cellular proteins COX-2, ERp57, Hsc70, NF-κB, Hsp70, PDI and PPARγ in intestinal villus cells, whereas NAC treatment of infected cells reduced Hsc70 and PDI to expression levels similar to those observed in villi from uninfected control mice[185]. The virus-associated increased expression of these cellular proteins adds evidence suggesting that rotavirus infection benefits from inducing oxidative stress and activating pro-inflammatory signaling in villus cells since treatment of rotavirus infected mice with NAC, NSAIDs or PPARγ agonists led to significantly reduced infection[185]. Inhibition of rotavirus infection by treatment with antioxidants, NSAIDs and PPARγ agonists are schematized in Figure 4.

The dependence of viruses on the host translation machinery imposes the recruitment of ribosomes for the translation of their functions and inhibition of the cellular innate defenses. More studies are needed to understand the detailed mechanisms involved in the strategy by which rotaviruses induce the shutoff of host protein synthesis machinery.

CONCLUSION

Despite the advances made over the past decade in the understanding of mechanisms explaining rotavirus infection, there are many unanswered questions regarding entry and internalization processes of rotavirus. A relevant question is whether rotaviruses have alternative entry pathways since inhibition of any of the proposed receptors failed to interfere completely with the viral infectivity. It is tempting to hypothesize that rotaviruses seem to have evolved to enter the target cell using three different types of cell surface molecules: (1) binding molecules represented by SA and some integrins; (2) chaperoning molecules including Hsc70 and other heat shock proteins; and (3) redox molecules such as PDI, Erp57 and other related thioredoxins. The current proposed receptors for rotavirus fall into these major categories of molecules supporting

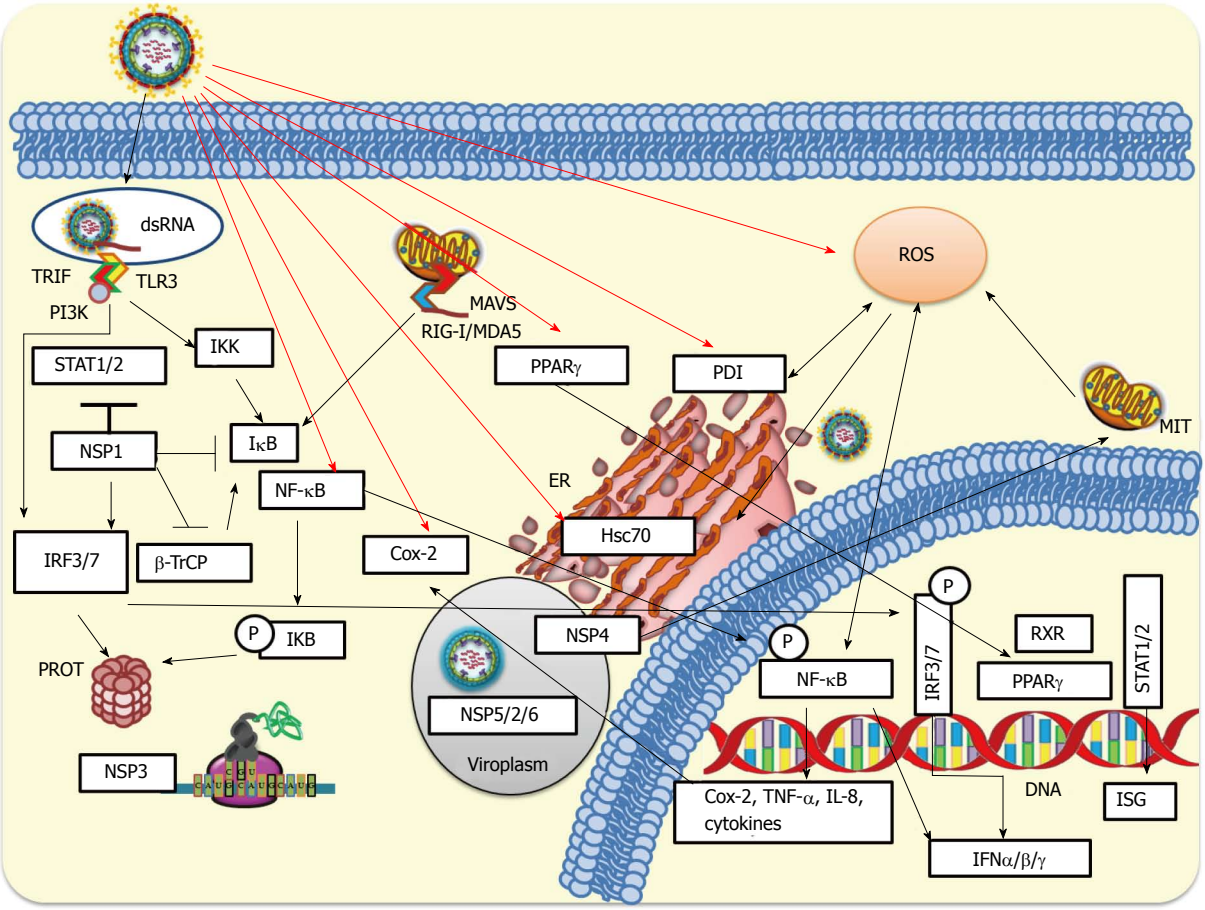

Figure 3 Cellular innate response to rotavirus infection. During rotavirus internalization viral nucleic acid may be exposed and recognized by either Toll-like receptors (TLR3) or intracellular RIG-I-like receptors (RLRs). Activated RLRs can bind and activate mitochondrial antiviral-signaling protein (MAVS), which recruits a signaling complex needed to activate cytoplasmic transcription factors including interferon regulatory factor 3 (IRF3) and nuclear factor-κB (NF-κB). On the other hand, activation of endosomal TLR3 facilitates the adaptor TRIF recruitment, which allows the recruitment of signaling molecules such as IKKs that phosphorylate IRF3 or NF-κB. Phosphorylated IRF3 is dimerized and then translocated to the nucleus. Signaling pathways induced by rotavirus infection produce phosphorylation of IκB (inhibitor of NF-κB) and its subsequent ubiquitination and proteasomal degradation mediated by SCFβ-TrCP E3 ligase. This signaling pathway leads to NF-κB translocation to the nucleus where, jointly with IRF3 and IRF7, binds to the interferon (IFN)-β promoter for transcription of IFN-β mRNA. Rotavirus can early counteract signaling pathways of innate response by NSP1-mediated degradation of IRF3 and IRF7. NSP1 encoded by some rotavirus strains can target SCFβ-TrCP for proteasomal degradation, whereas NSP1 from other strains has been implicated in the direct inhibition of the IFN-mediated STAT1 activation. NSP3 can interfere with the translation of cellular-encoded proteins including those induced by the IFN signaling. The viroplasm, which includes some viral non-structural proteins (NSP2/5/6), can protect viral RNAs from being recognized by some pattern-recognition receptors (RIG-I, MDA-5, among others) involved in antiviral response. MIT, ER and PROT are indicated. IKK: IκB kinase; MIT: Mitochondria; ER: Endoplasmic reticulum; PROT: Proteasome; TNF: Tumor necrosis factor.

entry mechanisms. It should not be excluded that other molecules, as yet undiscovered, could also perform the same functions in other cell types and for other rotavirus strains. Within this line of reasoning, it appears to be a universal mechanism for rotavirus entry, but the receptor molecules executing the entry mechanism might differ partially or wholly depending on the species, cell line and rotavirus strain. The receptor usage and tropism of rotaviruses would be determined by the relative abundance and physical proximity of the receptors in the host cell surface. Rotavirus structural proteins implicated in the early steps of the rotaviral life cycle are likely to be substrates of the cell surface molecules having oxidoreductase, thiol isomerase and chaperone activities which would be responsible for the conformational changes these viral interacting proteins need for ensuring internalization. Future research should emphasize the elucidation of the reason why many receptors are used by rotaviruses. The fact that rotaviruses induce oxidative stress and inflammatory signaling offers an opportunity for the development of novel therapeutic strategies aimed at interfering with rotavirus infection. The use of NAC, NSAIDs and PPARγ agonists to inhibit rotavirus infection opens a new way for treating the life-threatening rotavirus diarrhea and complementing vaccines. However, a major gap in the understanding of the rotavirus infectious strategy is the fact that rotavirus seems to antagonize the pro-inflammatory signaling in order to ensure replication but anti-inflammatory treatment inhibits virus infection. This gap poses a substantial challenge because a more detailed characterization of the molecular mechanisms underpinning rotavirus-induced inflammatory signaling is needed. Another unsolved issue is that the rotavirus-induced oxidative stress, seemingly at the same time, influences positively and negatively NF-κB signaling,

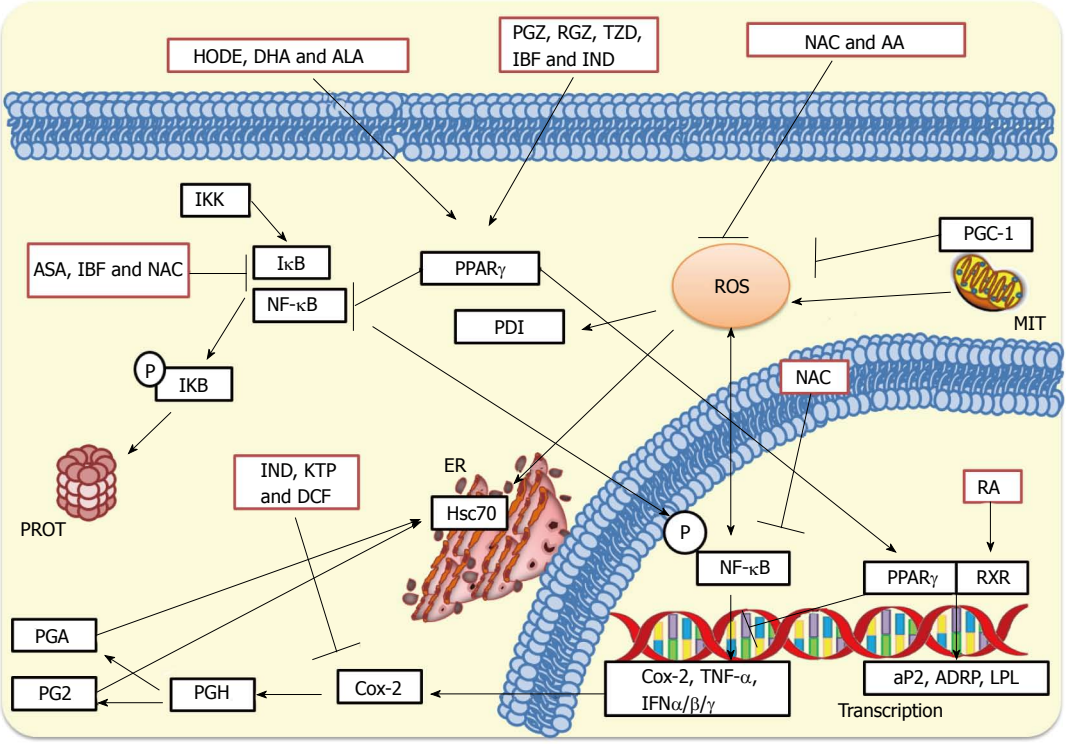

Figure 4 Inhibition of rotavirus infection by treatment with antioxidants, nonsteroidal antiinflammatory drugs and peroxisome proliferator-activated receptor gamma agonists. NAC and AA can inhibit the production of ROS, whereas NAC can also affect IκB preventing the cytoplasmic activation of NF-κB. NAC can further inhibit nuclear phosphorylated NF-κB preventing the transcription of pro-inflammatory genes. NSAIDs such as KTP, IND and DCF inhibit Cox-2 leading to a significant inhibition of prostaglandin accumulation. On the other hand, ASA and IBF inhibit activation of NF-κB suppressing the transcription of IFN-α, IFN-β and IFN-γ, cytokines and interleukins. These NSAID treatments significantly inhibit rotavirus infections in cultured cells and mice. PPARγ agonists such as 13(S)-hydroxyoctadecadienoic acid (HODE), ALA and DHA, and thiazolidinediones such as PGZ, RGZ, and 2, 4-thiazolidinedione (TZD) activate PPARγ leading to inhibition of cytoplasmic NF-κB. PPARγ can heterodimerize with the RA-activated RXR for promoting transcription of anti-inflammatory genes. This complex can also cause inhibition of phosphorylated NF-κB which in turn leads to decreased transcription of pro-inflammatory genes. MIT, ER, and PROT are indicated. NAC: N-acetylcysteine; NSAIDs: Nonsteroidal antiinflammatory drugs; PPARγ: Peroxisome proliferator-activated receptor gamma; AA: Ascorbic acid; ROS: Reactive oxygen species; NF-κB: Nuclear factor-κB; KTP: Ketoprofen; IND: Indomethacin; DCF: Diclofenac; Cox-2: Cyclooxygenase-2; ASA: Acetylsalicylic acid; IBF: Ibuprofen; IFN-α: Interferon-α; ALA: Alpha-linolenic acid; DHA: Docosahexaenoic acid; PGZ: Pioglitazone; RGZ: Rosiglitazone; RA: Retinoic acid; RXR: Retinoid X receptor; MIT: Mitochondria; ER: Endoplasmic reticulum; PROT: Proteasome.

whereas antioxidant treatment inhibits virus infection.

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REFERENCES

1 **Parashar UD**, Hummelman EG, Bresee JS, Miller MA, Glass RI. Global illness and deaths caused by rotavirus disease in children. *Emerg Infect Dis* 2003; **9**: 565-572 [PMID: 12737740 DOI: 10.3201/eid0905.020562]

2 **Tate JE**, Burton AH, Boschi-Pinto C, Steele AD, Duque J, Parashar UD. 2008 estimate of worldwide rotavirus-associated mortality in children younger than 5 years before the introduction of universal rotavirus vaccination programmes: a systematic review and meta-analysis. *Lancet Infect Dis* 2012; **12**: 136-141 [PMID: 22030330 DOI: 10.1016/s1473-3099(11)70253-5]

3 **Yeager M**, Dryden KA, Olson NH, Greenberg HB, Baker TS. Three-dimensional structure of rhesus rotavirus by cryoelectron microscopy and image reconstruction. *J Cell Biol* 1990; **110**: 2133-2144 [PMID: 2161857]

4 **Settembre EC**, Chen JZ, Dormitzer PR, Grigorieff N, Harrison SC. Atomic model of an infectious rotavirus particle. *EMBO J* 2011; **30**: 408-416 [PMID: 21157433 DOI: 10.1038/emboj.2010.322]

5 **McClain B**, Settembre E, Temple BR, Bellamy AR, Harrison SC. X-ray crystal structure of the rotavirus inner capsid particle at 3.8 A resolution. *J Mol Biol* 2010; **397**: 587-599 [PMID: 20122940 DOI: 10.1016/j.jmb.2010.01.055]

6 **Haselhorst T**, Fleming FE, Dyason JC, Hartnell RD, Yu X, Holloway G, Santegoets K, Kiefel MJ, Blanchard H, Coulson BS, von Itzstein M. Sialic acid dependence in rotavirus host cell invasion. *Nat Chem Biol* 2009; **5**: 91-93 [PMID: 19109595 DOI: 10.1038/nchembio.134]

7 **Guerrero CA**, Bouyssounade D, Zárate S, Isa P, López T, Espinosa R, Romero P, Méndez E, López S, Arias CF. Heat shock cognate protein 70 is involved in rotavirus cell entry. *J Virol* 2002; **76**: 4096-4102 [PMID: 11907249]

8 **Zárate S**, Cuadras MA, Espinosa R, Romero P, Juárez KO, Camacho-Nuez M, Arias CF, López S. Interaction of rotaviruses with Hsc70 during cell entry is mediated by VP5. *J Virol* 2003; **77**: 7254-7260 [PMID: 12805424]

9 **Zárate S**, Romero P, Espinosa R, Arias CF, López S. VP7 mediates the interaction of rotaviruses with integrin alphavbeta3 through a novel integrin-binding site. *J Virol* 2004; **78**: 10839-10847 [PMID: 15452204 DOI: 10.1128/JVI.78.20.10839-10847.2004]

10 **Graham KL**, Halasz P, Tan Y, Hewish MJ, Takada Y, Mackow ER, Robinson MK, Coulson BS. Integrin-using rotaviruses bind alpha2beta1 integrin alpha2 I domain via VP4 DGE sequence and recognize alphaXbeta2 and alphaVbeta3 by using VP7 during

cell entry. *J Virol* 2003; **77**: 9969-9978 [PMID: 12941907 DOI: 10.1128/JVI.77.18.9969-9978.2003]
11 **Graham KL**, Fleming FE, Halasz P, Hewish MJ, Nagesha HS, Holmes IH, Takada Y, Coulson BS. Rotaviruses interact with alpha4beta7 and alpha4beta1 integrins by binding the same integrin domains as natural ligands. *J Gen Virol* 2005; **86**: 3397-3408 [PMID: 16298987 DOI: 10.1099/vir.0.81102-0]
12 **Calderon MN**, Guerrero CA, Acosta O, Lopez S, Arias CF. Inhibiting rotavirus infection by membrane-impermeant thiol/disulfide exchange blockers and antibodies against protein disulfide isomerase. *Intervirology* 2012; **55**: 451-464 [PMID: 22398681 DOI: 10.1159/000335262]
13 **Lawton JA**, Estes MK, Prasad BV. Three-dimensional visualization of mRNA release from actively transcribing rotavirus particles. *Nat Struct Biol* 1997; **4**: 118-121 [PMID: 9033591]
14 **Petrie BL**, Greenberg HB, Graham DY, Estes MK. Ultrastructural localization of rotavirus antigens using colloidal gold. *Virus Res* 1984; **1**: 133-152 [PMID: 6099654]
15 **Campagna M**, Eichwald C, Vascotto F, Burrone OR. RNA interference of rotavirus segment 11 mRNA reveals the essential role of NSP5 in the virus replicative cycle. *J Gen Virol* 2005; **86**: 1481-1487 [PMID: 15831961 DOI: 10.1099/vir.0.80598-0]
16 **López T**, Camacho M, Zayas M, Nájera R, Sánchez R, Arias CF, López S. Silencing the morphogenesis of rotavirus. *J Virol* 2005; **79**: 184-192 [PMID: 15596814 DOI: 10.1128/jvi.79.1.184-192.2005]
17 **Estes M**, Kapikian A. Rotaviruses. In: Knipe D, Griffin D, Lamb R, Martin M, Roizman B, Straus S, editors. Fields of Virology. 5th ed. Philadelphia: Kluwer/Lippincott Williams and Wilkins, 2007: 1917-1975
18 **Cui H**, Kong Y, Zhang H. Oxidative stress, mitochondrial dysfunction, and aging. *J Signal Transduct* 2012; **2012**: 646354 [PMID: 21977319 DOI: 10.1155/2012/646354]
19 **Kregel KC**, Zhang HJ. An integrated view of oxidative stress in aging: basic mechanisms, functional effects, and pathological considerations. *Am J Physiol Regul Integr Comp Physiol* 2007; **292**: R18-R36 [PMID: 16917020 DOI: 10.1152/ajpregu.00327.2006]
20 **Valko M**, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 2007; **39**: 44-84 [PMID: 16978905 DOI: 10.1016/j.biocel.2006.07.001]
21 **Jorgenson TC**, Zhong W, Oberley TD. Redox imbalance and biochemical changes in cancer. *Cancer Res* 2013; **73**: 6118-6123 [PMID: 23878188 DOI: 10.1158/0008-5472.can-13-1117]
22 **Uttara B**, Singh AV, Zamboni P, Mahajan RT. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Curr Neuropharmacol* 2009; **7**: 65-74 [PMID: 19721819 DOI: 10.2174/157015909787602823]
23 **Oyinloye BE**, Adenowo AF, Kappo AP. Reactive oxygen species, apoptosis, antimicrobial peptides and human inflammatory diseases. *Pharmaceuticals* (Basel) 2015; **8**: 151-175 [PMID: 25850012 DOI: 10.3390/ph8020151]
24 **Jones DP**. Redefining oxidative stress. *Antioxid Redox Signal* 2006; **8**: 1865-1879 [PMID: 16987039 DOI: 10.1089/ars.2006.8.1865]
25 **Sies H**. Oxidative stress: Introductory remarks. In: Sies H, editor. Oxidative Stress. London: Academic Press, 1985: 1-8
26 **van der Flier LG**, Clevers H. Stem cells, self-renewal, and differentiation in the intestinal epithelium. *Annu Rev Physiol* 2009; **71**: 241-260 [PMID: 18808327 DOI: 10.1146/annurev.physiol.010908.163145]
27 **Circu ML**, Aw TY. Intestinal redox biology and oxidative stress. *Semin Cell Dev Biol* 2012; **23**: 729-737 [PMID: 22484611 DOI: 10.1016/j.semcdb.2012.03.014]
28 **Ramig RF**. Pathogenesis of intestinal and systemic rotavirus infection. *J Virol* 2004; **78**: 10213-10220 [PMID: 15367586 DOI: 10.1128/jvi.78.19.10213-10220.2004]
29 **Jones DP**, Go YM. Redox compartmentalization and cellular stress. *Diabetes Obes Metab* 2010; **12** Suppl 2: 116-125 [PMID: 21029308 DOI: 10.1111/j.1463-1326.2010.01266.x]
30 **Aw TY**, Williams MW. Intestinal absorption and lymphatic transport of peroxidized lipids in rats: effect of exogenous GSH. *Am J Physiol* 1992; **263**: G665-G672 [PMID: 1443140]
31 **Shan XQ**, Aw TY, Jones DP. Glutathione-dependent protection against oxidative injury. *Pharmacol Ther* 1990; **47**: 61-71 [PMID: 2195557 DOI: 10.1016/0163-7258(90)90045-4]
32 **Aw TY**, Wierzbicka G, Jones DP. Oral glutathione increases tissue glutathione in vivo. *Chem Biol Interact* 1991; **80**: 89-97 [PMID: 1913980 DOI: 10.1016/0009-2797(91)90033-4]
33 **Dahm LJ**, Jones DP. Rat jejunum controls luminal thiol-disulfide redox. *J Nutr* 2000; **130**: 2739-2745 [PMID: 11053515]
34 **Kawai T**, Akira S. Antiviral signaling through pattern recognition receptors. *J Biochem* 2007; **141**: 137-145 [PMID: 17190786 DOI: 10.1093/jb/mvm032]
35 **Kawai T**, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* 2010; **11**: 373-384 [PMID: 20404851 DOI: 10.1038/ni.1863]
36 **Katze MG**, He Y, Gale M. Viruses and interferon: a fight for supremacy. *Nat Rev Immunol* 2002; **2**: 675-687 [PMID: 12209136 DOI: 10.1038/nri888]
37 **Thompson AJ**, Locarnini SA. Toll-like receptors, RIG-I-like RNA helicases and the antiviral innate immune response. *Immunol Cell Biol* 2007; **85**: 435-445 [PMID: 17667934 DOI: 10.1038/sj.icb.7100100]
38 **Mason DR**, Beck PL, Muruve DA. Nucleotide-binding oligomerization domain-like receptors and inflammasomes in the pathogenesis of non-microbial inflammation and diseases. *J Innate Immun* 2012; **4**: 16-30 [PMID: 22067846 DOI: 10.1159/000334247]
39 **Gram AM**, Frenkel J, Ressing ME. Inflammasomes and viruses: cellular defence versus viral offence. *J Gen Virol* 2012; **93**: 2063-2075 [PMID: 22739062 DOI: 10.1099/vir.0.042978-0]
40 **O'Neill LA**, Bowie AG. Sensing and signaling in antiviral innate immunity. *Curr Biol* 2010; **20**: R328-R333 [PMID: 20392426 DOI: 10.1016/j.cub.2010.01.044]
41 **Yanai H**, Mizutani T, Inuzuka T, Honda K, Takaoka A, Taniguchi T. IRF family transcription factors in type I interferon induction. *International Congress* 2005; **1285**: 104-113 [DOI: 10.1016/j.ics.2005.09.010]
42 **Yanai H**, Chen HM, Inuzuka T, Kondo S, Mak TW, Takaoka A, Honda K, Taniguchi T. Role of IFN regulatory factor 5 transcription factor in antiviral immunity and tumor suppression. *Proc Natl Acad Sci USA* 2007; **104**: 3402-3407 [PMID: 17360658 DOI: 10.1073/pnas.0611559104]
43 **Santoro MG**, Rossi A, Amici C. NF-kappaB and virus infection: who controls whom. *EMBO J* 2003; **22**: 2552-2560 [PMID: 12773372 DOI: 10.1093/emboj/cdg267]
44 **Onoguchi K**, Yoneyama M, Fujita T. Retinoic acid-inducible gene-I-like receptors. *J Interferon Cytokine Res* 2011; **31**: 27-31 [PMID: 20950133 DOI: 10.1089/jir.2010.0057]
45 **Gupta SC**, Sundaram C, Reuter S, Aggarwal BB. Inhibiting NF-κB activation by small molecules as a therapeutic strategy. *Biochim Biophys Acta* 2010; **1799**: 775-787 [PMID: 20493977 DOI: 10.1016/j.bbagrm.2010.05.004]
46 **Karin M**, Greten FR. NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol* 2005; **5**: 749-759 [PMID: 16175180 DOI: 10.1038/nri1703]
47 **Magné N**, Toillon RA, Bottero V, Didelot C, Houtte PV, Gérard JP, Peyron JF. NF-kappaB modulation and ionizing radiation: mechanisms and future directions for cancer treatment. *Cancer Lett* 2006; **231**: 158-168 [PMID: 16399220 DOI: 10.1016/j.canlet.2005.01.022]
48 **Broquet AH**, Hirata Y, McAllister CS, Kagnoff MF. RIG-I/MDA5/MAVS are required to signal a protective IFN response in rotavirus-infected intestinal epithelium. *J Immunol* 2011; **186**: 1618-1626 [PMID: 21187438 DOI: 10.4049/jimmunol.1002862]
49 **Sen A**, Pruijssers AJ, Dermody TS, García-Sastre A, Greenberg HB. The early interferon response to rotavirus is regulated by PKR and depends on MAVS/IPS-1, RIG-I, MDA-5, and IRF3. *J Virol* 2011; **85**: 3717-3732 [PMID: 21307186 DOI: 10.1128/jvi.02634-10]

50 **Arnold MM**, Sen A, Greenberg HB, Patton JT. The battle between rotavirus and its host for control of the interferon signaling pathway. *PLoS Pathog* 2013; **9**: e1003064 [PMID: 23359266 DOI: 10.1371/journal.ppat.1003064]

51 **Deal EM**, Jaimes MC, Crawford SE, Estes MK, Greenberg HB. Rotavirus structural proteins and dsRNA are required for the human primary plasmacytoid dendritic cell IFNalpha response. *PLoS Pathog* 2010; **6**: e1000931 [PMID: 20532161 DOI: 10.1371/journal.ppat.1000931]

52 **Lopez-Guerrero DV**, Meza-Perez S, Ramirez-Pliego O, Santana-Calderon MA, Espino-Solis P, Gutierrez-Xicotencatl L, Flores-Romo L, Esquivel-Guadarrama FR. Rotavirus infection activates dendritic cells from Peyer's patches in adult mice. *J Virol* 2010; **84**: 1856-1866 [PMID: 20007263 DOI: 10.1128/JVI.02640-08]

53 **Pott J**, Stockinger S, Torow N, Smoczek A, Lindner C, McInerney G, Bäckhed F, Baumann U, Pabst O, Bleich A, Hornef MW. Age-dependent TLR3 expression of the intestinal epithelium contributes to rotavirus susceptibility. *PLoS Pathog* 2012; **8**: e1002670 [PMID: 22570612 DOI: 10.1371/journal.ppat.1002670]

54 **Azim T**, Zaki MH, Podder G, Sultana N, Salam MA, Rahman SM, Sefat-e-Khuda DA. Rotavirus-specific subclass antibody and cytokine responses in Bangladeshi children with rotavirus diarrhoea. *J Med Virol* 2003; **69**: 286-295 [PMID: 12683420 DOI: 10.1002/jmv.10280]

55 **Jiang B**, Snipes-Magaldi L, Dennehy P, Keyserling H, Holman RC, Bresee J, Gentsch J, Glass RI. Cytokines as mediators for or effectors against rotavirus disease in children. *Clin Diagn Lab Immunol* 2003; **10**: 995-1001 [PMID: 14607858]

56 **Vanden Broecke C**, Schwers A, Dagenais L, Goossens A, Maenhoudt M, Pastoret PP, Werenne J. Interferon response in colostrum-deprived newborn calves infected with bovine rotavirus: its possible role in the control of the pathogenicity. *Ann Rech Vet* 1984; **15**: 29-34 [PMID: 6207759]

57 **Broome RL**, Vo PT, Ward RL, Clark HF, Greenberg HB. Murine rotavirus genes encoding outer capsid proteins VP4 and VP7 are not major determinants of host range restriction and virulence. *J Virol* 1993; **67**: 2448-2455 [PMID: 8386262]

58 **Jessop CE**, Watkins RH, Simmons JJ, Tasab M, Bulleid NJ. Protein disulphide isomerase family members show distinct substrate specificity: P5 is targeted to BiP client proteins. *J Cell Sci* 2009; **122**: 4287-4295 [PMID: 19887585 DOI: 10.1242/jcs.059154]

59 **Hatahet F**, Ruddock LW. Protein disulfide isomerase: a critical evaluation of its function in disulfide bond formation. *Antioxid Redox Signal* 2009; **11**: 2807-2850 [PMID: 19476414 DOI: 10.1089/ars.2009.2466]

60 **Mamathambika BS**, Bardwell JC. Disulfide-linked protein folding pathways. *Annu Rev Cell Dev Biol* 2008; **24**: 211-235 [PMID: 18588487 DOI: 10.1146/annurev.cellbio.24.110707.175333]

61 **Oka OB**, Yeoh HY, Bulleid NJ. Thiol-disulfide exchange between the PDI family of oxidoreductases negates the requirement for an oxidase or reductase for each enzyme. *Biochem J* 2015; **469**: 279-288 [PMID: 25989104 DOI: 10.1042/bj20141423]

62 **Benham AM**. The protein disulfide isomerase family: key players in health and disease. *Antioxid Redox Signal* 2012; **16**: 781-789 [PMID: 22142258 DOI: 10.1089/ars.2011.4439]

63 **Coe H**, Michalak M. ERp57, a multifunctional endoplasmic reticulum resident oxidoreductase. *Int J Biochem Cell Biol* 2010; **42**: 796-799 [PMID: 20079872 DOI: 10.1016/j.biocel.2010.01.009]

64 **Turano C**, Coppari S, Altieri F, Ferraro A. Proteins of the PDI family: unpredicted non-ER locations and functions. *J Cell Physiol* 2002; **193**: 154-163 [PMID: 12384992 DOI: 10.1002/jcp.10172]

65 **Kozlov G**, Määttänen P, Thomas DY, Gehring K. A structural overview of the PDI family of proteins. *FEBS J* 2010; **277**: 3924-3936 [DOI: 10.1111/j.1742-4658.2010.07793.x]

66 **Ryser HJ**, Levy EM, Mandel R, DiSciullo GJ. Inhibition of human immunodeficiency virus infection by agents that interfere with thiol-disulfide interchange upon virus-receptor interaction. *Proc Natl Acad Sci USA* 1994; **91**: 4559-4563 [PMID: 8183947]

67 **Karala AR**, Ruddock LW. Bacitracin is not a specific inhibitor of protein disulfide isomerase. *FEBS J* 2010; **277**: 2454-2462 [PMID: 20477872 DOI: 10.1111/j.1742-4658.2010.07660.x]

68 **Zhao G**, Lu H, Li C. Proapoptotic activities of protein disulfide isomerase (PDI) and PDIA3 protein, a role of the Bcl-2 protein Bak. *J Biol Chem* 2015; **290**: 8949-8963 [PMID: 25697356 DOI: 10.1074/jbc.M114.619353]

69 **Laurindo FR**, Araujo TL, Abrahão TB. Nox NADPH oxidases and the endoplasmic reticulum. *Antioxid Redox Signal* 2014; **20**: 2755-2775 [PMID: 24386930 DOI: 10.1089/ars.2013.5605]

70 **Santos CX**, Stolf BS, Takemoto PV, Amanso AM, Lopes LR, Souza EB, Goto H, Laurindo FR. Protein disulfide isomerase (PDI) associates with NADPH oxidase and is required for phagocytosis of Leishmania chagasi promastigotes by macrophages. *J Leukoc Biol* 2009; **86**: 989-998 [PMID: 19564574 DOI: 10.1189/jlb.0608354]

71 **Fernandes DC**, Manoel AH, Wosniak J, Laurindo FR. Protein disulfide isomerase overexpression in vascular smooth muscle cells induces spontaneous preemptive NADPH oxidase activation and Nox1 mRNA expression: effects of nitrosothiol exposure. *Arch Biochem Biophys* 2009; **484**: 197-204 [PMID: 19402212 DOI: 10.1016/j.abb.2009.01.022]

72 **Parent R**, Qu X, Petit MA, Beretta L. The heat shock cognate protein 70 is associated with hepatitis C virus particles and modulates virus infectivity. *Hepatology* 2009; **49**: 1798-1809 [PMID: 19434724 DOI: 10.1002/hep.22852]

73 **Sullivan CS**, Pipas JM. The virus-chaperone connection. *Virology* 2001; **287**: 1-8 [PMID: 11504535 DOI: 10.1006/viro.2001.1038]

74 **Chromy LR**, Pipas JM, Garcea RL. Chaperone-mediated in vitro assembly of Polyomavirus capsids. *Proc Natl Acad Sci USA* 2003; **100**: 10477-10482 [PMID: 12928495 DOI: 10.1073/pnas.1832245100]

75 **Watanabe K**, Fuse T, Asano I, Tsukahara F, Maru Y, Nagata K, Kitazato K, Kobayashi N. Identification of Hsc70 as an influenza virus matrix protein (M1) binding factor involved in the virus life cycle. *FEBS Lett* 2006; **580**: 5785-5790 [PMID: 17022977 DOI: 10.1016/j.febslet.2006.09.040]

76 **Liu T**, Daniels CK, Cao S. Comprehensive review on the HSC70 functions, interactions with related molecules and involvement in clinical diseases and therapeutic potential. *Pharmacol Ther* 2012; **136**: 354-374 [PMID: 22960394 DOI: 10.1016/j.pharmthera.2012.08.014]

77 **Dastoor Z**, Dreyer J. Nuclear translocation and aggregate formation of heat shock cognate protein 70 (Hsc70) in oxidative stress and apoptosis. *J Cell Sci* 2000; **113** (Pt 16): 2845-2854 [PMID: 10910769]

78 **Chuang CK**, Yang TH, Chen TH, Yang CF, Chen WJ. Heat shock cognate protein 70 isoform D is required for clathrin-dependent endocytosis of Japanese encephalitis virus in C6/36 cells. *J Gen Virol* 2015; **96**: 793-803 [PMID: 25502019 DOI: 10.1099/jgv.0.000015]

79 **Lim JW**, Kim KH, Kim H. NF-kappaB p65 regulates nuclear translocation of Ku70 via degradation of heat shock cognate protein 70 in pancreatic acinar AR42J cells. *Int J Biochem Cell Biol* 2008; **40**: 2065-2077 [PMID: 18378183 DOI: 10.1016/j.biocel.2008.02.015]

80 **Ou W**, Silver J. Role of protein disulfide isomerase and other thiol-reactive proteins in HIV-1 envelope protein-mediated fusion. *Virology* 2006; **350**: 406-417 [PMID: 16507315 DOI: 10.1016/j.virol.2006.01.041]

81 **Auwerx J**, Isacsson O, Söderlund J, Balzarini J, Johansson M, Lundberg M. Human glutaredoxin-1 catalyzes the reduction of HIV-1 gp120 and CD4 disulfides and its inhibition reduces HIV-1 replication. *Int J Biochem Cell Biol* 2009; **41**: 1269-1275 [PMID: 19038358 DOI: 10.1016/j.biocel.2008.10.031]

82 **Reiser K**, François KO, Schols D, Bergman T, Jörnvall H, Balzarini J, Karlsson A, Lundberg M. Thioredoxin-1 and protein disulfide isomerase catalyze the reduction of similar disulfides in HIV gp120. *Int J Biochem Cell Biol* 2012; **44**: 556-562 [PMID: 22230366 DOI: 10.1016/j.biocel.2011.12.015]

83 **Wan SW**, Lin CF, Lu YT, Lei HY, Anderson R, Lin YS.

Endothelial cell surface expression of protein disulfide isomerase activates β1 and β3 integrins and facilitates dengue virus infection. *J Cell Biochem* 2012; **113**: 1681-1691 [PMID: 22422622 DOI: 10.1002/jcb.24037]
84 **Smith JG**, Cunningham JM. Receptor-induced thiolate couples Env activation to retrovirus fusion and infection. *PLoS Pathog* 2007; **3**: e198 [PMID: 18260686 DOI: 10.1371/journal.ppat.0030198]
85 **Abell BA**, Brown DT. Sindbis virus membrane fusion is mediated by reduction of glycoprotein disulfide bridges at the cell surface. *J Virol* 1993; **67**: 5496-5501 [PMID: 8350409]
86 **Abou-Jaoudé G**, Sureau C. Entry of hepatitis delta virus requires the conserved cysteine residues of the hepatitis B virus envelope protein antigenic loop and is blocked by inhibitors of thiol-disulfide exchange. *J Virol* 2007; **81**: 13057-13066 [PMID: 17898062 DOI: 10.1128/jvi.01495-07]
87 **Jain S**, McGinnes LW, Morrison TG. Thiol/disulfide exchange is required for membrane fusion directed by the Newcastle disease virus fusion protein. *J Virol* 2007; **81**: 2328-2339 [PMID: 17151113 DOI: 10.1128/jvi.01940-06]
88 **Jain S**, McGinnes LW, Morrison TG. Role of thiol/disulfide exchange in newcastle disease virus entry. *J Virol* 2009; **83**: 241-249 [PMID: 18922867 DOI: 10.1128/jvi.01407-08]
89 **Jain S**, McGinnes LW, Morrison TG. Overexpression of thiol/disulfide isomerases enhances membrane fusion directed by the Newcastle disease virus fusion protein. *J Virol* 2008; **82**: 12039-12048 [PMID: 18829746 DOI: 10.1128/jvi.01406-08]
90 **Gilbert J**, Ou W, Silver J, Benjamin T. Downregulation of protein disulfide isomerase inhibits infection by the mouse polyomavirus. *J Virol* 2006; **80**: 10868-10870 [PMID: 16928750 DOI: 10.1128/jvi.01117-06]
91 **Ali Khan H**, Mutus B. Protein disulfide isomerase a multifunctional protein with multiple physiological roles. *Front Chem* 2014; **2**: 70 [PMID: 25207270 DOI: 10.3389/fchem.2014.00070]
92 **Parakh S**, Atkin JD. Novel roles for protein disulphide isomerase in disease states: a double edged sword? *Front Cell Dev Biol* 2015; **3**: 30 [PMID: 26052512 DOI: 10.3389/fcell.2015.00030]
93 **Stegmann M**, Metcalfe C, Barclay AN. Immunoregulation through membrane proteins modified by reducing conditions induced by immune reactions. *Eur J Immunol* 2013; **43**: 15-21 [PMID: 23233323 DOI: 10.1002/eji.201242849]
94 **Calderón MN**, Guzmán F, Acosta O, Guerrero CA. Rotavirus VP4 and VP7-derived synthetic peptides as potential substrates of protein disulfide isomerase lead to inhibition of rotavirus infection. *Int J Pept Res Ther* 2012; **18**: 373-382 [DOI: 10.1007/s10989-012-9314-z]
95 **Lopez S**, Arias CF. Early steps in rotavirus cell entry. *Curr Top Microbiol Immunol* 2006; **309**: 39-66 [PMID: 16909896]
96 **Isa P**, Gutierrez M, Arias CF, Lopez S. Rotavirus cell entry. *Future Virol* 2008; **3**: 135-146 [DOI: 10.2217/17460794.3.2.135]
97 **Díaz-Salinas MA**, Silva-Ayala D, López S, Arias CF. Rotaviruses reach late endosomes and require the cation-dependent mannose-6-phosphate receptor and the activity of cathepsin proteases to enter the cell. *J Virol* 2014; **88**: 4389-4402 [PMID: 24501398 DOI: 10.1128/jvi.03457-13]
98 **Díaz-Salinas MA**, Romero P, Espinosa R, Hoshino Y, López S, Arias CF. The spike protein VP4 defines the endocytic pathway used by rotavirus to enter MA104 cells. *J Virol* 2013; **87**: 1658-1663 [PMID: 23175367 DOI: 10.1128/jvi.02086-12]
99 **Gutiérrez M**, Isa P, Sánchez-San Martin C, Pérez-Vargas J, Espinosa R, Arias CF, López S. Different rotavirus strains enter MA104 cells through different endocytic pathways: the role of clathrin-mediated endocytosis. *J Virol* 2010; **84**: 9161-9169 [PMID: 20631149 DOI: 10.1128/jvi.00731-10]
100 **Trask SD**, Kim IS, Harrison SC, Dormitzer PR. A rotavirus spike protein conformational intermediate binds lipid bilayers. *J Virol* 2010; **84**: 1764-1770 [PMID: 20007281 DOI: 10.1128/JVI.01682-09]
101 **Dormitzer PR**, Nason EB, Prasad BV, Harrison SC. Structural rearrangements in the membrane penetration protein of a non-enveloped virus. *Nature* 2004; **430**: 1053-1058 [PMID: 15329727 DOI: 10.1038/nature02836]
102 **Rodríguez JM**, Chichón FJ, Martín-Forero E, González-Camacho F, Carrascosa JL, Castón JR, Luque D. New insights into rotavirus entry machinery: stabilization of rotavirus spike conformation is independent of trypsin cleavage. *PLoS Pathog* 2014; **10**: e1004157 [PMID: 24873828 DOI: 10.1371/journal.ppat.1004157]
103 **Gualtero DF**, Guzmán F, Acosta O, Guerrero CA. Amino acid domains 280-297 of VP6 and 531-554 of VP4 are implicated in heat shock cognate protein hsc70-mediated rotavirus infection. *Arch Virol* 2007; **152**: 2183-2196 [PMID: 17876681]
104 **Isa P**, Arias CF, López S. Role of sialic acids in rotavirus infection. *Glycoconj J* 2006; **23**: 27-37 [PMID: 16575520 DOI: 10.1007/s10719-006-5435-y]
105 **Zárate S**, Espinosa R, Romero P, Méndez E, Arias CF, López S. The VP5 domain of VP4 can mediate attachment of rotaviruses to cells. *J Virol* 2000; **74**: 593-599 [PMID: 10623720]
106 **Zárate S**, Espinosa R, Romero P, Guerrero CA, Arias CF, López S. Integrin alpha2beta1 mediates the cell attachment of the rotavirus neuraminidase-resistant variant nar3. *Virology* 2000; **278**: 50-54 [PMID: 11112480 DOI: 10.1006/viro.2000.0660]
107 **Fleming FE**, Böhm R, Dang VT, Holloway G, Haselhorst T, Madge PD, Deveryshetty J, Yu X, Blanchard H, von Itzstein M, Coulson BS. Relative roles of GM1 ganglioside, N-acylneuraminic acids, and α2β1 integrin in mediating rotavirus infection. *J Virol* 2014; **88**: 4558-4571 [PMID: 24501414 DOI: 10.1128/jvi.03431-13]
108 **Dormitzer PR**, Sun ZY, Wagner G, Harrison SC. The rhesus rotavirus VP4 sialic acid binding domain has a galectin fold with a novel carbohydrate binding site. *EMBO J* 2002; **21**: 885-897 [PMID: 11867517 DOI: 10.1093/emboj/21.5.885]
109 **Venkataram Prasad BV**, Shanker S, Hu L, Choi JM, Crawford SE, Ramani S, Czako R, Atmar RL, Estes MK. Structural basis of glycan interaction in gastroenteric viral pathogens. *Curr Opin Virol* 2014; **7**: 119-127 [PMID: 25073118 DOI: 10.1016/j.coviro.2014.05.008]
110 **Hu L**, Crawford SE, Czako R, Cortes-Penfield NW, Smith DF, Le Pendu J, Estes MK, Prasad BV. Cell attachment protein VP8* of a human rotavirus specifically interacts with A-type histo-blood group antigen. *Nature* 2012; **485**: 256-259 [PMID: 22504179 DOI: 10.1038/nature10996]
111 **Liu Y**, Huang P, Tan M, Liu Y, Biesiada J, Meller J, Castello AA, Jiang B, Jiang X. Rotavirus VP8*: phylogeny, host range, and interaction with histo-blood group antigens. *J Virol* 2012; **86**: 9899-9910 [PMID: 22761376 DOI: 10.1128/jvi.00979-12]
112 **Huang P**, Xia M, Tan M, Zhong W, Wei C, Wang L, Morrow A, Jiang X. Spike protein VP8* of human rotavirus recognizes histo-blood group antigens in a type-specific manner. *J Virol* 2012; **86**: 4833-4843 [PMID: 22345472 DOI: 10.1128/JVI.05507-11]
113 **Guerrero CA**, Méndez E, Zárate S, Isa P, López S, Arias CF. Integrin alpha(v)beta(3) mediates rotavirus cell entry. *Proc Natl Acad Sci USA* 2000; **97**: 14644-14649 [PMID: 11114176 DOI: 10.1073/pnas.250299897]
114 **Hewish MJ**, Takada Y, Coulson BS. Integrins alpha2beta1 and alpha4beta1 can mediate SA11 rotavirus attachment and entry into cells. *J Virol* 2000; **74**: 228-236 [PMID: 10590110]
115 **Ren J**, Ding T, Zhang W, Song J, Ma W. Does Japanese encephalitis virus share the same cellular receptor with other mosquito-borne flaviviruses on the C6/36 mosquito cells? *Virol J* 2007; **4**: 83 [PMID: 17803826 DOI: 10.1186/1743-422x-4-83]
116 **Graham KL**, Takada Y, Coulson BS. Rotavirus spike protein VP5* binds alpha2beta1 integrin on the cell surface and competes with virus for cell binding and infectivity. *J Gen Virol* 2006; **87**: 1275-1283 [PMID: 16603530 DOI: 10.1099/vir.0.81580-0]
117 **Morimoto RI**. Dynamic remodeling of transcription complexes by molecular chaperones. *Cell* 2002; **110**: 281-284 [PMID: 12176314]
118 **Pérez-Vargas J**, Romero P, López S, Arias CF. The peptide-binding and ATPase domains of recombinant hsc70 are required to interact with rotavirus and reduce its infectivity. *J Virol* 2006; **80**: 3322-3331 [PMID: 16537599 DOI: 10.1128/jvi.80.7.3322-3331.2006]
119 **Delmas O**, Durand-Schneider AM, Cohen J, Colard O, Trugnan

G. Spike protein VP4 assembly with maturing rotavirus requires a postendoplasmic reticulum event in polarized caco-2 cells. *J Virol* 2004; **78**: 10987-10994 [PMID: 15452219 DOI: 10.1128/jvi.78.20.10987-10994.2004]

120 **Dowling W**, Denisova E, LaMonica R, Mackow ER. Selective membrane permeabilization by the rotavirus VP5* protein is abrogated by mutations in an internal hydrophobic domain. *J Virol* 2000; **74**: 6368-6376 [PMID: 10864647 DOI: 10.1128/JVI.74.14.6368-6376.2000]

121 **Coulson BS**, Londrigan SL, Lee DJ. Rotavirus contains integrin ligand sequences and a disintegrin-like domain that are implicated in virus entry into cells. *Proc Natl Acad Sci USA* 1997; **94**: 5389-5394 [PMID: 9144247 DOI: 10.1073/pnas.94.10.5389]

122 **López S**, Arias CF. Multistep entry of rotavirus into cells: a Versaillesque dance. *Trends Microbiol* 2004; **12**: 271-278 [PMID: 15165605 DOI: 10.1016/j.tim.2004.04.003]

123 **Trask SD**, Dormitzer PR. Assembly of highly infectious rotavirus particles recoated with recombinant outer capsid proteins. *J Virol* 2006; **80**: 11293-11304 [PMID: 16971442 DOI: 10.1128/jvi.01346-06]

124 **Mandel R**, Ryser HJ, Ghani F, Wu M, Peak D. Inhibition of a reductive function of the plasma membrane by bacitracin and antibodies against protein disulfide-isomerase. *Proc Natl Acad Sci USA* 1993; **90**: 4112-4116 [PMID: 8387210]

125 **Couët J**, de Bernard S, Loosfelt H, Saunier B, Milgrom E, Misrahi M. Cell surface protein disulfide-isomerase is involved in the shedding of human thyrotropin receptor ectodomain. *Biochemistry* 1996; **35**: 14800-14805 [PMID: 8942642 DOI: 10.1021/bi961359w]

126 **Orlandi PA**. Protein-disulfide isomerase-mediated reduction of the A subunit of cholera toxin in a human intestinal cell line. *J Biol Chem* 1997; **272**: 4591-4599 [PMID: 9020187]

127 **Santana AY**, Guerrero CA, Acosta O. Implication of Hsc70, PDI and integrin αvβ3 involvement during entry of the murine rotavirus ECwt into small-intestinal villi of suckling mice. *Arch Virol* 2013; **158**: 1323-1336 [PMID: 23404461 DOI: 10.1007/s00705-013-1626-6]

128 **Isa P**, Realpe M, Romero P, López S, Arias CF. Rotavirus RRV associates with lipid membrane microdomains during cell entry. *Virology* 2004; **322**: 370-381 [PMID: 15110534 DOI: 10.1016/j.virol.2004.02.018]

129 **Swiatkowska M**, Szymański J, Padula G, Cierniewski CS. Interaction and functional association of protein disulfide isomerase with alphaVbeta3 integrin on endothelial cells. *FEBS J* 2008; **275**: 1813-1823 [PMID: 18331351 DOI: 10.1111/j.1742-4658.2008.06339.x]

130 **Lahav J**, Wijnen EM, Hess O, Hamaia SW, Griffiths D, Makris M, Knight CG, Essex DW, Farndale RW. Enzymatically catalyzed disulfide exchange is required for platelet adhesion to collagen via integrin alpha2beta1. *Blood* 2003; **102**: 2085-2092 [PMID: 12791669 DOI: 10.1182/blood-2002-06-1646]

131 **Di Paolo NC**, Miao EA, Iwakura Y, Murali-Krishna K, Aderem A, Flavell RA, Papayannopoulou T, Shayakhmetov DM. Virus binding to a plasma membrane receptor triggers interleukin-1 alpha-mediated proinflammatory macrophage response in vivo. *Immunity* 2009; **31**: 110-121 [PMID: 19576795 DOI: 10.1016/j.immuni.2009.04.015]

132 **Browne A**, Tookman LA, Ingemarsdotter CK, Bouwman RD, Pirlo K, Wang Y, McNeish IA, Lockley M. Pharmacological Inhibition of β3 Integrin Reduces the Inflammatory Toxicities Caused by Oncolytic Adenovirus without Compromising Anticancer Activity. *Cancer Res* 2015; **75**: 2811-2821 [PMID: 25977332 DOI: 10.1158/0008-5472.can-14-3761]

133 **Li W**, Wang G, Liang W, Kang K, Guo K, Zhang Y. Integrin β3 is required in infection and proliferation of classical swine fever virus. *PLoS One* 2014; **9**: e110911 [PMID: 25340775 DOI: 10.1371/journal.pone.0110911]

134 **Ning P**, An L, Liang W, Zhang Y. Identification of inhibition of protein disulphide isomerase expression related to classical swine fever virus infection by using real-time PCR analysis. *Biotechnol Equip* 2015; **29**: 564-569 [DOI: 10.1080/13102818.2015.1018840]

135 **Zhang JL**, Wang JL, Gao N, Chen ZT, Tian YP, An J. Up-regulated expression of beta3 integrin induced by dengue virus serotype 2 infection associated with virus entry into human dermal microvascular endothelial cells. *Biochem Biophys Res Commun* 2007; **356**: 763-768 [PMID: 17382900 DOI: 10.1016/j.bbrc.2007.03.051]

136 **Halasz P**, Holloway G, Turner SJ, Coulson BS. Rotavirus replication in intestinal cells differentially regulates integrin expression by a phosphatidylinositol 3-kinase-dependent pathway, resulting in increased cell adhesion and virus yield. *J Virol* 2008; **82**: 148-160 [PMID: 17942548 DOI: 10.1128/jvi.01980-07]

137 **Wang C**, Li W, Ren J, Fang J, Ke H, Gong W, Feng W, Wang CC. Structural insights into the redox-regulated dynamic conformations of human protein disulfide isomerase. *Antioxid Redox Signal* 2013; **19**: 36-45 [PMID: 22657537 DOI: 10.1089/ars.2012.4630]

138 **Mor-Cohen R**. Disulfide Bonds as Regulators of Integrin Function in Thrombosis and Hemostasis. *Antioxid Redox Signal* 2016; **24**: 16-31 [PMID: 25314675 DOI: 10.1089/ars.2014.6149]

139 **Patton JT**, Hua J, Mansell EA. Location of intrachain disulfide bonds in the VP5* and VP8* trypsin cleavage fragments of the rhesus rotavirus spike protein VP4. *J Virol* 1993; **67**: 4848-4855 [PMID: 8392619]

140 **Svensson L**, Dormitzer PR, von Bonsdorff CH, Maunula L, Greenberg HB. Intracellular manipulation of disulfide bond formation in rotavirus proteins during assembly. *J Virol* 1994; **68**: 5204-5215 [PMID: 8035518]

141 **Aoki ST**, Settembre EC, Trask SD, Greenberg HB, Harrison SC, Dormitzer PR. Structure of rotavirus outer-layer protein VP7 bound with a neutralizing Fab. *Science* 2009; **324**: 1444-1447 [PMID: 19520960 DOI: 10.1126/science.1170481]

142 **Mathieu M**, Petitpas I, Navaza J, Lepault J, Kohli E, Pothier P, Prasad BV, Cohen J, Rey FA. Atomic structure of the major capsid protein of rotavirus: implications for the architecture of the virion. *EMBO J* 2001; **20**: 1485-1497 [PMID: 11285213 DOI: 10.1093/emboj/20.7.1485]

143 **Yoder JD**, Trask SD, Vo TP, Binka M, Feng N, Harrison SC, Greenberg HB, Dormitzer PR. VP5* rearranges when rotavirus uncoats. *J Virol* 2009; **83**: 11372-11377 [PMID: 19692464 DOI: 10.1128/jvi.01228-09]

144 **Cuadras MA**, Méndez E, Arias CF, López S. A new cysteine in rotavirus VP4 participates in the formation of an alternate disulfide bond. *J Gen Virol* 1998; **79** (Pt 11): 2673-2677 [PMID: 9820142]

145 **Butera D**, Cook KM, Chiu J, Wong JW, Hogg PJ. Control of blood proteins by functional disulfide bonds. *Blood* 2014; **123**: 2000-2007 [PMID: 24523239 DOI: 10.1182/blood-2014-01-549816]

146 **Arias CF**, Silva-Ayala D, López S. Rotavirus entry: a deep journey into the cell with several exits. *J Virol* 2015; **89**: 890-893 [PMID: 25378490 DOI: 10.1128/jvi.01787-14]

147 **Inoue T**, Moore P, Tsai B. How viruses and toxins disassemble to enter host cells. *Annu Rev Microbiol* 2011; **65**: 287-305 [PMID: 21682643 DOI: 10.1146/annurev-micro-090110-102855]

148 **Inoue T**, Tsai B. How viruses use the endoplasmic reticulum for entry, replication, and assembly. *Cold Spring Harb Perspect Biol* 2013; **5**: a013250 [PMID: 23284050 DOI: 10.1101/cshperspect.a013250]

149 **Cuadras MA**, Bordier BB, Zambrano JL, Ludert JE, Greenberg HB. Dissecting rotavirus particle-raft interaction with small interfering RNAs: insights into rotavirus transit through the secretory pathway. *J Virol* 2006; **80**: 3935-3946 [PMID: 16571810 DOI: 10.1128/jvi.80.8.3935-3946.2006]

150 **Charpilienne A**, Abad MJ, Michelangeli F, Alvarado F, Vasseur M, Cohen J, Ruiz MC. Solubilized and cleaved VP7, the outer glycoprotein of rotavirus, induces permeabilization of cell membrane vesicles. *J Gen Virol* 1997; **78** (Pt 6): 1367-1371 [PMID: 9191931]

151 **He B**. Viruses, endoplasmic reticulum stress, and interferon responses. *Cell Death Differ* 2006; **13**: 393-403 [PMID: 16397582 DOI: 10.1038/sj.cdd.4401833]

152 **Mirazimi A**, Svensson L. Carbohydrates facilitate correct disulfide bond formation and folding of rotavirus VP7. *J Virol* 1998; **72**: 3887-3892 [PMID: 9557673]

153 **Maruri-Avidal L**, López S, Arias CF. Endoplasmic reticulum chaperones are involved in the morphogenesis of rotavirus infectious particles. *J Virol* 2008; **82**: 5368-5380 [PMID: 18385250 DOI: 10.1128/jvi.02751-07]

154 **Chemello ME**, Aristimuño OC, Michelangeli F, Ruiz MC. Requirement for vacuolar H+ -ATPase activity and Ca2+ gradient during entry of rotavirus into MA104 cells. *J Virol* 2002; **76**: 13083-13087 [PMID: 12438636 DOI: 10.1128/JVI.76.24.13083-13087.2002]

155 **Gerasimenko JV**, Tepikin AV, Petersen OH, Gerasimenko OV. Calcium uptake via endocytosis with rapid release from acidifying endosomes. *Curr Biol* 1998; **8**: 1335-1338 [PMID: 9843688 DOI: 10.1016/S0960-9822(07)00565-9]

156 **Ruiz MC**, Abad MJ, Charpilienne A, Cohen J, Michelangeli F. Cell lines susceptible to infection are permeabilized by cleaved and solubilized outer layer proteins of rotavirus. *J Gen Virol* 1997; **78** (Pt 11): 2883-2893 [PMID: 9367375 DOI: 10.1099/0022-1317-78-11-2883]

157 **Beaulieu JF**. Differential expression of the VLA family of integrins along the crypt-villus axis in the human small intestine. *J Cell Sci* 1992; **102** (Pt 3): 427-436 [PMID: 1506425]

158 **Hamilton TE**, McClane SJ, Baldwin S, Burke C, Patel H, Rombeau JL, Raper SE. Efficient adenoviral-mediated murine neonatal small intestinal gene transfer is dependent on alpha(v) integrin expression. *J Pediatr Surg* 1997; **32**: 1695-1703 [PMID: 9434001 DOI: 10.1016/S0022-3468(97)90508-X]

159 **Beck MA**, Handy J, Levander OA. The role of oxidative stress in viral infections. *Ann N Y Acad Sci* 2000; **917**: 906-912 [PMID: 11268420]

160 **Djordjević VB**. Free radicals in cell biology. *Int Rev Cytol* 2004; **237**: 57-89 [PMID: 15380666 DOI: 10.1016/s0074-7696(04)37002-6]

161 **Schwarz KB**. Oxidative stress during viral infection: a review. *Free Radic Biol Med* 1996; **21**: 641-649 [PMID: 8891667]

162 **Zhang Y**, Wang Z, Chen H, Chen Z, Tian Y. Antioxidants: potential antiviral agents for Japanese encephalitis virus infection. *Int J Infect Dis* 2014; **24**: 30-36 [PMID: 24780919 DOI: 10.1016/j.ijid.2014.02.011]

163 **Randow F**, MacMicking JD, James LC. Cellular self-defense: how cell-autonomous immunity protects against pathogens. *Science* 2013; **340**: 701-706 [PMID: 23661752 DOI: 10.1126/science.1233028]

164 **Akaike T**. Role of free radicals in viral pathogenesis and mutation. *Rev Med Virol* 2001; **11**: 87-101 [PMID: 11262528 DOI: 10.1002/rmv.303]

165 **Dobmeyer TS**, Findhammer S, Dobmeyer JM, Klein SA, Raffel B, Hoelzer D, Helm EB, Kabelitz D, Rossol R. Ex vivo induction of apoptosis in lymphocytes is mediated by oxidative stress: role for lymphocyte loss in HIV infection. *Free Radic Biol Med* 1997; **22**: 775-785 [PMID: 9119245 DOI: 10.1016/S0891-5849(96)00403-0]

166 **Knobil K**, Choi AM, Weigand GW, Jacoby DB. Role of oxidants in influenza virus-induced gene expression. *Am J Physiol* 1998; **274**: L134-L142 [PMID: 9458811]

167 **Dikici I**, Mehmetoglu I, Dikici N, Bitirgen M, Kurban S. Investigation of oxidative stress and some antioxidants in patients with acute and chronic viral hepatitis B and the effect of interferon-alpha treatment. *Clin Biochem* 2005; **38**: 1141-1144 [PMID: 16300751 DOI: 10.1016/j.clinbiochem.2005.10.006]

168 **Korenaga M**, Wang T, Li Y, Showalter LA, Chan T, Sun J, Weinman SA. Hepatitis C virus core protein inhibits mitochondrial electron transport and increases reactive oxygen species (ROS) production. *J Biol Chem* 2005; **280**: 37481-37488 [PMID: 16150732 DOI: 10.1074/jbc.M506412200]

169 **Ano Y**, Sakudo A, Kimata T, Uraki R, Sugiura K, Onodera T. Oxidative damage to neurons caused by the induction of microglial NADPH oxidase in encephalomyocarditis virus infection. *Neurosci Lett* 2010; **469**: 39-43 [PMID: 19945511 DOI: 10.1016/j.neulet.2009.11.040]

170 **Mochizuki H**, Todokoro M, Arakawa H. RS virus-induced inflammation and the intracellular glutathione redox state in cultured human airway epithelial cells. *Inflammation* 2009; **32**: 252-264 [PMID: 19548075 DOI: 10.1007/s10753-009-9128-0]

171 **Olagnier D**, Peri S, Steel C, van Montfoort N, Chiang C, Beljanski V, Slifker M, He Z, Nichols CN, Lin R, Balachandran S, Hiscott J. Cellular oxidative stress response controls the antiviral and apoptotic programs in dengue virus-infected dendritic cells. *PLoS Pathog* 2014; **10**: e1004566 [PMID: 25521078 DOI: 10.1371/journal.ppat.1004566]

172 **Gullberg RC**, Jordan Steel J, Moon SL, Soltani E, Geiss BJ. Oxidative stress influences positive strand RNA virus genome synthesis and capping. *Virology* 2015; **475**: 219-229 [PMID: 25514423 DOI: 10.1016/j.virol.2014.10.037]

173 **Liao SL**, Raung SL, Chen CJ. Japanese encephalitis virus stimulates superoxide dismutase activity in rat glial cultures. *Neurosci Lett* 2002; **324**: 133-136 [PMID: 11988345]

174 **Sodhi CP**, Katyal R, Rana SV, Attri S, Singh V. Study of oxidative-stress in rotavirus infected infant mice. *Indian J Med Res* 1996; **104**: 245-249 [PMID: 8952176]

175 **Borghan MA**, Mori Y, El-Mahmoudy AB, Ito N, Sugiyama M, Takewaki T, Minamoto N. Induction of nitric oxide synthase by rotavirus enterotoxin NSP4: implication for rotavirus pathogenicity. *J Gen Virol* 2007; **88**: 2064-2072 [PMID: 17554041 DOI: 10.1099/vir.0.82618-0]

176 **Rodríguez-Díaz J**, Banasaz M, Istrate C, Buesa J, Lundgren O, Espinoza F, Sundqvist T, Rottenberg M, Svensson L. Role of nitric oxide during rotavirus infection. *J Med Virol* 2006; **78**: 979-985 [PMID: 16721855 DOI: 10.1002/jmv.20650]

177 **Gac M**, Bigda J, Vahlenkamp TW. Increased mitochondrial superoxide dismutase expression and lowered production of reactive oxygen species during rotavirus infection. *Virology* 2010; **404**: 293-303 [PMID: 20538313 DOI: 10.1016/j.virol.2010.05.018]

178 **Warner BB**, Stuart L, Gebb S, Wispé JR. Redox regulation of manganese superoxide dismutase. *Am J Physiol* 1996; **271**: L150-L158 [PMID: 8760145]

179 **Nogae C**, Makino N, Hata T, Nogae I, Takahashi S, Suzuki K, Taniguchi N, Yanaga T. Interleukin 1 alpha-induced expression of manganous superoxide dismutase reduces myocardial reperfusion injury in the rat. *J Mol Cell Cardiol* 1995; **27**: 2091-2099 [PMID: 8576926]

180 **Jones PL**, Ping D, Boss JM. Tumor necrosis factor alpha and interleukin-1beta regulate the murine manganese superoxide dismutase gene through a complex intronic enhancer involving C/EBP-beta and NF-kappaB. *Mol Cell Biol* 1997; **17**: 6970-6981 [PMID: 9372929]

181 **Haynes CM**, Titus EA, Cooper AA. Degradation of misfolded proteins prevents ER-derived oxidative stress and cell death. *Mol Cell* 2004; **15**: 767-776 [PMID: 15350220 DOI: 10.1016/j.molcel.2004.08.025]

182 **Kaneko M**, Takahashi T, Niinuma Y, Nomura Y. Manganese superoxide dismutase is induced by endoplasmic reticulum stress through IRE1-mediated nuclear factor (NF)-kappaB and AP-1 activation. *Biol Pharm Bull* 2004; **27**: 1202-1206 [PMID: 15305022]

183 **Kumar De U**, Mukherjee R, Nandi S, Patel BH, Dimri U, Ravishankar C, Verma AK. Alterations in oxidant/antioxidant balance, high-mobility group box 1 protein and acute phase response in cross-bred suckling piglets suffering from rotaviral enteritis. *Trop Anim Health Prod* 2014; **46**: 1127-1133 [PMID: 24848720 DOI: 10.1007/s11250-014-0616-3]

184 **Guerrero CA**, Murillo A, Acosta O. Inhibition of rotavirus infection in cultured cells by N-acetyl-cysteine, PPARγ agonists and NSAIDs. *Antiviral Res* 2012; **96**: 1-12 [PMID: 22842004 DOI: 10.1016/j.antiviral.2012.06.011]

185 **Guerrero CA**, Paula Pardo VR, Rafael Guerrero OA. Inhibition of rotavirus ECwt infection in ICR suckling mice by N-acetylcysteine, peroxisome proliferator-activated receptor gamma agonists and cyclooxygenase-2 inhibitors. *Mem Inst Oswaldo Cruz* 2013; **108**: 741-754 [PMID: 24037197 DOI: 10.1590/0074-0276108062013011]

186 **Guerrero CA**, Torres DP, García LL, Guerrero RA, Acosta O. N-Acetylcysteine treatment of rotavirus-associated diarrhea in children. *Pharmacotherapy* 2014; **34**: e333-e340 [PMID:

25251886 DOI: 10.1002/phar.1489]
187 **Luo K**, Cao SS. Endoplasmic reticulum stress in intestinal epithelial cell function and inflammatory bowel disease. *Gastroenterol Res Pract* 2015; **2015**: 328791 [PMID: 25755668 DOI: 10.1155/2015/328791]
188 **Kaufman RJ**. Regulation of mRNA translation by protein folding in the endoplasmic reticulum. *Trends Biochem Sci* 2004; **29**: 152-158 [PMID: 15003273 DOI: 10.1016/j.tibs.2004.01.004]
189 **Malhotra JD**, Miao H, Zhang K, Wolfson A, Pennathur S, Pipe SW, Kaufman RJ. Antioxidants reduce endoplasmic reticulum stress and improve protein secretion. *Proc Natl Acad Sci USA* 2008; **105**: 18525-18530 [PMID: 19011102 DOI: 10.1073/pnas.0809677105]
190 **Chaudhari N**, Talwar P, Parimisetty A, Lefebvre d'Hellencourt C, Ravanan P. A molecular web: endoplasmic reticulum stress, inflammation, and oxidative stress. *Front Cell Neurosci* 2014; **8**: 213 [PMID: 25120434 DOI: 10.3389/fncel.2014.00213]
191 **Bhandary B**, Marahatta A, Kim HR, Chae HJ. An involvement of oxidative stress in endoplasmic reticulum stress and its associated diseases. *Int J Mol Sci* 2012; **14**: 434-456 [PMID: 23263672 DOI: 10.3390/ijms14010434]
192 **Cao SS**, Kaufman RJ. Endoplasmic reticulum stress and oxidative stress in cell fate decision and human disease. *Antioxid Redox Signal* 2014; **21**: 396-413 [PMID: 24702237 DOI: 10.1089/ars.2014.5851]
193 **Hwang C**, Sinskey AJ, Lodish HF. Oxidized redox state of glutathione in the endoplasmic reticulum. *Science* 1992; **257**: 1496-1502 [PMID: 1523409]
194 **van der Vlies D**, Makkinje M, Jansens A, Braakman I, Verkleij AJ, Wirtz KW, Post JA. Oxidation of ER resident proteins upon oxidative stress: effects of altering cellular redox/antioxidant status and implications for protein maturation. *Antioxid Redox Signal* 2003; **5**: 381-387 [PMID: 13678525 DOI: 10.1089/152308603768295113]
195 **Sideraki V**, Gilbert HF. Mechanism of the antichaperone activity of protein disulfide isomerase: facilitated assembly of large, insoluble aggregates of denatured lysozyme and PDI. *Biochemistry* 2000; **39**: 1180-1188 [PMID: 10653666]
196 **Malhotra JD**, Kaufman RJ. Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? *Antioxid Redox Signal* 2007; **9**: 2277-2293 [PMID: 17979528 DOI: 10.1089/ars.2007.1782]
197 **Gross E**, Kastner DB, Kaiser CA, Fass D. Structure of Ero1p, source of disulfide bonds for oxidative protein folding in the cell. *Cell* 2004; **117**: 601-610 [PMID: 15163408]
198 **Harding HP**, Zhang Y, Zeng H, Novoa I, Lu PD, Calfon M, Sadri N, Yun C, Popko B, Paules R, Stojdl DF, Bell JC, Hettmann T, Leiden JM, Ron D. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol Cell* 2003; **11**: 619-633 [PMID: 12667446]
199 **Fung TS**, Liu DX. Coronavirus infection, ER stress, apoptosis and innate immunity. *Front Microbiol* 2014; **5**: 296 [PMID: 24987391 DOI: 10.3389/fmicb.2014.00296]
200 **Nieva JL**, Madan V, Carrasco L. Viroporins: structure and biological functions. *Nat Rev Microbiol* 2012; **10**: 563-574 [PMID: 22751485 DOI: 10.1038/nrmicro2820]
201 **Crawford SE**, Hyser JM, Utama B, Estes MK. Autophagy hijacked through viroporin-activated calcium/calmodulin-dependent kinase-β signaling is required for rotavirus replication. *Proc Natl Acad Sci USA* 2012; **109**: E3405-E3413 [PMID: 23184977 DOI: 10.1073/pnas.1216539109]
202 **Zhang L**, Wang A. Virus-induced ER stress and the unfolded protein response. *Front Plant Sci* 2012; **3**: 293 [PMID: 23293645 DOI: 10.3389/fpls.2012.00293]
203 **Jheng JR**, Ho JY, Horng JT. ER stress, autophagy, and RNA viruses. *Front Microbiol* 2014; **5**: 388 [PMID: 25140166 DOI: 10.3389/fmicb.2014.00388]
204 **Isler JA**, Skalet AH, Alwine JC. Human cytomegalovirus infection activates and regulates the unfolded protein response. *J Virol* 2005; **79**: 6890-6899 [PMID: 15890928 DOI: 10.1128/jvi.79.11.6890-6899.2005]
205 **Pavio N**, Romano PR, Graczyk TM, Feinstone SM, Taylor DR. Protein synthesis and endoplasmic reticulum stress can be modulated by the hepatitis C virus envelope protein E2 through the eukaryotic initiation factor 2alpha kinase PERK. *J Virol* 2003; **77**: 3578-3585 [PMID: 12610133]
206 **Tardif KD**, Mori K, Siddiqui A. Hepatitis C virus subgenomic replicons induce endoplasmic reticulum stress activating an intracellular signaling pathway. *J Virol* 2002; **76**: 7453-7459 [PMID: 12097557]
207 **Fung TS**, Torres J, Liu DX. The Emerging Roles of Viroporins in ER Stress Response and Autophagy Induction during Virus Infection. *Viruses* 2015; **7**: 2834-2857 [PMID: 26053926 DOI: 10.3390/v7062749]
208 **Mekahli D**, Bultynck G, Parys JB, De Smedt H, Missiaen L. Endoplasmic-reticulum calcium depletion and disease. *Cold Spring Harb Perspect Biol* 2011; **3**: pii: a004317 [PMID: 21441595 DOI: 10.1101/cshperspect.a004317]
209 **Hyser JM**, Collinson-Pautz MR, Utama B, Estes MK. Rotavirus disrupts calcium homeostasis by NSP4 viroporin activity. *MBio* 2010; **1**: pii: e00265-10 [PMID: 21151776 DOI: 10.1128/mBio.00265-10]
210 **Guo HC**, Sun SQ, Sun DH, Wei YQ, Xu J, Huang M, Liu XT, Liu ZX, Luo JX, Yin H, Liu DX. Viroporin activity and membrane topology of classic swine fever virus p7 protein. *Int J Biochem Cell Biol* 2013; **45**: 1186-1194 [PMID: 23583663 DOI: 10.1016/j.biocel.2013.03.021]
211 **Bhowmick R**, Halder UC, Chattopadhyay S, Chanda S, Nandi S, Bagchi P, Nayak MK, Chakrabarti O, Kobayashi N, Chawla-Sarkar M. Rotaviral enterotoxin nonstructural protein 4 targets mitochondria for activation of apoptosis during infection. *J Biol Chem* 2012; **287**: 35004-35020 [PMID: 22888003 DOI: 10.1074/jbc.M112.369595]
212 **Ao D**, Guo HC, Sun SQ, Sun DH, Fung TS, Wei YQ, Han SC, Yao XP, Cao SZ, Liu DX, Liu XT. Viroporin Activity of the Foot-and-Mouth Disease Virus Non-Structural 2B Protein. *PLoS One* 2015; **10**: e0125828 [PMID: 25946195 DOI: 10.1371/journal.pone.0125828]
213 **Berkova Z**, Crawford SE, Trugnan G, Yoshimori T, Morris AP, Estes MK. Rotavirus NSP4 induces a novel vesicular compartment regulated by calcium and associated with viroplasms. *J Virol* 2006; **80**: 6061-6071 [PMID: 16731945 DOI: 10.1128/jvi.02167-05]
214 **Trujillo-Alonso V**, Maruri-Avidal L, Arias CF, López S. Rotavirus infection induces the unfolded protein response of the cell and controls it through the nonstructural protein NSP3. *J Virol* 2011; **85**: 12594-12604 [PMID: 21937647 DOI: 10.1128/jvi.05620-11]
215 **Chan CP**, Siu KL, Chin KT, Yuen KY, Zheng B, Jin DY. Modulation of the unfolded protein response by the severe acute respiratory syndrome coronavirus spike protein. *J Virol* 2006; **80**: 9279-9287 [PMID: 16940539 DOI: 10.1128/jvi.00659-06]
216 **Yu CY**, Hsu YW, Liao CL, Lin YL. Flavivirus infection activates the XBP1 pathway of the unfolded protein response to cope with endoplasmic reticulum stress. *J Virol* 2006; **80**: 11868-11880 [PMID: 16987981 DOI: 10.1128/jvi.00879-06]
217 **Xuan B**, Qian Z, Torigoi E, Yu D. Human cytomegalovirus protein pUL38 induces ATF4 expression, inhibits persistent JNK phosphorylation, and suppresses endoplasmic reticulum stress-induced cell death. *J Virol* 2009; **83**: 3463-3474 [PMID: 19193809 DOI: 10.1128/jvi.02307-08]
218 **Ambrose RL**, Mackenzie JM. West Nile virus differentially modulates the unfolded protein response to facilitate replication and immune evasion. *J Virol* 2011; **85**: 2723-2732 [PMID: 21191014 DOI: 10.1128/jvi.02050-10]
219 **Field M**. Intestinal ion transport and the pathophysiology of diarrhea. *J Clin Invest* 2003; **111**: 931-943 [PMID: 12671039 DOI: 10.1172/jci18326]
220 **Tian P**, Estes MK, Hu Y, Ball JM, Zeng CQ, Schilling WP. The rotavirus nonstructural glycoprotein NSP4 mobilizes Ca2+ from the endoplasmic reticulum. *J Virol* 1995; **69**: 5763-5772 [PMID: 7637021]
221 **Ball JM**, Tian P, Zeng CQ, Morris AP, Estes MK. Age-dependent

diarrhea induced by a rotaviral nonstructural glycoprotein. *Science* 1996; **272**: 101-104 [PMID: 8600515]

222 **Ousingsawat J**, Mirza M, Tian Y, Roussa E, Schreiber R, Cook DI, Kunzelmann K. Rotavirus toxin NSP4 induces diarrhea by activation of TMEM16A and inhibition of Na+ absorption. *Pflugers Arch* 2011; **461**: 579-589 [PMID: 21399895 DOI: 10.1007/s00424-011-0947-0]

223 **Lorrot M**, Vasseur M. How do the rotavirus NSP4 and bacterial enterotoxins lead differently to diarrhea? *Virol J* 2007; **4**: 31 [PMID: 17376232 DOI: 10.1186/1743-422x-4-31]

224 **Buccigrossi V**, Laudiero G, Russo C, Miele E, Sofia M, Monini M, Ruggeri FM, Guarino A. Chloride secretion induced by rotavirus is oxidative stress-dependent and inhibited by Saccharomyces boulardii in human enterocytes. *PLoS One* 2014; **9**: e99830 [PMID: 24918938 DOI: 10.1371/journal.pone.0099830]

225 **Denning TL**, Takaishi H, Crowe SE, Boldogh I, Jevnikar A, Ernst PB. Oxidative stress induces the expression of Fas and Fas ligand and apoptosis in murine intestinal epithelial cells. *Free Radic Biol Med* 2002; **33**: 1641-1650 [PMID: 12488132]

226 **Atkuri KR**, Mantovani JJ, Herzenberg LA, Herzenberg LA. N-Acetylcysteine--a safe antidote for cysteine/glutathione deficiency. *Curr Opin Pharmacol* 2007; **7**: 355-359 [PMID: 17602868 DOI: 10.1016/j.coph.2007.04.005]

227 **Cotgreave IA**. N-acetylcysteine: pharmacological considerations and experimental and clinical applications. *Adv Pharmacol* 1997; **38**: 205-227 [PMID: 8895810]

228 **Aitio ML**. N-acetylcysteine -- passe-partout or much ado about nothing? *Br J Clin Pharmacol* 2006; **61**: 5-15 [PMID: 16390346 DOI: 10.1111/j.1365-2125.2005.02523.x]

229 **Sparks B**, Kesavan A. Treatment of a gastric lactobezoar with N-acetylcysteine. *Case Rep Gastrointest Med* 2014; **2014**: 254741 [PMID: 25505999 DOI: 10.1155/2014/254741]

230 **Song JW**, Shim JK, Soh S, Jang J, Kwak YL. Double-blinded, randomized controlled trial of N-acetylcysteine for prevention of acute kidney injury in high risk patients undergoing off-pump coronary artery bypass. *Nephrology* (Carlton) 2015; **20**: 96-102 [PMID: 25384603 DOI: 10.1111/nep.12361]

231 **Mahmoud KM**, Ammar AS. Effect of N-acetylcysteine on cardiac injury and oxidative stress after abdominal aortic aneurysm repair: a randomized controlled trial. *Acta Anaesthesiol Scand* 2011; **55**: 1015-1021 [PMID: 22092168 DOI: 10.1111/j.1399-6576.2011.02492.x]

232 **Sakamoto S**, Muramatsu Y, Satoh K, Ishida F, Kikuchi N, Sano G, Sugino K, Isobe K, Takai Y, Homma S. Effectiveness of combined therapy with pirfenidone and inhaled N-acetylcysteine for advanced idiopathic pulmonary fibrosis: a case-control study. *Respirology* 2015; **20**: 445-452 [PMID: 25639750 DOI: 10.1111/resp.12477]

233 **El Rahi C**, Thompson-Moore N, Mejia P, De Hoyos P. Successful use of N-acetylcysteine to treat severe hepatic injury caused by a dietary fitness supplement. *Pharmacotherapy* 2015; **35**: e96-e101 [PMID: 25823877 DOI: 10.1002/phar.1572]

234 **Aruoma OI**, Halliwell B, Hoey BM, Butler J. The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. *Free Radic Biol Med* 1989; **6**: 593-597 [PMID: 2546864 DOI: 10.1016/0891-5849(89)90066-X]

235 **De Flora S**, Grassi C, Carati L. Attenuation of influenza-like symptomatology and improvement of cell-mediated immunity with long-term N-acetylcysteine treatment. *Eur Respir J* 1997; **10**: 1535-1541 [PMID: 9230243 DOI: 10.1183/09031936.97.10071535]

236 **Cai J**, Chen Y, Seth S, Furukawa S, Compans RW, Jones DP. Inhibition of influenza infection by glutathione. *Free Radic Biol Med* 2003; **34**: 928-936 [PMID: 12654482 DOI: 10.1016/S0891-5849(03)00023-6]

237 **Garozzo A**, Tempera G, Ungheri D, Timpanaro R, Castro A. N-acetylcysteine synergizes with oseltamivir in protecting mice from lethal influenza infection. *Int J Immunopathol Pharmacol* 2007; **20**: 349-354 [PMID: 17624247]

238 **Ghezzi P**, Ungheri D. Synergistic combination of N-acetylcysteine and ribavirin to protect from lethal influenza viral infection in a mouse model. *Int J Immunopathol Pharmacol* 2004; **17**: 99-102 [PMID: 15000873]

239 **Lai KY**, Ng WY, Osburga Chan PK, Wong KF, Cheng F. High-dose N-acetylcysteine therapy for novel H1N1 influenza pneumonia. *Ann Intern Med* 2010; **152**: 687-688 [PMID: 20479037 DOI: 10.7326/0003-4819-152-10-201005180-00017]

240 **Geiler J**, Michaelis M, Naczk P, Leutz A, Langer K, Doerr HW, Cinatl J. N-acetyl-L-cysteine (NAC) inhibits virus replication and expression of pro-inflammatory molecules in A549 cells infected with highly pathogenic H5N1 influenza A virus. *Biochem Pharmacol* 2010; **79**: 413-420 [PMID: 19732754 DOI: 10.1016/j.bcp.2009.08.025]

241 **Garigliany MM**, Desmecht DJ. N-acetylcysteine lacks universal inhibitory activity against influenza A viruses. *J Negat Results Biomed* 2011; **10**: 5 [PMID: 21554703 DOI: 10.1186/1477-5751-10-5]

242 **Senanayake MP**, Jayamanne MD, Kankananarachchi I. N-acetylcysteine in children with acute liver failure complicating dengue viral infection. *Ceylon Med J* 2013; **58**: 80-82 [PMID: 23817939 DOI: 10.4038/cmj.v58i2.5684]

243 **Herzenberg LA**, De Rosa SC, Dubs JG, Roederer M, Anderson MT, Ela SW, Deresinski SC, Herzenberg LA. Glutathione deficiency is associated with impaired survival in HIV disease. *Proc Natl Acad Sci USA* 1997; **94**: 1967-1972 [PMID: 9050888 DOI: 10.1073/pnas.94.5.1967]

244 **Dröge W**, Breitkreutz R. N-acetyl-cysteine in the therapy of HIV-positive patients. *Curr Opin Clin Nutr Metab Care* 1999; **2**: 493-498 [PMID: 10678679 DOI: 10.1097/00075197-199911000-00011]

245 **De Rosa SC**, Zaretsky MD, Dubs JG, Roederer M, Anderson M, Green A, Mitra D, Watanabe N, Nakamura H, Tjioe I, Deresinski SC, Moore WA, Ela SW, Parks D, Herzenberg LA, Herzenberg LA. N-acetylcysteine replenishes glutathione in HIV infection. *Eur J Clin Invest* 2000; **30**: 915-929 [PMID: 11029607 DOI: 10.1046/j.1365-2362.2000.00736.x]

246 **Duh EJ**, Maury WJ, Folks TM, Fauci AS, Rabson AB. Tumor necrosis factor alpha activates human immunodeficiency virus type 1 through induction of nuclear factor binding to the NF-kappa B sites in the long terminal repeat. *Proc Natl Acad Sci USA* 1989; **86**: 5974-5978 [PMID: 2762307 DOI: 10.1073/pnas.86.15.5974]

247 **Roederer M**, Staal FJ, Raju PA, Ela SW, Herzenberg LA, Herzenberg LA. Cytokine-stimulated human immunodeficiency virus replication is inhibited by N-acetyl-L-cysteine. *Proc Natl Acad Sci USA* 1990; **87**: 4884-4888 [PMID: 2112750 DOI: 10.1073/pnas.87.12.4884]

248 **Staal FJ**, Roederer M, Herzenberg LA, Herzenberg LA. Intracellular thiols regulate activation of nuclear factor kappa B and transcription of human immunodeficiency virus. *Proc Natl Acad Sci USA* 1990; **87**: 9943-9947 [PMID: 2263644 DOI: 10.1073/pnas.87.24.9943]

249 **Schubert SY**, Neeman I, Resnick N. A novel mechanism for the inhibition of NF-kappaB activation in vascular endothelial cells by natural antioxidants. *FASEB J* 2002; **16**: 1931-1933 [PMID: 12368228 DOI: 10.1096/fj.02-0147fje]

250 **Sen R**, Baltimore D. Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. *Cell* 1986; **47**: 921-928 [PMID: 3096580]

251 **Flohé L**, Brigelius-Flohé R, Saliou C, Traber MG, Packer L. Redox regulation of NF-kappa B activation. *Free Radic Biol Med* 1997; **22**: 1115-1126 [PMID: 9034250]

252 **Hayakawa M**, Miyashita H, Sakamoto I, Kitagawa M, Tanaka H, Yasuda H, Karin M, Kikugawa K. Evidence that reactive oxygen species do not mediate NF-kappaB activation. *EMBO J* 2003; **22**: 3356-3366 [PMID: 12839997 DOI: 10.1093/emboj/cdg332]

253 **Marui N**, Offermann MK, Swerlick R, Kunsch C, Rosen CA, Ahmad M, Alexander RW, Medford RM. Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. *J Clin Invest* 1993; **92**: 1866-1874 [PMID: 7691889 DOI: 10.1172/jci116778]

254 **Faruqi RM**, Poptic EJ, Faruqi TR, De La Motte C, DiCorleto PE. Distinct mechanisms for N-acetylcysteine inhibition of cytokine-induced E-selectin and VCAM-1 expression. *Am J Physiol* 1997; **273**: H817-H826 [PMID: 9277499]

255 **Toborek M**, Barger SW, Mattson MP, McClain CJ, Hennig B. Role of glutathione redox cycle in TNF-alpha-mediated endothelial cell dysfunction. *Atherosclerosis* 1995; **117**: 179-188 [PMID: 8801863 DOI: 10.1016/0021-9150(95)05568-H]

256 **Bouloumie A**, Marumo T, Lafontan M, Busse R. Leptin induces oxidative stress in human endothelial cells. *FASEB J* 1999; **13**: 1231-1238 [PMID: 10385613]

257 **Hashimoto S**, Gon Y, Matsumoto K, Takeshita I, Horie T. N-acetylcysteine attenuates TNF-alpha-induced p38 MAP kinase activation and p38 MAP kinase-mediated IL-8 production by human pulmonary vascular endothelial cells. *Br J Pharmacol* 2001; **132**: 270-276 [PMID: 11156586 DOI: 10.1038/sj.bjp.0703787]

258 **Zafarullah M**, Li WQ, Sylvester J, Ahmad M. Molecular mechanisms of N-acetylcysteine actions. *Cell Mol Life Sci* 2003; **60**: 6-20 [PMID: 12613655]

259 **Kretzmann NA**, Chiela E, Matte U, Marroni N, Marroni CA. N-acetylcysteine improves antitumoural response of Interferon alpha by NF-kB downregulation in liver cancer cells. *Comp Hepatol* 2012; **11**: 4 [PMID: 23206959 DOI: 10.1186/1476-5926-11-4]

260 **Li YQ**, Zhang ZX, Xu YJ, Ni W, Chen SX, Yang Z, Ma D. N-Acetyl-L-cysteine and pyrrolidine dithiocarbamate inhibited nuclear factor-kappaB activation in alveolar macrophages by different mechanisms. *Acta Pharmacol Sin* 2006; **27**: 339-346 [PMID: 16490171 DOI: 10.1111/j.1745-7254.2006.00264.x]

261 **Arnold MM**, Barro M, Patton JT. Rotavirus NSP1 mediates degradation of interferon regulatory factors through targeting of the dimerization domain. *J Virol* 2013; **87**: 9813-9821 [PMID: 23824805 DOI: 10.1128/jvi.01146-13]

262 **Hu L**, Crawford SE, Hyser JM, Estes MK, Prasad BV. Rotavirus non-structural proteins: structure and function. *Curr Opin Virol* 2012; **2**: 380-388 [PMID: 22789743 DOI: 10.1016/j.coviro.2012.06.003]

263 **Barro M**, Patton JT. Rotavirus NSP1 inhibits expression of type I interferon by antagonizing the function of interferon regulatory factors IRF3, IRF5, and IRF7. *J Virol* 2007; **81**: 4473-4481 [PMID: 17301153 DOI: 10.1128/jvi.02498-06]

264 **Feng N**, Sen A, Nguyen H, Vo P, Hoshino Y, Deal EM, Greenberg HB. Variation in antagonism of the interferon response to rotavirus NSP1 results in differential infectivity in mouse embryonic fibroblasts. *J Virol* 2009; **83**: 6987-6994 [PMID: 19420080 DOI: 10.1128/jvi.00585-09]

265 **Sen A**, Feng N, Ettayebi K, Hardy ME, Greenberg HB. IRF3 inhibition by rotavirus NSP1 is host cell and virus strain dependent but independent of NSP1 proteasomal degradation. *J Virol* 2009; **83**: 10322-10335 [PMID: 19656876 DOI: 10.1128/jvi.01186-09]

266 **Graff JW**, Ettayebi K, Hardy ME. Rotavirus NSP1 inhibits NFkappaB activation by inducing proteasome-dependent degradation of beta-TrCP: a novel mechanism of IFN antagonism. *PLoS Pathog* 2009; **5**: e1000280 [PMID: 19180189 DOI: 10.1371/journal.ppat.1000280]

267 **Mansur DS**, Maluquer de Motes C, Unterholzner L, Sumner RP, Ferguson BJ, Ren H, Strnadova P, Bowie AG, Smith GL. Poxvirus targeting of E3 ligase β-TrCP by molecular mimicry: a mechanism to inhibit NF-κB activation and promote immune evasion and virulence. *PLoS Pathog* 2013; **9**: e1003183 [PMID: 23468625 DOI: 10.1371/journal.ppat.1003183]

268 **Holloway G**, Truong TT, Coulson BS. Rotavirus antagonizes cellular antiviral responses by inhibiting the nuclear accumulation of STAT1, STAT2, and NF-kappaB. *J Virol* 2009; **83**: 4942-4951 [PMID: 19244315 DOI: 10.1128/jvi.01450-08]

269 **Bagchi P**, Dutta D, Chattopadhyay S, Mukherjee A, Halder UC, Sarkar S, Kobayashi N, Komoto S, Taniguchi K, Chawla-Sarkar M. Rotavirus nonstructural protein 1 suppresses virus-induced cellular apoptosis to facilitate viral growth by activating the cell survival pathways during early stages of infection. *J Virol* 2010; **84**: 6834-6845 [PMID: 20392855 DOI: 10.1128/jvi.00225-10]

270 **Zijlstra RT**, McCracken BA, Odle J, Donovan SM, Gelberg HB, Petschow BW, Zuckermann FA, Gaskins HR. Malnutrition modifies pig small intestinal inflammatory responses to rotavirus. *J Nutr* 1999; **129**: 838-843 [PMID: 10203558]

271 **Rossen JW**, Bouma J, Raatgeep RH, Büller HA, Einerhand AW. Inhibition of cyclooxygenase activity reduces rotavirus infection at a postbinding step. *J Virol* 2004; **78**: 9721-9730 [PMID: 15331705 DOI: 10.1128/jvi.78.18.9721-9730.2004]

272 **Marnett LJ**, Kalgutkar AS. Cyclooxygenase 2 inhibitors: discovery, selectivity and the future. *Trends Pharmacol Sci* 1999; **20**: 465-469 [PMID: 10542447]

273 **Bartlett SR**, Sawdy R, Mann GE. Induction of cyclooxygenase-2 expression in human myometrial smooth muscle cells by interleukin-1beta: involvement of p38 mitogen-activated protein kinase. *J Physiol* 1999; **520** Pt 2: 399-406 [PMID: 10523409 DOI: 10.1111/j.1469-7793.1999.00399.x]

274 **Subbaramaiah K**, Hart JC, Norton L, Dannenberg AJ. Microtubule-interfering agents stimulate the transcription of cyclooxygenase-2. Evidence for involvement of ERK1/2 AND p38 mitogen-activated protein kinase pathways. *J Biol Chem* 2000; **275**: 14838-14845 [PMID: 10809726]

275 **Lee SM**, Gai WW, Cheung TK, Peiris JS. Antiviral effect of a selective COX-2 inhibitor on H5N1 infection in vitro. *Antiviral Res* 2011; **91**: 330-334 [PMID: 21798291 DOI: 10.1016/j.antiviral.2011.07.011]

276 **Carey MA**, Bradbury JA, Rebolloso YD, Graves JP, Zeldin DC, Germolec DR. Pharmacologic inhibition of COX-1 and COX-2 in influenza A viral infection in mice. *PLoS One* 2010; **5**: e11610 [PMID: 20657653 DOI: 10.1371/journal.pone.0011610]

277 **Subbaramaiah K**, Lin DT, Hart JC, Dannenberg AJ. Peroxisome proliferator-activated receptor gamma ligands suppress the transcriptional activation of cyclooxygenase-2. Evidence for involvement of activator protein-1 and CREB-binding protein/p300. *J Biol Chem* 2001; **276**: 12440-12448 [PMID: 11278336 DOI: 10.1074/jbc.M007237200]

278 **Straus DS**, Pascual G, Li M, Welch JS, Ricote M, Hsiang CH, Sengchanthalangsy LL, Ghosh G, Glass CK. 15-deoxy-delta 12,14-prostaglandin J2 inhibits multiple steps in the NF-kappa B signaling pathway. *Proc Natl Acad Sci USA* 2000; **97**: 4844-4849 [PMID: 10781090]

279 **Bassaganya-Riera J**, Song R, Roberts PC, Hontecillas R. PPAR-gamma activation as an anti-inflammatory therapy for respiratory virus infections. *Viral Immunol* 2010; **23**: 343-352 [PMID: 20712478 DOI: 10.1089/vim.2010.0016]

280 **Liu J**, Xia Q, Zhang Q, Li H, Zhang J, Li A, Xiu R. Peroxisome proliferator-activated receptor-gamma ligands 15-deoxy-delta(12,14)-prostaglandin J2 and pioglitazone inhibit hydroxyl peroxide-induced TNF-alpha and lipopolysaccharide-induced CXC chemokine expression in neonatal rat cardiac myocytes. *Shock* 2009; **32**: 317-324 [PMID: 19174742 DOI: 10.1097/SHK.0b013e31819c374c]

281 **Li M**, Pascual G, Glass CK. Peroxisome proliferator-activated receptor gamma-dependent repression of the inducible nitric oxide synthase gene. *Mol Cell Biol* 2000; **20**: 4699-4707 [PMID: 10848596]

282 **Jiang C**, Ting AT, Seed B. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. *Nature* 1998; **391**: 82-86 [PMID: 9422509 DOI: 10.1038/34184]

283 **Polvani S**, Tarocchi M, Galli A. PPARγ and Oxidative Stress: Con(β) Catenating NRF2 and FOXO. *PPAR Res* 2012; **2012**: 641087 [PMID: 22481913 DOI: 10.1155/2012/641087]

284 **Okuno Y**, Matsuda M, Miyata Y, Fukuhara A, Komuro R, Shimabukuro M, Shimomura I. Human catalase gene is regulated by peroxisome proliferator activated receptor-gamma through a response element distinct from that of mouse. *Endocr J* 2010; **57**: 303-309 [PMID: 20075562]

285 **Ren Y**, Sun C, Sun Y, Tan H, Wu Y, Cui B, Wu Z. PPAR gamma protects cardiomyocytes against oxidative stress and apoptosis via Bcl-2 upregulation. *Vascul Pharmacol* 2009; **51**: 169-174 [PMID: 19540934 DOI: 10.1016/j.vph.2009.06.004]

286 **Morgan MJ**, Liu ZG. Crosstalk of reactive oxygen species and

NF-κB signaling. *Cell Res* 2011; **21**: 103-115 [PMID: 21187859 DOI: 10.1038/cr.2010.178]
287 **Walsh D**, Mohr I. Viral subversion of the host protein synthesis machinery. *Nat Rev Microbiol* 2011; **9**: 860-875 [PMID: 22002165 DOI: 10.1038/nrmicro2655]
288 **Holcik M**, Sonenberg N. Translational control in stress and apoptosis. *Nat Rev Mol Cell Biol* 2005; **6**: 318-327 [PMID: 15803138 DOI: 10.1038/nrm1618]
289 **Walsh D**, Mathews MB, Mohr I. Tinkering with translation: protein synthesis in virus-infected cells. *Cold Spring Harb Perspect Biol* 2013; **5**: a012351 [PMID: 23209131 DOI: 10.1101/cshperspect.a012351]
290 **Firth AE**, Brierley I. Non-canonical translation in RNA viruses. *J Gen Virol* 2012; **93**: 1385-1409 [PMID: 22535777 DOI: 10.1099/vir.0.042499-0]
291 **Piron M**, Vende P, Cohen J, Poncet D. Rotavirus RNA-binding protein NSP3 interacts with eIF4GI and evicts the poly(A) binding protein from eIF4F. *EMBO J* 1998; **17**: 5811-5821 [PMID: 9755181 DOI: 10.1093/emboj/17.19.5811]
292 **Deo RC**, Groft CM, Rajashankar KR, Burley SK. Recognition of the rotavirus mRNA 3' consensus by an asymmetric NSP3 homodimer. *Cell* 2002; **108**: 71-81 [PMID: 11792322]
293 **Montero H**, Arias CF, Lopez S. Rotavirus Nonstructural Protein NSP3 is not required for viral protein synthesis. *J Virol* 2006; **80**: 9031-9038 [PMID: 16940515 DOI: 10.1128/jvi.00437-06]
294 **Arnold MM**, Brownback CS, Taraporewala ZF, Patton JT. Rotavirus variant replicates efficiently although encoding an aberrant NSP3 that fails to induce nuclear localization of poly(A)-binding protein. *J Gen Virol* 2012; **93**: 1483-1494 [PMID: 22442114 DOI: 10.1099/vir.0.041830-0]
295 **Rojas M**, Arias CF, López S. Protein kinase R is responsible for the phosphorylation of eIF2alpha in rotavirus infection. *J Virol* 2010; **84**: 10457-10466 [PMID: 20631127 DOI: 10.1128/JVI.00625-10]
296 **Corl BA**, Odle J, Niu X, Moeser AJ, Gatlin LA, Phillips OT, Blikslager AT, Rhoads JM. Arginine activates intestinal p70(S6k) and protein synthesis in piglet rotavirus enteritis. *J Nutr* 2008; **138**: 24-29 [PMID: 18156399]
297 **Rhoads JM**, Corl BA, Harrell R, Niu X, Gatlin L, Phillips O, Blikslager A, Moeser A, Wu G, Odle J. Intestinal ribosomal p70(S6K) signaling is increased in piglet rotavirus enteritis. *Am J Physiol Gastrointest Liver Physiol* 2007; **292**: G913-G922 [PMID: 17138969 DOI: 10.1152/ajpgi.00468.2006]
298 **Graves LM**, He Y, Lambert J, Hunter D, Li X, Earp HS. An intracellular calcium signal activates p70 but not p90 ribosomal S6 kinase in liver epithelial cells. *J Biol Chem* 1997; **272**: 1920-1928 [PMID: 8999881]
299 **Kirkby NS**, Zaiss AK, Wright WR, Jiao J, Chan MV, Warner TD, Herschman HR, Mitchell JA. Differential COX-2 induction by viral and bacterial PAMPs: Consequences for cytokine and interferon responses and implications for anti-viral COX-2 directed therapies. *Biochem Biophys Res Commun* 2013; **438**: 249-256 [PMID: 23850620 DOI: 10.1016/j.bbrc.2013.07.006]
300 **Steer SA**, Corbett JA. The role and regulation of COX-2 during viral infection. *Viral Immunol* 2003; **16**: 447-460 [PMID: 14733733 DOI: 10.1089/088282403771926283]
301 **Richardson JY**, Ottolini MG, Pletneva L, Boukhvalova M, Zhang S, Vogel SN, Prince GA, Blanco JC. Respiratory syncytial virus (RSV) infection induces cyclooxygenase 2: a potential target for RSV therapy. *J Immunol* 2005; **174**: 4356-4364 [PMID: 15778400 DOI: 10.4049/jimmunol.174.7.4356]
302 **Symensma TL**, Martinez-Guzman D, Jia Q, Bortz E, Wu TT, Rudra-Ganguly N, Cole S, Herschman H, Sun R. COX-2 induction during murine gammaherpesvirus 68 infection leads to enhancement of viral gene expression. *J Virol* 2003; **77**: 12753-12763 [PMID: 14610197 DOI: 10.1128/JVI.77.23.12753-12763.2003]
303 **Steer SA**, Moran JM, Maggi LB, Buller RM, Perlman H, Corbett JA. Regulation of cyclooxygenase-2 expression by macrophages in response to double-stranded RNA and viral infection. *J Immunol* 2003; **170**: 1070-1076 [PMID: 12517975 DOI: 10.4049/jimmunol.170.2.1070]
304 **Sainis I**, Angelidis C, Pagoulatos G, Lazaridis I. The hsc70 gene which is slightly induced by heat is the main virus inducible member of the hsp70 gene family. *FEBS Lett* 1994; **355**: 282-286 [PMID: 7988690 DOI: 10.1016/0014-5793(94)01210-5]
305 **Lyupina YV**, Zatsepina OG, Timokhova AV, Orlova OV, Kostyuchenko MV, Beljelarskaya SN, Evgen'ev MB, Mikhailov VS. New insights into the induction of the heat shock proteins in baculovirus infected insect cells. *Virology* 2011; **421**: 34-41 [PMID: 21982219 DOI: 10.1016/j.virol.2011.09.010]
306 **Xu H**, Yan F, Deng X, Wang J, Zou T, Ma X, Zhang X, Qi Y. The interaction of white spot syndrome virus envelope protein VP28 with shrimp Hsc70 is specific and ATP-dependent. *Fish Shellfish Immunol* 2009; **26**: 414-421 [PMID: 19138748 DOI: 10.1016/j.fsi.2009.01.001]

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MINIREVIEWS

Twenty years of human immunodeficiency virus care at the Mayo Clinic: Past, present and future

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Abstract

The Mayo human immunodeficiency virus (HIV) Clinic has been providing patient centered care for persons living with HIV in Minnesota and beyond for the past 20 years. Through multidisciplinary engagement, vital clinical outcomes such as retention in care, initiation of antiretroviral therapy and virologic suppression are maximized. In this commentary, we describe the history of the Mayo HIV Clinic and its best practices, providing a "Mayo Model" of HIV care that exceeds national outcomes and may be applicable in other settings.

Key words: Human immunodeficiency virus/acquired immune deficiency syndrome; Patient engagement; Care Cascade; Multidisciplinary care; Minimally disruptive medicine

Core tip: In this minireview, we describe the Mayo Clinic model of human immunodeficiency virus (HIV) care that has evolved over 20 years of caring for persons living with HIV. Multidisciplinary, team-based engagement at each clinic visit is essential to providing optimal longitudinal care of these patients.

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HUMAN IMMUNODEFICIENCY VIRUS IN MINNESOTA

Minnesota (MN) has a low prevalence of human

immunodeficiency virus (HIV) infection when compared to most other US States. However, new HIV diagnoses in MN occurred at a rate of 7.2 per 100000 persons in 2011, the highest rate in the upper mid-west states (North Dakota, South Dakota, Iowa and Wisconsin) (http://www.cdc.gov/hiv/pdf/statistics_2011_HIV_Surveillance_Report_vol_23.pdf#Page=68 accessed 6/10/15). Male to male sex remains the most common risk factor for HIV in MN; however, heterosexual transmission is the most common risk factor for women. Women are increasing in the HIV positive population in the US and MN, with 24% of persons living with HIV (PLHIV) in MN being female (http://www.health.state.mn.us/divs/idepc/diseases/hiv/hivstatistics.html accessed 3/26/2015). Nearly 20% of newly HIV-diagnosed persons are immigrants to the United States. Women of color (non-Caucasian) represent 17% of the female population in MN but comprise 73% of new HIV diagnoses among women. African born women are diagnosed with HIV at the highest rate of any ethnic group and accounted for one third of new diagnosis among women in 2013 (http://www.health.state.mn.us/divs/idepc/diseases/hiv/epiprofile/women.html accessed 6/10/15). MN and the nation have made great strides in preventing perinatal infection and each year an increasing number of HIV positive women are delivering uninfected babies. The rate of perinatal infection of babies born to HIV positive women in MN between 2010 and 2012 was 1.7% (http://www.health.state.mn.us/divs/idepc/diseases/hiv/hivstatistics.html accessed 3/26/2015), usually resulting from a lack of prenatal care or appropriate treatment of mothers prior to delivery.

Although deaths have significantly decreased from acquired immune deficiency syndrome (AIDS), the incidence of new AIDS diagnosis has remained stable. One third of all new HIV infection cases diagnosed in MN have AIDS or progress to AIDS within one year of diagnosis. Health disparities exist, with African born individuals and Hispanics being much more likely to be diagnosed later than Whites, and African Americans (http://www.health.state.mn.us/divs/idepc/diseases/hiv/hivstatistics.html accessed 3/26/2015). The lack of routine HIV screening upon immigration since 2010 has likely contributed to late diagnosis among African born individuals[1].

HISTORY OF THE MAYO HIV CLINIC

The evolution of HIV care at Mayo Clinic is comparable to other referral centers in the United States. Early cases of AIDS were seen, and the focus was on identifying their immune deficiency-related infections and conditions. There was fear of contagion by the public and medical professionals alike. There was no diagnostic test to identify the condition and the risk of transmission in the medical care setting was not defined in the early 1980s.

HIV was identified as the cause of AIDS in 1983, and the first HIV antibody test was licensed by the United States Food and Drug Administration in March 1985. Early in the epidemic, PLHIV seen at Mayo Clinic were primarily men who have sex with men. They had the typical opportunistic infections associated with HIV: Pneumocystis pneumonia, disseminated Mycobacterium avium complex disease, histoplasmosis, cytomegalovirus, and central nervous system toxoplasmosis. Patients were managed in the in-patient setting with the assistance of the infectious diseases consulting service. The management of their illness was restricted to treating the opportunistic infections, as there was no anti-retroviral therapy available at that time. Mortality was high; most PLHIV died within 6-12 mo of presenting to a medical center.

As the epidemic progressed, so did the knowledge about the virus that causes it, how it is transmitted, and how it causes diseases. The broad impact of the HIV epidemic on other risk groups, including persons with hemophilia, injection drug users, and those who acquire infection through heterosexual transmission was recognized. With the availability of diagnostic testing, more patients were identified at an earlier stage than in the prior years. Outpatient care began to expand. Until the establishment of the Mayo HIV Clinic formally in 1996, HIV care was provided by a handful of infectious diseases physicians, primary care physicians and hematologists. Among the first groups to be involved with HIV care at Mayo was the Infection Prevention and Control group, which formed an AIDS Committee, worked on infection precaution measures related to patients suspected with HIV, educated healthcare workers about HIV, and formulated a blood and body fluid exposure policy. The first multidisciplinary team approach to HIV care was established in the hemophilia clinic. When the HIV test became available, hemophiliac patients were tested for HIV, allowing for detection of HIV at an earlier stage. The HIV care team for these patients consisted of a hematologist, an infectious diseases physician, and a social worker.

The development of the first antiretroviral drugs brought much-needed hope to PLHIV. However, these early drugs were not as effective as current therapies and introduced additional complexity to HIV care-management of often severe adverse drug effects as well as increasing antiviral resistance. Potent antiretroviral drugs and the ability to monitor viral loads in the clinic setting became available in the mid-1990s. With the recognition that combination antiretroviral therapy (cART) is the right approach to treat HIV infection, the tide of the HIV epidemic began to turn. The recognition that the number of people infected and affected by HIV was large and expanding, and that HIV infection is a highly complex disease that requires a focused and multidisciplinary approach, led to the establishment of a formal and dedicated HIV clinic at Mayo Clinic in 1996.

CURRENT STRUCTURE AND ORGANIZATION

Population and demographics

Since the inception of the Mayo HIV Clinic, it has cared

for over 1400 PLHIV. Now, more than 400 individuals receive regular care at the Mayo HIV Clinic. The majority of the patients live in Central or Southern MN; however, a number of patients come from around the nation or the world. In addition to providing regular HIV care, the Mayo HIV Clinic also provides consultative care for PLHIV while they are at Mayo for treatment of other medical conditions.

Structure and organization of the Mayo HIV Clinic

The Mayo HIV Clinic is run by a multi-disciplinary team, which provides minimally disruptive care[2] to cater to each patient's needs. Each patient is assigned to an infectious disease fellow as his or her primary HIV provider. Seven consultant physicians who specialize in HIV treatment supervise fellows. As HIV has transformed into a chronic medical condition with which patients live for decades, they often require subspecialty medical care. Therefore, formal relationships have been established with providers in Obstetrics and Gynecology, Endocrinology, Nephrology and Colorectal Surgery who are knowledgeable in HIV and provide informed subspecialty care.

Medical evaluations of the patient at the first visit and subsequent visits generally follow United States national guidelines (https://aidsinfo.nih.gov/guidelines). On average, a patient who is doing well will be seen in the HIV Clinic every three months for laboratory testing, a physician visit with their fellow, and visits with other members of the multi-disciplinary team (discussed below). These additional services are supported through United States federal Ryan White Medical HIV Care Management and Ryan White Transportation grants administered through the Minnesota Department of Human Services.

Additional services provided by the Mayo HIV Clinic

When a person has been potentially exposed to the HIV virus, providing HIV post-exposure prophylaxis using combination antiretroviral therapy can significantly reduce the risk of transmission. Since HIV providers are facile with the risks of HIV transmission and HIV medications, any person potentially exposed to HIV as a result of a health care related blood or body fluid exposure or a sexual assault is provided an appointment in the HIV clinic within one business day of exposure for an evaluation, education and medications if needed as well as follow up testing. In addition, the Mayo HIV Clinic has a pre-exposure prophylaxis program, through which persons who are at high risk for acquiring HIV through sexual transmission or injection drug use are evaluated for starting anti-retroviral medicines to prevent HIV acquisition.

MULTIDISCIPLINARY ENGAGEMENT

The Mayo HIV Clinic relies on multi-disciplinary engagement from a team of providers including infectious disease fellows and consultants, a dedicated nurse, three social workers with expertise in HIV-case management, and a specialty pharmacist. Mental health care is available in the HIV clinic by a dedicated psychiatrist. This model of in-house mental health care facilitates the uptake of mental health care in our patients and communication between providers.

Many HIV clinics have such a team who are involved in the care of their patients. However, one of the unique features that the Mayo model utilizes is team-based multidisciplinary rounds for each patient. Patients have a visit with a nurse, a physician, a pharmacist, and a social worker. Cases are discussed at a round-table meeting that includes each of these providers. Insights and suggestions for optimizing patient care are shared, including factors affecting medication adherence, virologic suppression, and retention in care. Additionally, this model offers a system of "checks and balances" whereby each member of the team helps to improve quality of care and optimize patient outcomes (Figure 1). Privacy and confidentiality are very important parts of the multidisciplinary care provided by the clinic. To ensure confidentiality, only a limited number of individuals have access to protected health information, and these multidisciplinary interactions occur in a private work room in the clinic separate from the examination rooms.

The nurse assists providers with preventive health tasks, including immunizations which are administered in clinic, phone triage, and patient education. The social workers screen each new patient to the clinic. The social workers address mental health needs, education needs, support for families and significant others, financial needs, intimate partner violence, risk reduction counseling, legal issues, disclosing HIV-infection status to others, issues related to stigma, and assist the patient with identifying and accessing community resources. Patients who are at < 300% of the United States federally defined-poverty level and who have mental health, chemical dependency, financial needs, or other barriers to care qualify for intensive HIV case management through the Minnesota Department of Health. Patients who do not meet these criteria can still receive less intensive social work services based on individual needs.

The role of the pharmacist is critical to optimize adherence, maximize virologic suppression in our HIV population, and ultimately improve HIV patient outcomes. Within our care model, the pharmacist visits with every patient in conjunction with his or her physician appointment for routine HIV care. The pharmacist verifies the pharmacies where patients fill their HIV and non-HIV medications; performs medication reconciliation; screens for and identifies medication/supplement interactions; and verifies appropriate administration of cART. The pharmacist assesses adherence and identifies concerns that could affect routine adherence. Finally, the pharmacist provides adherence appropriate interventions and assistance with tools to enhance, optimize, or correct adherence problems. The pharmacist also provides a follow-up phone call one week after initiating or changing medication therapy to patients. The pharmacist aids the HIV care team by assisting with clinical and administrative tasks, including institutional formulary review of HIV medications upon request, selection of appropriate ART for salvage therapy,

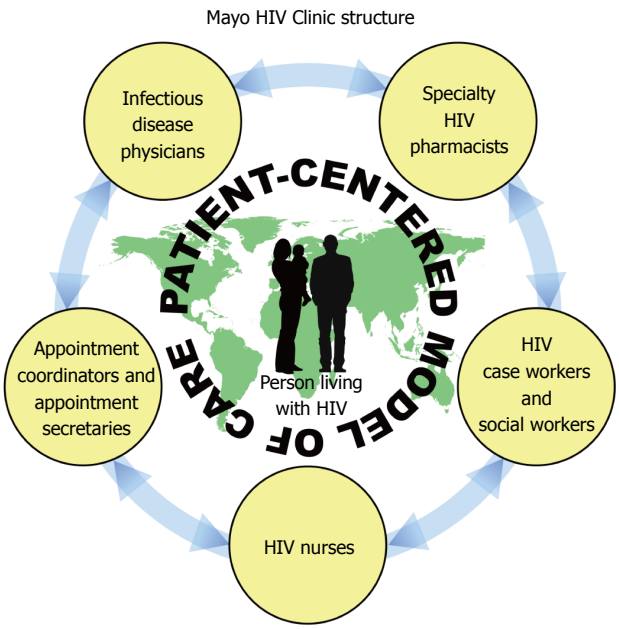

Figure 1 The Mayo human immunodeficiency virus Clinic structure. The Mayo human immunodeficiency virus (HIV) Clinic provides a model of multidisciplinary, patient-centered care.

collaborates with the care team to assist with drug therapy decisions for our patients, and provides drug therapy information/education for staff, residents, and fellows.

COLLABORATION WITH PRIMARY CARE PROVIDERS AND INTEGRATION ACROSS THE MIDWEST PRACTICE

The Mayo HIV Clinic provides both HIV management and some primary care services for patients within Central and Southeast MN. For patients not living within that region, our clinic delivers HIV care through developing co-management partnerships with patients' local primary care providers (PCPs). These local PCPs typically manage primary care and more emergent medical assessments. Most PCPs, however, are not equipped to provide specialty care to PLHIV. The Mayo HIV Clinic fulfills this role while informing the local PCP of the HIV management-care plans. The result is bidirectional and functional open lines of communication. Patients maintain a central role in reinforcing and supplementing the provider-to-provider communication strategies. Many patients cherish the relationships and trust they have developed with their local health providers; a diagnosis of HIV infection should not compromise this. The dialogue established between Mayo Clinic and local PCPs enables their inclusion into an expanded Mayo HIV "care team" for continued and optimized care of their patients.

In addition to its Rochester campus, Mayo Clinic also operates hospitals and outpatient clinics within the Mayo Clinic Health System, located in over 60 communities across the upper Midwest, and supports the Mayo Clinic Care Network, involving institutional partnerships with 30 other medical centers across the United States, including Puerto Rico and Mexico. Successful HIV care delivery within medical centers requires a close HIV team-collaboration among local PCPs and their continued vital roles in the care for their patients. Supplemental opportunities for PCPs in any location within the Mayo Clinic Health System and Mayo Clinic Care Network to connect with the Mayo HIV Clinic team include formal electronic consultations (eConsults), access to the web-based AskMayoExpert and through telemedicine consultations in select locations.

OUTCOMES AND THE HIV CARE CASCADE

cART saves lives, but unfortunately only approximately one quarter of PLHIV in the United States are successfully treated with cART and benefit from this lifesaving therapy. There are a number of biopsychosocial barriers to achieving this goal along the "HIV Care Cascade", which is defined as the critical steps in the identification and treatment of PLHIV. The elements of the Care Cascade include: (1) Diagnosis of HIV infection; (2) Referral to a specialist, or "Linkage to care"; (3) Regular engagement in clinical care; (4) Initiating cART; and (5) Virologic suppression, or therapeutic control of viral replication with effective cART. CDC statistics from 2011, the most recent year data was available and analyzed, revealed that 86% of PLHIV in the United States were aware of their diagnosis; 40% were engaged in care; 37% were prescribed ART; and 30% achieved viral suppression[3].

To define the Mayo HIV Clinic Cascade of Care, we conducted a retrospective review of incident HIV diagnoses based on first time positive HIV Western Blot or fourth generation enzyme-linked immunosorbent assay testing, or first time positive HIV nucleic acid testing, collected at Mayo Health System sites in Olmsted and the surrounding counties of Goodhue, Wabasha, Dodge, Winona, Mower, Fillmore and Houston, and performed at Mayo Medical Laboratories from 1/1/10-10/31/14. The study was approved by the Mayo Clinic Institutional Review Board (IRB# 14-006660), and medical records were reviewed (only if research authorization was not refused) according to institutional and state requirements. During the study time period, 50 new diagnoses of HIV infection were made in the local region. Forty-two (84%) were linked to care, as defined by referral for HIV specialty care and at least one clinic visit within 3 mo of diagnosis. Thirty-six (72%) were engaged in care, as defined by at least 2 clinic visits at least three months apart within the first year after diagnosis. Thirty-six (72%) were prescribed cART, and 30 (60%) achieved a plasma HIV RNA viral load < 50 copies/mL, *i.e.*, were virologically suppressed, within 6 mo of initiating therapy. Despite some variation in the absolute definitions of the steps along the Cascade of Care making direct comparisons challenging, these numbers far surpassed United States national levels noted above. Therefore, it is evident that the Mayo HIV Clinic excels in clinical outcomes for PLHIV through improved engagement in care and

penetrance of effective cART.

RESEARCH PARTICIPATION

The Mayo HIV Clinic regularly engages in both investigator and sponsor initiated research studies. These research studies range from biomedical discovery using patient-derived biologic samples to site participation in large multinational randomized clinical trials[4,5]. The Mayo HIV Clinic is a clinical site for several past and present International Network for Strategic Initiatives in Global HIV Trials clinical trials and prospective cohort studies. In general, the patients of the Mayo HIV Clinic are highly motivated, engaged and enthusiastic participants in research studies.

CONCLUSION

Over its twenty-year history, the Mayo HIV Clinic has developed a unique model of patient-centered care for PLHIV in Central and Southeast MN and elsewhere through multi-disciplinary engagement with patients and PCPs. While this multidisciplinary approach may be unique to the Mayo HIV Clinic, it is likely that adoption of a similar model, or portions thereof, by HIV providers in other locations may improve the health and quality of life for PLHIV outside of MN.

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REFERENCES

1 **Lowther SA**, Johnson G, Hendel-Paterson B, Nelson K, Mamo B, Krohn K, Pessoa-Brandão L, O'Fallon A, Stauffer W. HIV/AIDS and associated conditions among HIV-infected refugees in Minnesota, 2000-2007. *Int J Environ Res Public Health* 2012; **9**: 4197-4209 [PMID: 23202841 DOI: 10.3390/ijerph9114197]

2 **May C**, Montori VM, Mair FS. We need minimally disruptive medicine. *BMJ* 2009; **339**: b2803 [PMID: 19671932]

3 **Bradley H**, Hall HI, Wolitski RJ, Van Handel MM, Stone AE, LaFlam M, Skarbinski J, Higa DH, Prejean J, Frazier EL, Patel R, Huang P, An Q, Song R, Tang T, Valleroy LA. Vital Signs: HIV diagnosis, care, and treatment among persons living with HIV--United States, 2011. *MMWR Morb Mortal Wkly Rep* 2014; **63**: 1113-1117 [PMID: 25426654]

4 **El-Sadr WM**, Lundgren J, Neaton JD, Gordin F, Abrams D, Arduino RC, Babiker A, Burman W, Clumeck N, Cohen CJ, Cohn D, Cooper D, Darbyshire J, Emery S, Fätkenheuer G, Gazzard B, Grund B, Hoy J, Klingman K, Losso M, Markowitz N, Neuhaus J, Phillips A, Rappoport C. CD4+ count-guided interruption of antiretroviral treatment. *N Engl J Med* 2006; **355**: 2283-2296 [PMID: 17135583]

5 **Abrams D**, Lévy Y, Losso MH, Babiker A, Collins G, Cooper DA, Darbyshire J, Emery S, Fox L, Gordin F, Lane HC, Lundgren JD, Mitsuyasu R, Neaton JD, Phillips A, Routy JP, Tambussi G, Wentworth D. Interleukin-2 therapy in patients with HIV infection. *N Engl J Med* 2009; **361**: 1548-1559 [PMID: 19828532]

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MINIREVIEWS

Hepatitis C virus/human T lymphotropic virus 1/2 co-infection: Regional burden and virological outcomes in people who inject drugs

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Author contributions: Castro E and Roger E conceived and designed the study, and performed the literature review and analysis; Castro E wrote the manuscript.

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Abstract

This review analyses current data concerning co-infection with hepatitis C virus (HCV) and human T lymphotropic virus (HTLV)-1/2 in people who inject drugs (PWID), with a particular focus on disease burden and global implications for virological outcome. In addition, the available treatment options for HTLV-1/2 are summarized and the on-going and likely future research challenges are discussed. The data in this review was obtained from 34 articles on HCV/HTLV-1/2 co-infection in PWID retrieved from the PubMed literature database and published between 1997 and 2015. Despite unavailable estimates of the burden of HCV/HTLV-1/2 co-infection in general, the epidemiologic constellation of HTLV-1/2 shows high incidence in PWID with history of migration, incarceration, and other blood-borne infectious diseases such as HCV or human immunodeficiency virus. The most recent research data strongly suggest that HTLV-1 co-infection can influence HCV viral load, HCV sustained virological response to α-interferon treatment, and HCV-related liver disease progression. In short, outcome of HCV infection is worse in the context of HTLV-1 co-infection, yet more studies are needed to gain accurate estimations of the burden of HCV/HTLV-1/2 co-infections. Moreover, in the current era of new direct-acting antiviral treatments for HCV and proven HTLV-1/2 treatment options, prospective clinical and treatment studies should be carried out, with particular focus on the PWID patient population, with the aim of improving virological outcomes.

Key words: Hepatitis C virus; Human T lymphotropic virus; Hepatitis C virus/human T lymphotropic virus-1/2 co-infection; People who inject drugs; Human T lymphotropic virus-1/2 screening among people who inject drugs; Co-infection treatment

Core tip: People who inject drugs (PWID) are at higher risk of infection with blood-borne viruses and even co-

infections. Co-infections with human immunodeficiency virus and human T lymphotropic virus (HTLV)-1/2 are common, and well-studied, among PWID; however, the rise of HTLV-1/2 co-infections with hepatitis C virus (HCV) has gained much research attention and studies have shown that the former influences the chronic disease course of the latter. This review summarizes the data from 34 articles on HCV/HTLV-1/2 co-infection in the PWID patient population, including current treatment options and impact on virological outcome.

Castro E, Roger E. Hepatitis C virus/human T lymphotropic virus 1/2 co-infection: Regional burden and virological outcomes in people who inject drugs. *World J Virol* 2016; 5(2): 68-72 Available from: URL: http://www.wjgnet.com/2220-3249/full/v5/i2/68.htm DOI: http://dx.doi.org/10.5501/wjv.v5.i2.68

HEPATITIS C VIRUS AND HUMAN T LYMPHOTROPIC VIRUS-TYPES 1/2 CO-INFECTION BURDEN

The rate of hepatitis C virus (HCV) infection has reached the level of a global epidemic, with an estimated burden of 2.8% seroprevalence (anti-HCV antibody) in over 185 million individuals from both developed and developing nations[1]. In Europe and the United States, however, HCV transmission occurs mainly through intravenous drug use[2,3]. While this practice facilitates spread of blood-borne viruses, including the human immunodeficiency virus (HIV) as well as the hepatitis B virus, it allows transmission of HCV much more efficiently, as evidenced by the higher incidence rates of HCV in people who inject drugs (PWID) *vs* those with HIV[3]. Specifically, the 2011 estimate of global PWID seroprevalence for HCV was 67.0%[4]. PWID are a select population subgroup with extremely high seroprevalences of HCV; as such, they represent a primary driving force of the current HCV epidemic in high-resource settings, accounting for the majority of new (80%) and existing (60%) cases reportedly[5]. Yet, the high proportion of undiagnosed asymptomatic HCV carriers has precluded obtainment of an accurate estimate of chronic hepatitis C burden.

Human T lymphotropic virus (HTLV)-1 is an oncogenic retrovirus with a similar worldwide incidence. Although its founder effect remains unresolved, HTLV-1 shows high endemicity in Southwestern Japan, sub-Saharan Africa, South America, the Caribbean basin, the Middle East, and Australo-Melanesia[6]. The worldwide prevalence estimate of 20 million infected people is based on a serological screening from nearly 30 years ago, and an accurate estimate of the current global burden is unavailable[7]. The main transmission routes are contaminated blood products, sexual intercourse, and vertical transmission. In Europe, most HTLV-1 carriers are descendants of immigrants originally from regions with high endemicity and often with an HIV co-infection[6,7]. However, as reported for Spain, Italy and Ireland, PWID represent an especially affected population for HTLV-1 infection, even though HTLV-2 is much more prevalent[6]. In contrast, clinical onset of associated chronic illnesses, such as cancer [adult T-cell leukaemia/lymphoma (ATLL)] and neurological disorders [myelopathy and tropical spastic paraparesis (HAM/TSP)], has been reported in only 5%-10% of HTLV-1 carriers[8-10].

Similar to HTLV-1, HTLV-2 can be transmitted intravenously, sexually, or vertically. In the United States and Europe, needle sharing is a major route of HTLV-2 transmission among the PWID population[11-13]. Moreover, study of a cohort of PWID in the United States revealed significant associations between HTLV-2 infection and increased rates of pneumonia, acute bronchitis, urinary tract infection, and myelopathy[14], and the authors noted that the observed high correlation of HTLV-2 infection with HCV infection was suggestive of injection practices as a major route of transmission.

Studies of retroviral transmission carried out in various developing countries have identified incarceration as a risk factor, especially for HCV, suggesting that incarceration may be a surrogate marker for risky behaviour in general, such as needle sharing and unprotected sex[14]. In addition, our previous case report of HTLV in Eastern European countries indicated that the criminalization of drug use and lack of harm reduction strategies in prisons may also serve to increase risk for sexual and parental transmission[15].

Finally, the contribution of health care-associated infection (or "nosocomial") as a source of HCV and retrovirus transmission among migrant population originally coming from limited resources settings has been largely undervalued to date, with little research available[16]. The limited data reported has shown nosocomial rates ranging from as low as 5% and all the way up to 19%[16].

In conclusion, the epidemiological constellation of HCV/HTLV-1/2 co-infection is found within regions with high rates of PWID and history of other risk factors (Figure 1).

CLINICAL AND THERAPEUTIC IMPLICATIONS OF HCV/HTLV-1/2 CO-INFECTION

In order to gain a comprehensive overview of the current available knowledge on the clinical and therapeutic implications of HCV/HTLV-1/2 co-infection, we searched the PubMed (www.pubmed.gov) literature database for all articles affiliated with the terms "HTLV HCV", "HCV and HTLV coinfection", "HTLV burden", "HTLV treatment", and "HTLV migrants". Exclusion of articles published before January 1, 1990 left a total of 34 studies for review.

Clinical implications of HCV/HTLV-1/2 co-infection

A large-scale survey of residents of Iki Island in Japan, an endemic region for HTLV-1 infection, conducted by

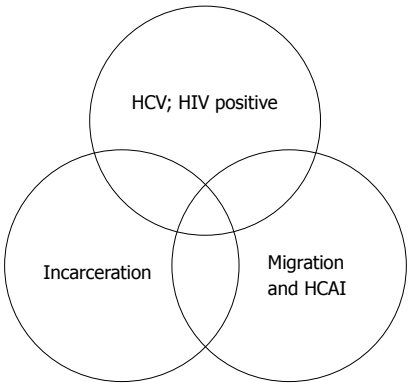

Figure 1 Global risk factors for co-infection with hepatitis C virus and human T lymphotropic virus-1/2 in people who inject drugs. Adapted from Roger and Castro, 2014[15]. In this model, each circle represents a risk factor of hepatitis C virus (HCV)/human T lymphotropic virus-1/2 co-infection in people who inject drugs in the context of global migration patterns and increased health care-associated infections (HCAI; also known as "nosocomial" or "hospital-based" infections) in settings with limited resources, incarceration (particularly in countries that lack harm-reduction programs for incarcerated populations), and in the background of human immunodeficiency virus (HIV) or HCV infection.

Kishihara *et al*[17] showed that individuals with HCV/HTLV-1 co-infection had a lower rate of natural clearance of HCV RNA and of sustained virological response to interferon (IFN) treatment than their counterparts with HCV infection alone; moreover, the co-infected population showed significantly higher HCV viremia ($P < 0.05$). Other Japanese studies of HCV/HTLV-1 co-infection in PWID showed associations with liver disease (6-fold increased risk)[18] and liver cancer mortality (2.6-fold increased risk)[19], leading to the hypothesis of an HTLV-1–induced immune modulation and inflammatory cytokine dysregulation that could affect HCV persistence and progression to liver disease[20,21]. In contrast to the Japanese findings, however, two Brazilian studies[22,23] of HCV/HTLV-1 co-infection provide epidemiological and immunological evidence of a higher rate of spontaneous clearance of HCV in patients with HIV/HTLV-1 co-infection as compared to patients harbouring only an HIV/HCV co-infection or an HCV mono-infection. The differences between HCV and HTLV-1 interaction outcomes in these two settings may be due to host genetic factors (*e.g.,* HLA genotypes), study design, or other unmeasured parameters of the study populations. Studies of the molecular underpinnings of the HCV and HTLV-1 interaction outcomes have shown that HTLV-1-infected T cells, together with viral gene expression and cellular signalling mechanisms, can trigger a strong virus-specific immune response and increased proinflammatory cytokine production[24,25]. Moreover, the cellular immune response has been implicated in the control of HTLV-1 infection as well as in the development of related inflammatory alterations in patients[26]. The cellular immune response involves $CD4^+$ T cells differentiating towards the Th1, Th2 and Th17 lineages, producing a variety of proinflammatory cytokines, chemokines, adhesion molecules and proinflammatory enzymes, which contribute to chronic inflammatory conditions and include reactive oxygen species (ROS), tumour necrosis factor alpha (TNFα), interleukins (IL1, 6, 8 and 18), nuclear factor-kappa B (NF-κB), hypoxia-inducible factor (HIF), IFNγ, and cyclooxygenase (COX)[27,28]. Moreover, contributions of different HTLV-1 oncogenic pathways related to viral proteins have been recently described recently[29]. Additionally, a study of 199 HTLV-1 infected subjects by Treviño *et al*[30] showed that the risk of developing TSP was 10-times higher among HTLV-1 carriers who harboured the IL B-28 CT and TT alleles than their counterparts who harboured the CC allele. The same study also showed an association between the CT polymorphism and increased HTLV-1 viral loads, and that the CC allele is found more frequently among asymptomatic carriers of HTLV-1 (62%). Collectively, these data strongly suggest that HTLV-1 co-infection plays a role in HCV viremia and evolution, attainment of HCV sustained virological response to α-interferon treatment, and HCV-related liver disease progression. Briefly, the current evidence supports postulation of worsening of HCV infection in the context of HTLV-1 co-infection.

Treatment implications of HCV/HTLV-1/2 co-infection

HTLV-1/2 asymptomatic carriers do not require treatment. However, for HTLV1/2 carriers who experience clinical onset of ATLL or HAM/TSP the current treatment options are limited and those available have a suboptimal range of efficacy. A meta-analysis of ATLL antiviral therapies showed that α-IFN and zidovudine (AZT) combination can induce complete remission and produce a high (82%) 5-year survival rate in ATLL patients[31]. Another ATLL therapeutic approach, specifically the α-interferon, arsenic and AZT combination, was evaluated in a later study of 16 patients and showed induction of a beneficial cytokine modulation response with a shift from the pre-treatment Treg/Th2 phenotype to the Th1 phenotype post-treatment[32]. Thus, this triple drug combination may be a useful treatment approach to restore an immuno-competent microenvironment, which will enhance the eradication of ATL cells and the prevention of opportunistic infections. Yet another study evaluated the combination of valproate (VPA) and AZT in patients with advanced HAM/TSP and found that the treatment may control viral replication through inhibition of the virus reverse-transcriptase and/or its associated molecular machinery[33]. The same strategy has been evaluated in non-human primates (*Papio papio*) naturally infected with the simian T cell lymphotropic virus type 1 (STLV-1; the equivalent of HTLV-1 which also causes simian ATLL). The animals were asymptomatic carriers and treatment with AZT/VPA induced a reduction of viral load which relapsed after treatment interruption[34]. A study of the HIV integrase inhibitor drug, raltegravir, as treatment for HTLV-1 (evaluating 5 carriers, including 2 with HAM and 3 asymptomatic) showed achievement of a transitory viral load reduction during the 24 wk of treatment but with no main clinical improvement[35]. Finally, Abad-Fernández

Table 1 Key features of hepatitis C virus and human T lymphotropic virus-1/2 co-infection

HTLV-1/2 infections are found in HCV co-infected PWID worldwide, as a consequence of unsafe injection practices
HTLV-1 infection induces chronic inflammation and oncogenic cellular changes
HTLV-1 co-infection of chronic hepatitis C carriers can increase HCV viral load, accelerate liver disease progression, and favour onset of liver cancer
Evidence suggests that HTLV-1/2 clinical presentations can be linked to higher viral loads in contrast to asymptomatic HTLV-1/2 carriers
Available treatment data shows that HTLV-1/2 viral load can be suppressed but not eradicated

HTLV: Human T lymphotropic virus; HCV: Hepatitis C virus; PWID: People who inject drugs.

et al[36] reported the only study to date in our collected articles from the PubMed literature to assess the evolution of HTLV co-infection (including with HIV, HTLV-2 and HCV) among patients who received treatment for HCV and showed reduction of HTLV-2 viral load in response to the α-IFN and ribavirin combination treatment.

DISCUSSION AND FUTURE PROSPECTS

The main features of HCV/HTLV-1/2 co-infection, based on evidence reported in the current literature, are summarized in Table 1. Briefly, they highlight the role of PWID as a core affected population and the negative immune modulation effect of HTLV-1 co-infection in patients with chronic hepatitis C. At the same time, HCV/HTLV-1/2 co-infection remains an unresolved clinical challenge; prospective studies looking at the HTLV-1/2 infection outcome in subjects receiving new direct-acting antiviral treatments targeting the HCV infection will likely provide further insights towards improvement.

The features listed in Table 1 are a source of new research questions to be addressed. In addition, they should challenge the clinical field to reflect on the pertinence of adding HTLV-1/2 screening for PWID patients and particularly in relation to caring for migrant populations from high endemic areas in different worldwide settings.

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REFERENCES

1 **Mohd Hanafiah K**, Groeger J, Flaxman AD, Wiersma ST. Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence. *Hepatology* 2013; **57**: 1333-1342 [PMID: 23172780 DOI: 10.1002/hep.26141]

2 **Esteban JI**, Sauleda S, Quer J. The changing epidemiology of hepatitis C virus infection in Europe. *J Hepatol* 2008; **48**: 148-162 [PMID: 18022726 DOI: 10.1016/j.jhep.2007.07.033]

3 **Alter MJ**. Epidemiology of hepatitis C virus infection. *World J Gastroenterol* 2007; **13**: 2436-2441 [PMID: 17552026 DOI: 10.3748/wjg.v13.i17.2436]

4 **Nelson PK**, Mathers BM, Cowie B, Hagan H, Des Jarlais D, Horyniak D, Degenhardt L. Global epidemiology of hepatitis B and hepatitis C in people who inject drugs: results of systematic reviews. *Lancet* 2011; **378**: 571-583 [PMID: 21802134 DOI: 10.1016/S0140-6736(11)61097-0]

5 **Grebely J**, Matthews GV, Lloyd AR, Dore GJ. Elimination of hepatitis C virus infection among people who inject drugs through treatment as prevention: feasibility and future requirements. *Clin Infect Dis* 2013; **57**: 1014-1020 [PMID: 23728143 DOI: 10.1093/cid/cit377]

6 **Gessain A**, Cassar O. Epidemiological Aspects and World Distribution of HTLV-1 Infection. *Front Microbiol* 2012; **3**: 388 [PMID: 23162541 DOI: 10.3389/fmicb.2012.00388]

7 **Manns A**, Hisada M, La Grenade L. Human T-lymphotropic virus type I infection. *Lancet* 1999; **353**: 1951-1958 [PMID: 10371587 DOI: 10.1016/S0140-6736(98)09460-4]

8 **Zehender G**, Colasante C, De Maddalena C, Bernini F, Savasi V, Persico T, Merli S, Ridolfo A, Santambrogio S, Moroni M, Galli M. High prevalence of human T-lymphotropic virus type 1 (HTLV-1) in immigrant male-to-female transsexual sex workers with HIV-1 infection. *J Med Virol* 2004; **74**: 207-215 [PMID: 15332268 DOI: 10.1002/jmv.20165]

9 **Gonçalves DU**, Proietti FA, Ribas JG, Araújo MG, Pinheiro SR, Guedes AC, Carneiro-Proietti AB. Epidemiology, treatment, and prevention of human T-cell leukemia virus type 1-associated diseases. *Clin Microbiol Rev* 2010; **23**: 577-589 [PMID: 20610824 DOI: 10.1128/CMR.00063-09]

10 **Proietti FA**, Carneiro-Proietti AB, Catalan-Soares BC, Murphy EL. Global epidemiology of HTLV-I infection and associated diseases. *Oncogene* 2005; **24**: 6058-6068 [PMID: 16155612 DOI: 10.1038/sj.onc.1208968]

11 **Hlela C**, Shepperd S, Khumalo NP, Taylor GP. The prevalence of human T-cell lymphotropic virus type 1 in the general population is unknown. *AIDS Rev* 2009; **11**: 205-214 [PMID: 19940947]

12 **Krook A**, Albert J, Andersson S, Biberfeld G, Blomberg J, Eklund I, Engström A, Julander I, Käll K, Martin C, Stendahl P, Struve J, Sönnerborg A. Prevalence and risk factors for HTLV-II infection in 913 injecting drug users in Stockholm, 1994. *J Acquir Immune Defic Syndr Hum Retrovirol* 1997; **15**: 381-386 [PMID: 9342259 DOI: 10.1097/00042560-199708150-00009]

13 **de la Fuente L**, Toro C, Soriano V, Brugal MT, Vallejo F, Barrio G, Jiménez V, Silva T. HTLV infection among young injection and non-injection heroin users in Spain: prevalence and correlates. *J Clin Virol* 2006; **35**: 244-249 [PMID: 16143565 DOI: 10.1016/j.jcv.2005.06.006]

14 **Zunt JR**, Tapia K, Thiede H, Lee R, Hagan H. HTLV-2 infection in injection drug users in King County, Washington. *Scand J Infect Dis* 2006; **38**: 654-663 [PMID: 16857611 DOI: 10.1080/00365540600617009]

15 **Roger E**, Castro E. Etude de cas: pertinence du dépistage de l' HTLV-1/2 chez les usagers de drogues IV en Europe. As oral presentation in proceedings of the IV Colloque international francophone sur le traitement de la dépendance aux opioïdes. TDO4, Brussels, Belgium. Available from: URL: http://www.tdo4.be/programme/

16 **World Health Organization 2010**. The burden of health care-associated infections worldwide. Available from: URL: http://www.who.int/gpsc/country_work/summary_20100430_en.pdf

17 **Kishihara Y**, Furusyo N, Kashiwagi K, Mitsutake A, Kashiwagi S, Hayashi J. Human T lymphotropic virus type 1 infection influences hepatitis C virus clearance. *J Infect Dis* 2001; **184**: 1114-1119 [PMID: 11598833 DOI: 10.1086/323890]

18 **Hisada M**, Chatterjee N, Zhang M, Battjes RJ, Goedert JJ. Increased hepatitis C virus load among injection drug users infected with human immunodeficiency virus and human T lymphotropic virus type II. *J Infect Dis* 2003; **188**: 891-897 [PMID: 12964121 DOI: 10.1086/377585]

19 **Boschi-Pinto C**, Stuver S, Okayama A, Trichopoulos D, Orav EJ, Tsubouchi H, Mueller N. A follow-up study of morbidity and mortality associated with hepatitis C virus infection and its interaction with human T lymphotropic virus type I in Miyazaki, Japan. *J Infect Dis* 2000; **181**: 35-41 [PMID: 10608748 DOI: 10.1086/315177]

20 **Casseb J**. Possible mechanism for positive interaction of human T cell leukemia type I on liver disease in a hepatitis C virus-infected Japanese cohort. *J Infect Dis* 2000; **182**: 379-380 [PMID: 10882633 DOI: 10.1086/315647]

21 **Tokunaga M**, Uto H, Oda K, Tokunaga M, Mawatari S, Kumagai K, Haraguchi K, Oketani M, Ido A, Ohnou N, Utsunomiya A, Tsubouchi H. Influence of human T-lymphotropic virus type 1 coinfection on the development of hepatocellular carcinoma in patients with hepatitis C virus infection. *J Gastroenterol* 2014; **49**: 1567-1577 [PMID: 24463696 DOI: 10.1007/s00535-013-0928-5]

22 **Bahia F**, Novais V, Evans J, Le Marchand C, Netto E, Page K, Brites C. The impact of human T-cell lymphotropic virus I infection on clinical and immunologic outcomes in patients coinfected with HIV and hepatitis C virus. *J Acquir Immune Defic Syndr* 2011; **57** Suppl 3: S202-S207 [PMID: 21857319 DOI: 10.1097/QAI.0b013e31821e9a1e]

23 **Le Marchand C**, Bahia F, Page K, Brites C. Hepatitis C virus infection and spontaneous clearance in HTLV-1 and HIV co-infected patients in Salvador, Bahia, Brazil. *Braz J Infect Dis* 2015; **19**: 486-491 [PMID: 26254690 DOI: 10.1016/j.bjid.2015.06.007]

24 **Ouaguia L**, Mrizak D, Renaud S, Moralès O, Delhem N. Control of the inflammatory response mechanisms mediated by natural and induced regulatory T-cells in HCV-, HTLV-1-, and EBV-associated cancers. *Mediators Inflamm* 2014; **2014**: 564296 [PMID: 25525301 DOI: 10.1155/2014/564296]

25 **Araya N**, Sato T, Yagishita N, Ando H, Utsunomiya A, Jacobson S, Yamano Y. Human T-lymphotropic virus type 1 (HTLV-1) and regulatory T cells in HTLV-1-associated neuroinflammatory disease. *Viruses* 2011; **3**: 1532-1548 [PMID: 21994794 DOI: 10.3390/v3091532]

26 **Leal FE**, Ndhlovu LC, Hasenkrug AM, Bruno FR, Carvalho KI, Wynn-Williams H, Neto WK, Sanabani SS, Segurado AC, Nixon DF, Kallas EG. Expansion in CD39+ CD4+ immunoregulatory t cells and rarity of Th17 cells in HTLV-1 infected patients is associated with neurological complications. *PLoS Negl Trop Dis* 2013; **7**: e2028 [PMID: 23409198 DOI: 10.1371/journal.pntd.0002028]

27 **Aggarwal BB**, Shishodia S, Sandur SK, Pandey MK, Sethi G. Inflammation and cancer: how hot is the link? *Biochem Pharmacol* 2006; **72**: 1605-1621 [PMID: 16889756 DOI: 10.1016/j.bcp.2006.06.029]

28 **Raval GU**, Bidoia C, Forlani G, Tosi G, Gessain A, Accolla RS. Localization, quantification and interaction with host factors of endogenous HTLV-1 HBZ protein in infected cells and ATL. *Retrovirology* 2015; **12**: 59 [PMID: 26140924 DOI: 10.1186/s12977-015-0186-0]

29 **Bidoia C**. Human T-lymphotropic virus proteins and post-translational modification pathways. *World J Virol* 2012; **1**: 115-130 [PMID: 24175216 DOI: 10.5501/wjv.v1.i4.115]

30 **Treviño A**, Lopez M, Vispo E, Aguilera A, Ramos JM, Benito R, Roc L, Eiros JM, de Mendoza C, Soriano V. Development of tropical spastic paraparesis in human T-lymphotropic virus type 1 carriers is influenced by interleukin 28B gene polymorphisms. *Clin Infect Dis* 2012; **55**: e1-e4 [PMID: 22460962 DOI: 10.1093/cid/cis343]

31 **Bazarbachi A**, Plumelle Y, Carlos Ramos J, Tortevoye P, Otrock Z, Taylor G, Gessain A, Harrington W, Panelatti G, Hermine O. Meta-analysis on the use of zidovudine and interferon-alfa in adult T-cell leukemia/lymphoma showing improved survival in the leukemic subtypes. *J Clin Oncol* 2010; **28**: 4177-4183 [PMID: 20585095 DOI: 10.1200/JCO.2010.28.0669]

32 **Kchour G**, Rezaee R, Farid R, Ghantous A, Rafatpanah H, Tarhini M, Kooshyar MM, El Hajj H, Berry F, Mortada M, Nasser R, Shirdel A, Dassouki Z, Ezzedine M, Rahimi H, Ghavamzadeh A, de Thé H, Hermine O, Mahmoudi M, Bazarbachi A. The combination of arsenic, interferon-alpha, and zidovudine restores an «immunocompetent-like» cytokine expression profile in patients with adult T-cell leukemia lymphoma. *Retrovirology* 2013; **10**: 91 [PMID: 23962110 DOI: 10.1186/1742-4690-10-91]

33 **Mahieux R**. [Virological aspects of HTLV-1 infection and new therapeutical concepts]. *Bull Soc Pathol Exot* 2011; **104**: 181-187 [PMID: 21607661 DOI: 10.1007/s13149-011-0161-5]

34 **Afonso PV**, Mekaouche M, Mortreux F, Toulza F, Moriceau A, Wattel E, Gessain A, Bangham CR, Dubreuil G, Plumelle Y, Hermine O, Estaquier J, Mahieux R. Highly active antiretroviral treatment against STLV-1 infection combining reverse transcriptase and HDAC inhibitors. *Blood* 2010; **116**: 3802-3808 [PMID: 20587783 DOI: 10.1182/blood-2010-02-270751]

35 **Treviño A**, Parra P, Bar-Magen T, Garrido C, de Mendoza C, Soriano V. Antiviral effect of raltegravir on HTLV-1 carriers. *J Antimicrob Chemother* 2012; **67**: 218-221 [PMID: 21965433 DOI: 10.1093/jac/dkr404]

36 **Abad-Fernández M**, Dronda F, Moreno A, Casado JL, Pérez-Elías MJ, Quereda C, Moreno S, Vallejo A. Brief Report: Reduced Cell-Associated HTLV-2 DNA in Antiretroviral Treated HIV-1-HCV-Coinfected Patients Who Either Received Interferon-α/Ribavirin-Based Hepatitis C Therapy or Had Spontaneous HCV RNA Clearance. *J Acquir Immune Defic Syndr* 2015; **69**: 286-290 [PMID: 26181704 DOI: 10.1097/QAI.0000000000000608]

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ORIGINAL ARTICLE

Retrospective Study

Active tracking of rejected dried blood samples in a large program in Nigeria

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Author contributions: Inalegwu A, Phillips S and Ndembi N designed and performed the research and wrote the first draft paper; Ndembi N supervised the research work; Datir R, Chime C, Ozumba P, Peters S, Ogbanufe O, Mensah C, Abimiku A and Dakum P revised the manuscript and contributed to the analysis.

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Institutional review board statement: The study was approved by the Institutional Review Board and Ethics Committee of the Institute of Human Virology, Nigeria and the National Human Research and Ethics Committee (NHREC Approval#NHREC /01/01/2007-15/08/2015). No patient identifying information was retained. Data analysis was unlinked and anonymous. With delinking of patient identifiers and confidentiality safeguards, the benefits of improved health care quality outweigh the minimal risks.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used secondary de-identified/anonymous clinical data that were obtained after each patient agreed to be enrolled in our treatment program.

Conflict-of-interest statement: The authors declare that they have no conflict of interest or no financial relationships to disclose.

Data sharing statement: No additional available data are available.

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Abstract

AIM: To study the impact of rejection at different levels of health care by retrospectively reviewing records of dried blood spot samples received at the molecular laboratory for human immunodeficiency virus (HIV) early infant diagnosis (EID) between January 2008 and December 2012.

METHODS: The specimen rejection rate, reasons for rejection and the impact of rejection at different levels of health care was examined. The extracted data were cleaned and checked for consistency and then de-duplicated using the unique patient and clinic identifiers. The cleaned data were ciphered and exported to SPSS version 19 (SPSS 2010 IBM Corp, New York, United States) for statistical analyses.

RESULTS: Sample rejection rate of 2.4% (n = 786/32552) and repeat rate of 8.8% (n = 69/786) were established. The mean age of infants presenting for first HIV molecular test among accepted valid samples was 17.83 wk (95%CI: 17.65-18.01) *vs* 20.30 wk (95%CI: 16.53-24.06) for repeated samples. HIV infection rate was 9.8% *vs* 15.9% for accepted and repeated samples. Compared to tertiary healthcare clinics, secondary and primary clinics had two-fold and three-fold higher likelihood of sample rejection, respectively (P < 0.05). We observed a significant increase in sample rejection rate with increasing number of EID clinics (r = 0.893, P = 0.041). The major reasons for rejection were improper sample collection (26.3%), improper labeling (16.4%) and insufficient blood (14.8%).

CONCLUSION: Programs should monitor pre-analytical variables and incorporate continuous quality improvement interventions to reduce errors associated with sample rejection and improve patient retention.

Key words: Human immunodeficiency virus; Prevention of mother-to-child transmission; Early infant diagnosis; Dried blood spot; Pre-analytical error; Sample rejection

Core tip: For early infant diagnosis of human immunodeficiency virus, the samples of choice are dried blood spots (DBS). DBS samples are received from over 100 health care centers at the Asokoro Laboratory Training Centre. When DBS arrives the laboratory, a technician receives the samples as well as all accompanying laboratory request forms and all relevant documentation. All routinely collected DBS samples are physically examined for quality and acceptability for molecular testing upon reception at the laboratory. Only samples that meet the laboratory acceptance criteria are usually tested. Samples which fail to meet the acceptance criteria are registered in the sample rejection logbook without being tested. All DBS samples accepted as fit-for-testing are electronically registered into the laboratory information management system (LIMS). The use of the LIMS reduces instances of transcriptional errors. DBS samples are processed using real-time PCR technology on the Cobas Taqman and Cobas ampliprep equipment. DBS spots are cut, eluted into solution, and then placed in the equipment where DNA extraction, amplification and detection is automatically carried out. Once results are ready, they are validated by the laboratory scientist for accuracy and completeness. If assay is judged to be a valid run, the assay is accepted with a click of a computer button.

Inalegwu A, Phillips S, Datir R, Chime C, Ozumba P, Peters S, Ogbanufe O, Mensah C, Abimiku A, Dakum P, Ndembi N. Active tracking of rejected dried blood samples in a large program in Nigeria. *World J Virol* 2016; 5(2): 73-81 Available from: URL: http://www.wjgnet.com/2220-3249/full/v5/i2/73.htm DOI: http://dx.doi.org/10.5501/wjv.v5.i2.73

INTRODUCTION

The recognition of prevention of mother-to-child transmission (PMTCT) as an essential tool for combating the human immunodeficiency virus (HIV) epidemic has led to its institution by the World Health Organization (WHO) as a global health agenda[1]. PMTCT programs can reduce the risk of MTCT to less than 2%, and is today the most efficacious tool for preventing pediatric HIV infection globally[2-5]. PMTCT programs have witnessed appreciable success in Nigeria with documented MTCT rates ranging from 1.3%-4.8% in mother-baby pairs who received antiretroviral therapy (ARV), compared to MTCT rates ranging from 39.8%-68.0% where no intervention was administered[6-9]. Nevertheless, MTCT is still a critical challenge of the HIV/AIDS pandemic in resource limited settings (RLS)[10-13]. According to UNGASS country reports, only 30.1% of HIV positive pregnant women in Nigeria received ARV to prevent MTCT in 2013, which resulted in MTCT rates as high as 27.3%. And in the same year, only 3.9% of exposed infants received a PCR diagnostic test within two months of birth[14]. This low level of diagnosis among HIV-exposed infants falls below the national target of ensuring that at least 90% of all HIV exposed infants have access to early diagnosis services by 2015[5]. Especially with an estimated 52125 to 104250 infants at risk of being infected with HIV without intervention[15].

Early testing of exposed infants from 4 to 6 wk of birth is recommended by the WHO to insure timely diagnosis and treatment of HIV positive children[1,16]. Without intervention HIV causes a 20% mortality rate in infected infants in RLS by 3 mo of age, which increases to an estimated 48% and 52% before ages one and two, respectively[16,17]. Despite this, the average age of initiation of ARV in pediatric HIV/AIDS patients in RLS remains high[17,18], and health-care systems often fail to meet the national demands for care[1]. In 2012 only 12% of children eligible for ARV received treatment in Nigeria[19]. Reports also show high rates of loss to follow-up (LTFU) of infants throughout the PMTCT cascade in RLS, with an over 30% rate of LTFU by 3 mo and more than 70% by 6 mo of birth[20-23]. It is estimated that only 0.5% to 52.8% of infants eligible for early infant diagnosis (EID) testing in RLS complete the care cascade and eventually access treatment[22]. Therefore, strategies for improving patient retention should be a critical focus of PMTCT programs with respect to the UNAIDS 90-90-90 targets[5]. A review of the PMTCT cascade is essential

to identify gaps towards achieving the goals of PMTCT services[24]. Careful consideration of the role of laboratory in ensuring early diagnosis and universal access to pediatric ARVs is also vital to ensure the widest possible coverage of PMTCT services[25].

EID is a vital intervention which allows countries to provide essential health services for all children and to continue to make progress in keeping children alive and healthy. Standard HIV antibody testing - as is done with adults and older children - cannot identify infected infants in their first year of life, as it also detects maternal HIV antibodies that are transferred to the baby during pregnancy (and subsequently decline slowly within the first year of life)[8,9]. More demanding testing methods that rely on detecting HIV-1, or virological tests are required for diagnosing infants[19]. HIV DNA PCR test is the most widely used initial assay for EID in industrialized countries[1]. Early HIV virological detection test at or after 6 wk of age for all HIV-exposed children identifies most children infected before, during and immediately after delivery[6-9].

The guideline for early infant diagnosis in Nigeria provides that all HIV exposed infants have a first HIV diagnostic test at 6 wk of age, a follow-up test at 6 wk after cessation of breastfeeding and a confirmatory HIV test at 18 mo[26-28]. Pre-analytical errors contribute an estimated 60%-70% of all mistakes in laboratory diagnostics and can render dried blood spots (DBS) untestable, leading to specimen rejection with a resultant negative impact on patients[29-31]. Common pre-analytical errors associated with DBS rejection include: Labeling errors, sample damage, missing or inconsistent data, and insufficient volume[32-35]. High risk for rapid disease progression and death necessitates the need for early identification and treatment of HIV positive infants[36]. The goal of the present study was to investigate the DBS sample rejection rate attributable to pre-analytical errors and its effect on patient care in the PMTCT cascade at the tertiary, secondary and primary levels of healthcare service delivery in Nigeria and provide strategies to reduce effectively to nil rejection at all levels of healthcare service delivery in Nigeria.

MATERIALS AND METHODS

Study setting and design

This is a cross-sectional descriptive study conducted among HIV-exposed babies from 150 health facilities using prospectively collected data from the molecular diagnostics laboratory of Institute of Human Virology, Nigeria (IHVN). The IHVN is a not-for-profit organization established in 2004 to scale up the US PEPFAR program in Nigeria and conduct research and training towards improving quality and promoting evidence based health system strengthening[37]. The IHVN currently has 10 out of the 26 molecular diagnostic laboratories across the six geopolitical regions of the country.

Laboratory data collected over a 5-year period from January 8, 2008 to December 19, 2012 were retrieved from the laboratory's information management Microsoft Excel database. The dataset included the following variables: (1) Date of sample collection; (2) Patient's hospital number; (3) Laboratory number; (4) Date specimen was received at the laboratory; (5) Specimen type; (6) Reason for DNA PCR test (first test for healthy exposed baby, first test for sick baby, follow-up test to confirm first test, follow-up test after cessation of breastfeeding); (7) Specimen suitability for analysis (accepted or rejected); and (8) Reasons for sample rejection and other demographic information. The demographic information included: (1) Patient's age; (2) Patient's sex; (3) PMTCT intervention administered to mother; (4) PMTCT intervention administered to patient (exposed infant); (5) Breastfeeding status; and (6) DBS collection clinic. The dataset included information on samples received at the molecular diagnostics laboratory from 150 healthcare centers including tertiary (n = 9; 6%), secondary (n = 101; 67%) and primary (n = 40, 27%) healthcare centers within the Northern region of Nigeria.

Sample history

All routinely collected DBS samples were examined for quality and acceptability for molecular testing upon reception at the laboratory. Valid specimens were accessioned and registered into the laboratory information management register and Microsoft Excel template. Only samples that met the laboratory acceptance criteria were tested. Samples which failed to meet the acceptance criteria were registered in the sample rejection log without being tested. The laboratory records for accepted (valid) and rejected samples were merged using the patient's hospital number and collection healthcare clinic identifiers.

Reasons for sample rejection

Reasons for sample rejection included: Sample quantity insufficient for testing; Sample not properly labeled with patient's name, patient's hospital number and the name of the collection clinic; Improperly collected sample. This includes all specimens which appeared diluted, had alcohol halo or serum ring around it and specimen which appeared abraded, over-saturated, clotted, caked or layered; Sample that appeared discolored or contaminated; Sample not properly packaged separately to avoid cross-contamination; Sample not allowed to dry completely before packaging and mailing; Sample for babies younger than 6 wk or older than 18 mo of age; and sample received without a patient/test request form.

Study variables

The sample rejection rate was the primary outcome variable in this study. The type and frequency of pre-analytical errors associated with sample rejection and the repeat rate for rejected samples were also determined relative to the type of healthcare center where the sample was collected. We also evaluated the HIV-1 positivity rate and the mean age among infants presenting for HIV-1

Table 1 Dried blood spot sample rejection rate by year

		Year					Total
		2008	2009	2010	2011	2012	
Rejected?	No	2117 (6.5%)	5186 (15.9%)	6634 (20.4%)	8759 (26.9%)	9070 (27.9%)	31766 (97.6%)
Count (%)	Yes	2 (0.1%)	62 (1.2%)	223 (3.3%)	166 (1.9%)	333 (3.5%)	786 (2.4%)
Total		2119 (6.5%)	5248 (16.1%)	6857 (21.1%)	8925 (27.4%)	9403 (28.9%)	32552 (100%)

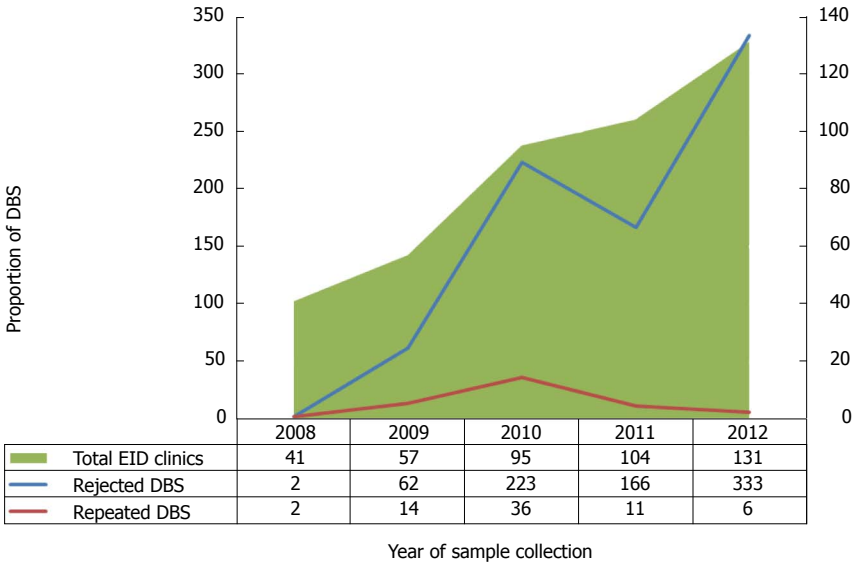

Figure 1 Annual early infant diagnosis collected at clinics *vs* proportion of rejected and repeated samples. EID: Early infant diagnosis; DBS: Dried blood spots.

DNA PCR test for accepted and repeated samples.

Statistical analysis

The extracted data were cleaned, checked for consistency and then de-duplicated using the unique patient and clinic identifiers. The cleaned data were ciphered and exported to SPSS version 19 (SPSS 2010 IBM Corp, New York, United States) for statistical analyses. We used descriptive statistics to establish the DBS sample rejection rate and the reasons for rejection; and to determine the mean age of infants presenting for first HIV-1 DNA PCR test and for a follow-up test. Logistic regression analysis was used to test the difference in sample rejection rate between the different types of healthcare centers providing care. Furthermore, we used Pearson correlation coefficients (r) to test the association between the annual sample rejection rate and the number of clinics providing EID services. A P-value < 0.05 was considered statistically significant. The statistical review of the study was performed by a biomedical statistician.

RESULTS

After the data cleaning process, 32552 sample data from laboratory records over the five-year study period were included in the analysis. A total of 6322/32552 (19.4%) samples were sent from tertiary health clinics, 24777/32552 (76.1%) from secondary health clinics, and 1453/32552 (4.5%) from primary health clinics. Based on the laboratory's sample rejection criteria, 786/32552 (2.4%) samples were found to have been rejected. Only 8.8% of rejected samples were repeated. Primary healthcare clinics had the highest rejection rate of 4.0%, while secondary and tertiary healthcare clinics had rejection rates of 2.6% and 1.3%, respectively. Secondary healthcare clinics had a twice greater probability (OR = 1.955; 95%CI: 1.557-2.455) and primary healthcare clinics had more than 3 times higher probability (OR = 3.051; 95%CI: 2.174-4.281) of DBS sample rejection when compared to tertiary health care clinics (P < 0.05). The repeat rates were 1.7%, 8.7%, and 14.1% for primary, secondary and tertiary healthcare centers, respectively.

As shown in Table 1, the cumulative sample rejection rate increased from 0.1% in 2008 to 3.5% in 2012, while the repeat rate of rejected samples decreased across the study period (Figure 1) from 2/2 (100%) to 6/333 (1.8%). The sample rejection rate also increased with increasing number of EID DBS collection clinics (Figure 2) in the PMTCT program (r = 0.893, P = 0.041).

We observed a high mean age of 17.83 wk (SD = 15.29; 95%CI: 17.65-18.01) for infants presenting for first EID test in the program. A higher mean age of 20.30 wk (SD = 14.31; 95%CI: 16.53-24.06) was recorded for repeated samples among infants presenting for a first EID test. The mean age of infants for all repeated samples including patients presenting for first test and follow-up test was 22.32 wk (SD = 15.49; 95%CI: 18.60-26.05) *vs* 19.95 wk (SD = 16.43; 95%CI: 19.77-20.14) among samples that were accepted at first collection. Additionally, the mean

Table 2 Reasons for sample rejection by type of healthcare care center (n = 786)

Reason for rejection count (%)	Tertiary	Secondary	Primary	Total
No DBS card	5 (0.6)	76 (9.7)	4 (0.5)	85 (10.8)
Insufficient quantity of sample	15 (1.9)	72 (9.2)	29 (3.7)	116 (14.8)
No request form	6 (0.8)	39 (5.0)	0 (0.0)	45 (5.7)
Improper collection	25 (3.2)	171 (21.9)	11 (1.4)	207 (26.3)
Baby over age (⩾ 18 mo)	6 (0.8)	58 (7.4)	0 (0.0)	64 (8.1)
Improper labeling	17 (2.2)	110 (14.0)	2 (0.3)	129 (16.4)
Improper packaging	4 (0.5)	53 (6.7)	8 (1.0)	65 (8.3)
Contaminated sample	0 (0.0)	5 (0.6)	0 (0.0)	5 (0.6)
Baby under age (< 6 wk)	2 (0.3)	19 (2.4)	2 (0.3)	23 (2.9)
Reason unknown	6 (0.8)	40 (5.1)	1 (0.1)	47 (6.0)
Total	86 (10.9)	643 (81.8)	57 (7.3)	786 (100.0)

DBS: Dried blood spot.

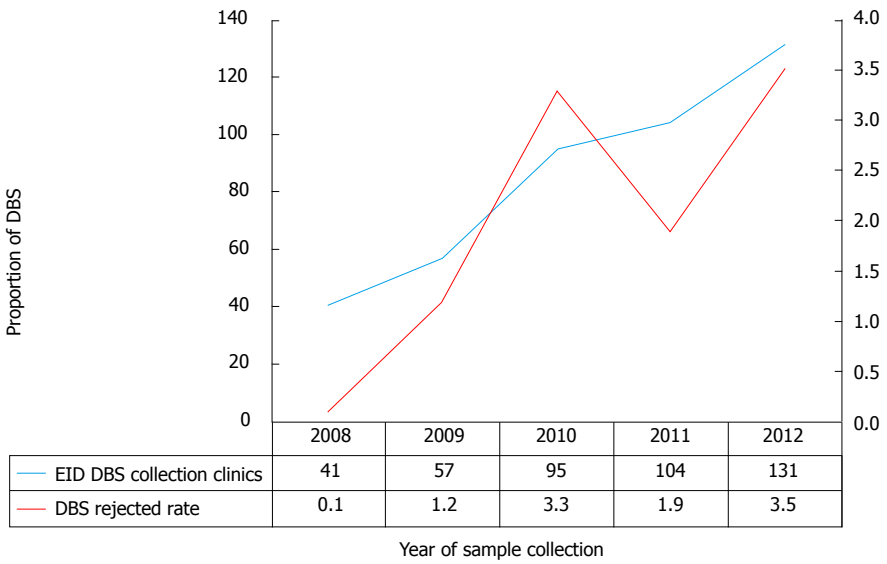

Figure 2 Annual total early infant diagnosis collected at clinics *vs* sample rejection rate. EID: Early infant diagnosis; DBS: Dried blood spots.

age was 33.02 wk (SD = 17.70; 95%CI: 21.13-44.91) for those presenting for a follow-up test among repeated samples *vs* 35.55 wk (SD = 16.09; 95%CI: 35.03-36.08) for accepted samples. We established a cumulative positive rate of 9.8% for all accepted samples routinely tested over the 5-year period while the positive rate for repeated samples was 15.9%.

The average turn-around time from sample collection at the health facility to receipt of sample at the laboratory was 3.82 wk ± 3.63 (95%CI: 3.69-3.95). Overall, the most frequently occurring errors associated with sample rejection were improper sample collection (n = 207/786; 26.3%), improper labeling (n = 129/786; 16.4%) and insufficient blood (n = 116/786; 14.8%). Other reasons for rejection included improper packaging, no sample sent, no test request form sent, baby over-age (> 18 mo), baby under-age (< 6 wk) and contaminated sample (Table 2).

DISCUSSION

The mean age of infants at first HIV DNA PCR test in this study is far beyond the recommended age of 4-6 wk for EID testing[1]. Without treatment, HIV related mortality in infected infants peaks at 8 to 12 wk[38]. Delay in presentation for EID averts the opportunities to administer ARV and reduce MTCT[6,7,39], thereby permitting the emergence of more severe clinical manifestation of HIV infection in pediatric patients[40]. Strategies that enhance awareness of PMTCT and EID services, promote partner involvement, provide economic incentives and offer close follow-up to HIV positive women during pregnancy and after delivery have been shown to be effective[41,42]. Active tracking of HIV positive mothers using support groups and mobile applications have also been shown to increase uptake of services and retention of the mother-baby pair in PMTCT programs[41,43,44].

Establishing an accurate link between rejected samples and the impact on clinical outcome is difficult[32]. However, the observed high rejection and low repeat rates in addition to the higher mean age of infants at the time of specimen recollection in this study suggest that sample rejection further delays HIV diagnosis in infants while emphasizing the importance of standardization and monitoring of pre-analytical variables[30]. Our study agrees with other investigations where pre-analytical

errors are implicated in delayed diagnosis of infant HIV[33-35,45]. Other adverse patient outcomes due to sample rejection include demand for patient revisits for specimen recollection, discomfort to the patient, test abandonment or LTFU and time lost in waiting for results with the accompanying cost implications associated with multiple clinic visits[22,46,47]. The extended delay in results may also have contributed to the high attrition and low repeat rates among rejected samples.

Due to the importance of accurate and timely diagnosis in the care and treatment of HIV positive children and the increased risk for postnatal transmission, morbidity and early mortality in untreated HIV[6,7,48], greater attention to sample quality, clear guidelines on the responsibility and protocols for sample collection, error reporting and initiating patient follow-up for timely specimen recollection should be established. The high turn-around time of 3.82 wk ± 3.63 wk from sample collection to receipt at the testing laboratory also suggests the need for improved systems for rapid sample transportation[49]. Lack of standardized protocols for laboratory processes including sample collection, specimen acquisition, management and storage contributes up to 93% of errors in diagnostics[50]. Implementing standardized protocols for reporting and managing non-conformance events can also improve service performance[46,47].

The majority of the samples in our study were rejected due to improper collection, a factor attributable to personnel error and is seen to be highest in secondary health clinics where the number of patients presenting for EID testing is highest. A recent study reported that staff sensitization on patient preparation, test request forms, and sample management significantly reduced pre-analytical error rate from 19.07% to 6.76%[47]. Thus, programs should intensify monitoring of pre-analytical staff, processes and performance towards improving sample quality[25,30,46,51].

Significant correlations between the annual number of DBS sample collection clinics and the annual sample rejection rate also suggests that an increasing number of EID clinics can put a strain on the program. Increased focus on site-based EID training and mentoring activities through 2011 is thought to be responsible for the decline in DBS sample rejection observed in that year. The shift to accelerated scale-up and decentralization of PMTCT services to primary health clinics where Community Health Extension Workers constitute a greater percentage of the workforce may have contributed to the peak in sample rejection recorded in succeeding year, 2012[52-54]. This may also explain the higher relative risk of sample rejection in primary health clinics.

Lapses in control, monitoring and supervision in the pre-analytical phase of clinical laboratory services and sample collection by non-laboratory personnel have been implicated as red flags for error propagation[55,56].

In the present study the infection rate among accepted samples and repeated samples (9.8% *vs* 15.9%) is in agreement with previous findings that LTFU can lead to low levels of detection of HIV infection in infants and missed opportunities for care[22,57]. Active patient tracking systems that use social workers to track patients have been applied in Kenya to reduce LTFU among HIV, PMTCT and tuberculosis patients from 21% to 15%[43]. In other studies, peer-based strategies that engage expert and or mentor-mothers in educating and motivating HIV positive mothers to access PMTCT services using their own experience, have been instrumental in improving retention of mother-baby pair in care[58,59]. Interventions should therefore seek to educate mothers and guardians on the grave importance of early diagnosis in pediatric HIV.

Although Quality Management System (QMS) is still seeing little application in Nigeria, an effective QMS is critical to the success of the laboratory testing networks[28,29]. Recent studies report that application of Quality Improvement tools such as Rapid Results Initiative and Continuous Quality Improvement interventions that seek to identify and correct system defects can significantly reduce sample rejection and increase patient retention in PMTCT programs in the similar setting[41,44].

Limitations

The current study is a retrospective analysis of laboratory records which are often incomplete as evidenced by the proportion of rejected samples with unknown reasons for rejection. This can introduce misclassification or information bias. Also it is often difficult to accurately interpret retrospective data and the quality of data collected over time. We did not investigate the reason for requesting an HIV test for samples collected for a follow-up test among repeated samples. This then does not reflect the actual mean age of infants presenting for a follow-up test among rejected samples as we could not determine if the tests were follow-up due to sample repeat or true follow-up tests.

Given the small size of the rejected samples compared to the total number of routinely collected samples, we did not test the statistical significance of the comparative analysis between these groups. Additionally, due to incomplete documentation we could not determine the mean age of infants presenting for HIV-1 DNA PCR test for the rejected samples.

In conclusion, the study demonstrates that DBS sample rejection can further delay HIV-1 EID testing, contributes to LTFU and adversely impacts program and patient outcomes at various levels of healthcare. An integrated multidisciplinary approach which engages social support groups, health personnel, quality improvement interventions as well as electronic and mobile communication tools is needed to improve uptake of PMTCT services and the overall health outcome of HIV positive mothers and their infants. Intensified training and monitoring of personnel, quality policies for sample collection and patient follow-up should be integrated into the scale-up agenda to prevent sample rejection

and promote recollection when errors occur. Other considerations should include continuous counseling and active tracking of mothers and care givers to improve patient retention and achieve the goals of PMTCT programs.

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COMMENTS

Background

Studies reveal that antiretroviral therapy can reduce rate of mother-to-child transmission of human immunodeficiency virus (HIV) to less than 2%. However, over 30% of HIV-exposed infants in resource limited settings are lost to follow-up by 3 mo of life and only 0.5%-52.8% of these infants are successfully enrolled into care and treatment.

Research frontiers

Eight countries (Nigeria, South Africa, India, Mozambique, Tanzania, Zimbabwe, Uganda and Kenya) accounted for 58% of the global acquired immunodeficiency syndrome (AIDS)-related deaths in 2013. Without antiretroviral preventive interventions for prevention of mother-to-child (PMTC), the risk of perinatal HIV transmission has varied between 15% and 45%, depending on maternal risk factors and whether breastfeeding is practiced. Nigeria has the highest number of children contracting the HIV, in the world (UNAIDS 2012). Early testing of exposed infants from 4 to 6 wk of birth is recommended by the World Health Organization to insure timely diagnosis and treatment of HIV positive children. An investigation of gaps in the PMTC transmission (PMTCT) cascade is important to identify improvement areas for optimizing linkage of HIV/AIDS infants into care and treatment.

Innovations and breakthroughs

An investigation of gaps in the PMTCT cascade is important to identify improvement areas for optimizing linkage of HIV/AIDS infants into care and treatment. The use of SMS printers and laboratory information system are major innovations that have been shown to reduce TAT and enhance tracking of rejected dried blood spot samples.

Applications

The shift to accelerated scale-up and decentralization of PMTCT services to primary health clinics where Community Health Extension Workers constitute a greater percentage of the workforce may have contributed to the peak in sample rejection recorded. An integrated multidisciplinary approach which engages social support groups, health personnel, quality improvement interventions as well as electronic and mobile communication tools is needed to improve uptake of PMTCT services and the overall health outcome of HIV positive mothers and their infants. Intensified training and monitoring of personnel, quality policies for sample collection and patient follow-up should be integrated into the scale-up agenda to prevent sample rejection and promote recollection when errors occur.

Terminology

EID: Early infant diagnosis; PMTCT: Prevention of mother-to-child transmission; PCR: Polymerase chain reaction.

Peer-review

This work by Inalegwu *et al*, addresses an important problem of enhanced tracking of rejected dried blood spot samples, which dramatically affects the PMTCT of HIV. The paper is well written, and the data are convincing since they are analyzed with appropriate statistical tools.

REFERENCES

1 **World Health Organization**. PMTCT Strategic Vision 2010-2015. Prevention of mother-to-child transmission of HIV to reach the UNGASS and millennium development goals. moving towards elimination of paediatric HIV. 2010. Available from: URL: http://whqlibdoc.who.int/publications/2010/9789241599030_eng.pdf

2 **Cooper ER**, Charurat M, Mofenson L, Hanson IC, Pitt J, Diaz C, Hayani K, Handelsman E, Smeriglio V, Hoff R, Blattner W. Combination antiretroviral strategies for the treatment of pregnant HIV-1-infected women and prevention of perinatal HIV-1 transmission. *J Acquir Immune Defic Syndr* 2002; **29**: 484-494 [PMID: 11981365]

3 **Dorenbaum A**, Cunningham CK, Gelber RD, Culnane M, Mofenson L, Britto P, Rekacewicz C, Newell ML, Delfraissy JF, Cunningham-Schrader B, Mirochnick M, Sullivan JL. Two-dose intrapartum/newborn nevirapine and standard antiretroviral therapy to reduce perinatal HIV transmission: a randomized trial. *JAMA* 2002; **288**: 189-198 [PMID: 12095383]

4 **Sam-Agudu NA**, Cornelius LJ, Okundaye JN, Adeyemi OA, Isah HO, Wiwa OM, Adejuyigbe E, Galadanci H, Afe AJ, Jolaoso I, Bassey E, Charurat ME. The impact of mentor mother programs on PMTCT service uptake and retention-in-care at primary health care facilities in Nigeria: a prospective cohort study (MoMent Nigeria). *J Acquir Immune Defic Syndr* 2014; **67** Suppl 2: S132-S138 [PMID: 25310119 DOI: 10.1097/QAI.0000000000000331]

5 **Aliyu MH**, Blevins M, Megazzini KM, Audet CM, Dunlap J, Sodangi IS, Gebi UI, Shepherd BE, Wester CW, Vermund SH. Correlates of suboptimal entry into early infant diagnosis in rural north central Nigeria. *J Acquir Immune Defic Syndr* 2014; **67**: e19-e26 [PMID: 24853310 DOI: 10.1097/QAI.0000000000000215]

6 **Ugochukwu EF**, Kanu SO. Early infant diagnosis of HIV infection in southeastern Nigeria: prevalence of HIV infection among HIV-exposed babies. *West Afr J Med* 2010; **29**: 3-7 [PMID: 20496330]

7 **Anoje C**, Aiyenigba B, Suzuki C, Badru T, Akpoigbe K, Odo M, Odafe S, Adedokun O, Torpey K, Chabikuli ON. Reducing mother-to-child transmission of HIV: findings from an early infant diagnosis program in south-south region of Nigeria. *BMC Public Health* 2012; **12**: 184 [PMID: 22410161 DOI: 10.1186/1471-2458-12-184]

8 **Imade GE**, Sagay AS, Musa J, Ocheke AN, Adeniyi DS, Idighri M, Powl R, Sendeht A, Ogwuche JP, Elujoba M, Egbodo CO, Oyebode T, Daru PH, Agbaji O, Pam IC, Meloni ST, Okonkwo P, Kanki PJ. Declining rate of infection with maternal human immunodeficiency virus at delivery units in north-central Nigeria. *Afr J Reprod Health* 2013; **17**: 138-145 [PMID: 24689325]

9 **Onankpa B**, Airede L, Paul I, Dorcas I. Pattern of pediatric HIV/AIDS: a five-year experience in a tertiary hospital. *J Natl Med Assoc* 2008; **100**: 821-825 [PMID: 18672559]

10 **Okusanya BO**, Ashimi AO, Aigere EO, Salawu SE, Hassan R. Scaling up prevention of mother to child transmission of HIV infection to primary health facilities in Nigeria: findings from two primary health centres in Northwest Nigeria. *Afr J Reprod Health* 2013; **17**: 130-137 [PMID: 24689324]

11 **Short SE**, Goldberg RE. Children Living with HIV-Infected Adults: Estimates for 23 Countries in sub-Saharan Africa. *PLoS One* 2015; **10**: e0142580 [PMID: 26575484 DOI: 10.1371/journal.pone.0142580]

12 **De Cock KM**, Fowler MG, Mercier E, de Vincenzi I, Saba J, Hoff E, Alnwick DJ, Rogers M, Shaffer N. Prevention of mother-to-child HIV transmission in resource-poor countries: translating research into policy and practice. *JAMA* 2000; **283**: 1175-1182 [PMID: 10703780]

13 **van Lettow M**, Bedell R, Landes M, Gawa L, Gatto S, Mayuni I, Chan AK, Tenthani L, Schouten E. Uptake and outcomes of a prevention-of mother-to-child transmission (PMTCT) program in Zomba district, Malawi. *BMC Public Health* 2011; **11**: 426 [PMID: 21639873 DOI: 10.1186/1471-2458-11-426]

14 **National Agency for the Control of AIDS**. Fact sheet: PMTCT

in Nigeria 2011 report. NACA 2011. Available from: URL: http://naca.gov.ng/content/view/399/lang,en/

15 **UNAIDS**. Report on the global AIDS epidemic 2012. Available from: URL: www.unaids.org/en/media/unaids/contentassets/documents/epidemiology/2012/gr2012/20121120_UNAIDS_Global_Report_2012_en.pdf

16 **Marston M**, Becquet R, Zaba B, Moulton LH, Gray G, Coovadia H, Essex M, Ekouevi DK, Jackson D, Coutsoudis A, Kilewo C, Leroy V, Wiktor S, Nduati R, Msellati P, Dabis F, Newell ML, Ghys PD. Net survival of perinatally and postnatally HIV-infected children: a pooled analysis of individual data from sub-Saharan Africa. *Int J Epidemiol* 2011; **40**: 385-396 [PMID: 21247884 DOI: 10.1093/ije/dyq255]

17 **Newell ML**, Coovadia H, Cortina-Borja M, Rollins N, Gaillard P, Dabis F. Mortality of infected and uninfected infants born to HIV-infected mothers in Africa: a pooled analysis. *Lancet* 2004; **364**: 1236-1243 [PMID: 15464184]

18 **Sutcliffe CG**, van Dijk JH, Bolton C, Persaud D, Moss WJ. Effectiveness of antiretroviral therapy among HIV-infected children in sub-Saharan Africa. *Lancet Infect Dis* 2008; **8**: 477-489 [PMID: 18652994 DOI: 10.1016/S1473-3099(08)70180-4]

19 **UNAIDS**. 2013 Progress Report on the Global Plan. Towards the elimination of new HIV infections among children by 2015 and keeping their mothers alive. UNAIDS 2013. Available from: URL: http://www.unaids.org/sites/default/files/ media_asset/ 20130625_progress_global_ plan_en_0.pdf

20 **Black V**, Hoffman RM, Sugar CA, Menon P, Venter F, Currier JS, Rees H. Safety and efficacy of initiating highly active antiretroviral therapy in an integrated antenatal and HIV clinic in Johannesburg, South Africa. *J Acquir Immune Defic Syndr* 2008; **49**: 276-281 [PMID: 18845949 DOI: 10.1097/QAI.0b013e318189a769]

21 **Braitstein P**, Katshcke A, Shen C, Sang E, Nyandiko W, Ochieng VO, Vreeman R, Yiannoutsos CT, Wools-Kaloustian K, Ayaya S. Retention of HIV-infected and HIV-exposed children in a comprehensive HIV clinical care programme in Western Kenya. *Trop Med Int Health* 2010; **15**: 833-841 [PMID: 20487430 DOI: 10.1111/j.1365-3156.2010.02539.x]

22 **Ciaranello AL**, Park JE, Ramirez-Avila L, Freedberg KA, Walensky RP, Leroy V. Early infant HIV-1 diagnosis programs in resource-limited settings: opportunities for improved outcomes and more cost-effective interventions. *BMC Med* 2011; **9**: 59 [PMID: 21599888 DOI: 10.1186/1741-7015-9-59]

23 **Namukwaya Z**, Mudiope P, Kekitiinwa A, Musoke P, Matovu J, Kayma S, Salmond W, Bitarakwate E, Mubiru M, Maganda A, Galla M, Byamugisha J, Fowler MG. The impact of maternal highly active antiretroviral therapy and short-course combination antiretrovirals for prevention of mother-to-child transmission on early infant infection rates at the Mulago national referral hospital in Kampala, Uganda, January 2007 to May 2009. *J Acquir Immune Defic Syndr* 2011; **56**: 69-75 [PMID: 21099692 DOI: 10.1097/QAI.0b013e3181fdb4a8]

24 **Chi BH**, Tih PM, Zanolini A, Stinson K, Ekouevi DK, Coetzee D, Welty TK, Bweupe M, Shaffer N, Dabis F, Stringer EM, Stringer JS. Implementation and Operational Research: Reconstructing the PMTCT Cascade Using Cross-sectional Household Survey Data: The PEARL Study. *J Acquir Immune Defic Syndr* 2015; **70**: e5-e9 [PMID: 26068722 DOI: 10.1097/QAI.0000000000000718]

25 **Stevens W**, Sherman G, Downing R, Parsons LM, Ou CY, Crowley S, Gershy-Damet GM, Fransen K, Bulterys M, Lu L, Homsy J, Finkbeiner T, Nkengasong JN. Role of the laboratory in ensuring global access to ARV treatment for HIV-infected children: consensus statement on the performance of laboratory assays for early infant diagnosis. *Open AIDS J* 2008; **2**: 17-25 [PMID: 18923696 DOI: 10.2174/1874613600802010017]

26 **Mehta N**, Trzmielina S, Nonyane BA, Eliot MN, Lin R, Foulkes AS, McNeal K, Ammann A, Eulalievyolo V, Sullivan JL, Luzuriaga K, Somasundaran M. Low-cost HIV-1 diagnosis and quantification in dried blood spots by real time PCR. *PLoS One* 2009; **4**: e5819 [PMID: 19503790 DOI: 10.1371/journal.pone.0005819]

27 **Federal Government of Nigeria**. National guidelines for prevention of mother to child transmission of HIV in Nigeria 2010. Federal Ministry of Health Nigeria. 2011. Available from: URL: http://www.emtct-iatt.org/wp content/uploads/2013/04/Nigeria_National- PMTCT- Guidelines_2010.pdf

28 **Lippi G**, Chance JJ, Church S, Dazzi P, Fontana R, Giavarina D, Grankvist K, Huisman W, Kouri T, Palicka V, Plebani M, Puro V, Salvagno GL, Sandberg S, Sikaris K, Watson I, Stankovic AK, Simundic AM. Preanalytical quality improvement: from dream to reality. *Clin Chem Lab Med* 2011; **49**: 1113-1126 [PMID: 21517699 DOI: 10.1515/CCLM.2011.600]

29 **Justman JE**, Koblavi-Deme S, Tanuri A, Goldberg A, Gonzalez LF, Gwynn CR. Developing laboratory systems and infrastructure for HIV scale-up: A tool for health systems strengthening in resource-limited settings. *J Acquir Immune Defic Syndr* 2009; **52** Suppl 1: S30-S33 [PMID: 19858935 DOI: 10.1097/QAI.0b013e3181bbc9f5]

30 **Jacobsz LA**, Zemlin AE, Roos MJ, Erasmus RT. Chemistry and haematology sample rejection and clinical impact in a tertiary laboratory in Cape Town. *Clin Chem Lab Med* 2011; **49**: 2047-2050 [PMID: 21995606 DOI: 10.1515/CCLM.2011.743]

31 **Creek T**, Tanuri A, Smith M, Seipone K, Smit M, Legwaila K, Motswere C, Maruping M, Nkoane T, Ntumy R, Bile E, Mine M, Lu L, Tebele G, Mazhani L, Davis MK, Roels TH, Kilmarx PH, Shaffer N. Early diagnosis of human immunodeficiency virus in infants using polymerase chain reaction on dried blood spots in Botswana's national program for prevention of mother-to-child transmission. *Pediatr Infect Dis J* 2008; **27**: 22-26 [PMID: 18162933]

32 **Menzies NA**, Homsy J, Chang Pitter JY, Pitter C, Mermin J, Downing R, Finkbeiner T, Obonyo J, Kekitiinwa A, Tappero J, Blandford JM. Cost-effectiveness of routine rapid human immunodeficiency virus antibody testing before DNA-PCR testing for early diagnosis of infants in resource-limited settings. *Pediatr Infect Dis J* 2009; **28**: 819-825 [PMID: 20050391]

33 **Lofgren SM**, Morrissey AB, Chevallier CC, Malabeja AI, Edmonds S, Amos B, Sifuna DJ, von Seidlein L, Schimana W, Stevens WS, Bartlett JA, Crump JA. Evaluation of a dried blood spot HIV-1 RNA program for early infant diagnosis and viral load monitoring at rural and remote healthcare facilities. *AIDS* 2009; **23**: 2459-2466 [PMID: 19741481 DOI: 10.1097/QAD.0b013e328331f702]

34 **Obimbo EM**, Mbori-Ngacha DA, Ochieng JO, Richardson BA, Otieno PA, Bosire R, Farquhar C, Overbaugh J, John-Stewart GC. Predictors of early mortality in a cohort of human immunodeficiency virus type 1-infected african children. *Pediatr Infect Dis J* 2004; **23**: 536-543 [PMID: 15194835]

35 **Becquet R**, Marston M, Dabis F, Moulton LH, Gray G, Coovadia HM, Essex M, Ekouevi DK, Jackson D, Coutsoudis A, Kilewo C, Leroy V, Wiktor SZ, Nduati R, Msellati P, Zaba B, Ghys PD, Newell ML. Children who acquire HIV infection perinatally are at higher risk of early death than those acquiring infection through breastmilk: a meta-analysis. *PLoS One* 2012; **7**: e28510 [PMID: 22383946 DOI: 10.1371/journal.pone.0028510]

36 **Bourne DE**, Thompson M, Brody LL, Cotton M, Draper B, Laubscher R, Abdullah MF, Myers JE. Emergence of a peak in early infant mortality due to HIV/AIDS in South Africa. *AIDS* 2009; **23**: 101-106 [PMID: 19065753]

37 **Nogueira SA**, Abreu T, Oliveira R, Araújo L, Costa T, Andrade M, Garcia Psic MF, Machado K, Mercadante R, Fernandes I, Sapia MC, Lambert JS. Successful prevention of hiv transmission from mother to infant in Brazil using a multidisciplinary team approach. *Braz J Infect Dis* 2001; **5**: 78-86 [PMID: 11493413]

38 **Frizzera Dias C**, Moreira-Silva SF, Reis MA, Ribeiro Patrício L, Biancardi Gavioli CF, Miranda AE. Late diagnosis and HIV infection in children attending a service of specialized care for pediatric AIDS in Brazil. *Rev Soc Bras Med Trop* 2014; **47**: 93-96 [PMID: 24749159]

39 **Dillabaugh LL**, Lewis Kulzer J, Owuor K, Ndege V, Oyanga A, Ngugi E, Shade SB, Bukusi E, Cohen CR. Towards Elimination of Mother-to-Child Transmission of HIV: The Impact of a Rapid Results Initiative in Nyanza Province, Kenya. *AIDS Res Treat* 2012;

2012: 602120 [PMID: 22548155 DOI: 10.1155/2012/602120]

40 **Taylor NK**, Buttenheim AM. Improving utilization of and retention in PMTCT services: can behavioral economics help? *BMC Health Serv Res* 2013; **13**: 406 [PMID: 24112440 DOI: 10.1186/1472-6963-13-406]

41 **Thomson KA**, Cheti EO, Reid T. Implementation and outcomes of an active defaulter tracing system for HIV, prevention of mother to child transmission of HIV (PMTCT), and TB patients in Kibera, Nairobi, Kenya. *Trans R Soc Trop Med Hyg* 2011; **105**: 320-326 [PMID: 21511317 DOI: 10.1016/j.trstmh.2011.02.011]

42 **Ghadrshenas A**, Ben Amor Y, Chang J, Dale H, Sherman G, Vojnov L, Young P, Yogev R. Improved access to early infant diagnosis is a critical part of a child-centric prevention of mother-to-child transmission agenda. *AIDS* 2013; **27** Suppl 2: S197-S205 [PMID: 24361629 DOI: 10.1097/QAD.0000000000000104]

43 **Khamadi S**, Okoth V, Lihana R, Nabwera J, Hungu J, Okoth F, Lubano K, Mwau M. Rapid identification of infants for antiretroviral therapy in a resource poor setting: the Kenya experience. *J Trop Pediatr* 2008; **54**: 370-374 [PMID: 18511477 DOI: 10.1093/tropej/fmn036]

44 **Agarwal R**, Chaturvedi S, Chhillar N, Goyal R, Pant I, Tripathi CB. Role of intervention on laboratory performance: evaluation of quality indicators in a tertiary care hospital. *Indian J Clin Biochem* 2012; **27**: 61-68 [PMID: 23277714 DOI: 10.1007/s12291-011-0182-7]

45 **Dikmen ZG**, Pinar A, Akbiyik F. Specimen rejection in laboratory medicine: Necessary for patient safety? *Biochem Med* (Zagreb) 2015; **25**: 377-385 [PMID: 26527231 DOI: 10.11613/BM.2015.037]

46 **Nyandiko WM**, Otieno-Nyunya B, Musick B, Bucher-Yiannoutsos S, Akhaabi P, Lane K, Yiannoutsos CT, Wools-Kaloustian K. Outcomes of HIV-exposed children in western Kenya: efficacy of prevention of mother to child transmission in a resource-constrained setting. *J Acquir Immune Defic Syndr* 2010; **54**: 42-50 [PMID: 20224420 DOI: 10.1097/QAI.0b013e3181d8ad51]

47 **Nkengasong JN**, Nsubuga P, Nwanyanwu O, Gershy-Damet GM, Roscigno G, Bulterys M, Schoub B, DeCock KM, Birx D. Laboratory systems and services are critical in global health: time to end the neglect? *Am J Clin Pathol* 2010; **134**: 368-373 [PMID: 20716791 DOI: 10.1309/AJCPMPSINQ9BRMU6]

48 **Lippi G**, Guidi GC, Mattiuzzi C, Plebani M. Preanalytical variability: the dark side of the moon in laboratory testing. *Clin Chem Lab Med* 2006; **44**: 358-365 [PMID: 16599826]

49 **Atay A**, Demir L, Cuhadar S, Saglam G, Unal H, Aksun S, Arslan B, Ozkan A, Sutcu R. Clinical biochemistry laboratory rejection rates due to various types of preanalytical errors. *Biochem Med* (Zagreb) 2014; **24**: 376-382 [PMID: 25351356 DOI: 10.11613/BM.2014.040]

50 **Federal Ministry of Health**. National scale up plan towards elimination of mother-to-child transmission of HIV in Nigeria 2010-2015. Abuja, Nigeria: Federal Ministry of Health, 2010. Available from: URL: http://www.emtct-iatt.org/wp-content/uploads/2013/04/Nigeria_National-PMTCT-Guidelines_2010.pdf

51 **Federal Ministry of Health**. National Health Sector Strategic Plan and Implementation Plan for HIV/AIDS 2010-2015. HIV/AIDS Division. Abuja, Nigeria: Department of Public Health, Federal Ministry of Health, 2010

52 **National Agency for the Control of AIDS (NACA) PMTCT demand creation for accelerated uptake of services**. A national prevention of mother-to-child transmission (PMTCT) of HIV communication strategy. Nigeria: 2014. Available from: URL: https://c-changeprogram.org/sites/default/ files/CChange_Nigeria_PMTCT_v9_web.pdf

53 **Agboghoroma CO**, Sagay SA, Ikechebelu JI. Nigerian prevention of mother to child transmission of human immunodeficiency virus programme: the journey so far. *J HIV Hum Reprod* 2013; **1**: 1-7

54 **Garcia A**, Subbarao S, Zhang G, Parsons L, Nkengasong J, Ou CY, Ellenberger D. Impact of proficiency testing program for laboratories conducting early diagnosis of HIV-1 infection in infants in low- to middle-income countries. *J Clin Microbiol* 2014; **52**: 773-780 [PMID: 24353004 DOI: 10.1128/JCM.03097-13]

55 **WHO**, UNICEF, UNAIDS. Global HIV/AIDS response: epidemic update and health sector progress towards universal access: progress report 2011. Geneva: WHO, 2011. Available from: URL: http://www.who.int/hiv/pub/progress_report2011/en/

56 **Decroo T**, Van Damme W, Kegels G, Remartinez D, Rasschaert F. Are Expert Patients an Untapped Resource for ART Provision in Sub-Saharan Africa? *AIDS Res Treat* 2012; **2012**: 749718 [PMID: 22577527 DOI: 10.1155/2012/749718]

57 **Abrams EJ**, Simonds RJ, Modi S, Rivadeneira E, Vaz P, Kankasa C, Tindyebwa D, Phelps BR, Bowsky S, Teasdale CA, Koumans E, Ruff AJ. PEPFAR scale-up of pediatric HIV services: innovations, achievements, and challenges. *J Acquir Immune Defic Syndr* 2012; **60** Suppl 3: S105-S112 [PMID: 22797731 DOI: 10.1097/QAI.0b013e31825cf4f5]

58 **Audu RA**, Sylvester-Ikondu U, Onwuamah CK, Salu OB. Experience of Quality Management System in a Clinical Laboratory in Nigeria. *Afr J Lab Med* 2011; **1**: 1-5 [DOI: 10.4102/ajlm.v1i1.18]

59 **Jegede FE**, Mbah HA, Yakubu TN, Adedokun O, Negedu-momoh OR, Torpey K. Laboratory quality audit in 25 anti-retroviral therapy facilities in north west of Nigeria. *Open J Clin Diagn* 2014; **4**: 193-204 [DOI: 10.4236/ojcd.2014.44028]

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LETTERS TO THE EDITOR

Viral outbreaks and communicable health hazards due to devastating floods in Pakistan

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Abstract

Pakistan is a developing country that has a population of 190 million people and faces a huge burden of viral diseases. Every year during monsoon season heavy rain fall and lack of disaster management skills potentially increase the transmission of waterborne diseases, vector borne diseases and viral outbreaks. Due to severe flooding, thousands of people lose their lives and millions are displaced each year. In most of the cases the children who lose their family members are forced into illegal professions of begging, child labor and prostitution which make them prone to sexually transmitted infections. Up to date, no scientific study has been conducted nationwide to illustrate epidemiological patterns of waterborne diseases, vector borne diseases and viral epidemics during flash flood. Mosquito sprays would not be a sufficient approach for dengue eradication; mass awareness, larvicide and biological control by Guppy fishes are also effective strategies to overcome dengue problem. International health bodies and non-governmental organizations must take note of this alerting situation and take adequate steps such as financial/medical aid in order to defeat the after-effects of flood.

Key words: Health hazards; Viral outbreak; Dengue; Flood; Waterborne diseases

Core tip: In Pakistan every year monsoon brings havoc in term of devastating flood. Lack of management skills results in increased transmission of waterborne diseases, vector borne diseases and viral outbreaks. Due to severe flooding, thousands of people lose their lives and millions are displaced each year. In most of the cases the children who lose their family members are forced into illegal professions of begging, child labor and prostitution which make them prone to sexually transmitted infections.

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TO THE EDITOR

In Pakistan, heavy rain fall and lack of disaster management skills potentially increase transmission of waterborne, vector borne diseases and viral epidemics. Communicable diseases with increased risk of transmission during flood includes viral hepatitis A, cholera, typhoid fever, leptospirosis, malaria, West Nile fever, yellow fever, dengue and dengue haemorrhagic fever. The country suffers loss of numerous lives each year due to unprepared set of mind. It has been reported that over the last three years, due to severe flooding, thousands of people have lost their lives and millions have been displaced. Many people lost their conscious state of mind and mental health was seriously disturbed. Sometimes children who lost their family members are forced into illegal professions of child labor, begging and prostitution which make them prone to sexually transmitted infections. If the similar situations remain persistent during the upcoming years, serious viral epidemics, acting as threatening viral time bomb could wipe out the entire nation. Policy-makers must provide wider opportunities for the dissemination of awareness and knowledge related to silent routes of viral transmission and focus on epidemiological patterns associated with emerging viral infections in Pakistan[1,2].

National Disaster Management has reported 118 deaths, above 800 injuries and loss of 325000 acres of crops land, due to a flash flood which affected nearly 1700 villages[3]. The healthcare facilities are mainly administered by private sectors in Pakistan. Healthcare and sanitation systems are inadequate at urban sectors and very poor in rural areas[4]. The government of Pakistan has provided limited healthcare facilities as compared to rapidly increasing population. It has been reported that there are 127859 doctors and 12804 healthcare infrastructures in Pakistan to cater for more than 175 million people[5]. Among various hospitals, due to in-appropriate facilities, patients travel from hundreds of kilometers for the sake of basic healthcare facilities. During flood, sometimes travel to only few kilometers, is almost impossible. Pakistan again and again faced serious flood problems in major provinces, which caused hundreds of deaths and massive displacements due to sheer negligence of National Disaster Management. Many domestic animals were also infected by various diseases due to flood. Heavy flood coming from India adds to flooding misery. India released more than 170000 cusecs of water which severely damaged catchments areas of Sutlej River near Kasur, affecting hundreds of thousands of people[6]. In Punjab, heavy rainfall swept 187000 acres of land and affected more than 165000 people in Rajanpur. In Sindh, Lyari and Malir have been seriously affected by flooding. In Khyber Pakhtunkhwa, regions heavily damaged by flash floods, in term of damaged houses, infrastructures, loss of many precious lives, water irrigation, and electricity, includes Peshawar, Bannu, Chitral, Tank and Lakki Marwat. In Balochistan, extensive damages have been reported from Jaffarabad, Harnai, Jhal Magsi, Sibi and Loralai districts[7]. Previously our research group identified and isolated a virulent phage (from sewerage water samples) against multiple drug resistant pseudomonas aeruginosa responsible for bacteremia, respiratory system infections, gastrointestinal infections, dermatitis, soft tissue infections, urinary tract infections, bone and joint infections and a variety of systemic infections[8]. The bacterial infections which are resistant to antibiotics can also be reduced by using bacteriophage therapy. The risk of communicable disease (including viral hepatitis A, cholera, typhoid fever, leptospirosis, malaria, West Nile fever, yellow fever, dengue and dengue haemorrhagic fever) from flooding can be reduced *via* chlorination of water to ensure safe drinking water, vaccination against hepatitis A, malaria prevention, health education and proper handling corpses. The nature contains hidden remedies against multiple diseases and there is a strong need to identify therapeutic potentials of natural entities[9,10].

Due to heavy rainfall and river overflow, in many regions of Pakistan, standing water becomes breeding sites for mosquitoes. It has been reported that more than 21204 people were infected with dengue in November 2010 after a worst flood in Punjab[11]. The prevalence of viral infections is unfortunately increasing day by day in developing countries due to limited awareness among the general population[12,13]. Although a new vaccine for dengue fever has proven safe in nonhuman primates, a lot of efforts are required to supply effective vaccines at minimal cost. Up to now there is no vaccine against dengue haemorrhagic fever in Pakistan. Although the government of Pakistan took crucial steps to manage the devastating situation through organizing awareness programs at offices and educational institutes, and many spraying teams for fumigating, spraying and fogging affected areas, this problem survived for a few months due to the complexity of this issue. The dengue infection reoccurred in 2011, 2012 and 2013 due to heavy rain fall of monsoon. In Karachi on average 700, 858 and 630 deaths were reported due to dengue infections in 2010, 2011 and 2012, respectively. But in 2013 the number of deaths due to dengue has increased to 2706 cases which depict a 323.4% increase in dengue cases compared to 2012[14]. It has been reported that allied hospitals in capital twin cities (Islamabad and Rawalpindi) of Pakistan were receiving almost 25 fresh dengue cases every day. In October 2013 it has been reported from Rawalpindi that almost 722 suspected cases have been reported since September 2013. It was further disclosed that the provincial health department was hiding the actual number of deaths only due to hiding the incompetence of the department[15]. Mosquito sprays would not be a sufficient approach for dengue eradication, and mass awareness, larvicide and biological control by Guppy fishes are also effective strategies to overcome this problem. Lessons should be learned from our previous mistakes of poor flood management. The government of Pakistan has to cope with the ongoing impact of Pakistan's flood and the resulting displacement of populations. Each year Pakistan suffers lose water due to the absence of water

storage capacities. The water storage in deep wells and dams will not only prevent flash flood, but it will also be a positive step towards generation of electricity. There is a strong need to improve surveillance at local, national, and international levels to develop Disaster-Preparedness Programmes and Early Warning Systems. International health bodies and non-governmental organizations must take note of this alarming situation and take appropriate steps like financial/medical aid to defeat the after-effects of flood.

REFERENCES

1 **Saeed U**, Waheed Y, Manzoor S, Ashraf M. Identification of novel silent HIV propagation routes in Pakistan. *World J Virol* 2013; **2**: 136-138 [PMID: 24255884 DOI: 10.5501/wjv.v2.i3.136]

2 **Saeed U**, Mazoor S, Jalal N, Zahid Piracha Z. Contemplating the Importance of Toll-like Receptors I and II Regarding Human Viral Pathogenesis. *Jundishapur J Microbiol* 2015; **8**: e13348 [PMID: 25763131 DOI: 10.5812/jjm.13348]

3 **SOS Children Village**. Pakistan is Unprepared for Flooding. Available from: URL: http://www.soschildrensvillages.org.uk/about-our-charity/news/pakistan-is-unprepared-for-flooding-in-2013-1

4 Wikipedia, Healthcare in Pakistan 2015. Available from: URL: http://en.wikipedia.org/wiki/Healthcare_in_Pakistan

5 **Southasia One World**. Available from: URL: http://southasia.oneworld.net/todaysheadlines/healthcare-in-pakistan-too-expensive-to-afford

6 India's release of water adds to flooding misery. Pakistan Today. Available from: URL: http://www.pakistantoday.com.pk/2013/08/19/news/national/indias-release-of-water-adds-to-flooding-misery/#sthash.gGdkfzky.dpuf

7 **World Health Organization**. Situation Report Pakistan floods-2013. Issue # 1. Available from: URL: http://www.who.int/hac/crises/pak/sitreps/pakistan_sitrep_5august2013.pdf

8 **Piracha ZZ**, Saeed U, Khursheed A, Chauhdry WN. Isolation and Partial Characterization of Virulent Phage Specific against Pseudomonas aeruginosa. *Global J Med Res* 2014; **14**: 1-9

9 **Saeed U**. In silico identification of BIM-1 (2-methyl-1H-indol-3-yl) as a potential therapeutic agent against elevated protein kinase C beta associated diseases. *African J Biotech* 2012; **11**: 4434-4441 [DOI: 10.5897/AJB11.3192]

10 **Saeed U**, Jalal N, Ashraf M. Roles of Cyclin Dependent Kinase and Cdk- Activating Kinase in Cell Cycle Regulation: Contemplation of Intracellular Interactions and Functional Characterization. *Global J Med Res* 2012; **12**: 47-52

11 Dengue deaths reach 31; over 5,000 infected. ARY NEWS. Available from: URL: http://research.omicsgroup.org/index.php/2011_dengue_outbreak_in_Pakistan

12 **Saeed U**, Waheed Y, Ashraf M. Hepatitis B and hepatitis C viruses: a review of viral genomes, viral induced host immune responses, genotypic distributions and worldwide epidemiology. *Asian Pac J Trop Dis* 2014; **4**: 88-96 [DOI: 10.1016/S2222-1808(14)60322-4]

13 **Saeed U**, Waheed Y, Ashraf M, Waheed U, Anjum S, Afzal MS. Estimation of Hepatitis B Virus, Hepatitis C Virus, and Different Clinical Parameters in the Thalassemic Population of Capital Twin Cities of Pakistan. *Virology* (Auckl) 2015; **6**: 11-16 [PMID: 26568681 DOI: 10.4137/VRT.S31744]

14 Staff Report. 323.4% increase in dengue cases compared to 2012. Daily Times. Available from: URL: http://www.dailytimes.com.pk/default.asp?page=2013%20%20story_26-10-%202013_pg12_5

15 **Wasif S**, Ali F. Dengue outbreak: Disease becoming epidemic in Rawalpindi. The Express Tribune. Available from: URL: http://tribune.com.pk/story/623874/dengue-outbreak-disease-becoming-epidemic-in-rawalpindi/

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LETTERS TO THE EDITOR

Determination of 50% endpoint titer using a simple formula

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Abstract

Two commonly used methods for calculating 50% endpoint using serial dilutions are Spearman-Karber method and Reed and Muench method. To understand/apply the above formulas, moderate statistical/mathematical skills are necessary. In this paper, a simple formula/method for calculating 50% endpoints has been proposed. The formula yields essentially similar results as those of the Spearman-Karber method. The formula has been rigorously evaluated with several samples.

Key words: Endpoint dilution; TCID50; Spearman-Karber; Reed and Muench

Core tip: The formula described in this manuscript can be used to calculate 50% endpoint titre such as TCID50%, LD50, TD50, *etc*., in addition to the currently existing methods. The proposed formula can be applied without the help of calculator or computer.

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TO THE EDITOR

Currently, there are two methods (formulas) *viz*., Reed and Muench[1] and Spearman-Karber[2,3] are commonly employed for the calculation of 50% endpoint by serial dilution. To understand/apply these methods, moderate mathematical skills along with calculator or computer are essential. Here, I have proposed a simple formula to calculate the 50% endpoint titre and this formula can be used in addition to Reed and Muench or Spearman-Karber, methods but not exclusively at this point. In the following section, the newly proposed method is compared with two commonly used methods viz., Reed and Muench and Spearman-Karber.

Reed and Muench method

$\log_{10}$ 50% end point dilution = $\log_{10}$ of dilution showing a mortality next above 50% - (difference of logarithms × logarithm of dilution factor).

Generally, the following formula is used to calculate "difference of logarithms" (difference of logarithms is also known as "proportionate distance" or "interpolated

Table 1 Calculation of virus titre in mice using the Reed and Muench method

Log_{10} virus dilution	Mice		Cumulative total			Percent mortality
	Died	Survived	Died	Survived	Total	
-1	10	0	57	0	57	57/57 × 100 = 100
-2	10	0	47	0	47	47/47 × 100 = 100
-3	10	0	37	0	37	37/37 × 100 = 100
-4	10	0	27	0	27	27/27 × 100 = 100
-5	10	0	17	0	17	17/17 × 100 = 100
-6	6	4	7	4	11	7/11 × 100 = 63
-7	1	9	1	13	14	1/14 × 100 = 7

Difference of logarithms = (63-50)/(63-7) = 0.23; log_{10} 50% end point dilution = -6 - (0.23 × 1) = -6.23; 50% end point dilution = $10^{-6.23}$; the titre of the virus = $10^{6.23}$ LD_{50}/mL.

Table 2 Calculation of virus titre in mice using the Spearman-Karber method

Log_{10} virus dilution	Mice	
	Died	Inoculated
-1	10	10
-2	10	10
-3	10	10
-4	10	10
-5	10	10
-6	6	10
-7	1	10

x_0 = 5; d = 1; log_{10} of 50% endpoint dilution = - [5 - ½ + 1 (17/10)] = -6.2; 50% end point dilution = $10^{-6.2}$; the titre of the virus = $10^{6.2}$ LD_{50}/mL.

value"): Difference of logarithms = [(mortality at dilution next above 50%)-50%]/[(mortality next above 50%)-(mortality next below 50%)].

Spearman-Karber method

log_{10} 50% end point dilution = - (x_0 - d/2 + d ∑ r_i/n_i)

x_0 = log_{10} of the reciprocal of the highest dilution (lowest concentration) at which all animals are positive;

d = log_{10} of the dilution factor;

n_i = number of animals used in each individual dilution (after discounting accidental deaths);

r_i = number of positive animals (out of n_i).

Summation is started at dilution x_0.

Newly proposed method

Formula 1:

log_{10} 50% end point dilution = -[(total number of animals died/number of animals inoculated per dilution) + 0.5] × log dilution factor.

Formula 2 (if any accidental death occurred):

log_{10} 50% end point dilution = -(total death score + 0.5) × log dilution factor.

Table 3 Calculation of virus titre in mice using the new method

Log_{10} virus dilution	Mice		Death score
	Died	Inoculated	
-1	10	10	10/10 = 1
-2	10	10	10/10 = 1
-3	10	10	10/10 = 1
-4	10	10	10/10 = 1
-5	10	10	10/10 = 1
-6	6	10	6/10 = 0.6
-7	1	10	1/10 = 0.1
Total	57		5.7

By using formula 1: log_{10} 50% end point dilution = - (57/10 + 0.5) × 1 = -6.2; 50% end point dilution = $10^{-6.2}$; the titre of the virus = $10^{6.2}$ LD_{50}/mL. By using formula 2: log_{10} 50% end point dilution = - (5.7 + 0.5) × 1 = -6.2; 50% end point dilution = $10^{-6.2}$.

Comparison of the newly proposed and existing methods with an example of virus titration in mice: For simplicity, it is assumed that 1 mL of each dilution was inoculated (Tables 1-3).

The newly proposed formula has been intensively validated with several samples and essentially yields the same results as those by the Spearman-Karber method. Therefore, the newly proposed method can be used in addition to the existing methods but not exclusively at this point.

REFERENCES

1 **Reed LJ**, Muench H. A simple method of estimating fifty per cent endpoints. *Am J Hyg* 1938; **27**: 493-497

2 **Kärber G**. Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. *Archiv f experiment Pathol u Pharmakol* 1931; **162**: 480-483 [DOI: 10.1007/BF01863914]

3 **Spearman C**. The Method of "Right and Wrong Cases" (Constant Stimuli) without Gauss's Formula. *Br J Psychol* 1908; **2**: 227-242 [DOI: 10.1037/h0063767]

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REVIEW

Intrinsic host restriction factors of human cytomegalovirus replication and mechanisms of viral escape

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Abstract

Before a pathogen even enters a cell, intrinsic immune defenses are active. This first-line defense is mediated by a variety of constitutively expressed cell proteins collectively termed "restriction factors" (RFs), and they form a vital element of the immune response to virus infections. Over time, however, viruses have evolved in a variety ways so that they are able to overcome these RF defenses *via* mechanisms that are specific for each virus. This review provides a summary of the universal characteristics of RFs, and goes on to focus on the strategies employed by some of the most important RFs in their attempt to control human cytomegalovirus (HCMV) infection. This is followed by a discussion of the counter-restriction mechanisms evolved by viruses to circumvent the host cell's intrinsic immune defenses. RFs include nuclear proteins IFN-γ inducible protein 16 (IFI16) (a Pyrin/HIN domain protein), Sp100, promyelocytic leukemia, and hDaxx; the latter three being the keys elements of nuclear domain 10 (ND10). IFI16 inhibits the synthesis of virus DNA by down-regulating UL54 transcription - a gene encoding a CMV DNA polymerase; in response, the virus antagonizes IFI16 *via* a process involving viral proteins UL97 and pp65 (pUL83), which results in the mislocalizing of IFI16 into the cytoplasm. In contrast, viral regulatory proteins, including pp71 and IE1, seek to modify or disrupt the ND10 proteins and thus block or reverse their inhibitory effects upon virus replication. All in all, detailed knowledge of these HCMV counter-restriction mechanisms will be fundamental for the future development of new strategies for combating HCMV infection and for identifying novel therapeutic agents.

Key words: Human cytomegalovirus; Intrinsic immunity; Restriction factors; Viral escape mechanisms; DNA sensors

Core tip: Cellular "restriction factors", active before human cytomegalovirus (HCMV) enters the cells, form a component of the intrinsic resistance to virus infection. Examples of such factors are hDaxx, promyelocytic leukemia, Sp100 - components of ND10 - and IFN-γ inducible protein 16 (IFI16), an Interferon-inducible protein of the Pyrin/HIN domain protein family. Over time, viruses have developed mechanisms to counteract ND10 and IFI16 through viral proteins, such as IE1 and pp71, or UL97 and pp65, respectively. Detailed knowledge of these mechanisms will provide new competencies useful to control HCMV infection and, in turn, contribute to the development of novel therapeutic approaches.

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INTRODUCTION

Viral replication in the infected cell is the result of complex interactions between host and viral proteins. Indeed, in the course of evolution, mammalian immune systems have evolved to response *via* an array of cellular defense mechanisms, which include both innate and adaptive immune responses, designed to protect against and remove invading pathogens[1]. The innate immune system, mediated by specialized cells such as natural killer cells (NK), dendritic cells, and macrophages[2], is the first to respond, but it is not very specific and does not lead to a long-lasting memory of the response to the pathogen. A specific form of innate immunity, termed "intrinsic immunity", has also been identified of late, thus generating a third branch of the immune system that was until now considered a bipartite system. Intrinsic immunity involved a set of defense mechanisms that operate on the cellular level[3], realized by cellular proteins known as "restriction factors" (RFs), as they can interfere with various steps of the virus replication cycle[4,5]. The word restriction factor was first coined by research groups studying the murine immune response to retroviruses. Work conducted over 40 years ago revealed that "friend virus susceptibility factor-1" was responsible for conferring resistance to infection by retroviruses[6]. Retroviruses consequently became a model system for investigating intrinsic immunity and have been instrumental in deepening our knowledge of the interaction between viruses and their hosts[7,8]. Over time, the notion of "intrinsic immunity" get up from the finding that the cells attacked by primate lentiviruses are able to resist infection, despite the fact that no signaling event appeared to be necessary for this form of defense, and from the finding that these cells constitutively express prototype human antiretroviral RFs, including the APOBEC3 family of cytidine deaminases[9,10], TRIM5a[11], Tetherin[7], SAMHD1[12], and BST-2[13,14]. RFs are thus germline-encoded proteins mediating the intrinsic cellular immune response against viral replication. Type Ⅰ interferons (IFN) have been demonstrated to increase the expression of RFs, however cells targeted by IFN do not rely on its activity for constitutive antiretroviral activity[15].

INTRINSIC IMMUNITY

The sensing of "pathogen-associated molecular patterns" (PAMPs) - typically microbe nucleic acids and proteins (usually absent from healthy hosts and thus hallmarks of infection) - by germline encoded proteins serving as "pattern recognition receptors" (PRRs) constitutes the earliest step in the innate immune response[2,16]. Viral nucleic acid including DNA containing CpG motifs, and RNA species, including both double-stranded and single-stranded RNA, can be detected by Toll-like receptors TLR3, TLR7-8 and TLR9, or PRR in the cytoplasmatic or the nuclear compartment[17,18]. Two different innate immunity signaling cascades are triggered by detecting exogenous nucleic acid. In the first, transcription factors (TFs) are activated, such as NF-κB and IRF3, culminating in the production of chemokines, cytokines, and IFN-type Ⅰ [19,20]. The second signaling cascade leads to inflammasome complex formation; this activates caspase-1, an enzyme that generates active cytokines set for secretion by proteolytically cutting pro-IL-1β and pro-IL-18[21-23]. Whereas PRRs activate signals that inhibit infection indirectly, RFs provide front-line defense by interfering directly with the activity of genes essential for the virus's replication. Indeed, this is often computed before the production of antiviral cytokines has even been activated. Thus, the properties of RFs are clearly distinct to those of PRRs. First and foremost, while RFs are basally expressed in many cell types, their expression may be increased by IFN signaling. Second, isolated RFs have been shown to exhibit antiviral activity in cells, maintaining their capacity to inhibit precise steps in the viral life cycle. Third, viral proteins have evolved to antagonize certain RFs. Finally, genetic selection driven by host *vs* pathogen coevolution has undoubtedly operated on the genes for RFs[3]. Thus, according to the concept of intrinsic immunity, we can define cell as either "restrictive" or "permissive" depending on viruses ability to replicate efficiently within them[5,24]. Retroviruses have presented a model that has played a pivotal role in the development of our understanding of virus-host interactions[4,8,14,15,18]. However, evidence now shows that several other viruses are also counteracted by intrinsic immunity, including herpesviruses[25,26].

Here, we focus on the newest findings about human cytomegalovirus (HCMV), which belongs to the Herpesviridae family[27,28], and provide a summary of the RFs that perturb its replication (Table 1). Interestingly, HCMV appears to have evolved a number of mechanisms to counteract the action of restriction factors, ultimately leading to the successful replication of viruses in cells[29-32].

Table 1 Overview of host restriction factors for human cytomegalovirus

Host restriction factors PYHIN family	Regulation	CMV inhibition	HCMV counter measure	Ref.
IFI16	Type I IFN inducible	HCMV-DNA sensing in the nucleus Interaction with HCMV pp65 to inhibit UL54 promoter Antiviral cytokine expression	Sequestration by HCMV pp65 for MIEP activation Protection from proteasome degradation by pp65 Delocalization upon phosphorylation by HCMV UL97	[42,43,46,60,72]
AIM2	Type I IFN inducible	MCMV-DNA sensing in the cytoplasm Inflammasome activation	Not known	[66]
ND10 family PML hDaxx Sp100	Cell cycle dependent	Transcriptionally inactive chromatin state of MIEP induction	Targeting HCMV IE1 for degradation hDaxx binding by pp71 for proteasome degradation	[93-116]
KDMs	Cell cycle dependent	Inhibition of HCMV latency	Prevention of KDM association with the MIEP by HCMV UL138	[117]

CMV: Cytomegalovirus; HCMV: Human cytomegalovirus; MCMV: Murine cytomegalovirus; IFI16: IFN-γ inducible protein 16; PYHIN: Pyrin/HIN domain; AIM2: Absent in melanoma 2; KDMs: Lysine-specific demethylases; MIEP: Major immediate early promoter.

THE IFN-γ INDUCIBLE PROTEIN 16 PROTEIN

The IFN inducible IFI16 protein is a member of the Pyrin and HIN domain containing proteins (PYHIN) family; it is coded by an IFN-inducible group of genes residing on chromosome 1q23[33-35]. In humans, this family includes five PYHIN proteins: The recently discovered "Pyrin domain only protein 3", "Pyrin and HIN domain family member 1" (PYHIN1), "absent in melanoma 2" (AIM2), "myeloid cell nuclear differentiation antigen" (MNDA), and "IFN-γ inducible protein 16" (IFI16)[36,37]. All five of these proteins possess an N-terminal PYRIN domain, and at least four possess a conserved domain of 200-amino acid repeats (HIN-200) within the C-terminal region (in single or tandem copies), thus they are collectively known as PYHIN. The PYD (or PAAD or DAPIN) domain is a member of the death domain family and consists of an α-helical motif that interacts with other PYD-containing proteins[38]. The HIN domain contains consensus motifs encompassing the 200-amino acid repeats, according to which it is classified into 3 subtypes, designated A, B, C[39,40]. PYHIN1, MNDA, and IFI16 all contain nuclear localization sequences located within their N-termini, and as such are primarily expressed in the cell nucleus[35,39,41]. However, in response to environmental stimuli, such as viral infection, they undergo post-translational modifications, *i.e.,* acetylation, and translocate into the cytoplasm[42,43]. Alternative splicing of the *IFI16* gene produces three isoforms[39]; each isoform is made up of two domains, designated A and B, each 200-amino acid long. These domains are divided by a spacer region that may vary in its length. The B isoform is the most predominant and has been detected in an array of histologically distinct cell types (*i.e.*, immune, endothelial, and epithelial cells[44]). The IFI16 N-terminal region display a bi-partite "nuclear localization signal"[45], responsible for its nuclear subcellular localization in quiescent cells, such as: Fibroblasts[46], endothelial cells, and keratinocytes (for a review see[47]). It is of interest that IFI16 protein has also been identified within the nucleolus[34]. However, in fibroblasts, macrophages, and keratinocytes, IFI16 is able to relocate from the nucleus to the cytoplasm. In fibroblasts and macrophages, this occurs following infection by herpesvirus[42,43]; while in keratinocytes, exposure to ultraviolet B light is able to trigger this redistribution[48,49]. In herpesvirus infection, IFI16 redistribution is associated with inflammasome, and after UVB exposure, it is associated with apoptosis. IFI16 is able to form homodimers or bind to other proteins to form heterodimers; its partners include: p53[38,50,51], Rb[52], BRCA1[53], ASC[54] and STING[55]. Indeed, protein-protein interactions are now thought to determine the subcellular localization of proteins; however, the molecular mechanisms regulating the redistribution of IFI16 from the nuclear to the cytoplasmic compartment remain unknown. Finally, a role of viral DNA sensor has also been attributed to cytoplasmic IFI16[47,56]. Indeed, the capacity of IFI16 to bind to viral DNA has been confirmed both *in vitro* and *in vivo*[40,55-60]. It is now believed that IFI16 may actually tune the innate immune response by stimulating IFN-type I release[47,56]. Thus, in addition to the various types of protein-protein interactions involving IFI16, another factor that may lead or contribute to IFI16 redistribution within the cell is its binding to microbial DNA.

Inhibition of HCMV replication by IFI16 and viral evasion

AIM2 and IFI16 are the two PYHIN members that have been demonstrated to act as PRRs of intracellular DNA of virus origin[51,60-66]. In particular, in cells infected with Kaposi Sarcoma Associated herpesvirus, IFI16 was revealed to form a functional inflammasome by interacting with ASC together with procaspase-1[54]. Moreover, this virus triggered NF-κB and IRF3 expression and activation [TFs routinely observed to be activated

after DNA transfection or the infection with herpes simplex virus type 1 (HSV-1)] could be inhibited by reducing IFI16 expression (or its mouse counterpart p204) using siRNA[67-70]. Besides its role as a PRR, IFI16 had previously been recognized to carry out a variety of other functions in the cell, although none in relation to antiviral activity (reviewed in[35]). However, our understanding of the functions of IFI16 in the cell has dramatically changed over modern years; this is largely due to the results gained from the application of two different experimental approaches (reviewed in[42]). The first involves IFI16 knockdown through the use of specific siRNA or IFI16 inactivation achieved by transfecting cells with a lentivirus carrying a dominant negative mutant form of the protein[71]. For example, eliminating functional IFI16 protein in fibroblasts isolated from human embryo lung (HELFs), *via* either methodology, was shown to significantly increase herpesvirus replication, including HCMV. The second approach involves augmenting the quantity of IFI16. The overexpression of IFI16 in HELFs infected with HCMV was associated with a 2.5 log reduction in viral yield. However, light had yet to be shed on the molecular mechanisms responsible for the antiviral role of IFI16, prompting an investigation into the consequences of overexpression on the distinct phases of virus replication. Exploiting the luciferase reporter gene methodology, transfection experiments were used to study the effects of deleting the viral polymerase (UL54) or UL44 promoters or introducing mutated forms of the two[72]. These studies indicated the IR-1 locus (inverted repeat element 1), located upstream of the polymerase transcription start-site, to be the object of IFI16-induced virus suppression. Chromatin immunoprecipitation and EMSA revealed that Sp1-like factors were effectively blocked by IFI16 and that this in turn led to UL54 suppression. This result was confirmed by deleting the element within the UL44 promoter responsive to Sp1, which accordingly eliminated the suppressive effect of IFI16 on HCMV replication (UL44 protein associates with UL54 during viral DNA replication). Thus, in addition to confirming IFI16's role as a DNA sensor, for the first time IFI16 had also been demonstrated to act as a restriction factor of herpesvirus replication[72] (Figure 1).

HCMV is nevertheless able to replicate in host cells despite the restrictive capacity of IFI16. This suggests that HCMV has developed evasion strategies to respond to the effects of IFI16[42,43]. The first evidence sustaining a plausible HCMV evasion strategy was obtained by infecting fibroblasts with a BAC mutant unable to express UL97 phosphoprotein[42]. Early on during infection, IFI16 binds to virus DNA, but at a later time point during viral DNA synthesis, IFI16 undergoes relocalization from the nucleus to the cytoplasm. It was also revealed that this virus-induced movement of IFI16 out of the nucleus required that UL97 (a viral protein kinase) bound to IFI16. Upon binding to UL97 phosphoprotein, IFI16 undergoes phosphorylation, which in turn promotes its nucleo-cytoplasmic relocalization. IFI16's ensuing transfer into the virus assembly complex is regulated by the endosomal sorting complex required for transport machinery. Finally, IFI16 becomes integrated into newly assembled virions during the process of virus maturation and budding, effectively expelling IFI16 from the infected host[42]. However, recent studies have revealed that IFI16 phosphorylation by UL97 is not the only mechanism for HCMV escape from IFI16 restriction activity. Using a BAC mutant virus unable to express the tegument protein pp65 (pUL83) Biolatti *et al*[73] (2016, unpublished results) have demonstrated that IFI16 interacts with pp65 targeting early gene promoters including that of the viral DNA polymerase pUL54. The capability of IFI16 to downregulate virus growth was found to depend on its interaction with pp65 at the UL54 promoter, as shown by the growth properties of the HMCV mutant v65Stop in IFI16 knockdown cells. Interestingly, at later time points of HCMV infection, IFI16 was not degraded, as observed in HSV-1 - infected cells, but it was protected by its interaction with pp65. These data reveal a dual role for pp65. Initially it modulates IFI16 activity at the promoter of immediate-early and early genes, and subsequently, it delocalizes IFI16 from the nucleus, thereby protecting it from proteasomal degradation. Overall, these data identify a novel activity displayed by the pp65/IFI16 interactome in the regulation of *UL54* gene expression and IFI16 protein stability during HCMV replication.

In summary, these experiments point toward IFI16 nuclear egression, subsequent to its binding to UL97 and pp65, as the mechanism through which HCMV is successfully able to evade IFI16 restriction activity; this removal of IFI16 from its site of restriction activity is finalized with its incorporation into newly formed virions and expulsion from the cell altogether[42]. This is most likely the event that, to all intents and purposes, underlies the HMCV's successful evasion of IFI16 antiviral activity (Figure 1, Table 1).

NUCLEAR DOMAIN 10

The nuclear matrix, hypothesized by some to organize and regulate a number of nuclear functions within the nucleus of eukaryotes[74,75], contains discrete bodies designated "nuclear domain 10" (ND10), "promyelocytic leukemia (PML) nuclear bodies", or PODS. These bodies appear as sphere-like, measuring between 0.1-1 μm in diameter, and in some circumstances they present a granular center. ND10 can be found within the nucleoplasmic domains collectively termed the interchromosomal space, often next to proteinaceous bodies. Sp100, hDaxx, and PML protein are three of the protein constituents of ND10. These proteins recruit additional proteins that are SUMOylated[76,77]. One of these additional proteins is SUMO, a protein related to ubiquitin, and its conjugation to PML is implicated in the further recruitment of yet more binding partners[76,77]. ND10 are devoid of RNA or DNA and they typically gather into clusters of 5 to 15[78]. PML protein forms the outer "casing" of the structure, and its protein partners are usually concentrated inside. Functionally, ND10 play a regulatory role, influencing

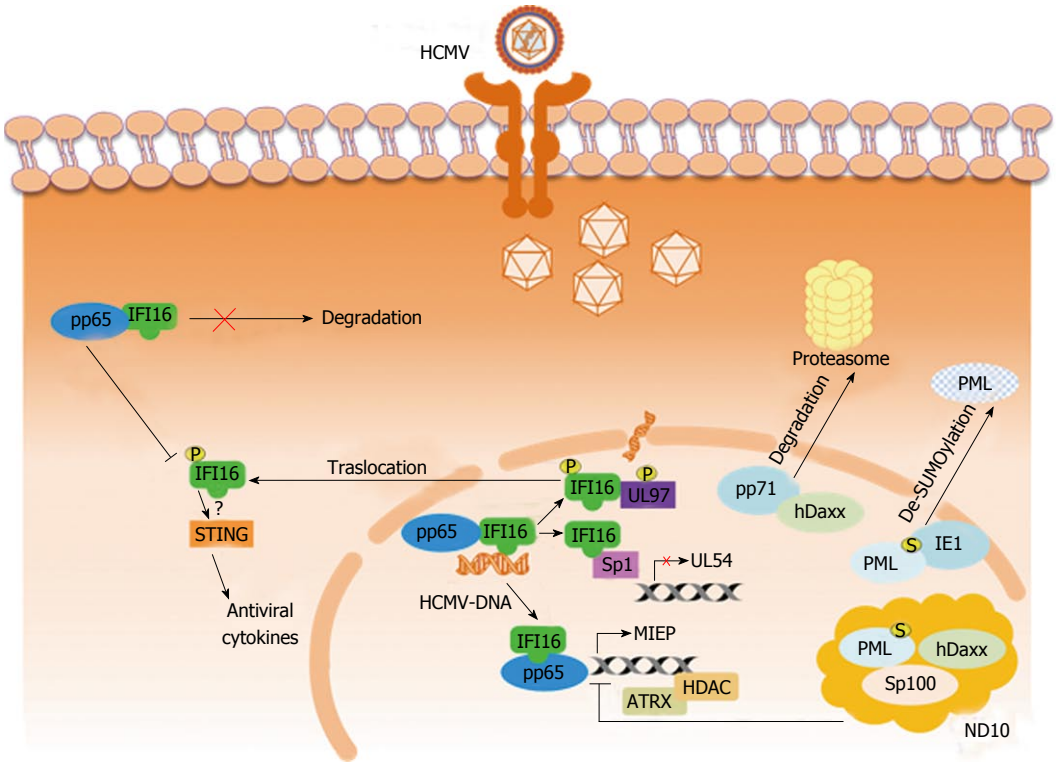

Figure 1 Simplified model of host restrictions against human cytomegalovirus and mechanisms of viral escape. IFI16 recognizes HCMV-DNA in the nucleus. In the early phases of infection, HCMV-pp65 hijacks IFI16 to activate MIEP expression; later on the complex IFI16-pp65 blocks HCMV replication by inhibiting the action of Sp1 on UL54 promoter. IFI16 is not degraded, but it was stabilized by its interaction with pp65. To evade IFI16 antiviral activity, HCMV induces nuclear translocation of IFI16 upon recruitment of HCMV kinase UL97 to IFI16. In the cytoplasm, IFI16 activates STING-mediated antiviral cytokine expression. ND10 components (hDaxx, Sp100, PML) limit viral replication by silencing viral *IE* genes, inducing a transcriptionally inactive chromatin state of the MIEP *via* recruitment of ATRX or HDACs to the viral DNA. The viral pp71 binds to hDaxx for proteasome degradation and relieves MIEP repression. Degradation of hDaxx is preceded by the release of ATRX from ND10. IE1 promotes the dispersion of the ND10, by de-SUMOylation, with the consequent inhibition of PML oligomerization. HCMV: Human cytomegalovirus; IFI16: IFN-γ inducible protein 16; PYHIN: Pyrin/HIN domain; AIM2: Absent in melanoma 2; MIEP: Major immediate early promoter; ND10: Nuclear domain 10; ATRX: Alpha thalassemia and mental retardation syndrome X-linked; PML: Promyelocytic leukemia.

diverse key processes, such as DNA damage repair[79], oncogenesis[80-82], apoptosis[83-85], senescence[86,87], and gene expression regulation[75,76]. The fact that ND10 are subject to profound biochemical modification during virus infection is of particular interest[81,87-90]. During the infection of quiescent cells, evidence indicates that NB10 accumulates viral DNA within their central core and/or at their periphery. Moreover, considering the fact that IFNs stimulate an increase expression of PML, hDaxx, and Sp100[91-93], it would appear that ND10 play a key role in the innate antiviral response.

ND10 restriction of HCMV replication vs viral evasion tactics

Although aggregates of HCMV *IE* gene transcripts only form adjacent to ND10 - originally considered the ideal cellular location for HCMV to initiate its program of *IE* gene expression - it was soon realized that early during infection HCMV actually targets ND10 in order to destroy them[94-97] (Figure 1, Table 1). The viral regulatory protein IE1 is responsible for this activity; moreover ND10 destruction by IE1 correlates with efficient lytic replication[98]. ND10 are now understood to be key cellular restriction factors playing an effective biological role in the inhibition of virus replication. The silencing of PML expression by siRNA and thus depletion of this ND10 constituent, for instance, was shown to increase the susceptibility of "human primary foreskin fibroblasts" (HFF) to HCMV infection[92]. Indeed, the capacity of ND10 bodies to restrict HCMV is achieved by down-regulating *IE* gene expression[93]. Of note, when PML-null HFF were used, HCMV infection resulted in the formation of Sp100 and hDaxx[99]. It has since emerged that it is actually the virus itself that stimulates the recruitment of ND10 constituents to nucleoprotein complex of HCMV[98]. What is more, studies suggest that this ND10-instigated intrinsic immune response probably entails other ND10 associated proteins, like Sp100 and hDaxx[100-102].

In summary, the results of studies carried out by various groups together depict a scenario where all the three key ND10 proteins - *i.e.,* Sp100, hDaxx, and PML- are involved in restricting viral replication through the silencing of viral *IE* gene expression[97,102-104]. In addition, infection experiments comparing the effects of single *vs* double knock-down of ND10 constituents found that *HCMV* gene expression was actually lower in the single knock-down cells compared with the respective double knock-down conditions[101,102]. This undoubtedly points toward the individual proteins playing independent roles in HCMV restriction. Epigenetic mechanisms able to

block viral genome transcription and replication may be involved[105,106]. During early infection, viral DNA exists in a repressive chromatin state, a result of posttranslational modifications of histones[106]. Through its ability to recruit to the viral DNA chromatin modifying enzymes, including the histone deacetylases, or chromatin remodeling protein "alpha thalassemia and mental retardation syndrome X-linked" (ATRX)[105,107], hDaxx has been demonstrated to convert the HCMV immediate-early enhancer/promoter (MIEP) into a transcriptionally inactive chromatin state[108]. Sp100 and PML have similarly been confirmed to interact with enzymes that modify chromatin; once again implicating the contribution of possible epigenetic modifications in the intrinsic immune repression of *IE* gene expression[109,110].

Until now, the role of ND10 has largely been studied in the context of productive HCMV infection. Interestingly, Saffert and Kalejta[110], using three different cellular settings, including NT2 and THP-1 cells, primary human $CD34^+$ cells, and two myeloblastic cell lines (Kasumi-3 and KG-1), provided evidence that hDaxx is also involved in *IE* gene silencing in latent HCMV infections. By contrast, the group led by Sinclair provide evidence indicating that hDaxx protein is only marginally involved in MIEP regulation during latent infection, since its knockdown in NT2 cells block *IE* gene expression[105]. To solve this apparent discrepancy, Stamminger's group[111] used the THP-1 monocytes recognized as reliable *in vitro* latency model of HCMV. In non-differentiated THP-1 monocytes, HCMV undergoes latency; while in THP-1 cells induced to undergo differentiation towards a macrophage-like phenotype, achieved using PMA, HCMV enters its lytic cycle[111]. The results obtained showed that the silencing of PML, hDaxx, or Sp100 expression by small hairpin RNA in non-differentiated THP-1 monocytes did not have an effect on *IE* gene expression. In contrast, the silencing of ND10 in differentiated THP-1 significantly augmented cells positive for *IE* gene expression. Altogether, these conclusions indicate that hDaxx, PML, and Sp100 serve as restriction factors of *IE* gene expression, but are only marginally involved in the establishment of HCMV latency[111].

Nevertheless, we know that HCMV is still able to undergo successful lytic replication in spite of the restrictive behavior of the ND10 constituent proteins; this tells us that HCMV has co-evolved to circumvent this aspect of the innate immune response. Indeed, we now know that shortly after virus penetration, pp71 (the viral transactivator tegument protein) moves to ND10 bodies where it interacts with ND10 constituent proteins[112]; in particular, it associates with hDaxx, which, as discussed above, is capable of down-regulating *IE* gene expression by silencing MIEP[113]. The interaction between pp71 and hDaxx results in the latter being directed down the path of proteasome degradation, thereby relieving MIEP repression[112]. However, investigations by other groups have recently shown that the scenario is actually much more complex. Degradation of hDaxx is preceded by the pp71-stimulated release of ATRX from ND10, and it is the displacement of ATRX that alleviates the repression of *IE* gene expression[113].

While pp71 is fundamental to counteract the capacity of both ATRX and hDaxx to silence virus genes, it is the action of IE1 protein that seems to eliminate the restrictive effects of PML protein. Indeed, IEI stimulates ND10 body dispersal, with the associated displacement of both PML and Sp100. At low MOI, IE1 synergizes with IE2 and promotes the activation of various viral gene expression[114,115]. However, only IE1 is required for ND10 dispersal. Moreover, the disruption of PML by IE1 is not followed by degradation of PML *via* proteosome; instead it becomes de-SUMOylated; which effectively inhibits PML oligomerization and thus its ability to re-associate within ND10 bodies[116] (Figure 1, Table 1).

LYSINE-SPECIFIC DEMETHYLASES

Lysine-specific demethylases (KDMs) inhibit the establishment of HCMV latency by getting rid of epigenetic "tags" present on histones associated with the repression of MIEP. Interestingly, the viral UL138 protein counteracts this defense by interfering with the association of KDMs with the MIEP[117] (Table 1).

Thus, the presence of viral factors neutralizing cognate host restriction factors indicate that HCMV has developed multiple escape strategies over lifelong colonization at the cellular level.

CONCLUSION

In conclusion, frontline cell defense against HCMV replication is now known to be accomplished by different proteins through different pathways. Moreover, the viral countermeasures to overcome these restriction factors are now clearly understood to involve a number of viral proteins, including pp71, IE1, UL97, and pp65. Ongoing research is presently being focused at compiling a more in-depth picture of the molecular mechanisms involving ND10 that underlie the host cell's restrictive response the viral evasion strategies.

REFERENCES

1 **McCormick AL**, Mocarski ES. Viral modulation of the host response to infection. In: Arvin A, Campadelli-Fiume G, Mocarski E, Moore PS, Roizman B, Whitley R, Yamanishi K. Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis. Cambridge: Cambridge University Press. [accessed 2016 Apr 19]. Available from: URL: http//www.ncbi.nlm.nih.gov/books/NBK47417

2 **Takeuchi O**, Akira S. Pattern recognition receptors and inflammation. *Cell* 2010; **140**: 805-820 [PMID: 20303872 DOI: 10.1016/j.cell.2010.01.022]

3 **Bieniasz PD**. Intrinsic immunity: a front-line defense against viral attack. *Nat Immunol* 2004; **5**: 1109-1115 [PMID: 15496950 DOI: 10.1038/ni1125]

4 **Bieniasz PD**. Restriction factors: a defense against retroviral infection. *Trends Microbiol* 2003; **11**: 286-291 [PMID: 12823946]

5 **Johnson WE**. Rapid adversarial co-evolution of viruses and cellular restriction factors. *Curr Top Microbiol Immunol* 2013; **371**: 123-151

[PMID: 23686234 DOI: 10.1007/978-3-642-37765-5_5]
6 **Pincus T**, Rowe WP, Lilly F. A major genetic locus affecting resistance to infection with murine leukemia viruses. II. Apparent identity to a major locus described for resistance to friend murine leukemia virus. *J Exp Med* 1971; **133**: 1234-1241 [PMID: 4325133]
7 **Neil SJ**, Zang T, Bieniasz PD. Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature* 2008; **451**: 425-430 [PMID: 18200009 DOI: 10.1038/nature06553]
8 **Simon V**, Bloch N, Landau NR. Intrinsic host restrictions to HIV-1 and mechanisms of viral escape. *Nat Immunol* 2015; **16**: 546-553 [PMID: 25988886 DOI: 10.1038/ni.3156]
9 **Compton AA**, Hirsch VM, Emerman M. The host restriction factor APOBEC3G and retroviral Vif protein coevolve due to ongoing genetic conflict. *Cell Host Microbe* 2012; **11**: 91-98 [PMID: 22264516 DOI: 10.1016/j.chom.2011.11.010]
10 **Malim MH**. APOBEC proteins and intrinsic resistance to HIV-1 infection. *Philos Trans R Soc Lond B Biol Sci* 2009; **364**: 675-687 [PMID: 19038776 DOI: 10.1098/rstb.2008.0185]
11 **Grütter MG**, Luban J. TRIM5 structure, HIV-1 capsid recognition, and innate immune signaling. *Curr Opin Virol* 2012; **2**: 142-150 [PMID: 22482711 DOI: 10.1016/j.coviro.2012.02.003]
12 **Yan J**, Kaur S, DeLucia M, Hao C, Mehrens J, Wang C, Golczak M, Palczewski K, Gronenborn AM, Ahn J, Skowronski J. Tetramerization of SAMHD1 is required for biological activity and inhibition of HIV infection. *J Biol Chem* 2013; **288**: 10406-10417 [PMID: 23426366 DOI: 10.1074/jbc.M112.443796]
13 **Hammonds J**, Wang JJ, Spearman P. Restriction of Retroviral Replication by Tetherin/BST-2. *Mol Biol Int* 2012; **2012**: 424768 [PMID: 22811908 DOI: 10.1155/2012/424768]
14 **Jakobsen MR**, Olagnier D, Hiscott J. Innate immune sensing of HIV-1 infection. *Curr Opin HIV AIDS* 2015; **10**: 96-102 [PMID: 25485569 DOI: 10.1097/COH.0000000000000129]
15 **Neil S**, Bieniasz P. Human immunodeficiency virus, restriction factors, and interferon. *J Interferon Cytokine Res* 2009; **29**: 569-580 [PMID: 19694548 DOI: 10.1089/jir.2009.0077]
16 **Mogensen TH**. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin Microbiol Rev* 2009; **22**: 240-73, Table of Contents [PMID: 19366914 DOI: 10.1128/CMR.00046-08]
17 **Thaiss CA**, Levy M, Itav S, Elinav E. Integration of Innate Immune Signaling. *Trends Immunol* 2016; **37**: 84-101 [PMID: 26755064 DOI: 10.1016/j.it.2015.12.003]
18 **Thompson MR**, Kaminski JJ, Kurt-Jones EA, Fitzgerald KA. Pattern recognition receptors and the innate immune response to viral infection. *Viruses* 2011; **3**: 920-940 [PMID: 21994762 DOI: 10.3390/v3060920]
19 **Paludan SR**, Bowie AG. Immune sensing of DNA. *Immunity* 2013; **38**: 870-880 [PMID: 23706668 DOI: 10.1016/j.immuni.2013.05.004]
20 **Unterholzner L**. The interferon response to intracellular DNA: why so many receptors? *Immunobiology* 2013; **218**: 1312-1321 [PMID: 23962476 DOI: 10.1016/j.imbio.2013.07.007]
21 **Guo H**, Callaway JB, Ting JP. Inflammasomes: mechanism of action, role in disease, and therapeutics. *Nat Med* 2015; **21**: 677-687 [PMID: 26121197 DOI: 10.1038/nm.3893]
22 **Latz E**, Xiao TS, Stutz A. Activation and regulation of the inflammasomes. *Nat Rev Immunol* 2013; **13**: 397-411 [PMID: 23702978 DOI: 10.1038/nri3452]
23 **Xiao TS**. The nucleic acid-sensing inflammasomes. *Immunol Rev* 2015; **265**: 103-111 [PMID: 25879287 DOI: 10.1111/imr.12281]
24 **Dempsey A**, Bowie AG. Innate immune recognition of DNA: A recent history. *Virology* 2015; **479-480**: 146-152 [PMID: 25816762 DOI: 10.1016/j.virol.2015.03.013]
25 **Diner BA**, Lum KK, Cristea IM. The emerging role of nuclear viral DNA sensors. *J Biol Chem* 2015; **290**: 26412-26421 [PMID: 26354430 DOI: 10.1074/jbc.R115.652289]
26 **Paludan SR**, Bowie AG, Horan KA, Fitzgerald KA. Recognition of herpesviruses by the innate immune system. *Nat Rev Immunol* 2011; **11**: 143-154 [PMID: 21267015 DOI: 10.1038/nri2937]
27 **Boeckh M**, Geballe AP. Cytomegalovirus: pathogen, paradigm, and puzzle. *J Clin Invest* 2011; **121**: 1673-1680 [PMID: 21659716 DOI: 10.1172/JCI45449]
28 **Griffiths P**, Baraniak I, Reeves M. The pathogenesis of human cytomegalovirus. *J Pathol* 2015; **235**: 288-297 [PMID: 25205255 DOI: 10.1002/path.4437]
29 **Beck K**, Meyer-König U, Weidmann M, Nern C, Hufert FT. Human cytomegalovirus impairs dendritic cell function: a novel mechanism of human cytomegalovirus immune escape. *Eur J Immunol* 2003; **33**: 1528-1538 [PMID: 12778470 DOI: 10.1002/eji.200323612]
30 **Browne EP**, Shenk T. Human cytomegalovirus UL83-coded pp65 virion protein inhibits antiviral gene expression in infected cells. *Proc Natl Acad Sci USA* 2003; **100**: 11439-11444 [PMID: 12972646 DOI: 10.1073/pnas.1534570100]
31 **Child SJ**, Hakki M, De Niro KL, Geballe AP. Evasion of cellular antiviral responses by human cytomegalovirus TRS1 and IRS1. *J Virol* 2004; **78**: 197-205 [PMID: 14671101]
32 **Marshall EE**, Geballe AP. Multifaceted evasion of the interferon response by cytomegalovirus. *J Interferon Cytokine Res* 2009; **29**: 609-619 [PMID: 19708810 DOI: 10.1089/jir.2009.0064]
33 **Trapani JA**, Dawson M, Apostolidis VA, Browne KA. Genomic organization of IFI16, an interferon-inducible gene whose expression is associated with human myeloid cell differentiation: correlation of predicted protein domains with exon organization. *Immunogenetics* 1994; **40**: 415-424 [PMID: 7959953]
34 **Dawson MJ**, Trapani JA. The interferon-inducible autoantigen, IFI 16: localization to the nucleolus and identification of a DNA-binding domain. *Biochem Biophys Res Commun* 1995; **214**: 152-162 [PMID: 7545391 DOI: 10.1006/bbrc.1995.2269]
35 **Gariglio M**, Mondini M, De Andrea M, Landolfo S. The multifaceted interferon-inducible p200 family proteins: from cell biology to human pathology. *J Interferon Cytokine Res* 2011; **31**: 159-172 [PMID: 21198352 DOI: 10.1089/jir.2010.0106]
36 **Connolly DJ**, Bowie AG. The emerging role of human PYHIN proteins in innate immunity: implications for health and disease. *Biochem Pharmacol* 2014; **92**: 405-414 [PMID: 25199457 DOI: 10.1016/j.bcp.2014.08.031]
37 **Jakobsen MR**, Paludan SR. IFI16: At the interphase between innate DNA sensing and genome regulation. *Cytokine Growth Factor Rev* 2014; **25**: 649-655 [PMID: 25027602 DOI: 10.1016/j.cytogfr.2014.06.004]
38 **Liao JC**, Lam R, Brazda V, Duan S, Ravichandran M, Ma J, Xiao T, Tempel W, Zuo X, Wang YX, Chirgadze NY, Arrowsmith CH. Interferon-inducible protein 16: insight into the interaction with tumor suppressor p53. *Structure* 2011; **19**: 418-429 [PMID: 21397192 DOI: 10.1016/j.str.2010.12.015]
39 **Johnstone RW**, Kershaw MH, Trapani JA. Isotypic variants of the interferon-inducible transcriptional repressor IFI 16 arise through differential mRNA splicing. *Biochemistry* 1998; **37**: 11924-11931 [PMID: 9718316 DOI: 10.1021/bi981069a]
40 **Jin T**, Perry A, Jiang J, Smith P, Curry JA, Unterholzner L, Jiang Z, Horvath G, Rathinam VA, Johnstone RW, Hornung V, Latz E, Bowie AG, Fitzgerald KA, Xiao TS. Structures of the HIN domain: DNA complexes reveal ligand binding and activation mechanisms of the AIM2 inflammasome and IFI16 receptor. *Immunity* 2012; **36**: 561-571 [PMID: 22483801 DOI: 10.1016/j.immuni.2012.02.014]
41 **Gariglio M**, Azzimonti B, Pagano M, Palestro G, De Andrea M, Valente G, Voglino G, Navino L, Landolfo S. Immunohistochemical expression analysis of the human interferon-inducible gene IFI16, a member of the HIN200 family, not restricted to hematopoietic cells. *J Interferon Cytokine Res* 2002; **22**: 815-821 [PMID: 12184920 DOI: 10.1089/107999002320271413]
42 **Dell'Oste V**, Gatti D, Gugliesi F, De Andrea M, Bawadekar M, Lo Cigno I, Biolatti M, Vallino M, Marschall M, Gariglio M, Landolfo S. Innate nuclear sensor IFI16 translocates into the cytoplasm during the early stage of in vitro human cytomegalovirus infection and is entrapped in the egressing virions during the late stage. *J Virol* 2014; **88**: 6970-6982 [PMID: 24696486 DOI: 10.1128/JVI.00384-14]
43 **Li T**, Diner BA, Chen J, Cristea IM. Acetylation modulates cellular distribution and DNA sensing ability of interferon-inducible protein IFI16. *Proc Natl Acad Sci USA* 2012; **109**: 10558-10563

[PMID: 22691496 DOI: 10.1073/pnas.1203447109]
44 **Wei W**, Clarke CJ, Somers GR, Cresswell KS, Loveland KA, Trapani JA, Johnstone RW. Expression of IFI 16 in epithelial cells and lymphoid tissues. *Histochem Cell Biol* 2003; **119**: 45-54 [PMID: 12548405 DOI: 10.1007/s00418-002-0485-0]
45 **Briggs LJ**, Johnstone RW, Elliot RM, Xiao CY, Dawson M, Trapani JA, Jans DA. Novel properties of the protein kinase CK2-site-regulated nuclear- localization sequence of the interferon-induced nuclear factor IFI 16. *Biochem J* 2001; **353**: 69-77 [PMID: 11115400]
46 **Cristea IM**, Moorman NJ, Terhune SS, Cuevas CD, O'Keefe ES, Rout MP, Chait BT, Shenk T. Human cytomegalovirus pUL83 stimulates activity of the viral immediate-early promoter through its interaction with the cellular IFI16 protein. *J Virol* 2010; **84**: 7803-7814 [PMID: 20504932 DOI: 10.1128/JVI.00139-10]
47 **Veeranki S**, Choubey D. Interferon-inducible p200-family protein IFI16, an innate immune sensor for cytosolic and nuclear double-stranded DNA: regulation of subcellular localization. *Mol Immunol* 2012; **49**: 567-571 [PMID: 22137500 DOI: 10.1016/j.molimm.2011.11.004]
48 **Bawadekar M**, De Andrea M, Gariglio M, Landolfo S. Mislocalization of the interferon inducible protein IFI16 by environmental insults: implications in autoimmunity. *Cytokine Growth Factor Rev* 2015; **26**: 213-219 [PMID: 25466628 DOI: 10.1016/j.cytogfr.2014.10.003]
49 **Costa S**, Borgogna C, Mondini M, De Andrea M, Meroni PL, Berti E, Gariglio M, Landolfo S. Redistribution of the nuclear protein IFI16 into the cytoplasm of ultraviolet B-exposed keratinocytes as a mechanism of autoantigen processing. *Br J Dermatol* 2011; **164**: 282-290 [PMID: 20973769 DOI: 10.1111/j.1365-2133.2010.10097.x]
50 **Gugliesi F**, Mondini M, Ravera R, Robotti A, de Andrea M, Gribaudo G, Gariglio M, Landolfo S. Up-regulation of the interferon-inducible IFI16 gene by oxidative stress triggers p53 transcriptional activity in endothelial cells. *J Leukoc Biol* 2005; **77**: 820-829 [PMID: 15728246 DOI: 10.1189/jlb.0904507]
51 **Johnstone RW**, Wei W, Greenway A, Trapani JA. Functional interaction between p53 and the interferon-inducible nucleoprotein IFI 16. *Oncogene* 2000; **19**: 6033-6042 [PMID: 11146555 DOI: 10.1038/sj.onc.1204005]
52 **Xin H**, Curry J, Johnstone RW, Nickoloff BJ, Choubey D. Role of IFI 16, a member of the interferon-inducible p200-protein family, in prostate epithelial cellular senescence. *Oncogene* 2003; **22**: 4831-4840 [PMID: 12894224 DOI: 10.1038/sj.onc.1206754]
53 **Aglipay JA**, Lee SW, Okada S, Fujiuchi N, Ohtsuka T, Kwak JC, Wang Y, Johnstone RW, Deng C, Qin J, Ouchi T. A member of the Pyrin family, IFI16, is a novel BRCA1-associated protein involved in the p53-mediated apoptosis pathway. *Oncogene* 2003; **22**: 8931-8938 [PMID: 14654789 DOI: 10.1038/sj.onc.1207057]
54 **Kerur N**, Veettil MV, Sharma-Walia N, Bottero V, Sadagopan S, Otageri P, Chandran B. IFI16 acts as a nuclear pathogen sensor to induce the inflammasome in response to Kaposi Sarcoma-associated herpesvirus infection. *Cell Host Microbe* 2011; **9**: 363-375 [PMID: 21575908 DOI: 10.1016/j.chom.2011.04.008]
55 **Unterholzner L**, Keating SE, Baran M, Horan KA, Jensen SB, Sharma S, Sirois CM, Jin T, Latz E, Xiao TS, Fitzgerald KA, Paludan SR, Bowie AG. IFI16 is an innate immune sensor for intracellular DNA. *Nat Immunol* 2010; **11**: 997-1004 [PMID: 20890285 DOI: 10.1038/ni.1932]
56 **Brázda V**, Coufal J, Liao JC, Arrowsmith CH. Preferential binding of IFI16 protein to cruciform structure and superhelical DNA. *Biochem Biophys Res Commun* 2012; **422**: 716-720 [PMID: 22618232 DOI: 10.1016/j.bbrc.2012.05.065]
57 **Ni X**, Ru H, Ma F, Zhao L, Shaw N, Feng Y, Ding W, Gong W, Wang Q, Ouyang S, Cheng G, Liu ZJ. New insights into the structural basis of DNA recognition by HINa and HINb domains of IFI16. *J Mol Cell Biol* 2016; **8**: 51-61 [PMID: 26246511 DOI: 10.1093/jmcb/mjv053]
58 **Sharma S**, DeOliveira RB, Kalantari P, Parroche P, Goutagny N, Jiang Z, Chan J, Bartholomeu DC, Lauw F, Hall JP, Barber GN, Gazzinelli RT, Fitzgerald KA, Golenbock DT. Innate immune recognition of an AT-rich stem-loop DNA motif in the Plasmodium falciparum genome. *Immunity* 2011; **35**: 194-207 [PMID: 21820332 DOI: 10.1016/j.immuni.2011.05.016]
59 **Stratmann SA**, Morrone SR, van Oijen AM, Sohn J. The innate immune sensor IFI16 recognizes foreign DNA in the nucleus by scanning along the duplex. *Elife* 2015; **4**: e11721 [PMID: 26673078 DOI: 10.7554/eLife.11721]
60 **Horan KA**, Hansen K, Jakobsen MR, Holm CK, Søby S, Unterholzner L, Thompson M, West JA, Iversen MB, Rasmussen SB, Ellermann-Eriksen S, Kurt-Jones E, Landolfo S, Damania B, Melchjorsen J, Bowie AG, Fitzgerald KA, Paludan SR. Proteasomal degradation of herpes simplex virus capsids in macrophages releases DNA to the cytosol for recognition by DNA sensors. *J Immunol* 2013; **190**: 2311-2319 [PMID: 23345332 DOI: 10.4049/jimmunol.1202749]
61 **Ansari MA**, Singh VV, Dutta S, Veettil MV, Dutta D, Chikoti L, Lu J, Everly D, Chandran B. Constitutive interferon-inducible protein 16-inflammasome activation during Epstein-Barr virus latency I, II, and III in B and epithelial cells. *J Virol* 2013; **87**: 8606-8623 [PMID: 23720728 DOI: 10.1128/JVI.00805-13]
62 **Johnson KE**, Chikoti L, Chandran B. Herpes simplex virus 1 infection induces activation and subsequent inhibition of the IFI16 and NLRP3 inflammasomes. *J Virol* 2013; **87**: 5005-5018 [PMID: 23427152 DOI: 10.1128/JVI.00082-13]
63 **Jakobsen MR**, Bak RO, Andersen A, Berg RK, Jensen SB, Tengchuan J, Laustsen A, Hansen K, Ostergaard L, Fitzgerald KA, Xiao TS, Mikkelsen JG, Mogensen TH, Paludan SR. IFI16 senses DNA forms of the lentiviral replication cycle and controls HIV-1 replication. *Proc Natl Acad Sci USA* 2013; **110**: E4571-80 [PMID: 24154727 DOI: 10.1073/pnas.1311669110]
64 **Lo Cigno I**, De Andrea M, Borgogna C, Albertini S, Landini MM, Peretti A, Johnson KE, Chandran B, Landolfo S, Gariglio M. The Nuclear DNA Sensor IFI16 Acts as a Restriction Factor for Human Papillomavirus Replication through Epigenetic Modifications of the Viral Promoters. *J Virol* 2015; **89**: 7506-7520 [PMID: 25972554 DOI: 10.1128/JVI.00013-15]
65 **Man SM**, Karki R, Kanneganti TD. AIM2 inflammasome in infection, cancer, and autoimmunity: Role in DNA sensing, inflammation, and innate immunity. *Eur J Immunol* 2016; **46**: 269-280 [PMID: 26626159 DOI: 10.1002/eji.201545839]
66 **Rathinam VA**, Jiang Z, Waggoner SN, Sharma S, Cole LE, Waggoner L, Vanaja SK, Monks BG, Ganesan S, Latz E, Hornung V, Vogel SN, Szomolanyi-Tsuda E, Fitzgerald KA. The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nat Immunol* 2010; **11**: 395-402 [PMID: 20351692 DOI: 10.1038/ni.1864]
67 **Conrady CD**, Zheng M, Fitzgerald KA, Liu C, Carr DJ. Resistance to HSV-1 infection in the epithelium resides with the novel innate sensor, IFI-16. *Mucosal Immunol* 2012; **5**: 173-183 [PMID: 22236996 DOI: 10.1038/mi.2011.63]
68 **Orzalli MH**, DeLuca NA, Knipe DM. Nuclear IFI16 induction of IRF-3 signaling during herpesviral infection and degradation of IFI16 by the viral ICP0 protein. *Proc Natl Acad Sci USA* 2012; **109**: E3008-E3017 [PMID: 23027953 DOI: 10.1073/pnas.1211302109]
69 **Orzalli MH**, Conwell SE, Berrios C, DeCaprio JA, Knipe DM. Nuclear interferon-inducible protein 16 promotes silencing of herpesviral and transfected DNA. *Proc Natl Acad Sci USA* 2013; **110**: E4492-E4501 [PMID: 24198334 DOI: 10.1073/pnas.1316194110]
70 **Søby S**, Laursen RR, Ostergaard L, Melchjorsen J. HSV-1-induced chemokine expression via IFI16-dependent and IFI16-independent pathways in human monocyte-derived macrophages. *Herpesviridae* 2012; **3**: 6 [PMID: 23062757 DOI: 10.1186/2042-4280-3-6]
71 **Gariano GR**, Dell'Oste V, Bronzini M, Gatti D, Luganini A, De Andrea M, Gribaudo G, Gariglio M, Landolfo S. The intracellular DNA sensor IFI16 gene acts as restriction factor for human cytomegalovirus replication. *PLoS Pathog* 2012; **8**: e1002498 [PMID: 22291595 DOI: 10.1371/journal.ppat.1002498]
72 **Rivera-Molina YA**, Martínez FP, Tang Q. Nuclear domain 10 of the viral aspect. *World J Virol* 2013; **2**: 110-122 [PMID: 24255882 DOI: 10.5501/wjv.v2.i3.110]

73 **Biolatti M**, Dell'Oste V, Pautasso S, von Einem J, Marschall M, Plachter B, Gariglio M, De Andrea M, Landolfo S. Regulatory Interaction between the Cellular Restriction Factor IFI16 and Viral pp65 (pUL83) Modulates Viral Gene Expression and IFI16 Protein Stability. *J Virol* 2016; Epub ahead of print [PMID: 27384655 DOI: 10.1128/JVI.00923-16]

74 **Stuurman N**, Meijne AM, van der Pol AJ, de Jong L, van Driel R, van Renswoude J. The nuclear matrix from cells of different origin. Evidence for a common set of matrix proteins. *J Biol Chem* 1990; **265**: 5460-5465 [PMID: 2180926]

75 **Bernardi R**, Pandolfi PP. Structure, dynamics and functions of promyelocytic leukaemia nuclear bodies. *Nat Rev Mol Cell Biol* 2007; **8**: 1006-1016 [PMID: 17928811 DOI: 10.1038/nrm2277]

76 **Shen TH**, Lin HK, Scaglioni PP, Yung TM, Pandolfi PP. The mechanisms of PML-nuclear body formation. *Mol Cell* 2006; **24**: 331-339 [PMID: 17081985 DOI: 10.1016/j.molcel.2006.09.013]

77 **Boisvert FM**, Hendzel MJ, Bazett-Jones DP. Promyelocytic leukemia (PML) nuclear bodies are protein structures that do not accumulate RNA. *J Cell Biol* 2000; **148**: 283-292 [PMID: 10648561]

78 **Gurrieri C**, Capodieci P, Bernardi R, Scaglioni PP, Nafa K, Rush LJ, Verbel DA, Cordon-Cardo C, Pandolfi PP. Loss of the tumor suppressor PML in human cancers of multiple histologic origins. *J Natl Cancer Inst* 2004; **96**: 269-279 [PMID: 14970276]

79 **Koken MH**, Linares-Cruz G, Quignon F, Viron A, Chelbi-Alix MK, Sobczak-Thépot J, Juhlin L, Degos L, Calvo F, de Thé H. The PML growth-suppressor has an altered expression in human oncogenesis. *Oncogene* 1995; **10**: 1315-1324 [PMID: 7731682]

80 **Terris B**, Baldin V, Dubois S, Degott C, Flejou JF, Hénin D, Dejean A. PML nuclear bodies are general targets for inflammation and cell proliferation. *Cancer Res* 1995; **55**: 1590-1597 [PMID: 7882370]

81 **Everett RD**. Interactions between DNA viruses, ND10 and the DNA damage response. *Cell Microbiol* 2006; **8**: 365-374 [PMID: 16469050 DOI: 10.1111/j.1462-5822.2005.00677.x]

82 **Bernardi R**, Pandolfi PP. Role of PML and the PML-nuclear body in the control of programmed cell death. *Oncogene* 2003; **22**: 9048-9057 [PMID: 14663483 DOI: 10.1038/sj.onc.1207106]

83 **Bernardi R**, Papa A, Pandolfi PP. Regulation of apoptosis by PML and the PML-NBs. *Oncogene* 2008; **27**: 6299-6312 [PMID: 18931695 DOI: 10.1038/onc.2008.305]

84 **Guo A**, Salomoni P, Luo J, Shih A, Zhong S, Gu W, Pandolfi PP. The function of PML in p53-dependent apoptosis. *Nat Cell Biol* 2000; **2**: 730-736 [PMID: 11025664 DOI: 10.1038/35036365]

85 **Bischof O**, Kirsh O, Pearson M, Itahana K, Pelicci PG, Dejean A. Deconstructing PML-induced premature senescence. *EMBO J* 2002; **21**: 3358-3369 [PMID: 12093737 DOI: 10.1093/emboj/cdf341]

86 **Salomoni P**, Pandolfi PP. The role of PML in tumor suppression. *Cell* 2002; **108**: 165-170 [PMID: 11832207]

87 **Carvalho T**, Seeler JS, Ohman K, Jordan P, Pettersson U, Akusjärvi G, Carmo-Fonseca M, Dejean A. Targeting of adenovirus E1A and E4-ORF3 proteins to nuclear matrix-associated PML bodies. *J Cell Biol* 1995; **131**: 45-56 [PMID: 7559785]

88 **Everett RD**. DNA viruses and viral proteins that interact with PML nuclear bodies. *Oncogene* 2001; **20**: 7266-7273 [PMID: 11704855 DOI: 10.1038/sj.onc.1204759]

89 **Maul GG**, Guldner HH, Spivack JG. Modification of discrete nuclear domains induced by herpes simplex virus type 1 immediate early gene 1 product (ICP0). *J Gen Virol* 1993; **74** (Pt 12): 2679-2690 [PMID: 8277273 DOI: 10.1099/0022-1317-74-12-2679]

90 **Szekely L**, Pokrovskaja K, Jiang WQ, de The H, Ringertz N, Klein G. The Epstein-Barr virus-encoded nuclear antigen EBNA-5 accumulates in PML-containing bodies. *J Virol* 1996; **70**: 2562-2568 [PMID: 8642686]

91 **Negorev DG**, Vladimirova OV, Maul GG. Differential functions of interferon-upregulated Sp100 isoforms: herpes simplex virus type 1 promoter-based immediate-early gene suppression and PML protection from ICP0-mediated degradation. *J Virol* 2009; **83**: 5168-5180 [PMID: 19279115 DOI: 10.1128/JVI.02083-08]

92 **Regad T**, Chelbi-Alix MK. Role and fate of PML nuclear bodies in response to interferon and viral infections. *Oncogene* 2001; **20**: 7274-7286 [PMID: 11704856 DOI: 10.1038/sj.onc.1204854]

93 **Ahn JH**, Hayward GS. The major immediate-early proteins IE1 and IE2 of human cytomegalovirus colocalize with and disrupt PML-associated nuclear bodies at very early times in infected permissive cells. *J Virol* 1997; **71**: 4599-4613 [PMID: 9151854]

94 **Kelly C**, Van Driel R, Wilkinson GW. Disruption of PML-associated nuclear bodies during human cytomegalovirus infection. *J Gen Virol* 1995; **76** (Pt 11): 2887-2893 [PMID: 7595400 DOI: 10.1099/0022-1317-76-11-2887]

95 **Kim YE**, Lee JH, Kim ET, Shin HJ, Gu SY, Seol HS, Ling PD, Lee CH, Ahn JH. Human cytomegalovirus infection causes degradation of Sp100 proteins that suppress viral gene expression. *J Virol* 2011; **85**: 11928-11937 [PMID: 21880768 DOI: 10.1128/JVI.00758-11]

96 **Tavalai N**, Adler M, Scherer M, Riedl Y, Stamminger T. Evidence for a dual antiviral role of the major nuclear domain 10 component Sp100 during the immediate-early and late phases of the human cytomegalovirus replication cycle. *J Virol* 2011; **85**: 9447-9458 [PMID: 21734036 DOI: 10.1128/JVI.00870-11]

97 **Korioth F**, Maul GG, Plachter B, Stamminger T, Frey J. The nuclear domain 10 (ND10) is disrupted by the human cytomegalovirus gene product IE1. *Exp Cell Res* 1996; **229**: 155-158 [PMID: 8940259 DOI: 10.1006/excr.1996.0353]

98 **Tavalai N**, Papior P, Rechter S, Leis M, Stamminger T. Evidence for a role of the cellular ND10 protein PML in mediating intrinsic immunity against human cytomegalovirus infections. *J Virol* 2006; **80**: 8006-8018 [PMID: 16873257 DOI: 10.1128/JVI.00743-06]

99 **Glass M**, Everett RD. Components of promyelocytic leukemia nuclear bodies (ND10) act cooperatively to repress herpesvirus infection. *J Virol* 2013; **87**: 2174-2185 [PMID: 23221561 DOI: 10.1128/JVI.02950-12]

100 **Tavalai N**, Stamminger T. New insights into the role of the subnuclear structure ND10 for viral infection. *Biochim Biophys Acta* 2008; **1783**: 2207-2221 [PMID: 18775455 DOI: 10.1016/j.bbamcr.2008.08.004]

101 **Tavalai N**, Papior P, Rechter S, Stamminger T. Nuclear domain 10 components promyelocytic leukemia protein and hDaxx independently contribute to an intrinsic antiviral defense against human cytomegalovirus infection. *J Virol* 2008; **82**: 126-137 [PMID: 17942542 DOI: 10.1128/JVI.01685-07]

102 **Adler M**, Tavalai N, Müller R, Stamminger T. Human cytomegalovirus immediate-early gene expression is restricted by the nuclear domain 10 component Sp100. *J Gen Virol* 2011; **92**: 1532-1538 [PMID: 21471311 DOI: 10.1099/vir.0.030981-0]

103 **Everett RD**, Chelbi-Alix MK. PML and PML nuclear bodies: implications in antiviral defence. *Biochimie* 2007; **89**: 819-830 [PMID: 17343971 DOI: 10.1016/j.biochi.2007.01.004]

104 **Lukashchuk V**, McFarlane S, Everett RD, Preston CM. Human cytomegalovirus protein pp71 displaces the chromatin-associated factor ATRX from nuclear domain 10 at early stages of infection. *J Virol* 2008; **82**: 12543-12554 [PMID: 18922870 DOI: 10.1128/JVI.01215-08]

105 **Woodhall DL**, Groves IJ, Reeves MB, Wilkinson G, Sinclair JH. Human Daxx-mediated repression of human cytomegalovirus gene expression correlates with a repressive chromatin structure around the major immediate early promoter. *J Biol Chem* 2006; **281**: 37652-37660 [PMID: 17035242 DOI: 10.1074/jbc.M604273200]

106 **Preston CM**, Nicholl MJ. Role of the cellular protein hDaxx in human cytomegalovirus immediate-early gene expression. *J Gen Virol* 2006; **87**: 1113-1121 [PMID: 16603511 DOI: 10.1099/vir.0.81566-0]

107 **Reeves M**, Woodhall D, Compton T, Sinclair J. Human cytomegalovirus IE72 protein interacts with the transcriptional repressor hDaxx to regulate LUNA gene expression during lytic infection. *J Virol* 2010; **84**: 7185-7194 [PMID: 20444888 DOI: 10.1128/JVI.02231-09]

108 **Kim EJ**, Park JI, Nelkin BD. IFI16 is an essential mediator of growth inhibition, but not differentiation, induced by the leukemia inhibitory factor/JAK/STAT pathway in medullary thyroid

carcinoma cells. *J Biol Chem* 2005; **280**: 4913-4920 [PMID: 15572361 DOI: 10.1074/jbc.M410542200]

109 **Shin HJ**, Kim YE, Kim ET, Ahn JH. The chromatin-tethering domain of human cytomegalovirus immediate-early (IE) 1 mediates associations of IE1, PML and STAT2 with mitotic chromosomes, but is not essential for viral replication. *J Gen Virol* 2012; **93**: 716-721 [PMID: 22158879 DOI: 10.1099/vir.0.037986-0]

110 **Saffert RT**, Kalejta RF. Inactivating a cellular intrinsic immune defense mediated by Daxx is the mechanism through which the human cytomegalovirus pp71 protein stimulates viral immediate-early gene expression. *J Virol* 2006; **80**: 3863-3871 [PMID: 16571803 DOI: 10.1128/JVI.80.8.3863-3871.2006]

111 **Wagenknecht N**, Reuter N, Scherer M, Reichel A, Müller R, Stamminger T. Contribution of the Major ND10 Proteins PML, hDaxx and Sp100 to the Regulation of Human Cytomegalovirus Latency and Lytic Replication in the Monocytic Cell Line THP-1. *Viruses* 2015; **7**: 2884-2907 [PMID: 26057166 DOI: 10.3390/v7062751]

112 **Hofmann H**, Sindre H, Stamminger T. Functional interaction between the pp71 protein of human cytomegalovirus and the PML-interacting protein human Daxx. *J Virol* 2002; **76**: 5769-5783 [PMID: 11992005]

113 **Cantrell SR**, Bresnahan WA. Interaction between the human cytomegalovirus UL82 gene product (pp71) and hDaxx regulates immediate-early gene expression and viral replication. *J Virol* 2005; **79**: 7792-7802 [PMID: 15919932 DOI: 10.1128/JVI.79.12.7792-7802.2005]

114 **Greaves RF**, Mocarski ES. Defective growth correlates with reduced accumulation of a viral DNA replication protein after low-multiplicity infection by a human cytomegalovirus ie1 mutant. *J Virol* 1998; **72**: 366-379 [PMID: 9420235]

115 **Gawn JM**, Greaves RF. Absence of IE1 p72 protein function during low-multiplicity infection by human cytomegalovirus results in a broad block to viral delayed-early gene expression. *J Virol* 2002; **76**: 4441-4455 [PMID: 11932411]

116 **Xu Y**, Ahn JH, Cheng M, apRhys CM, Chiou CJ, Zong J, Matunis MJ, Hayward GS. Proteasome-independent disruption of PML oncogenic domains (PODs), but not covalent modification by SUMO-1, is required for human cytomegalovirus immediate-early protein IE1 to inhibit PML-mediated transcriptional repression. *J Virol* 2001; **75**: 10683-10695 [PMID: 11602710 DOI: 10.1128/JVI.75.22.10683-10695.2001]

117 **Lee SH**, Albright ER, Lee JH, Jacobs D, Kalejta RF. Cellular defense against latent colonization foiled by human cytomegalovirus UL138 protein. *Sci Adv* 2015; **1**: e1501164 [PMID: 26702450 DOI: 10.1126/sciadv.1501164]

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REVIEW

Microbiology laboratory and the management of mother-child varicella-zoster virus infection

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Abstract

Varicella-zoster virus, which is responsible for varicella (chickenpox) and herpes zoster (shingles), is ubiquitous and causes an acute infection among children, especially those aged less than six years. As 90% of adults have had varicella in childhood, it is unusual to encounter an infected pregnant woman but, if the disease does appear, it can lead to complications for both the mother and fetus or newborn. The major maternal complications include pneumonia, which can lead to death if not treated. If the virus passes to the fetus, congenital varicella syndrome, neonatal varicella (particularly serious if maternal rash appears in the days immediately before or after childbirth) or herpes zoster in the early years of life may occur depending on the time of infection. A Microbiology laboratory can help in the diagnosis and management of mother-child infection at four main times: (1) when a pregnant woman has been exposed to varicella or herpes zoster, a prompt search for specific antibodies can determine whether she is susceptible to, or protected against infection; (2) when a pregnant woman develops clinical symptoms consistent with varicella, the diagnosis is usually clinical, but a laboratory can be crucial if the symptoms are doubtful or otherwise unclear (atypical patterns in immunocompromised subjects, patients with post-vaccination varicella, or subjects who have received immunoglobulins), or if there is a need for a differential diagnosis between varicella and other types of dermatoses with vesicle formation; (3) when a prenatal diagnosis of uterine infection is required in order to detect cases of congenital varicella syndrome after the onset of varicella in the mother; and (4) when the baby is born and it is necessary to confirm a diagnosis of varicella (and its complications), make a differential diagnosis between varicella and other diseases with similar symptoms, or confirm a causal relationship between maternal varicella and malformations in a newborn.

Key words: Mother-child infection; Congenital varicella syndrome; Varicella-zoster virus; Neonatal varicella; Microbiology laboratory

Core tip: Although varicella during pregnancy is infrequent and congenital varicella syndrome (CVS) is rare, every available means should be used to prevent and diagnose them. Microbiology laboratories can be crucial in these situations: Evaluating a mother's immune status with sensitive and specific tests for the detection of antibodies; allowing a rapid diagnosis with molecular biology tests when a clinical manifestation may be due to different etiologies; following pregnant women with varicella for the prenatal diagnosis of CVS with close collaboration between molecular biology investigators and specialists in imaging diagnostics.

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INTRODUCTION

Clinical features of varicella-zoster virus

Varicella-zoster virus (VZV) is responsible for varicella (chickenpox) and herpes zoster (shingles). Varicella is typically a childhood disease. Children experience a slight fever, fatigue and the appearance of typical vesicles on the skin[1]. Except in children aged < 1 year, complications are rare (2%-6%), but include *Staphylococcus* and *Streptococcus* super-infections of the lower and upper respiratory tract (pneumonia is more frequent in children aged < 1 year), conjunctivitis, corneal infections, meningo-encephalitis and occasionally death[2-5]. The disease is infrequent in immunocompetent children and adults, but complications are 25-40 times more frequent than in infants, probably because of a lower cell-mediated immune response than children[5-9]. These complications include a high fever, hepatitis, encephalitis, and especially viral or bacterial pneumonia (the latter in 10%-20% of cases), and the mortality rate without treatment may be as high as 20%-45%[10-12]. In general, adult varicella accounts for only 5%-7% of the total number of reported cases, but the mortality rate is about 35%[5]. Furthermore, 36% of immunocompromised subjects may experience serious and deadly disease with visceral dissemination and other complications (pneumonia, meningo-encephalitis)[5,13-15].

The infection may be transmitted by air (inhalation of the virus from respiratory tract secretions or vesicular fluid), fomites (*e.g.*, skin cells, hair, clothing, and bedding), or direct contact[16-18]. The incubation period is usually 14-16 d (range 10-21 d), but may be up to 28 d in subjects treated with immunoglobulins, and even longer in immunocompromised subjects[5,9,19]. The virus enters the body through various mucous membranes (the nasopharynx, conjunctiva) and passes into regional lymph nodes where it replicates[9,20]. Primary viremia occurs 4-6 d after infection, with dissemination to, and replication in other organs (liver, spleen, the sensory ganglia); secondary viremia appears after about 14 d (range 10-21 d) and is expressed in the form of a skin infection and the characteristic rash[5,21,22]. The macules quickly progress to papules and vesicles, and then to scabs. The lesions appear at various stages of development at the same time, and have a central distribution, mainly on the trunk and face, and less on the limbs[5,7]. The vesicles contain many viruses, and may therefore be the most important route of transmission[23-25]. Patients are usually infectious from two days before the rash until the formation of scabs generally five days after[26,27].

Herpes zoster (shingles) is caused by virus reactivation years after the first infection, during which the virus migrates to the sensory nerve ganglia of the dorsal root and establishes latent infection in neuronal cells[13,28]. Its reactivation years or decades later causes the reappearance of the lytic infection in up to 15%-30% of the population[29-33]. The virus spreads unilaterally along the dermatomes and produces vesicles confined to a single dermatome of the skin, giving rise to a particularly painful rash[34-36]. Post-herpetic neuralgia usually lasts 2-3 wk but, in some cases, it can last for months or even years after the rash has disappeared[9,37].

Reactivation is caused by a decline in cell-mediated immunity (CMI) (particularly in specific T lymphocytes), or immunosuppression due to diseases, transplantations or medical therapies[9,18,38], although other possible predisposing factors may be considered as gender, seasonality, race, stress, exposure to immunotoxic substances, trauma and genetic susceptibility[39,40]. It is more frequent in the elderly (50% of people aged > 85 years of age experience episodes of herpes), and in subjects with CMI disorders (lymphoproliferative cancers, organ transplantation, AIDS, *etc.*)[18,41,42].

The virus

VZV or human herpesvirus 3 is a DNA virus belonging to the order *Herpesvirales*, family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genera *Varicellovirus*[43]. It is exclusively human, and has a diameter of about 175 nm[9,44]. Of the seven known genotypes (E1, E2, J, M1, M2, M3, M4), five are phylogenetically circulating groups, and two (M1 and M2) are probably recombinants of E1 and J[30].

The genome consists of a unique long region of 107836 bp flanked by inverted repeat regions (the long terminal repeat and internal repeat long and a unique short region flanked by repeated internal regions (the short terminal repeat and inverted repeat short)[45].

The DNA contains 70 genes that are expressed sequentially during the lytic cycle with the production of immediate early non-structural and late structural proteins[45,46]. The structural proteins form an icosahedral capsid (162 capsomeres) containing the DNA, linked to the outer lipoprotein envelope by a tegument (amor-

phous-protein structure)[9,47]. The envelope is made up of glycoproteins (gB, gE, gH, gI, gK) and is important in virion attachment, entry, envelopment, cell-to-cell spread and egress[48]. After entering a cell, the virus replicates in the nucleus where the pre-formed DNA is incorporated into capsids that leave the nucleus through the nuclear membrane. After passing the inner nuclear membrane, a first enveloped virus is formed in the perinuclear space and, by means of the fusion of this envelope with the outer leaflet of the nuclear membrane, the nucleocapsid is released into the cytoplasm. The nucleocapsids are re-wrapped in the Golgi complex, and the mature virions are released into the outer space after the fusion of the vesicle membranes with the cell membrane[49]. Unenveloped virions can also pass from cell to cell, thus contributing to the spread of the virus, for example in the skin[9,40]. The latency phase is characterised by the expression of only a small number of proteins, and the resumption of the lytic cycle after latency leads to the manifestation of herpes zoster[40,50].

Natural immunity

Natural infection induces a long-lasting immunity that is maintained and enhanced by internal reactivation and/or external boosting after exposure to VZV[9,34,51,52]. A person who has had varicella is usually protected against the disease unless he or she is immunosuppressed[5,9,53,54]. People with a history of varicella who are re-exposed to the virus may develop a new infection but it is usually asymptomatic and can only be detected on the basis of an increase in antibody titres[5,55,56], but it may sometimes be mildly symptomatic in the case of a failure to develop or maintain cell memory (leading to a reduction in specific T lymphocytes), or if the viral load is too high[57,58]. Some studies have reported 4.5%-13% of symptomatic cases in children with a reported previous history of varicella, and there have also been cases of varicella during pregnancy in the presence of weak antibody positivity[58-63].

Humoral immunity does not prevent latent viral infection or subsequent reactivation in the form of herpes zoster, but CMI is important to contain the virus because the virus spreads through the human body intracellularly[9,64-67]. If CMI is working correctly, the absence of antibodies after infection does not automatically imply susceptibility to re-infection, but a poor or absent response increases incidence of herpes zoster even in the presence of antibodies as has been reported in the elderly[9,34,68,69]. Finally, in addition to antibody-dependent cellular cytotoxicity, natural killer cell cytotoxicity is also important[70].

Humoral immunity is expressed as the production of antibodies against components of the capsomere and surface glycoproteins[71-78]. The antibodies against the surface glycoprotein components (anti-gE, anti-gB, anti-gI and anti-gH) are particularly neutralising[79-81].

In the acute phase, IgM antibodies appear 1-7 d after the rash, peak after 14 d (range 7-30), and generally disappear during convalescence, although they may persist for several months[82-84]. They may also be found during re-infection and reactivation, appearing 8-10 d after the rash and peaking after 18-19 d in 50% of herpes zoster cases[30,82,85]. However, IgM is not always found in cases of full-blown varicella and so their absence does not indicate the absence of infection[30].

IgA antibodies have the same trend as IgM and, in the case of re-exposure or reactivation, reappear (or increase) in 50%-99% of cases of herpes zoster[83,85,86].

IgG antibodies appear about 9-10 d after the beginning of the rash, peak after about 60-70 d, and then decrease to lower levels for the rest of the infected subject's lifetime[82,87]. External boosters (contacts with exogenous virus) or internal reactivation can increase their titres over time[9,34,51,52].

Epidemiology

Varicella is a ubiquitous disease: In countries with a temperate climate such as North America, European countries, more than 90% of the population contract the infection before the age of 15 years[54,88-93]. It mainly affects children with especially winter/spring seasonality: It is estimated that 52%-78% of all reported cases of varicella occur in children aged < 6 years, and 89%-96% of cases in children aged < 14 years; only 5%-7% of cases occur in adults[5,94,95]. The population incidence is about 1300-1600 cases per 100000 inhabitants per year[87], but varies with geographical location: It is 300-1291 cases per 100000 in Western Europe[96-98], 164-1240 cases per 100000 in Southern Europe[99-111], 350 cases per 100000 in Eastern Europe[112,113], and 1500-1600 cases per 100000 in the United States (pre-vaccine era)[114]. The reported mortality rate is 0.04-0.06 per 100000 inhabitants, and 2-4 per 100000 cases of varicella, including 35% of adult cases[5,30,115-121].

Epidemiology is different in tropical countries, where there is no marked seasonality[5]. The infection is acquired in childhood less than in temperate countries, with only 25%-85% of subjects contracting primary infection before the age of 15 years[122-131]. Various hypotheses have been postulated to explain this difference, including viral inactivation due to high ambient temperatures, race, interference from other more prevalent viruses and a lack of exposure due to the rural living conditions in the tropics, where VZV does not circulate very much[123,132-134]. Advanced age of infection and the severity of adult disease may be responsible for the increased morbidity and mortality due to varicella and complications in these areas[59,135-137].

However, epidemiology in industrialised countries is changing because of the increase in immigration and/or greater vaccination coverage insofar as the incidence of infection has decreased by 57%-95% in all age groups in the places in which vaccination is widespread[5,138-140].

As regards the herpes zoster, the reported annual incidence is 120-480 per 100000 inhabitants (all ages), and 720-1180 per 100000/year in people aged > 60 years[18,27,141,142].

Consequences of an infection contracted during pregnancy

As varicella is a widespread disease, especially among children, the global risk of exposure during pregnancy has been estimated to be 12%-24%[54,56,143]. Fortunately, as most adults in temperate countries are already protected, the possibility of contracting varicella during pregnancy among susceptible women is much lower, and the estimated incidence of varicella is 0.1-3 per 1000 pregnancies[8,26,90,117,144-150].

Contracting varicella during pregnancy can be very serious for a mother, especially if it is contracted in the third trimester[10,151]. In fact varicella can be more severe in all adults than in children as it may lead to complications such as meningoencephalitis, hepatitis and, especially, pneumonia[16,152]. The incidence of pneumonia during pregnancy does not seem to be higher than in non-pregnant adults, but it is more severe in pregnant than in non-pregnant women especially if it appears in the third trimester of pregnancy[6,10,19,26,56,121,144,153-157]. Pneumonia can appear in 5%-20% of pregnant women[54,121,152,158] and leads to death in 20%-45% of cases unless appropriate treatment is started, but with appropriate treatment and better breathing management, the mortality rate drops to 0%-14%[10,16,152-154,157,159-163].

During pregnancy, the infection can also be transmitted to the fetus and, depending on the time of transmission, may cause congenital varicella syndrome (CVS), neonatal varicella, or childhood herpes zoster. It has been estimated that transmission from mother to fetus occurs in 8%-24% of cases[56,90,145,164]. Early studies using IgM in the newborn as a sign of infection (now considered a very insensitive marker) indicated transmission rates of 5% in the first trimester, 10% in the second, and 25% in the third[145].

The crossing of the placental barrier seems to occur both viremic phases of incubation, but secondary viremia seems to play a greater role in fetal transmission[21,22,26]. Furthermore, infection arising from a lesioned birth canal can cause intrauterine infection[165].

CVS: The characteristic defects include skin scarring with a dermatomeric distribution; eye defects (microphthalmia, chorioretinitis, corneal alterations, cataracts); limb hypoplasia with muscle hypoplasia; neurological abnormalities (microcephaly, cortical atrophy, mental retardation or bowel and bladder sphincter dysfunction, cerebral calcifications); and (less frequently) ear, cardiac, gastrointestinal and genitourinary abnormalities, slow fetal growth and a pre-term delivery[26,145,166-168].

It has been reported that the mortality rate during the first month of life is 30%, but infants who survive this period can have a good long-term outcome even though there is a 15% risk of developing herpes zoster between the fourth and forty-first month of life[26,53,145,147,159,169,170].

CVS is probably due to herpes zoster-like reactivation *in utero* rather than initial varicella, and this view is supported by the fact that the skin lesions have a dermatomal distribution similar to that of herpes zoster[26,167,171]. The short period between primary infection and reactivation may be due to an immature VZV-specific cell-mediated immune response in the fetus[172]. As 65%-85% of the infants born with CVS are female, it has been hypothesised that there is a higher rate of fetal death among males[26,145,147,168,171].

It has been estimated that 8%-25% of the cases of viral transmission to the fetus occur during the first two trimesters of pregnancy, but only 12% of these cases actually develop CVS, and so the incidence of CVS in different studies ranges from 0% to 2.63%[53,56,145,152,164,166,173-178]. A meta-analysis of studies published between 1986 and 2002 calculated a total incidence of 0.70% (0.55% in the first trimester, 1.4% in the second, and 0% in the third)[26]. The most important period of transmission is between the fifth and twenty-eighth week of pregnancy, particularly up to the twentieth week, when the incidence of 0.91%[26,145,168,179]. Cases of CVS are rare between the twenty-first and twenty-eighth week, and are mainly described in individual case reports[152,179-186]. The probability of observing cases before 3-5 wk and after 28 wk of gestation is practically zero (in the latter case because of fetal maturation)[26,145,159]. Considering an average risk of two cases per 1000 pregnancies, the number if expected cases per year was estimated to be 41 in the United States, four in Canada, 2-10 in the United Kingdom, and seven in Germany[26,54,90,187,188].

The risk of a miscarriage is debated in the literature: Some authors have indicated a 3% risk during the first trimester, and an 8% risk during the second, whereas others have found that the risk does not seem to be any greater than the risk in uninfected pregnant women[16,145,159,166,173,176,189,190].

Neonatal varicella: In the case of mothers who contract varicella in the four weeks before birth, there is a 50% possibility of infection in the newborns, 20%-30% of whom will actually develop the disease[16,191]. If the maternal varicella appears 20-7 d before the birth, the varicella in the newborn (if it develops) generally has a benign and non-fatal course because newly born infants have their mothers' antibodies and the risk of complications is low[5,26,131,165,191-193].

In the case of maternal varicella between seven days before and seven after delivery, 2/3 of the infants become infected and 50% have severe symptoms because they do not have maternal antibodies and their cell-mediated response is insufficiently mature[26,145,169,191,194,195]. During this period, the most dangerous situation is when maternal varicella appears between five days before and two days after delivery because, in the absence of adequate therapy, the neonatal mortality rate may be as high as 20%-30% and, even with therapy, may still be 7%[5,153,157,159,165,191,196-198].

Maternal varicella may be transmitted transplacentally, or by means of ascending infection during birth, or by means of respiratory droplets or direct contact with infectious lesions after birth, and neonatal disease may manifest itself with skin lesions, ulcerated

necrotic or hemorrhagic lesions, and/or systemic disease (pneumonia, liver failure, encephalitis or coagulopathy)[16,26,53,159,165]. As the incubation period of varicella transmitted *in utero* from the initial maternal rash is 10-12 d (but may be as short as four days), the varicella observed in infants in the first 10-12 d of life is considered of intra-uterine origin, and that appearing later as probably post-natal[53,90,145,159,165].

In conclusion, if the newborn has passively acquired maternal antibodies, post-natal varicella contracted from the mother or from people other than the mother is rare and more benign[193]. However, if the baby is premature (< 28 wk or < 1000 g), he or she is still at high risk in the first six weeks of life because of the non-acquisition of maternal antibodies as a result of the reduced period of gestation[131,192,199]. Consequently, in the case of planned childbirth, it is advisable to avoid delivery during the 5-7 d after the onset of the rash in order to allow the passive transmission of antibodies[16,200,201].

Herpes zoster in the first years of life: Maternal varicella occurring after the twenty-fourth week of gestation leads to asymptomatic fetal seroconversion and the birth of an asymptomatic newborn; however, herpes zoster may develop in the first years of life (especially in the first or second year) because of viral reactivation[202]. The short latency period is due to the infant's immature cell-mediated response[203]. The course of the disease is normally uncomplicated, but it is recommended to check children with herpes zoster for other clinical signs of intra-uterine infection especially at ophthalmological signs[147,203].

The incidence of herpes zoster is 4%-20% in the case of documented *in utero* infection but, if this is not the case and the mother had varicella during the pregnancy, the incidence decreases to 0.8%-1.7% depending on whether the maternal varicella appeared in the second or third trimester[145,159,164,165,203].

Herpes zoster may also occur after 2-41 mo of life in 15% of newborns with CVS if the onset of maternal varicella was between the eighth and twenty-fourth week of gestation[147].

Prevention and treatment

Vaccination: The first live attenuated vaccine contained wild-type OKA VZV cultivated human embryonic lung fibroblasts (WI-38) and propagated embryonic guinea pig fibroblasts[204,205], and has been used in the United States since March 1995. Commercial versions all use the OKA strain but differ in terms of the number of passages in guinea pig and human cells (with additional passages in MRC5 cells), the viral load in each dose, excipients and other patented aspects[9]. It is currently available as a vaccine to be administered alone, or in a quadrivalent vaccine against measles, mumps, rubella and varicella (MMRV)[5,206].

It has been shown that a single dose is effective in preventing varicella in 80%-85% of cases and severe forms in 95%-100%, especially in children aged < 10 years of age, but also in 74% of adults[5,30,206-209]; vaccinated subjects are also at lower risk of developing herpes zoster[115,210]. The vaccination was initially administered as a single dose and, as it was not 100% effective, failed to protect about 15% of treated subjects[211-214]. There have been reports of cases of breakthrough varicella (BV) in 10% of vaccinated healthcare workers and 15%-20% of vaccinated children after one dose due to primary (no take) or secondary vaccine failure (immune response decreasing over time), and that 30% of vaccinated subjects gradually lose antibodies after the first dose[5,9,30,215,216].

BV occurs more than 42 d after vaccination and is due to wild-type virus[217]. Its symptoms are milder than those of classic varicella as there is no fever and < 50 skin lesions, which take the form of papules and tend not to progress to vesicles[5,218-220]. Classic varicella is associated with 250-500 lesions, but one study found that 56% of the patients with BV had < 50 lesions, 33% had 50-300 lesions, and only 11% had > 300 lesions[5,221]. These occurrences of BV led to the indication to administer two doses but, although the second dose has minimised primary vaccination failure, BV may still occur[5,206,222,223].

Vaccination induces a humoral immune response with the production of antibodies that appear after 3-5 wk, a cell-mediated response that appears after four days in 50% of vaccinated subjects[224]. The immunity persists for at least 20 years, thus providing long-term protection, and the subjects who remain seronegative after vaccination still have the chance of acquiring CMI[9,30,165,225-228].

The rate of seroconversion in healthy children is about 87%-100% after the first dose and 97%-100% after two doses[5,9] and consequently, antibody testing is not considered necessary after two doses[5]. Vaccination is generally stronger in children[229,230]: In adults, 78% respond after the first dose and 94%-99% after the second[5]. As it is a live attenuated vaccine, vaccination is not recommended in immunocompromised patients, but may be considered in some categories of patients (patients with human immunodeficiency virus infection and normal CD4 cell counts, patients with leukemia, candidates for transplantation, *etc.*)[5,88,231].

Antibody titres gradually decline after the initial peak, but can be restored by an external booster[30,226]. Like the wild-type virus, the vaccine virus may also become latent and then be reactivated to cause herpes zoster in both healthy and immunosuppressed subjects[30]. However, the risk is lower and the symptoms are milder and without complications[5,232], probably because the attenuated vaccine virus is less likely to be reactivated and cause the a rash that allows the virus to travel to the dorsal root and establish latency[25]. Nevertheless, herpes zoster is more frequent if there is a post-vaccination rash[233], which may occur in 5% of the subjects receiving the first dose, and in 1% after the second[5]. It must also be borne in mind that herpes zoster in vaccinated subjects may be due to the vaccine virus or

the wild-type virus, and there also the possibility of their recombination[5,30,221,234,235].

The vaccine may also be useful in preventing varicella if administered within 3-5 d of exposure to infection due to contact, even if not entirely prevents it[5,236-238]. However, as it is a live attenuated vaccine, it is not recommended for pregnant women exposed to infection[19]. Furthermore, pregnancy should be avoided for at least one month[199,206,239,240], although it may be offered to susceptible women before conception or *post partum*, and it is not contraindicated during breast-feeding[5,169,241].

Finally, a vaccine against herpes zoster that contains a higher titre of the OKA strain used in the MMRV vaccine has been approved for subjects aged > 50 years, and is recommended for those aged > 60 years[5,242].

Immunoglobulins: It has been found that, in order to prevent the most serious consequences of infection in susceptible pregnant women, immunoglobulins (VZIGs) can be administered within 72-96 h of exposure (within 10 d according to the United Kingdom guidelines)[16,145,188,199,206,243-249]. The difference in the timing has been attributed to the different formulation of the VZIGs[21]. The protection lasts for about three weeks, and any further exposure requires additional administrations[16,19,199,250].

The rationale underlying the administration of VZIGs is that it can prevent or mitigate varicella by as much as 75%, although some authors insist that it mitigates rather than prevents varicella[16,145,159,165,206,245,251-253]. It has been reported that 50% of the mothers treated with VZIGs present an uncomplicated or mildly evolving form of varicella, and 5%-25% a sub-clinical form[8,54,90,253]. Fetal transmission also seems to be reduced[254]. As the virus crosses the placenta during the two viremic phases of incubation, it has been suggested that passive prophylaxis may be effective if administered before the first[21,22,26]. It has been reported that ZVIGs can reduce intra-uterine transmission from 12.3% to 1.1%, and reduce or practically eliminate the risk of developing CVS[16,54,90,145,164,254]. There are in fact very few published cases of CVS[166], but it is unclear whether VZIGs prevent CVS because they prevent fetal viremia or prevent CVS even in the presence of fetal viremia, and so there is still some uncertainty concerning their real effectiveness in this regard[26,159]. It has also been pointed out that, as CVS is a rare disease, it is numerically and ethically difficult to carry out studies[54]. In any case, the guidelines indicate the administration of VZIGs after exposure at any time during pregnancy, and after childbirth if the delivery takes place within 10 d of exposure, but not after the appearance of the rash[188,199,255-257].

The administration of VZIGs to newborns is indicated if the mother had varicella symptoms between seven days before and seven days after childbirth, and especially between five days before and two days afterwards, but is probably not necessary if maternal varicella appeared before or after this period[5,16,165,174,247,258-260]. In general, the combined neonatal administration of VZIGs (and antiviral agents) alters the clinical course of the disease, but does not completely prevent it, and 50% of the infants may still experience clinical symptoms[87,191]; however, mortality is reduced (even if not entirely eliminated)[16,153,191,261,262].

VZIGs are also recommended in premature infants (born after 28 wk or later) who have been exposed in the neonatal period and have mothers without any evidence of immunity (*i.e.*, there has been no passive transmission of antibodies from the mother), and in those born before 28 wk (or weighing ⩾ 1000 g) who have been exposed neonatally regardless of the immune status of the mother (because the reduced gestational period means that they cannot have acquired maternal antibodies)[5,26,165,199,263,264].

However, once again, infant administration is not indicated if varicella has already appeared[261,262].

Therapy: The most widely used drug, acyclovir (and its precursor valacyclovir) is a synthetic nucleoside analogue of guanine that is highly specific for cells infected by herpesvirus and does not interfere with human DNA[169]. When phosphorylated by cellular enzymes (thymidine kinases), acyclovir triphosphate inhibits viral DNA synthesis by competing with deoxyguanosine triphosphate as a substrate for viral DNA polymerase. Incorporation of acyclovir triphosphate into viral DNA results in obligate chain termination. Viral DNA polymerase is tightly associated with the terminated DNA chain and is functionally inactivated[53]. Acyclovir crosses the placental barrier, and can be found in fetal tissues, cord blood and amniotic fluid[19].

It has been suggested that viral treatment after exposure is a means of preventing or mitigating the infection because, if administered within 24 h of the appearance of the rash, its use in immunocompromised children and immunocompetent adults reduces the symptoms[21,26,252,256,265,266]. Consequently, it has been suggested as prophylaxis in pregnant women (especially if VZIGs are not available)[169], particularly if administered within seven days of exposure and within 24 h of the onset of the rash[26,54,252,265,266]. The use of acyclovir during pregnancy has been widely debated in the literature: There is general consensus concerning its use when the mother's life is in danger and as a means of reducing the severity of complications occurring in late pregnancy[19,53,151,156,256,267], but its therapeutic and CVS prophylactic use before the twentieth week of gestation is more controversial; some authors favour its administration always, and others only after 20 wk[10,16,19,53,54,151,156,169,252,256,267-271].

As the virus crosses the placenta during both viremic phases of incubation, the second of which seems to play a greater role in fetal transmission, it has been suggested that secondary viremia can be prevented or minimised by acyclovir provided that it is not given too early[21,22,26,265]. However, there are still doubts as to whether inhibiting maternal viral replication can inhibit or limit trans-placental transmission, thus reducing fetal

mortality and morbidity[26,53,154,199,245,272,273]. It is important to note that the use of acyclovir during pregnancy does not seem to increase the risk of fetal malformations, and that international registries show that the incidence of embryopathies in women treated during the first trimester of pregnancy is equal to that in the general population[16,269,274-277].

Finally the use of acyclovir in infants with varicella can reduce the severity of the disease and the complications[153,259,278,279]. The are also a few case reports indicating that it can block the ophthalmic and neurological progression CVS in newborns[279,280].

THE ROLE OF THE MICROBIOLOGY LABORATORY

Microbiology laboratories play an essential role in relation to the general management of four aspects of maternal/infantile VZV infection: Determining immune status after VZV exposure; diagnosing varicella in pregnant women; prenatally diagnosing intrauterine VZV infection and/or CVS; and diagnosing neonatal infection.

Anamnesis

In the case that a pregnant woman comes into contact with someone (usually a child) affected by varicella, the first step is to record her medical history as this may be important to establish the risk that she will contract the infection. The main points to consider are the risk of transmission, which depends on the source of infection and the immune status of the pregnant woman.

Risk of transmission: This depends on whether the source of exposure is someone with varicella, someone with herpes zoster, someone with BV (due to wild-type virus), or someone with post-vaccination rash (due to the OKA strain).

In subjects with varicella, the horizontal transmission rate is 61%-100% of susceptible contacts[5,34,281]. Infection can occur 1-2 d before the appearance of vesicles, lasts for 5-7 d after the onset of the rash, and continues to be infectious until the vesicles are crusted over[1,26,282]. The virus is transmitted by means of direct contact or through the air (respiratory tract secretions or the inhalation of aerosols from the vesicular fluid of the skin lesions)[5]. Infection normally occurs as a result of close face-to-face contact or simultaneous presence in the same room for 15-60 min[16,19]: Most authors believe 15 min are sufficient, whereas others specify 5 min of face-to-face contact and 15 min in the same room[16,159,165]. The risk of congenital varicella in a mother who develops varicella during pregnancy has been described above. Transmission due to asymptomatic infection or re-infection has been hypothesised, but not convincingly documented[169,283].

In subjects with herpes zoster, the horizontal transmission rate is 16% of susceptible contacts[284]. Transmission primarily arises from the exposed skin lesions of immunocompromised subjects or subjects with disseminated zoster, and is rare if the lesions are not exposed[16,17,285-289]. The risk of fetal infection is virtually zero: Localised maternal zoster (the few published data estimate 0.15-2 cases of herpes/1000 pregnancies[90,147,290,291]) does not seem to be associated with fetal or *in utero* infection, nor with post-natal infection because newborns are protected by passively transmitted maternal antibodies[56,90,145,147,166,173,191]. There are published case reports of fetal malformations in mothers affected by localised zoster during pregnancy but without any laboratory evidence of intra-uterine infection[159,290,292]. Instead one case of a confirmed VZV-infected newborn with CVS, whose mother had disseminated herpes zoster after 12 wk of pregnancy, was reported[171].

In subjects with BV, the disease is milder, fever free, and with fewer skin lesions, which are generally atypical with papules that tend not to progress to vesicles, and so the subjects are not considered infectious until new lesions appear[30,218]. Transmission affects 12%-37% of susceptible contacts but, if the number of lesions is > 50, the wild virus transmission rate is the same as that of classic varicella in unvaccinated subjects[293,294]. In the case of BV, the risk of fetal varicella is considered to lower than that in unvaccinated subjects, but the only data come from reports of cases without fetal consequences[159,295]. In any case, the prophylactic and therapeutic measures are the same as those for unvaccinated women[159,165].

In subjects with post-vaccination rash (OKA strain) as a complication of vaccination, the rash appears in 4%-6% of the subjects vaccinated with one dose, and 1% of those receiving the second dose[5]. Horizontal transmission is rare[16,206]: There are very few reported cases, and it only occurs in the presence of rash[5,206]. The contacts have asymptomatic infections (seroconversion) or only mild symptoms[5], but it is, important to check whether the varicella, that develops within 42 d of vaccination is due to the wild-type or vaccine virus. Anti-VZV vaccinations should be avoided in pregnant women because they involve a live attenuated vaccine, and vaccinated women should avoid pregnancy for at least one month, but no cases of fetal malformations have been reported in inadvertently vaccinated pregnant women[16,54,156,199,206,239,240,296,297]. There is one case of transmission from a vaccinated child to a pregnant woman without subsequent fetal infection[298]. Furthermore, no viral genome has been found in breast milk after vaccination, and there have been no reports of transmission through the breast milk of women vaccinated after giving birth, who can therefore continue to breastfeed[16,241,299].

Finally, no cases of transmission from patients vaccinated with zoster vaccine have been reported[5].

Immune status: An anamnesis can provide important information about a subject's immune status because a history of varicella is 90%-99% predictive of the presence

Table 1 Anti-varicella-zoster virus seroprevalence rates in women in some countries with a temperate climate

Country	Population	Age	Anti-VZV seroprevalence (%)	Ref.
United Kingdom	Pregnant women	Mean 28 yr	95.8	[301]
United Kingdom	Pregnant women	Mean 28 yr	94.8	[308]
Spain	Women	19-39 yr	92.3	[309]
Spain	Women	Not reported	95.3	[313]
Holland	Women	Mean 47 yr	93	[311]
Holland	Women	Mean 29 yr	100	[312]
Slovenia	Women of childbearing age	15-49 yr	97.2	[314]
Croatia	Women (12% pregnant)	Not reported	84.3	[315]
France	Women	Mean 30.4 yr	98.8	[316]
Germany	Pregnant women	Mean 28 yr	96.7	[317]
Finland	Pregnant women	Mean 30 yr	95	[318]
Italy	Women	15-39 yr	87.4	[321]
Italy	Pregnant women	15-49 yr	89.4	[310]

VZV: Varicella-zoster virus.

Table 2 Seroprevalence rates in some tropical countries

Country	Population	Seroprevalence (%)	Ref.
St.Lucia (Caribbean)	40-year-old	> 70	[123]
Tropical islands	Pregnant women	84	[131]
Bolivia	Women of child-bearing age	88.4	[322]
Singapore	15-24 yr	40	[95]
	25-34 yr	> 80	
India	17-20 yr	30	[130]
India	Adults (rural areas)	68.9	[134]
	Adults (urban areas)	96.6	
Iran	Pregnant women	78.5	[323]
Saudi Arabia	Pregnant women	74.4	[324]
Singapore	15-24 yr	40	[515]
	25-34 yr	> 80	

of antibodies, although the time between vaccination and exposure should be carefully considered as the antibodies appear 3-5 wk after vaccination[54,199,224,300-334].

If there is no reported history of varicella, antibodies are in any case present in 70%-90% of cases[54,302-306]. The seroprevalence rate is high in industrialised and/or temperate climate countries[99,150,301,307-321] (Table 1). However, a reported history of varicella is rather less predictive in women from tropical countries where the seroprevalence rate is lower[123,129-132,134,150,300,322-324] (Table 2).

The intense population movements of recent times should encourage a careful evaluation of the epidemiology of a woman's region of origin. Furthermore, a previous vaccination (preferably formally documented by a vaccination certificate) may be important for evaluating antibody protective: As 78% of adults respond to the first dose and 99% to the second, women who have received two doses are considered protected, an antibody search is not necessary, and immunoglobulins are not indicated[5,159]. Vaccination policies vary from country to country, and so it is necessary to consider a woman's country of origin in the case of recent immigration[325,326].

It has been estimated that at least 80% of women can be reassured after a careful history has been taken; the others need to be referred for laboratory tests[304].

Labotoristic determination of immune status

In the case of women of foreign origin or those with a negative history of varicella, as well as if there are doubts any kind, it is necessary to search for specific antibodies in order to determine immune status[8,16,139,151,159,247]. This should be done as soon as possible because the results of serological tests should be expected before 24-48 h and, if they are negative, VZIGs must be administered within 96 h[16,54,244,327]. The antibodies detected within 7-10 d of a contact with someone who is infected are considered as having been acquired before exposure[328].

It is possible to investigate both cell-mediated and humoral immunity. The tests that have been used to detect cell-mediated responses are ELISPOT[329-332], flow cytometry[333,334], hypersensitivity skin tests[306,335-337], and T cell cytokine response tests[338], but these have mainly been used to test the response to vaccination[294,339-341]. For example, a positive response to ELISPOT has been found in 90% of subjects one year after vaccination, and in 87% after five years[338]; the response is greater after two doses than after one dose[294,339,340]. A positive response to a hypersensitivity skin test has been found in 100% of subjects 5-7 wk after vaccination[337]. However, CMI tests cannot usually be used for routine purposes as they require special instruments and the time necessary is not compatible with a rapid response.

It is much quicker and easier to analyse antibody responses, and many tests have been used to predict disease susceptibility and the immune response to vaccination. These include complement fixation (CF), anti-complement immunofluorescence (ACIF), immune adherence hemagglutination (IAHA), passive hemagglutination (PHA), radioimmunoassays (RIAs), neutralisation tests (Nt), latex agglutination (LA), indirect fluorescent antibody tests (IFA), fluorescent antibody to membrane antigen (FAMA), time-resolved fluorescent immunoassays (TRFIAs), immunoblotting (IB), enzyme-

linked immunosorbent assays (ELISAs) or enzyme immunoassays (EIAs), glycoprotein ELISAs (gpELISAs), chemiluminescence immunoassays (CLIAs), and enzyme-linked fluorescent assays.

CF: One of the first tests was CF, but it is not very sensitive, difficult to automate, and can only be used to diagnose recent infection[342-349].

ACIF, IAHA, PHA, RIA: The sensitivity and specificity of tests such as ACIF[350], IAHA[351-356], PHA[357-359], and RIAs[344,360-363] have been compared with other methods with varying results[364], but they are generally impractical and difficult to use for routine testing. Although RIAs are more practical and sensitive[344,346,361], they are no longer usable because of the need for radioisotopes.

Nt: Nt measure the antibodies against virus glycoproteins, and can therefore highlight the ability to neutralise the infectiousness of the virus[342,343,365-375]. A preparation of free virus from cells is incubated with dilutions of the examined serum in order allow a virus/antibody interaction. Inoculation of the mixture in a cell indicator (MRC-5) allows the cytotoxic effect to develop (in the absence of neutralising antibodies) or not (in their presence). Sensitivity has been improved by adding guinea pig complement (C-enhanced neutralisation test) and/or anti-immunoglobulins (Ig-enhanced neutralisation test), although some prozone effect has been observed[366,369,372,376]. A titre of 1:2-1:16 (conventional test) or 1:4 (enhanced test) is considered protective against varicella, but not against re-infection, which leads to a rise in antibody titre in the absence of clinical symptoms[343,365,376]. Nt (primarily the C-enhanced tests) have been found to be as sensitive as FAMA in some studies, but they cannot be automated and, as they are laborious and take a long time, they are difficult to use in routine practice and are therefore only used in reference centres[348, 366,367,376,377].

LA: LA is simple, cheap and rapid[377-380]. The sensitivity of some commercial tests is comparable with that of ELISAs in subjects with natural infection, but they may give false positive reports and may not be sufficiently sensitive in vaccinated subjects[5]. Furthermore, they cannot be automated easily.

IFA: Indirect immunofluorescence antibody tests allow the detection of IgG or IgM antibodies which binds to a spot of virus infected cells on a slide by a fluorescent-labled secondary antibody and can be detected using a fluorescence microscope[348,381-383]. They are sensitive and rapid tests, but manual and difficult to automate, subjectively read, and unsuitable for large numbers[348,384].

FAMA: FAMA is considered the gold standard[347, 376,379,385-388]. It is highly sensitive because the preservation of the structure of the surface glycoproteins on infected cells allows neutralising antibodies to be detected[389]. It has been used to assess protection against varicella as a value of ⩾ 4 correlates with protection[215,347,376,390-392]. Its limitations are that it is semi-quantitative, cannot be automated, and requires specialised personnel and specific equipment; reading is subjective and subject to inter-laboratory variability[30]. It is generally only used in specialised centres[54].

TRFIA: TRFIA is a quantitative test that has been well standardised using an international calibrator and allows the results to be expressed mIU/mL[30,253,380]. Some Authors have indicated a cut-off value 150 mIU/mL as the threshold of protection (positive > 150 mIU/mL, doubtful 100-150 mIU/mL and negative < 100 mIU/mL)[380], and others have reported that a cut-off value of 130 mIU/mL is capable of discriminating infection-naive subjective (those that have never come into contact with the virus) from those who have previously had the infection among vaccinated subjects[393]. It is more sensitive and specific than many commercial ELISAs but requires special equipment and is only used in a few specialised centres[54,380,394].

IB: Some studies have used IB, particularly Western blotting, which has been used to study difference in the presence and intensity of the bands in patients with varicella or herpes zoster, but its usefulness in discriminating primary infection from an anamnestic response is a matter of controversy[75,82,362,395].

ELISAs and EIAs: ELISAs or EIAs, which use antigens of the complete virus, are widely used because of their simplicity and the possibility of automation[328,364,396-401]. However there is considerable variation in the sensitivity of the different tests[380,394]. They are generally sufficiently sensitive in subjects with a history of natural infection, but not sufficiently sensitive to detect seroconversion after vaccination as the antibody titres induced by vaccination are a logarithm lower than in the case of natural infection[5,30,54,380,393,397]. In vaccinated subjects, the tests are less sensitive than FAMA as studies have found that 58%-88% of the subjects who were negative or borderline at ELISA were positive to FAMA, which uses surface antigens[91,96,402,403].

GpELISA: The gpELISAs are ELISAs that use the external glycoproteins (typically gE, gB, gH) as antigens of the solid phase[219,339,371,404-412]. They are sensitive enough to detect low antibody levels after vaccination, and have been used to evaluate the level of protection[407]: In one study, 95% of the vaccinated subjects had a gpELISA value of ⩾ 5 U/ mL six weeks after immunisation, whereas those with values of < 5 were at three times greater risk of developing BV[413]. However, these tests were not easy to find[30].

Quantitative CLIAs: The need for more sensitive and

standardised tests has led to the production of CLIAs against international standards. The proposed cut-off values are 150 mIU/mL[414] or 100 mIU/mL (negative < 50 mIU/mL; doubtful 50-100 mIU/mL; positive > 100 mIU/mL)[327,403]. Their ability to give a quantitative result makes it easier to distinguish protected and unprotected subjects: For example, when they were used before VZIG administration to test women exposed to VZV during pregnancy, it was found that the women with CLIA (or TRFIA) values of < 100 mIU/mL were more likely to develop varicella than those with values of > 100 mIU/mL[253]. A value of 100 mIU/mL may therefore differentiate women susceptible to infection from those protected against exposure[253]. However, some of the international guidelines, while stressing the importance of this value, point out that this cut-off value may vary depending on vaccinations, ethnicity or age[54].

Microarrays: Finally, there are also some serological microarray tests for the simultaneous screening of antibodies against VZV and viruses such as herpes simplex virus (HSV-1 and HSV-2), cytomegalovirus, mumps, rubella or measles[415-419].

Diagnosing varicella in pregnant women

Varicella can still occur during pregnancy because the woman may not realise she has been exposed or may have consulted a doctor too late, or because the administration of VZIG does not provide 100% protection[159].

Classic varicella is usually diagnosed clinically, but a laboratory diagnosis may be crucial in doubtful cases such as BV, in immunocompromised women, or varicella in women who have received VZIG, or when it is important to make a differential diagnosis with other exanthematic diseases (especially HSV or enterovirus), arthropod bites or stings, allergic reactions (Stevens-Johnson syndrome), pityriasis lichenoides et varioliformis acuta, and guttate psoriasis[5,30,87].

Technological advances over the years have led to the development of increasing sensitive and specific tests for the diagnosis of varicella.

Tzanck smear tests: Cytology (the Tzanck test) was initially important as it can detect the cytopathic effect of herpesvirus infection morphologically[420-426]. Cells taken by scraping the base of the vesicles or pustules of skin lesions during the rash are placed on slides and, after Giemsa-Wright, hematoxylin-eosin or Papanicolaou staining, are examined by means of light microscopy for the cytopathic alterations typical of herpesvirus (multinucleated giant cells, syncytia and ballooning cell degeneration). Although this test is easy to perform, rapid and cheap, its sensitivity is limited (only 40%-50% in comparison with cell cultures)[422,427]. It is also influenced by the stage of the lesion as it is more sensitive to material taken from fresh vesicles rather than from pustules or scabs[421,428] and, above all, cannot distinguish the lesions caused by HSV and those caused by VZV[429].

Electron microscopy: Electron microscopy has been used, but it is laborious and does not distinguish HSV and VZV[332,424,430,431]. It is more specific when using antibodies conjugated with colloidal gold, although there may still be cross-reactivity with other alphaherpesviruses[430,432]. It is therefore impractical for routine screening.

Virus isolation: Virus isolation in tissue cultures was long considered the gold standard, and was used to evaluate the sensitivity and specificity of other tests[423,425,432-435]. It uses permissive cell lines in which material is deposited from skin or mucocutaneous lesions, or other biological fluids. The various cell lines include human diploid lung fibroblasts (MRC-5, WI-38), human lung carcinoma cells (A549) and primary rhesus monkey kidney cells, which have different yields in terms of viral replication[434,436,437]. However, the method suffers from the need to particular sampling techniques, and special sample transport and storage conditions as the material must be obtained from the base of fresh vesicles during the first 3-4 d, and immediately transported to the laboratory because the virus is labile[429]. In general, viral culture sensitivity is good if specific, fluorescein - conjugated monoclonal antibodies are added, but is still less than that of the latest molecular biology tests[423,434,438,439]. Viral cultures are also laborious, require trained personnel and specially equipped laboratories, and the results are subject to subjective interpretation. They also take a very long time[434]: Tube viral cultures (the standard culture) require seven days to two weeks, but this can be shortened 16-18 times by using shell vial cultures as a centrifuge facilitates virus adherence to the monolayer on round cover-slips, although there are different opinions concerning the sensitivity[348,440]. Viral isolation can be confirmed using immunofluorescence and anti-VZV monoclonal antibodies[348]. Viral cultures may still be useful in determining the antiviral activity of anti-VZV drugs[441,442].

Direct fluorescent antibody assay: The direct fluorescent antibody (DFA) assay uses specific fluorescein-conjugated, polyclonal or monoclonal VZV antibodies to detect VZV antigens on slides with cells scraped from identified skin lesions[235,423,425,433,434,438,443-447], and there is also an immunoperoxidase version[348]. The monoclonal antibodies of choice are those against cell membrane-associated viral antigens[446]. The assay is highly specific and more sensitive than Tzanck smears or viral cultures[423,434,448], and as specific as but less sensitive (73.6%-86%) than molecular biology tests[348,428], although it can be used if molecular biology tests are not available[5]. It is also rapid (about two hours), but it needs special equipment that mean it can only be used in limited areas[348,349], and requires material collected from the skin lesions, which have to be swabbed in order to avoid any bleeding (any antibodies present in the blood can stop the reaction and lead to false negative results)[30].

Molecular biology tests: Molecular biology tests based on in vitro nucleic acid amplification (PCR) are now considered the new platinum tests[20,30,429,449,450]. Various types of PCR are used to diagnose varicella and herpes zoster[13,421,422,450-456], including nested PCR, which is particularly sensitive, but susceptible to contamination leading to false positive results[348]. However, the latest real-time PCR tests are not only rapid, easy to perform, and as sensitive as nested PCR, but have also reduced the risk of contamination[348]. Furthermore, they can be automated tests and used on a wide variety of materials. They are more sensitive than viral cultures, DFA and Tzanck smears particularly when primers for genes 28 and 29 are used[30,428,429,440,449,450,457]. Molecular biology tests are also useful in the case of varicella appearing 7-42 d after vaccination, in the case of herpes zoster appearing 42 d after vaccination and, in the case of the suspected transmission of the vaccine virus, can distinguish virus vaccine, wild-type virus and potential recombinants of vaccine and wild-type viruses[30,451,452,454,455,458-463], although some tests have proved to be less appropriate over time for these purposes[30].

There are also some multiplex assays that can differentiate HSV-1, HSV-2 and VZV in cutaneous and mucocutaneous samples, making them very useful in the case of skin lesions of unclear etiology or immunosuppressed patients[429,464,465]. Multiplex tests are rapid, need only a small volume of sample, and can simultaneously detect different viral agents at the same time in a single, closed-tube reaction[466-468]. They are also robust enough to avoid the need for an extraction step, thus reducing the time of execution at the expense of only small decrease in sensitivity[468-470].

Microarray and electrospray ionisation mass spectrometry techniques have also been developed for the detection of amplification products[471,472].

The materials that can be used include skin swabs, cerebrospinal fluid (CSF), broncho-alveolar lavage (BAL) fluid, nasopharyngeal secretions, urine, saliva, blood, intraocular fluid, amniotic fluid, follicular fluid, corneal tissue and other body fluids. BAL is important[159] as pneumonia is found in 5%-20% of pregnant women with varicella[54,121,158,163], although it can also be caused by bacterial superinfection. Saliva can be useful for diagnosing neurological diseases without rash[473], and CSF for diagnosing encephalitis[429]. The materials of choice in the case of varicella are the vesicles and scabs[23,449,450,474-476] and so, once the lesions have resolved, the probability of finding viral DNA by means of PCR is virtually zero[30]. Leung *et al*[474] (simultaneously considered the use of skin lesions (vesicles, macular and/or papular lesions, and scabs), buccal and throat swabs, oral fluid, urine and blood in patients at the time of the beginning of the rash and two weeks later, and found that all of the material taken from skin lesions at the time of the (and not just vesicles and scabs) are suitable for search purposes, even the macules and papules that have always been regarded critically[30,408], This is important because some atypical symptoms occur only with macules/papules, as in the case of BV[222,477]. Sensitivity therefore does not vary depending on the type of lesion and, during the rash, any skin lesion may be suitable. Of the other tested materials, only the oral samples were sufficiently sensitive to be used at the beginning of symptoms.

Serology: Serology has been used to diagnose varicella, but it has many limitations because it can be negative in immunosuppressed subjects, falsely positive in transfused subjects, or cross-reactive with HSV[364,478-480]. IgM and IgA antibodies have been used as indicators of recent or current infection, but they may also be present during re-infections and reactivations[30,64,153,410]. In general, neither direct or capture IgM tests are as sensitive as molecular biology tests[5,30,410,443,446], and they may also be non-specific in the presence of high IgG titres[5,30,348]. It is probable that the time of sampling may be important because Leung *et al*[474] found that only 25% of their PCR-positive patients with varicella showed IgM in samples taken 0-3 d after the onset of the rash, whereas other authors found that the percentage rose to 48% after four days[85] or 77% after 1-7 d[410]. Samples may therefore be IgM negative if they are taken too soon.

IgG positivity in the absence of IgM usually indicates past infection, and a search has limited usefulness in the acute phase of the disease. However, it may be of help when assessing seroconversion or a 4-fold increase in titre between acute and convalescent serum samples taken 7-10 d apart, although the need for two samples postpones the diagnosis[74,408,410]. Furthermore, people with a history of varicella or vaccination may have higher levels of basic IgG, and a 4-fold increase may therefore not be noticeable in the case of re-infection or BV[30], especially in the elderly[30,474], and IgG may sometimes no longer be present after two weeks of rash, thus lengthening seroconversion times[481]. It has also been found that differences in titres during the acute phase may be found in only 33% of PCR-positive cases[474]. However, a search for IgG in CSF may be useful to evaluate intrathecal synthesis in cases of VZV-induced encephalitis[482,483].

IgG avidity: The determination of IgG avidity may be useful, and is usually done by separating low and high antibody avidity using denaturing agents in an EIA, IFA or CLIA test[58]. Avidity is generally expressed as a percentage of antibody titre by comparing the results with and without the denaturing agent[484-486], which can be helpful in differentiating primary infection (low avidity) and past infection, reactivations or re-infections (high avidity), and then discriminating varicella and herpes zoster[287,393,454,484,487].

It has also been used to differentiate patients with past infection and naïve patients after vaccination[393], although a number of points need to be borne in mind. IgG maturation times are approximately 40-80 d depending on the test used[393,484,486,487], and this needs to be taken into account. Antibodies against some

nuclear proteins (p32 and p36) mature differently from those against surface glycoproteins (they are more likely to have low avidity)[484], and establishing a cut-off value can sometimes be arbitrary[30]. The results are different in immunocompromised and immunocompetent subjects[488,489], and age may also be a factor[490,491]: Low avidity may be found during re-infections in the elderly and in some cases of recurrent infections in children[485,492]. Other things to consider are the time since vaccination (first or second dose), and the time since exposure or the onset of rash: Individual situations (low responders to vaccination can maintain lower antibody titres and low avidity over time)[393]; and the type of infection (*e.g.*, it is not useful in cases of BV because it is likely that vaccinated subjects already have high avidity in response to the vaccination itself)[30].

Prenatal infection and the diagnosis of CVS

CVS is diagnosed using ultrasonography, computed axial tomography (CAT) or magnetic resonance imaging (MRI) and a search for VZV DNA in amniotic fluid[164], placental villi[348], and/or fetal blood[164,493,494].

Ultrasonography is particularly useful for detecting fetal malformations (limb deformity, microcephaly, hydrocephalus, polydramnios, soft tissue calcification, and intra-uterine growth restriction)[90,180,495,496], and is recommended after 16-22 wk of gestation or five weeks after infection[16,187,493,497]. It is not sensitive enough to detect congenital defects before the fourth week[16,493,497]. The limitation of ultrasonography is that it is not very much sensitive or specific[169], and so cannot detect all abnormalities[498]; however, its predictive value is better if there is a diagnosis of fetal infection with positive VZV DNA[499].

Fetal MRI and cranial CAT may sometimes be useful for further investigating the morphological abnormalities detected by ultrasonography[180,181,497].

VZV DNA can be sought in amniotic fluid, which is considered the material of choice, but this should not be done less than a month after maternal infection in order to avoid false negative results[53,164,187]. VZV DNA can persist for weeks in the peripheral blood of infected pregnant mothers and, as a false positive result in amniotic fluid due to maternal contamination has been reported, it has been suggested that aminiocentesis should performed after maternal viremia has become negative[498]. The main limitation of searching for VZV DNA in amniotic fluid is that the presence of viremia does not automatically mean the presence of fetal damage[164,500]. Only a small percentage of VZV DNA-positive fetuses present CVS at birth[164], and so amniocentesis has a good negative predictive value but poor positive predictive value[16].

Given the limitations of ultrasonography and a search for VZV DNA in amniotic fluid, Enders *et al*[90] studied the risk of CVS by combining both methods during pregnancy (Table 3). In literature, their use over time was evaluated separating, which can be approached in two ways[169]. It may be better to perform an amniocentesis first (but in any case not until the mother's skin lesions have disappeared) because, if it is negative, the mother can be reassured she is carrying healthy fetus in 90% of cases, although there is still the small risk of miscarriage associated with amniocentesis[16,501]. If the amniocentesis is positive, ultrasonography can be used for confirmation, although it is possible that a mother knowing the amniocentesis findings would prefer to undergo an immediate abortion[169]. The alternative is to use amniocentesis to confirm ultrasonographic findings of anomalies, although it must be remembered that ultrasonography is not very sensitive or specific, and so it does not detect all malformations[169,498]. Some authors recommend following-up mothers who have contracted varicella during pregnancy by means of ultrasonography, and then searching for viral DNA in the case of malformations[19,159], insisting on always searching for VZV DNA because other micro-organisms such as Coxsackie B and HSV can cause congenital lesions similar to those of CVS[502-504]. There is a report of a case of fetal malformations due to HSV2 and not VZV in a mother who contracted varicella during pregnancy[502], and conditions such as microphthalmia dermal aplasia scleroderma (MIDAS) or microphthalmia with linear skin defects (MLS) may also lead to malformations, with maternal varicella being just a coincidence[505,506].

There are few and unconvincing data concerning chorionic villi as the PCR-detected presence of viral DNA is not necessarily associated with an infected fetus, but may due to false positivity caused by maternal contamination or a placental infection not transmitted to the fetus[169,506,507].

In relation to other tests used in the past, viral cultures in amniotic fluid and a search for IgM in fetal blood are less sensitive[164,500]. The detection of IgM (which was used before the advent of PCR)[508,509] was positive in only 25% of the cases in a post-natal study[145]. Furthermore, it can only be detected in fetal blood after 24 wk of gestation and so, if the infection occurs earlier, the fetus may not show an immune response[164,348]. Finally, sampling is more invasive and less safe than amniocentesis.

Diagnosis in newborns

In most cases, a baby born after maternal infection is healthy and there are no long-term consequences in terms of intellectual performance and neurodevelopment[152,169]. Previous infection may possibly be demonstrated by the presence of IgM at birth, but as the search is positive in only 25% of cases, it more likely to be negative[53,145,169]. As the IgG antibodies passively passed from mother to child have a limited half-life, their detection more than seven months after birth may be the only indication of intrauterine infection[131,192]. However, it is important to monitor the child in order to identify the occurrence of herpes zoster in the first 1-2 years of life[510].

The morbidity neonatal varicella is generally low in the children of immune mothers because of the presence of maternal antibodies[193] but in their absence, the children

Table 3 Prenatal congenital varicella syndrome diagnosis combining ultrasoonography and a search for varicella-zoster virus DNA in amniotic fluid[90]

Weeks of pregnancy	VZV DNA in amniocentesis	Ultrasound	Risk of CVS
Initial			
17-21	Positive	Normal	Uncertain
Follow-up			
23-24	Positive	Normal	Unlikely
	Positive	Abnormal	High
15-22/> 23	Negative	Normal	Low

VZV: Varicella-zoster virus; CVS: Congenital varicella syndrome.

are at risk of contracting primary infection during the first months of life from their mothers or other people[16]. Premature infants born after 28 wk of gestation are at risk in the first six weeks of life because the reduced gestational period means that they do not have maternal antibodies[5,88], and a diagnosis of varicella in the newborn should therefore be related to maternal varicella in the last month of pregnancy. Neonatal varicella is more severe if varicella appears in the mother between seven days before and seven days after delivery, especially between five days before and two days after delivery when the neonatal mortality rate can be as high as 30%[153,169,196]. A diagnosis of neonatal varicella is typically clinical but the contribution of a microbiology laboratory may be important using molecular biological tests (PCR) on vesicles or other biological liquids such as CSF in cases of encephalitis[429]. Also it is crucial in the differential diagnoses with similar clinical manifestations, such as those caused by HSV or enterovirus, syphilis or incontinentia pigmenti[87,147,165,511].

In the absence of a prenatal diagnosis (*e.g.*, in the case of sub-clinical maternal infection), and an infant born with typical malformations of CVS the relationship between these malformations and maternal infection should be confirmed[283,512]. A search for VZV DNA using molecular biology techniques on neonatal tissues (*e.g.*, skin lesions, CSF) can provide evidence of intrauterine infection[182,494,512]. Viral cultures are not recommended because they are insensitive[26,147,159,165,169], as is the presence of IgM in blood (only 25% positive)[145,147]. Molecular biology tests are also indicated in the case of rare or uncharacteristic malformations, or when the relationship between maternal infection and congenital malformations is doubtful[513]. Consideration should be given to differential diagnoses of congenital varicella with rubella, cytomegalovirus, HSV, Coxsackie virus, *Toxoplasma gondii*, and MIDAS or MLS[165,502-504,514].

CONCLUSION

Although varicella during pregnancy is fortunately infrequent and CVS is even rarer, every available means should be used to prevent and diagnose them. Microbiology laboratories can be crucial in both situations because the development of increasingly sensitive and specific tests for the detection of antibodies makes it possible to evaluate a mother's immune status more precisely. Quantitative ELISAs and CLIAs can help to determine whether the detected antibody level is protective or not on the basis of proposed cut-off values because, although further studies are needed to assess whether these can be extended universally to all pregnant women regardless of age, geographical origin or race, they are certainly valuable in case assessment. However, it is important to stress the need for a widespread information campaign to ensure that pregnant women are aware of the risks of exposure, and promptly consult a doctor quickly because, for example, specific immunoglobulins need to be administered to susceptible women as soon as possible after exposure.

The tests for the diagnosis of varicella have also become increasingly sensitive and specific. The introduction of molecular biology tests has opened up new scenerios in all fields of microbiological diagnostics, and the availability of simple and rapid multiplex tests capable of simultaneously detecting multiple microorganisms simultaneously can allow a rapid diagnosis when a clinical manifestation may be due to different viruses, as in the case of the skin lesions caused by HSV and VZV.

Finally, close collaboration between molecular biology investigators (VZV DNA) and specialists in imaging diagnostics (ultrasonography) is important in the follow-up of pregnant woman with varicella because this makes it possible to make a prenatal diagnosis of CVS.

REFERENCES

1 **Brunell PA**, Shehab ZM. Varicella-Zoster. In: Rose NR, Friedman H, Fahey JL. Manual of Clinical Laboratory Immunology. Washington DC: ASM, 1986: 502-503

2 **Giacchino R**, Losurdo G, Castagnola E. Decline in mortality with varicella vaccination. *N Engl J Med* 2005; **352**: 1819 [PMID: 15858196 DOI: 10.1056/NEJM200504283521718]

3 **Losurdo G**, Bertoluzzo L, Canale F, Timitilli A, Bondi E, Castagnola E, Giacchino R. Varicella and its complications as cause of hospitalization. *Infez Med* 2005; **13**: 229-234 [PMID: 16388278]

4 **Bertoluzzo L**, Castagnola E, Losurdo G, Bondi E, Canale F, Giacchino R. The hospitalization because of varicella in a tertiary care pediatric hospital during 10 year study period. *JPMH* 2005; **46**: 169-172

5 **Center for Disease Control and Prevention (CDC)**. Varicella. Epidemiology and Prevention of Vaccine-Preventable Diseases. The Pink Book: Course Textbook - 13th ed 2015. [accessed 2016 Apr 4]. Available from: URL: http://www.cdc.gov/vaccines/pubs/pinkbook/varicella.html

6 **Centers for Disease Control and Prevention (CDC)**. Varicella-related deaths among adults--United States, 1997. *MMWR Morb Mortal Wkly Rep* 1997; **46**: 409-412 [PMID: 9162840]

7 **Guess HA**, Broughton DD, Melton LJ, Kurland LT. Population-based studies of varicella complications. *Pediatrics* 1986; **78**: 723-727 [PMID: 3763290]

8 **Miller E**. Marshall R. Vurdien J. Epidemiology, outcome and control of varicella-zoster infection. *Rev Med Microbiol* 1993; **4**: 222-230 [DOI: 10.1097/00013542-199310000-00006]

9 **Gabutti G**. VZV infection: epidemiology and prevention. *J Prev Med Hyg* 2007; **48**: 65-71 [PMID: 18274340]

10 **Smego RA**, Asperilla MO. Use of acyclovir for varicella pneumonia during pregnancy. *Obstet Gynecol* 1991; **78**: 1112-1116 [PMID: 1945218]

11 **Baren JM**, Henneman PL, Lewis RJ. Primary varicella in adults:

pneumonia, pregnancy, and hospital admission. *Ann Emerg Med* 1996; **28**: 165-169 [PMID: 8759580 DOI: 10.1016/S0196-0644(96)70057-4]

12 **Steele RW**. Immunology of Varicella-Zoster Virus. In: Nahmias AJ, O'Reilly RJ. Immunology of Human Infection. Vol 9 Part II, New York: Springer, 1982: 73-88 [DOI: 10.1007/978-1-4684-1012-9_3]

13 **Gilden DH**, Kleinschmidt-DeMasters BK, LaGuardia JJ, Mahalingam R, Cohrs RJ. Neurologic complications of the reactivation of varicella-zoster virus. *N Engl J Med* 2000; **342**: 635-645 [PMID: 10699164 DOI: 10.1056/NEJM200003023420906]

14 **Schmidt NJ**. Varicella-Zoster Virus. In: Manual of Clinical Microbiology. 4th ed. Lennette EH, Balows A, Hausler WJ, Shadomy HJ. Washington DC: American Society of Microbiology (ASM), 1985: 720-727

15 **Takahashi M**. Herpesviridae: varicella zoster virus. In EM Lennette, P Halonen, FA Murphy Laboratory diagnosis of infectious diseases: principles and practice. Viral, rickettsial, and chlamydial diseases. Vol 2. New York: Springer-Verlag, 1988: 261-275 [DOI: 10.1007/978-1-4612-3900-0_14]

16 **Royal Colege of Obstetricians and Ginaecologists**. Chickenpox in Pregnancy: Green-top Guideline N.13. 2015. [accessed 2016 Apr 4]. Available from: URL: https://www.rcog.org.uk/en/guidelines-research-services/guidelines/gtg13/

17 **Sawyer MH**, Chamberlin CJ, Wu YN, Aintablian N, Wallace MR. Detection of varicella-zoster virus DNA in air samples from hospital rooms. *J Infect Dis* 1994; **169**: 91-94 [PMID: 8277202 DOI: 10.1093/infdis/169.1.91]

18 **Gnann JW**, Whitley RJ. Clinical practice. Herpes zoster. *N Engl J Med* 2002; **347**: 340-346 [PMID: 12151472 DOI: 10.1056/NEJMcp013211]

19 **Shrim A**, Koren G, Yudin MH, Farine D. Management of varicella infection (chickenpox) in pregnancy. *J Obstet Gynaecol Can* 2012; **34**: 287-292 [PMID: 22385673 DOI: 10.1016/S1701-2163(16)35190-8]

20 **Kido S**, Ozaki T, Asada H, Higashi K, Kondo K, Hayakawa Y, Morishima T, Takahashi M, Yamanishi K. Detection of varicella-zoster virus (VZV) DNA in clinical samples from patients with VZV by the polymerase chain reaction. *J Clin Microbiol* 1991; **29**: 76-79 [PMID: 1847154]

21 **Grose C**. Varicella infection during pregnancy. *Herpes* 1999; **6**: 33-37

22 **Goldblatt D**. The immunology of chickenpox. A review prepared for the UK Advisory Group on Chickenpox on behalf of the British Society for the Study of Infection. *J Infect* 1998; **36** Suppl 1: 11-16 [PMID: 9514103 DOI: 10.1016/S0163-4453(98)80150-3]

23 **Koropchak CM**, Graham G, Palmer J, Winsberg M, Ting SF, Wallace M, Prober CG, Arvin AM. Investigation of varicella-zoster virus infection by polymerase chain reaction in the immunocompetent host with acute varicella. *J Infect Dis* 1991; **163**: 1016-1022 [PMID: 1850441 DOI: 10.1093/infdis/163.5.1016]

24 **Tsolia M**, Gershon AA, Steinberg SP, Gelb L. Live attenuated varicella vaccine: evidence that the virus is attenuated and the importance of skin lesions in transmission of varicella-zoster virus. National Institute of Allergy and Infectious Diseases Varicella Vaccine Collaborative Study Group. *J Pediatr* 1990; **116**: 184-189 [PMID: 2153790 DOI: 10.1016/S0022-3476(05)82872-0]

25 **Chen JJ**, Zhu Z, Gershon AA, Gershon MD. Mannose 6-phosphate receptor dependence of varicella zoster virus infection in vitro and in the epidermis during varicella and zoster. *Cell* 2004; **119**: 915-926 [PMID: 15620351 DOI: 10.1016/j.cell.2004.11.007]

26 **Tan MP**, Koren G. Chickenpox in pregnancy: revisited. *Reprod Toxicol* 2006; **21**: 410-420 [PMID: 15979274 DOI: 10.1016/j.reprotox.2005.04.011]

27 **Ragozzino MW**, Melton LJ, Kurland LT, Chu CP, Perry HO. Population-based study of herpes zoster and its sequelae. *Medicine* (Baltimore) 1982; **61**: 310-316 [PMID: 6981045 DOI: 10.1097/00005792-198209000-00003]

28 **Lungu O**, Annunziato PW, Gershon A, Staugaitis SM, Josefson D, LaRussa P, Silverstein SJ. Reactivated and latent varicella-zoster virus in human dorsal root ganglia. *Proc Natl Acad Sci USA* 1995; **92**: 10980-10984 [PMID: 7479921 DOI: 10.1073/pnas.92.24.10980]

29 **Cohen JI**, Brunell PA, Straus SE, Krause PR. Recent advances in varicella-zoster virus infection. *Ann Intern Med* 1999; **130**: 922-932 [PMID: 10375341 DOI: 10.7326/0003-4819-130-11-199906010-00017]

30 **Schmid DS**, Jumaan AO. Impact of varicella vaccine on varicella-zoster virus dynamics. *Clin Microbiol Rev* 2010; **23**: 202-217 [PMID: 20065330 DOI: 10.1128/CMR.00031-09]

31 **Weller TH**. Varicella and herpes zoster: a perspective and overview. *J Infect Dis* 1992; **166** Suppl 1: S1-S6 [PMID: 1320645]

32 **Gershon MD**, Gershon AA. VZV infection of keratinocytes: production of cell-free infectious virions in vivo. *Curr Top Microbiol Immunol* 2010; **342**: 173-188 [PMID: 20225011 DOI: 10.1007/82_2010_13]

33 **Straus SE**, Ostrove JM, Inchauspé G, Felser JM, Freifeld A, Croen KD, Sawyer MH. NIH conference. Varicella-zoster virus infections. Biology, natural history, treatment, and prevention. *Ann Intern Med* 1988; **108**: 221-237 [PMID: 2829675 DOI: 10.7326/0003-4819-108-2-221]

34 **Hope-Simpson RE**. The nature of herpes zoster: a long-term study and a new hypothesis. *Proc R Soc Med* 1965; **58**: 9-20 [PMID: 14267505]

35 **Weller TH**, Witton HM, Bell EJ. The etiologic agents of varicella and herpes zoster; isolation, propagation, and cultural characteristics in vitro. *J Exp Med* 1958; **108**: 843-868 [PMID: 13598816 DOI: 10.1084/jem.108.6.8430]

36 **Katz J**, Cooper EM, Walther RR, Sweeney EW, Dworkin RH. Acute pain in herpes zoster and its impact on health-related quality of life. *Clin Infect Dis* 2004; **39**: 342-348 [PMID: 15307000 DOI: 10.1086/421942]

37 **Dworkin RH**. Post-herpetic neuralgia. *Herpes* 2006; **13** Suppl 1: 21A-27A

38 **Buchbinder SP**, Katz MH, Hessol NA, Liu JY, O'Malley PM, Underwood R, Holmberg SD. Herpes zoster and human immunodeficiency virus infection. *J Infect Dis* 1992; **166**: 1153-1156 [PMID: 1308664 DOI: 10.1093/infdis/166.5.1153]

39 **Thomas SL**, Hall AJ. What does epidemiology tell us about risk factors for herpes zoster? *Lancet Infect Dis* 2004; **4**: 26-33 [PMID: 14720565 DOI: 10.1016/S1473-3099(03)00857-0]

40 **Hambleton S**, Gershon AA. Preventing varicella-zoster disease. *Clin Microbiol Rev* 2005; **18**: 70-80 [PMID: 15653819 DOI: 10.1128/CMR.18.1.70-80.2005]

41 **Volpi A**. Varicella immunization and herpes zoster. *Herpes* 2005; **12**: 59 [PMID: 16393520]

42 **Gershon AA**. Varicella and Herpes zoster. Clinical diseases and complications. *Herpes* 2006; **13** Suppl 1: 4A-8A

43 **Quinlivan M**, Breuer J. Molecular studies of Varicella zoster virus. *Rev Med Virol* 2006; **16**: 225-250 [PMID: 16791838 DOI: 10.1002/rmv.502]

44 **Nagler FP**, Rake G. The Use of the Electron Microscope in Diagnosis of Variola, Vaccinia, and Varicella. *J Bacteriol* 1948; **55**: 45-51 [PMID: 16561433]

45 **Davison AJ**, Scott JE. The complete DNA sequence of varicella-zoster virus. *J Gen Virol* 1986; **67** (Pt 9): 1759-1816 [PMID: 3018124 DOI: 10.1099/0022-1317-67-9-1759]

46 **Cohrs RJ**, Hurley MP, Gilden DH. Array analysis of viral gene transcription during lytic infection of cells in tissue culture with Varicella-Zoster virus. *J Virol* 2003; **77**: 11718-11732 [PMID: 14557657 DOI: 10.1128/JVI.77.21.11718-11732.2003]

47 **Davison AJ**. Varicella-zoster virus. The Fourteenth Fleming lecture. *J Gen Virol* 1991; **72** (Pt 3): 475-486 [PMID: 1848588 DOI: 10.1099/0022-1317-72-3-475]

48 **Cohen JI**, Straus SS, Arvin A. Varicella-zoster virus, replication, pathogenesis and management. In: Knipe DM, Howley PM. Fields virology. Philadelphia: Lippincott-Raven Publishers, 2007: 2774-2818

49 **Mettenleiter TC**. Budding events in herpesvirus morphogenesis. *Virus Res* 2004; **106**: 167-180 [PMID: 15567495 DOI: 10.1016/j.virusres.2004.08.013]

50 **Gershon AA**. Varicella. In: Crovari P, Principi N. Le vaccinazioni. Pisa: Pacini Editore, 2000: 555-576

51 **Ahmed R**, Gray D. Immunological memory and protective immunity: understanding their relation. *Science* 1996; **272**: 54-60 [PMID: 8600537 DOI: 10.1126/science.272.5258.54]

52 **Arvin A**. Aging, immunity, and the varicella-zoster virus. *N Engl J Med* 2005; **352**: 2266-2267 [PMID: 15930416 DOI: 10.1056/NEJMp058091]

53 **Lamont RF**, Sobel JD, Carrington D, Mazaki-Tovi S, Kusanovic JP, Vaisbuch E, Romero R. Varicella-zoster virus (chickenpox) infection in pregnancy. *BJOG* 2011; **118**: 1155-1162 [PMID: 21585641 DOI: 10.1111/j.1471-0528.2011.02983.x]

54 **Bailey H**. Screening for varicella in pregnancy. External review against programme appraisal criteria for the UK National Screening Committee (UKNSC). NSC UK National Screening Committee. [updated 2015 Jul]. Available from: URL: http://legacy.screening.nhs.uk/policydb_download.php?doc=558&rct=j&frm=1&q=&esrc=s&sa=U&ved=0ahUKEwi28oe7yPzLAhUEuBQKHceBCDEQFggWMAA&usg=AFQjCNHexcTA2NK7dh1kCnynm5aHnhiIwA

55 **Krause PR**, Klinman DM. Varicella vaccination: evidence for frequent reactivation of the vaccine strain in healthy children. *Nat Med* 2000; **6**: 451-454 [PMID: 10742154 DOI: 10.1038/74715]

56 **Paryani SG**, Arvin AM. Intrauterine infection with varicella-zoster virus after maternal varicella. *N Engl J Med* 1986; **314**: 1542-1546 [PMID: 3012334 DOI: 10.1056/NEJM198606123142403]

57 **Gershon AA**. Chickenpox, Measles, and Mumps. In: Remington JS, Klein JO. Infectious Diseases of the Fetus and Newborn Infant. Philadelphia: W.B Saunders Company, 2001: 683-732

58 **Martin KA**, Junker AK, Thomas EE, Van Allen MI, Friedman JM. Occurrence of chickenpox during pregnancy in women seropositive for varicella-zoster virus. *J Infect Dis* 1994; **170**: 991-995 [PMID: 7930746 DOI: 10.1093/infdis/170.4.991]

59 **Terada K**, Kawano S, Shimada Y, Yagi Y, Kataoka N. Recurrent chickenpox after natural infection. *Pediatr Infect Dis J* 1996; **15**: 179-181 [PMID: 8822299 DOI: 10.1097/00006454-199602000-00022]

60 **Junker AK**, Angus E, Thomas EE. Recurrent varicella-zoster virus infections in apparently immunocompetent children. *Pediatr Infect Dis J* 1991; **10**: 569-575 [PMID: 1891288 DOI: 10.1097/00006454-199108000-00004]

61 **Gershon AA**, Steinberg SP, Gelb L. Clinical reinfection with varicella-zoster virus. *J Infect Dis* 1984; **149**: 137-142 [PMID: 6321605 DOI: 10.1093/infdis/149.2.137]

62 **Gershon AA**. Varicella-zoster virus: prospects for control. *Adv Pediatr Infect Dis* 1995; **10**: 93-124 [PMID: 7718215]

63 **Hall S**, Maupin T, Seward J, Jumaan AO, Peterson C, Goldman G, Mascola L, Wharton M. Second varicella infections: are they more common than previously thought? *Pediatrics* 2002; **109**: 1068-1073 [PMID: 12042544 DOI: 10.1542/peds.109.6.1068]

64 **Arvin AM**. Immune responses to varicella-zoster virus. *Infect Dis Clin North Am* 1996; **10**: 529-570 [PMID: 8856351 DOI: 10.1016/S0891-5520(05)70312-3]

65 **Marin M**, Nguyen HQ, Keen J, Jumaan AO, Mellen PM, Hayes EB, Gensheimer KF, Gunderman-King J, Seward JF. Importance of catch-up vaccination: experience from a varicella outbreak, Maine, 2002-2003. *Pediatrics* 2005; **115**: 900-905 [PMID: 15805362 DOI: 10.1542/peds.2004-1162]

66 **Straus SE**. Overview: the biology of varicella-zoster virus infection. *Ann Neurol* 1994; **35** Suppl: S4-S8 [PMID: 8185296 DOI: 10.1002/ana.410350704]

67 **Gershon AA**, Gershon MD. Pathogenesis and current approaches to control of varicella-zoster virus infections. *Clin Microbiol Rev* 2013; **26**: 728-743 [PMID: 24092852 DOI: 10.1128/CMR.00052-13]

68 **Castle SC**. Clinical relevance of age-related immune dysfunction. *Clin Infect Dis* 2000; **31**: 578-585 [PMID: 10987724 DOI: 10.1086/313947]

69 **Vossen MT**, Gent MR, Weel JF, de Jong MD, van Lier RA, Kuijpers TW. Development of virus-specific CD4+ T cells on reexposure to Varicella-Zoster virus. *J Infect Dis* 2004; **190**: 72-82 [PMID: 15195245 DOI: 10.1086/421277]

70 **Etzioni A**, Eidenschenk C, Katz R, Beck R, Casanova JL, Pollack S. Fatal varicella associated with selective natural killer cell deficiency. *J Pediatr* 2005; **146**: 423-425 [PMID: 15756234 DOI: 10.1016/j.jpeds.2004.11.022]

71 **van Loon AM**, van der Logt JT, Heessen FW, Heeren MC, Zoll J. Antibody-capture enzyme-linked immunosorbent assays that use enzyme-labelled antigen for detection of virus-specific immunoglobulin M, A and G in patients with varicella or herpes zoster. *Epidemiol Infect* 1992; **108**: 165-174 [PMID: 1312479 DOI: 10.1017/S095026880004961X]

72 **Terada K**, Niizuma T, Ogita S, Kataoka N. Responses of varicella zoster virus (VZV)-specific immunity in seropositive adults after inhalation of inactivated or live attenuated varicella vaccine. *Vaccine* 2002; **20**: 3638-3643 [PMID: 12399190 DOI: 10.1016/S0264-410X(02)00408-5]

73 **Bogger-Goren S**, Bernstein JM, Gershon AA, Ogra PL. Mucosal cell-mediated immunity to varicella zoster virus: role in protection against disease. *J Pediatr* 1984; **105**: 195-199 [PMID: 6086865 DOI: 10.1016/S0022-3476(84)80112-2]

74 **Arvin AM**, Koropchak CM. Immunoglobulins M and G to varicella-zoster virus measured by solid-phase radioimmunoassay: antibody responses to varicella and herpes zoster infections. *J Clin Microbiol* 1980; **12**: 367-374 [PMID: 6260833]

75 **Dubey L**, Steinberg SP, LaRussa P, Oh P, Gershon AA. Western blot analysis of antibody to varicella-zoster virus. *J Infect Dis* 1988; **157**: 882-888 [PMID: 2834466 DOI: 10.1093/infdis/157.5.882]

76 **Echevarría JM**, Téllez A, Martínez-Martín P. Subclass distribution of the serum and intrathecal IgG antibody response in varicella-zoster virus infections. *J Infect Dis* 1990; **162**: 621-626 [PMID: 2167334 DOI: 10.1093/infdis/162.3.621]

77 **Palumbo PE**, Arvin AM, Koropchak CM, Wittek AE. Investigation of varicella-zoster virus-infected cell proteins that elicit antibody production during primary varicella using the immune transfer method. *J Gen Virol* 1984; **65** (Pt 12): 2141-2147 [PMID: 6096492 DOI: 10.1099/0022-1317-65-12-2141]

78 **Schmidt NJ**, Gallo D. Class-specific antibody responses to early and late antigens of varicella and herpes simplex viruses. *J Med Virol* 1984; **13**: 1-12 [PMID: 6319583 DOI: 10.1002/jmv.1890130102]

79 **Forghani B**, Dupuis KW, Schmidt NJ. Epitopes functional in neutralization of varicella-zoster virus. *J Clin Microbiol* 1990; **28**: 2500-2506 [PMID: 1701445]

80 **Haumont M**, Jurdan M, Kangro H, Jacquet A, Massaer M, Deleersnyder V, Garcia L, Bosseloir A, Bruck C, Bollen A, Jacobs P. Neutralizing antibody responses induced by varicella-zoster virus gE and gB glycoproteins following infection, reactivation or immunization. *J Med Virol* 1997; **53**: 63-68 [PMID: 9298734 DOI: 10.1002/(SICI)1096-9071(199709)53:1<63::AID-JMV11>3.0.CO;2-Y]

81 **Vafai A**, Wellish M, Wroblewska Z, Cisco M, Gilden D. Induction of antibody against in vitro translation products encoded by varicella-zoster virus glycoprotein genes. *Virus Res* 1987; **7**: 325-333 [PMID: 3039752 DOI: 10.1016/0168-1702(87)90046-3]

82 **Harper DR**, Kangro HO, Heath RB. Serological responses in varicella and zoster assayed by immunoblotting. *J Med Virol* 1988; **25**: 387-398 [PMID: 2844983 DOI: 10.1002/jmv.1890250403]

83 **Cradock-Watson JE**, Ridehalgh MK, Bourne MS. Specific immunoglobulin responses after varicella and herpes zoster. *J Hyg* (Lond) 1979; **82**: 319-336 [PMID: 219110 DOI: 10.1017/S0022172400025730]

84 **Kangro HO**, Ward A, Argent S, Heath RB, Cradock-Watson JE, Ridehalgh MK. Detection of specific IgM in varicella and herpes zoster by antibody-capture radioimmunoassay. *Epidemiol Infect* 1988; **101**: 187-195 [PMID: 3402547 DOI: 10.1017/S0950268800029344]

85 **Sauerbrei A**, Eichhorn U, Schacke M, Wutzler P. Laboratory diagnosis of herpes zoster. *J Clin Virol* 1999; **14**: 31-36 [PMID: 10548128 DOI: 10.1016/S1386-6532(99)00042-6]

86 **Wittek AE**, Arvin AM, Koropchak CM. Serum immunoglobulin A antibody to varicella-zoster virus in subjects with primary varicella and herpes zoster infections and in immune subjects. *J Clin Microbiol* 1983; **18**: 1146-1149 [PMID: 6315766]

87 **Heininger U**, Seward JF. Varicella. *Lancet* 2006; **368**: 1365-1376 [PMID: 17046469 DOI: 10.1016/S0140-6736(06)69561-5]

88 **Centers for Disease Control (CDC)**. Prevention of varicella. Update recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recomm Rep* 1999; **48**: 1-5 [PMID: 10366137]

89 **Enders G**. Varicella-zoster virus infection in pregnancy. *Prog Med Virol* 1984; **29**: 166-196 [PMID: 6322232]

90 **Enders G**, Miller E. Varicella and herpes zoster in pregnancy and the newborn. In: Arvin AM, Gershon AA. Varicella-Zoster Virus Virology and Clinical Mangement. Cambridge: University Press, 2000: 317-347 [DOI: 10.1017/CBO9780511601194.018]

91 **Heininger U**, Braun-Fahrländer C, Desgrandchamps D, Glaus J, Grize L, Wutzler P, Schaad UB. Seroprevalence of varicella-zoster virus immunoglobulin G antibodies in Swiss adolescents and risk factor analysis for seronegativity. *Pediatr Infect Dis J* 2001; **20**: 775-778 [PMID: 11734740 DOI: 10.1097/00006454-200108000-00011]

92 **World Health Organization**. Epidemiology of chickenpox. 1977-1990. *Wkly Epidemiol Rec* 1992; **67**: 118-119 [PMID: 1571238]

93 **Wutzler P**, Färber I, Wagenpfeil S, Bisanz H, Tischer A. Seroprevalence of varicella-zoster virus in the German population. *Vaccine* 2001; **20**: 121-124 [PMID: 11567755 DOI: 10.1016/S0264-410X(01)00276-6]

94 **Bonanni P**, Breuer J, Gershon A, Gershon M, Hryniewicz W, Papaevangelou V, Rentier B, Rümke H, Sadzot-Delvaux C, Senterre J, Weil-Olivier C, Wutzler P. Varicella vaccination in Europe - taking the practical approach. *BMC Med* 2009; **7**: 26 [PMID: 19476611 DOI: 10.1186/1741-7015-7-26]

95 **EUVAC.NET**. A surveillance community network for vaccine preventable infectious diseases. Varicella vaccination overview in European countries. Varicella sureveillance report 2010. Available from: URL: http://ecdc.europa.eu/en/publications/Publications/varicella_report_2010_euvacnet.pdf

96 **Wutzler P**, Neiss A, Banz K, Goertz A, Bisanz H. Can varicella be eliminated by vaccination? Potential clinical and economic effects of universal childhood varicella immunisation in Germany. *Med Microbiol Immunol* 2002; **191**: 89-96 [PMID: 12410347 DOI: 10.1007/s00430-002-0123-4]

97 **Waaijenborg S**, Hahné SJ, Mollema L, Smits GP, Berbers GA, van der Klis FR, de Melker HE, Wallinga J. Waning of maternal antibodies against measles, mumps, rubella, and varicella in communities with contrasting vaccination coverage. *J Infect Dis* 2013; **208**: 10-16 [PMID: 23661802 DOI: 10.1093/infdis/jit143]

98 **de Melker H**, Berbers G, Hahné S, Rümke H, van den Hof S, de Wit A, Boot H. The epidemiology of varicella and herpes zoster in The Netherlands: implications for varicella zoster virus vaccination. *Vaccine* 2006; **24**: 3946-3952 [PMID: 16564115 DOI: 10.1016/j.vaccine.2006.02.017]

99 **Gabutti G**, Rota MC, Guido M, De Donno A, Bella A, Ciofi degli Atti ML, Crovari P. The epidemiology of Varicella Zoster Virus infection in Italy. *BMC Public Health* 2008; **8**: 372 [PMID: 18954432 DOI: 10.1186/1471-2458-8-372]

100 **Fleming DM**, Schellevis FG, Falcao I, Alonso TV, Padilla ML. The incidence of chickenpox in the community. Lessons for disease surveillance in sentinel practice networks. *Eur J Epidemiol* 2001; **17**: 1023-1027 [PMID: 12380716 DOI: 10.1023/A: 1020066806544]

101 **Díez-Domingo J**, Aristegui J, Calbo F, Gonzalez-Hachero J, Moraga F, Peña Guitian J, Ruiz Contreras J, Torrellas A. Epidemiology and economic impact of varicella in immunocompetent children in Spain. A nation-wide study. *Vaccine* 2003; **21**: 3236-3239 [PMID: 12804853 DOI: 10.1016/S0264-410X(03)00264-0]

102 **Ciofi Degli Atti ML**, Salmaso S, Bella A, Arigliani R, Gangemi M, Chiamenti G, Brusoni G, Tozzi AE. Pediatric sentinel surveillance of vaccine-preventable diseases in Italy. *Pediatr Infect Dis J* 2002; **21**: 763-768 [PMID: 12192166 DOI: 10.1097/00006454-200208000-00013]

103 **Baldo V**, Baldovin T, Russo F, Busana MC, Piovesan C, Bordignon G, Giliberti A, Trivello R. Varicella: epidemiological aspects and vaccination coverage in the Veneto Region. *BMC Infect Dis* 2009; **9**: 150 [PMID: 19737419 DOI: 10.1186/1471-2334-9-150]

104 **García Cenoz M**, Castilla J, Montes Y, Morán J, Salaberri A, Elía F, Floristán Y, Rodrígo I, Irisarri F, Arriazu M, Zabala A, Barricarte A. [Varicella and herpes zoster incidence prior to the introduction of systematic child vaccination in Navarre, 2005-2006]. *An Sist Sanit Navar* 2008; **31**: 71-80 [PMID: 18496581]

105 **Valerio L**, Escribá JM, Fernández-Vázquez J, Roca C, Milozzi J, Solsona L, Molina I. Biogeographical origin and varicella risk in the adult immigration population in Catalonia, Spain (2004-2006). *Euro Surveill* 2009; **14**: pii: 19332 [PMID: 19761736]

106 **Bonsignori F**, Chiappini E, Frenos S, Peraldo M, Galli L, de Martino M. Hospitalization rates for complicated and uncomplicated chickenpox in a poorly vaccined pediatric population. *Infection* 2007; **35**: 444-450 [PMID: 18034210 DOI: 10.1007/s15010-007-6248-z]

107 **Giaquinto C**, Sturkenboom M, Mannino S, Arpinelli F, Nicolosi A, Cantarutti L. [Epidemiology and outcomes of varicella in Italy: results of a prospective study of children (0-14 years old) followed up by pediatricians (Pedianet study)]. *Ann Ig* 2002; **14**: 21-27 [PMID: 12389301]

108 **Socan M**, Kraigher A, Pahor L. Epidemiology of varicella in Slovenia over a 20-year period (1979-98). *Epidemiol Infect* 2001; **126**: 279-283 [PMID: 11349978 DOI: 10.1017/S0950268801005350]

109 **Socan M**, Blaško M. Surveillance of varicella and herpes zoster in Slovenia, 1996-2005. *Euro Surveill* 2007; **12**: 13-16

110 **Pérez-Farinós N**, Ordobás M, García-Fernández C, García-Comas L, Cañellas S, Rodero I, Gutiérrez-Rodríguez A, García-Gutiérrez J, Ramírez R. Varicella and herpes zoster in Madrid, based on the Sentinel General Practitioner Network: 1997-2004. *BMC Infect Dis* 2007; **7**: 59 [PMID: 17570859 DOI: 10.1186/1471-2334-7-59]

111 **Nicolosi A**, Sturkenboom M, Mannino S, Arpinelli F, Cantarutti L, Giaquinto C. The incidence of varicella: correction of a common error. *Epidemiology* 2003; **14**: 99-102 [PMID: 12500056 DOI: 10.1097/00001648-200301000-00024]

112 **National Institute of Public Health**. Reports on cases of infectious diseases and poisonings in Poland. Available from: URL: http://wwwold.pzh.gov.pl/oldpage/epimeld/index_a.html

113 **Arama V**, Rafila A, Streinu-Cercel A, Pistol A, Bacruban R, Sandu R, Pitigoi D, Negoita A. Varicella in Romania: epidemiological trends, 1986-2004. *Euro Surveill* 2005; **10**: E050811.6 [PMID: 16785684]

114 **Wharton M**. The epidemiology of varicella-zoster virus infections. *Infect Dis Clin North Am* 1996; **10**: 571-581 [PMID: 8856352 DOI: 10.1016/S0891-5520(05)70313-5]

115 **Brisson M**, Edmunds WJ, Gay NJ, Law B, De Serres G. Modelling the impact of immunization on the epidemiology of varicella zoster virus. *Epidemiol Infect* 2000; **125**: 651-669 [PMID: 11218215 DOI: 10.1017/S0950268800004714]

116 **Meyer PA**, Seward JF, Jumaan AO, Wharton M. Varicella mortality: trends before vaccine licensure in the United States, 1970-1994. *J Infect Dis* 2000; **182**: 383-390 [PMID: 10915066 DOI: 10.1086/315714]

117 **Bramley JC**, Jones IG. Epidemiology of chickenpox in Scotland: 1981 to 1998. *Commun Dis Public Health* 2000; **3**: 282-287 [PMID: 11280260]

118 **Fairley CK**, Miller E. Varicella-zoster virus epidemiology--a changing scene? *J Infect Dis* 1996; **174** Suppl 3: S314-S319 [PMID: 8896538]

119 **Boëlle PY**, Hanslik T. Varicella in non-immune persons: incidence, hospitalization and mortality rates. *Epidemiol Infect* 2002; **129**: 599-606 [PMID: 12558344 DOI: 10.1017/S0950268802007720]

120 **Chant KG**, Sullivan EA, Burgess MA, Ferson MJ, Forrest JM, Baird LM, Tudehope DI, Tilse M. Varicella-zoster virus infection in Australia. *Aust N Z J Public Health* 1998; **22**: 413-418 [PMID: 9659764 DOI: 10.1111/j.1467-842X.1998.tb01405.x]

121 **Rawson H**, Crampin A, Noah N. Deaths from chickenpox in England and Wales 1995-7: analysis of routine mortality data. *BMJ* 2001; **323**: 1091-1093 [PMID: 11701571 DOI: 10.1136/

bmj.323.7321.1091]
122 **Akram DS**, Qureshi H, Mahmud A, Khan AA, Kundi Z, Shafi S, N-ur-Rehman B, Weil J, Bock H, Yazdani I. Seroepidemiology of varicella-zoster in Pakistan. *Southeast Asian J Trop Med Public Health* 2000; **31**: 646-649 [PMID: 11414405]
123 **Garnett GP**, Cox MJ, Bundy DA, Didier JM, St Catharine J. The age of infection with varicella-zoster virus in St Lucia, West Indies. *Epidemiol Infect* 1993; **110**: 361-372 [PMID: 8386097 DOI: 10.1017/S0950268800068308]
124 **Lokeshwar MR**, Agrawal A, Subbarao SD, Chakraborty MS, Ram Prasad AV, Weil J, Bock HL, Kanwal S, Shah RC, Shah N. Age related seroprevalence of antibodies to varicella in India. *Indian Pediatr* 2000; **37**: 714-719 [PMID: 10906803]
125 **Lolekha S**, Tanthiphabha W, Sornchai P, Kosuwan P, Sutra S, Warachit B, Chup-Upprakarn S, Hutagalung Y, Weil J, Bock HL. Effect of climatic factors and population density on varicella zoster virus epidemiology within a tropical country. *Am J Trop Med Hyg* 2001; **64**: 131-136 [PMID: 11442207]
126 **Poveda JD**, Babin M, Bonnici JF, du Pasquier P, Fleury HJ. [Serological study of the occurrence of Herpesviridae in French Guyana]. *Bull Soc Pathol Exot Filiales* 1986; **79**: 207-212 [PMID: 3015441]
127 **Sixl W**, Schneeweiss WD, Withalm H, Schuhmann G, Rosegger H. Serological testing of human blood samples for infectious diseases in the Abeokuta and the Minna Hospitals/Nigeria. *J Hyg Epidemiol Microbiol Immunol* 1987; **31**: 490-492 [PMID: 3443750]
128 **Sixl W**, Sixl-Voigt B. Serological screenings of various infectious diseases on the Cape Verde Islands (West Africa). *J Hyg Epidemiol Microbiol Immunol* 1987; **31**: 469-471 [PMID: 3443744]
129 **Lee BW**. Review of varicella zoster seroepidemiology in India and Southeast Asia. *Trop Med Int Health* 1998; **3**: 886-890 [PMID: 9855401 DOI: 10.1046/j.1365-3156.1998.00316.x]
130 **Venkitaraman AR**, John TJ. The epidemiology of varicella in staff and students of a hospital in the tropics. *Int J Epidemiol* 1984; **13**: 502-505 [PMID: 6519892 DOI: 10.1093/ije/13.4.502]
131 **Gershon AA**, Raker R, Steinberg S, Topf-Olstein B, Drusin LM. Antibody to Varicella-Zoster virus in parturient women and their offspring during the first year of life. *Pediatrics* 1976; **58**: 692-696 [PMID: 185578]
132 **Dworkin RH**. Racial differences in herpes zoster and age at onset of varicella. *J Infect Dis* 1996; **174**: 239-241 [PMID: 8656005 DOI: 10.1093/infdis/174.1.239]
133 **Sinha DP**. Chickenpox--a disease predominantly affecting adults in rural West Bengal, India. *Int J Epidemiol* 1976; **5**: 367-374 [PMID: 1010666 DOI: 10.1093/ije/5.4.367]
134 **Mandal BK**, Mukherjee PP, Murphy C, Mukherjee R, Naik T. Adult susceptibility to varicella in the tropics is a rural phenomenon due to the lack of previous exposure. *J Infect Dis* 1998; **178** Suppl 1: S52-S54 [PMID: 9852974 DOI: 10.1086/514262]
135 **Poulsen A**, Cabral F, Nielsen J, Roth A, Lisse IM, Vestergaard BF, Aaby P. Varicella zoster in Guinea-Bissau: intensity of exposure and severity of infection. *Pediatr Infect Dis J* 2005; **24**: 102-107 [PMID: 15702036 DOI: 10.1097/01.inf.0000151034.15747.4a]
136 **Welgama U**, Wickramasinghe C, Perera J. Varicella-zoster virus infection in the Infectious Diseases Hospital, Sri Lanka. *Ceylon Med J* 2003; **48**: 119-121 [PMID: 15125402 DOI: 10.4038/cmj.v48i4.3327]
137 **Weller TH**. Varicella: historical perspective and clinical overview. *J Infect Dis* 1996; **174** Suppl 3: S306-S309 [PMID: 8896536 DOI: 10.1093/infdis/174.Supplement_3.S306]
138 **Leikin E**, Figueroa R, Bertkau A, Lysikiewicz A, Visintainer P, Tejani N. Seronegativity to varicella-zoster virus in a tertiary care obstetric population. *Obstet Gynecol* 1997; **90**: 511-513 [PMID: 9380306 DOI: 10.1016/S0029-7844(97)00353-0]
139 **Morgan-Capner P**, Crowcroft NS. Guidelines on the management of, and exposure to, rash illness in pregnancy (including consideration of relevant antibody screening programmes in pregnancy). *Commun Dis Public Health* 2002; **5**: 59-71 [PMID: 12070980]
140 **Centers for Disease Control and Prevention (CDC)**. Decline in annual incidence of varicella--selected states, 1990-2001. *MMWR Morb Mortal Wkly Rep* 2003; **52**: 884-885 [PMID: 13679791]
141 **Donahue JG**, Choo PW, Manson JE, Platt R. The incidence of herpes zoster. *Arch Intern Med* 1995; **155**: 1605-1609 [PMID: 7618983 DOI: 10.1001/archinte.1995.00430150071008]
142 **Skull SA**, Wang EE. Varicella vaccination--a critical review of the evidence. *Arch Dis Child* 2001; **85**: 83-90 [PMID: 11466178 DOI: 10.1136/adc.85.2.83]
143 **Troughton JA**, Crealey G, Crawford V, Coyle PV. Management of varicella contacts in pregnancy: VZIG or vaccination? *J Clin Virol* 2009; **46**: 345-348 [PMID: 19828369 DOI: 10.1016/j.jcv.2009.09.014]
144 **Stagno S**, Whitley RJ. Herpesvirus infections of pregnancy. Part II: Herpes simplex virus and varicella-zoster virus infections. *N Engl J Med* 1985; **313**: 1327-1330 [PMID: 3903503 DOI: 10.1056/NEJM198511213132105]
145 **Enders G**, Miller E, Cradock-Watson J, Bolley I, Ridehalgh M. Consequences of varicella and herpes zoster in pregnancy: prospective study of 1739 cases. *Lancet* 1994; **343**: 1548-1551 [PMID: 7802767 DOI: 10.1016/S0140-6736(94)92943-2]
146 **Sever J**, White LR. Intrauterine viral infections. *Annu Rev Med* 1968; **19**: 471-486 [PMID: 4172728 DOI: 10.1146/annurev.me.19.020168.002351]
147 **Sauerbrei A**, Wutzler P. The congenital varicella syndrome. *J Perinatol* 2000; **20**: 548-554 [PMID: 11190597 DOI: 10.1038/sj.jp.7200457]
148 **Mirlesse V**, Lebon P. [Chickenpox during pregnancy]. *Arch Pediatr* 2003; **10**: 1113-1118 [PMID: 14643554 DOI: 10.1016/j.arcped.2003.09.036]
149 **McKendrick MW**, Lau J, Alston S, Bremner J. VZV infection in pregnancy: a retrospective review over 5 years in Sheffield and discussion on the potential utilisation of varicella vaccine in prevention. *J Infect* 2007; **55**: 64-67 [PMID: 17418420 DOI: 10.1016/j.jinf.2007.02.003]
150 **Talukder YS**, Kafatos G, Pinot de Moira A, Aquilina J, Parker SP, Crowcroft NS, Brown DW, Breuer J. The seroepidemiology of varicella zoster virus among pregnant Bangladeshi and white British women in the London Borough of Tower Hamlets, UK. *Epidemiol Infect* 2007; **135**: 1344-1353 [PMID: 17445317 DOI: 10.1017/S0950268807008497]
151 **Daley AJ**, Thorpe S, Garland SM. Varicella and the pregnant woman: prevention and management. *Aust N Z J Obstet Gynaecol* 2008; **48**: 26-33 [PMID: 18275568 DOI: 10.1111/j.1479-828X.2007.00797.x]
152 **Harger JH**, Ernest JM, Thurnau GR, Moawad A, Thom E, Landon MB, Paul R, Miodovnik M, Dombrowski M, Sibai B, Van Dorsten P, McNellis D. Frequency of congenital varicella syndrome in a prospective cohort of 347 pregnant women. *Obstet Gynecol* 2002; **100**: 260-265 [PMID: 12151147 DOI: 10.1097/00006250-200208000-00010]
153 **Sauerbrei A**, Wutzler P. Neonatal varicella. *J Perinatol* 2001; **21**: 545-549 [PMID: 11774017 DOI: 10.1038/sj.jp.7210599]
154 **Landsberger EJ**, Hager WD, Grossman JH. Successful management of varicella pneumonia complicating pregnancy. A report of three cases. *J Reprod Med* 1986; **31**: 311-314 [PMID: 3746781]
155 **Harris RE**, Rhoades ER. Varicella pneumonia complicating pregnancy. report of a case and review of literature. *Obstet Gynecol* 1965; **25**: 734-740 [PMID: 14289538]
156 **Gardella C**, Brown ZA. Managing varicella zoster infection in pregnancy. *Cleve Clin J Med* 2007; **74**: 290-296 [PMID: 17438678 DOI: 10.3949/ccjm.74.4.290]
157 **Schutte TJ**, Rogers LC, Copas PR. Varicella pneumonia complicating pregnancy: a report of seven cases. *Infect Dis Obstet Gynecol* 1996; **4**: 338-346 [PMID: 18476122 DOI: 10.1155/S1064744996000683]
158 **Smith CK**, Arvin AM. Varicella in the fetus and newborn. *Semin Fetal Neonatal Med* 2009; **14**: 209-217 [PMID: 19097954 DOI: 10.1016/j.siny.2008.11.008]
159 **Sauerbrei A**. Review of varicella-zoster virus infections in pregnant women and neonates. *Health* 2010; **2**: 143-152 [DOI: 10.4236/health.2010.22022]

160 **Broussard RC**, Payne DK, George RB. Treatment with acyclovir of varicella pneumonia in pregnancy. *Chest* 1991; **99**: 1045-1047 [PMID: 2009766 DOI: 10.1378/chest.99.4.1045]

161 **Greffe BS**, Dooley SL, Deddish RB, Krasny HC. Transplacental passage of acyclovir. *J Pediatr* 1986; **108**: 1020-1021 [PMID: 3012053 DOI: 10.1016/S0022-3476(86)80954-4]

162 **Chandra PC**, Patel H, Schiavello HJ, Briggs SL. Successful pregnancy outcome after complicated varicella pneumonia. *Obstet Gynecol* 1998; **92**: 680-682 [PMID: 9764662 DOI: 10.1097/00006250-199810001-00018]

163 **Harger JH**, Ernest JM, Thurnau GR, Moawad A, Momirova V, Landon MB, Paul R, Miodovnik M, Dombrowski M, Sibai B, Van Dorsten P. Risk factors and outcome of varicella-zoster virus pneumonia in pregnant women. *J Infect Dis* 2002; **185**: 422-427 [PMID: 11865393 DOI: 10.1086/338832]

164 **Mouly F**, Mirlesse V, Méritet JF, Rozenberg F, Poissonier MH, Lebon P, Daffos F. Prenatal diagnosis of fetal varicella-zoster virus infection with polymerase chain reaction of amniotic fluid in 107 cases. *Am J Obstet Gynecol* 1997; **177**: 894-898 [PMID: 9369842 DOI: 10.1016/S0002-9378(97)70291-6]

165 **Sauerbrei A**, Wutzler P. Herpes simplex and varicella-zoster virus infections during pregnancy: current concepts of prevention, diagnosis and therapy. Part 2: Varicella-zoster virus infections. *Med Microbiol Immunol* 2007; **196**: 95-102 [PMID: 17180380 DOI: 10.1007/s00430-006-0032-z]

166 **Pastuszak AL**, Levy M, Schick B, Zuber C, Feldkamp M, Gladstone J, Bar-Levy F, Jackson E, Donnenfeld A, Meschino W. Outcome after maternal varicella infection in the first 20 weeks of pregnancy. *N Engl J Med* 1994; **330**: 901-905 [PMID: 8114861 DOI: 10.1056/NEJM199403313301305]

167 **Birthistle K**, Carrington D. Fetal varicella syndrome--a reappraisal of the literature. A review prepared for the UK Advisory Group on Chickenpox on behalf of the British Society for the Study of Infection. *J Infect* 1998; **36** Suppl 1: 25-29 [PMID: 9514105]

168 **Alkalay AL**, Pomerance JJ, Rimoin DL. Fetal varicella syndrome. *J Pediatr* 1987; **111**: 320-323 [PMID: 3625399 DOI: 10.1016/S0022-3476(87)80447-X]

169 **Mandelbrot L**. Fetal varicella - diagnosis, management, and outcome. *Prenat Diagn* 2012; **32**: 511-518 [PMID: 22514124 DOI: 10.1002/pd.3843]

170 **Schulze A**, Dietzsch HJ. The natural history of varicella embryopathy: a 25-year follow-up. *J Pediatr* 2000; **137**: 871-874 [PMID: 11113846 DOI: 10.1067/mpd.2000.109005]

171 **Higa K**, Dan K, Manabe H. Varicella-zoster virus infections during pregnancy: hypothesis concerning the mechanisms of congenital malformations. *Obstet Gynecol* 1987; **69**: 214-222 [PMID: 3027637]

172 **Grose C**. Congenital varicella-zoster virus infection and the failure to establish virus-specific cell-mediated immunity. *Mol Biol Med* 1989; **6**: 453-462 [PMID: 2560525]

173 **Sauerbrei A**, Wutzler P. Varicella-Zoster Virus Infections During Pregnancy: Epidemiology, Clinical Symptoms, Diagnosis, Prevention and Therapy. *Curr Pediat Rev* 2005; **1**: 205-215 [DOI: 10.2174/157339605774574962]

174 **Prober CG**, Gershon AA, Grose C, McCracken GH, Nelson JD. Consensus: varicella-zoster infections in pregnancy and the perinatal period. *Pediatr Infect Dis J* 1990; **9**: 865-869 [PMID: 2277741 DOI: 10.1097/00006454-199012000-00001]

175 **Balducci J**, Rodis JF, Rosengren S, Vintzileos AM, Spivey G, Vosseller C. Pregnancy outcome following first-trimester varicella infection. *Obstet Gynecol* 1992; **79**: 5-6 [PMID: 1727585]

176 **Jones KL**, Johnson KA, Chambers CD. Offspring of women infected with varicella during pregnancy: a prospective study. *Teratology* 1994; **49**: 29-32 [PMID: 8171394 DOI: 10.1002/tera.1420490106]

177 **Dufour P**, de Bièvre P, Vinatier D, Tordjeman N, Da Lage B, Vanhove J, Monnier JC. Varicella and pregnancy. *Eur J Obstet Gynecol Reprod Biol* 1996; **66**: 119-123 [PMID: 8735731 DOI: 10.1016/0301-2115(96)02395-0]

178 **Figueroa-Damian R**, Arredondo-Garcia JL. Perinatal outcome of pregnancies complicated with varicella infection during the first 20 weeks of gestation. *Am J Perinatol* 1997; **14**: 411-414 [PMID: 9263561 DOI: 10.1055/s-2007-994170]

179 **Bai PV**, John TJ. Congenital skin ulcers following varicella in late pregnancy. *J Pediatr* 1979; **94**: 65-67 [PMID: 758425 DOI: 10.1016/S0022-3476(79)80354-6]

180 **Kerkering KW**. Abnormal cry and intracranial calcifications: clues to the diagnosis of fetal varicella-zoster syndrome. *J Perinatol* 2001; **21**: 131-135 [PMID: 11324360 DOI: 10.1038/sj.jp.7200503]

181 **Deasy NP**, Jarosz JM, Cox TC, Hughes E. Congenital varicella syndrome: cranial MRI in a long-term survivor. *Neuroradiology* 1999; **41**: 205-207 [PMID: 10206169 DOI: 10.1007/s002340050736]

182 **Michie CA**, Acolet D, Charlton R, Stevens JP, Happerfield LC, Bobrow LG, Kangro H, Gau G, Modi N. Varicella-zoster contracted in the second trimester of pregnancy. *Pediatr Infect Dis J* 1992; **11**: 1050-1053 [PMID: 1461697 DOI: 10.1097/00006454-199211120-00012]

183 **Lambert SR**, Taylor D, Kriss A, Holzel H, Heard S. Ocular manifestations of the congenital varicella syndrome. *Arch Ophthalmol* 1989; **107**: 52-56 [PMID: 2910286 DOI: 10.1001/archo pht.1989.01070010054026]

184 **Salzman MB**, Sood SK. Congenital anomalies resulting from maternal varicella at 25 1/2 weeks of gestation. *Pediatr Infect Dis J* 1992; **11**: 504-505 [PMID: 1608698 DOI: 10.1097/00006454-199206000-00023]

185 **Ong CL**, Daniel ML. Antenatal diagnosis of a porencephalic cyst in congenital varicella-zoster virus infection. *Pediatr Radiol* 1998; **28**: 94 [PMID: 9472052 DOI: 10.1007/s002470050301]

186 **Forrest J**, Mego S, Burgess M. Congenital and neonatal varicella in Australia. *J Paediatr Child Health* 2000; **36**: 108-113 [PMID: 10760005 DOI: 10.1046/j.1440-1754.2000.00474.x]

187 **Koren G**. Congenital varicella syndrome in the third trimester. *Lancet* 2005; **366**: 1591-1592 [PMID: 16271630 DOI: 10.1016/S0140-6736(05)67643-X]

188 **Health Protection Agency**. Guidance on Viral Rash in Pregnancy: Investigation, Diagnosis and Management of Viral Rash Illnes or Exposure to Viral Rash Illnesss, in Pregnancy. Version 1, January 2011. 4.1.4. Chichenpox: page 20. [accessed 2016 Apr 4]. Available from: URL: https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/322688/Viral_rash_in_pregnancy_guidance.pdf

189 **Connan L**, Ayoubi J, Icart J, Halasz A, Thene M, Berrebi A. Intrauterine fetal death following maternal varicella infection. *Eur J Obstet Gynecol Reprod Biol* 1996; **68**: 205-207 [PMID: 8886708 DOI: 10.1016/0301-2115(96)02394-9]

190 **Siegel M**. Congenital malformations following chickenpox, measles, mumps, and hepatitis. Results of a cohort study. *JAMA* 1973; **226**: 1521-1524 [PMID: 4800931 DOI: 10.1001/jama. 226.13.1521]

191 **Miller E**, Cradock-Watson JE, Ridehalgh MK. Outcome in newborn babies given anti-varicella-zoster immunoglobulin after perinatal maternal infection with varicella-zoster virus. *Lancet* 1989; **2**: 371-373 [PMID: 2569560 DOI: 10.1016/S0140-6736(89)90547-3]

192 **Akisu M**, Yalaz M, Aksu G, Arslanoglu S, Genel F, Kutukculer N, Kultursay N. Maternally acquired varicella-zoster virus antibodies disappear at 6 months of age in prematurely born children. *Panminerva Med* 2003; **45**: 155-156 [PMID: 12855941]

193 **Heidl M**. Varicella-Zoster-Virus-Infektion in der Schwangerschaft, beim Neugeborenen un jungen Säugling. *Z Lin Med* 1985; **40**: 245-250

194 **Yu HR**, Chang JC, Chen RF, Chuang H, Hong KC, Wang L, Yang KD. Different antigens trigger different Th1/Th2 reactions in neonatal mononuclear cells (MNCs) relating to T-bet/GATA-3 expression. *J Leukoc Biol* 2003; **74**: 952-958 [PMID: 12960249 DOI: 10.1189/jlb.0902474]

195 **Maródi L**. Neonatal innate immunity to infectious agents. *Infect Immun* 2006; **74**: 1999-2006 [PMID: 16552028 DOI: 10.1128/IAI.74.4.1999-2006.2006]

196 **Meyers JD**. Congenital varicella in term infants: risk reconsidered. *J Infect Dis* 1974; **129**: 215-217 [PMID: 4129828 DOI: 10.1093/

infdis/129.2.215]
197 **Gershon AA**. Varicella in mother and infants: problems old and new. In: Krugman S, Gershon AA. Infections of the fetus and the newborn infant: progress in clinical and biological research. Vol 3. New York: Alan R Liss Inc, 1985: 79-85
198 **Cox SM**, Cunningham FG, Luby J. Management of varicella pneumonia complicating pregnancy. *Am J Perinatol* 1990; **7**: 300-301 [PMID: 2222616 DOI: 10.1055/s-2007-999508]
199 **Centers for Disease Control and Prevention (CDC)**. Prevention of varicella: Recommendations of the Advisory Committee on Immunization Practices (ACIP). Centers for Disease Control and Prevention. *MMWR Recomm Rep* 1996; **45**: 1-36 [PMID: 8668119]
200 **Paulman PM**, McLellan R. Varicella during pregnancy: the timing of effective treatment. *J Am Board Fam Pract* 1990; **3**: 121-123 [PMID: 2333759]
201 **Zieger W**, Friese K, Weigel M, Becker KP, Melchert F. Varicella infection at birth. *Z Geburtshilfe Perinatol* 1994; **198**: 134-137
202 **Rodríguez-Fanjul X**, Noguera A, Vicente A, González-Enseñat MA, Jiménez R, Fortuny C. Herpes zoster in healthy infants and toddlers after perinatal exposure to varicella-zoster virus: a case series and review of the literature. *Pediatr Infect Dis J* 2010; **29**: 574-576 [PMID: 20516836 DOI: 10.1097/INF.0b013e3181d76f7f]
203 **Sauerbrei A**, Wutzler P. Das fetale Varizellensyndrom. *Monatsschr Kinderheilkd* 2003; **151**: 209-213 [DOI: 10.1007/s00112-002-0478-z]
204 **Takahashi M**, Otsuka T, Okuno Y, Asano Y, Yazaki T. Live vaccine used to prevent the spread of varicella in children in hospital. *Lancet* 1974; **2**: 1288-1290 [PMID: 4139526 DOI: 10.1016/S0140-6736(74)90144-5]
205 **Takahashi M**, Baba K, Horiuchi K, Kamiya H, Asano Y. A live varicella vaccine. *Adv Exp Med Biol* 1990; **278**: 49-58 [PMID: 1963045 DOI: 10.1007/978-1-4684-5853-4_6]
206 **Marin M**, Güris D, Chaves SS, Schmid S, Seward JF. Prevention of varicella: recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recomm Rep* 2007; **56**: 1-40 [PMID: 17585291]
207 **Nguyen HQ**, Jumaan AO, Seward JF. Decline in mortality due to varicella after implementation of varicella vaccination in the United States. *N Engl J Med* 2005; **352**: 450-458 [PMID: 15689583 DOI: 10.1056/NEJMoa042271]
208 **Marin M**, Meissner HC, Seward JF. Varicella prevention in the United States: a review of successes and challenges. *Pediatrics* 2008; **122**: e744-e751 [PMID: 18762511 DOI: 10.1542/peds.2008-0567]
209 **Guris D**, Jumaan AO, Mascola L, Watson BM, Zhang JX, Chaves SS, Gargiullo P, Perella D, Civen R, Seward JF. Changing varicella epidemiology in active surveillance sites--United States, 1995-2005. *J Infect Dis* 2008; **197** Suppl 2: S71-S75 [PMID: 18419413 DOI: 10.1086/522156]
210 **Gidding HF**, Brisson M, Macintyre CR, Burgess MA. Modelling the impact of vaccination on the epidemiology of varicella zoster virus in Australia. *Aust N Z J Public Health* 2005; **29**: 544-551 [PMID: 16366065 DOI: 10.1111/j.1467-842X.2005.tb00248.x]
211 **Seward JF**, Watson BM, Peterson CL, Mascola L, Pelosi JW, Zhang JX, Maupin TJ, Goldman GS, Tabony LJ, Brodovicz KG, Jumaan AO, Wharton M. Varicella disease after introduction of varicella vaccine in the United States, 1995-2000. *JAMA* 2002; **287**: 606-611 [PMID: 11829699 DOI: 10.1001/jama.287.5.606]
212 **Seward JF**, Marin M, Vázquez M. Varicella vaccine effectiveness in the US vaccination program: a review. *J Infect Dis* 2008; **197** Suppl 2: S82-S89 [PMID: 18419415 DOI: 10.1086/522145]
213 **Dayan GH**, Panero MS, Debbag R, Urquiza A, Molina M, Prieto S, Del Carmen Perego M, Scagliotti G, Galimberti D, Carroli G, Wolff C, Schmid DS, Loparev V, Guris D, Seward J. Varicella seroprevalence and molecular epidemiology of varicella-zoster virus in Argentina, 2002. *J Clin Microbiol* 2004; **42**: 5698-5704 [PMID: 15583301 DOI: 10.1128/JCM.42.12.5698-5704.2004]
214 **Vázquez M**, LaRussa PS, Gershon AA, Niccolai LM, Muehlenbein CE, Steinberg SP, Shapiro ED. Effectiveness over time of varicella vaccine. *JAMA* 2004; **291**: 851-855 [PMID: 14970064 DOI: 10.1001/jama.291.7.851]
215 **Saiman L**, LaRussa P, Steinberg SP, Zhou J, Baron K, Whittier S, Della-Latta P, Gershon AA. Persistence of immunity to varicella-zoster virus after vaccination of healthcare workers. *Infect Control Hosp Epidemiol* 2001; **22**: 279-283 [PMID: 11428437 DOI: 10.1086/501900]
216 **Ampofo K**, Saiman L, LaRussa P, Steinberg S, Annunziato P, Gershon A. Persistence of immunity to live attenuated varicella vaccine in healthy adults. *Clin Infect Dis* 2002; **34**: 774-779 [PMID: 11830801 DOI: 10.1086/338959]
217 **Watson B**. Varicella: a vaccine preventable disease? *J Infect* 2002; **44**: 220-225 [PMID: 12099727]
218 **Vázquez M**, Shapiro ED. Varicella vaccine and infection with varicella-zoster virus. *N Engl J Med* 2005; **352**: 439-440 [PMID: 15689581 DOI: 10.1056/NEJMp048320]
219 **White CJ**, Kuter BJ, Hildebrand CS, Isganitis KL, Matthews H, Miller WJ, Provost PJ, Ellis RW, Gerety RJ, Calandra GB. Varicella vaccine (VARIVAX) in healthy children and adolescents: results from clinical trials, 1987 to 1989. *Pediatrics* 1991; **87**: 604-610 [PMID: 1850506]
220 **Bernstein HH**, Rothstein EP, Watson BM, Reisinger KS, Blatter MM, Wellman CO, Chartrand SA, Cho I, Ngai A, White CJ. Clinical survey of natural varicella compared with breakthrough varicella after immunization with live attenuated Oka/Merck varicella vaccine. *Pediatrics* 1993; **92**: 833-837 [PMID: 8233746]
221 **Sharrar RG**, LaRussa P, Galea SA, Steinberg SP, Sweet AR, Keatley RM, Wells ME, Stephenson WP, Gershon AA. The post-marketing safety profile of varicella vaccine. *Vaccine* 2000; **19**: 916-923 [PMID: 11115716 DOI: 10.1016/S0264-410X(00)00297-8]
222 **Chaves SS**, Zhang J, Civen R, Watson BM, Carbajal T, Perella D, Seward JF. Varicella disease among vaccinated persons: clinical and epidemiological characteristics, 1997-2005. *J Infect Dis* 2008; **197** Suppl 2: S127-S131 [PMID: 18419385 DOI: 10.1086/522150]
223 **Bonanni P**, Gershon A, Gershon M, Kulcsár A, Papaevangelou V, Rentier B, Sadzot-Delvaux C, Usonis V, Vesikari T, Weil-Olivier C, de Winter P, Wutzler P. Primary versus secondary failure after varicella vaccination: implications for interval between 2 doses. *Pediatr Infect Dis J* 2013; **32**: e305-e313 [PMID: 23838789 DOI: 10.1097/INF.0b013e31828b7def]
224 **Laboratory Diagnosis of Varicella Zoster Virus Infections**. [accessed 2016 Apr 4]. Available from: URL: http://virology-online.com/viruses/VZV5.htm
225 **Johnson CE**, Stancin T, Fattlar D, Rome LP, Kumar ML. A long-term prospective study of varicella vaccine in healthy children. *Pediatrics* 1997; **100**: 761-766 [PMID: 9346974]
226 **Asano Y**, Suga S, Yoshikawa T, Kobayashi I, Yazaki T, Shibata M, Tsuzuki K, Ito S. Experience and reason: twenty-year follow-up of protective immunity of the Oka strain live varicella vaccine. *Pediatrics* 1994; **94**: 524-526 [PMID: 7936864]
227 **Kuter B**, Matthews H, Shinefield H, Black S, Dennehy P, Watson B, Reisinger K, Kim LL, Lupinacci L, Hartzel J, Chan I. Ten year follow-up of healthy children who received one or two injections of varicella vaccine. *Pediatr Infect Dis J* 2004; **23**: 132-137 [PMID: 14872179 DOI: 10.1097/01.inf.0000109287.97518.67]
228 **Ludwig B**, Kraus FB, Allwinn R, Keim S, Doerr HW, Buxbaum S. Loss of varicella zoster virus antibodies despite detectable cell mediated immunity after vaccination. *Infection* 2006; **34**: 222-226 [PMID: 16896582 DOI: 10.1007/s15010-006-5616-9]
229 Immunization of health-care workers: recommendations of the Advisory Committee on Immunization Practices (ACIP) and the Hospital Infection Control Practices Advisory Committee (HICPAC). *MMWR Recomm Rep* 1997; **46**: 1-42 [PMID: 9427216]
230 **Gershon AA**, LaRussa PS. Varicella vaccine. *Pediatr Infect Dis J* 1998; **17**: 248-249 [PMID: 9535255 DOI: 10.1097/00006454-199509000-00003]
231 **Rubin LG**, Levin MJ, Ljungman P, Davies EG, Avery R, Tomblyn M, Bousvaros A, Dhanireddy S, Sung L, Keyserling H, Kang I. 2013 IDSA clinical practice guideline for vaccination of the immunocompromised host. *Clin Infect Dis* 2014; **58**: 309-318 [PMID: 24421306 DOI: 10.1093/cid/cit816]

232 **White CJ**. Clinical trials of varicella vaccine in healthy children. *Infect Dis Clin North Am* 1996; **10**: 595-608 [PMID: 8856354 DOI: 10.1016/S0891-5520(05)70315-9]

233 **Suzuki K**, Yoshikawa T, Tomitaka A, Matsunaga K, Asano Y. Detection of aerosolized varicella-zoster virus DNA in patients with localized herpes zoster. *J Infect Dis* 2004; **189**: 1009-1012 [PMID: 14999603 DOI: 10.1086/382029]

234 **Barrett-Muir W**, Scott FT, Aaby P, John J, Matondo P, Chaudhry QL, Siqueira M, Poulsen A, Yaminishi K, Breuer J. Genetic variation of varicella-zoster virus: evidence for geographical separation of strains. *J Med Virol* 2003; **70** Suppl 1: S42-S47 [PMID: 12627486 DOI: 10.1002/jmv.10319]

235 **Peters GA**, Tyler SD, Grose C, Severini A, Gray MJ, Upton C, Tipples GA. A full-genome phylogenetic analysis of varicella-zoster virus reveals a novel origin of replication-based genotyping scheme and evidence of recombination between major circulating clades. *J Virol* 2006; **80**: 9850-9860 [PMID: 16973589 DOI: 10.1128/JVI.00715-06]

236 **Ferson MJ**. Varicella vaccine in post-exposure prophylaxis. *Commun Dis Intell* 2001; **25**: 13-15 [PMID: 11280194]

237 **Watson B**, Seward J, Yang A, Witte P, Lutz J, Chan C, Orlin S, Levenson R. Postexposure effectiveness of varicella vaccine. *Pediatrics* 2000; **105**: 84-88 [PMID: 10617709 DOI: 10.1542/peds.105.1.84]

238 **Macartney K**, McIntyre P. Vaccines for post-exposure prophylaxis against varicella (chickenpox) in children and adults. *Cochrane Database Syst Rev* 2008; **16**: CD001833 [PMID: 18646079 DOI: 10.1002/14651858.CD001833.pub2]

239 **American Academy of Pediatrics**. Committee on Infectious Diseases. Varicella vaccine update. *Pediatrics* 2000; **105**: 136-141 [PMID: 10617719]

240 **Australian Technical Advisory Group on Immunisation**. The Australian Immunisation Handbook 10th. 4.22: Varicella. [updated 2015 Jun; accessed 2016 Apr 4]. Available from: URL: http://www.immunise.health.gov.au/internet/immunise/publishing.nsf/Content/Handbook10-home~handbook10part4~handbook10-4-22

241 **Bohlke K**, Galil K, Jackson LA, Schmid DS, Starkovich P, Loparev VN, Seward JF. Postpartum varicella vaccination: is the vaccine virus excreted in breast milk? *Obstet Gynecol* 2003; **102**: 970-977 [PMID: 14672472 DOI: 10.1016/S0029-7844(03)00860-3]

242 **Oxman MN**, Levin MJ, Johnson GR, Schmader KE, Straus SE, Gelb LD, Arbeit RD, Simberkoff MS, Gershon AA, Davis LE, Weinberg A, Boardman KD, Williams HM, Zhang JH, Peduzzi PN, Beisel CE, Morrison VA, Guatelli JC, Brooks PA, Kauffman CA, Pachucki CT, Neuzil KM, Betts RF, Wright PF, Griffin MR, Brunell P, Soto NE, Marques AR, Keay SK, Goodman RP, Cotton DJ, Gnann JW, Loutit J, Holodniy M, Keitel WA, Crawford GE, Yeh SS, Lobo Z, Toney JF, Greenberg RN, Keller PM, Harbecke R, Hayward AR, Irwin MR, Kyriakides TC, Chan CY, Chan IS, Wang WW, Annunziato PW, Silber JL. A vaccine to prevent herpes zoster and postherpetic neuralgia in older adults. *N Engl J Med* 2005; **352**: 2271-2284 [PMID: 15930418 DOI: 10.1056/NEJMoa051016]

243 **Koch-Institut R**. Empfehlungen der Ständingen Impfkommission (STIKO) am Robert Koch-Institut/Stand. *Epidemiol Bull* 2004; **30**: 235-250

244 **Center for Disease Control (CDC)**. Manual for the Surveillance of Vaccine-Preventable Diseases Chapter 17: Varicella. [updated 2015 Oct; accessed 2016 Apr 4]. Available from: URL: http://www.cdc.gov/vaccines/pubs/surv-manual/chpt17-varicella.html

245 **Ogilvie MM**. Antiviral prophylaxis and treatment in chickenpox. A review prepared for the UK Advisory Group on Chickenpox on behalf of the British Society for the Study of Infection. *J Infect* 1998; **36** Suppl 1: 31-38 [PMID: 9514106 DOI: 10.1016/S0163-4453(98)80153-9]

246 **Department of Health, Joint Committee of Vaccination and Immunisation**. Immunisation against infectious disease. Stationery Office Books. 2016. [updated 2008 Jul 30; accessed 2016 Apr 4]. Available from: URL: http://webarchive.nationalarchives.gov.uk/20080910134953/http://dh.gov.uk/en/publichealth/healthprotection/immunisation/greenbook/dh_4097254

247 **Public Health England**. Varicella: the green book, chapter 34. [updated 2015 Aug 26; accessed 2016 Apr 4]. Available from: URL: https://www.gov.uk/government/publications/varicella-the-green-book-chapter-34

248 **Grose C**. Varicella vaccination of children in the United States: assessment after the first decade 1995-2005. *J Clin Virol* 2005; **33**: 89-95; discussion 96-98 [PMID: 15911422 DOI: 10.1016/j.jcv.2005.02.003]

249 **Salisbury D**, Ramsay M, Noakes K. Immunisation Against Infectious Disease-The Green Book 3. London: Varicella, 2006; 421-442. [accessed 2016 Apr 4]. Available from: URL: http://webarchive.nationalarchives.gov.uk/ /www.dh.gov.uk/prod_consum_dh/groups/dh_digitalassets/documents/digitalasset/dh_113539.pdf

250 **Snoeck R**, Andrei G, De Clercq E. Current pharmacological approaches to the therapy of varicella zoster virus infections: a guide to treatment. *Drugs* 1999; **57**: 187-206 [PMID: 10188760 DOI: 10.2165/00003495-199957020-00005]

251 **Brunell PA**. Varicella in pregnancy, the fetus, and the newborn: problems in management. *J Infect Dis* 1992; **166** Suppl 1: S42-S47 [PMID: 1624811 DOI: 10.1093/infdis/166.Supplement_1.S42]

252 **Heuchan AM**, Isaacs D. The management of varicella-zoster virus exposure and infection in pregnancy and the newborn period. Australasian Subgroup in Paediatric Infectious Diseases of the Australasian Society for Infectious Diseases. *Med J Aust* 2001; **174**: 288-292 [PMID: 11297117]

253 **Boxall EH**, Maple PA, Rathod P, Smit E. Follow-up of pregnant women exposed to chicken pox: an audit of relationship between level of antibody and development of chicken pox. *Eur J Clin Microbiol Infect Dis* 2011; **30**: 1193-1200 [PMID: 21455665 DOI: 10.1007/s10096-011-1211-4]

254 **Cohen A**, Moschopoulos P, Stiehm RE, Koren G. Congenital varicella syndrome: the evidence for secondary prevention with varicella-zoster immune globulin. *CMAJ* 2011; **183**: 204-208 [PMID: 21262937 DOI: 10.1503/cmaj.100615]

255 **Gruslin A**, Steben M, Halperin S, Money DM, Yudin MH. Immunization in pregnancy. *J Obstet Gynaecol Can* 2009; **31**: 1085-1101 [PMID: 20175349 DOI: 10.1016/S1701-2163(16) 34354-7]

256 **Nathwani D**, Maclean A, Conway S, Carrington D. Varicella infections in pregnancy and the newborn. A review prepared for the UK Advisory Group on Chickenpox on behalf of the British Society for the Study of Infection. *J Infect* 1998; **36** Suppl 1: 59-71 [PMID: 9514109 DOI: 10.1016/S0163-4453(98)80156-4]

257 **Centers for Disease Control and Prevention (CDC)**. A new product (VariZIG) for postexposure prophylaxis of varicella available under an investigational new drug application expanded access protocol. *MMWR Morb Mortal Wkly Rep* 2006; **55**: 209-210 [PMID: 16511443]

258 **Sharland M**, Cant A, Davies EG, Elliman DA, Esposito S, Finn A, Gray J, Heath PT, Lyall H, Pollard AJ, Ramsay ME, Riordan A, Shingadiaet D. Chicken Pox—Varicella Zoster. In: Manual of Childhood Infections. The Blue Book. Oxford: Oxford University Press, 2011: 467-474

259 **McIntosh D**, Isaacs D. Varicella zoster virus infection in pregnancy. *Arch Dis Child* 1993; **68**: 1-2 [PMID: 8192711 DOI: 10.1136/adc.68.1_Spec_No.1]

260 **Miller E**. Varicella zoster virus infection in pregnancy. *Arch Dis Child Fetal Neonatal Ed* 1994; **70**: F157-F158 [PMID: 8154911 DOI: 10.1136/fn.70.2.F157-b]

261 **Holland P**, Isaacs D, Moxon ER. Fatal neonatal varicella infection. *Lancet* 1986; **2**: 1156 [PMID: 2877295 DOI: 10.1016/S0140-6736(86)90556-8]

262 **King SM**, Gorensek M, Ford-Jones EL, Read SE. Fatal varicella-zoster infection in a newborn treated with varicella-zoster immunoglobulin. *Pediatr Infect Dis* 1986; **5**: 588-589 [PMID: 3020522 DOI: 10.1097/00006454-198609000-00020]

263 **Lipton SV**, Brunell PA. Management of varicella exposure in a neonatal intensive care unit. *JAMA* 1989; **261**: 1782-1784 [PMID: 2537441 DOI: 10.1001/jama.1989.03420120120037]

264 **Deutsche Gesellschaft für Pädiatrische Infektiologie**. Handbuch 2003: Infektionen im Kindesalter, Munchen: Futuramed, 2003:

732-739
265 **Suga S**, Yoshikawa T, Ozaki T, Asano Y. Effect of oral acyclovir against primary and secondary viraemia in incubation period of varicella. *Arch Dis Child* 1993; **69**: 639-642; discussion 642-643 [PMID: 8285774 DOI: 10.1136/adc.69.6.639]
266 **Wallace MR**, Bowler WA, Murray NB, Brodine SK, Oldfield EC. Treatment of adult varicella with oral acyclovir. A randomized, placebo-controlled trial. *Ann Intern Med* 1992; **117**: 358-363 [PMID: 1323943 DOI: 10.7326/0003-4819-117-5-358]
267 **Kempf W**, Meylan P, Gerber S, Aebi C, Agosti R, Büchner S, Coradi B, Garweg J, Hirsch H, Kind C, Lauper U, Lautenschlager S, Reusser P, Ruef C, Wunderli W, Nadal D. Swiss recommendations for the management of varicella zoster virus infections. *Swiss Med Wkly* 2007; **137**: 239-251 [PMID: 17557214]
268 **Tunbridge AJ**, Breuer J, Jeffery KJ. Chickenpox in adults - clinical management. *J Infect* 2008; **57**: 95-102 [PMID: 18555533 DOI: 10.1016/j.jinf.2008.03.004]
269 **Stone KM**, Reiff-Eldridge R, White AD, Cordero JF, Brown Z, Alexander ER, Andrews EB. Pregnancy outcomes following systemic prenatal acyclovir exposure: Conclusions from the international acyclovir pregnancy registry, 1984-1999. *Birth Defects Res A Clin Mol Teratol* 2004; **70**: 201-207 [PMID: 15108247 DOI: 10.1002/bdra.20013]
270 **Mohsen AH**, McKendrick M. Varicella pneumonia in adults. *Eur Respir J* 2003; **21**: 886-891 [PMID: 12765439 DOI: 10.1183/09031936.03.00103202]
271 **Martínez Segura JM**, Gutiérrez Oliver A, Maraví Poma E, Jiménez Urra I. [Severe chickenpox pneumonia]. *Rev Clin Esp* 2003; **203**: 591-594 [PMID: 14622509 DOI: 10.1016/S0014-2565(03)71365-4]
272 **Henderson GI**, Hu ZQ, Johnson RF, Perez AB, Yang Y, Schenker S. Acyclovir transport by the human placenta. *J Lab Clin Med* 1992; **120**: 885-892 [PMID: 1453110]
273 **Haddad J**, Simeoni U, Paira M, Lokiec F, Messer J, Willard D. [Transplacental passage of acyclovir]. *Presse Med* 1987; **16**: 1864 [PMID: 2962128]
274 **Ratanajamit C**, Vinther Skriver M, Jepsen P, Chongsuvivatwong V, Olsen J, Sørensen HT. Adverse pregnancy outcome in women exposed to acyclovir during pregnancy: a population-based observational study. *Scand J Infect Dis* 2003; **35**: 255-259 [PMID: 12839155 DOI: 10.1080/00365540310000229]
275 **Pasternak B**, Hviid A. Use of acyclovir, valacyclovir, and famciclovir in the first trimester of pregnancy and the risk of birth defects. *JAMA* 2010; **304**: 859-866 [PMID: 20736469 DOI: 10.1001/jama.2010.1206]
276 **Mills JL**, Carter TC. Acyclovir exposure and birth defects: an important advance, but more are needed. *JAMA* 2010; **304**: 905-906 [PMID: 20736478 DOI: 10.1001/jama.2010.1214]
277 **Acyclovir Pregnancy Registry and Valacyclovir Pregnancy Registry**. Final study report. 1 June 1984 through 30 April 1999. Glaxo Welcome, 1999
278 **Carter PE**, Duffy P, Lloyd DJ. Neonatal varicella infection. *Lancet* 1986; **2**: 1459-1460 [PMID: 2878305 DOI: 10.1016/S0140-6736(86)92766-2]
279 **Schulze-Oechtering F**, Roth B, Enders G, Grosser R. [Congenital varicella syndrome - is it infectious?]. *Z Geburtshilfe Neonatol* 2004; **208**: 25-28 [PMID: 15039888 DOI: 10.1055/s-2004-815519]
280 **Sauerbrei A**, Pawlak J, Luger C, Wutzler P. Intracerebral varicella-zoster virus reactivation in congenital varicella syndrome. *Dev Med Child Neurol* 2003; **45**: 837-840 [PMID: 14667077 DOI: 10.1017/S0012162203001555]
281 **Ross AH**. Modification of chicken pox in family contacts by administration of gamma globulin. *N Engl J Med* 1962; **267**: 369-376 [PMID: 14494142 DOI: 10.1056/NEJM196208232670801]
282 **Tyring SK**. Natural history of varicella zoster virus. *Semin Dermatol* 1992; **11**: 211-217 [PMID: 1390036]
283 **Mustonen K**, Mustakangas P, Valanne L, Professor MH, Koskiniemi M. Congenital varicella-zoster virus infection after maternal subclinical infection: clinical and neuropathological findings. *J Perinatol* 2001; **21**: 141-146 [PMID: 11324362 DOI: 10.1038/sj.jp.7200508]
284 **Seiler HE**. A study of herpes zoster particularly in its relationship to chickenpox. *J Hyg* (Lond) 1949; **47**: 253-262 [PMID: 15408424 DOI: 10.1017/S002217240001456X]
285 **Breuer J**. Herpes zoster: new insights provide an important wake-up call for management of nosocomial transmission. *J Infect Dis* 2008; **197**: 635-637 [PMID: 18260760 DOI: 10.1086/527421]
286 **Josephson A**, Gombert ME. Airborne transmission of nosocomial varicella from localized zoster. *J Infect Dis* 1988; **158**: 238-241 [PMID: 3392417 DOI: 10.1093/infdis/158.1.238]
287 **Lopez AS**, Burnett-Hartman A, Nambiar R, Ritz L, Owens P, Loparev VN, Guris D, Schmid DS. Transmission of a newly characterized strain of varicella-zoster virus from a patient with herpes zoster in a long-term-care facility, West Virginia, 2004. *J Infect Dis* 2008; **197**: 646-653 [PMID: 18260757 DOI: 10.1086/527419]
288 **Suzuki K**, Yoshikawa T, Tomitaka A, Suzuki K, Matsunaga K, Asano Y. Detection of varicella-zoster virus DNA in throat swabs of patients with herpes zoster and on air purifier filters. *J Med Virol* 2002; **66**: 567-570 [PMID: 11857538 DOI: 10.1002/jmv.2182]
289 **Yoshikawa T**, Ihira M, Suzuki K, Suga S, Tomitaka A, Ueda H, Asano Y. Rapid contamination of the environments with varicella-zoster virus DNA from a patient with herpes zoster. *J Med Virol* 2001; **63**: 64-66 [PMID: 11130889 DOI: 10.1002/1096-9071(200101)63:1<64::AID-JMV1009>3.3.CO;2-Y]
290 **Brazin SA**, Simkovich JW, Johnson WT. Herpes zoster during pregnancy. *Obstet Gynecol* 1979; **53**: 175-181 [PMID: 418971]
291 **Eyal A**, Friedman M, Peretz BA, Paldi E. Pregnancy complicated by herpes zoster. A report of two cases and literature review. *J Reprod Med* 1983; **28**: 600-603 [PMID: 6313918]
292 **Webster MH**, Smith CS. Congenital abnormalities and maternal herpes zoster. *Br Med J* 1977; **2**: 1193 [PMID: 412551 DOI: 10.1136/bmj.2.6096.1193]
293 **Seward JF**, Zhang JX, Maupin TJ, Mascola L, Jumaan AO. Contagiousness of varicella in vaccinated cases: a household contact study. *JAMA* 2004; **292**: 704-708 [PMID: 15304467 DOI: 10.1001/jama.292.6.704]
294 **Watson BM**, Piercy SA, Plotkin SA, Starr SE. Modified chickenpox in children immunized with the Oka/Merck varicella vaccine. *Pediatrics* 1993; **91**: 17-22 [PMID: 8416499]
295 **Picone O**, Vauloup-Fellous C, Senat MV, Frydman R, Grangeot-Keros L. Maternal varicella infection during pregnancy in a vaccinated patient. *Prenat Diagn* 2008; **28**: 971-972 [PMID: 18814191 DOI: 10.1002/pd.2083]
296 **Shields KE**, Galil K, Seward J, Sharrar RG, Cordero JF, Slater E. Varicella vaccine exposure during pregnancy: data from the first 5 years of the pregnancy registry. *Obstet Gynecol* 2001; **98**: 14-19 [PMID: 11430950 DOI: 10.1097/00006250-200107000-00004]
297 **Wilson E**, Goss MA, Marin M, Shields KE, Seward JF, Rasmussen SA, Sharrar RG. Varicella vaccine exposure during pregnancy: data from 10 Years of the pregnancy registry. *J Infect Dis* 2008; **197** Suppl 2: S178-S184 [PMID: 18419394 DOI: 10.1086/522136]
298 **Salzman MB**, Sharrar RG, Steinberg S, LaRussa P. Transmission of varicella-vaccine virus from a healthy 12-month-old child to his pregnant mother. *J Pediatr* 1997; **131**: 151-154 [PMID: 9255208 DOI: 10.1016/S0022-3476(97)70140-9]
299 **Dolbear GL**, Moffat J, Falkner C, Wojtowycz M. A pilot study: is attenuated varicella virus present in breast milk after postpartum immunization? *Obstet Gynecol* 2003; **101** Suppl 4: 47S [DOI: 10.1097/00006250-200304001-00108]
300 **MacMahon E**, Brown LJ, Bexley S, Snashall DC, Patel D. Identification of potential candidates for varicella vaccination by history: questionnaire and seroprevalence study. *BMJ* 2004; **329**: 551-552 [PMID: 15273160 DOI: 10.1136/bmj.38170.691956.AE]
301 **Ayres KL**, Talukder Y, Breuer J. Humoral immunity following chickenpox is influenced by geography and ethnicity. *J Infect* 2010; **61**: 244-251 [PMID: 20600297 DOI: 10.1016/j.jinf.2010.06.012]
302 **Field N**, Amirthalingam G, Waight P, Andrews N, Ladhani SN, van Hoek AJ, Maple PA, Brown KE, Miller E. Validity of a reported history of chickenpox in targeting varicella vaccination at susceptible adolescents in England. *Vaccine* 2014; **32**: 1213-1217 [PMID: 23871823 DOI: 10.1016/j.vaccine.2013.06.098]

303 **Watson B**, Civen R, Reynolds M, Heath K, Perella D, Carbajal T, Mascola L, Jumaan A, Zimmerman L, James A, Quashi C, Schmid S. Validity of self-reported varicella disease history in pregnant women attending prenatal clinics. *Public Health Rep* 2007; **122**: 499-506 [PMID: 17639653]

304 **McGregor JA**, Mark S, Crawford GP, Levin MJ. Varicella zoster antibody testing in the care of pregnant women exposed to varicella. *Am J Obstet Gynecol* 1987; **157**: 281-284 [PMID: 3039845 DOI: 10.1016/S0002-9378(87)80151-5]

305 **Inocencion G**, Loebstein R, Lalkin A, Geist R, Petric M, Koren G. Managing exposure to chickenpox during pregnancy. New program. *Can Fam Physician* 1998; **44**: 745-747 [PMID: 9585843]

306 **LaRussa P**, Steinberg SP, Seeman MD, Gershon AA. Determination of immunity to varicella-zoster virus by means of an intradermal skin test. *J Infect Dis* 1985; **152**: 869-875 [PMID: 2995511 DOI: 10.1093/infdis/152.5.869]

307 **Kilgore PE**, Kruszon-Moran D, Seward JF, Jumaan A, Van Loon FP, Forghani B, McQuillan GM, Wharton M, Fehrs LJ, Cossen CK, Hadler SC. Varicella in Americans from NHANES III: implications for control through routine immunization. *J Med Virol* 2003; **70** Suppl 1: S111-S118 [PMID: 12627498 DOI: 10.1002/jmv.10364]

308 **Pembrey L**, Raynor P, Griffiths P, Chaytor S, Wright J, Hall AJ. Seroprevalence of cytomegalovirus, Epstein Barr virus and varicella zoster virus among pregnant women in Bradford: a cohort study. *PLoS One* 2013; **8**: e81881 [PMID: 24312372 DOI: 10.1371/journal.pone.0081881]

309 **González-Escalada A**, García-García L, Viguera-Ester P, Marín-García P, García J, Gil-de-Miguel A, Gil-Prieto R. Seroprevalence of antibodies against measles, rubella, mumps, varicella-zoster, and B. Pertussis in young adults of Madrid, Spain. *Hum Vaccin Immunother* 2013; **9**: 1918-1925 [PMID: 23793571 DOI: 10.4161/hv.25127]

310 **Guido M**, Tinelli A, De Donno A, Quattrocchi M, Malvasi A, Campilongo F, Zizza A. Susceptibility to varicella-zoster among pregnant women in the province of Lecce, Italy. *J Clin Virol* 2012; **53**: 72-76 [PMID: 22074933 DOI: 10.1016/j.jcv.2011.10.007]

311 **van Rijckevorsel GG**, Damen M, Sonder GJ, van der Loeff MF, van den Hoek A. Seroprevalence of varicella-zoster virus and predictors for seronegativity in the Amsterdam adult population. *BMC Infect Dis* 2012; **12**: 140 [PMID: 22721551 DOI: 10.1186/1471-2334-12-140]

312 **van Rijckevorsel GG**, Bovée LP, Damen M, Sonder GJ, Schim van der Loeff MF, van den Hoek A. Increased seroprevalence of IgG-class antibodies against cytomegalovirus, parvovirus B19, and varicella-zoster virus in women working in child day care. *BMC Public Health* 2012; **12**: 475 [PMID: 22726391 DOI: 10.1186/1471-2458-12-475]

313 **Urbiztondo L**, Bayas JM, Broner S, Costa J, Esteve M, Campins M, Borrás E, Domínguez A. Varicella-zoster virus immunity among health care workers in Catalonia. *Vaccine* 2014; **32**: 5945-5948 [PMID: 25219564 DOI: 10.1016/j.vaccine.2014.08.055]

314 **Socan M**, Berginc N, Lajovic J. Varicella susceptibility and transmission dynamics in Slovenia. *BMC Public Health* 2010; **10**: 360 [PMID: 20573202 DOI: 10.1186/1471-2458-10-360]

315 **Vilibic-Cavlek T**, Ljubin-Sternak S, Kolaric B, Kaic B, Sviben M, Kos L, Mlinaric-Galinovic G. Immunity to varicella-zoster virus in Croatian women of reproductive age targeted for serology testing. *Arch Gynecol Obstet* 2012; **286**: 901-904 [PMID: 22678561 DOI: 10.1007/s00404-012-2398-z]

316 **Saadatian-Elahi M**, Mekki Y, Del Signore C, Lina B, Derrough T, Caulin E, Thierry J, Vanhems P. Seroprevalence of varicella antibodies among pregnant women in Lyon-France. *Eur J Epidemiol* 2007; **22**: 405-409 [PMID: 17534728 DOI: 10.1007/s10654-007-9136-z]

317 **Sauerbrei A**, Prager J, Bischoff A, Wutzler P. [Antibodies against vaccine-preventable diseases in pregnant women and their offspring. Measles, mumps, rubella, poliomyelitis, and varicella]. *Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz* 2004; **47**: 10-15 [PMID: 15205818 DOI: 10.1007/s00103-003-0689-z]

318 **Alanen A**, Kahala K, Vahlberg T, Koskela P, Vainionpää R. Seroprevalence, incidence of prenatal infections and reliability of maternal history of varicella zoster virus, cytomegalovirus, herpes simplex virus and parvovirus B19 infection in South-Western Finland. *BJOG* 2005; **112**: 50-56 [PMID: 15663397 DOI: 10.1111/j.1471-0528.2004.00320.x]

319 **Tafuri S**, Gallone MS, Cappelli MG, Gallone MF, Larocca AM, Germinario C. A seroprevalence survey on varicella among adults in the vaccination era in Apulia (Italy). *Vaccine* 2014; **32**: 6544-6547 [PMID: 25236583 DOI: 10.1016/j.vaccine.2014.08.088]

320 **Plans P**, Costa J, Espuñes J, Plasència A, Salleras L. Prevalence of varicella-zoster antibodies in pregnant women in Catalonia (Spain). Rationale for varicella vaccination of women of childbearing age. *BJOG* 2007; **114**: 1122-1127 [PMID: 17666097 DOI: 10.1111/j.1471-0528.2007.01454.x]

321 **Nardone A**, de Ory F, Carton M, Cohen D, van Damme P, Davidkin I, Rota MC, de Melker H, Mossong J, Slacikova M, Tischer A, Andrews N, Berbers G, Gabutti G, Gay N, Jones L, Jokinen S, Kafatos G, de Aragón MV, Schneider F, Smetana Z, Vargova B, Vranckx R, Miller E. The comparative sero-epidemiology of varicella zoster virus in 11 countries in the European region. *Vaccine* 2007; **25**: 7866-7872 [PMID: 17919788 DOI: 10.1016/j.vaccine.2007.07.036]

322 **Bartoloni A**, Bartalesi F, Roselli M, Mantella A, Dini F, Carballo ES, Barron VP, Paradisi F. Seroprevalence of varicella zoster and rubella antibodies among rural populations of the Chaco region, south-eastern Bolivia. *Trop Med Int Health* 2002; **7**: 512-517 [PMID: 12031073 DOI: 10.1046/j.1365-3156.2002.00852.x]

323 **Mamani M**, Zamani M, Hashemi SH, Akhtari M, Niayesh, Seroepidemiology of varicella-virus among pregnant women in Hamedan, Iran. *Afr J Microbiol Res* 2012; **6**: 1829-1832 [DOI: 10.5897/AJMR11.1525]

324 **Ghazi HO**, Telmesani AM, Mahomed MF. TORCH agents in pregnant Saudi women. *Med Princ Pract* 2002; **11**: 180-182 [PMID: 12424411 DOI: 10.1159/000065813]

325 **Pandolfi E**, Chiaradia G, Moncada M, Rava L, Tozzi AE. Prevention of congenital rubella and congenital varicella in Europe. *Euro Surveill* 2009; **14**: 16-20 [PMID: 19317971]

326 **Pinot de Moira A**, Nardone A. Varicella zoster virus vaccination policies and surveillance strategies in Europe. *Euro Surveill* 2005; **10**: 43-45 [PMID: 15701939]

327 **Koch-Institut R**. Recommendations of the Standing Committee on Vaccinations: status. *Epidemiol Bull* 2001; **8**: 55-62

328 **Sauerbrei A**, Schäfler A, Hofmann J, Schacke M, Gruhn B, Wutzler P. Evaluation of three commercial varicella-zoster virus IgG enzyme-linked immunosorbent assays in comparison to the fluorescent-antibody-to-membrane-antigen test. *Clin Vaccine Immunol* 2012; **19**: 1261-1268 [PMID: 22718131 DOI: 10.1128/CVI.00183-12]

329 **Smith JG**, Liu X, Kaufhold RM, Clair J, Caulfield MJ. Development and validation of a gamma interferon ELISPOT assay for quantitation of cellular immune responses to varicella-zoster virus. *Clin Diagn Lab Immunol* 2001; **8**: 871-879 [PMID: 11527795 DOI: 10.1128/CDLI.8.5.871-879.2001]

330 **Smith JG**, Levin M, Vessey R, Chan IS, Hayward AR, Liu X, Kaufhold RM, Clair J, Chalikonda I, Chan C, Bernard M, Wang WW, Keller P, Caulfield MJ. Measurement of cell-mediated immunity with a Varicella-Zoster Virus-specific interferon-gamma ELISPOT assay: responses in an elderly population receiving a booster immunization. *J Med Virol* 2003; **70** Suppl 1: S38-S41 [PMID: 12627485 DOI: 10.1002/jmv.10318]

331 **Smith JG**, Joseph HR, Green T, Field JA, Wooters M, Kaufhold RM, Antonello J, Caulfield MJ. Establishing acceptance criteria for cell-mediated-immunity assays using frozen peripheral blood mononuclear cells stored under optimal and suboptimal conditions. *Clin Vaccine Immunol* 2007; **14**: 527-537 [PMID: 17376862 DOI: 10.1128/CVI.00435-06]

332 **Levin MJ**, Smith JG, Kaufhold RM, Barber D, Hayward AR, Chan CY, Chan IS, Li DJ, Wang W, Keller PM, Shaw A, Silber JL, Schlienger K, Chalikonda I, Vessey SJ, Caulfield MJ. Decline

in varicella-zoster virus (VZV)-specific cell-mediated immunity with increasing age and boosting with a high-dose VZV vaccine. *J Infect Dis* 2003; **188**: 1336-1344 [PMID: 14593591 DOI: 10.1086/379048]

333 **van Besouw NM**, Verjans GM, Zuijderwijk JM, Litjens NH, Osterhaus AD, Weimar W. Systemic varicella zoster virus reactive effector memory T-cells impaired in the elderly and in kidney transplant recipients. *J Med Virol* 2012; **84**: 2018-2025 [PMID: 23080511 DOI: 10.1002/jmv.23427]

334 **Lafer MM**, Weckx LY, de Moraes-Pinto MI, Garretson A, Steinberg SP, Gershon AA, LaRussa PS. Comparative study of the standard fluorescent antibody to membrane antigen (FAMA) assay and a flow cytometry-adapted FAMA assay to assess immunity to varicella-zoster virus. *Clin Vaccine Immunol* 2011; **18**: 1194-1197 [PMID: 21613463 DOI: 10.1128/CVI.05130-11]

335 **Baba K**, Shiraki K, Kanesaki T, Yamanishi K, Ogra PL, Yabuuchi H, Takahashi M. Specificity of skin test with varicella-zoster virus antigen in varicella-zoster and herpes simplex virus infections. *J Clin Microbiol* 1987; **25**: 2193-2196 [PMID: 2826535]

336 **Sato H**, Yamamura Ji, Kageyama S, Kurokawa M, Shiraki K. Superiority of varicella skin test antigen over purified varicella-zoster virus glycoproteins in monitoring booster response to Oka varicella vaccine. *Vaccine* 2003; **22**: 15-20 [PMID: 14604566 DOI: 10.1016/S0264-410X(03)00542-5]

337 **Takahashi M**, Kamiya H, Asano Y, Shiraki K, Baba K, Otsuka T, Hirota T, Yamanishi K. Immunization of the elderly to boost immunity against varicella-zoster virus (VZV) as assessed by VZV skin test reaction. *Arch Virol Suppl* 2001; **17**: 161-172 [PMID: 11339545 DOI: 10.1007/978-3-7091-6259-0_17]

338 **Zerboni L**, Nader S, Aoki K, Arvin AM. Analysis of the persistence of humoral and cellular immunity in children and adults immunized with varicella vaccine. *J Infect Dis* 1998; **177**: 1701-1704 [PMID: 9607852 DOI: 10.1086/517426]

339 **Watson B**, Boardman C, Laufer D, Piercy S, Tustin N, Olaleye D, Cnaan A, Starr SE. Humoral and cell-mediated immune responses in healthy children after one or two doses of varicella vaccine. *Clin Infect Dis* 1995; **20**: 316-319 [PMID: 7742436 DOI: 10.1093/clinids/20.2.316]

340 **Watson B**, Rothstein E, Bernstein H, Arbeter A, Arvin A, Chartrand S, Clements D, Kumar ML, Reisinger K, Blatter M. Safety and cellular and humoral immune responses of a booster dose of varicella vaccine 6 years after primary immunization. *J Infect Dis* 1995; **172**: 217-219 [PMID: 7797914 DOI: 10.1093/infdis/172.1.217]

341 **Nader S**, Bergen R, Sharp M, Arvin AM. Age-related differences in cell-mediated immunity to varicella-zoster virus among children and adults immunized with live attenuated varicella vaccine. *J Infect Dis* 1995; **171**: 13-17 [PMID: 7798653 DOI: 10.1093/infdis/171.1.13]

342 **Asano Y**, Nakayama H, Yazaki T, Kato R, Hirose S. Protection against varicella in family contacts by immediate inoculation with live varicella vaccine. *Pediatrics* 1977; **59**: 3-7 [PMID: 190583]

343 **Asano Y**, Nakayama H, Yazaki T, Ito S, Isomura S. Protective efficacy of vaccination in children in four episodes of natural varicella and zoster in the ward. *Pediatrics* 1977; **59**: 8-12 [PMID: 190584]

344 **Heath RB**, Malpas JS. Experience with the live Oka-strain varicella vaccine in children with solid tumours. *Postgrad Med J* 1985; **61** Suppl 4: 107-111 [PMID: 3014467]

345 **Gerna G**, Achilli G, Chambers RW. Determination of neutralizing antibody and IgG antibody to varicella-zoster virus and of IgG antibody to membrane antigens by the immunoperoxidase technique. *J Infect Dis* 1977; **135**: 975-979 [PMID: 194001 DOI: 10.1093/infdis/135.6.975]

346 **Wreghitt TG**, Tedder RS, Nagington J, Ferns RB. Antibody assays for varicella-zoster virus: comparison of competitive enzyme-linked immunosorbent assay (ELISA), competitive radioimmunoassay (RIA), complement fixation, and indirect immunofluorescence assays. *J Med Virol* 1984; **13**: 361-370 [PMID: 6330290 DOI: 10.1002/jmv.1890130407]

347 **Williams V**, Gershon A, Brunell PA. Serologic response to varicella-zoster membrane antigens measured by direct immunofluorescence. *J Infect Dis* 1974; **130**: 669-672 [PMID: 4372275 DOI: 10.1093/infdis/130.6.669]

348 **Mendelson E**, Aboudy Y, Smetana Z, Tepperberg M, Grossman Z. Laboratory assessment and diagnosis of congenital viral infections: Rubella, cytomegalovirus (CMV), varicella-zoster virus (VZV), herpes simplex virus (HSV), parvovirus B19 and human immunodeficiency virus (HIV). *Reprod Toxicol* 2006; **21**: 350-382 [PMID: 16564672 DOI: 10.1016/j.reprotox.2006.02.001]

349 **Grist NR**, Bell E J, Follett EAC, Urquhart GED. Diagnostic methods in clinical virology. 3rd ed. Oxford: Blackwell Scientific Publications, 1979: 95-115

350 **Gallo D**, Schmidt NJ. Comparison of anticomplement immunofluorescence and fluorescent antibody-to-membrane antigen tests for determination of immunity status to varicella-zoster virus and for serodifferentiation of varicella-zoster and herpes simplex virus infections. *J Clin Microbiol* 1981; **14**: 539-543 [PMID: 6273453]

351 **Forghani B**, Schmidt NJ, Dennis J. Antibody assays for varicella-zoster virus: comparison of enzyme immunoassay with neutralization, immune adherence hemagglutination, and complement fixation. *J Clin Microbiol* 1978; **8**: 545-552 [PMID: 215602]

352 **Gershon AA**, Kalter ZG, Steinberg S, Kuhns WJ. Detection of antibody to Varicella-Zoster virus by immune adherence hemagglutination. *Proc Soc Exp Biol Med* 1976; **151**: 762-765 [PMID: 177988 DOI: 10.3181/00379727-151-39302]

353 **Gillani A**, Spence L. Immune adherence hemagglutination test applied to the study of herpes simplex and varicella-zoster virus infections. *J Clin Microbiol* 1978; **7**: 114-117 [PMID: 204662]

354 **Takahashi M**. Current status and prospects of live varicella vaccine. *Vaccine* 1992; **10**: 1007-1014 [PMID: 1335196 DOI: 10.1016/0264-410X(92)90109-W]

355 **Wong CL**, Castriciano S, Chernesky MA, Rawls WE. Quantitation of antibodies to varicella-zoster virus by immune adherence hemagglutination. *J Clin Microbiol* 1978; **7**: 6-11 [PMID: 203604]

356 **Weibel RE**, Kuter BJ, Neff BJ, Rothenberger CA, Fitzgerald AJ, Connor KA, Morton D, McLean AA, Scolnick EM. Live Oka/Merck varicella vaccine in healthy children. Further clinical and laboratory assessment. *JAMA* 1985; **254**: 2435-2439 [PMID: 2995697 DOI: 10.1001/jama.254.17.2435]

357 **Chung A**, Naylor DH. Detection of anti-varicella-zoster virus antibodies in blood donors by automated passive haemagglutination. *Vox Sang* 1981; **41**: 245-248 [PMID: 6278772 DOI: 10.1111/j.1423-0410.1981.tb01044.x]

358 **Kino Y**, Minamishima Y. Diagnosis of zoster and evaluation of varicella vaccine with a passive haemagglutination assay. *Vaccine* 1993; **11**: 1151-1153 [PMID: 8249435 DOI: 10.1016/0264-410X(93)90078-C]

359 **Kino Y**, Minamishima Y. Passive hemagglutination assays for the detection of antibodies to herpes viruses. *Microbiol Immunol* 1993; **37**: 365-368 [PMID: 8394981 DOI: 10.1111/j.1348-0421.1993.tb03223.x]

360 **Arvin AM**, Kinney-Thomas E, Shriver K, Grose C, Koropchak CM, Scranton E, Wittek AE, Diaz PS. Immunity to varicella-zoster viral glycoproteins, gp I (gp 90/58) and gp III (gp 118), and to a nonglycosylated protein, p 170. *J Immunol* 1986; **137**: 1346-1351 [PMID: 3016094]

361 **Campbell-Benzie A**, Kangro HO, Heath RB. The development and evaluation of a solid-phase radioimmunoassay (RIA) procedure for the determination of susceptibility to varicella. *J Virol Methods* 1981; **2**: 149-158 [PMID: 6268649 DOI: 10.1016/0166-0934(81)90033-1]

362 **Harper DR**, Grose C. IgM and IgG responses to varicella-zoster virus p32/p36 complex after chickenpox and zoster, congenital and subclinical infections, and vaccination. *J Infect Dis* 1989; **159**: 444-451 [PMID: 2536788 DOI: 10.1093/infdis/159.3.444]

363 **Richman DD**, Cleveland PH, Oxman MN, Zaia JA. A rapid radioimmunoassay using 125I-labeled staphylococcal protein A for antibody to varicella-zoster virus. *J Infect Dis* 1981; **143**: 693-699 [PMID: 6263987 DOI: 10.1093/infdis/143.5.693]

364 **Krah DL**. Assays for antibodies to varicella-zoster virus. *Infect Dis Clin North Am* 1996; **10**: 507-527 [PMID: 8856350 DOI: 10.1016/S0891-5520(05)70311-1]

365 **Asano Y**, Takahashi M. Clinical and serologic testing of a live varicella vaccine and two-year follow-up for immunity of the vaccinated children. *Pediatrics* 1977; **60**: 810-814 [PMID: 202916]

366 **Asano Y**, Albrecht P, Stagno S, Takahashi M. Potentiation of neutralization of Varicella-Zoster virus to antibody to immunoglobulin. *J Infect Dis* 1982; **146**: 524-529 [PMID: 6288808 DOI: 10.1093/infdis/146.4.524]

367 **Asano Y**, Albrecht P, Vujcic LK, Quinnan GV, Kawakami K, Takahashi M. Five-year follow-up study of recipients of live varicella vaccine using enhanced neutralization and fluorescent antibody membrane antigen assays. *Pediatrics* 1983; **72**: 291-294 [PMID: 6310478]

368 **Caunt AE**, Shaw DG. Neutralization tests with varicella-zoster virus. *J Hyg* (Lond) 1969; **67**: 343-352 [PMID: 4183291 DOI: 10.1017/S0022172400041747]

369 **Krah DL**, Provost PJ, Ellis RW. Combined use of complement and anti-immunoglobulin in an enhanced neutralization assay for antibodies to varicella-zoster virus. *J Virol Methods* 1995; **53**: 176-187 [PMID: 7673386 DOI: 10.1016/0166-0934(95)00013-K]

370 **Ozaki T**, Nagayoshi S, Morishima T, Isomura S, Suzuki S, Asano Y, Takahashi M. Use of a live varicella vaccine for acute leukemic children shortly after exposure in a children's ward. *Biken J* 1978; **21**: 69-72 [PMID: 219841]

371 **Provost PJ**, Krah DL, Kuter BJ, Morton DH, Schofield TL, Wasmuth EH, White CJ, Miller WJ, Ellis RW. Antibody assays suitable for assessing immune responses to live varicella vaccine. *Vaccine* 1991; **9**: 111-116 [PMID: 1647574 DOI: 10.1016/0264-410X(91)90266-9]

372 **Schmidt NJ**, Lennette EH. Neutralizing antibody responses to varicella-zoster virus. *Infect Immun* 1975; **12**: 606-613 [PMID: 170206]

373 **Ueda K**, Yamada I, Goto M, Nanri T, Fukuda H, Katsuta M, Otsuka T, Takahashi M. Use of a live varicella vaccine to prevent the spread of varicella in handicapped or immunosuppressed children including MCLS (muco-cutaneous lymphnode syndrome) patients in hospitals. *Biken J* 1977; **20**: 117-123 [PMID: 208503]

374 **Asano Y**, Takahashi M. Studies on neutralization of varicella-zoster virus and serological follow-up of cases of varicella and zoster. *Biken J* 1978; **21**: 15-23 [PMID: 208504]

375 **Cremer NE**, Cossen CK, Shell G, Diggs J, Gallo D, Schmidt NJ. Enzyme immunoassay versus plaque neutralization and other methods for determination of immune status to measles and varicella-zoster viruses and versus complement fixation for serodiagnosis of infections with those viruses. *J Clin Microbiol* 1985; **21**: 869-874 [PMID: 2989325]

376 **Grose C**, Edmond BJ, Brunell PA. Complement-enhanced neutralizing antibody response to varicella-zoster virus. *J Infect Dis* 1979; **139**: 432-437 [PMID: 220336 DOI: 10.1093/infdis/139.4.432]

377 **Steinberg SP**, Gershon AA. Measurement of antibodies to varicella-zoster virus by using a latex agglutination test. *J Clin Microbiol* 1991; **29**: 1527-1529 [PMID: 1653268]

378 **Gershon AA**, Larussa P, Steinberg S. Detection of antibodies to varicella-zoster virus using a latex agglutination assay. *Clin Diagn Virol* 1994; **2**: 271-277 [PMID: 15566772 DOI: 10.1016/0928-0197(94)90051-5]

379 **Landry ML**, Ferguson D. Comparison of latex agglutination test with enzyme-linked immunosorbent assay for detection of antibody to varicella-zoster virus. *J Clin Microbiol* 1993; **31**: 3031-3033 [PMID: 8263191]

380 **Chris Maple PA**, Gunn A, Sellwood J, Brown DW, Gray JJ. Comparison of fifteen commercial assays for detecting Varicella Zoster virus IgG with reference to a time resolved fluorescence immunoassay (TRFIA) and the performance of two commercial assays for screening sera from immunocompromised individuals. *J Virol Methods* 2009; **155**: 143-149 [PMID: 18996415 DOI: 10.1016/j.jviromet.2008.09.032]

381 **Ndumbe PM**, Cradock-Watson J, Levinsky RJ. Natural and artificial immunity to varicella zoster virus. *J Med Virol* 1988; **25**: 171-178 [PMID: 2839610 DOI: 10.1002/jmv.1890250207]

382 **Schmidt NJ**, Lennette EH, Woodie JD, Ho HH. Immunofluorescent staining in the laboratory diagnosis of varicella-zoster virus infections. *J Lab Clin Med* 1965; **66**: 403-412 [PMID: 5319786]

383 **Vafai A**. Antibody-binding sites on truncated forms of varicella-zoster virus gpI(gE) glycoprotein. *Vaccine* 1994; **12**: 1265-1269 [PMID: 7531921 DOI: 10.1016/S0264-410X(94)80030-4]

384 **Sauerbrei A**, Färber I, Brandstädt A, Schacke M, Wutzler P. Immunofluorescence test for sensitive detection of varicella-zoster virus-specific IgG: an alternative to fluorescent antibody to membrane antigen test. *J Virol Methods* 2004; **119**: 25-30 [PMID: 15109817 DOI: 10.1016/j.jviromet.2004.02.012]

385 **Mazur MH**, Whitley RJ, Dolin R. Serum antibody levels as risk factors in the dissemination of herpes zoster. *Arch Intern Med* 1979; **139**: 1341-1345 [PMID: 229783 DOI: 10.1001/archinte.1979.03630490011007]

386 **Paryani SG**, Arvin AM, Koropchak CM, Dobkin MB, Wittek AE, Amylon MD, Budinger MD. Comparison of varicella zoster antibody titers in patients given intravenous immune serum globulin or varicella zoster immune globulin. *J Pediatr* 1984; **105**: 200-205 [PMID: 6086866 DOI: 10.1016/S0022-3476(84)80113-4]

387 **Zaia JA**, Oxman MN. Antibody to varicella-zoster virus-induced membrane antigen: immunofluorescence assay using monodisperse glutaraldehyde-fixed target cells. *J Infect Dis* 1977; **136**: 519-530 [PMID: 198487 DOI: 10.1093/infdis/136.4.519]

388 **Park R**, Hwang JY, Lee KI, Namkoong S, Choi SK, Park S, Park H, Park J. Measurement of antibodies to varicella-zoster virus using a virus-free fluorescent-antibody-to-membrane-antigen (FAMA) test. *J Microbiol Biotechnol* 2015; **25**: 268-273 [PMID: 25248983 DOI: 10.4014/jmb.1408.08048]

389 **Grose C**, Edwards DP, Friedrichs WE, Weigle KA, McGuire WL. Monoclonal antibodies against three major glycoproteins of varicella-zoster virus. *Infect Immun* 1983; **40**: 381-388 [PMID: 6299963]

390 **Gershon AA**, Steinberg SP, LaRussa P, Ferrara A, Hammerschlag M, Gelb L. Immunization of healthy adults with live attenuated varicella vaccine. *J Infect Dis* 1988; **158**: 132-137 [PMID: 2839577 DOI: 10.1093/infdis/158.1.132]

391 **Iltis JP**, Castellano GA, Gerber P, Le C, Vujcic LK, Quinnan GV. Comparison of the Raji cell line fluorescent antibody to membrane antigen test and the enzyme-linked immunosorbent assay for determination of immunity to varicella-zoster virus. *J Clin Microbiol* 1982; **16**: 878-884 [PMID: 6759530]

392 **Michalik DE**, Steinberg SP, Larussa PS, Edwards KM, Wright PF, Arvin AM, Gans HA, Gershon AA. Primary vaccine failure after 1 dose of varicella vaccine in healthy children. *J Infect Dis* 2008; **197**: 944-949 [PMID: 18419532 DOI: 10.1086/529043]

393 **McDonald SL**, Maple PA, Andrews N, Brown KE, Ayres KL, Scott FT, Al Bassam M, Gershon AA, Steinberg SP, Breuer J. Evaluation of the time resolved fluorescence immunoassay (TRFIA) for the detection of varicella zoster virus (VZV) antibodies following vaccination of healthcare workers. *J Virol Methods* 2011; **172**: 60-65 [PMID: 21192976 DOI: 10.1016/j.jviromet.2010.12.021]

394 **Maple PA**, Gray J, Breuer J, Kafatos G, Parker S, Brown D. Performance of a time-resolved fluorescence immunoassay for measuring varicella-zoster virus immunoglobulin G levels in adults and comparison with commercial enzyme immunoassays and Merck glycoprotein enzyme immunoassay. *Clin Vaccine Immunol* 2006; **13**: 214-218 [PMID: 16467328 DOI: 10.1128/CVI.13.2.214-218.2006]

395 **Craig WY**, Poulin SE, Dorsett PH, Ledue TB, Ritchie RF. Application of checkerboard immunoblotting (CBIB) to the detection of anti-viral IgG in human serum. *J Clin Lab Anal* 1993; **7**: 203-208 [PMID: 8360795 DOI: 10.1002/jcla.1860070403]

396 **Kim YH**, Hwang JY, Shim HM, Lee E, Park S, Park H. Evaluation of a commercial glycoprotein enzyme-linked immunosorbent assay for measuring vaccine immunity to varicella. *Yonsei Med J* 2014; **55**: 459-466 [PMID: 24532518 DOI: 10.3349/ymj.2014.55.2.459]

397 **Maple PA**, Breuer J, Quinlivan M, Kafatos G, Brown KE.

Comparison of a commercial Varicella Zoster glycoprotein IgG enzyme immunoassay with a reference time resolved fluorescence immunoassay (VZV TRFIA) for measuring VZV IgG in sera from pregnant women, sera sent for confirmatory testing and pre and post vOka vaccination sera from healthcare workers. *J Clin Virol* 2012; **53**: 201-207 [PMID: 22261123 DOI: 10.1016/j.jcv.2011.12.010]

398 **Gershon AA**, Frey HM, Steinberg SP, Seeman MD, Bidwell D, Voller A. Determination of immunity to varicella using an enzyme-linked-immunosorbent-assay. *Arch Virol* 1981; **70**: 169-172 [DOI: 10.1007/BF01315011]

399 **Larussa P**, Steinberg S, Waithe E, Hanna B, Holzman R. Comparison of five assays for antibody to varicella-zoster virus and the fluorescent-antibody-to-membrane-antigen test. *J Clin Microbiol* 1987; **25**: 2059-2062 [PMID: 2826532]

400 **Weinberg A**, Hayward AR, Masters HB, Ogu IA, Levin MJ. Comparison of two methods for detecting varicella-zoster virus antibody with varicella-zoster virus cell-mediated immunity. *J Clin Microbiol* 1996; **34**: 445-446 [PMID: 8789035]

401 **Doern GV**, Robbie L, St Amand R. Comparison of the Vidas and Bio-Whittaker enzyme immunoassays for detecting IgG reactive with varicella-zoster virus and mumps virus. *Diagn Microbiol Infect Dis* 1997; **28**: 31-34 [PMID: 9218916 DOI: 10.1016/S0732-8893(97)89156-0]

402 **Rolando L**, Schneider WJ, Steinberg S, Low S, Stiles J, Gomez L, Gershon AA, Brown AE. Effect of varicella-zoster virus (VZV) fluorescent-antibody-to-membrane-antigen (FAMA) testing on sensitivity of determining VZV immunity in healthcare workers and on furlough days. *Infect Control Hosp Epidemiol* 2010; **31**: 972-974 [PMID: 20666603 DOI: 10.1086/655840]

403 **de Ory F**, Echevarría JM, Kafatos G, Anastassopoulou C, Andrews N, Backhouse J, Berbers G, Bruckova B, Cohen DI, de Melker H, Davidkin I, Gabutti G, Hesketh LM, Johansen K, Jokinen S, Jones L, Linde A, Miller E, Mossong J, Nardone A, Rota MC, Sauerbrei A, Schneider F, Smetana Z, Tischer A, Tsakris A, Vranckx R. European seroepidemiology network 2: Standardisation of assays for seroepidemiology of varicella zoster virus. *J Clin Virol* 2006; **36**: 111-118 [PMID: 16616612 DOI: 10.1016/j.jcv.2006.01.017]

404 **Keller PM**, Lonergan K, Neff BJ, Morton DA, Ellis RW. Purification of individual varicella-zoster virus (VZV) glycoproteins gpI, gpII, and gpIII and their use in ELISA for detection of VZV glycoprotein-specific antibodies. *J Virol Methods* 1986; **14**: 177-188 [PMID: 3021804 DOI: 10.1016/0166-0934(86)90048-0]

405 **Wasmuth EH**, Miller WJ. Sensitive enzyme-linked immunosorbent assay for antibody to varicella-zoster virus using purified VZV glycoprotein antigen. *J Med Virol* 1990; **32**: 189-193 [PMID: 2177782 DOI: 10.1002/jmv.1890320310]

406 **Watson B**, Gupta R, Randall T, Starr S. Persistence of cell-mediated and humoral immune responses in healthy children immunized with live attenuated varicella vaccine. *J Infect Dis* 1994; **169**: 197-199 [PMID: 8277182 DOI: 10.1093/infdis/169.1.197]

407 **White CJ**, Kuter BJ, Ngai A, Hildebrand CS, Isganitis KL, Patterson CM, Capra A, Miller WJ, Krah DL, Provost PJ, Ellis RW, Calandra GB. Modified cases of chickenpox after varicella vaccination: correlation of protection with antibody response. *Pediatr Infect Dis J* 1992; **11**: 19-23 [PMID: 1312704 DOI: 10.1097/00006454-199201000-00006]

408 **Centers for Disease Control and Prevention (CDC)**. Chickenpox (Varicella). Interpreting Laboratory Tests. [accessed 2016 Apr 4]. Available from: URL: http://www.cdc.gov/chickenpox/hcp/lab-tests.html

409 **Hammond O**, Wang Y, Green T, Antonello J, Kuhn R, Motley C, Stump P, Rich B, Chirmule N, Marchese RD. The optimization and validation of the glycoprotein ELISA assay for quantitative varicella-zoster virus (VZV) antibody detection. *J Med Virol* 2006; **78**: 1679-1687 [PMID: 17063506 DOI: 10.1002/jmv.20754]

410 **Weinmann S**, Chun C, Mullooly JP, Riedlinger K, Houston H, Loparev VN, Schmid DS, Seward JF. Laboratory diagnosis and characteristics of breakthrough varicella in children. *J Infect Dis* 2008; **197** Suppl 2: S132-S138 [PMID: 18419386 DOI: 10.1086/522148]

411 **Krah DL**, Cho I, Schofield T, Ellis RW. Comparison of gpELISA and neutralizing antibody responses to Oka/Merck live varicella vaccine (Varivax) in children and adults. *Vaccine* 1997; **15**: 61-64 [PMID: 9041667 DOI: 10.1016/S0264-410X(96)00107-7]

412 **Sauerbrei A**, Wutzler P. Serological detection of varicella-zoster virus-specific immunoglobulin G by an enzyme-linked immunosorbent assay using glycoprotein antigen. *J Clin Microbiol* 2006; **44**: 3094-3097 [PMID: 16954232 DOI: 10.1128/JCM.00719-06]

413 **Li S**, Chan IS, Matthews H, Heyse JF, Chan CY, Kuter BJ, Kaplan KM, Vessey SJ, Sadoff JC. Inverse relationship between six week postvaccination varicella antibody response to vaccine and likelihood of long term breakthrough infection. *Pediatr Infect Dis J* 2002; **21**: 337-342 [PMID: 12075766 DOI: 10.1097/00006454-200204000-00014]

414 **Maple PA**, Rathod P, Smit E, Gray J, Brown D, Boxall EH. Comparison of the performance of the LIAISON VZV-IgG and VIDAS automated enzyme linked fluorescent immunoassays with reference to a VZV-IgG time-resolved fluorescence immunoassay and implications of choice of cut-off for LIAISON assay. *J Clin Virol* 2009; **44**: 9-14 [PMID: 18823815 DOI: 10.1016/j.jcv.2008.08.012]

415 **Binnicker MJ**, Jespersen DJ, Rollins LO. Evaluation of the Bio-Rad BioPlex Measles, Mumps, Rubella, and Varicella-Zoster Virus IgG multiplex bead immunoassay. *Clin Vaccine Immunol* 2011; **18**: 1524-1526 [PMID: 21795463 DOI: 10.1128/CVI.05207-11]

416 **Dhiman N**, Jespersen DJ, Rollins LO, Harring JA, Beito EM, Binnicker MJ. Detection of IgG-class antibodies to measles, mumps, rubella, and varicella-zoster virus using a multiplex bead immunoassay. *Diagn Microbiol Infect Dis* 2010; **67**: 346-349 [PMID: 20638602 DOI: 10.1016/j.diagmicrobio.2010.03.008]

417 **Smits GP**, van Gageldonk PG, Schouls LM, van der Klis FR, Berbers GA. Development of a bead-based multiplex immunoassay for simultaneous quantitative detection of IgG serum antibodies against measles, mumps, rubella, and varicella-zoster virus. *Clin Vaccine Immunol* 2012; **19**: 396-400 [PMID: 22237896 DOI: 10.1128/CVI.05537-11]

418 **Jääskeläinen AJ**, Moilanen K, Bühler S, Lappalainen M, Vapalahti O, Vaheri A, Piiparinen H. Serological microarray for detection of HSV-1, HSV-2, VZV, and CMV antibodies. *J Virol Methods* 2009; **160**: 167-171 [PMID: 19477202 DOI: 10.1016/j.jviromet.2009.05.013]

419 **Ardizzoni A**, Capuccini B, Baschieri MC, Orsi CF, Rumpianesi F, Peppoloni S, Cermelli C, Meacci M, Crisanti A, Steensgaard P, Blasi E. A protein microarray immunoassay for the serological evaluation of the antibody response in vertically transmitted infections. *Eur J Clin Microbiol Infect Dis* 2009; **28**: 1067-1075 [PMID: 19415353 DOI: 10.1007/s10096-009-0748-y]

420 **Cohen PR**. Tests for detecting herpes simplex virus and varicella-zoster virus infections. *Dermatol Clin* 1994; **12**: 51-68 [PMID: 8143385]

421 **Nahass GT**, Goldstein BA, Zhu WY, Serfling U, Penneys NS, Leonardi CL. Comparison of Tzanck smear, viral culture, and DNA diagnostic methods in detection of herpes simplex and varicella-zoster infection. *JAMA* 1992; **268**: 2541-2544 [PMID: 1328700 DOI: 10.1001/jama.1992.03490180073029]

422 **Ozcan A**, Senol M, Saglam H, Seyhan M, Durmaz R, Aktas E, Ozerol IH. Comparison of the Tzanck test and polymerase chain reaction in the diagnosis of cutaneous herpes simplex and varicella zoster virus infections. *Int J Dermatol* 2007; **46**: 1177-1179 [PMID: 17988338 DOI: 10.1111/j.1365-4632.2007.03337.x]

423 **Schirm J**, Meulenberg JJ, Pastoor GW, van Voorst Vader PC, Schröder FP. Rapid detection of varicella-zoster virus in clinical specimens using monoclonal antibodies on shell vials and smears. *J Med Virol* 1989; **28**: 1-6 [PMID: 2542440]

424 **Folkers E**, Vreeswijk J, Oranje AP, Duivenvoorden JN. Rapid diagnosis in varicella and herpes zoster: re-evaluation of direct smear (Tzanck test) and electron microscopy including colloidal gold immuno-electron microscopy in comparison with virus isolation. *Br J Dermatol* 1989; **121**: 287-296 [PMID: 2553095 DOI: 10.1111/j.1365-2133.1989.tb01421.x]

425 **Sadick NS**, Swenson PD, Kaufman RL, Kaplan MH. Comparison of detection of varicella-zoster virus by the Tzanck smear, direct immunofluorescence with a monoclonal antibody, and virus isolation. *J Am Acad Dermatol* 1987; **17**: 64-69 [PMID: 2440920 DOI: 10.1016/S0190-9622(87)70172-8]

426 **Solomon AR**, Rasmussen JE, Weiss JS. A comparison of the Tzanck smear and viral isolation in varicella and herpes zoster. *Arch Dermatol* 1986; **122**: 282-285 [PMID: 3006599 DOI: 10.1001/archderm.1986.01660150060016]

427 **Sullivan M**, Sams R, Jamieson B, Holt J. Clinical inquiries. What is the best test to detect herpes in skin lesions? *J Fam Pract* 2006; **55**: 346, 348 [PMID: 16608675]

428 **Frisch S**, Guo AM. Diagnostic methods and management strategies of herpes simplex and herpes zoster infections. *Clin Geriatr Med* 2013; **29**: 501-526 [PMID: 23571042 DOI: 10.1016/j.cger.2013.01.003]

429 **Fan F**, Day S, Lu X, Tang YW. Laboratory diagnosis of HSV and varicella zoster virus infections. *Future Virol* 2014; **9**: 721-731 [DOI: 10.2217/fvl.14.61]

430 **Vreeswijk J**, Folkers E, Wagenaar F, Kapsenberg JG. The use of colloidal gold immunoelectron microscopy to diagnose varicella-zoster virus (VZV) infections by rapid discrimination between VZV, HSV-1 and HSV-2. *J Virol Methods* 1988; **22**: 255-271 [PMID: 2851604 DOI: 10.1016/0166-0934(88)90108-5]

431 **Williams MG**, Almeida JD, Howatson AF. Electron microscope studies on viral skin lesions. A simple and rapid method of identifying virus particles. *Arch Dermatol* 1962; **86**: 290-297 [PMID: 14007203 DOI: 10.1001/archderm.1962.01590090032010]

432 **Folkers E**, Vreeswijk J, Oranje AP, Wagenaar F, Duivenvoorden JN. Improved detection of HSV by electron microscopy in clinical specimens using ultracentrifugation and colloidal gold immunoelectron microscopy: comparison with viral culture and cytodiagnosis. *J Virol Methods* 1991; **34**: 273-289 [PMID: 1660490 DOI: 10.1016/0166-0934(91)90106-A]

433 **Drew WL**, Mintz L. Rapid diagnosis of varicella-zoster virus infection by direct immunofluorescence. *Am J Clin Pathol* 1980; **73**: 699-701 [PMID: 6990743 DOI: 10.1093/ajcp/73.5.699]

434 **Coffin SE**, Hodinka RL. Utility of direct immunofluorescence and virus culture for detection of varicella-zoster virus in skin lesions. *J Clin Microbiol* 1995; **33**: 2792-2795 [PMID: 8567930]

435 **Sefcovicová L**. Varicella-zoster virus cultivation in cell cultures of non-primate origin. *Acta Virol* 1971; **15**: 171-173 [PMID: 4396416]

436 **Brinker JP**, Doern GV. Comparison of MRC-5 and A-549 cells in conventional culture tubes and shell vial assays for the detection of varicella-zoster virus. *Diagn Microbiol Infect Dis* 1993; **17**: 75-77 [PMID: 8395374 DOI: 10.1016/0732-8893(93)90075-I]

437 **Huang YT**, Hite S, Duane V, Yan H. CV-1 and MRC-5 mixed cells for simultaneous detection of herpes simplex viruses and varicella zoster virus in skin lesions. *J Clin Virol* 2002; **24**: 37-43 [PMID: 11744427 DOI: 10.1016/S1386-6532(01)00230-X]

438 **Gleaves CA**, Lee CF, Bustamante CI, Meyers JD. Use of murine monoclonal antibodies for laboratory diagnosis of varicella-zoster virus infection. *J Clin Microbiol* 1988; **26**: 1623-1625 [PMID: 2846644]

439 **West PG**, Aldrich B, Hartwig R, Haller GJ. Increased detection rate for varicella-zoster virus with combination of two techniques. *J Clin Microbiol* 1988; **26**: 2680-2681 [PMID: 2852678]

440 **Leland DS**, Ginocchio CC. Role of cell culture for virus detection in the age of technology. *Clin Microbiol Rev* 2007; **20**: 49-78 [PMID: 17223623 DOI: 10.1128/CMR.00002-06]

441 **Kodama E**, Mori S, Shigeta S. Analysis of mutations in the thymidine kinase gene of varicella zoster virus associated with resistance to 5-iodo-2'-deoxyuridine and 5-bromo-2'-deoxyuridine. *Antiviral Res* 1995; **27**: 165-170 [PMID: 7486953 DOI: 10.1016/0166-3542(94)00077-L]

442 **Andrei G**, Snoeck R, Reymen D, Liesnard C, Goubau P, Desmyter J, De Clercq E. Comparative activity of selected antiviral compounds against clinical isolates of varicella-zoster virus. *Eur J Clin Microbiol Infect Dis* 1995; **14**: 318-329 [PMID: 7649195]

443 **Dahl H**, Marcoccia J, Linde A. Antigen detection: the method of choice in comparison with virus isolation and serology for laboratory diagnosis of herpes zoster in human immunodeficiency virus-infected patients. *J Clin Microbiol* 1997; **35**: 347-349 [PMID: 9003593]

444 **Olding-Stenkvist E**, Grandien M. Early diagnosis of virus-caused vesicular rashes by immunofluorescence on skin biopsies. I. Varicella, zoster and herpes simplex. *Scand J Infect Dis* 1976; **8**: 27-35 [PMID: 178050]

445 **Schmidt NJ**, Gallo D, Devlin V, Woodie JD, Emmons RW. Direct immunofluorescence staining for detection of herpes simplex and varicella-zoster virus antigens in vesicular lesions and certain tissue specimens. *J Clin Microbiol* 1980; **12**: 651-655 [PMID: 6268653]

446 **Pérez JL**, García A, Niubò J, Salvà J, Podzamczer D, Martín R. Comparison of techniques and evaluation of three commercial monoclonal antibodies for laboratory diagnosis of varicella-zoster virus in mucocutaneous specimens. *J Clin Microbiol* 1994; **32**: 1610-1613 [PMID: 8077417]

447 **Rawlinson WD**, Dwyer DE, Gibbons VL, Cunningham AL. Rapid diagnosis of varicella-zoster virus infection with a monoclonal antibody based direct immunofluorescence technique. *J Virol Methods* 1989; **23**: 13-18 [PMID: 2536379 DOI: 10.1016/0166-0934(89)90084-0]

448 **Zirn JR**, Tompkins SD, Huie C, Shea CR. Rapid detection and distinction of cutaneous herpesvirus infections by direct immunofluorescence. *J Am Acad Dermatol* 1995; **33**: 724-728 [PMID: 7593769 DOI: 10.1016/0190-9622(95)91808-6]

449 **Espy MJ**, Teo R, Ross TK, Svien KA, Wold AD, Uhl JR, Smith TF. Diagnosis of varicella-zoster virus infections in the clinical laboratory by LightCycler PCR. *J Clin Microbiol* 2000; **38**: 3187-3189 [PMID: 10970354]

450 **Stránská R**, Schuurman R, de Vos M, van Loon AM. Routine use of a highly automated and internally controlled real-time PCR assay for the diagnosis of herpes simplex and varicella-zoster virus infections. *J Clin Virol* 2004; **30**: 39-44 [PMID: 15072752 DOI: 10.1016/j.jcv.2003.08.006]

451 **Campsall PA**, Au NH, Prendiville JS, Speert DP, Tan R, Thomas EE. Detection and genotyping of varicella-zoster virus by TaqMan allelic discrimination real-time PCR. *J Clin Microbiol* 2004; **42**: 1409-1413 [PMID: 15070981 DOI: 10.1128/JCM.42.4.1409-1413.2004]

452 **LaRussa P**, Lungu O, Hardy I, Gershon A, Steinberg SP, Silverstein S. Restriction fragment length polymorphism of polymerase chain reaction products from vaccine and wild-type varicella-zoster virus isolates. *J Virol* 1992; **66**: 1016-1020 [PMID: 1346169]

453 **Engelmann I**, Petzold DR, Kosinska A, Hepkema BG, Schulz TF, Heim A. Rapid quantitative PCR assays for the simultaneous detection of herpes simplex virus, varicella zoster virus, cytomegalovirus, Epstein-Barr virus, and human herpesvirus 6 DNA in blood and other clinical specimens. *J Med Virol* 2008; **80**: 467-477 [PMID: 18205230 DOI: 10.1002/jmv.21095]

454 **Loparev VN**, Argaw T, Krause PR, Takayama M, Schmid DS. Improved identification and differentiation of varicella-zoster virus (VZV) wild-type strains and an attenuated varicella vaccine strain using a VZV open reading frame 62-based PCR. *J Clin Microbiol* 2000; **38**: 3156-3160 [PMID: 10970349]

455 **Loparev VN**, McCaustland K, Holloway BP, Krause PR, Takayama M, Schmid DS. Rapid genotyping of varicella-zoster virus vaccine and wild-type strains with fluorophore-labeled hybridization probes. *J Clin Microbiol* 2000; **38**: 4315-4319 [PMID: 11101557]

456 **Parker SP**, Quinlivan M, Taha Y, Breuer J. Genotyping of varicella-zoster virus and the discrimination of Oka vaccine strains by TaqMan real-time PCR. *J Clin Microbiol* 2006; **44**: 3911-3914 [PMID: 17088366 DOI: 10.1128/JCM.00346-06]

457 **Espy MJ**, Uhl JR, Sloan LM, Buckwalter SP, Jones MF, Vetter EA, Yao JD, Wengenack NL, Rosenblatt JE, Cockerill FR, Smith TF. Real-time PCR in clinical microbiology: applications for routine laboratory testing. *Clin Microbiol Rev* 2006; **19**: 165-256 [PMID: 16418529 DOI: 10.1128/CMR.19.1.165-256.2006]

458 **Cobo F**. Application of molecular diagnostic techniques for viral testing. *Open Virol J* 2012; **6**: 104-114 [PMID: 23248732 DOI: 10.2174/1874357901206010104]

459 **Ihira M**, Higashimoto Y, Kawamura Y, Sugata K, Ohashi M,

Asano Y, Yoshikawa T. Cycling probe technology to quantify and discriminate between wild-type varicella-zoster virus and Oka vaccine strains. *J Virol Methods* 2013; **193**: 308-313 [PMID: 23820238 DOI: 10.1016/j.jviromet.2013.06.031]

460 **Tang YW**, Allawi HT, DeLeon-Carnes M, Li H, Day SP, Schmid D. Detection and differentiation of wild-type and vaccine mutant varicella-zoster viruses using an Invader Plus method. *J Clin Virol* 2007; **40**: 129-134 [PMID: 17728179 DOI: 10.1016/j.jcv.2007.07.007]

461 **Thiele S**, Borschewski A, Küchler J, Bieberbach M, Voigt S, Ehlers B. Molecular analysis of varicella vaccines and varicella-zoster virus from vaccine-related skin lesions. *Clin Vaccine Immunol* 2011; **18**: 1058-1066 [PMID: 21562115 DOI: 10.1128/CVI.05021-11]

462 **Toi CS**, Dwyer DE. Differentiation between vaccine and wild-type varicella-zoster virus genotypes by high-resolution melt analysis of single nucleotide polymorphisms. *J Clin Virol* 2008; **43**: 18-24 [PMID: 18479962 DOI: 10.1016/j.jcv.2008.03.027]

463 **Sauerbrei A**, Uebe B, Wutzler P. Molecular diagnosis of zoster post varicella vaccination. *J Clin Virol* 2003; **27**: 190-199 [PMID: 12829041 DOI: 10.1016/S1386-6532(03)00071-4]

464 **Fan F**, Stiles J, Mikhlina A, Lu X, Babady NE, Tang YW. Clinical validation of the Lyra direct HSV 1+2/VZV assay for simultaneous detection and differentiation of three herpesviruses in cutaneous and mucocutaneous lesions. *J Clin Microbiol* 2014; **52**: 3799-3801 [PMID: 25078915 DOI: 10.1128/JCM.02098-14]

465 **Tan HH**, Goh CL. Viral infections affecting the skin in organ transplant recipients: epidemiology and current management strategies. *Am J Clin Dermatol* 2006; **7**: 13-29 [PMID: 16489840 DOI: 10.2165/00128071-200607010-00003]

466 **Bennett S**, Carman WF, Gunson RN. The development of a multiplex real-time PCR for the detection of herpes simplex virus 1 and 2, varizella zoster virus, adenovirus and Chlamydia trachomatis from eye swabs. *J Virol Methods* 2013; **189**: 143-147 [PMID: 23000752 DOI: 10.1016/j.jviromet.2012.08.020]

467 **Tan TY**, Zou H, Ong DC, Ker KJ, Chio MT, Teo RY, Koh MJ. Development and clinical validation of a multiplex real-time PCR assay for herpes simplex and varicella zoster virus. *Diagn Mol Pathol* 2013; **22**: 245-248 [PMID: 24193006 DOI: 10.1097/PDM.0b013e3182914291]

468 **Buelow DR**, Bankowski MJ, Fofana D, Gu Z, Pounds S, Hayden RT. Comparison of two multiplexed PCR assays for the detection of HSV-1, HSV-2, and VZV with extracted and unextracted cutaneous and mucosal specimens. *J Clin Virol* 2013; **58**: 84-88 [PMID: 23751960 DOI: 10.1016/j.jcv.2013.05.008]

469 **Sakai K**, Wakasugi S, Muchemwa FC, Ihn H. Quick detection of herpes viruses from skin vesicles and exudates without nucleic acid extraction using multiplex PCR. *Biosci Trends* 2008; **2**: 164-168 [PMID: 20103923]

470 **Binkhamis K**, Al-Siyabi T, Heinstein C, Hatchette TF, LeBlanc JJ. Molecular detection of varicella zoster virus while keeping an eye on the budget. *J Virol Methods* 2014; **202**: 24-27 [PMID: 24607430 DOI: 10.1016/j.jviromet.2014.02.009]

471 **Legoff J**, Feghoul L, Mercier-Delarue S, Dalle JH, Scieux C, Chérot J, de Fontbrune FS, Baruchel A, Socié G, Simon F. Broad-range PCR-electrospray ionization mass spectrometry for detection and typing of adenovirus and other opportunistic viruses in stem cell transplant patients. *J Clin Microbiol* 2013; **51**: 4186-4192 [PMID: 24108617 DOI: 10.1128/JCM.01978-13]

472 **Lévêque N**, Legoff J, Mengelle C, Mercier-Delarue S, N'guyen Y, Renois F, Tissier F, Simon F, Izopet J, Andréoletti L. Virological diagnosis of central nervous system infections by use of PCR coupled with mass spectrometry analysis of cerebrospinal fluid samples. *J Clin Microbiol* 2014; **52**: 212-217 [PMID: 24197874 DOI: 10.1128/JCM.02270-13]

473 **Mehta SK**, Tyring SK, Gilden DH, Cohrs RJ, Leal MJ, Castro VA, Feiveson AH, Ott CM, Pierson DL. Varicella-zoster virus in the saliva of patients with herpes zoster. *J Infect Dis* 2008; **197**: 654-657 [PMID: 18260763 DOI: 10.1086/527420]

474 **Leung J**, Harpaz R, Baughman AL, Heath K, Loparev V, Vázquez M, Watson BM, Schmid DS. Evaluation of laboratory methods for diagnosis of varicella. *Clin Infect Dis* 2010; **51**: 23-32 [PMID: 20504232 DOI: 10.1086/653113]

475 **Beards G**, Graham C, Pillay D. Investigation of vesicular rashes for HSV and VZV by PCR. *J Med Virol* 1998; **54**: 155-157 [PMID: 9515761 DOI: 10.1002/(SICI)1096-9071(199803)54: 3<155::AID-JMV1>3.0.CO;2-4]

476 **Nahass GT**, Mandel MJ, Cook S, Fan W, Leonardi CL. Detection of herpes simplex and varicella-zoster infection from cutaneous lesions in different clinical stages with the polymerase chain reaction. *J Am Acad Dermatol* 1995; **32**: 730-733 [PMID: 7722016 DOI: 10.1016/0190-9622(95)91450-1]

477 **Vázquez M**. Varicella infections and varicella vaccine in the 21st century. *Pediatr Infect Dis J* 2004; **23**: 871-872 [PMID: 15361729 DOI: 10.1097/01.inf.0000140786.15816.38]

478 **American Academy of Pediatrics**. Varicella-Zoster Infections. In: Pickering LK, Baker CJ, Long SS, McMillan JA. Red Book: 2006 Report of the Committee on Infectious Diseases. 27th ed. Elk Grove Village, IL: American Academy of Pediatrics, 2006: 711-725 Available from: URL: https://redbook.solutions.aap.org/DocumentLibrary/2006 RB.pdf

479 **Shiraki K**, Okuno T, Yamanishi K, Takahashi M. Polypeptides of varicella-zoster virus (VZV) and immunological relationship of VZV and herpes simplex virus (HSV). *J Gen Virol* 1982; **61** (Pt 2): 255-269 [PMID: 6288857 DOI: 10.1099/0022-1317-61-2-255]

480 **Oladepo DK**, Klapper PE, Percival D, Vallely PJ. Serological diagnosis of varicella-zoster virus in sera with antibody-capture enzyme-linked immunosorbent assay of IgM. *J Virol Methods* 2000; **84**: 169-173 [PMID: 10680966 DOI: 10.1016/S0166-0934(99)00139-1]

481 **Watson B**, Keller PM, Ellis RW, Starr SE. Cell-mediated immune responses after immunization of healthy seronegative children with varicella vaccine: kinetics and specificity. *J Infect Dis* 1990; **162**: 794-799 [PMID: 2169495 DOI: 10.1093/infdis/162.4.794]

482 **Nagel MA**, Forghani B, Mahalingam R, Wellish MC, Cohrs RJ, Russman AN, Katzan I, Lin R, Gardner CJ, Gilden DH. The value of detecting anti-VZV IgG antibody in CSF to diagnose VZV vasculopathy. *Neurology* 2007; **68**: 1069-1073 [PMID: 17287447 DOI: 10.1212/01.wnl.0000258549.13334.16]

483 **Grahn A**, Studahl M, Nilsson S, Thomsson E, Bäckström M, Bergström T. Varicella-zoster virus (VZV) glycoprotein E is a serological antigen for detection of intrathecal antibodies to VZV in central nervous system infections, without cross-reaction to herpes simplex virus 1. *Clin Vaccine Immunol* 2011; **18**: 1336-1342 [PMID: 21697341 DOI: 10.1128/CVI.05061-11]

484 **Kangro HO**, Manzoor S, Harper DR. Antibody avidity following varicella-zoster virus infections. *J Med Virol* 1991; **33**: 100-105 [PMID: 1646852 DOI: 10.1002/jmv.1890330207]

485 **Schoub BD**, Blackburn NK, Johnson S, McAnerney JM, Miller B. Low antibody avidity in elderly chickenpox patients. *J Med Virol* 1992; **37**: 113-115 [PMID: 1321222 DOI: 10.1002/jmv.1890370207]

486 **Thomas HI**, Morgan-Capner P, Meurisse EV. Studies on the avidity of IgG1 subclass antibody specific for varicella-zoster virus. *Serodiagn immunother Infect Dis* 1990; **4**: 371-377 [DOI: 10.1016/0888-0786(90)90027-L]

487 **Kneitz RH**, Schubert J, Tollmann F, Zens W, Hedman K, Weissbrich B. A new method for determination of varicella-zoster virus immunoglobulin G avidity in serum and cerebrospinal fluid. *BMC Infect Dis* 2004; **4**: 33 [PMID: 15355548 DOI: 10.1186/1471-2334-4-33]

488 **L'Huillier AG**, Ferry T, Courvoisier DS, Aebi C, Cheseaux JJ, Kind C, Rudin C, Nadal D, Hirschel B, Sottas C, Siegrist CA, Posfay-Barbe KM. Impaired antibody memory to varicella zoster virus in HIV-infected children: low antibody levels and avidity*. *HIV Med* 2012; **13**: 54-61 [PMID: 21722287 DOI: 10.1111/j.1468-1293.2011.00936.x]

489 **Prelog M**, Schönlaub J, Jeller V, Almanzar G, Höfner K, Gruber S, Eiwegger T, Würzner R. Reduced varicella-zoster-virus (VZV)-specific lymphocytes and IgG antibody avidity in solid organ transplant recipients. *Vaccine* 2013; **31**: 2420-2426 [PMID: 23583889 DOI: 10.1016/j.vaccine.2013.03.058]

490 **Ridings J**, Nicholson IC, Goldsworthy W, Haslam R, Roberton DM, Zola H. Somatic hypermutation of immunoglobulin genes in

human neonates. *Clin Exp Immunol* 1997; **108**: 366-374 [PMID: 9158112 DOI: 10.1046/j.1365-2249.1997.3631264.x]

491 **Ridings J**, Dinan L, Williams R, Roberton D, Zola H. Somatic mutation of immunoglobulin V(H)6 genes in human infants. *Clin Exp Immunol* 1998; **114**: 33-39 [PMID: 9764600 DOI: 10.1046/j.1365-2249.1998.00694.x]

492 **Junker AK**, Tilley P. Varicella-zoster virus antibody avidity and IgG-subclass patterns in children with recurrent chickenpox. *J Med Virol* 1994; **43**: 119-124 [PMID: 8083659 DOI: 10.1002/jmv.1890430204]

493 **Pretorius DH**, Hayward I, Jones KL, Stamm E. Sonographic evaluation of pregnancies with maternal varicella infection. *J Ultrasound Med* 1992; **11**: 459-463 [PMID: 1337112]

494 **Scharf A**, Scherr O, Enders G, Helftenbein E. Virus detection in the fetal tissue of a premature delivery with a congenital varicella syndrome. A case report. *J Perinat Med* 1990; **18**: 317-322 [PMID: 2175786]

495 **Bruder E**, Ersch J, Hebisch G, Ehrbar T, Klimkait T, Stallmach T. Fetal varicella syndrome: disruption of neural development and persistent inflammation of non-neural tissues. *Virchows Arch* 2000; **437**: 440-444 [PMID: 11097371 DOI: 10.1007/s004280000236]

496 **Petignat P**, Vial Y, Laurini R, Hohlfeld P. Fetal varicella-herpes zoster syndrome in early pregnancy: ultrasonographic and morphological correlation. *Prenat Diagn* 2001; **21**: 121-124 [PMID: 11241539 DOI: 10.1002/1097-0223(200102)21:2<121::AID-PD996>3.0.CO;2-P]

497 **Verstraelen H**, Vanzieleghem B, Defoort P, Vanhaesebrouck P, Temmerman M. Prenatal ultrasound and magnetic resonance imaging in fetal varicella syndrome: correlation with pathology findings. *Prenat Diagn* 2003; **23**: 705-709 [PMID: 12975778 DOI: 10.1002/pd.669]

498 **Mirlesse V**, Solé Y, Jacquemard F, Delhommeau F, Daffos F. Persistent maternal viremia after varicella infection during pregnancy as a possible cause of false positive prenatal diagnosis of fetal infection on amniotic fluid. *BJOG* 2004; **111**: 885-887 [PMID: 15270944 DOI: 10.1111/j.1471-0528.2004.00189.x]

499 **Guerra B**, Simonazzi G, Puccetti C, Lanari M, Farina A, Lazzarotto T, Rizzo N. Ultrasound prediction of symptomatic congenital cytomegalovirus infection. *Am J Obstet Gynecol* 2008; **198**: 380.e1-380.e7 [PMID: 18191802 DOI: 10.1016/j.ajog.2007.09.052]

500 **Lécuru F**, Taurelle R, Bernard JP, Parrat S, Lafay-pillet MC, Rozenberg F, Lebon P, Dommergues M. Varicella zoster virus infection during pregnancy: the limits of prenatal diagnosis. *Eur J Obstet Gynecol Reprod Biol* 1994; **56**: 67-68 [DOI: 10.1016/0028-2243(94)90156-2]

501 **Weisz B**, Book M, Lipitz S, Katorza E, Achiron R, Grossman Z, Shrim A. Fetal outcome and amniocentesis results in pregnancies complicated by varicella infection. *J Obstet Gynaecol Can* 2011; **33**: 720-724 [PMID: 21749748 DOI: 10.1016/S1701-2163(16)34957-X]

502 **Johansson AB**, Rassart A, Blum D, Van Beers D, Liesnard C. Lower-limb hypoplasia due to intrauterine infection with herpes simplex virus type 2: possible confusion with intrauterine varicella-zoster syndrome. *Clin Infect Dis* 2004; **38**: e57-e62 [PMID: 15034848 DOI: 10.1086/382673]

503 **Koskimies O**, Lapinleimu K, Saxén L. Infections and other maternal factors as risk indicators for congenital malformations: a case-control study with paired serum samples. *Pediatrics* 1978; **61**: 832-837 [PMID: 209394]

504 **Sauerbrei A**, Glück B, Jung K, Bittrich H, Wutzler P. Congenital skin lesions caused by intrauterine infection with coxsackievirus B3. *Infection* 2000; **28**: 326-328 [PMID: 11073143 DOI: 10.1007/s150100070029]

505 **Sanchez MA**, Bello-Munoz JC, Cebrecos I, Sanz TH, Martinez JS, Moratonas EC, Roura LC. The prevalence of congenital varicella syndrome after a maternal infection, but before 20 weeks of pregnancy: a prospective cohort study. *J Matern Fetal Neonatal Med* 2011; **24**: 341-347 [PMID: 20670093 DOI: 10.3109/14767058.2010.497567]

506 **Kustermann A**, Zoppini C, Tassis B, Della Morte M, Colucci G, Nicolini U. Prenatal diagnosis of congenital varicella infection. *Prenat Diagn* 1996; **16**: 71-74 [PMID: 8821856 DOI: 10.1002/(SICI)1097-0223(199601)16:1<71::AID-PD806>3.0.CO;2-Z]

507 **Isada NB**, Paar DP, Johnson MP, Evans MI, Holzgreve W, Qureshi F, Straus SE. In utero diagnosis of congenital varicella zoster virus infection by chorionic villus sampling and polymerase chain reaction. *Am J Obstet Gynecol* 1991; **165**: 1727-1730 [PMID: 1661069]

508 **Cuthbertson G**, Weiner CP, Giller RH, Grose C. Prenatal diagnosis of second-trimester congenital varicella syndrome by virus-specific immunoglobulin M. *J Pediatr* 1987; **111**: 592-595 [PMID: 2821214 DOI: 10.1016/S0022-3476(87)80128-2]

509 **Liesnard C**, Donner C, Brancart F, Rodesch F. Varicella in pregnancy. *Lancet* 1994; **344**: 950-951 [PMID: 7802821 DOI: 10.1016/S0140-6736(94)92299-3]

510 **Katz G**, Pfau A. Congenital varicella causing neurogenic bladder and anal dysfunction. *Urology* 1986; **28**: 424-425 [PMID: 3787907 DOI: 10.1016/0090-4295(86)90078-6]

511 **Gershon AA**. Varicella-zoster virus. In: Feign RD, Cherry JD. Textbook of pediatric infectious diseases. Philadelphia: WB Saunders Company, 1998: 1769-1777

512 **Sauerbrei A**, Müller D, Eichhorn U, Wutzler P. Detection of varicella-zoster virus in congenital varicella syndrome: a case report. *Obstet Gynecol* 1996; **88**: 687-689 [PMID: 8841253 DOI: 10.1016/0029-7844(96)00253-0]

513 **Al-Katawee YA**, Al-Hasoun YA, Taha MN, Al-Moslem K. Congenital varicella-zoster virus infection. A rare case of severe brain and ocular malformations without limb or cutaneous involvement in a newborn after maternal subclinical infection. *Saudi Med J* 2005; **26**: 869-871 [PMID: 15951887]

514 **Spranger S**, Stute H, Blankenagel A, Jauch A, Hager D, Tariverdian G. MIDAS-Syndrom-Eine X-chromosomale Erkrankung. Differenzialdiagnose zum Kongenitalen Varizellensyndrom. *Monatsschr Kinderheilkd* 1998; **146**: 761-765 [DOI: 10.1007/s001120050319]

515 **Ooi PL**, Goh KT, Doraisingham S, Ling AE. Prevalence of varicella-zoster virus infection in Singapore. *Southeast Asian J Trop Med Public Health* 1992; **23**: 22-25 [PMID: 1523475]

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ORIGINAL ARTICLE

Basic Study

Genotyping and pathotyping of diversified strains of infectious bronchitis viruses circulating in Egypt

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Abstract

AIM: To characterize the circulating infectious bronchitis virus (IBV) strains in Egypt depending on the sequence of the spike-1 (*S1*) gene [hypervariable region-3 (HVR-3)] and to study the pathotypic features of these strains.

METHODS: In this work, twenty flocks were sampled for IBV detection using RRT-PCR and isolation of IBV in specific pathogen free (SPF) chicks during the period from 2010 to 2015. Partial sequencing and phylogenetic analysis of 400 bp representing the HVR-3 of the *S1* gene was conducted. Pathotypic characterization of one selected virus from each group (Egy/Var-Ⅰ, Egy/Var-Ⅱ and classic) was evaluated in one day old SPF chicks. The chicks were divided into 4 groups 10 birds each including the negative control group. Birds were inoculated at one day by intranasal instillation of 10^5EID$_{50}$/100 μL of IBV viruses [IBV-EG/1212B-2012 (Egy/Var-Ⅱ), IBV/EG/IBV1-2011 (Egy/Var-Ⅰ) and IBV-EG/11539F-2011 (classic)], while the remaining negative control group was kept uninfected. The birds were observed for clinical signs, gross lesions and virus pathogenicity. The real-time rRT-PCR test was performed for virus detection in the tissues. Histopathological examinations were evaluated in both trachea and kidneys.

RESULTS: The results revealed that these viruses were separated into two distinct groups; variant (GI-23) and

classic (GI-1), where 16 viruses belonged to a variant group, including 2 subdivisions [Egy/Var-Ⅰ(6 isolates) and Egy/Var-Ⅱ (10 isolates)] and 4 viruses clustered to the classic group (Mass-like). IBV isolates in the variant group were grouped with other IBV strains from the Middle East. The variant subgroup (Egy/Var-Ⅰ) was likely resembling the original Egyptian variant strain (Egypt/Beni-Suif/01) and the Israeli strain (IS/1494/2006). The second subgroup (Egy/Var-Ⅱ) included the viruses circulating in the Middle East (Ck/EG/BSU-2 and Ck/EG/BSU-3/2011) and the Israeli strain (IS/885/00). The two variant subgroups (Egy/Var-Ⅰ and Egy/Var-Ⅱ) found to be highly pathogenic to SPF chicks with mortalities up to 50% than those of the classic group which was of low virulence (10% mortality). Pathogenicity indices were 25 (Egy/Var-Ⅱ), 24 (Egy/Var-Ⅰ) and 8 (classic); with clinical scores 3, 2 and 1 respectively.

CONCLUSION: These findings indicated that the recent circulating Egyptian IBVs have multiple heterogeneous origins in marked diversifying nature of their spread, with high pathotype in specific pathogen free chicks.

Key words: Infectious bronchitis virus; Phylogenetic analysis; Variant infectious bronchitis virus; *S1* gene; HVR-3 Sequencing; Egypt

Core tip: Infectious bronchitis became enzootic in Egypt with frequent outbreaks of different variant in broiler chickens in spite of intensive vaccination programs used causing severe infections. These manuscripts discuss the prevalence of these different variants with pathotyping of these variants in specific pathogen free chicks.

Zanaty A, Arafa AS, Hagag N, El-Kady M. Genotyping and pathotyping of diversified strains of infectious bronchitis viruses circulating in Egypt. *World J Virol* 2016; 5(3): 125-134 Available from: URL: http://www.wjgnet.com/2220-3249/full/v5/i3/125.htm DOI: http://dx.doi.org/10.5501/wjv.v5.i3.125

INTRODUCTION

Infectious bronchitis virus (IBV) is a highly contagious viral disease causing severe economic losses in the commercial poultry industry and is ubiquitous in most parts of the world. IBV targets primarily the upper respiratory tract; however the nephropathogenic strains have a predilection for the kidney of young chickens causing nephritis that can produce significant mortalities[1-3]. In layers and breeders, a decrease in egg production and egg quality has been documented[4].

IBV is a coronavirus of genus *Gammacoronavirus*; family *Coronaviridae* order *Nidovirales*[5]. IBV is an enveloped virus and has a linear positive sense non-segmented single-stranded RNA genome, approximately 27.6 kb in length. Four main structural proteins construct the IBV particles; namely the phosphorylated nucleoprotein (N), the membrane protein (M), the spike (S) glycoprotein and the small membrane protein (E). The S glycoprotein is proteolitically cleaved into 2 separate subunits; the S1 and S2 polypeptide[6]. The S1 subunit is attached to the viral envelope and is responsible for fusion of virus envelope and the host cell membrane. It carries virus-neutralizing and serotype-specific determinants that located in the hypervariable regions (HVRs) of the S1 subunit[7]. Furthermore, S1 reveals high sequence variability than S2 subunit[8]. Hence, the evolutionary characterization and detection of IBV is mainly targeting the analysis of the variable *S1* gene or the expressed S1 protein[9].

IBV variants are distributed worldwide, there are more than 20 IBV serotypes differentiated globally[10]. Genomic insertions, deletions, point mutations, substitutions and RNA recombination of the *S1* gene are associated with the emergence of new variants[5,11]. Different serotypes of newly evolved variants from chickens may cause partially efficacious vaccines or even vaccine breaks[12].

In Egypt, IBV strains continue to spread everywhere in the country, and have been isolated from both vaccinated and non-vaccinated flocks[13]. Different genotypes were isolated from poultry flocks and they were similar to Massachusetts, D3128, D274, D08880, 793B (4/91 and CR88), IS/885/00 and Egypt/Beni-Suef/01[14-16]. In 2011, two Egyptian strains, named Egy/Var-Ⅱ (Ck/Eg/BSU-2, 3/2011), were reported as a new IBV variant resembling IS/885/00 strain according to sequence of the HVR-3[16]. Recently in 2016, depending on the full S1 sequence, Valastro *et al*[9] clustered the Egyptian variant strains in the GI-23 lineage which represents the unique wild-type cluster geographically confined to the middle East.

Herein, twenty chicken flocks suffering from IBV-like symptoms were genetically and phylogenetically analyzed based on the *HVR-3* of *S1* gene and compared to the previously isolated Egyptian viruses and others from neighboring countries along with common vaccine viruses used. In addition, pathotyping of three viruses was carried out to determine the pathogenic type of isolates. The resulting information will provide a guide for the matching level between field and vaccine viruses and that will help for optimal use of existing live vaccines and plan for future vaccine strategy.

MATERIALS AND METHODS

Samples and flock history

Samples were collected from twenty broiler farms from 12 governorates in Egypt, showing mild-to-severe respiratory signs, in the period between 2010 and 2015 (Table 1). Samples were delivered to the Reference Laboratory for Quality control on Poultry production (RLQP), Egypt. The chickens were vaccinated with H120 strain of IBV at one day of age. Chickens showed

Table 1 Phylogenetic grouping of infectious bronchitis virus in the study

Isolate No.	Isolate name	Age of birds (d)	Governorate	GeneBank accession number	Phylogenetic group
1	IBV-EG/12773F(3)-2012	20	Beni-Suef	KC608180	Classic[1]
2	IBV-EG/11539F-2011	23	Kafr-El-Shikh	JQ839289	Classic
3	IBV-EG/10643F(1-7)-2010	19	Sharqia	KC608171	Classic
4	IBV-EG/116F-1(1)-2011	30	Gharbia	KC608176	Classic
5	IBV-EG/11673F-2011	20	Alexandria	KC608173	Egy Var I
6	IBV-EG/11413F-2011	25	Sharqia	KC608177	Egy Var I
7	IBV-EG/10324F-2010	17	Giza	KC608172	Egy Var I
8	IBV-EG/1299B-2012	18	Fayoum	KC608182	Egy Var I
9	IBV-EG/1196F-2011	15	Behira	KC608174	Egy Var I
10	IBV/EG/IBV1-2011	25	Suez	JQ839288	Egy Var I
11	IBV-EG/1138F-4-2011	18	Dakahlia	KC608175	Egy Var II
12	IBV-EG/Qalyobia/121-2012	22	Qaliobeya	KC608181	Egy Var II
13	IBV-EG/1212B-2012	14	Al Behira	JQ839287	Egy Var II
14	IBV-EG/1293B-2012	21	Fayoum	KC608178	Egy Var II
15	IBV-EG/1262F(3)-2012	28	Ismalia	KC608179	Egy Var II
16	CH/EGYPT/13200F/2013	20	Giza	KT832805	Egy Var II
17	CH/EGYPT/13950F/2013	25	Alexandria	KT832806	Egy Var II
18	CH/EGYPT/14251F/2014	22	Suez	KT832807	Egy Var II
19	CH/EGYPT/141107F/2014	32	Giza	KT832808	Egy Var II
20	CH/EGYPT/15919F/2015	26	Dakahlia	KT832809	Egy Var II

[1]Massachusetts - like strain.

respiratory symptoms such as gasping, coughing, sneezing and tracheal rales with white diarrhea in some cases. Necropsy showed mild to severe tracheitis with congested lung. In addition, birds were suffering from kidney lesions such as enlargement, congestion, and uroletheasis. The samples were collected as pooled homogenate from trachea and kidney. Further, the samples were prepared as 10% w/v suspensions in PBS (pH 7.4) and centrifuged at 3000 rpm for 10 min; the supernatants were then collected for further analysis.

Virus detection and isolation

Viral RNA was extracted directly from the samples by using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), following the kit manufacturer's instructions. The virus identification was confirmed by real-time reverse transcription-polymerase chain reaction (rRT-PCR) for the presence of the neucloprotein (*NP*) gene sequence of the IBV using Quantitect probe RT-PCR kit (Qiagen, Hilden, Germany), with specific primers and probe targeting the *NP* gene[17].

Viral isolation from trachea and kidney was performed according to Momayez *et al*[18]. Nine-eleven day old SPF chicken eggs were inoculated *via* the chorioallantoic route. Dead embryos were investigated for the presence of embryo stunting, curling, dwarfing, subcutaneous hemorrhage and ureate deposition in the mesonephros. The allantoic fluids from each sample were screened using rRT-PCR for further confirmation.

Sequencing of the S1 gene and phylogenetic analysis

Positive virus screening was further tested using a specific primer set for the amplifications of the *HVR-3* of the *S1* gene using Qiagen one-step RT-PCR (Qiagen, Hilden, Germany), according to the manufacturer's protocol[19]. Amplificates of 400 bp in size were excised and purified from gels using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The purified RT-PCR products were sequenced using Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer, Foster City, CA) and Applied Biosystems 3130 genetic analyzer (ABI, United States). Sequences similarities and relationships of the *HVR-3* of the *S1* gene from the 20 samples in this study were compared with previously published IBV vaccine and reference strains available in the public database (NCBI, United States). Amino acids phylogenetic tree was drawn for the sequenced isolates along with other vaccine and reference strains available in the GenBank database using MEGA version 6[20]. A comparative analysis of deduced amino acids and nucleotide sequences of the HVR-3 was created using the CLUSTAL W Multiple Sequence Alignment Program, version 1.83 of MegAlign module of Lasergene DNAStar software[21]. Sequences generated in the frame of this study were submitted to the GenBank database with accession numbers showed in Table 1.

Pathogenicity study

Forty SPF chicks of one-day-old were obtained from the Nile SPF company, Kom-Oshim, Fayoum, Egypt. The birds were housed in separate bio-safety level-3 chicken isolators in RLQP under strict hygienic conditions. Chickens were tagged with wing bands for identification and randomly divided into 4 groups 10 birds each including the negative control group. The first three groups were assigned for determination of pathogenicity. Birds were inoculated at one day by intranasal instillation of 10^5EID$_{50}$/100 μL of IBV viruses (IBV-EG/1212B-2012, IBV/EG/IBV1-2011 and IBV-EG/11539F-2011) according to Purcell *et al*[22], while the remaining negative control group was kept uninfected.

The birds were observed for clinical signs, gross lesions and virus pathogenicity. The clinical signs were

Table 2 Nucleotide and deduced amino acid identities of infectious bronchitis virus Egyptian isolates with other selected references and vaccine strains from different serotypes

Strain name nucleotide identity (%) (upper right) amino acid identity (%) (lower left)	Egypt/Beni-Suef/01	IB-isolate-variant-2-S1	IBV-IS-1494-06-S1	IBV-isolate-IS-885-S1	QXIBV	IBV-H120	IBV-Ma5	IBV-M41	IBV-variant-1-S1-(1/96)	IBV-4-91	IBV-CR12188	IBV-D274	IBV-Eg/11539F-2011 (Classic)	IBV-Eg/IBV1-2011 (EGY-Var-1)	IBV-Eg/1212B-2012 (EGY-Var-2)
Egypt/Beni-Suef/01		72	73	70	86	65	65	64	81	68	80	82	68	98	89
IB-isolate-variant-2-S1	97		99	92	91	87	87	87	63	90	63	66	84	70	67
IBV-IS-1494-06-S1	99	98		94	91	87	87	87	63	90	63	66	84	71	68
IBV-isolate-IS-885-S1	90	88	90		90	86	86	86	63	89	62	65	83	69	69
QXIBV	86	85	86	84		85	85	85	63	92	63	65	82	67	66
IBV-H120	79	79	79	76	76		100	100	61	83	60	65	95	65	64
IBV-Ma5	79	79	79	76	76	100		100	61	83	60	65	95	65	64
IBV-M41	79	79	79	76	76	99	99		61	83	60	65	95	65	64
IBV-variant-1-S1 (1/96)	80	80	80	79	85	72	72	71		69	98	82	63	80	81
IBV-4-91	83	83	83	82	87	74	74	73	95		68	66	80	66	65
IBV-CR12188	82	81	82	80	86	72	72	71	96	95		83	62	79	80
IBV-D274	82	82	83	80	83	78	78	77	80	83	83		68	82	81
IBV-Eg/11539F-2011 (Classic)	77	77	77	74	74	98	98	97	71	73	71	76		81	78
IBV-Eg/IBV1-2011 (EGY-Var-1)	97	94	96	88	84	81	81	81	78	81	80	80	79		88
IBV-Eg/1212B-2012 (EGY-Var-2)	89	87	89	90	83	80	80	80	81	83	81	82	78	87	

IBV: Infectious bronchitis virus.

recorded daily for up to 14 d post-infection, according to clinical scoring formula presented by Wang *et al*[1]. Gross lesions in the trachea and kidney as well as pathogenicity index were calculated based on the criteria described in Wang *et al*[1]. The presence of IBV was checked in samples obtained from the inoculated groups at 14 d post-infection. The real-time rRT-PCR test was performed for the detection of virus in the tissues. Histopathological examinations were carried out on both trachea and kidneys, according to Bancroft *et al*[23].

RESULTS

Virus screening and isolation

Samples representing the twenty flocks in this study showed positive results for detection of IBV using rRT-PCR. Virus isolation was obtained from the homogenate pool of the trachea and kidney from each flock. The allantoic fluid from the 3rd passage of each sample further confirmed positive using rRT-PCR.

Genetic and phylogenetic characterization

The 400 bp amplified PCR products of the *HVR-3* of *S1* gene were obtained from the 20 positive samples, then the partial sequencing of HVR-3 and sequence analysis was conducted. Phylogenetic tree was constructed from the amino acid sequences of HVR-3 of the S1 protein (Figure 1). The results indicated that Egyptian IBV viruses in this study were divided into two distinct groups (classic and variant). Sixteen isolates, isolate numbers 5 to 20 (Table 1), were found to be closely related to the variant group and 4 isolates, numbers 1 to 4 (Table 1), were genetically related to the classic genotype of Massachusetts strain.

The sixteen variant isolates were further subdivided into two subgroups: IBV Egy/Var-Ⅰand Ⅱ. Where, virus isolates, numbers from 5-10 (Table 1), were found to be related to Egy/Var-Ⅰand they were very close to both the ancestral Egyptian virus (Egypt/Beni-Suef/01, Genbank accession number JX174183.1) and other viruses of Egy/Var-Ⅰavailable in the GenBank, also to IBV-IS-1494-06. They share amino acid identities from 96% to 100% with each other (Table 2). Virus isolates, numbers from 11 to 20, were clustered within the Egy/Var-Ⅱsubgroup (Ck/Eg/BSU-2/2011, Ck/Eg/BSU-3/2011 and IBV/IS/885-00) and other Egyptian related strains in the GenBank (Table 2, Figure 1).

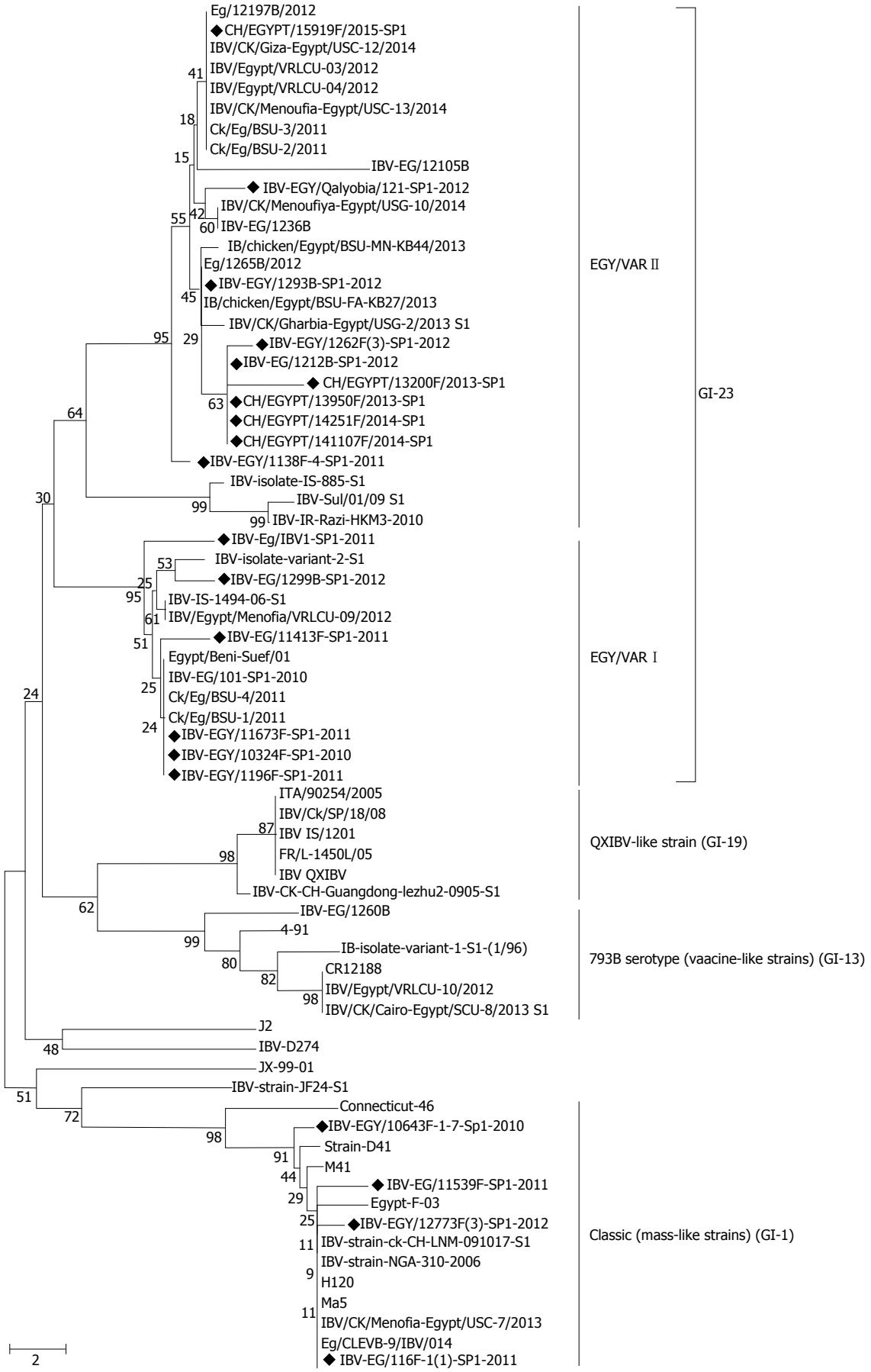

Figure 1 Phylogenetic tree representing the partial amino acid sequences of the *S1* gene for 20 infectious bronchitis virus isolates (marked with black diamond) with other related infectious bronchitis virus and reference strains.

Table 3 Necropsy findings of specific pathogen free chicks infected with infectious bronchitis viruses at one-day-old and the examined survivor and dead birds during the 14 d observation

Group	IBV isolates code	Necropsy							
		Trachea				Kidney			
		No Lesion	Slight mucin	Excessive mucin	Mucosal congestion	No Lesion	Swelling	Ureate	Congestion
1	IBV-EG/1212B-2012	0	2	5	3	0	6	2	2
2	IBV-EG/IBV1-2011	0	5	3	2	0	5	2	3
3	IBV-EG/11539F-2011	3	6	0	1	10	0	0	0
4	Negative control	10	0	0	0	10	0	0	0

Kidney and trachea score = no of chicks with lesion score of ⩾ 1. IBV: Infectious bronchitis virus.

Table 4 Pathogenicity index based on clinical scoring and necropsy of kidney and trachea of specific pathogen free chicks infected at one-day-old with infectious bronchitis viruses with examining survivors and dead birds during 14 d observation

Group	IBV isolates code	Observation record			Clinical score[1]	Gross lesion score[2]		Pathogenicity index[3]	Pathotyp[4]
		Dead	Survived	Mortality (%)		Trachea	Kidney		
1	IBV-EG/1212B-2012	5/10	5/10	50	3	10	10	25	High
2	IBV-EG/IBV1-2011	4/10	6/10	40	2	10	10	24	High
3	IBV-EG/11539F-2011	1/10	9/10	10	1	7	0	8	Low
4	Negative control	0/10	10/10	0	0	0	0	0	No

[1]Clinical score[2,28]: Score 0: No clinical signs; score 1: Lacrimation, slight shaking of head, watery feces; score 2: Lacrimation, presence of nasal exudate, depression, watery feces; score 3: Strong lacrimation, presence of nasal exudates, severe watery feces; [2]kidney and trachea score: No of chicks with lesion score of ⩾ 1; [3]pathogenicity index: No of chicks with lesion score ⩾ 1 + 1 point for every 10% mortality; [4]pathotypes: Low (pathogenicity index value 1-9), intermediate (pathogenicity index value 10-18), high (pathogenicity index value ⩾ 19). IBV: Infectious bronchitis virus.

Table 5 Virus detection from various organs of chicks post-inoculated with different infectious bronchitis viruses using reverse transcription-polymerase chain reaction

Group	IBV isolates	Phylogenetic group	Virus detection (number of positive/total)		
			Trachea	Kidney	Lung
1	IBV-EG/1212B-2012	Egy Var Ⅱ	10/10	9/10	6/10
2	IBV-EG/IBV1-2011	Egy Var Ⅰ	8/10	10/10	0/10
3	IBV-EG/11539F-2011	Classic (mass-like strain)	5/10	0/10	0/10
4	Negative control		0/10	0/10	0/10

IBV: Infectious bronchitis virus.

Alignment analysis of the S1-HVR3 for both nucleotide and deduced amino acid were performed and compared with the previously published reference and vaccine IBV strains commonly used in the field (H120, Ma5, D274, 4/91, CR88121 and 1/96) as well as original Egyptian viruses Egy/Var-Ⅰ and Egy/Var-Ⅱ. One virus represents the consensus of each group was selected for the comparative analysis (Table 2). Classic Egyptian IBV isolates (1 to 4) showed amino acid identities reached up to 98% with the Mass-like strains (H120, Ma5 and M41), while they showed only about 78% amino acid identities with the variant group. In comparison to vaccine strains used in Egypt, the viruses isolated in this study share different amino acid identities: For Egy/Var-Ⅰ strains, including strain number from 5 to 10 they showed identity from 78% to 81% for strains H120, Ma5, 4/91, CR88, D274 and 1/96 (Table 2). In the meantime, Egy/Var-Ⅱ strains shared 80% to 83% amino acid with them (Table 2). The strains included in the classic group (1 to 4) showed only about 71% to 76% with 4/91, CR88, D274 and 1/96 serotypes (Table 2).

Pathogenicity

The pathogenicity of the IBV strains was evaluated by a standard pathogenicity assay using SPF chicken. For each group, the clinical and pathogenicity scores were recorded (Tables 3 and 4). In group 1 and 2 (inoculated with variant strains) sick chicks showed varying degrees of coughing, sneezing, tracheal rales, and watery feces. The severity of the signs increases in group 1 (Egy/Var-Ⅱ) than group 2 (Egy/Var-Ⅰ); however, for group 3 (classic strain) only mild respiratory signs were observed. The clinical scores were recorded in Table 4. The mortalities were 50%, 40%, and 10% and the recorded clinical scores were 3, 2 and 1 in the three groups 1, 2 and 3, respectively (Table 4).

Pathogenicity indices were 25, 24 and 8 for the isolates in groups 1 (Egy/Var-Ⅱ), 2 (Egy/Var-Ⅰ) and 3 (classic), respectively (Table 4). Based on the lesion in both kidney and trachea, isolates could be classified according to the pathogenicity index to high virulent (Egy/Var-Ⅱ and Egy/Var-Ⅰ) and low virulent (classic) (Table 4). The main commonly reported macroscopic lesions

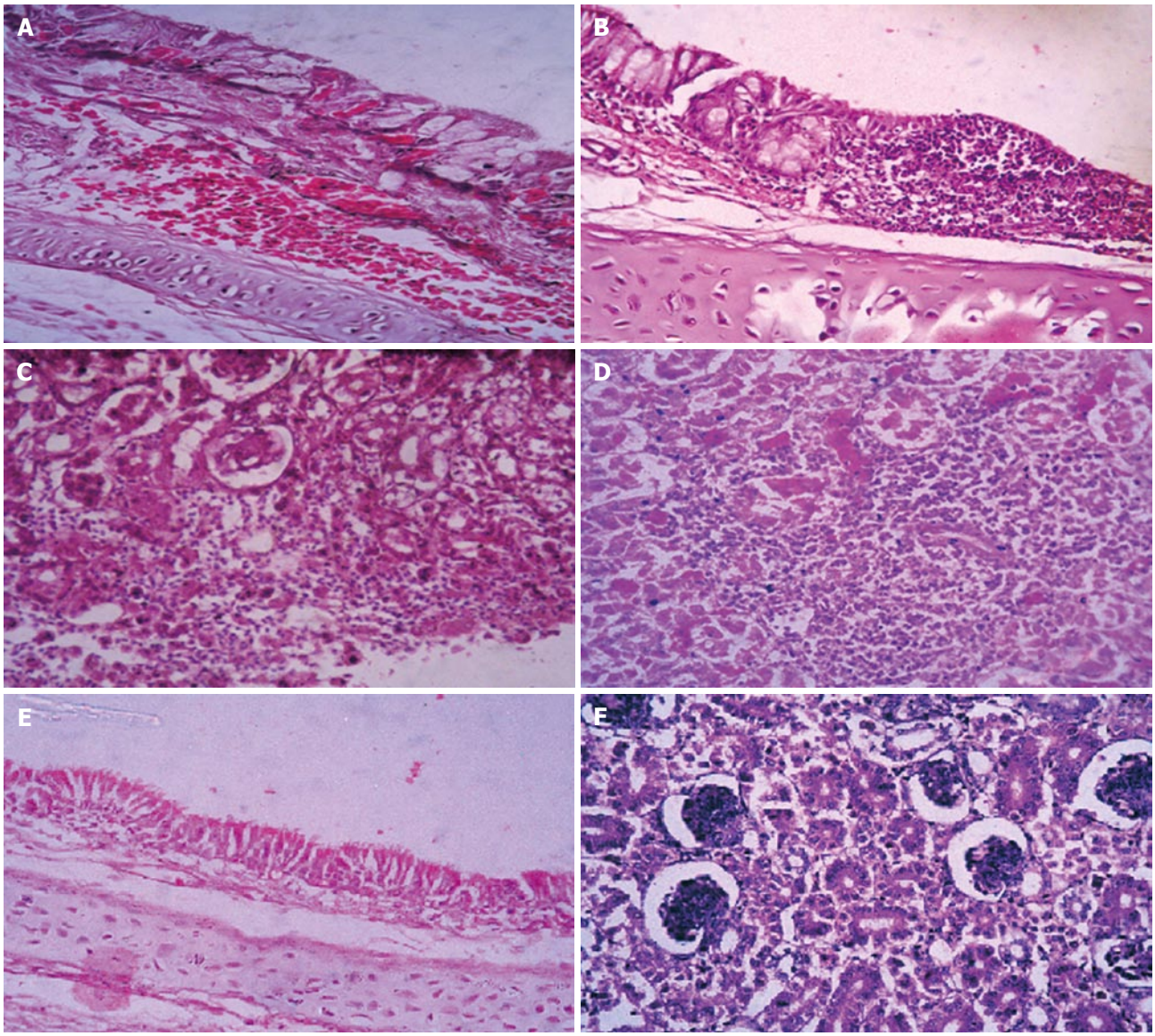

Figure 2 Histopathology illustration of the trachea and kidney from experimentally infected chickens. A: Trachea (from Group 1) showed subepithelial hemorrhage accompanied with goblet cell activation, inflammatory cells and edema; B: Trachea (from Group 2) with focal aggregation of lymphocytic cells and epithelial desquamation, ulceration accompanied with goblet cell hypertrophy; C: Kidney (from Group 2) showed glomerular edema and glomerulonephritis with extravasation of blood vessels between renal tubules; D: Kidney (from Group 1) with severe necrosis of renal tubules and focal lymphocytic aggregation; E: Trachea of negative control group; F: Kidney of negative control group (H and E, × 20).

included: Congestion with casious plug in the trachea, congested lung, swollen and congested kidneys with ureters distended with ureate. Grossly, the kidney lesions were more severe in Egy/Var-Ⅱ group than in Egy/Var-Ⅰ group; however, the lungs were only affected in Egy/Var-Ⅱ group (Tables 3 and 4). While in the classic group the tracheal lesions were less severe than the other 2 variant groups.

Virus distribution in tissues following virus inoculation of one-day-old SPF-chicks with 3 IBV isolated strains: IBV-Eg/1212B-2012 (Egy/Var-Ⅱ), IBV-Eg/IBV1-2011 (Egy/Var-Ⅰ) and IBV-Eg/11539F-2011 (Mass-like) were described (Table 5). The virus was found in the trachea and kidney in groups infected with variant strains (Egy/Var-Ⅰ, Egy/Var-Ⅱ). The virus was not detected in the lungs of Egy/Var-Ⅰ group also from lung and kidney of the classic group. The negative control group had no virus in tissues (Table 5).

Microscopic examination of sacrificed birds at 14 d Post- infection and freshly dead birds revealed marked loss of cilia, epithelial desquamation, lymphocytic cell infiltration, epithelial hyperplasia and congested trachea in both 2 subgroups of variant viruses (Egy/Var-Ⅰ, Egy/Var-Ⅱ) (Figure 2A and B). Kidneys showed severe changes, including hemorrhages, degeneration in renal tubules and hyper cellularity of the renal glomeruli as well as focal infiltration of inflammatory lymphocytes in group 1 (Egy/Var-Ⅱ) of variant viruses and to lesser extent in group 2 (Egy/Var-Ⅰ,) (Figure 2C and D). The lesion for the classic group 3 of Eg/11539F-2011 (Mass-like) showed the lowest severity in trachea and kidneys in comparison to variant groups (data not shown).

DISCUSSION

One of the main problems of IBV is the frequent emergence of several IBV serotypes or antigenic variants due to high rates of *S1* gene mutation[2,24]. Therefore, it is important to detect these new emerging viruses and to choose an appropriate vaccine against IBV infection. In the *S1* gene, there are three HVRs located within amino acids 38-67 (HVR-1), 91-141 (HVR-2) and 274-387 (HVR-3)[25]. Genotyping of IBV based on *S1* gene sequencing, especially the HVRs, is the most reliable way to classify IBV isolates. Usually IBV serotypes have a wide range of genetic variations in the *S1* gene ranged from 2% to 25%[6,26]. In Egypt, many strains of live attenuated and inactivated vaccines used to control IBV. However, the outbreaks of the disease have continued to cause severe infections[13].

In this work, 20 IBV isolates from commercial broiler chicken flocks from 12 governorates were genotyped by sequencing of the HVR-3 of the *S1* gene. The molecular data indicated that the IBVs isolated in Egypt during the last five years evolved into two groups; variant (GI-23) and classic (GI-1). The strains of the variant group (GI-23) are indigenous and predominate in the Middle East[9], and in Egypt were subdivided into 2 subgroups according to the sequence of the HVR-3[14,16]. The first subgroup of Egy/Var-Ⅰ, represented by 6 viruses (Table 1), is very close to the original Egyptian strain Egypt/Beni-Suef/01 and to Ck/EG/BSU-1,4,5/2011 isolated in 2011[16]. In this work, viruses of Egy/Var-Ⅰ subgroup were also found to be closely related to IS/1494/2006 with 96%-97% amino acid identity. The second subgroup Egy/Var-Ⅱ included 10 viruses and they were mostly related to IS/885/00 strain with 90% amino acid identities (Tables 1 and 2, Figure 1). Accordingly, Egy/Var-Ⅱ subgroup was shown to be widely spread in Egypt during the last 5 years[15,16]. However, full genome sequencing will provide more accurate information about different subgroups[9]. In the meantime, the 4 isolates in the classic group (Mass-like strain of GI-1) were genetically related to Massachusetts strains and had the same phylogenetic origin. It showed high amino acid similarity (98%) to H120 and Ma5 vaccine strains with 95% nucleotide identities (Table 2, Figure 1). The four isolates of Mass-like strain were obtained from vaccinated farms with the same strain. Furthermore, previous studies have confirmed that live IBV vaccines persist in chicks for many weeks after administration with virus isolation[27-29].

Phylogenetic analysis revealed that the variant IBV isolates (Egy/Var-Ⅰ and Egy/Var-Ⅱ) had a distant relation to vaccine strains commonly used in Egypt, including Ma5, H120, M41, 4/91, CR88, D274 and 1/96 (Table 2, Figure 1). The new IBV variants frequently emerge as a result of a few changes in the amino acid structure along the S1 protein[6]. These changes may be due to immunological pressure caused by the wide use of live vaccines of different strain types along with field virus infection. This can lead to genetic alterations and recombinations allowing new field strains to evolve[3].

In this work, the three IBV strains (IBV-Eg/1212B-2012 of Egy/Var-Ⅱ, IBV-EG/IBV1-2011 of Egy/Var-Ⅰ and IBV-EG/11539F-2011 of classic Mass-like genotype) were able to induce respiratory signs post-inoculation with clinical scores of 3, 2 and 1; respectively (Table 4). In addition, respiratory and renal lesions were recorded (Tables 3 and 4). The virulence of the three strains (Egy/Var-Ⅱ, Egy/Var-Ⅰ and classic Mass-like) was assessed in one-day-old SPF chicks for comparison. The three IBV strains were able to produce 50%, 40% and 10% deaths; respectively. It is well known that the most severe clinical symptoms of IBV appear in very young chicks and the severity decreased in older chickens[30]. This fact explains the high mortality rate observed in infected chicks with IBV-Eg/1212B-2012 (50%) and IBV/EG/IBV1-2011 (40%) (Table 4). These findings matched with Wang *et al*[31], who reported mortality rates ranged from 10% to 60% in experimentally infected chicks with QX strain of IBV. Furthermore, these findings agreed with Ignjatović *et al*[32], who found that strains of IBV differed in their virulence for the respiratory tract, kidney or oviduct. The majority of IBV strains, including those of the Mass-like serotype produce prominent respiratory disease[33]. The possibility of re-isolations of H120 vaccine strain cannot be excluded. Although vaccine strains induced lesions in chickens, but the mortality did not exceed 10%[31].

Trachea, lung and kidney were collected from infected birds after intra-nasal inoculation and the virus tropism was detected by rRT-PCR, the results were shown in Table 5. IBV nucleic acids were detected more frequently in the tracheal tissues than in the lungs, and kidney (Table 5). Terregino *et al*[34] isolated IBV from kidney, trachea, ovary, and oviduct following infection with the QX-IBV strain.

The presence of acute interstitial nephritis and gross renal ureates deposition and histological lesions in the experimental chicks at day 14 post infection indicated that IBV-Eg/1212B-2012 and IBV-EG/IBV1-2011 were nephrogenic viruses. Severe renal hemorrhages were observed grossly and histopathologically in dead birds for the two viruses denoting that the deaths might be resulted from acute renal failure rather than the respiratory distress (Figure 2). Similarly, variants of IBV were reported as nephrogenic strains in Egypt: D274, D3896, D1559, Egypt/Beni-Suef/01, 720/99 Israel, 4/91, IS/1494[13,29]. The microscopic picture of the renal tubules matched with the general findings recorded with nephrogenic IBV strains[35].

Regarding microscopic lesions in trachea associated with IBV infection in day old SPF chicks and examined at 14 d pi, the findings appeared similar to those previously recorded by Cavanagh *et al*[33], including: Deciliation, degenerative changes and edema of the tracheal mucosa, irregular loss of epithelium, desquamation of the epithelium in the tracheal lumen, goblet cell activation and focal aggregation to diffuse massive lymphocytic infiltration (Figure 2).

In conclusion, our results provide evidence of evolving

the recent Egyptian IBV strains and showed two groups of variants are co-circulating in Egypt with high mortality in SPF chicks. The distinctive dissimilarity between these variants and the widely used IBV vaccine reveal that the antigenic drift is likely to occur under the long-term immune pressure. Further epidemiological surveillance studies are needed in order to explain the mechanism of emergence of variants and their biological properties, including pathogenicity and vaccine trails to help in disease control.

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COMMENTS

Background

Infectious bronchitis virus (IBV), a major pathogen of commercial poultry flocks, circulates in the form of different genotypes. Three IBV genotypes were isolated from broiler chickens showing severe respiratory and renal lesions in vaccinated and non-vaccinated flocks.

Research frontiers

Different research articles discuss the current situation of IBV depending on the sequence of either hypervariable region (HVR)-1, 2 or HVR-3 of the *S1* gene. While other characterize IBV depending on the full *S1* gene sequence.

Innovations and breakthroughs

In this study three genotypes were characterized depending on the sequence of the HVR 3 of the *S1* gene. The virulence of the three genotypes (Egy/Var-Ⅱ, Egy/Var- Ⅰ and classic) was assessed in one-day-old SPF chicks for comparison.

Applications

This work includes detection of the currently circulating strains of IBV. The high genetic variation between these variants and the widely used IBV vaccine reveal that the antigenic drift is likely to occur under the long-term immune pressure. Also, this work focus on the high mortality caused by the variant strains of IBV in the poultry flocks.

Terminology

HVR-3, a part of the *S1* gene used intensively to characterize IBV genotype.

Peer-review

In this study, Ali Zanaty *et al* isolated 20 IBV isolates from 20 chicken flocks in Egypt and characterized the genotypes of the isolates to be two groups (classic and variant, and the latter was further classified into two subgroups) and tested the pathogenicity of three selected isolates to SPF chicks. The work contributed to the epidemiology and biology of IBV. The experiments were well designed and the manuscript is well organized.

REFERENCES

1 **Wang CH**, Huang YC. Relationship between serotypes and genotypes based on the hypervariable region of the S1 gene of infectious bronchitis virus. *Arch Virol* 2000; **145**: 291-300 [PMID: 10752554 DOI: 10.1007/s007050050024]

2 **Lee CW**, Hilt DA, Jackwood MW. Typing of field isolates of infectious bronchitis virus based on the sequence of the hypervariable region in the S1 gene. *J Vet Diagn Invest* 2003; **15**: 344-348 [PMID: 12918815 DOI: 10.1177/104063870301500407]

3 **Liu S**, Kong X. A new genotype of nephropathogenic infectious bronchitis virus circulating in vaccinated and non-vaccinated flocks in China. *Avian Pathol* 2004; **33**: 321-327 [PMID: 15223561 DOI: 10.1080/0307945042000220697]

4 **Gelb J**, Wolff JB, Moran CA. Variant serotypes of infectious bronchitis virus isolated from commercial layer and broiler chickens. *Avian Dis* 1991; **35**: 82-87 [PMID: 1851422 DOI: 10.2307/1591298]

5 **Carstens E**. Report from the 40th meeting of the Executive Committee of the International Committee of Taxonomy of Viruses, 2009: 1571–1574 [DOI: 10.1007/s00705-009-0458-x]

6 **Cavanagh D**, Davis PJ, Cook JK, Li D, Kant A, Koch G. Location of the amino acid differences in the S1 spike glycoprotein subunit of closely related serotypes of infectious bronchitis virus. *Avian Pathol* 1992; **21**: 33-43 [PMID: 18670913 DOI: 10.1080/03079459208418816]

7 **Casais R**, Dove B, Cavanagh D, Britton P. Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. *J Virol* 2003; **77**: 9084-9089 [PMID: 12885925 DOI: 10.1128/JVI.77.16.9084-9089.2003]

8 **Boursnell ME**, Brown TD, Foulds IJ, Green PF, Tomley FM, Binns MM. Completion of the sequence of the genome of the coronavirus avian infectious bronchitis virus. *J Gen Virol* 1987; **68** (Pt 1): 57-77 [PMID: 3027249 DOI: 10.1099/0022-1317-68-1-57]

9 **Valastro V**. Holmes EC. Britton P. Fusaro A. Jackwood MW, Cattoli G, Monne I. S1 gene-based phylogeny of infectious bronchitis virus: An attempt to harmonize virus classification. *Infect Genet Evol* 2016; **39**: 349-364

10 **Sjaak de Wit JJ**, Cook JK, van der Heijden HM. Infectious bronchitis virus variants: a review of the history, current situation and control measures. *Avian Pathol* 2011; **40**: 223-235 [PMID: 21711181 DOI: 10.1080/03079457.2011.56626]

11 **Alvarado IR**, Villegas P, Mossos N, Jackwood MW. Molecular characterization of avian infectious bronchitis virus strains isolated in Colombia during 2003. *Avian Dis* 2005; **49**: 494-499 [PMID: 16404989 DOI: 10.1637/7202-050304R.1]

12 **Cavanagh D**. Severe acute respiratory syndrome vaccine development: experiences of vaccination against avian infectious bronchitis coronavirus. *Avian Pathol* 2003; **32**: 567-582 [PMID: 14676007 DOI: 10.1080/03079450310001621198]

13 **Abd El Rahman S**, Hoffmann M, Lueschow D, Eladl A, Hafez HM. Isolation and characterization of new variant strains of infectious bronchitis virus in Northern Egypt. *Adv Anim Vet Sci* 2015; **3**: 362-371

14 **Ganapathy K**, Ball C, Forrester A. Genotypes of infectious bronchitis viruses circulating in the Middle East between 2009 and 2014. *Virus Res* 2015; **210**: 198-204 [PMID: 26226233 DOI: 10.1016/j.viruses.2015.07.019]

15 **Sultan H**, Abdel-Razik AG, Shehata AA, Ibrahim M, Talaat S, Abo-Elkhair M, Bazid AE, Moharam IM and Vahlenkamp T. Characterization of Infectious Bronchitis Viruses Circulating in Egyptian chickens during 2012 and 2013. *J Vet Sci Med Diagn* 2015; **4**: 5 [DOI: 10.4172/2325-9590.1000180]

16 **Abdel-Moneim AS**, Afifi MA, El-Kady MF. Emergence of a novel genotype of avian infectious bronchitis virus in Egypt. *Arch Virol* 2012; **157**: 2453-2457 [PMID: 22903394 DOI: 10.1007/s00705-012-1445]

17 **Callison SA**, Jackwood MW, Hilt DA. Molecular characterization of infectious bronchitis virus isolates foreign to the United States and comparison with United States isolates. *Avian Dis* 2001; **45**: 492-499 [PMID: 11417834 DOI: 10.2307/1592994]

18 **Momayez R**, Pourbakhsh SA, Khodashenas M, Banani M. Isolation and Identification of Infectious Bronchitis Virus from Commercial Chickens. *Arch Razi Ins* 2002; **53**: 1

19 **Adzhar A**, Gough RE, Haydon D, Shaw K, Britton P, Cavanagh D. Molecular analysis of the 793/B serotype of infectious bronchitis virus in Great Britain. *Avian Pathol* 1997; **26**: 625-640 [PMID: 18483932 DOI: 10.1080/03079459708419239]

20 **Tamura K**, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* 2013; **30**: 2725-2729 [PMID: 24132122 DOI: 10.1093/molbev/mst197]

21 **Ziegler AF**, Ladman BS, Dunn PA, Schneider A, Davison S, Miller PG, Lu H, Weinstock D, Salem M, Eckroade RJ, Gelb J. Nephropathogenic infectious bronchitis in Pennsylvania chickens 1997-2000. *Avian Dis* 2002; **46**: 847-858 [PMID: 12495045 DOI: 10.1637/0005-2086(2002)046[0847: NIBIPC]2.0.CO; 2]

22 **Purcell DA**, Tham VL, Surman PG. The histopathology of infectious bronchitis in fowls infected with a nephrotropic "T" strain of virus. *Aust Vet J* 1976; **52**: 85-91 [PMID: 186001 DOI: 10.1111/j.1751-0813.1976.tb13864.x]

23 **Bancroft JD**, Stevens A. Theory and practices of histologic techniques 2nd Eds. Churchill, Living Stone Edingburgh, London Melborne and New York, 1977

24 **Li M**, Mo ML, Huang BC, Fan WS, Wei ZJ, Wei TC, Li KR, Wei P. Continuous evolution of avian infectious bronchitis virus resulting in different variants co-circulating in Southern China. *Arch Virol* 2013; **158**: 1783-1786 [PMID: 23474985 DOI: 10.1007/s00705-013-1656-0]

25 **Moore KM**, Jackwood MW, Hilt DA. Identification of amino acids involved in a serotype and neutralization specific epitope within the s1 subunit of avian infectious bronchitis virus. *Arch Virol* 1997; **142**: 2249-2256 [PMID: 9672590]

26 **Kingham BF**, Keeler CL, Nix WA, Ladman BS, Gelb J. Identification of avian infectious bronchitis virus by direct automated cycle sequencing of the S-1 gene. *Avian Dis* 2000; **44**: 325-335 [PMID: 10879913 DOI: 10.2307/1592547]

27 **Alvarado IR**, Villegas P, El-Attrache J, Jackwood MW. Detection of Massachusetts and Arkansas serotypes of infectious bronchitis virus in broilers. *Avian Dis* 2006; **50**: 292-297 [PMID: 16863085 DOI: 10.1637/7458-101805R.1]

28 **Worthington KJ**, Currie RJ, Jones RC. A reverse transcriptase-polymerase chain reaction survey of infectious bronchitis virus genotypes in Western Europe from 2002 to 2006. *Avian Pathol* 2008; **37**: 247-257 [PMID: 18568650]

29 **Abdel-Moneim AS**, El-Kady MF, Ladman BS, Gelb J. S1 gene sequence analysis of a nephropathogenic strain of avian infectious bronchitis virus in Egypt. *Virol J* 2006; **3**: 78 [PMID: 16987422 DOI: 10.1186/1743-422X-3-78]

30 **Dhinakar Raj G**, Jones RC. Protectotypic differentiation of avian infectious bronchitis viruses using an in vitro challenge model. *Vet Microbiol* 1996; **53**: 239-252 [PMID: 9008335 DOI: 10.1016/S0378-1135(96)01258-8]

31 **Wang CH**, Hsieh MC, Chang PC. Isolation, pathogenicity, and H120 protection efficacy of infectious bronchitis viruses isolated in Taiwan. *Avian Dis* 1996; **40**: 620-625 [PMID: 8883793]

32 **Ignjatović J**, Sapats S. Avian infectious bronchitis virus. *Rev Sci Tech* 2000; **19**: 493-508 [PMID: 10935276 DOI: 10.20506/rst.19.2.1228]

33 **Cavanagh D**, Naqi SA. Infectious bronchitis. In B.W. Calnek, H.J. Barnes, C.W. Bearol, L.R. Mc Daugald, and Y.M. Saif (eds). Disease of Poultry 10th Ed. Lawa University Press: Ames, 1997: 511-526

34 **Terregino C**, Toffan A, Beato MS, De Nardi R, Vascellari M, Meini A, Ortali G, Mancin M, Capua I. Pathogenicity of a QX strain of infectious bronchitis virus in specific pathogen free and commercial broiler chickens, and evaluation of protection induced by a vaccination programme based on the Ma5 and 4/91 serotypes. *Avian Pathol* 2008; **37**: 487-493 [PMID: 18798022 DOI: 10.1080/03079450802356938]

35 **Albassam MA**, Winterfield RW, Thacker HL. Comparison of the nephropathogenicity of four strains of infectious bronchitis virus. *Avian Dis* 1986; **30**: 468-476 [PMID: 3021097 DOI: 10.2307/1590408]

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REVIEW

Neurological manifestations of Zika virus infection

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Abstract

Zika virus (ZIKV) is a flavivirus (*Flaviviridae* family) transmitted mainly by *Aedes* mosquitoes. The virus was restricted to the African continent until its spread to south-east Asia in the 1980's, the Micronesia in 2007, the French Polynesia in 2013 and, more recently in the Americas in 2015, where, up to date, the World Health Organization (WHO) has estimated about 3-4 million total cases of ZIKV infection. During outbreaks in the French Polynesia and Brazil in 2013 and 2015, respectively, national health authorities reported potential neurological complications of ZIKV disease, chiefly an upsurge in Guillain-Barré syndrome, which coincided with ZIKV outbreaks. On the other hand, the emergence of ZIKV in Brazil has been associated with a striking increase in the number of reported cases of microcephaly in fetus and newborns, twenty times higher than in that reported in previous years. While investigations are currently assessing whether there is an actual association between neurological complications and ZIKV infections, the evidence was enough worrisome for WHO to declare a public health emergency of international concern. Here we present an updated review addressing what is currently known about the possible association between ZIKV infection and the development of severe neurological disorders.

Key words: Zika virus; Flavivirus; Microcephaly; Guillain-Barré syndrome; Transmission routes

Core tip: Zika virus (ZIKV), a mosquito-borne flavivirus, was restricted to Africa until its spread to south-east Asia, the Pacific, and, finally, to the Americas, where an estimated 4 million cases of ZIKV infection have been recorded, and where a worrisome possible association of ZIKV with the development of severe neurological disorders, such as Guillain-Barré Syndrome and microcephaly, have been reported. In this contribution we present an updated review addressing what is currently known about the possible association between ZIKV infection and the development of severe neurological disorders, remarking the urgent need for further investigations to clearly resolve this point.

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THE VIRUS

Zika virus (ZIKV) is a mosquito-borne *Flavivirus* classified into the *Flaviviridae* family. It is closely related to other important pathogens that affect human and animal health such as Japanese encephalitis virus, dengue virus (DENV), yellow fever virus (YFV), West Nile virus (WNV) or St. Louis encephalitis virus[1]. ZIKV was first isolated in 1947 from the serum of a febrile sentinel rhesus monkey in the Zika Forest (Uganda) during the investigations performed to study the enzootic cycle of YFV. The virus was isolated for the second time from *Aedes africanus* mosquitoes collected at the same site one year later. In both cases, the virus was isolated by intracranial inoculation into infant mice[2].

ZIKV genome is constituted by a positive polarity RNA molecule of about 11 kb in length, comprising two untranslated regions flanking an open reading frame coding for a polyprotein of about 3420 amino acids. Similar to other flaviviruses, the ZIKV single polyprotein is expected to be post-translationally cleaved by host and viral proteases into three structural proteins [capsid (C), pre-membrane (prM), and envelope (E)] and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A NS4B, and NS5)[3] (Figure 1). The structure of mature ZIKV particle has been recently described[4] (Figure 2), and the virus particle has been observed to be structurally stable even at 40 ℃[5].

Phylogenetic analyses of the virus confirm its inclusion within the mosquito-borne flavivirus cluster with the presence of two major lineages: One includes the African strains, which is divided into two groups, the East and the West African clusters, and the other gathers the Asian and American strains[1]. ZIKV life cycle, as any other arbovirus, has several barriers to accumulate mutations as a consequence of the intrinsic constraints associated with dual replication in mammalian and invertebrate hosts, thus driving to a relatively slow fixation of mutations[1]. For instance, ZIKV strains collected over a few years interval in Central African Republic show minimal changes on their sequences[6].

Even though ZIKV strains from different continents and outbreaks showed up to 99% identity[1], nonsynonymous nucleotide differences have been described among them that, in other flaviviruses, have been implicated in viral infectivity. For instance, a full-length ZIKV genome amplified from fetal tissues obtained during the Brazilian outbreak presented five nonsynonymous mutations when compared with the French Polynesian isolate[7]. Three of these amino acid changes were found in NS1, implicated in immune evasion in the case of DENV[8], one in NS4B, related to the inhibition of type I interferon signaling in other flaviviruses[9,10], and one in a NS5 domain which has been shown to mask the viral RNAs from host recognition in the case of WNV[11,12]. In this line, it has been hypothesized the possible adaptation of the ZIKV virus to the human host by changes in non-structural proteins[13]. Thereby, Asian strains of ZIKV differ significantly from the African ones in codon usage in the NS1 region of the genome[14]. Codon usage by the pandemic strain is optimized for adaptation to human housekeeping cells, which could facilitate viral replication in human cells. In fact, codon optimization could result in higher viral titers and increased infectivity for mosquito vectors, as seen in other viruses[15].

Analysis of the polyprotein sequence predicted the presence of potential N-glycosylation sites in the ZIKV proteins prM, E and NS1[4,16-18]. Noteworthy, a 4 amino acid deletion corresponding to the envelope protein 154 glycosylation motif was found in several ZIKV strains, in a similar way to many other flaviviruses, such as West Nile virus strains[6]. Glycosylation has been associated in some instances with virulence[19,20], even though the functional importance of the N-glycosylations is not clear in related flaviviruses, since flaviviruses presenting or not this N-glycosylation can maintain the same antigenicity[21]. Additionally, glycosylation could play a role in replication and maturation[22]. In fact, it has been suggested that extensive mouse brain or cell culture passage could lead to the deletion of the potential glycosylation site, since there are differences on this site even between ZIKV isolates with different passage history, such as those of the prototypic strain ZIKV MR766[23,24]. Even more, it has been suggested that ZIKV may have experienced recombination in nature and that a loss of the N154 glycosylation site in the envelope protein was a possible adaptive response to the vector[25]. Therefore, a detailed analysis of whether and how these differences are directly related to virulence and pathogenicity has to be clearly elucidated for a better control of ZIKV infection.

TRANSMISSION

ZIKV is transmitted by mosquitoes of the genus *Aedes,* mainly of *Aedes aegypti* and *Aedes albopictus*, although the virus has been isolated from other genus such as *Anopheles, Culex,* and *Mansonia spp*[1]. Both *Ae. aegypti* and *Ae. albopictus* have a history of global expansion associated with trade and travel and are widely distributed[26].

Non-human primates are considered to serve as reservoir hosts for ZIKV, although the primary species have not been identified. ZIKV natural transmission cycle has been described to involve *Cercopithecus aethiops* and *Erythrocebus patas* monkeys in Africa[27], while ZIKV antibodies have been found among semi-captive and wild orangutans in Asia[28] (Figure 3). There is no current evidence of other animals than humans and non-human primates acting as amplifying hosts for ZIKV[29]. However, antibodies against ZIKV have been

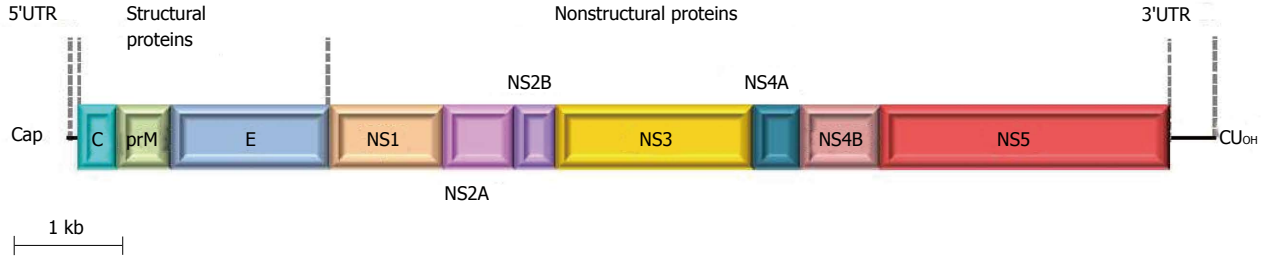

Figure 1 Schematic representation of Zika virus genome organization. The single open reading fram (boxes) that encodes both structural and non-structural proteins is flanked by two untranslated regions.

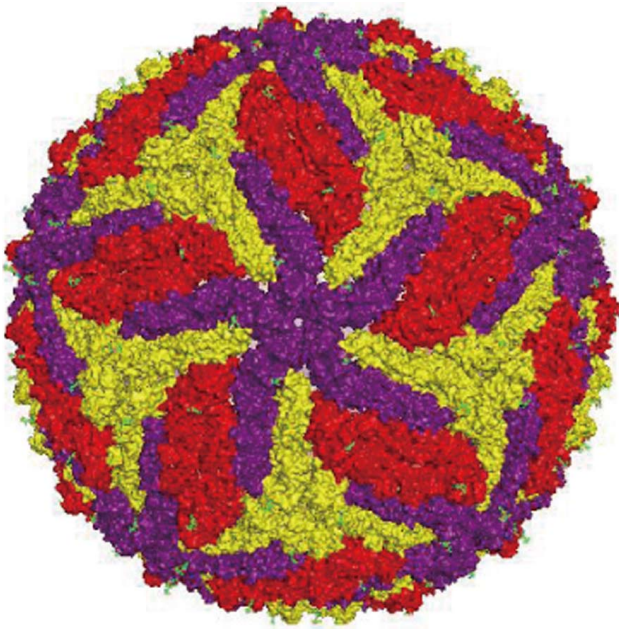

Figure 2 Schematic representation of Zika virus particle based on cryo-electron microscopy data[4].

found in many other vertebrate species, such as sheep, goats, cattle birds, rodents and even reptiles[1].

Even though mosquito transmission is the main cause of ZIKV outbreaks, other additional routes of transmission have been proposed: Breastfeeding, perinatal, sexual or by blood transfusion (Figure 3).

Horizontal transmission

The potential for viral transmission through blood transfusion was first suggested during the French Polynesia outbreak. Almost 3% of blood donors, who were asymptomatic at the time of donation, were found positive for acute ZIKV infection by specific reverse transcriptase polymerase chain reaction (RT-PCR)[30]. Moreover, in a very recent prospective study carried out in 72 pregnant women in Brazil, 26 tested positive for ZIKV RNA in blood samples[31]. These data point to the need for implementation of measures to prevent this way of infection in endemic areas, and, in other zones free of ZIKV, to advice people coming back from affected areas to delay blood donations[1].

Besides blood transfusion, sexual activity could be another risk factor for horizontal transmission. In this regard, ZIKV RNA and replicative virus have been found in semen[32-34]. In 2008, a case of sexual transmission was suspected to occur from an American scientist, who contracted ZIKV infection in Senegal, to his wife. Even though she had not left the United States during the previous year, she also developed clinical symptoms related to ZIKV infection. Even though, ZIKV was not investigated in the semen of the patient, virus infection was serologically confirmed in both[35]. A recent retrospective study in Italy detected ZIKV specific neutralizing antibodies in the sera of a couple with a suspected DENV infection, of which the female had not travelled to tropical areas during the previous year[36]. Later on, in early February 2016, the case of a ZIKV infected person after sexual contact in the United States has been reported[37]. In this line of investigations, the CDC received reports of 14 cases of suspected sexual transmission of ZIKV during February 2016, of which only two were laboratory-confirmed and four classified as probable cases of Zika disease. All reported cases belonged to women which only known risk factor was to have had sexual intercourse with symptomatic partners recently returned from an area with ongoing ZIKV circulation[38]. Up to date, and according to WHO, five countries have reported locally acquired infection in the absence of any known mosquito vectors, probably through sexual transmission (Argentina, France, Italy, New Zealand and the United States). Additionally, ZIKV RNA and infectious ZIKV in urine[39] and saliva[40] have been reported. All these data suggest that sexual transmission could play a role on ZIKV infection and transmission, even though this route seems unlikely to play a major role in ZIKV spread. In any case, the CDC have considered that ZIKV sexual transmission is of particular concern and, consequently, have published an interim guideline for prevention of sexual transmission of ZIKV[41].

Vertical transmission

ZIKV RNA in breast milk was first detected during the outbreak in the French Polynesia[42] and, more recently, the presence of infective ZIKV particles, with substantial viral loads, in breast milk has also been described[43]. Nevertheless, since there is no evidence supporting viral transmission to babies by lactation, the CDC

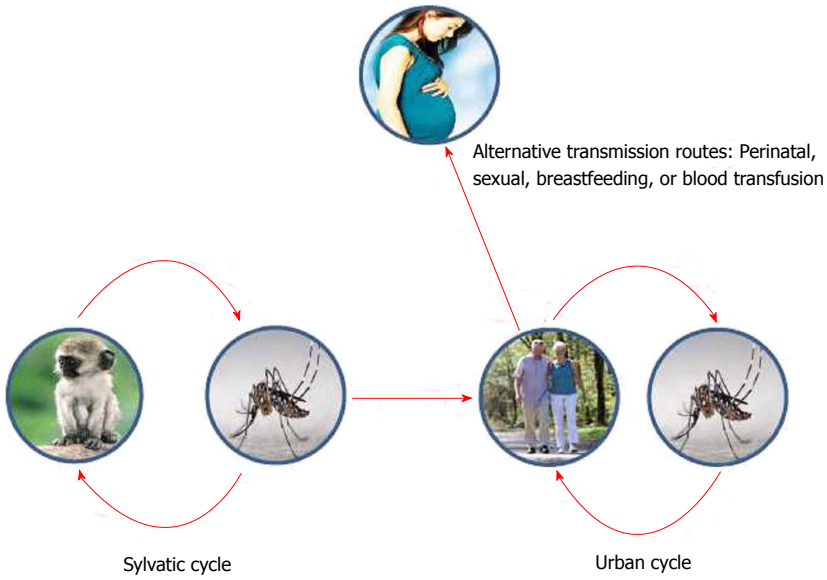

Figure 3 Schematic representation of Zika virus transmission cycle, with a sylvatic natural cycle between mosquitoes and monkeys, and an urban cycle between mosquitoes and human population.

encourage mothers to breastfeed their children, arguing that the benefits of it outweigh the risk of transmission (http://www.cdc.gov/zika/transmission/), as so do the Pan American Health Organization (PAHO/WHO) (http://www.paho.org), and several national health authorities. However, it should be noted that breast milk transmission has been previously documented in humans and experimentation animal models in other flaviviruses, such as DENV or WNV[44,45].

In any case, the most worrying aspect of recent ZIKV outbreaks is the increasing evidence pointing to mother-to-child viral transmission, which can lead to infants neurological disorders. As mentioned early, perinatal transmission was documented for the first time during the French Polynesia outbreak[42]. Sera from two mothers and their newborns were RT-PCR tested positive for ZIKV, although contamination during delivery could not be discarded. Later on, during the outbreak in Brazil, RT-PCR detection and histopathologic findings in tissue samples from two newborns with microcephaly who died within 20 h of birth and two miscarriages showed the presence of ZIKV. All four mothers had clinical signs of ZIKV infection during the first trimester of pregnancy, but not at the time of delivery or miscarriage[46]. Further reports in Brazil have described the presence of ZIKV RNA in fetuses and amniotic fluids[31,47,48]. Even though sporadic vertical transmission in humans has been previously reported in other members of the *Flaviviridae* family, such DENV[49] or YFV[50], the surprisingly high number of infants born with microcephaly in Brazil during the current outbreak, which could probably be the result of a possible vertical transmission, has urged the WHO to publish some advice for women who are pregnant, or planning to become pregnant, to take extra care to protect themselves from the bites of the mosquitoes that transmits ZIKV (http://www.who.int/features/qa/zika-pregnancy/en/).

Clinical features of the disease

ZIKV infection has been described to be symptomatic only in around 18% of the cases[51], causing a mild, self-limiting illness with an incubation period of up to 10 d[52]. Signs and symptoms generally include an onset of fever, maculopapular rash, arthralgia, myalgia, and conjunctivitis, and can be often mistaken with other arboviral infections, like dengue or chikungunya (Table 1). However, severe disease with hospitalization has not been commonly needed until now[1]. However, and even though a causal link has not been yet established, there seem to be growing evidences linking ZIKV infection to Guillain-Barré syndrome (GBS) and microcephaly in newborns. So that, due to this unexpectedly upsurge of severe neuronal complications, a case definition for ZIKV disease has been established by the WHO (http://www.who.int/csr/disease/zika/case-definition/en/) for the purpose of providing global standardization for classification and reporting of ZIKV cases. These interim guidelines distinguish between suspected cases, probable cases, and confirmed cases of ZIKV disease, showing the essential requirements for each of them[12] (Table 2).

GBS is a clinical syndrome of multiple autoimmune etiologies, which involve idiopathic peripheral neuropathy manifested as a progressive paralysis over 1-3 wk, with a 5% death rate and up to 20% of patients left with a significant disability[53-55]. Severe manifestation of GBS with respiratory failure affects 20%-30% of cases[56]. GBS is the most common and severe acute paralytic neuropathy, with en estimate incidence ranging 0.8-1.9 cases per 100000 people per year, with a 70% of these cases associated with previous infectious diseases. The syndrome was also first associated with ZIKV infection

Table 1 Clinical features of Zika virus disease

Mild symptoms	Other complications of the disease
Fever	Guillain-Barré syndrome
Rash	Microcephaly in fetuses and newborns
Joint pain	
Conjunctivitis	
Muscle pain	
Headache	

during the French Polynesian outbreak in 2013[57], where the incidence rate of GBS cases was about 20-fold higher than expected[58]. Likewise, in Colombia, during the ongoing outbreak, a three times higher number of GBS cases than the averaged expected cases during the 6 previous years has been reported. An association between the increase of GBS cases and ZIKV infection has also been reported in Venezuela (http://www.who.int/csr/don/12-february-2016-gbs-colombia-venezuela/en/). Very recently, two cases of GBS with confirmed ZIKV infection have been notified from the United States to the PAHO/WHO (http://www.who.int/csr/don/21-march-2016-gbs-usa/en/). According to the WHO, and in the context of ZIKV circulation, twelve countries or territories have reported an increased incidence of GBS and/or laboratory confirmation of a ZIKV infection among GBS cases (http://www.who.int/emergencies/zika-virus/situation-report/17-march-2016/en/). These data point to an alarming increase in the potential clinical severity of ZIKV infection[59].

In a case-control study performed during the French Polynesia outbreak, 42 patients were diagnosed with GBS at the Centre Hospitalier de Polynésie Française (Papeete, Tahiti, French Polynesia). Study control cohorts were age-matched, sex-matched, and residence-matched patients who presented at the hospital with a non-febrile illness (control group 1; n = 98) and age-matched patients with acute ZIKV disease and no neurological symptoms (control group 2; n = 70). Up to 98% of the patients with GBS had anti-ZIKV IgM or IgG, compared with 56% in control group 1[60]. Even though in this study a history of past dengue virus infection seemed not to differ significantly between patients with GBS and those in the two control groups, other reports have suggested that the simultaneous increase in dengue and chikungunya infections in the region may have contribute to the registered increase in GBS incidence[61]. The 42 GBS cases reported in the French Polynesia between November 2013 and February 2014 contrasted with the less than ten cases per year recorded during the previous four years (http://ecdc.europa.eu/en/publications/Publications/Zika-virus-French-Polynesia-rapid-risk-assessment.pdf), and suggests a possible association between ZIKV and GBS[60]. GBS was also the first important ZIKV-associated condition documented in Brazil, with 121 cases during the first half of 2015 (http://portalsaude.saude.gov.br/index.php/o-ministerio/principal/secretarias/svs/noticias-svs/19139-evento-desaude-publica-relacionado-aos-casos-de-febre-do-zika).

Table 2 Zika virus disease interim case definitions according to World Health Organization

Suspected case	Probable case	Confirmed case
A person presenting with rash and/or fever and at least one of the following signs or symptoms: Arthralgia, or Arthritis, or Conjunctivitis (non-purulent/hyperaemic)	A suspected case with presence of: IgM antibody against Zika virus (with no evidence of infection with other flaviviruses) and An epidemiological link (contact with a confirmed case, or a history of residing in or travelling to an area with local transmission of Zika virus within 2 wk prior to onset of symptoms)	A person with laboratory confirmation of recent Zika virus infection: Presence of Zika virus RNA or antigen in serum or other samples, or IgM antibody against Zika virus positive and PRNT90 for Zika virus with titre ⩾ 20 and Zika virus PRNT90 titre ratio ⩾ 4 compared to other tested flaviviruses, and Exclusion of other flaviviruses

Available from: URL: http://www.who.int/csr/disease/zika/case-definition/en.

Even though GBS has also been associated to other arboviral infections, such as DENV[62,63], WNV[64], or CHIKV[65], it is believed to be a rare event. The onset of GBS presumably involves an autoimmune process[66], and although the possible factors determining the association of GBS and ZIKV have not yet been established, it has been suggested that sequential arbovirus infections may exacerbate the immune response and trigger an immunopathogenic process attacking peripheral nerves, and thus leading to the onset of GBS[58].

No matter what, the most concerning manifestation of ZIKV infection is the dramatic increase of reported cases of microcephaly in Brazil. Microcephaly is a head size smaller than expected for age, and is associated to different genetic factors, maternal malnutrition, intrauterine infection (including toxoplasmosis, cytomegalovirus, or rubella), and exposure to toxins during gestation (http://www.cdc.gov/ncbddd/birthdefects/microcephaly.html). Microcephaly is defined as an occipitofrontal head circumference below the third centile, or more than 2 standard deviations (SD) below the mean for sex, age, and ethnicity[67]. Anyway, the possible link of microcephaly with ZIKV is not still clear among researchers. The Latin American Collaborative Study of Congenital Malformations (ECLAMC) suggested that this increase in reported cases of microcephaly might largely be due to the intense search for cases of the birth defect, and to misdiagnoses, that arose from heightened awareness in the wake of the possible link with ZIKV; and the WHO had also stated that the causal relation of these disorders with ZIKV infection had not yet been scientifically proven[68].

According to the WHO data, between October 2015 and January 2016, Brazil reported 4783 cases of microcephaly and/or central nervous system malformation, while during the fifteen previous years the average number of cases reported in the country was 163 per year[69]. Although most of the Brazilian cases have not yet been confirmed, as only a few studies have investigated in detail the possible link between ZIKV infection and fetus cerebral damage, an increase in microcephaly and other fetal malformations has been widely reported in Brazil[31,70,71] and the French Polynesia[72]. In a retrospective analysis of data performed from the ZIKV outbreak in French Polynesia, eight cases of microcephaly were identified between September 2013 and July 2015. Seven of them occurred in a 4-mo period around the end of the ZIKV outbreak. With the development of a mathematical model, the study estimated a prevalence of risk of microcephaly associated with ZIKV infection in the first trimester of pregnancy of 95 out of 10000 infected women (around 1%) *vs* a baseline prevalence of microcephaly of 2 out of 10000[73]. Two additional cases, linked to a stay in Brazil, were detected in the United States[74] and Slovenia[7]. Even though no such a high increase has been observed in ZIKV Brazil endemic neighboring countries, a very recent report has diagnosed, for the first time in Colombia, one newborn with microcephaly and two with congenital brain abnormalities, which tested positive for ZIKV[75], and Panama has recently reported to the WHO a newborn with microcephaly and occipital encephalocoele who died a few hours after birth and also tested positive for ZIKV by RT-PCR.

It is also noteworthy to mention that first experimental studies with ZIKV infection in two mouse model revealed that virus replication is mainly performed in brain cells, such as neurons and astroglial cells[76,77], which would be in line with a possible physiological mechanism linking ZIKV infection with microcephaly. Otherwise, a very recent study have showed that ZIKV infection of human cortical neural progenitors cells derived from induced pluripotent stem cell produced an attenuation of their growth, pointing to a possible mechanistic link between ZIKV and microcephaly[78]. On the other hand, it has been hypothesized that infection could damage the fetus either by evading the natural immunoprotective response of the placenta by direct transmission of the virus to the early embryo or fetus, or by the placenta itself provoking a response to the exposure, and thus contributing to, or causing, the brain defects[79]. In any case, the mechanism by which ZIKV may cause fetal microcephaly is still unknown and, thus, this point need to be clearly established.

Public health measures and future considerations

As in most flaviviral infections, there is no current specific antiviral treatment, vaccine or prophylaxis available for ZIKV. Treatment is generally symptomatic and based on analgesics, antipyretics, and antihistamines. This lack of specific measures against the virus emphasizes the importance of vector control strategies (Table 3). ZIKV is principally spread by mosquitoes, and not by person-to-person contact, although a limited number of cases of sexual transmission has been reported. Accordingly, vector control measures are analogous to those suggested in other mosquito-transmitted diseases[3], such as removing sources of standing water, insecticide application, avoidance of mosquito exposure, and implementation of accurate mosquito control programs. Besides these vector control approaches, development of effective ZIKV vaccines, and search for specific antiviral drugs are current challenges for Zika disease.

Table 3 Preventive measures

Vector control measures	Personal preventive measures
Removal of sources of standing water Implementation of accurate mosquito control programs	Avoidance of mosquito exposure Insecticide application Prevention of sexual transmission by use of preventive measures Travelling avoidance to risk countries during pregnancy

Since the WHO declared a public health emergency of international concern on the 1st of February of 2016, a list of preventive guidelines has been assessed, particularly during pregnancy. Recommendations for pregnant women considering travel to an area with ZIKV circulation and recommendations for screening, testing, and management of pregnant returning travelers are included in the CDC interim guidelines[80]. However, it should be taken into account that, even though ZIKV has been identified in a few cases in fetuses with microcephaly, this association does not demonstrate causality, and it will be necessary careful assessment to find the causal link between ZIKV infection and microcephaly[1,81]. Furthermore, in the case of newborns with microcephaly, the lack of data on short or long-term outcomes of neonatal or infant infection makes it difficult to take into consideration more subtle effects of ZIKV infection in the brain until later stages of childhood. Therefore, systematic and longer-term follow-up is mandatory to assess this point and to determine whether there are more fetal effects.

On the other hand, Zika's association with other viral infections in humans, such as dengue and chikungunya, has raised questions about the potential roles of these other viruses as cofactors for the more serious complications of ZIKV infection[82]. As the current ZIKV expansion is occurring in regions where dengue is endemic, pre-existing dengue immunity can cause increased ZIKV replication in patients, resulting in increased viremia and increased infectivity. In this sense, the possibility of immune enhancement by pre-existing heterologous anti-flavivirus antibodies, like DENV, has been hypothesized to increase viral replication[13]. Immune enhancement has been reported to play a major role in the pathogenesis of severe dengue infections[83]. In fact, ZIKV replication in cell culture were shown to be

enhanced by heterologous flavivirus antibodies[4]. In any case, the potential role of this immune enhancement by previous infection with other flaviviruses as cofactors for the more serious complications associated with ZIKV should be addressed in future research.

Beyond the considerable efforts exerted by the scientific community and the national and international health authorities focused on improving the knowledge on ZIKV infection, sufficient resources should be allocated to provide the necessary tools for assessing the potential mechanisms of ZIKV association to severe neurological diseases, such as GBS or microcephaly, as well as the development of more systematic diagnostic tools, vaccines, and design of antiviral therapies.

REFERENCES

1 **Saiz JC**, Vázquez-Calvo Á, Blázquez AB, Merino-Ramos T, Escribano-Romero E, Martín-Acebes MA. Zika Virus: the Latest Newcomer. *Front Microbiol* 2016; **7**: 496 [PMID: 27148186 DOI: 10.3389/fmicb.2016.00496]

2 **Dick GW**, Kitchen SF, Haddow AJ. Zika virus. I. Isolations and serological specificity. *Trans R Soc Trop Med Hyg* 1952; **46**: 509-520 [PMID: 12995440]

3 **Martín-Acebes MA**, Saiz JC. West Nile virus: A re-emerging pathogen revisited. *World J Virol* 2012; **1**: 51-70 [PMID: 24175211 DOI: 10.5501/wjv.v1.i2.51]

4 **Sirohi D**, Chen Z, Sun L, Klose T, Pierson TC, Rossmann MG, Kuhn RJ. The 3.8 Å resolution cryo-EM structure of Zika virus. *Science* 2016; **352**: 467-470 [PMID: 27033547 DOI: 10.1126/science.aaf5316]

5 **Kostyuchenko VA**, Lim EX, Zhang S, Fibriansah G, Ng TS, Ooi JS, Shi J, Lok SM. Structure of the thermally stable Zika virus. *Nature* 2016; **533**: 425-428 [PMID: 27093288 DOI: 10.1038/nature17994]

6 **Berthet N**, Nakouné E, Kamgang B, Selekon B, Descorps-Declère S, Gessain A, Manuguerra JC, Kazanji M. Molecular characterization of three Zika flaviviruses obtained from sylvatic mosquitoes in the Central African Republic. *Vector Borne Zoonotic Dis* 2014; **14**: 862-865 [PMID: 25514122 DOI: 10.1089/vbz.2014.1607]

7 **Mlakar J**, Korva M, Tul N, Popović M, Poljšak-Prijatelj M, Mraz J, Kolenc M, Resman Rus K, Vesnaver Vipotnik T, Fabjan Vodušek V, Vizjak A, Pižem J, Petrovec M, Avšič Županc T. Zika Virus Associated with Microcephaly. *N Engl J Med* 2016; **374**: 951-958 [PMID: 26862926 DOI: 10.1056/NEJMoa1600651]

8 **Scaturro P**, Cortese M, Chatel-Chaix L, Fischl W, Bartenschlager R. Dengue Virus Non-structural Protein 1 Modulates Infectious Particle Production via Interaction with the Structural Proteins. *PLoS Pathog* 2015; **11**: e1005277 [PMID: 26562291 DOI: 10.1371/journal.ppat.1005277]

9 **Morrison J**, Aguirre S, Fernandez-Sesma A. Innate immunity evasion by Dengue virus. *Viruses* 2012; **4**: 397-413 [PMID: 22590678 DOI: 10.3390/v4030397]

10 **Nitta S**, Sakamoto N, Nakagawa M, Kakinuma S, Mishima K, Kusano-Kitazume A, Kiyohashi K, Murakawa M, Nishimura-Sakurai Y, Azuma S, Tasaka-Fujita M, Asahina Y, Yoneyama M, Fujita T, Watanabe M. Hepatitis C virus NS4B protein targets STING and abrogates RIG-I-mediated type I interferon-dependent innate immunity. *Hepatology* 2013; **57**: 46-58 [PMID: 22911572 DOI: 10.1002/hep.26017]

11 **Daffis S**, Szretter KJ, Schriewer J, Li J, Youn S, Errett J, Lin TY, Schneller S, Zust R, Dong H, Thiel V, Sen GC, Fensterl V, Klimstra WB, Pierson TC, Buller RM, Gale M, Shi PY, Diamond MS. 2'-O methylation of the viral mRNA cap evades host restriction by IFIT family members. *Nature* 2010; **468**: 452-456 [PMID: 21085181 DOI: 10.1038/nature09489]

12 **Weaver SC**, Costa F, Garcia-Blanco MA, Ko AI, Ribeiro GS, Saade G, Shi PY, Vasilakis N. Zika virus: History, emergence, biology, and prospects for control. *Antiviral Res* 2016; **130**: 69-80 [PMID: 26996139 DOI: 10.1016/j.antiviral.2016.03.010]

13 **Russell PK**. The Zika Pandemic - A Perfect Storm? *PLoS Negl Trop Dis* 2016; **10**: e0004589 [PMID: 26991663 DOI: 10.1371/journal.pntd.0004589]

14 **Freire CC**, Iamarino A, Neto DF, Sall AA, Zanotto PM. Spread of the pandemic Zika virus lineage is associated with NS1 codon usage adaptation in humans. BioRxiv, 2016

15 **Andersen KG**, Shapiro BJ, Matranga CB, Sealfon R, Lin AE, Moses LM, Folarin OA, Goba A, Odia I, Ehiane PE, Momoh M, England EM, Winnicki S, Branco LM, Gire SK, Phelan E, Tariyal R, Tewhey R, Omoniwa O, Fullah M, Fonnie R, Fonnie M, Kanneh L, Jalloh S, Gbakie M, Saffa S, Karbo K, Gladden AD, Qu J, Stremlau M, Nekoui M, Finucane HK, Tabrizi S, Vitti JJ, Birren B, Fitzgerald M, McCowan C, Ireland A, Berlin AM, Bochicchio J, Tazon-Vega B, Lennon NJ, Ryan EM, Bjornson Z, Milner DA, Lukens AK, Broodie N, Rowland M, Heinrich M, Akdag M, Schieffelin JS, Levy D, Akpan H, Bausch DG, Rubins K, McCormick JB, Lander ES, Günther S, Hensley L, Okogbenin S, Schaffner SF, Okokhere PO, Khan SH, Grant DS, Akpede GO, Asogun DA, Gnirke A, Levin JZ, Happi CT, Garry RF, Sabeti PC. Clinical Sequencing Uncovers Origins and Evolution of Lassa Virus. *Cell* 2015; **162**: 738-750 [PMID: 26276630 DOI: 10.1016/j.cell.2015.07.020]

16 **Baronti C**, Piorkowski G, Charrel RN, Boubis L, Leparc-Goffart I, de Lamballerie X. Complete coding sequence of zika virus from a French polynesia outbreak in 2013. *Genome Announc* 2014; **2**: [PMID: 24903869 DOI: 10.1128/genomeA.00500-14]

17 **Kuno G**, Chang GJ. Full-length sequencing and genomic characterization of Bagaza, Kedougou, and Zika viruses. *Arch Virol* 2007; **152**: 687-696 [PMID: 17195954 DOI: 10.1007/s00705-006-0903-z]

18 **Fagbami AH**, Halstead SB, Marchette NJ, Larsen K. Cross-infection enhancement among African flaviviruses by immune mouse ascitic fluids. *Cytobios* 1987; **49**: 49-55 [PMID: 3028713]

19 **Mondotte JA**, Lozach PY, Amara A, Gamarnik AV. Essential role of dengue virus envelope protein N glycosylation at asparagine-67 during viral propagation. *J Virol* 2007; **81**: 7136-7148 [PMID: 17459925 DOI: 10.1128/JVI.00116-07]

20 **Shirato K**, Miyoshi H, Goto A, Ako Y, Ueki T, Kariwa H, Takashima I. Viral envelope protein glycosylation is a molecular determinant of the neuroinvasiveness of the New York strain of West Nile virus. *J Gen Virol* 2004; **85**: 3637-3645 [PMID: 15557236 DOI: 10.1099/vir.0.80247-0]

21 **Winkler G**, Heinz FX, Kunz C. Studies on the glycosylation of flavivirus E proteins and the role of carbohydrate in antigenic structure. *Virology* 1987; **159**: 237-243 [PMID: 2441520]

22 **Li J**, Bhuvanakantham R, Howe J, Ng ML. The glycosylation site in the envelope protein of West Nile virus (Sarafend) plays an important role in replication and maturation processes. *J Gen Virol* 2006; **87**: 613-622 [PMID: 16476982 DOI: 10.1099/vir.0.81320-0]

23 **Adams SC**, Broom AK, Sammels LM, Hartnett AC, Howard MJ, Coelen RJ, Mackenzie JS, Hall RA. Glycosylation and antigenic variation among Kunjin virus isolates. *Virology* 1995; **206**: 49-56 [PMID: 7530394]

24 **Haddow AD**, Schuh AJ, Yasuda CY, Kasper MR, Heang V, Huy R, Guzman H, Tesh RB, Weaver SC. Genetic characterization of Zika virus strains: geographic expansion of the Asian lineage. *PLoS Negl Trop Dis* 2012; **6**: e1477 [PMID: 22389730 DOI: 10.1371/journal.pntd.0001477]

25 **Faye O**, Freire CC, Iamarino A, Faye O, de Oliveira JV, Diallo M, Zanotto PM, Sall AA. Molecular evolution of Zika virus during its emergence in the 20(th) century. *PLoS Negl Trop Dis* 2014; **8**: e2636 [PMID: 24421913 DOI: 10.1371/journal.pntd.0002636]

26 **Kraemer MU**, Sinka ME, Duda KA, Mylne AQ, Shearer FM, Barker CM, Moore CG, Carvalho RG, Coelho GE, Van Bortel W, Hendrickx G, Schaffner F, Elyazar IR, Teng HJ, Brady OJ, Messina JP, Pigott DM, Scott TW, Smith DL, Wint GR, Golding N, Hay SI. The global distribution of the arbovirus vectors Aedes aegypti and Ae. albopictus. *Elife* 2015; **4**: e08347 [PMID: 26126267 DOI:

10.7554/eLife.08347]
27 **Faye O**, Faye O, Diallo D, Diallo M, Weidmann M, Sall AA. Quantitative real-time PCR detection of Zika virus and evaluation with field-caught mosquitoes. *Virol J* 2013; **10**: 311 [PMID: 24148652 DOI: 10.1186/1743-422X-10-311]
28 **Wolfe ND**, Kilbourn AM, Karesh WB, Rahman HA, Bosi EJ, Cropp BC, Andau M, Spielman A, Gubler DJ. Sylvatic transmission of arboviruses among Bornean orangutans. *Am J Trop Med Hyg* 2001; **64**: 310-316 [PMID: 11463123]
29 **Lazear HM**, Diamond MS. Zika Virus: New Clinical Syndromes and Its Emergence in the Western Hemisphere. *J Virol* 2016; **90**: 4864-4875 [PMID: 26962217 DOI: 10.1128/JVI.00252-16]
30 **Musso D**, Nhan T, Robin E, Roche C, Bierlaire D, Zisou K, Shan Yan A, Cao-Lormeau VM, Broult J. Potential for Zika virus transmission through blood transfusion demonstrated during an outbreak in French Polynesia, November 2013 to February 2014. *Euro Surveill* 2014; **19**: pii: 20761 [PMID: 24739982]
31 **Brasil P**, Pereira JP, Raja Gabaglia C, Damasceno L, Wakimoto M, Ribeiro Nogueira RM, Carvalho de Sequeira P, Machado Siqueira A, Abreu de Carvalho LM, Cotrim da Cunha D, Calvet GA, Neves ES, Moreira ME, Rodrigues Baião AE, Nassar de Carvalho PR, Janzen C, Valderramos SG, Cherry JD, Bispo de Filippis AM, Nielsen-Saines K. Zika Virus Infection in Pregnant Women in Rio de Janeiro - Preliminary Report. *N Engl J Med* 2016 Mar 4; Epub ahead of print [PMID: 26943629 DOI: 10.1056/NEJMoa1602412]
32 **Atkinson B**, Hearn P, Afrough B, Lumley S, Carter D, Aarons EJ, Simpson AJ, Brooks TJ, Hewson R. Detection of Zika Virus in Semen. *Emerg Infect Dis* 2016; **22**: 940 [PMID: 27088817 DOI: 10.3201/eid2205.160107]
33 **Mansuy JM**, Dutertre M, Mengelle C, Fourcade C, Marchou B, Delobel P, Izopet J, Martin-Blondel G. Zika virus: high infectious viral load in semen, a new sexually transmitted pathogen? *Lancet Infect Dis* 2016; **16**: 405 [PMID: 26949027 DOI: 10.1016/S1473-3099(16)00138-9]
34 **Musso D**. Zika Virus Transmission from French Polynesia to Brazil. *Emerg Infect Dis* 2015; **21**: 1887 [PMID: 26403318 DOI: 10.3201/eid2110.151125]
35 **Foy BD**, Kobylinski KC, Chilson Foy JL, Blitvich BJ, Travassos da Rosa A, Haddow AD, Lanciotti RS, Tesh RB. Probable non-vector-borne transmission of Zika virus, Colorado, USA. *Emerg Infect Dis* 2011; **17**: 880-882 [PMID: 21529401 DOI: 10.3201/eid1705.101939]
36 **Venturi G**, Zammarchi L, Fortuna C, Remoli ME, Benedetti E, Fiorentini C, Trotta M, Rizzo C, Mantella A, Rezza G, Bartoloni A. An autochthonous case of Zika due to possible sexual transmission, Florence, Italy, 2014. *Euro Surveill* 2016; **21**: [PMID: 26939607 DOI: 10.2807/1560-7917.ES.2016.21.8.30148]
37 **McCarthy M**. Zika virus was transmitted by sexual contact in Texas, health officials report. *BMJ* 2016; **352**: i720 [PMID: 26848011]
38 **Hills SL**, Russell K, Hennessey M, Williams C, Oster AM, Fischer M, Mead P. Transmission of Zika Virus Through Sexual Contact with Travelers to Areas of Ongoing Transmission - Continental United States, 2016. *MMWR Morb Mortal Wkly Rep* 2016; **65**: 215-216 [PMID: 26937739 DOI: 10.15585/mmwr.mm6508e2]
39 **Gourinat AC**, O'Connor O, Calvez E, Goarant C, Dupont-Rouzeyrol M. Detection of Zika virus in urine. *Emerg Infect Dis* 2015; **21**: 84-86 [PMID: 25530324 DOI: 10.3201/eid2101.140894]
40 **Musso D**, Roche C, Nhan TX, Robin E, Teissier A, Cao-Lormeau VM. Detection of Zika virus in saliva. *J Clin Virol* 2015; **68**: 53-55 [PMID: 26071336 DOI: 10.1016/j.jcv.2015.04.021]
41 **Oster AM**, Russell K, Stryker JE, Friedman A, Kachur RE, Petersen EE, Jamieson DJ, Cohn AC, Brooks JT. Update: Interim Guidance for Prevention of Sexual Transmission of Zika Virus--United States, 2016. *MMWR Morb Mortal Wkly Rep* 2016; **65**: 323-325 [PMID: 27032078 DOI: 10.15585/mmwr.mm6512e3]
42 **Besnard M**, Lastere S, Teissier A, Cao-Lormeau V, Musso D. Evidence of perinatal transmission of Zika virus, French Polynesia, December 2013 and February 2014. *Euro Surveill* 2014; **19**: pii: 20751 [PMID: 24721538]
43 **Dupont-Rouzeyrol M**, Biron A, O'Connor O, Huguon E, Descloux E. Infectious Zika viral particles in breastmilk. *Lancet* 2016; **387**: 1051 [PMID: 26944028 DOI: 10.1016/S0140-6736(16)00624-3]
44 **Barthel A**, Gourinat AC, Cazorla C, Joubert C, Dupont-Rouzeyrol M, Descloux E. Breast milk as a possible route of vertical transmission of dengue virus? *Clin Infect Dis* 2013; **57**: 415-417 [PMID: 23575200 DOI: 10.1093/cid/cit227]
45 **Blázquez AB**, Sáiz JC. West Nile virus (WNV) transmission routes in the murine model: intrauterine, by breastfeeding and after cannibal ingestion. *Virus Res* 2010; **151**: 240-243 [PMID: 20438776 DOI: 10.1016/j.virusres.2010.04.009]
46 **Martines RB**, Bhatnagar J, Keating MK, Silva-Flannery L, Muehlenbachs A, Gary J, Goldsmith C, Hale G, Ritter J, Rollin D, Shieh WJ, Luz KG, Ramos AM, Davi HP, Kleber de Oliveria W, Lanciotti R, Lambert A, Zaki S. Notes from the Field: Evidence of Zika Virus Infection in Brain and Placental Tissues from Two Congenitally Infected Newborns and Two Fetal Losses--Brazil, 2015. *MMWR Morb Mortal Wkly Rep* 2016; **65**: 159-160 [PMID: 26890059 DOI: 10.15585/mmwr.mm6506e1]
47 **de Oliveira CS**, da Costa Vasconcelos PF. Microcephaly and Zika virus. *J Pediatr* (Rio J) 2016; **92**: 103-105 [PMID: 27036749 DOI: 10.1016/j.jped.2016.02.003]
48 **Sarno M**, Sacramento GA, Khouri R, do Rosário MS, Costa F, Archanjo G, Santos LA, Nery N, Vasilakis N, Ko AI, de Almeida AR. Zika Virus Infection and Stillbirths: A Case of Hydrops Fetalis, Hydranencephaly and Fetal Demise. *PLoS Negl Trop Dis* 2016; **10**: e0004517 [PMID: 26914330 DOI: 10.1371/journal.pntd.0004517]
49 **Ribeiro CF**, Lopes VG, Brasil P, Coelho J, Muniz AG, Nogueira RM. Perinatal transmission of dengue: a report of 7 cases. *J Pediatr* 2013; **163**: 1514-1516 [PMID: 23916226 DOI: 10.1016/j.jpeds.2013.06.040]
50 **Bentlin MR**, de Barros Almeida RA, Coelho KI, Ribeiro AF, Siciliano MM, Suzuki A, Fortaleza CM. Perinatal transmission of yellow fever, Brazil, 2009. *Emerg Infect Dis* 2011; **17**: 1779-1780 [PMID: 21888828 DOI: 10.3201/eid1709.110242]
51 **Duffy MR**, Chen TH, Hancock WT, Powers AM, Kool JL, Lanciotti RS, Pretrick M, Marfel M, Holzbauer S, Dubray C, Guillaumot L, Griggs A, Bel M, Lambert AJ, Laven J, Kosoy O, Panella A, Biggerstaff BJ, Fischer M, Hayes EB. Zika virus outbreak on Yap Island, Federated States of Micronesia. *N Engl J Med* 2009; **360**: 2536-2543 [PMID: 19516034 DOI: 10.1056/NEJMoa0805715]
52 **Macnamara FN**. Zika virus: a report on three cases of human infection during an epidemic of jaundice in Nigeria. *Trans R Soc Trop Med Hyg* 1954; **48**: 139-145 [PMID: 13157159]
53 **Domínguez-Moreno R**, Tolosa-Tort P, Patiño-Tamez A, Quintero-Bauman A, Collado-Frías DK, Miranda-Rodríguez MG, Canela-Calderón OJ, Hurtado-Valadez P, de Gante-Castro R, Ortiz-Guillén KM, Estañol-Vidal B, Sentíes-Madrid H, García-Ramos G, Cantú-Brito C, Ruiz-Sandoval JL, Chiquete E. Mortality associated with a diagnosis of Guillain-Barré syndrome in adults of Mexican health institutions. *Rev Neurol* 2014; **58**: 4-10 [PMID: 24343535]
54 **Smith DW**, Mackenzie J. Zika virus and Guillain-Barré syndrome: another viral cause to add to the list. *Lancet* 2016; **387**: 1486-1488 [PMID: 26948432 DOI: 10.1016/S0140-6736(16)00564-X]
55 **van den Berg B**, Bunschoten C, van Doorn PA, Jacobs BC. Mortality in Guillain-Barre syndrome. *Neurology* 2013; **80**: 1650-1654 [PMID: 23576619 DOI: 10.1212/WNL.0b013e3182904fcc]
56 **Willison HJ**, Jacobs BC, van Doorn PA. Guillain-Barré syndrome. *Lancet* 2016; **388**: 717-727 [PMID: 26948435 DOI: 10.1016/S0140-6736(16)00339-1]
57 **Cao-Lormeau VM**, Roche C, Teissier A, Robin E, Berry AL, Mallet HP, Sall AA, Musso D. Zika virus, French polynesia, South pacific, 2013. *Emerg Infect Dis* 2014; **20**: 1085-1086 [PMID: 24856001 DOI: 10.3201/eid2006.140138]
58 **Oehler E**, Watrin L, Larre P, Leparc-Goffart I, Lastere S, Valour F, Baudouin L, Mallet H, Musso D, Ghawche F. Zika virus infection complicated by Guillain-Barre syndrome--case report, French Polynesia, December 2013. *Euro Surveill* 2014; **19**: pii: 20720 [PMID: 24626205]
59 **Roth A**, Mercier A, Lepers C, Hoy D, Duituturaga S, Benyon E, Guillaumot L, Souares Y. Concurrent outbreaks of dengue, chikungunya and Zika virus infections - an unprecedented epidemic

wave of mosquito-borne viruses in the Pacific 2012-2014. *Euro Surveill* 2014; **19**: pii: 20929 [PMID: 25345518]

60 **Cao-Lormeau VM**, Blake A, Mons S, Lastère S, Roche C, Vanhomwegen J, Dub T, Baudouin L, Teissier A, Larre P, Vial AL, Decam C, Choumet V, Halstead SK, Willison HJ, Musset L, Manuguerra JC, Despres P, Fournier E, Mallet HP, Musso D, Fontanet A, Neil J, Ghawché F. Guillain-Barré Syndrome outbreak associated with Zika virus infection in French Polynesia: a case-control study. *Lancet* 2016; **387**: 1531-1539 [PMID: 26948433 DOI: 10.1016/S0140-6736(16)00562-6]

61 **Malone RW**, Homan J, Callahan MV, Glasspool-Malone J, Damodaran L, Schneider Ade B, Zimler R, Talton J, Cobb RR, Ruzic I, Smith-Gagen J, Janies D, Wilson J. Zika Virus: Medical Countermeasure Development Challenges. *PLoS Negl Trop Dis* 2016; **10**: e0004530 [PMID: 26934531 DOI: 10.1371/journal.pntd.0004530]

62 **Garg RK**, Malhotra HS, Jain A, Malhotra KP. Dengue-associated neuromuscular complications. *Neurol India* 2015; **63**: 497-516 [PMID: 26238884 DOI: 10.4103/0028-3886.161990]

63 **Simon O**, Billot S, Guyon D, Daures M, Descloux E, Gourinat AC, Molko N, Dupont-Rouzeyrol M. Early Guillain-Barré Syndrome associated with acute dengue fever. *J Clin Virol* 2016; **77**: 29-31 [PMID: 26895226 DOI: 10.1016/j.jcv.2016.01.016]

64 **Sejvar JJ**. West Nile virus and "poliomyelitis". *Neurology* 2004; **63**: 206-207 [PMID: 15277609]

65 **Wielanek AC**, Monredon JD, Amrani ME, Roger JC, Serveaux JP. Guillain-Barré syndrome complicating a Chikungunya virus infection. *Neurology* 2007; **69**: 2105-2107 [PMID: 18040016 DOI: 10.1212/01.wnl.0000277267.07220.88]

66 **Yuki N**, Hartung HP. Guillain-Barré syndrome. *N Engl J Med* 2012; **366**: 2294-2304 [PMID: 22694000 DOI: 10.1056/NEJMra1114525]

67 **von der Hagen M**, Pivarcsi M, Liebe J, von Bernuth H, Didonato N, Hennermann JB, Bührer C, Wieczorek D, Kaindl AM. Diagnostic approach to microcephaly in childhood: a two-center study and review of the literature. *Dev Med Child Neurol* 2014; **56**: 732-741 [PMID: 24617602 DOI: 10.1111/dmcn.12425]

68 **Rodrigues LC**. Microcephaly and Zika virus infection. *Lancet* 2016; **387**: 2070-2072 [PMID: 26993880 DOI: 10.1016/S0140-6736(16)00742-X]

69 **Zwizwai R**. Infection disease surveillance update. *Lancet Infect Dis* 2016; **16**: 157 [PMID: 26867462 DOI: 10.1016/S1473-3099(16)00023-2]

70 **Calvet G**, Aguiar RS, Melo AS, Sampaio SA, de Filippis I, Fabri A, Araujo ES, de Sequeira PC, de Mendonça MC, de Oliveira L, Tschoeke DA, Schrago CG, Thompson FL, Brasil P, Dos Santos FB, Nogueira RM, Tanuri A, de Filippis AM. Detection and sequencing of Zika virus from amniotic fluid of fetuses with microcephaly in Brazil: a case study. *Lancet Infect Dis* 2016; **16**: 653-660 [PMID: 26897108 DOI: 10.1016/S1473-3099(16)00095-5]

71 **Kleber de Oliveira W**, Cortez-Escalante J, De Oliveira WT, do Carmo GM, Henriques CM, Coelho GE, Araújo de França GV. Increase in Reported Prevalence of Microcephaly in Infants Born to Women Living in Areas with Confirmed Zika Virus Transmission During the First Trimester of Pregnancy - Brazil, 2015. *MMWR Morb Mortal Wkly Rep* 2016; **65**: 242-247 [PMID: 26963593 DOI: 10.15585/mmwr.mm6509e2]

72 **Jouannic JM**, Friszer S, Leparc-Goffart I, Garel C, Eyrolle-Guignot D. Zika virus infection in French Polynesia. *Lancet* 2016; **387**: 1051-1052 [PMID: 26944027 DOI: 10.1016/S0140-6736(16)00625-5]

73 **Cauchemez S**, Besnard M, Bompard P, Dub T, Guillemette-Artur P, Eyrolle-Guignot D, Salje H, Van Kerkhove MD, Abadie V, Garel C, Fontanet A, Mallet HP. Association between Zika virus and microcephaly in French Polynesia, 2013-15: a retrospective study. *Lancet* 2016; **387**: 2125-2132 [PMID: 26993883 DOI: 10.1016/S0140-6736(16)00651-6]

74 **Driggers RW**, Ho CY, Korhonen EM, Kuivanen S, Jääskeläinen AJ, Smura T, Rosenberg A, Hill DA, DeBiasi RL, Vezina G, Timofeev J, Rodriguez FJ, Levanov L, Razak J, Iyengar P, Hennenfent A, Kennedy R, Lanciotti R, du Plessis A, Vapalahti O. Zika Virus Infection with Prolonged Maternal Viremia and Fetal Brain Abnormalities. *N Engl J Med* 2016; **374**: 2142-2151 [PMID: 27028667 DOI: 10.1056/NEJMoa1601824]

75 **Butler D**. First Zika-linked birth defects detected in Colombia. *Nature* 2016; **531**: 153 [PMID: 26961637 DOI: 10.1038/nature.2016.19502]

76 **Bell TM**, Field EJ, Narang HK. Zika virus infection of the central nervous system of mice. *Arch Gesamte Virusforsch* 1971; **35**: 183-193 [PMID: 5002906]

77 **Weinbren MP**, Williams MC. Zika virus: further isolations in the Zika area, and some studies on the strains isolated. *Trans R Soc Trop Med Hyg* 1958; **52**: 263-268 [PMID: 13556872]

78 **Tang H**, Hammack C, Ogden SC, Wen Z, Qian X, Li Y, Yao B, Shin J, Zhang F, Lee EM, Christian KM, Didier RA, Jin P, Song H, Ming GL. Zika Virus Infects Human Cortical Neural Progenitors and Attenuates Their Growth. *Cell Stem Cell* 2016; **18**: 587-590 [PMID: 26952870 DOI: 10.1016/j.stem.2016.02.016]

79 **Adibi JJ**, Marques ET, Cartus A, Beigi RH. Teratogenic effects of the Zika virus and the role of the placenta. *Lancet* 2016; **387**: 1587-1590 [PMID: 26952548 DOI: 10.1016/S0140-6736(16)00650-4]

80 **Petersen EE**, Staples JE, Meaney-Delman D, Fischer M, Ellington SR, Callaghan WM, Jamieson DJ. Interim Guidelines for Pregnant Women During a Zika Virus Outbreak--United States, 2016. *MMWR Morb Mortal Wkly Rep* 2016; **65**: 30-33 [PMID: 26796813 DOI: 10.15585/mmwr.mm6502e1]

81 **Frank C**, Faber M, Stark K. Causal or not: applying the Bradford Hill aspects of evidence to the association between Zika virus and microcephaly. *EMBO Mol Med* 2016; **8**: 305-307 [PMID: 26976611 DOI: 10.15252/emmm.201506058]

82 **Nabel GJ**, Zerhouni EA. Once and future epidemics: Zika virus emerging. *Sci Transl Med* 2016; **8**: 330ed2 [PMID: 27089202 DOI: 10.1126/scitranslmed.aaf4548]

83 **Clyde K**, Kyle JL, Harris E. Recent advances in deciphering viral and host determinants of dengue virus replication and pathogenesis. *J Virol* 2006; **80**: 11418-11431 [PMID: 16928749 DOI: 10.1128/JVI.01257-06]

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MINIREVIEWS

Regulation of Wnt/β-catenin signaling by herpesviruses

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Abstract

The Wnt/β-catenin signaling pathway is instrumental in successful differentiation and proliferation of mammalian cells. It is therefore not surprising that the herpesvirus family has developed mechanisms to interact with and manipulate this pathway. Successful coexistence with the host requires that herpesviruses establish a lifelong infection that includes periods of latency and reactivation or persistence. Many herpesviruses establish latency in progenitor cells and viral reactivation is linked to host-cell proliferation and differentiation status. Importantly, Wnt/β-catenin is tightly connected to stem/progenitor cell maintenance and differentiation. Numerous studies have linked Wnt/β-catenin signaling to a variety of cancers, emphasizing the importance of Wnt/β-catenin pathways in development, tissue homeostasis and disease. This review details how the alpha-, beta-, and gammaherpesviruses interact and manipulate the Wnt/β-catenin pathway to promote a virus-centric agenda.

Key words: Herpesvirus; Herpes simplex virus-1; Varicella zoster virus; Cytomegalovirus; Epstein-Barr virus; Kaposi's sarcoma-associated herpesvirus; Wnt/β-catenin; Glycogen synthase kinase-3; Axin

Core tip: The Wnt/β-catenin signaling pathway is essential for many host cell functions. Herpesviruses have evolved to manipulate and control this vital pathway to promote viral propagation, evade host immune recognition and maintain latency.

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INTRODUCTION

Herpesviruses have been coevolving with vertebrates

for millions of years and have developed multiple mechanisms to avoid immune recognition and manipulate host signaling pathways to promote efficient viral replication. This is evident in the ability of herpesviruses to persist for the lifetime of the host while causing limited adverse effects[1]. In general, severe symptoms are only seen in those individuals who are immunocompromised[2]. Accumulating evidence suggests that herpesviruses interact with the Wnt/β-catenin pathway to regulate viral gene expression and alter host cell gene expression by manipulating downstream signaling components during both active infection and latency.

The Wnt/β-catenin pathway is responsible for a signaling cascade that is required during embryonic development and continues throughout the life of an organism. Nearly every tissue and organ depends on this signaling cascade for normal function. Correct Wnt/β-catenin signaling is crucial in the development of many organs including the brain, heart, lung, bone, liver, kidney and gut among others[3,4]. Many of these essential roles continue in adulthood in relation to tissue homeostasis, regeneration, maintenance and repair functions. Additionally, Wnt/β-catenin has been shown to be important in cell migration, genetic stability and apoptosis[5-8]. With such widespread influence on many diverse signaling cascades, dysfunctional Wnt/β-catenin signaling can have deleterious effects. Unregulated Wnt/β-catenin signaling was first linked to human disease in the 1990s when adenomatous polyposis coli (APC) protein was found to interact with β-catenin[9,10]. Since then, Wnt/β-catenin signaling has been implicated in many cancers[11-15], fibrosis[16,17], and metabolic disease[18].

Although conclusive data on the importance of Wnt/β-catenin signaling during the complete replication cycle of all herpesvirus members are lacking, accumulating data are beginning to reveal the importance of this pathway to viral replication, latency and pathogenesis. The potential to target the Wnt/β-catenin pathway for therapeutic intervention is enormous but is compounded by the complexity of the signaling cascade, the number of potential players involved during signaling activation and its importance to cellular homeostasis. Understanding how herpesviruses manipulate this pathway has increased our knowledge of this important pathway and may ultimately lead to novel antiviral therapies.

THE WNT/β-CATENIN SIGNALING CASCADE

Wnts are lipid-modified glycoprotein ligands that act in an autocrine or paracrine manner. Wnt signaling can be divided into three main signaling cascades: Canonical Wnt and two β-catenin-independent pathways, the non-canonical planar cell pathway[19] and the non-canonical Wnt/calcium pathway[20,21]. This review will focus on the canonical Wnt pathway but crosstalk of the three signaling cascades has been reported and is therefore unavoidable. Briefly, in the absence of Wnt stimulation, cytoplasmic β-catenin is phosphorylated and degraded by the ubiquitin-proteasome system (Figure 1). Upon binding of Wnt, phosphorylation of β-catenin is blocked allowing it to translocate to the nucleus where it complexes with transcription factors to upregulate Wnt target gene transcription (Figure 1). Canonical Wnt signaling is initiated when Wnts bind to a heterodimeric transmembrane receptor complex consisting of Frizzled (Fz) receptor and the co-receptors low-density lipoprotein receptor-related protein 5 (LRP5) and LRP6. The ligand interaction induces conformational changes and subsequent phosphorylation of target proteins. This results in recruitment and signaling through the scaffold protein Dishevelled promoting the inhibition of the destruction complex, which contains Axin, APC, β-catenin, casein kinase Iα/β (CKI Iα/β), and glycogen synthase kinase-3α/β (GSK-3α/β). APC directly interacts with β-catenin and Axin. Axin binds to the cytoplasmic tail of LRP6 and this complex is regulated through phosphorylation by GSK-3 and CK1. When the destruction complex is intact, Axin associated β-catenin is phosphorylated by CKI and GSK-3β at N-terminal Ser/Thr residues. Phosphorylated β-catenin is then recognized by the E3 ubiquitin ligase complex β-TrCP (Beta-Transducin Repeat Containing E3 Ubiquitin Protein Ligase) and targeted for degradation by the proteasome. In the presence of Wnt ligand, signaling results in the dissociation of the destruction complex and loss of GSK-3 mediated phosphorylation of β-catenin. Axin is recruited to the phosphorylated tail of LRP preventing β-catenin phosphorylation and ubiquitination. As a result, β-catenin is free to accumulate and translocate to the nucleus where it interacts with members of the T cell factor/lymphoid enhancer-binding factor (TCF/LEF) family of transcription factors and transcriptional coactivators such as CREB-binding protein (CBP), E1A-associated protein p300, and Pygopus to initiate Wnt target gene expression[22]. β-catenin can also interact with many other transcription factors not linked to the TCF/LEF family but that do play important roles in cell maintenance and differentiation[23-25]. For more in depth reviews on Wnt/β-catenin signaling, the reader is referred to many of the excellent reviews available[23,26-29].

HERPESVIRUSES

The taxonomic order *Herpesvirales* includes over 130 herpesviruses divided into three virus families: *Herpesviridae* that can infect mammals, birds and reptiles; *Alloherpesviridae* that infect amphibians and bony fish; and *Malacoherpesviridae* that infects some invertebrates, including molluscs[30-32]. These classifications are based on genome size/structure and biological function. *Herpesviridae* is a family of

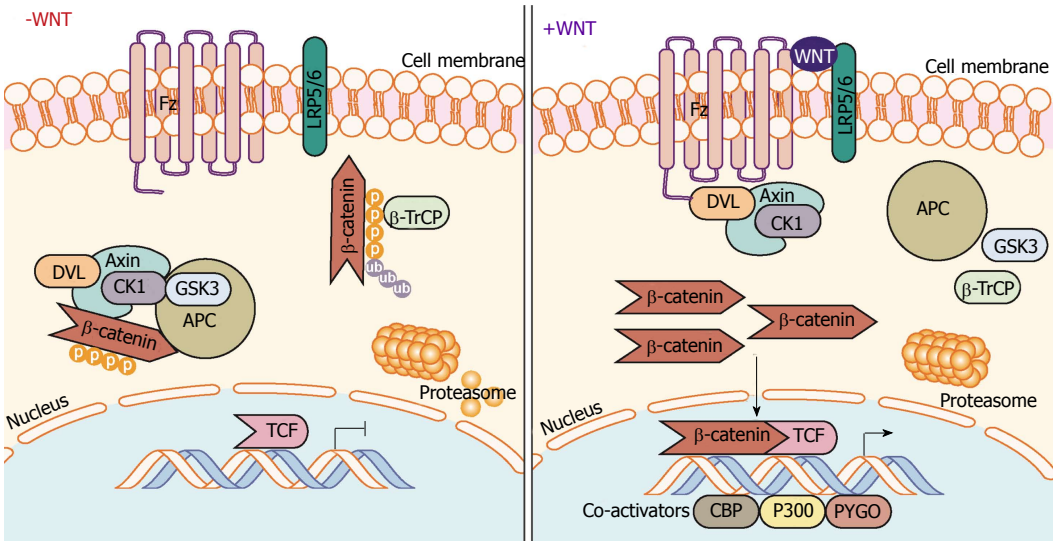

Figure 1 Canonical Wnt/β-catenin signaling pathway. In the absence of Wnt ligand stimulation, the β-catenin destruction complex - consisting of the proteins Axin, CK1, GSK-3α/β, APC, and DVL - phosphorylate β-catenin allowing β-TrCP to ubiquitinate β-catenin marking it for proteasomal degradation. When stimulated by Wnt ligands, engagement of the Fz receptor and co-receptors LRP5/6, induces signaling through DVL inhibiting the action of the destruction complex. This frees β-catenin from degradation pathways allowing β-catenin to translocate and accumulate in the nucleus. β-catenin mediated interaction with TCF family transcription factors and co-activators (CBP, *etc.*) and initiates transcription of target genes. APC: Adenomatous polyposis coli; β-TrCP: Beta-transducin repeat containing E3 ubiquitin protein ligase; CBP: CREB-binding protein; CK1: Casein kinase 1; DVL: Dishevelled; Fz: Frizzled receptor; GSK-3: Glycogen synthase kinase 3; LRP: Low-density lipoprotein receptor-related protein; TCF/LEF-1: T-cell factor/lymphoid enhancer-binding factor 1.

enveloped, DNA viruses that is further divided into 3 subfamilies (*Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*). A criterion for inclusion in the *Herpesviridae* family morphologically is centered on the virion structure[33]. The virion is spherical in shape and includes a core, capsid, tegument and envelope. The core contains the viral genome, which is a linear, double-stranded DNA molecule. The core is surrounded by an icosahedral capsid that is enclosed within a proteinaceous layer called the tegument. Finally, a lipid bilayer envelope surrounds the exterior of the tegument and completes the structure of the virion.

Humans can be infected by eight different herpesviruses. Herpesvirus infections are typically systemic, although some may be localized. Gene expression is tightly regulated and orchestrated in a temporal manner. Simplistically, immediate-early genes encoding regulatory proteins are expressed soon after infection, followed by expression of early genes that are important for replication of viral DNA. Finally, late genes encoding structural proteins are expressed. Due to various host immune evasion strategies, herpesviruses establish life-long latent infections in infected individuals. In an oversimplified model in regards to human infection, *Alphaherpesvirinae* establish latency in neurons, *Betaherpesvirinae* in monocytes and *Gammaherpesvirinae* in lymphocytes, monocytes, and macrophages[1,32,34].

HUMAN ALPHAHERPESVIRUSES

The subfamily *Alphaherpesvirinae* includes three members. The human herpesviruses 1 and 2 (HHV-1/2) also known as herpes simplex virus (HSV) (type 1/2) belong in the genus Simplexvirus while HHV-3 or Varicella-zoster virus (VZV) is classified in the genus Varicellovirus[32,33]. Infection can result in skin vesicles or mucosal ulcers and on rare occasions meningitis and encephalitis[2].

HHV-1 (HSV-1)

To date there have been no focused, thorough investigations of the role of Wnt/β-catenin on HSV-1/2. The studies that have been completed implicate individual members of the Wnt/β-catenin signaling cascade in viral pathogenesis. An example of this is the upregulation of the antiviral cytokine interferon-β (IFN-β) during HSV-1 infection. In adult immunocompetent mice, macrophages are essential for clearing HSV-1 from the blood; however, it was observed that macrophages from Akt$^{-/-}$ mice display poor clearance of HSV-1. The Akt1 family of serine/threonine kinases was shown to phosphorylate β-catenin at serine 552 allowing accumulation and β-catenin mediated induction of IFN-β[35]. Akt1 classically has been described as a β-catenin transcriptional promoter, exerting its effects by repressing GSK-3 mediated β-catenin proteasomal degradation[36]. Interestingly, the serine 552-phosphorylation site is distinct from the site typically targeted by GSK-3. The authors conclude that Akt1 is responsible for inhibiting GSK-3 phosphorylation of β-catenin on Ser9 and also for direct phosphorylation of β-catenin at serine 552 allowing for stabilization, enhanced nuclear translocation and transcriptional activity of β-catenin (Figure 2).

In a second study, Choi *et al*[37] observed that HSV-1 infection and replication was more efficient in a fibroblast-like murine cell line, L929. Knocking down Axin or treatment with Wnt3a conditioned media reduces

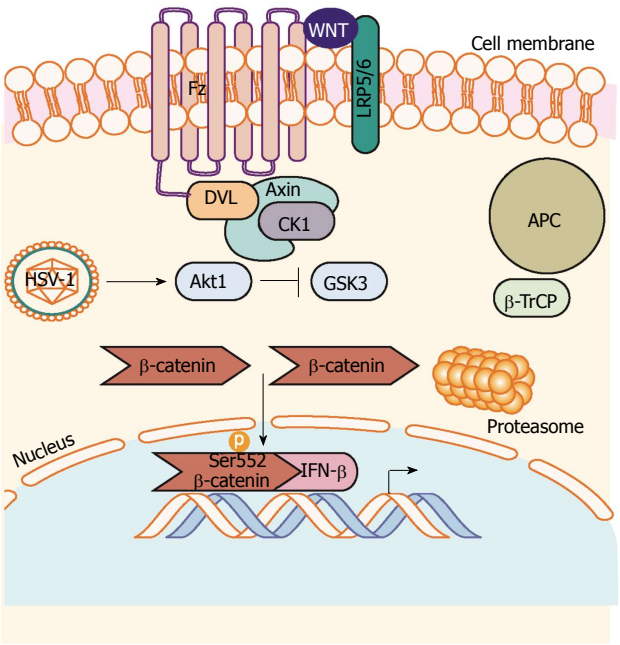

Figure 2 β-catenin mediated antiviral interferon response during herpes simplex virus 1 infection. HSV-1 infection induces activation of Akt1 activity. Akt1 phosphorylates β-catenin on Serine 552 inhibiting degradation signaling through GSK-3-mediated phosphorylation of β-catenin on Serine 9. β-catenin can then accumulate in the nucleus to induce transcription of β-catenin target genes such as the antiviral cytokine IFN-β. Akt1: Protein kinase B; HSV-1: Herpes simplex virus 1.

HSV-1 replication in L929 cells. They further showed that Axin expression minimalizes HSV-1 induced cell death, which in turn promotes increased HSV-1 replication. In a follow up study, this group observed that HSV-1 infection also induced autophagy but this is delayed in L929 cells ectopically expressing L-Axin[38]. The authors concluded that delay in induction of autophagy favors HSV-1 viral replication likely by suppression of HSV-1 mediated cell death. The implication is that HSV-1 replication is inversely related to Wnt signaling.

Lastly, Piacentini *et al*[39] demonstrated that HSV-1 infection disrupts synaptic function in cultured murine cortical neurons through GSK-3 activation and intracellular accumulation of amyloid-β protein. In a previous study this group showed that HSV-1 mediated increases in intracellular Ca^{2+} is the main mechanism for activation of GSK-3 in this model[39]. These studies suggest a possible link between HSV-1 pathogenesis and Alzheimer's disease.

To date, the involvement of Wnt/β-catenin signaling during VZV infection has been underinvestigated. Markus *et al*[40] observed an increase in canonical Wnt pathway transcription in infection of neurons derived from human embryonic stem cells. The Wnt pathway was unaffected during late VZV infection of fibroblasts. Intriguingly, like HSV-1 and -2, VZV will enter latency in neurons but will lytically replicate in fibroblasts suggesting a differing need for Wnt pathway modification by the virus in different stages of the viral life cycle[40]. Given the limited studies on Wnt/β-catenin signaling during alphaherpesvirus infections, how vital Wnt/β-catenin signaling is to viral replication and pathogenesis remains unknown. The studies mentioned above seem to portray a conflicting role of β-catenin in viral replication. More thorough studies using defined cell types and carefully delineated "branches" of the Wnt pathway will provide a clearer understanding.

HUMAN BETAHERPESVIRUS

The human *Betaherpesvirinae* subfamily consists of the three viruses: HHV-5 known as human cytomegalovirus (HCMV), HHV-6A/B, and HHV-7 (the latter two are commonly referred to as Roseolovirus)[32,33]. Infection is usually asymptomatic but infectious mononucleosis like symptoms are seen in HCMV infections and the development of a rash is associated with Roseolovirus. In immunocompromised individuals (organ transplant patients, HIV positive individuals, *etc*.) or during pregnancy, infection and/or reactivation of β-herpesvirus can have life-threatening consequences. Of these three viruses, HCMV is the most studied and is considered the prototypical betaherpesvirus. As little is known about Wnt/β-catenin regulation during infection by the polyphyletic Roseolovirus group, this portion of the review will focus exclusively on HCMV.

HHV-5 (HCMV)

The Wnt/β-catenin pathway is one of the many cellular pathways manipulated by HCMV to likely facilitate lytic viral replication. By dysregulating the physiological condition of the Wnt/β-catenin pathway, HCMV inhibits or severely hampers the processes of cellular replication, movement/migration, and differentiation among others[41,42].

HCMV infection of the placenta may cause impaired invasion of placental-derived cells toward maternal spiral arteries leading to shallow placentation and a deficit in oxygen/nutrient flow to the developing fetus[43]. The Wnt/β-catenin pathway is important in the differentiation of placental cytotrophoblasts into extravillous trophoblasts, the invasive lineage of cells that remodel maternal spiral arteries to establish blood flow to the placenta[44-46]. Using an *in vitro* model of first trimester cytotrophoblasts (SGHPL-4) infected with HCMV, Angelova *et al*[41] demonstrated that β-catenin protein levels decrease significantly during the late stages of infection roughly corresponding to expression of late proteins and packaging of nucleocapsids into an envelope to produce mature virions. This decrease in β-catenin protein is dependent on proteasomal degradation and occurs in all cellular pools including membrane, cytoplasm and nucleus. Remaining β-catenin, aggregates near the viral assembly compartment, a juxtanuclear region present during infection involved in virion assembly and egress; however, the reasons for this are currently unclear. Transcriptional targets of β-catenin, such as Dickkopf-related protein 1 (Dkk1) and Cyclin D, also exhibit transcriptional repression as a result. However,

β-catenin mRNA levels actually increase in the same timeframe[41]. Consistent with these results, Ueland *et al*[47] showed that plasma levels of DKK-1 were significantly lower in solid organ transplant patients with HCMV DNAemia. In contrast, Langemeijer *et al*[48] reported that HCMV infection increases transcriptional activation of β-catenin in a glioblastoma cell line that is dependent on expression of the virally encoded G-protein coupled receptor, US28. These different results may be explained by the use of different cell types and methods to detect β-catenin activity.

The mechanism by which HCMV depletes membrane stores of β-catenin is currently unknown although infection extensively remodels cellular membranes[49]. As for cytoplasmic and nuclear stores of β-catenin, HCMV exerts control at the level of the β-catenin destruction complex as disruption of this complex with lithium chloride (LiCl), a GSK-3β inhibitor can inhibit the degradation and depletion of β-catenin during infection. It should be noted that inhibition of β-catenin degradation does not rescue transcriptional function of β-catenin[41]. This may be due to further regulation of transcriptional activity of β-catenin, for example through regulation of β-catenin coactivators like TCF/LEF-1, by the virus or due to undetected post-translational modification of β-catenin. Viral regulation of the destruction complex appears to be mostly mediated at Axin1, the rate-limiting protein in the β-catenin destruction complex in the cytoplasm. PolyADP Ribose Polymerase 5a and 5b (PARP5a/b), also called Tankyrase (TNKS as a combination of isoforms 1 and 2), PARsylates Axin1 leading to degradation through the ubiquitin proteasome pathway. During HCMV infection, TNKS PARsylation activity is inhibited allowing for stabilization of Axin1 and stabilization of the β-catenin destruction complex leading to the degradation of β-catenin seen during infection (Figure 3)[50]. This suggests that HCMV requires a complete and competent β-catenin destruction complex for degradation of β-catenin.

The non-canonical pathways of Wnt signaling, although lacking direct β-catenin regulation, seem to play a role in regulation of the canonical Wnt/β-catenin pathway during HCMV infection. Wnt5a interacts with the tyrosine-like orphan kinase 2 ROR2 and physiologically activates the Wnt/Planar Cell Polarity pathway and Wnt/Ca^{2+} pathway[42]. During HCMV infection, infected cells become insensitive to normal Wnt5a ligand signaling but ROR2 expression is significantly increased. Uninfected trophoblasts invade toward a Wnt5a gradient *in vitro* but are incapable of doing so when infected despite the increased presence of ROR2. The increase in ROR2 expression inhibits canonical signaling by repressing β-catenin TCF/LEF-1 transcriptional activity. Knockdown of non-canonical ROR2 that is overexpressed during infection can rescue some function of the canonical Wnt/β-catenin pathway in trophoblasts suggesting that the canonical and non-canonical Wnt pathways are deeply intertwined, especially during HCMV infection[42].

Targeting of Wnt/β-catenin signaling with select pharmacological inhibitors can inhibit viral replication suggesting that some level of β-catenin or a member of the canonical Wnt pathway may be necessary for viral replication[51]. Why HCMV infection overrides normal Wnt/β-catenin signaling is unknown, but some research indicates involvement of repurposing the molecular members of the pathway to further HCMV replication. Activity of GSK-3 has been implicated in assembly of the viral nucleocapsid in simian CMV (infecting Chimpanzees and Orangutans). Phosphorylation of the viral assembly protein precursor (pAP) by GSK-3 may induce conformational changes in the protein and stabilize pAP interaction with the major capsid protein during capsid assembly[52]. Additionally GSK-3 (along with other members of the β-catenin destruction complex) has been identified as a target for phosphorylation by the viral kinase UL97[53]. However, inhibition of UL97 activity during infection does not seem to rescue β-catenin degradation suggesting that UL97 phosphorylation of GSK-3 is not the primary mechanism by which HCMV depletes β-catenin stores (our unpublished data). Further research must be conducted to determine the importance of molecular mechanisms of Wnt/β-catenin on viral replication itself.

HCMV infection has recently been associated with a diverse array of diseases and disorders such as diabetes[54], atherosclerosis[55], and some cancers (reviewed in[56,57]), along with the abovementioned issues with infection during pregnancy on the placenta and developing fetus. As data show that HCMV infection undermines normal functioning of canonical Wnt/β-catenin and non-canonical Wnt signaling in diverse ways, differing perhaps by infection of a multitude of diverse cell types, it becomes key to better characterize this viral regulation.

HUMAN GAMMAHERPESVIRUSES

The human *Gammaherpesvirinae* family includes two members: Human herpesvirus 4 (HHV-4) commonly known as Epstein-Barr virus (EBV) and HHV-8 or Kaposi's sarcoma-associated herpesvirus (KSHV)[32,33]. They are further classified under the genera Lymphocryptovirus and Rhadinovirus, respectively. EBV was one of the first viruses to be associated with human cancer when it was originally identified in Burkitt's lymphoma. Since then, it has become associated with B cell malignancies and epithelial cell associated cancers. KSHV was discovered in 1994 when samples from AIDS-associated Kaposi's sarcoma came back positive for viral DNA sequences[58]. Diseases associated with KSHV include B cell malignancy primary effusion lymphoma (PEL), Castleman's disease and the endothelial lesion, Kaposi's sarcoma.

HHV-4 (EBV)

The accumulation of β-catenin is seen in EBV-infected epithelial and B cells. In the earliest report, Shackelford *et al*[59] reported that β-catenin was not degraded in

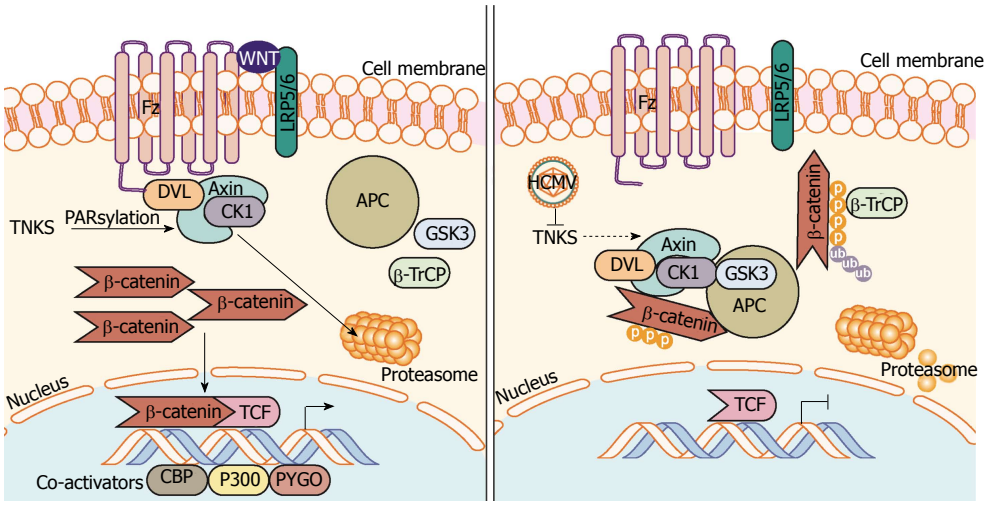

Figure 3 Human cytomegalovirus inhibits PARsylation activity of tankyrase 1 and 2 (PARP5a/b) to enhance infection. Regulation of Axin is the rate-limiting step in the assembly and function of the β-catenin destruction complex. PARsylation, a process driven by NAD^+, marks Axin for proteasomal degradation, inhibiting β-catenin destruction complex formation resulting in β-catenin accumulation and transcription in the nucleus. HCMV infection causes inhibition of TNKS (PARP5a/b) PARsylation activity, which inhibits PARsylation of Axin and increases its stability. Further inhibition of TNKS PARsylation activity or knockdown of TNKS significantly aids in HCMV replication. An increase in stable Axin permits β-catenin destruction complex formation, increased β-catenin degradation, and subsequent inhibition of β-catenin-mediated transcription. HCMV: Human cytomegalovirus; NAD^+: Nicotinamide adenine dinucleotide; PARsylation: Poly-ADP ribose modification; PARP5a/b: Poly-ADP ribose polymerase 5a/b; TNKS: Tankyrase 1 and 2 (PARP5a/b).

lymphoid cells during an EBV type Ⅲ latent infection. The authors postulate that these observations may be due to ubiquitinating enzymes or the dysregulation of other oncogenes. Interestingly, this effect was not observed during EBV type Ⅰ latency infection[59]. Shortly after, a second group showed that telomerase-immortalized human foreskin keratinocytes have increased β-catenin accumulation after infection with EBV[60]. The mechanism was shown to be dependent on latent membrane protein 2A (LMP2A) activation of Akt and Akt-mediated inactivation of GSK-3, independent of phosphorylation at Ser9. Treatment with LiCl led to β-catenin accumulation in the cytoplasm, translocation into the nucleus and activation of a TCF-responsive reporter. In a follow-up study, the immunoreceptor tyrosine-based activation and PY motifs of LMP2A were found to mediate the accumulation and nuclear translocation of β-catenin[61]. Using LMP2A ΔPY mutants, they showed that β-catenin levels and translocation to the nucleus decreased along with epithelial cell differentiation. The authors concluded that LMP2A mediated epithelial cell differentiation appears to be inversely correlated with β-catenin activation in this model.

EBV latent membrane protein 1 (LMP1) has also been associated with an increase in β-catenin levels in EBV-infected BL cells[62]. Jang *et al*[63] reported that an E3 ubiquitin ligase, a human homolog of *Drosophilia* seven in absentia (Siah-1), is repressed by LMP1. Siah-1 binds APC and in a GSK-3 independent manner, degrades β-catenin. However, another study using transient and stable expression of LMP1 sequences failed to find evidence that LMP1 induces Wnt/β-catenin signaling or promotes the accumulation of β-catenin[64]. To further verify their observations, they proceeded to show that there was little evidence for interactions between LMP1 and β-catenin. The authors proposed that differences in cell lines and LMP1 sequences used may account for the conflicting results in these two studies.

Lastly, EBV-mediated dysregulation of Wnt/β-catenin was associated with idiopathic pulmonary fibrosis (IPF)[65]. EBV detection in alveolar epithelial cells has been associated with poor prognosis. Pathogenesis is believed to occur in IPF due to repetitive epithelial cell injury that may be mediated by EBV. Using transcriptomic data, the authors identified altered Wnt/β-catenin pathway transcripts. Specifically, Wnt5b expression was altered. The authors conclude that EBV may be using a non-canonical Wnt/β-catenin pathway that includes CUX1 and the EBV early gene Rta.

HHV-8 (KSHV)

Fujimuro *et al*[66] first observed the association between Wnt/β-catenin and KSHV in 2003. They made the observation that in latently KSHV-infected B cell lines derived from PEL, β-catenin accumulated at high levels in the cytoplasm. KSHV infection of PEL cells results in a high KSHV latency rate suggesting that the increased levels of β-catenin may be linked to expression of KSHV latency associated proteins. The latency-associated nuclear antigen (LANA) protein proved to be the protein responsible, as siRNA transient knockdown specific to LANA, decreased levels of LANA and β-catenin[67]. LANA was originally shown to be involved in the tethering of KSHV episomal genomes to host chromosomes to aid in viral DNA replication[68,69]. Using a yeast-two hybrid system, paired with coimmunoprecipitation assays, LANA was also found to possess the ability to bind to GSK-3α and GSK-3β[67].

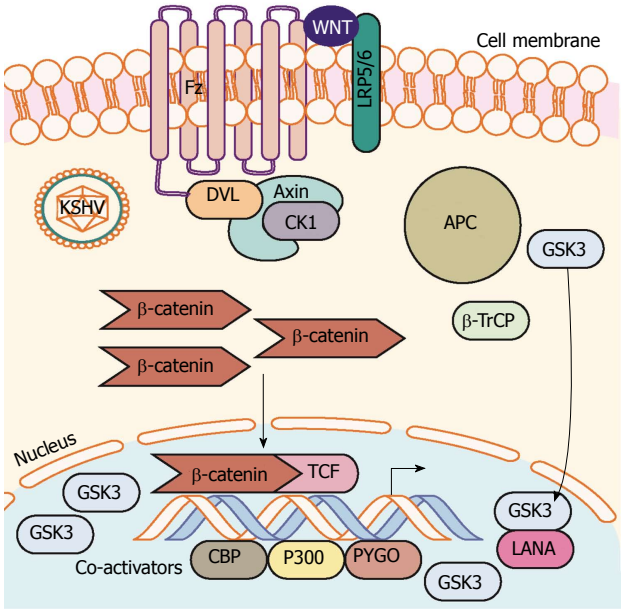

Figure 4 Kaposi's sarcoma-associated herpesvirus latency upregulates β-catenin. Establishment of KSHV latency involves expression of the latency associated protein LANA. LANA binds with and translocates GSK-3 into the nucleus after phosphorylation by GSK-3. This translocation of cytoplasmic pools of GSK-3 prevents β-catenin destruction complex formation and stability of cytoplasmic β-catenin. β-catenin translocation to the nucleus occurs resulting in increased β-catenin-mediated transcription. LANA: Latency-associated nuclear antigen; KSHV: Kaposi's sarcoma-associated herpesvirus.

In addition to mediating the phosphorylation of β-catenin as part of the destruction complex in the cytoplasm, GSK-3 also translocates to the nucleus during apoptotic stimuli in a cell cycle-dependent manner. Nuclear levels of GSK-3 protein increase in the nucleus of PEL cells specifically during the S phase of the cell cycle[66]. This results in lower GSK-3 within the destruction complex and more unphosphoryated β-catenin that translocates to the nucleus and activates target gene expression (Figure 4). The authors proposed that LANA promotes the accumulation of GSK-3 in the nucleus, reducing the total amount of GSK-3 in the cytoplasm.

Further studies revealed that the C-terminal region of LANA displayed limited homology to the domain of Axin that binds GSK-3β and is functionally similar to Axin[67]. LANA protein mutants were used to study the binding potential between LANA and GSK-3β[70]. These studies showed that changing Phe^{291} in the coding sequence of LANA to Leu (F291L mutant), leads to a reduction in binding to GSK-3 by 90%. The interaction of the various components of the destruction complex is mediated by phosphorylation, which also mediates the interaction of LANA and GSK-3. GSK-3 and LANA interactions require the LANA C-terminal GSK-3 interacting domain and GSK-3 phosphorylation of the LANA N-terminus. Within this region are four consensus GSK-3 phosphorylation sites [(Ser/Thr)xxx(Ser/Thr)p]. Mutation of the four consensus sites prevented GSK-3 binding to LANA, suggesting that this is a phosphorylation mediated event[70]. Additionally, as GSK-3 substrates typically must be primed prior to phosphorylation by GSK-3, a mutant (R96A) was used to determine if GSK-3 phosphorylation of LANA could proceed without priming. Results showed that, under *in vitro* conditions, GSK-3 phosphorylation of LANA requires priming kinases. The reader is referred to two comprehensive reviews detailing the manipulation of GSK-3 by KSHV[71,72].

Additional studies revealed that CKI and mitogen-activated protein kinase could each function as priming kinases for GSK-3 phosphorylation of LANA[73]. To summarize, KSHV latency protein LANA, promotes nuclear accumulation of GSK-3 to promote dysregulation of β-catenin. Functionally, there is increased expression of cyclin D1, and when β-catenin reporters have been tested, there is increased activity[74]. Surprisingly, it was also determined that most of the GSK-3 in the nucleus of LANA-expressing cells is in an inactive phosphorylated form suggesting that despite increased GSK-3 present in the nucleus, there is a decrease in nuclear GSK-3 activity. Inhibitor of the MyoD family a (I-mfa) and the human I-mfa domain-containing protein (HIC) has been shown to be negative inhibitors of the Wnt pathway. Kusano *et al*[75] showed that LANA interacts with HIC and I-mfa in the 995-1102 amino acid region of LANA. This site is located near the GSK-3 binding site and inhibits the LANA mediated transactivation of a β-catenin construct. Furthermore, this interaction decreases LANA-GSK-3 complex formation resulting in a decrease in Wnt/β-catenin signaling associated transcription. Thus manipulation of the Wnt/β-catenin pathway may play a key role in LANA-mediated oncogenesis in KSHV-infected cells.

Lastly, a recent paper reports that KSHV viral IFN regulatory factor 4 (vIRF4) targets the β-catenin/TCF transcription complex[76]. Using a TOPFlash system, the data suggests that LANA and vIRF4 are negative regulators of each other. Expression of LANA alone resulted in increased β-catenin protein and transcriptional levels, but introducing vIRF4 reduced the levels of LANA-mediated β-catenin/TCF activation. The authors also observed that that this effect was not dependent on β-catenin protein stability. In conclusion, the study suggests that KSHV employs vIRF4 to block the progression of the cell cycle at the G_1-S phase to aid in viral replication.

It has been proposed that dysregulation of the viral gene program leads to nonlytic expression[4]. Angelova *et al*[77] show a novel pathway that KSHV uses to upregulate the Wnt/β-catenin pathway. The KSHV virally-encoded G-protein coupled receptor (vGPCR) inserted into a retroviral vector was transduced into endothelial cells. The authors observed increased cyclin D1, Wnt7A and pygopus 1 (Pygo) expression in vGPCR expressing cells as compared to non-expressing control cells. Additionally, β-catenin was found to accumulate in the nucleus of vGPCR expressing cells and β-catenin/LEF1-dependent TOPFlash reporter constructs displayed increased activity. Initial data suggests that vGPCR-

Table 1 Wnt/β-catenin molecular manipulations by human *Herpesviridae*

Virus	Pathway component	Stabilization, activation or inhibition of pathway component	Outcome	Ref.
Alphaherpesvirinae				
HSV-1	β-catenin	Stabilized	β-catenin stabilized, increased transcriptional activity of β-catenin	[35]
	Axin	Stabilized	Reduced host cell apoptosis	[37,38]
	GSK-3	Stabilized	Phosphorylation of APP	[39]
Betaherpesvirinae				
HCMV	β-catenin	Inhibited	β-catenin degradation, decrease in β-catenin transcriptional targets	[41]
	Axin	Stabilized	TNKS PARsylation activity inhibited resulting in β-catenin degradation	[50]
	ROR2	Activated	Repression of β-catenin TCF/LEF-1 transcriptional activity	[42]
	GSK-3	Stabilized	Stabilization of pAP and promotion of HCMV replication	[52,53]
Gammaherpesvirinae				
EBV	β-catenin	Stabilized	Accumulation of β-catenin in type III latency	[59]
	GSK-3	Inhibited	LMP2A activation of Akt inactivates GSK-3 resulting in β-catenin accumulation	[60,61]
	APC	Activated/inhibited (conflicting results)	LMP1 represses Siah-1 promoting β-catenin accumulation. LMP1 does not promote β-catenin stabilization	[63,64]
KSHV	β-catenin	Stabilized/inhibited (dependent on viral stage?)	Increased transcriptional activity, induction of viral latency/inhibition of LANA mediated transactivation of β-catenin	[66,76,77]
	GSK-3	Inhibited	LANA promotes nuclear accumulation of GSK-3	[67,70]

GSK-3: Glycogen synthase kinase 3; HCMV: Human cytomegalovirus; TNKS: Tankyrase 1 and 2 (PARP5a/b); TCF/LEF-1: T-cell factor/lymphoid enhancer-binding factor 1; pAP: Protein precursor; LMP2A: Latent membrane protein 2A; APC: Adenomatous polyposis coli; LMP1: Latent membrane protein 1; LANA: Latency-associated nuclear antigen; KSHV: Kaposi's sarcoma-associated herpesvirus.

induced activation of the Wnt/β-catenin is through the PI3K/Akt pathway, similar to what is seen in HSV and EBV. This conclusion was contrary to prior work suggesting that this effect may be mediated through COX2 activity; it was found that PI3K/Akt inhibition potently inhibited Wnt/β-catenin activity in endothelial cells and prevented formation of capillary endothelial tubes *in vitro*[77].

SPECULATION AND QUESTIONS

Despite numerous studies addressing the role of Wnt/β-catenin signaling in herpesviruses, there are still many questions to address. It seems at odds that the gammaherpesviruses would institute a program promoting the accumulation of β-catenin whilst the other family members inhibit the accumulation of β-catenin. The range and complexity of the Wnt/β-catenin pathway makes a simple answer unlikely; but factors such as stage of viral infection and cell type are obvious candidates. As we understand more about herpesviruses, it is conceivable that the herpesvirus family can change the regulation and function of such an important pathway at different times during the viral life cycle. Control over apoptosis, cytoskeletal rearrangement, migration and differentiation are all vital components of viral control over the host cell that would be required at different times post infection.

As mentioned previously, dysregulation of the Wnt/β-catenin pathway is tightly associated with numerous human cancers. In fact, most of the human herpesviruses can be thought of as oncomodulators, whether in a direct manner such as in the expression of oncogenic viral proteins in gammaherpesvirinae infection or through indirect generation of oncostimulatory microenvironments by virally induced inflammation or cellular metabolic shifts caused by alpha- and betaherpesvirinae infection. Why would an evolutionarily successful viral family induce cancer in its host? Ultimately, herpesviruses are successful because they coexist with their host. The development of cancer due to the persistence of a herpesvirus is likely an infrequent event that is complicated by others factors such as altered host cell metabolism and possibly the presence of other pathogens. For example, HCMV is now known to alter host cell metabolism during infection[78-81]. The changes are very similar to the Warburg-effect first identified in cancer cells.

Interestingly, a recent publication may bridge the different actions of viruses on the Wnt/β-catenin pathway during different stages of infection. Data from Marcato *et al*[82] suggests that the TCF/β-catenin complex is instrumental in mounting an effective antiviral response. They linked two observations, namely, that IFN-β is needed during the innate antiviral response and that murine models lacking IFN-β are susceptible to viral infections. In this paper, the authors show that inhibiting GSK-3 using LiCl increases IFN-β expression if β-catenin interacts with the IFN-β promoter by recruitment of TCF/β-catenin complexes to the promoter region. Using Rift Valley fever virus, a RNA virus belonging to the Bunyaviridae family, they showed pathogenicity is correlated to viral targeting of the β-catenin pathway.

Viral manipulation of Wnt/β-catenin signaling may be impeded using small molecules inhibitors that target the Wnt/β-catenin pathway. In fact, Chan *et al*[83] have shown results displaying the potential of this treatment. The authors used ICG-001, a small molecular Wnt modulator (CBP/β-catenin antagonist) to inhibit the growth of tumor spheres in a model of nasopharyngeal

carcinoma. This epithelial malignancy is associated with EBV latent infection. It is hypothesized that ICG-001 targets the cancer stem cells within the tumor reducing growth due to alterations in signaling cascades. To date, no studies have looked at the direct effects of small molecule inhibitors as antivirals, but targeting the Wnt pathway is being explored in many other diseases and should be examined in the context of herpesvirus infection (reviewed in[28]).

CONCLUSION

Human herpesviruses exploit the Wnt/β-catenin signaling pathway to ensure successful replication and survival in host cells (Table 1). The manipulation of such an important signaling cascade by herpesviruses should not be surprising as this pathway dictates the expression of many essential transcriptional pathways. The current literature provides an incomplete picture of why herpesviruses alter the Wnt/β-catenin pathway when they do. A deeper understanding of why herpesviruses induce changes in the Wnt/β-catenin pathway when they do, would provide vital information about the viral purpose of manipulating this pathway and how to interfere with this host manipulation controlled by the virus. As we understand more about virally induced aberrant Wnt/β-catenin we can develop better antivirals and possibly apply this knowledge to other human diseases associated with the Wnt/β-catenin pathway.

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REFERENCES

1 **Grinde B**. Herpesviruses: latency and reactivation - viral strategies and host response. *J Oral Microbiol* 2013; **5** [PMID: 24167660 DOI: 10.3402/jom.v5i0.22766]

2 **Evans CM**, Kudesia G, McKendrick M. Management of herpesvirus infections. *Int J Antimicrob Agents* 2013; **42**: 119-128 [PMID: 23820015 DOI: 10.1016/j.ijantimicag.2013.04.023]

3 **Logan CY**, Nusse R. The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* 2004; **20**: 781-810 [PMID: 15473860 DOI: 10.1146/annurev.cellbio.20.010403.113126]

4 **van Amerongen R**, Nusse R. Towards an integrated view of Wnt signaling in development. *Development* 2009; **136**: 3205-3214 [PMID: 19736321 DOI: 10.1242/dev.033910]

5 **Webster MR**, Weeraratna AT. A Wnt-er migration: the confusing role of β-catenin in melanoma metastasis. *Sci Signal* 2013; **6**: pe11 [PMID: 23532332 DOI: 10.1126/scisignal.2004114]

6 **Hoffmeyer K**, Raggioli A, Rudloff S, Anton R, Hierholzer A, Del Valle I, Hein K, Vogt R, Kemler R. Wnt/β-catenin signaling regulates telomerase in stem cells and cancer cells. *Science* 2012; **336**: 1549-1554 [PMID: 22723415 DOI: 10.1126/science.1218370]

7 **Alberici P**, Fodde R. The role of the APC tumor suppressor in chromosomal instability. *Genome Dyn* 2006; **1**: 149-170 [PMID: 18724059 DOI: 10.1159/000092506]

8 **Frisch SM**, Schaller M, Cieply B. Mechanisms that link the oncogenic epithelial-mesenchymal transition to suppression of anoikis. *J Cell Sci* 2013; **126**: 21-29 [PMID: 23516327 DOI: 10.1242/jcs.120907]

9 **Rubinfeld B**, Souza B, Albert I, Müller O, Chamberlain SH, Masiarz FR, Munemitsu S, Polakis P. Association of the APC gene product with beta-catenin. *Science* 1993; **262**: 1731-1734 [PMID: 8259518 DOI: 10.1126/science.8259518]

10 **Su LK**, Vogelstein B, Kinzler KW. Association of the APC tumor suppressor protein with catenins. *Science* 1993; **262**: 1734-1737 [PMID: 8259519 DOI: 10.1126/science.8259519]

11 **Porfiri E**, Rubinfeld B, Albert I, Hovanes K, Waterman M, Polakis P. Induction of a beta-catenin-LEF-1 complex by wnt-1 and transforming mutants of beta-catenin. *Oncogene* 1997; **15**: 2833-2839 [PMID: 9419974 DOI: 10.1038/sj.onc.1201462]

12 **Webster MR**, Kugel CH, Weeraratna AT. The Wnts of change: How Wnts regulate phenotype switching in melanoma. *Biochim Biophys Acta* 2015; **1856**: 244-251 [PMID: 26546268 DOI: 10.1016/j.bbcan.2015.10.002]

13 **Kinzler KW**, Nilbert MC, Su LK, Vogelstein B, Bryan TM, Levy DB, Smith KJ, Preisinger AC, Hedge P, McKechnie D. Identification of FAP locus genes from chromosome 5q21. *Science* 1991; **253**: 661-665 [PMID: 1651562 DOI: 10.1126/science.1651562]

14 **Kinzler KW**, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell* 1996; **87**: 159-170 [PMID: 8861899 DOI: 10.1016/S0092-8674(00)81333-1]

15 **Rubinfeld B**, Robbins P, El-Gamil M, Albert I, Porfiri E, Polakis P. Stabilization of beta-catenin by genetic defects in melanoma cell lines. *Science* 1997; **275**: 1790-1792 [PMID: 9065403 DOI: 10.1126/science.275.5307.1790]

16 **Chilosi M**, Poletti V, Zamò A, Lestani M, Montagna L, Piccoli P, Pedron S, Bertaso M, Scarpa A, Murer B, Cancellieri A, Maestro R, Semenzato G, Doglioni C. Aberrant Wnt/beta-catenin pathway activation in idiopathic pulmonary fibrosis. *Am J Pathol* 2003; **162**: 1495-1502 [PMID: 12707032 DOI: 10.1016/S0002-9440(10)64282-4]

17 **Dees C**, Distler JH. Canonical Wnt signalling as a key regulator of fibrogenesis - implications for targeted therapies? *Exp Dermatol* 2013; **22**: 710-713 [PMID: 24118232 DOI: 10.1111/exd.12255]

18 **Schinner S**. Wnt-signalling and the metabolic syndrome. *Horm Metab Res* 2009; **41**: 159-163 [PMID: 19214925 DOI: 10.1055/s-0028-1119408]

19 **Wang Y**, Nathans J. Tissue/planar cell polarity in vertebrates: new insights and new questions. *Development* 2007; **134**: 647-658 [PMID: 17259302 DOI: 10.1242/dev.02772]

20 **Veeman MT**, Axelrod JD, Moon RT. A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. *Dev Cell* 2003; **5**: 367-377 [PMID: 12967557 DOI: 10.1016/S1534-5807(03)00266-1]

21 **Kohn AD**, Moon RT. Wnt and calcium signaling: beta-catenin-independent pathways. *Cell Calcium* 2005; **38**: 439-446 [PMID: 16099039 DOI: 10.1016/j.ceca.2005.06.022]

22 **Li VS**, Ng SS, Boersema PJ, Low TY, Karthaus WR, Gerlach JP, Mohammed S, Heck AJ, Maurice MM, Mahmoudi T, Clevers H. Wnt signaling through inhibition of β-catenin degradation in an intact Axin1 complex. *Cell* 2012; **149**: 1245-1256 [PMID: 22682247 DOI: 10.1016/j.cell.2012.05.002]

23 **Valenta T**, Hausmann G, Basler K. The many faces and functions of β-catenin. *EMBO J* 2012; **31**: 2714-2736 [PMID: 22617422 DOI: 10.1038/emboj.2012.150]

24 **Tang Y**, Liu Z, Zhao L, Clemens TL, Cao X. Smad7 stabilizes beta-catenin binding to E-cadherin complex and promotes cell-cell adhesion. *J Biol Chem* 2008; **283**: 23956-23963 [PMID: 18593713 DOI: 10.1074/jbc.M800351200]

25 **Scholtysek C**, Katzenbeisser J, Fu H, Uderhardt S, Ipseiz N, Stoll C, Zaiss MM, Stock M, Donhauser L, Böhm C, Kleyer A, Hess A, Engelke K, David JP, Djouad F, Tuckermann JP, Desvergne B, Schett G, Krönke G. PPARβ/δ governs Wnt signaling and bone turnover. *Nat Med* 2013; **19**: 608-613 [PMID: 23542786 DOI: 10.1038/nm.3146]

26 **Clevers H**, Nusse R. Wnt/β-catenin signaling and disease. *Cell* 2012; **149**: 1192-1205 [PMID: 22682243 DOI: 10.1016/j.cell.2012.05.012]

27 **Cruciat CM**. Casein kinase 1 and Wnt/β-catenin signaling. *Curr Opin Cell Biol* 2014; **31**: 46-55 [PMID: 25200911 DOI: 10.1016/

j.ceb.2014.08.003]
28 **Kahn M**. Can we safely target the WNT pathway? *Nat Rev Drug Discov* 2014; **13**: 513-532 [PMID: 24981364 DOI: 10.1038/nrd4233]
29 **MacDonald BT**, Tamai K, He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell* 2009; **17**: 9-26 [PMID: 19619488 DOI: 10.1016/j.devcel.2009.06.016]
30 **McGeoch DJ**, Rixon FJ, Davison AJ. Topics in herpesvirus genomics and evolution. *Virus Res* 2006; **117**: 90-104 [PMID: 16490275 DOI: 10.1016/j.virusres.2006.01.002]
31 **Brown JC**, Newcomb WW. Herpesvirus capsid assembly: insights from structural analysis. *Curr Opin Virol* 2011; **1**: 142-149 [PMID: 21927635 DOI: 10.1016/j.coviro.2011.06.003]
32 **Knipe DM**, Howley PM. Fields virology. 6th ed. Philadelphia, PA: Wolters Kluwer/Lippincott Williams & Wilkins Health, 2013: 1
33 Human herpesviruses: Biology, therapy, and immunoprophylaxis. In: Arvin A, Campadelli-Fiume G, Mocarski E, Moore PS, Roizman B, Whitley R, Yamanishi K, editors. Cambridge: Cambridge University Press, 2007
34 **Monini P**, Colombini S, Stürzl M, Goletti D, Cafaro A, Sgadari C, Buttò S, Franco M, Leone P, Fais S, Leone P, Melucci-Vigo G, Chiozzini C, Carlini F, Ascherl G, Cornali E, Zietz C, Ramazzotti E, Ensoli F, Andreoni M, Pezzotti P, Rezza G, Yarchoan R, Gallo RC, Ensoli B. Reactivation and persistence of human herpesvirus-8 infection in B cells and monocytes by Th-1 cytokines increased in Kaposi's sarcoma. *Blood* 1999; **93**: 4044-4058 [PMID: 10361101]
35 **Gantner BN**, Jin H, Qian F, Hay N, He B, Ye RD. The Akt1 isoform is required for optimal IFN-β transcription through direct phosphorylation of β-catenin. *J Immunol* 2012; **189**: 3104-3111 [PMID: 22904301 DOI: 10.4049/jimmunol.1201669]
36 **Hay N**. Interplay between FOXO, TOR, and Akt. *Biochim Biophys Acta* 2011; **1813**: 1965-1970 [PMID: 21440577 DOI: 10.1016/j.bbamcr.2011.03.013]
37 **Choi EJ**, Kim S, Jho EH, Song KJ, Kee SH. Axin expression enhances herpes simplex virus type 1 replication by inhibiting virus-mediated cell death in L929 cells. *J Gen Virol* 2013; **94**: 1636-1646 [PMID: 23535572 DOI: 10.1099/vir.0.051540-0]
38 **Choi EJ**, Kee SH. Axin expression delays herpes simplex virus-induced autophagy and enhances viral replication in L929 cells. *Microbiol Immunol* 2014; **58**: 103-111 [PMID: 24329555 DOI: 10.1111/1348-0421.12123]
39 **Piacentini R**, Li Puma DD, Ripoli C, Marcocci ME, De Chiara G, Garaci E, Palamara AT, Grassi C. Herpes Simplex Virus type-1 infection induces synaptic dysfunction in cultured cortical neurons via GSK-3 activation and intraneuronal amyloid-β protein accumulation. *Sci Rep* 2015; **5**: 15444 [PMID: 26487282 DOI: 10.1038/srep15444]
40 **Markus A**, Waldman Ben-Asher H, Kinchington PR, Goldstein RS. Cellular transcriptome analysis reveals differential expression of pro- and antiapoptosis genes by varicella-zoster virus-infected neurons and fibroblasts. *J Virol* 2014; **88**: 7674-7677 [PMID: 24741086 DOI: 10.1128/JVI.00500-14]
41 **Angelova M**, Zwezdaryk K, Ferris M, Shan B, Morris CA, Sullivan DE. Human cytomegalovirus infection dysregulates the canonical Wnt/β-catenin signaling pathway. *PLoS Pathog* 2012; **8**: e1002959 [PMID: 23071438 DOI: 10.1371/journal.ppat.1002959]
42 **van Zuylen WJ**, Ford CE, Wong DD, Rawlinson WD. Human Cytomegalovirus Modulates Expression of Noncanonical Wnt Receptor ROR2 To Alter Trophoblast Migration. *J Virol* 2015; **90**: 1108-1115 [PMID: 26559837 DOI: 10.1128/JVI.02588-15]
43 **Tabata T**, Petitt M, Zydek M, Fang-Hoover J, Larocque N, Tsuge M, Gormley M, Kauvar LM, Pereira L. Human cytomegalovirus infection interferes with the maintenance and differentiation of trophoblast progenitor cells of the human placenta. *J Virol* 2015; **89**: 5134-5147 [PMID: 25741001 DOI: 10.1128/JVI.03674-14]
44 **Pollheimer J**, Loregger T, Sonderegger S, Saleh L, Bauer S, Bilban M, Czerwenka K, Husslein P, Knöfler M. Activation of the canonical wingless/T-cell factor signaling pathway promotes invasive differentiation of human trophoblast. *Am J Pathol* 2006; **168**: 1134-1147 [PMID: 16565489 DOI: 10.2353/ajpath.2006.050686]
45 **Sonderegger S**, Haslinger P, Sabri A, Leisser C, Otten JV, Fiala C, Knöfler M. Wingless (Wnt)-3A induces trophoblast migration and matrix metalloproteinase-2 secretion through canonical Wnt signaling and protein kinase B/AKT activation. *Endocrinology* 2010; **151**: 211-220 [PMID: 19887570 DOI: 10.1210/en.2009-0557]
46 **Knöfler M**, Pollheimer J. Human placental trophoblast invasion and differentiation: a particular focus on Wnt signaling. *Front Genet* 2013; **4**: 190 [PMID: 24133501 DOI: 10.3389/fgene.2013.00190]
47 **Ueland T**, Rollag H, Hartmann A, Jardine AG, Humar A, Michelsen AE, Bignamini AA, Åsberg A, Aukrust P. Secreted Wnt antagonists during eradication of cytomegalovirus infection in solid organ transplant recipients. *Am J Transplant* 2014; **14**: 210-215 [PMID: 24224707 DOI: 10.1111/ajt.12506]
48 **Langemeijer EV**, Slinger E, de Munnik S, Schreiber A, Maussang D, Vischer H, Verkaar F, Leurs R, Siderius M, Smit MJ. Constitutive β-catenin signaling by the viral chemokine receptor US28. *PLoS One* 2012; **7**: e48935 [PMID: 23145028 DOI: 10.1371/journal.pone.0048935]
49 **Tabata T**, McDonagh S, Kawakatsu H, Pereira L. Cytotrophoblasts infected with a pathogenic human cytomegalovirus strain dysregulate cell-matrix and cell-cell adhesion molecules: a quantitative analysis. *Placenta* 2007; **28**: 527-537 [PMID: 16822542 DOI: 10.1016/j.placenta.2006.05.006]
50 **Roy S**, Liu F, Arav-Boger R. Human Cytomegalovirus Inhibits the PARsylation Activity of Tankyrase--A Potential Strategy for Suppression of the Wnt Pathway. *Viruses* 2015; **8**: pii: E8 [PMID: 26729153 DOI: 10.3390/v8010008]
51 **Kapoor A**, He R, Venkatadri R, Forman M, Arav-Boger R. Wnt modulating agents inhibit human cytomegalovirus replication. *Antimicrob Agents Chemother* 2013; **57**: 2761-2767 [PMID: 23571549 DOI: 10.1128/AAC.00029-13]
52 **Casaday RJ**, Bailey JR, Kalb SR, Brignole EJ, Loveland AN, Cotter RJ, Gibson W. Assembly protein precursor (pUL80.5 homolog) of simian cytomegalovirus is phosphorylated at a glycogen synthase kinase 3 site and its downstream "priming" site: phosphorylation affects interactions of protein with itself and with major capsid protein. *J Virol* 2004; **78**: 13501-13511 [PMID: 15564461 DOI: 10.1128/JVI.78.24.13501-13511.2004]
53 **Oberstein A**, Perlman DH, Shenk T, Terry LJ. Human cytomegalovirus pUL97 kinase induces global changes in the infected cell phosphoproteome. *Proteomics* 2015; **15**: 2006-2022 [PMID: 25867546 DOI: 10.1002/pmic.201400607]
54 **Mohammad AA**, Rahbar A, Lui WO, Davoudi B, Catrina A, Stragliotto G, Mellbin L, Hamsten A, Rydén L, Yaiw KC, Söderberg-Nauclér C. Detection of circulating hcmv-miR-UL112-3p in patients with glioblastoma, rheumatoid arthritis, diabetes mellitus and healthy controls. *PLoS One* 2014; **9**: e113740 [PMID: 25462570 DOI: 10.1371/journal.pone.0113740]
55 **Simanek AM**, Dowd JB, Pawelec G, Melzer D, Dutta A, Aiello AE. Seropositivity to cytomegalovirus, inflammation, all-cause and cardiovascular disease-related mortality in the United States. *PLoS One* 2011; **6**: e16103 [PMID: 21379581 DOI: 10.1371/journal.pone.0016103]
56 **Herbein G**, Kumar A. The oncogenic potential of human cytomegalovirus and breast cancer. *Front Oncol* 2014; **4**: 230 [PMID: 25202681 DOI: 10.3389/fonc.2014.00230]
57 **Chen HP**, Chan YJ. The oncomodulatory role of human cytomegalovirus in colorectal cancer: implications for clinical trials. *Front Oncol* 2014; **4**: 314 [PMID: 25452935 DOI: 10.3389/fonc.2014.00314]
58 **Chang Y**, Cesarman E, Pessin MS, Lee F, Culpepper J, Knowles DM, Moore PS. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* 1994; **266**: 1865-1869 [PMID: 7997879 DOI: 10.1126/science.7997879]
59 **Shackelford J**, Maier C, Pagano JS. Epstein-Barr virus activates beta-catenin in type III latently infected B lymphocyte lines: association with deubiquitinating enzymes. *Proc Natl Acad Sci USA* 2003; **100**: 15572-15576 [PMID: 14663138 DOI: 10.1073/pnas.2636947100]
60 **Morrison JA**, Klingelhutz AJ, Raab-Traub N. Epstein-Barr virus

latent membrane protein 2A activates beta-catenin signaling in epithelial cells. *J Virol* 2003; **77**: 12276-12284 [PMID: 14581564 DOI: 10.1128/JVI.77.22.12276-12284.2003]

61 **Morrison JA**, Raab-Traub N. Roles of the ITAM and PY motifs of Epstein-Barr virus latent membrane protein 2A in the inhibition of epithelial cell differentiation and activation of {beta}-catenin signaling. *J Virol* 2005; **79**: 2375-2382 [PMID: 15681438 DOI: 10.1128/JVI.79.4.2375-2382.2005]

62 **Everly DN**, Kusano S, Raab-Traub N. Accumulation of cytoplasmic beta-catenin and nuclear glycogen synthase kinase 3beta in Epstein-Barr virus-infected cells. *J Virol* 2004; **78**: 11648-11655 [PMID: 15479806 DOI: 10.1128/JVI.78.21.11648-11655.2004]

63 **Jang KL**, Shackelford J, Seo SY, Pagano JS. Up-regulation of beta-catenin by a viral oncogene correlates with inhibition of the seven in absentia homolog 1 in B lymphoma cells. *Proc Natl Acad Sci USA* 2005; **102**: 18431-18436 [PMID: 16344472 DOI: 10.1073/pnas.0504054102]

64 **Webb N**, Connolly G, Tellam J, Yap AS, Khanna R. Epstein-Barr virus associated modulation of Wnt pathway is not dependent on latent membrane protein-1. *PLoS One* 2008; **3**: e3254 [PMID: 18806872 DOI: 10.1371/journal.pone.0003254]

65 **Malizia AP**, Lacey N, Walls D, Egan JJ, Doran PP. CUX1/Wnt signaling regulates epithelial mesenchymal transition in EBV infected epithelial cells. *Exp Cell Res* 2009; **315**: 1819-1831 [PMID: 19361498 DOI: 10.1016/j.yexcr.2009.04.001]

66 **Fujimuro M**, Wu FY, ApRhys C, Kajumbula H, Young DB, Hayward GS, Hayward SD. A novel viral mechanism for dysregulation of beta-catenin in Kaposi's sarcoma-associated herpesvirus latency. *Nat Med* 2003; **9**: 300-306 [PMID: 12592400 DOI: 10.1038/nm829]

67 **Fujimuro M**, Hayward SD. The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus manipulates the activity of glycogen synthase kinase-3beta. *J Virol* 2003; **77**: 8019-8030 [PMID: 12829841 DOI: 10.1128/JVI.77.14.8019-8030.2003]

68 **Ballestas ME**, Chatis PA, Kaye KM. Efficient persistence of extrachromosomal KSHV DNA mediated by latency-associated nuclear antigen. *Science* 1999; **284**: 641-644 [PMID: 10213686 DOI: 10.1126/science.284.5414.641]

69 **Cotter MA**, Robertson ES. The latency-associated nuclear antigen tethers the Kaposi's sarcoma-associated herpesvirus genome to host chromosomes in body cavity-based lymphoma cells. *Virology* 1999; **264**: 254-264 [PMID: 10562490 DOI: 10.1006/viro.1999.9999]

70 **Fujimuro M**, Liu J, Zhu J, Yokosawa H, Hayward SD. Regulation of the interaction between glycogen synthase kinase 3 and the Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen. *J Virol* 2005; **79**: 10429-10441 [PMID: 16051835 DOI: 10.1128/JVI.79.16.10429-10441.2005]

71 **Fujimuro M**, Hayward SD. Manipulation of glycogen-synthase kinase-3 activity in KSHV-associated cancers. *J Mol Med* (Berl) 2004; **82**: 223-231 [PMID: 14991150 DOI: 10.1007/s00109-003-0519-7]

72 **Hayward SD**, Liu J, Fujimuro M. Notch and Wnt signaling: mimicry and manipulation by gamma herpesviruses. *Sci STKE* 2006; **2006**: re4 [PMID: 16705130 DOI: 10.1126/stke.3352006re4]

73 **Price MA**. CKI, there's more than one: casein kinase I family members in Wnt and Hedgehog signaling. *Genes Dev* 2006; **20**: 399-410 [PMID: 16481469 DOI: 10.1101/gad.1394306]

74 **An FQ**, Compitello N, Horwitz E, Sramkoski M, Knudsen ES, Renne R. The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus modulates cellular gene expression and protects lymphoid cells from p16 INK4A-induced cell cycle arrest. *J Biol Chem* 2005; **280**: 3862-3874 [PMID: 15525642 DOI: 10.1074/jbc.M407435200]

75 **Kusano S**, Eizuru Y. Human I-mfa domain proteins specifically interact with KSHV LANA and affect its regulation of Wnt signaling-dependent transcription. *Biochem Biophys Res Commun* 2010; **396**: 608-613 [PMID: 20417616 DOI: 10.1016/j.bbrc.2010.04.111]

76 **Lee HR**, Mitra J, Lee S, Gao SJ, Oh TK, Kim MH, Ha T, Jung JU. Kaposi's Sarcoma-Associated Herpesvirus Viral Interferon Regulatory Factor 4 (vIRF4) Perturbs the G1-S Cell Cycle Progression via Deregulation of the cyclin D1 Gene. *J Virol* 2015; **90**: 1139-1143 [PMID: 26491150 DOI: 10.1128/JVI.01897-15]

77 **Angelova M**, Ferris M, Swan KF, McFerrin HE, Pridjian G, Morris CA, Sullivan DE. Kaposi's sarcoma-associated herpesvirus G-protein coupled receptor activates the canonical Wnt/β-catenin signaling pathway. *Virol J* 2014; **11**: 218 [PMID: 25514828 DOI: 10.1186/s12985-014-0218-8]

78 **Munger J**, Bajad SU, Coller HA, Shenk T, Rabinowitz JD. Dynamics of the cellular metabolome during human cytomegalovirus infection. *PLoS Pathog* 2006; **2**: e132 [PMID: 17173481 DOI: 10.1371/journal.ppat.0020132]

79 **Munger J**, Bennett BD, Parikh A, Feng XJ, McArdle J, Rabitz HA, Shenk T, Rabinowitz JD. Systems-level metabolic flux profiling identifies fatty acid synthesis as a target for antiviral therapy. *Nat Biotechnol* 2008; **26**: 1179-1186 [PMID: 18820684 DOI: 10.1038/nbt.1500]

80 **Chambers JW**, Maguire TG, Alwine JC. Glutamine metabolism is essential for human cytomegalovirus infection. *J Virol* 2010; **84**: 1867-1873 [PMID: 19939921 DOI: 10.1128/JVI.02123-09]

81 **Yu Y**, Maguire TG, Alwine JC. ChREBP, a glucose-responsive transcriptional factor, enhances glucose metabolism to support biosynthesis in human cytomegalovirus-infected cells. *Proc Natl Acad Sci USA* 2014; **111**: 1951-1956 [PMID: 24449882 DOI: 10.1073/pnas.1310779111]

82 **Marcato V**, Luron L, Laqueuvre LM, Simon D, Mansuroglu Z, Flamand M, Panthier JJ, Souès S, Massaad C, Bonnefoy E. β-Catenin Upregulates the Constitutive and Virus-Induced Transcriptional Capacity of the Interferon Beta Promoter through T-Cell Factor Binding Sites. *Mol Cell Biol* 2016; **36**: 13-29 [PMID: 26459757 DOI: 10.1128/MCB.00641-15]

83 **Chan KC**, Chan LS, Ip JC, Lo C, Yip TT, Ngan RK, Wong RN, Lo KW, Ng WT, Lee AW, Tsao GS, Kahn M, Lung ML, Mak NK. Therapeutic targeting of CBP/β-catenin signaling reduces cancer stem-like population and synergistically suppresses growth of EBV-positive nasopharyngeal carcinoma cells with cisplatin. *Sci Rep* 2015; **5**: 9979 [PMID: 25897700 DOI: 10.1038/srep09979]

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ORIGINAL ARTICLE

Basic Study

Antiretroviral naive and treated patients: Discrepancies of B cell subsets during the natural course of human immunodeficiency virus type 1 infection

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Abstract

AIM

To evaluate alterations of memory B cell subpopulations during a 48-wk period in human immunodeficiency virus type 1 (HIV-1) patients.

METHODS

Forty-one antiretroviral naïve and 41 treated HIV-1 patients matched for age and duration of HIV infection were recruited. All clinical, epidemiological and laboratory data were recorded or measured. The different B cell subsets were characterized according to their

surface markers: Total B cells ($CD19^+$), memory B cells ($CD19^+CD27^+$, BMCs), resting BMCs ($CD19^+CD27^+CD21^{high}$, RM), exhausted BMCs ($CD19^+CD21^{low}CD27^-$, EM), IgM memory B ($CD19^+CD27^+IgM^{high}$), isotype-switched BMCs ($CD19^+CD27^+IgM^-$, ITS) and activated BMCs ($CD19^+CD21^{low+}CD27^+$, AM) at baseline on week 4 and week 48.

RESULTS

Mean counts of BMCs were higher in treated patients. There was a marginal upward trend of IgM memory B cell proportions which differed significantly in the treated group (overall trend, P = 0.004). ITS BMC increased over time significantly in all patients. Naive patients had of lower levels of EM B cells compared to treated, with a downward trend, irrespectively of highly active antiretroviral therapy (HAART) intake. Severe impairment of EM B cells was recorded to both treated (P = 0.024) and naive (P = 0.023) and patients. Higher proportions of RM cells were noted in HAART group, which differed significantly on week 4th (P = 0.017) and 48th (P = 0.03). Higher levels of AM were preserved in HAART naive group during the whole study period (week 4: P = 0.018 and 48: P = 0.035). HIV-RNA viremia strongly correlated with AM B cells (r = 0.54, P = 0.01) and moderately with RM cells (r = -0.45, P = 0.026) at baseline.

CONCLUSION

HIV disrupts memory B cell subpopulations leading to impaired immunologic memory over time. BMC, RM, EM and ITS BMC were higher in patients under HAART. Activated BMCs (AM) were higher in patients without HAART. Viremia correlated with AM and RM. Significant depletion was recorded in EM B cells irrespectively of HAART intake. Perturbations in BMC-populations are not fully restored by antiretrovirals.

Key words: B cell subpopulations; Time-trend; Memory cells; Human immunodeficiency virus infection; Highly active antiretroviral therapy

Core tip: During the progress of human immunodeficiency virus (HIV) infection and viral replication functional irritations of memory B-cell (BMC) compartment occur. Depletion of BMCs is one hallmark of deregulation in HIV-1 infection. Diminished levels of IgM^+ BMCs are also noted. Additionally, resting BMCs are severely impaired and defective B-cell subsets, like exhausted and activated BMCs circulate in peripheral blood. Significant fluctuations of these B cells' frequencies are recorded over time and antiretroviral therapy may play a role on this observation. Assessing these populations could potentially lead to improvement in assessing vaccine responses and tracing vulnerable patients to certain infections.

Tsachouridou O, Skoura L, Zebekakis P, Margariti A, Georgiou A, Bougiouklis D, Pilalas D, Galanos A, Daniilidis M, Metallidis S. Antiretroviral naive and treated patients: Discrepancies of B cell subsets during the natural course of human immunodeficiency virus type 1 infection. *World J Virol* 2016; 5(4): 155-160 Available from: URL: http://www.wjgnet.com/2220-3249/full/v5/i4/155.htm DOI: http://dx.doi.org/10.5501/wjv.v5.i4.155

INTRODUCTION

During the chronic human immunodeficiency virus (HIV) infection and viral proliferation functional irritations of B-cells take place like impairment of isotype switching, polyclonal activation, divergences in the frequencies of circulating B cell-populations and diminished immune reactions to immunization[1-3].

The deprivation of memory B cells ($CD19^+CD21^+$-$CD27^+$, BMC) reflects some dysfunction in HIV patients[3,4]. Reduced IgM^+ BMCs ($CD19^+CD27^+IgM^{high}$) is also observed[2,3]. During the course of the infection, resting BMCs ($CD19^+CD21^{high}CD27^+$, RM) are severely impaired[5]. Furthermore, dysfunctional B-cell subsets, accounting for activated BMCs ($CD19^+CD21^{low}CD27^+$, AM) and exhausted BMCs ($CD19^+CD21^{low}CD27^-$, EM), rise in these patients, while they appear at very low frequencies in healthy subjects[4].

The phenotype of B cells that serves the immune response to several antigens has been ambivalent[6-9]. The conflict primarily is targeted on the surface markers of the B cells that respond to the antigens[3,6,7,10-12]. It is currently challenged that IgM BMCs are merely in charge of antibody production; since other memory subsets such as isotype switched B cells ($CD19^+CD27^+IgM^-$) also produce anti-polysaccharide antibodies *in vitro*[2].

Highly active antiretroviral therapy (HAART) reduces polyclonal B-cell activation but has only a constricted effect on the remediation of B-cells and remains to be elucidated which certain perturbations can be repaired[3,5]. The loss of memory is confirmed by in the reduction of antigen specific BMCs post vaccine administration, which is not reconstituted by HAART[13,14]. RM cells are preserved if HAART is initiated immediately in HIV confirmation of infection[14].

Aim of this study was to record and evaluate alterations of BMC subpopulations during a 48-wk period in HIV-1 patients. Moreover, we prospectively studied the impact of HAART on these cell populations seeking for significant changes.

MATERIALS AND METHODS

This is a longitudinal study including 82 HIV patients matched for age and duration of HIV infection, 41 of whom were antiretroviral naive and 41 were under HAART, with successful viral suppression (HIV-1 viral load < 34 copies/mL). All rest data, including epidemiological (age, gender, HIV-1 transmission route, co morbidities) and laboratory results, HIV-1 viral load, current CD4 T-cell count, nadir CD4 cell count were

Table 1 Patients' characteristics at enrollment

	HAART naïve patients (n = 41)	Treated patients (n = 41)	P value
Age (yr)	31.76 ± 7.16	34.15 ± 6.17	0.168
Gender (male/female), n (%)	41 (100.0)/0 (0.0)	36 (87.8)/ 4 (12.2)	0.065
Years on HIV infection	3.27 ± 2.78	3.6 = 98 ± 4.41	0.415
Nadir CD4 cell count	573.6 ± 223.4	326 ± 187.3	0.0005
ART duration in months	NA	34.4 ± 14.18	NA
HCV infection, n (%)	2 (4.8)	3 (7.3)	0.345
HBV infection, n (%)	6 (14.6)	4 (9.7)	0.167

All quantitative data are presented as mean ± SD, median (IQR). HAART: Highly active antiretroviral therapy; VL: HIV RNA viral load; HIV: Human immunodeficiency virus; ART: Antiretroviral therapy; NA: Not applicable; HCV: Hepatitis C virus; HBV: Hepatitis B virus.

recorded.

The Aristotle's University Ethical Committee approved the protocol and a written informed consent was obtained from all participants. All study individuals were asked to give blood sample on day 0 and on week 4 and 48.

Mouse anti-human fluorochrome-conjugated monoclonal antibodies of Immunostep Company® were used: CD19-PerCP, CD27-PE, IgM-FITC, IgD-FITC and CD21-FITC to count total B cells and BMC subsets combined properly. One hundred microliter of blood samples after adding 10 μL of the above combined monoclonal antibodies were incubated in the dark for ten minutes. In turn, red blood cells were thawed upon ingestion of 2 mL of Lysis Buffer (BD Biosciences, San Jose, CA) and incubated for another twenty minutes in room temperature. B cells were assessed pre vaccination. The different B cell subsets were characterized as follows: Total B cells ($CD19^+$), BMCs ($CD19^+CD27^+$), EM ($CD19^+CD21^{low}CD27^-$), IgM memory B ($CD19^+CD27^+IgM^{high}$), RM ($CD19^+CD27^+CD21$high), IgM memory B ($CD19^+CD27^+IgM^{high}$), AM ($CD19^+$-$CD21^{low+}CD27^+$) and ITS ($CD19^+CD27^+IgM^-$) at baseline and on weeks four and forty eight. Results were expressed as B cell absolute counts or as a percentage of the total B cell population.

Sample processing and result extraction was performed in the XL Epics cytometer (Beckman Coulter™ Company, Florida, United States). The input capture, cell staining and flow cytometry were performed immediately in a blinded manner.

Statistical analysis

The comparison of variables at each time point was performed using the independent samples t test or the Mann-Whitney test in case of violation of normality. To indicate the trend in the one year period, the median percentage changes after 4 wk and 48 wk respectively were calculated. All tests are two-sided, a P-value of < 0.05 was used to denote statistical significance. All analyses were carried out using the statistical package SPSS vr 16.00 (Statistical Package for the Social Sciences, SPSS Inc., Chicago, Ill., United States).

RESULTS

The demographics, clinical and rest data of eighty two HIV individuals are illustrated in Table 1.

In order to confirm whether the percentages of B-cell subsets were altered among treated and antiretroviral naive HIV-1 individuals, the percentages of memory B, activated memory, resting memory, exhausted memory as well as isotype-switched and total B-cells were assessed. Significant differences were observed between the groups in relation to B cell subsets.

Mean counts of BMCs ($CD19^+CD27^+$) were higher in treated patients throughout the 48 wk (P = 0.987, NS), with a gradual declining trend by the end of the 48th week. Mean fraction of IgM memory B ($CD19^+CD27^+IgM^{high}$) cell-population found higher in the treated group at baseline. There was a marginal upward trend of the proportions which differed significantly in the treated group (overall trend, P = 0.004) (Figure 1). Isotype-switched BMC ($CD19^+CD27^+IgM^-$) were slightly elevated in patients without HAART compared to the other group. The time trend variation was equivalent in both groups, irrelevantly of HAART intake (P = 0.808). ITS B cell compartment raised significantly in all patients, concerning baseline levels (overall significance, P = 0.0005) (Figure 1).

HAART patients preserved higher proportions of EM B cells ($CD19^+CD21^{low}CD27^-$) compared those without treatment, with a downward trend along with the progression of the disease, irrespectively of HAART intake. These changes were not significant among groups (overall significance, P = 0.876). Significant depletion of EM B cells was recorded to both ART-naive (P = 0.023) and rest individuals (P = 0.024) (Figure 1). The fraction of RM cells ($CD19^+CD21^{high+}CD27^+$) in patients under HAART were higher and significantly different on week 4th (P = 0.017) and 48th (P = 0.03). The fluctuation over time of RM was nearly the same though, in both groups (P = 0.201) with treated patients having a significant overall increase (P = 0.003). Patients HAART-naive maintained higher levels of AM ($CD19^+CD21^{low+}CD27^+$) during the whole study period, with the downward trend being significant in the treated group (P = 0.004) (Figure 1).

HIV-RNA viremia strongly correlated with AM B cells (r = 0.54, P = 0.01) and moderately with RM B cells (r = -0.45, P = 0.026) at baseline, supporting the impact of viral replication on these subsets (data not shown).

DISCUSSION

HIV infection impels to a broad amplitude of B cell defects, like cell switching, depleted numbers of B cells, production of uncommon B cell populations and disfunctional immune responses even in patients under HAART[3,6,7]. Furthermore, it is generally accepted to augments the risk of several infections. Very scarce and

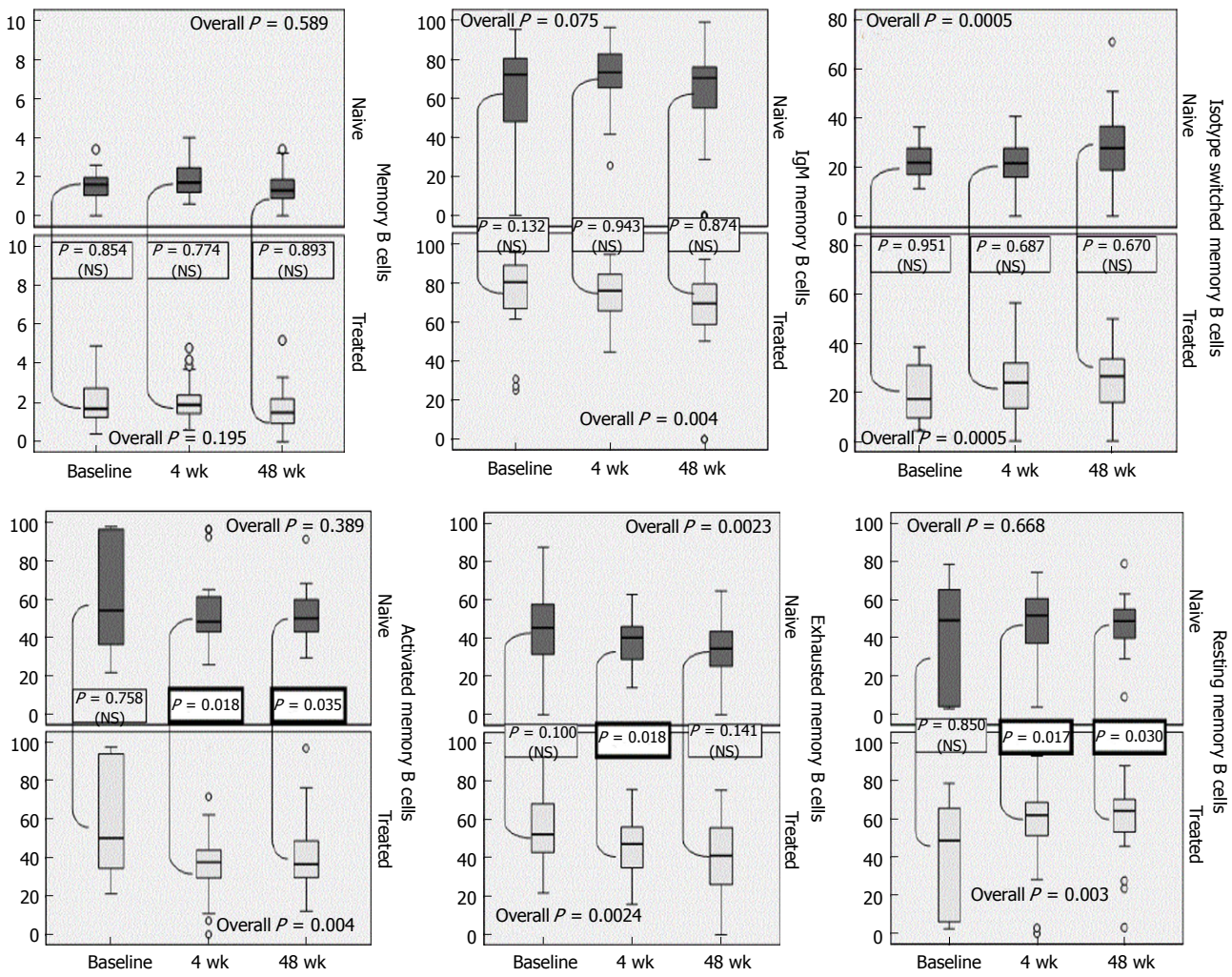

Figure 1 Time trend alterations of memory B cells.

conflicting data are currently available illustrating the significance of B cell subsets that mediate satisfactory and protective immune responses[6,7].

Our study promotes the scouting and assessment of specific BMC subpopulations interfered in humoral responses, confirming few other authors claiming that, not solely ITS and IgM BMC, but also AM and RM, might contribute to impaired protection against certain bacteria[7-12,14]. Recent studies focus on B cell memory and immunological response post immunizations, though most constitute solely cross-sectional studies[8-12,14].

BMCs were increased in patients on HAART compared to naive on an annual basis, as confirmed in other studies as well[2,15]. We showed rise of BMCs in both groups, which depleted throughout time in a similar pattern, that is in line with previous studies[7].

Interestingly, slight rise in IgM BMC was confirmed in all patients. Patients on treatment preserved high frequencies of them, confirming authors suggesting that HAART preserves the levels of this specific subset[15]. Both treated and naive patients maintained their IgM BMC over time, which is controversial in literature[15].

EM B cells are believed to be increased in naive patients[2,6]. Although in our study we did not confirmed the former observation, gradual decrease has been recorded irrespectively of HAART. Patients under HAART had decreased AM cells compared to those without treatment, explaining the effect of HAART which restricts their expansion during the progress of HIV infection[2]. We additionally confirmed that AM B cells are preserved in continuous viral replication[13]. Even though effective HAART is regarded to have no impact on RM, in our study maintenance of high levels especially in the treated group, implies that some restoration may be feasible upon HAART initiation, regardless being not during primary infection[5,14].

Studies have shown that isotype switched B cells are not affected in healthy individuals[7], but these are dramatically impaired in HIV infection irrespectively of HAART[15]. Despite few studies that confirmed high frequencies of ITS B cells in patients under HAART[2,15], our study confirmed more recent authors[15] showing no effect of HAART, which underlies the need for further investigation on this specific memory subset.

HAART introduction lead to further investigation on these populations, concluding that most divergences are reversible, implying that viremia has a causal relationship. Viremia has been associated with the

elevated frequency of AM cells[16]. However, the impact of HIV viremia has not been fully explained, apart from in limited studies[14]. Our study in lineage with other authors has shown that viremia was linked to certain B cell populations[14].

In conclusion, the data of our study points out that significant divergences occur in specific BMC populations in HIV patients. Natural course of HIV infection has an immunological impact on distinct B cells, sparking modifications on their absolute numbers and functions in the peripheral blood of HIV adults. Furthermore, HAART administration affects subsets like RM and AM which are significant in secondary immune responses, while has controversial implication in other BMC-compartments. We propose that evaluation of BMC might implicate in immunization and have clinical utility in forecasting all susceptible HIV adults to bacterial and other viral infections.

The significance of the paper lies to the fact that HAART prompt initiation may alter few of the disturbances that HIV infection itself promotes. Similar findings for the significance of immediate initiation of antivirals have been published recently, which insist that HAART is necessary to be started once the diagnosis of HIV infections has been confirmed[17].

Additionally, concerning the HIV vaccine development design, new scientific trends lean towards the role of B cells in HIV pathogenesis and their possible use to design and develop a proper vaccine for preventing HIV infections. Multiple studies try to assess and isolate the responsible B cell subsets that interfere to the pathogenesis of HIV infection and will lead to the vaccine development[18,19].

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COMMENTS

Background

Human immunodeficiency virus (HIV) causes several phenotypic and functional perturbations on B lymphocytes, like hyperactivation leading to hypergammaglobulinemia. Simultaneously, B cells also display hyporesponsiveness to vaccines. Memory B cell (BMC) react and secrete antibodies specific to the antigen faced, with improved pertinence when meeting the same antigen twice, offering protection against several infections. Highly active antiretroviral therapy (HAART) partially restores B cell perturbation, especially when initiation is prompt. Moreover, antivirals are cannot retrieve humoral response in HIV adults, and re-immunization might be mandatory. Optimizing vaccine strategy may improve BMC responses to immunizations. The role of some recently described BMC subsets is not fully understood and remains to be elucidated. Health care providers should consider prompt HAART initiation in order to restrict HIV-associated BMC impairment.

Research frontiers

Previous studies have assessed the role of early antiretroviral therapy (ART) administration showing that some populations may be benefited and protected from functional and phenotypic perturbations, with conflicting results.

Innovations and breakthroughs

This study aimed to assess over time the fluctuation of significant BMC subsets for humoral responses in HIV patients and not in single time slot. The study revealed certain time-trend differences among antiretroviral naive and treated patients.

Applications

Literature is still ambiguous concerning the precise role of certain BMC populations and the significance of HAART in restoring or preventing some disturbances on them. ART intake affects subsets like resting BMC (RM) and activated BMC (AM) which are significant in secondary immune responses, while has controversial implication in other BMC. Evaluation of BMCs might intervene in immunizations and have clinical utility in pointing out the susceptible HIV adults to bacterial and other infections.

Terminology

$CD27^+$ BMC comprise of $CD21^+$ cells (RM) and $CD21^-$ cells (AM). RM are depleted while AM rise during the natural course of HIV infection. The classical $CD27^+$ BMC are classified as ITS and un-switched subpopulations, while the isotype-switched BMC subset represent BMC that have switched their immunoglobulin from IgM and IgD to other classes. TLM ($CD19^+CD10^-$ $CD27^-$ $CD21^{low}$), rise in HIV infected patients.

Peer-review

The article is well prepared and makes a pleasant and useful reading for those in the field.

REFERENCES

1 **Shen X**, Tomaras GD. Alterations of the B-cell response by HIV-1 replication. *Curr HIV/AIDS Rep* 2011; **8**: 23-30 [PMID: 21161615 DOI: 10.1007/s11904-010-0064-2]

2 **Moir S**, Buckner CM, Ho J, Wang W, Chen J, Waldner AJ, Posada JG, Kardava L, O'Shea MA, Kottilil S, Chun TW, Proschan MA, Fauci AS. B cells in early and chronic HIV infection: evidence for preservation of immune function associated with early initiation of antiretroviral therapy. *Blood* 2010; **116**: 5571-5579 [PMID: 20837780 DOI: 10.1182/blood-2010-05-285528]

3 **Hu Z**, Luo Z, Wan Z, Wu H, Li W, Zhang T, Jiang W. HIV-associated memory B cell perturbations. *Vaccine* 2015; **33**: 2524-2529 [PMID: 25887082 DOI: 10.1016/j.vaccine.2015.04.008]

4 **Moir S**, Fauci AS. Pathogenic mechanisms of B-lymphocyte dysfunction in HIV disease. *J Allergy Clin Immunol* 2008; **122**: 12-19; quiz 20-21 [PMID: 18547629 DOI: 10.1016/j.jaci.2008.04.034]

5 **Moir S**, Malaspina A, Ho J, Wang W, Dipoto AC, O'Shea MA, Roby G, Mican JM, Kottilil S, Chun TW, Proschan MA, Fauci AS. Normalization of B cell counts and subpopulations after antiretroviral therapy in chronic HIV disease. *J Infect Dis* 2008; **197**: 572-579 [PMID: 18240953 DOI: 10.1086/526789]

6 **Moir S**, Ho J, Malaspina A, Wang W, DiPoto AC, O'Shea MA, Roby G, Kottilil S, Arthos J, Proschan MA, Chun TW, Fauci AS. Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals. *J Exp Med* 2008; **205**: 1797-1805 [PMID: 18625747 DOI: 10.1084/jem.20072683]

7 **Hart M**, Steel A, Clark SA, Moyle G, Nelson M, Henderson DC, Wilson R, Gotch F, Gazzard B, Kelleher P. Loss of discrete memory B cell subsets is associated with impaired immunization responses in HIV-1 infection and may be a risk factor for invasive pneumococcal disease. *J Immunol* 2007; **178**: 8212-8220 [PMID: 17548660 DOI: 10.4049/jimmunol.178.12.8212]

8 **Lee KY**, Tsai MS, Kuo KC, Tsai JC, Sun HY, Cheng AC, Chang SY, Lee CH, Hung CC. Pneumococcal vaccination among HIV-infected adult patients in the era of combination antiretroviral therapy. *Hum Vaccin Immunother* 2014; **10**: 3700-3710 [PMID: 25483681 DOI: 10.4161/hv.32247]

9 **Leggat DJ**, Iyer AS, Ohtola JA, Kommoori S, Duggan JM, Georgescu CA, Khuder SA, Khaskhely NM, Westerink MJ. Response to Pneumococcal Polysaccharide Vaccination in Newly Diagnosed HIV-Positive Individuals. *J AIDS Clin Res* 2015; **6**: pii: 419 [PMID: 25908995 DOI: 10.4172/2155-6113.1000419]

10 **Zhang L**, Li Z, Wan Z, Kilby A, Kilby JM, Jiang W. Humoral immune responses to Streptococcus pneumoniae in the setting of HIV-1 infection. *Vaccine* 2015; **33**: 4430-4436 [PMID: 26141012 DOI: 10.1016/j.vaccine.2015.06.077]

11 **Tsachouridou O**, Skoura L, Zebekakis P, Margariti A, Metallidis S. Memory B Cell Divergences upon Immunization Against Streptococcus pneumoniae in HIV-1-Infected Adults. *AIDS Res Hum Retroviruses* 2015; **31**: 1053-1054 [PMID: 26535799 DOI: 10.1089/aid.2015.0148]

12 **Tsachouridou O**, Skoura L, Zebekakis P, Margariti A, Georgiou A, Daniilidis M, Malisiovas N, Metallidis S. The controversial impact of B cells subsets on immune response to pneumococcal vaccine in HIV-1 patients. *Int J Infect Dis* 2015; **38**: 24-31 [PMID: 26192868 DOI: 10.1016/j.ijid.2015.07.008]

13 **Moir S**, Fauci AS. Insights into B cells and HIV-specific B-cell responses in HIV-infected individuals. *Immunol Rev* 2013; **254**: 207-224 [PMID: 23772622 DOI: 10.1111/imr.12067]

14 **Pensieroso S**, Galli L, Nozza S, Ruffin N, Castagna A, Tambussi G, Hejdeman B, Misciagna D, Riva A, Malnati M, Chiodi F, Scarlatti G. B-cell subset alterations and correlated factors in HIV-1 infection. *AIDS* 2013; **27**: 1209-1217 [PMID: 23343911 DOI: 10.1097/QAD.0b013e32835edc47]

15 **D'Orsogna LJ**, Krueger RG, McKinnon EJ, French MA. Circulating memory B-cell subpopulations are affected differently by HIV infection and antiretroviral therapy. *AIDS* 2007; **21**: 1747-1752 [PMID: 17690573 DOI: 10.1097/QAD.0b013e32828642c7]

16 **Amu S**, Ruffin N, Rethi B, Chiodi F. Impairment of B-cell functions during HIV-1 infection. *AIDS* 2013; **27**: 2323-2334 [PMID: 23595152 DOI: 10.1097/QAD.0b013e328361a427]

17 **Lundgren JD**, Babiker AG, Gordin F, Emery S, Grund B, Sharma S, Avihingsanon A, Cooper DA, Fätkenheuer G, Llibre JM, Molina JM, Munderi P, Schechter M, Wood R, Klingman KL, Collins S, Lane HC, Phillips AN, Neaton JD. Initiation of Antiretroviral Therapy in Early Asymptomatic HIV Infection. *N Engl J Med* 2015; **373**: 795-807 [PMID: 26192873 DOI: 10.1056/NEJMoa1506816]

18 **Haynes BF**, Moody MA, Liao HX, Verkoczy L, Tomaras GD. B cell responses to HIV-1 infection and vaccination: pathways to preventing infection. *Trends Mol Med* 2011; **17**: 108-116 [PMID: 21112250 DOI: 10.1016/j.molmed.2010.10.008]

19 **Zhang R**, Verkoczy L, Wiehe K, Munir Alam S, Nicely NI, Santra S, Bradley T, Pemble CW, Zhang J, Gao F, Montefiori DC, Bouton-Verville H, Kelsoe G, Larimore K, Greenberg PD, Parks R, Foulger A, Peel JN, Luo K, Lu X, Trama AM, Vandergrift N, Tomaras GD, Kepler TB, Moody MA, Liao HX, Haynes BF. Initiation of immune tolerance-controlled HIV gp41 neutralizing B cell lineages. *Sci Transl Med* 2016; **8**: 336ra62 [PMID: 27122615 DOI: 10.1126/scitranslmed.aaf0618]

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ORIGINAL ARTICLE

Basic Study

Role of RNA secondary structure in emergence of compartment specific hepatitis B virus immune escape variants

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Author contributions: Datta S designed the study to test the hypothesis, performed predictions, analyzed the models and wrote the manuscript; Chakravarty R reviewed the results and edited the manuscript.

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Abstract

AIM

To investigate the role of subgenotype specific RNA secondary structure in the compartment specific selection of hepatitis B virus (HBV) immune escape mutations.

METHODS

This study was based on the analysis of the specific observation of HBV subgenotype A1 in the serum/plasma, while subgenotype A2 with G145R mutation in the peripheral blood leukocytes (PBLs). Genetic variability found among the two subgenotypes was used for prediction and comparison of the full length pregenomic RNA (pgRNA) secondary structure and base pairings. RNA secondary structures were predicted for 37 ℃ using the Vienna RNA fold server, using default parameters. Visualization and detailed analysis was done using RNA shapes program.

RESULTS

In this analysis, using similar algorithm and conditions, entirely different pgRNA secondary structures for subgenotype A1 and subgenotype A2 were predicted, suggesting different base pairing patterns within the

two subgenotypes of genotype A, specifically, in the HBV genetic region encoding the major hydrophilic loop. We observed that for subgenotype A1 specific pgRNA, nucleotide 358^{U} base paired with 1738^{A} and nucleotide 587^{G} base paired with 607^{C}. However in sharp contrast, in subgenotype A2 specific pgRNA, nucleotide 358^{U} was opposite to nucleotide 588^{G}, while 587^{G} was opposite to 359^{U}, hence precluding correct base pairing and thereby lesser stability of the stem structure. When the nucleotides at 358^{U} and 587^{G} were replaced with 358^{C} and 587^{A} respectively (as observed specifically in the PBL associated A2 sequences), these nucleotides base paired correctly with 588^{G} and 359^{U}, respectively.

CONCLUSION

The results of this study show that compartment specific mutations are associated with HBV subgenotype specific alterations in base pairing of the pgRNA, leading to compartment specific selection and preponderance of specific HBV subgenotype with unique mutational pattern.

Key words: Hepatitis B; Compartmentalization; Peripheral blood leukocytes; pgRNA; RNA secondary structure; G145R

Core tip: We have previously shown that, in our study population, distribution of hepatitis B virus (HBV) subgenotypes A1 and A2 is highly biased in the serum/plasma and peripheral blood leukocyte (PBL) compartments respectively. Analysing the predicted base pairing patterns of pregenomic RNAs (pgRNAs), specific for HBV subgenotype A1 and A2, we demonstrate that the potent immune escape mutation G145R evolves specifically in the context of HBV subgenotype A2. The PBL compartment is exposed to strong anti-HBs immunity, and thus G145R is highly advantageous for the virus to persist. This explains the exclusive preponderance of subgenotype A2 in the PBL compartment, sharply contrasting the prevalence of subgenotype A1 in the serum/plasma.

Datta S, Chakravarty R. Role of RNA secondary structure in emergence of compartment specific hepatitis B virus immune escape variants. *World J Virol* 2016; 5(4): 161-169 Available from: URL: http://www.wjgnet.com/2220-3249/full/v5/i4/161.htm DOI: http://dx.doi.org/10.5501/wjv.v5.i4.161

INTRODUCTION

Viral compartmentalization signify infection, persistence and replication of viruses in off-target cells/tissues or anatomical compartments of the host, and this phenomenon is now believed to be a crucial event in many important viral infections, including hepatitis B virus (HBV), human immunodeficiency virus, hepatitis C virus, *etc.*[1-5]. Recent molecular evolutionary studies have demonstrated that viruses evolve independently under the influence of unique immunological milieu in a given compartment, leading to the selection and emergence of specific viral variants, which endow the virus with an advantage to survive and persist in that particular compartment[2,6,7]. Such compartment specific viral evolution have extremely important implications in emergence and re-emergence of immune-escape mutants, antiviral resistant mutants, their long term persistence and transmission through different non-conventional routes[1,7].

HBV is the prototype member of the *Hepadnaviridae* family of enveloped viruses with a very unique partially double-stranded DNA genome[8]. Despite having a DNA genome, HBV exclusively uses an RNA intermediate (the *pregenomic* RNA or the *pgRNA*) and a virus encoded reverse transcriptase to replicate its genome through a complex mechanism of primer shifting[8]. Even though HBV is classically considered to be a hepatotropic virus, HBV related nucleic acids and proteins have long been detected in different tissues, suggesting that it replicates and propagates in various non-hepatic tissues[1]. Interestingly, some of these extrahepatic sites have been shown to act as reservoirs and also the source of reinfection after surgical and therapeutic interventions[9,10]. Recently, ours and other research groups have provided convincing evidences that the HBV strains and their mutational signature pattern present in different extra-hepatic compartments, are often characteristically distinct from the HBV strains circulating in the serum/plasma/hepatic compartments and that immune escape/drug resistance mutations are significantly more frequent in different extrahepatic compartments in HBV carriers[2,4,11].

In our previous studies, we have recognized the subgenotype A1 (*Afro-Asian* subgenotype) as the predominant subgenotype of HBV genotype A circulating in the sera/plasma of our study population and that the occurrence of G145R mutation therein was sporadic[12-14]. In sharp contrast, we documented the confined and exclusive existence of HBV subgenotype A2 with the potent "immune escape" mutation G145R within the peripheral blood leukocytes (PBL), across the study population, irrespective of the HBV genotype/subgenotype circulating in the serum/plasma of the respective individual[2]. G145R is the mutation signifying Glycine to Arginine substitution at amino acid residue 145 in the major hydrophilic loop (MHL), a B-cell epitope of the hepatitis B surface antigen (HBsAg), which provides a strong immune escape property. These observations strongly signify that viral mutants with G145R does have an explicit replicative advantage within the PBLs, that are exposed to strong anti-HBs immunity and that this mutation emerges specifically in the perspective of subgenotype A2, but not in subgenotype A1. Moreover, all the subgenotype specific nucleotide substitutions in the MHL encoding region of A1 (505^{C}, 514^{C}, 616^{A} and 619^{T}) and A2 (505^{T}, 514^{A}, 616^{G} and 619^{C}) are

Table 1 Comparison of nucleotides at phylogenetically informative sites of subgenotypes A1 and A2 in GenBank sequences and in sequences isolated from our study population

Nucleotide Position of the HBV genome[1]	Base present in reference GenBank sequences			Base in genotype A1 sequences isolated from serum/ plasma[2]	Base in genotype A2 sequences isolated from PBL[2]
	Aa/A1 (Asia)	Aa/A1 (South Africa)	Ae/A2 (Europe/ United States)		
505	c	t	t	c	t
514	c	c	a	c	a
616	a	a	g	a	g
619	t	t	c	t	c

[1]Nucleotide positions indicate distance from the unique EcoRI site in the HBV genome; [2]Sequences isolated from our study population. HBV: Hepatitis B virus; PBL: Peripheral blood leukocyte.

synonymous in nature, which aptly rules out the possibility that the predilection of the subgenotype A2 in the PBL is due to the subgenotype specific epitopic difference in the HBsAg[2]. Taken together, the above observations led us to hypothesize that the subgenotype specific nucleotide substitutions might modulate the base pairing of the A2 specific pgRNA in a way, which favours the emergence of G145R.

In the present work, we compared the changes in the base pairing of the pgRNA due to subgenotype A1 and A2 specific substitutions in the MHL encoding region. Based on the RNA secondary structure predictions, we demonstrate that the selection and emergence of G145R within HBV subgenotype A2 sequences in the PBL compartment occurs due to the differential base pairing characteristics in the subgenotype A2 specific pgRNA.

MATERIALS AND METHODS

Sequences for analysis

HBV surface gene sequences, corresponding to the nucleotide 341 to 660 of the HBV genome (nucleotide position counted from the unique EcoRI site in the HBV genome), that code for the epitopic MHL for both subgenotype A1 sequences (isolated from serum/ plasma) and subgenotype A2 sequences (isolated from PBL), obtained during our previous studies were used in this analysis[2,14]. Using alignment of these sequences along with other reference GenBank HBV sequences[15], subgenotype A1 and A2 specific nucleotide substitutions (summarised in the Table 1) were determined earlier[2]. These nucleotide differences were consequently used for studying the alterations in the subgenotype specific base-pairing and folding of the pgRNA.

For subgenotype A1 and A2 specific pgRNA secondary structure predictions, template sequences were generated separately by editing two well defined full length sequences, namely-GenBank accession number DQ315784 (India) for subgenotype A1 and GenBank accession number AJ309370 (France) for subgenotype A2, respectively, following the method described previously for generation of full length pgRNA sequence[16]. The pgRNA templates so generated were unpolyadenylated and included the terminal redundancy. These two sequences served as the base sequences for prediction of secondary structures, to which nucleotide substitutions observed in the MHL encoding and flanking regions of serum associated A1 and PBL associated A2 (as mentioned in the previous section) were substituted respectively at appropriate nucleotide positions. Finally, these two template sequences (approximately 3.3 kb) were subjected to RNA secondary structure prediction and comparison.

Prediction of RNA structure

For prediction of the secondary structures, the pgRNA sequences generated as stated above were submitted to the Vienna RNA secondary structure server[17,18]. The server predicts the minimum free energy *(mfe)* secondary structures for single RNA sequences using an algorithm proposed by Zuker and Stiegler, and also calculates the equilibrium base-pairing probabilities by means of partition function (*pf*) algorithm proposed by McCaskill[19,20]. Apart from the *mfe* and *pf*, the server also provides a centroid structure, which indicates the reliability of the predictions, while the dot-plot which provides information on base-pairing probabilities of all the possible predicted structures[17,21]. All the secondary structure predictions were performed for a temperature of 37 ℃, keeping all the other parameters to default[22]. Visualization, annotation and analysis of the *mfe* structures were performed using the RNAshapes program[23]. As the present study was focused on the genetic variability of the HBV genome encoding the MHL region of the surface gene, we restricted our detailed analysis of base pairing pattern to the secondary structure of the part of pgRNA, corresponding to the MHL encoding sequence.

RESULTS

Changes in the nucleotide base pairing of the predicted secondary structure of pgRNA

The gross structural features of the subgenotype A1 and A2 specific pgRNA were found to be entirely distinct (Figure 1). The difference in the pgRNA structure was also evident from the mountain plots showing the mfe, pf, centroid, entropy and the dot plot of the two subgenotypes. The difference in other features are summarised in the Table 2. Detailed scrutiny of the pgRNA secondary structures corresponding to the MHL encoding genetic regions, revealed entirely distinct pgRNA secondary structures with discrete intramolecular base pairing patterns due to the subgenotype specific and variations between A1 and A2 sequences (Figures 2 and 3). Interestingly, when we focussed on the base pairing of the nucleotides encoding the MHL region, we noted that in subgenotype A1 specific pgRNA, nucleotide 358^{U} base paired with 1738^{A} and nucleotide 587^{G} base paired with 607^{C} (Figure 2).

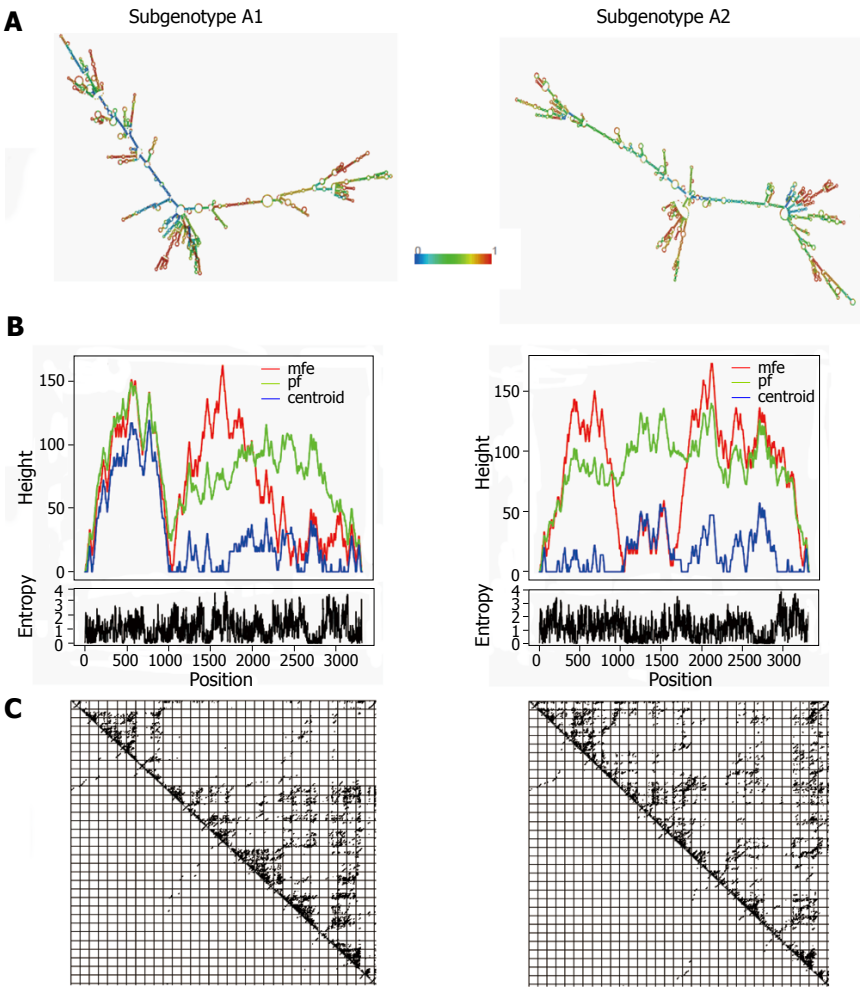

Figure 1 Comparative diagram showing the differences between different aspects of the predicted secondary structures of the pgRNA, specific for HBV subgenotype A1 and subgenotype A2. A: Predicted minimum free energy (mfe) structures, coloured by base-pairing probabilities (according to the rainbow scale shown in the middle, denoting base pair probabilities from 0 to 1). Colour of the unpaired regions denotes the probability of being unpaired; B: Mountain plot representing the mfe structure (red line), the thermodynamic ensemble of RNA structures (green line), and the centroid structure (blue line). Positional entropy for each position is presented below the mountain plot; C: Dot-plot showing the base-pairing probabilities of the two predictions.

Table 2 Comparison of the thermodynamic characteristics of the minimum free energy secondary structure predictions for subgenotypes A1 and A2 pgRNA

Features	Subgenotype A1	Subgenotype A2
Minimum free energy of the optimal secondary structure	-1052.10 kcal/mol	-1049.50 kcal/mol
Free energy of the thermodynamic ensemble	-1099.56 kcal/mol	-1098.93 kcal/mol
Minimum free energy of the centroid secondary structure	-722.20 kcal/mol	-679.21 kcal/mol
Ensemble diversity	863.25	954.99

However in sharp contrast, in subgenotype A2 specific pgRNA, nucleotide 358^{U} was opposite to nucleotide 588^{G}, while 587^{G} was opposite to 359^{U}, hence precluding correct base pairing and thereby less stability of the stem structure. When the nucleotides at 358^{U} and 587^{G} were replaced with 358^{C} and 587^{A} respectively (as observed specifically in the PBL associated A2 sequences), these nucleotides base paired correctly with 588^{G} and 359^{U}, respectively (Figure 3), forming a correctly paired stem-loop structure, hence stabilizing the local conformation. Nevertheless, the effects of other substitutions were not as influential as these two changes. The exclusive detection of subgenotype A2 sequences with the abovementioned substitutions in the PBL clearly suggest the selective advantage of the pgRNA with 358^{C} and 587^{A}, and in turn the importance of G145R immune escape mutation in the PBL compartment.

DISCUSSION

In this study, we present interesting observations about the possible mechanism of compartment specific selection of immune escape HBV mutants. Based on our previous studies done on serum/plasma isolated HBV genotypes, we have documented the predominance of at least three distinct HBV genotypes in our study population, namely genotype D (most abundant) followed by genotypes C and A[13,14]. However, when we investigated the paired HBV sequences isolated from

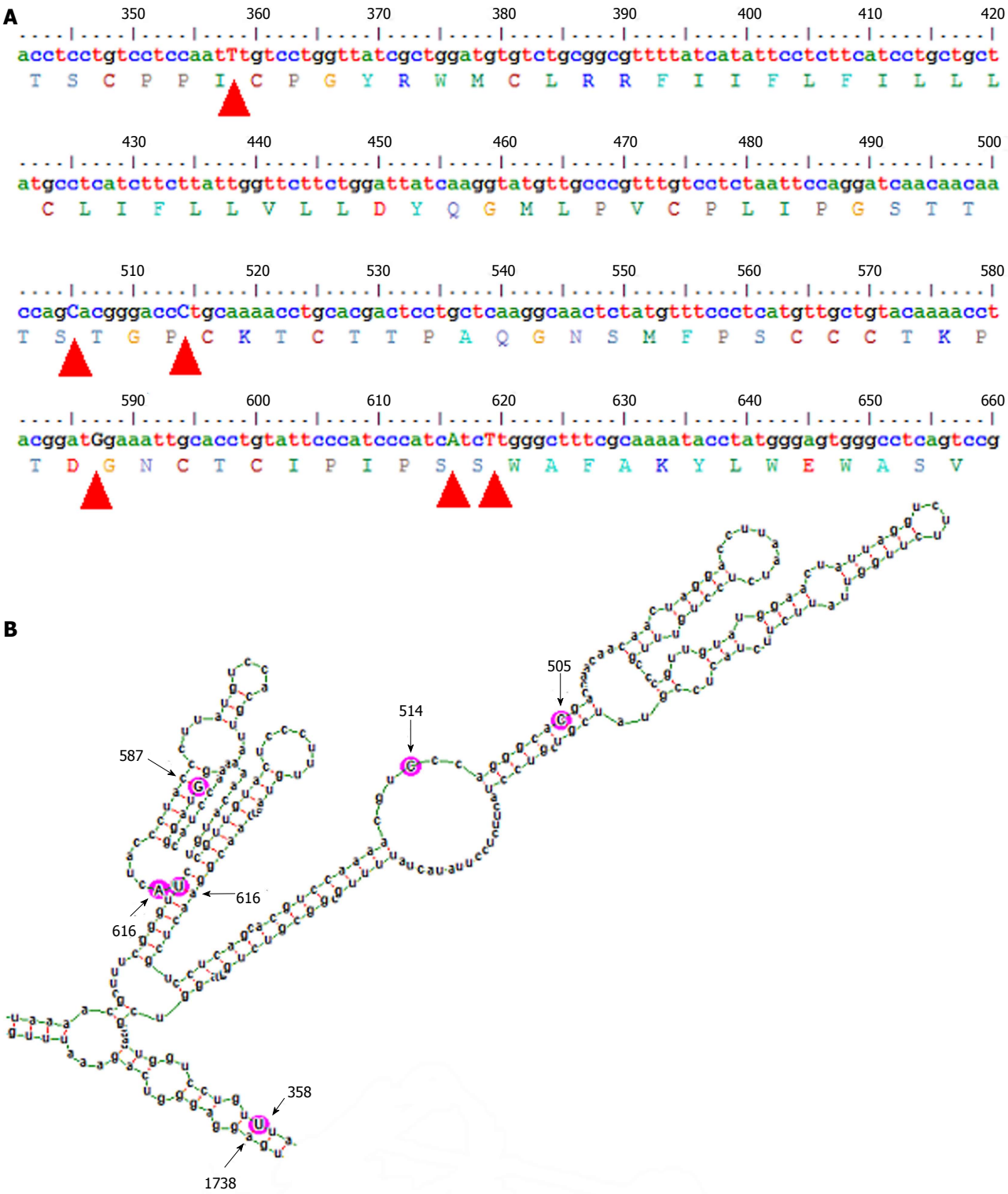

Figure 2 Diagram showing the genetic variability and the part of the predicted mfe structure, corresponding to the genetic region encoding the major hydrophilic loop of subgenotype A1. A: Consensus nucleotide sequence (corresponding to positions 341 to 660 of the HBV genome) and predicted amino acid sequence (corresponding to residues 63 to 168 of the HBsAg). Subgenotype A1 specific nucleotides (505^{C}, 514^{C}, 616^{A} and 619^{T}) and two variable nucleotides (358^{T} and 587^{G}) are indicated by arrowheads; B: Detailed base pairing pattern of the mfe pgRNA structure, specific for subgenotype A1. Aforementioned variable sites are encircled by pink circles and indicated by arrows and numbers correspond to their nucleotide position in the HBV genome. HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen.

serum/plasma and the PBLs, we surprisingly observed the exclusive preponderance of the genotype A in the PBL, irrespective of the HBV genotypes circulating in the serum/plasma of any given individual[2]. More interestingly, the genotype A sequences isolated from the PBL was found to be markedly distinct from that of genotype A sequences isolated from serum/plasma, in terms of subgenotype and specific nucleotide sub-

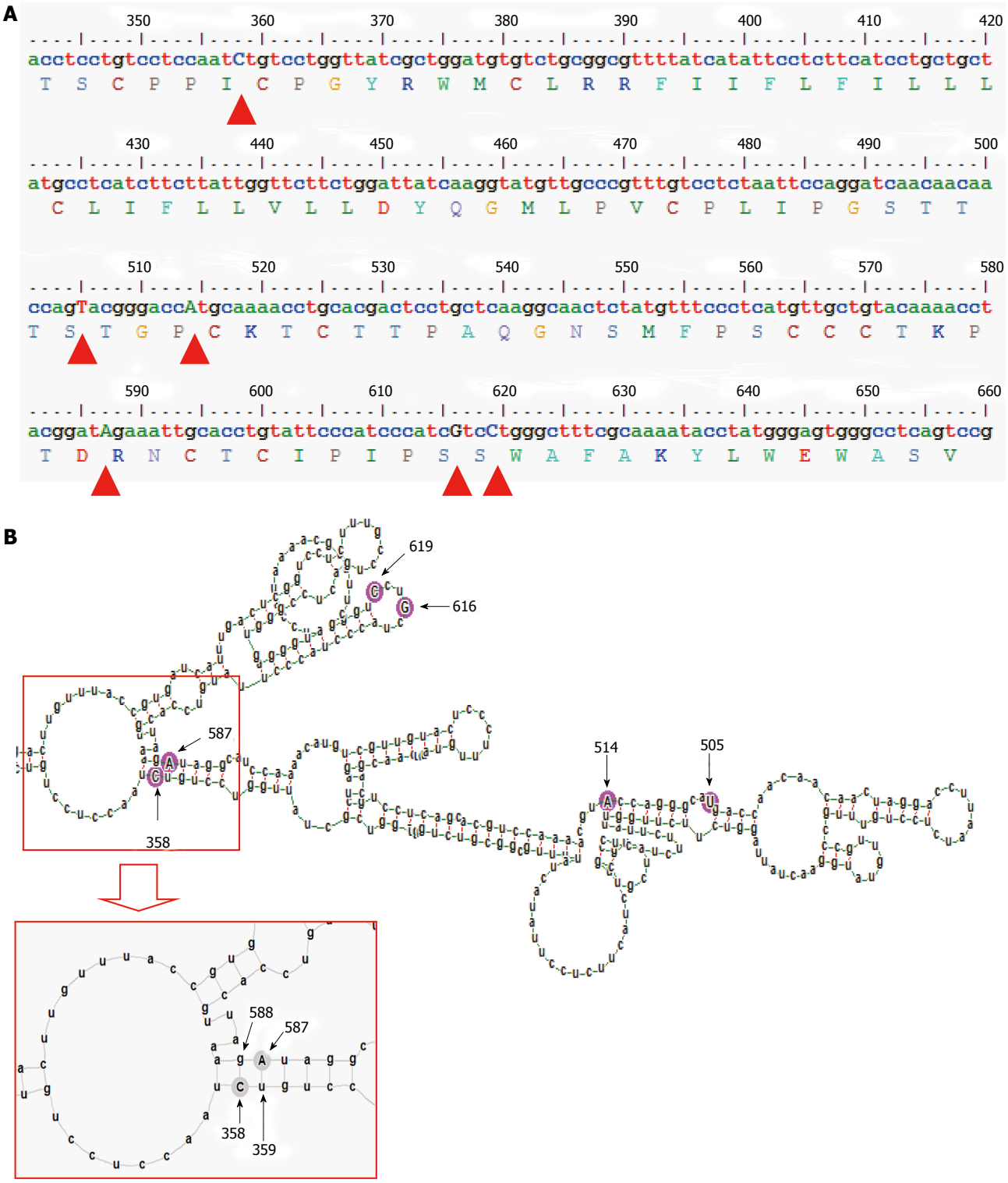

Figure 3 Diagram showing the genetic variability and the part of the predicted mfe structure, corresponding to the genetic region encoding the major hydrophilic loop of subgenotype A2. A: Consensus nucleotide sequence (corresponding to positions 341 to 660 of the HBV genome) and predicted amino acid sequence (corresponding to residues 63 to 168 of the HBsAg). Subgenotype A2 specific nucleotides (505^{T}, 514^{A}, 616^{G} and 619^{C}) and two co-evolving nucleotides (358^{C} and 587^{A}) are indicated by arrowheads; B: Detailed base pairing pattern of the mfe pgRNA structure, specific for subgenotype A2. Aforementioned variable sites are encircled by pink circles and indicated by arrows and numbers correspond to their nucleotide position in the HBV genome. Part of the mfe structure is amplified in the inset for better visualization of the base pairing. HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen.

stitution patterns. More precisely, subgenotype A1 of HBV genotype A was prevalent in the serum/plasma while in sharp contrast; subgenotype A2 was solely isolated from the PBL[2].

In the present study, we sought to examine the selective advantage of subgenotype A2 in the PBL compartment with the help of advance computational prediction and analysis programs. We focussed our

analysis on the examination of nucleotide sequences encoding the dominant B-cell epitope (MHL) of the HBV surface antigen, since in a number of other viruses, analogous genetic regions (epitope regions of the envelope protein) have been shown to undergo faster evolution to facilitate the emergence of compartment specific immune-escape variants[2]. Interestingly, when we compared serum/plasma circulating subgenotype A1 sequences with PBL confined subgenotype A2 sequences, we found that only four subgenotype specific nucleotide substitutions differentiate both sequences[2]. However, all these four subgenotype specific nucleotide substitutions in the MHL encoding region were found to be synonymous in nature (*i.e.*, the sequence of amino acids in the MHL remains same between subgenotypes A1 and A2), suggestive of the fact that MHL epitope diversity might not be directly relevant to the selection of subgenotype A2 over subgenotype A1 in PBL. On the other hand, in addition to these four subgenotype specific nucleotide substitutions, two additional nucleotide substitutions (358^{C} and 587^{A}) were evident with PBL associated A2 sequences, across the study population, which we have earlier shown to be co-evolving in the PBL[2]. Interestingly, we further noted that nucleotide substitution 358^{C} was also synonymous, while substitution 587^{A} was non-synonymous and translated into the potent immune escape G145R mutation of HBsAg. Earlier studies have demonstrated that by virtue of its definite advantages, G145R mutation helps HBV to dynamically evade anti-HBs specific immune response, thereby ensuring viral persistence in anatomical compartments, which are exposed to strong anti-HBs immunity[2]. The association of these five synonymous nucleotide substitutions and a potent immune escape mutation with PBL associated Ae/A2 sequences led us to hypothesize that the advantageous 587^{A} (G145R) might be selected at the pgRNA base pairing level and to verify this hypothesis, this comparative study was undertaken.

On comparison of the pgRNA secondary structures, we observed that the invariable association of subgenotype A2 with the selection of nucleotide 587^{A} (causing G145R) most possibly occur in the context of genotype A2 specific altered pgRNA base pairing patterns. Fascinatingly, we observed that substitution of a uracil (U, corresponding to Thymidine, T in DNA sequence) to cytosine (C) at position 358 altogether changed the local base pairing pattern of the pgRNA (358^{C} paired with nucleotide 588^{G} instead of the normal pairing with 1738^{A} in subgenotype A1). In the context of this altered base pairing, a single nucleotide change (U to C) at 359 was found to stabilize the stem structures by pairing with the wild type 587^{G}, just opposite to it. However, the nucleotide at position 359 encodes a Cysteine residue at amino acid position 69 of HBsAg, which is extremely essential for the generation of subviral 20 nm HBsAg particles, and thus, any non-synonymous substitution at this position is most likely to be detrimental for the virus persistence[24]. Therefore, based on the predicted secondary structures, we hypothesized that, instead of selecting an altered nucleotide at this exceptionally essential position (359), a compensatory alteration of a single nucleotide (G to A) at position 587 is expected to serve dual purpose, firstly it may help stabilize the stem structure (by pairing with the highly conserved $359^{T/U}$) and secondly it results in the emergence of a potent immune escape G145R mutation, both of which appears to be highly advantageous for the virus.

The HBV polymerase lacking proof reading function has been implicated in the generation of random mutations and generation of "quasi-species", of which the genomes (viral DNA) or pregenomes (pgRNA) having mutations useful for escaping the immune response of the host are gradually selected and subsequently become the prevalent viral population[25]. Apart from viral polymerase induced random mutations, host PBL associated APOBEC (apolipoprotein B mRNA editing enzyme, catalytic polypeptide) family of cellular cytidine deaminases have also been shown to induce hypermutation (G to A mutations) in the HBV genome, which has been suggested to cause genetic diversification and consequently selective evolution among the divergent genomes[26-28]. Nevertheless, the finding of selective predominance of the point mutation leading to G145R in the present study is highly significant in the context of PBL, since PBLs are exposed to strong anti-HBs humoral immune response and HBV variants with G145R are capable of strongly neutralizing this immune response, without any compromise in the replicative competence, thereby ensuring viral perseverance[29,30]. Whatever is the source of genetic diversification, in the present work we describe a probable mechanism of RNA folding, through which divergent viral genomes/pregenomes having favourable mutations are selected for propagation.

We acknowledge that the RNA folding predictions are based on statistical/mathematical algorithms and the biological relevance of these predictions are based on their corroboration with the biological data. Interestingly, the results of the present RNA folding predictions beautifully elucidate the observed co-evolution of the mutations at positions 358 and 587, which supports the biological relevance of the observed predictions. The results of the present study further implies that, certain HBV mutations are selected at the subgenomic RNA level (as they are synonymous at the protein level), which may significantly alter the base pairing of the pgRNA, which in turn may hasten the selection of mutations at other sites. Interestingly, the mechanism suggested in this work is very much similar to the mechanism described for HBV genotype specific selection of the most widely studied HBV precore mutation (1896^{A}), which emerges to stabilize the stem-loop structure of the epsilon "ε" signal of pgRNA[31]. Altogether the present study, support the findings of Kidd-Ljunggren *et al*[16], that demonstrate the implications of genotype specific differences in the pgRNA secondary structures in the emergence of genotype specific variations in the HBV genome.

In conclusion, our results based on the predicted RNA secondary structures suggest the role of HBV genotype/subgenotype specific base pairing patterns of the pgRNA in selection/emergence of advantageous mutations. Furthermore, the observed association of a potent immune escape mutation with a particular HBV subgenotype, confined in a specific anatomic compartment indicate the possible mechanism of genotype/subgenotype specific compartmentalization of HBV, which may have important implications in extrahepatic maintenance and transmission of HBV through hitherto unknown routes.

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COMMENTS

Background

In our previous study we have observed highly compartment specific prevalence of hepatitis B virus (HBV) genotype A. In particular, HBV subgenotype A1 was detected in serum/plasma isolates, while HBV subgenotype A2 was predominant in the peripheral blood leukocytes (PBLs). Apart from subgenotype specific differences, G145R - a potent immune escape mutation was specifically observed in the PBL related subgenotype A2 isolates. The authors undertook this study to understand the possible mechanism of the compartment specific distribution of HBV subgenotypes and immune escape mutation.

Research frontiers

Compartment specific evolution and emergence of HBV mutations is a poorly understood area. A few recent studies, including the authors have provided evidences, indicating that HBV independently evolves in different anatomical compartments, depending upon the immune selection pressure on that compartment.

Innovations and breakthroughs

Using computational prediction methods, the authors show that the PBL specific emergence of G145R occurs in the context of HBV subgenotype A2, due to altered base-pairing patterns, as compared to the HBV subgenotype A1.

Applications

The findings of the present study are important in the long term persistence, evolution and transmission of different HBV genotypes/subgenotypes/mutations in different anatomical compartments. These findings have important implications in transmission of HBV.

Terminology

Compartmentalization: Compartmentalization is the process of compartment specific infection, evolution and persistence of viral variants (genotypes/subgenotypes) in different anatomically distinct sites. Due to difference in the immune selection pressure, viral variants with alterations advantageous under the given immune pressure are gradually selected, leading to their divergence from the circulating strains. Compartmentalization has been well studied in human immunodeficiency virus, hepatitis C virus, Epstein-Barr virus, *etc.* in comparison, studies on HBV compartmentalization are scanty and the mechanisms of emergence of mutations is poorly understood.

Peer-review

This study has shown that HBV subtype A1 and A2 have entirely different pgRNA secondary structure, which may explain compartment specific selection and preponderance of specific HBV subgenotype with unique mutational pattern. This study has novel findings.

REFERENCES

1 **Datta S**. Compartmentalization of hepatitis B virus: Looking beyond the liver. *World J Hepatol* 2015; **7**: 2241-2244 [PMID: 26380649 DOI: 10.4254/wjh.v7.i20.2241]

2 **Datta S**, Panigrahi R, Biswas A, Chandra PK, Banerjee A, Mahapatra PK, Panda CK, Chakrabarti S, Bhattacharya SK, Biswas K, Chakravarty R. Genetic characterization of hepatitis B virus in peripheral blood leukocytes: evidence for selection and compartmentalization of viral variants with the immune escape G145R mutation. *J Virol* 2009; **83**: 9983-9992 [PMID: 19420079 DOI: 10.1128/JVI.01905-08]

3 **Bednar MM**, Sturdevant CB, Tompkins LA, Arrildt KT, Dukhovlinova E, Kincer LP, Swanstrom R. Compartmentalization, Viral Evolution, and Viral Latency of HIV in the CNS. *Curr HIV/AIDS Rep* 2015; **12**: 262-271 [PMID: 25914150 DOI: 10.1007/s11904-015-0265-9]

4 **Ene L**, Duiculescu D, Tardei G, Ruta S, Smith DM, Mehta S, Letendre S, Achim CL. Hepatitis B virus compartmentalization in the cerebrospinal fluid of HIV-infected patients. *Clin Microbiol Infect* 2015; **21**: 387.e5-387.e8 [PMID: 25658525 DOI: 10.1016/j.cmi.2014.11.012]

5 **Ramirez S**, Perez-Del-Pulgar S, Carrion JA, Costa J, Gonzalez P, Massaguer A, Fondevila C, Garcia-Valdecasas JC, Navasa M, Forns X. Hepatitis C virus compartmentalization and infection recurrence after liver transplantation. *Am J Transplant* 2009; **9**: 1591-1601 [PMID: 19459796 DOI: 10.1111/j.1600-6143.2009.02666.x]

6 **Gismondi MI**, Díaz Carrasco JM, Valva P, Becker PD, Guzmán CA, Campos RH, Preciado MV. Dynamic changes in viral population structure and compartmentalization during chronic hepatitis C virus infection in children. *Virology* 2013; **447**: 187-196 [PMID: 24210114 DOI: 10.1016/j.virol.2013.09.002]

7 **Blackard JT**. HIV compartmentalization: a review on a clinically important phenomenon. *Curr HIV Res* 2012; **10**: 133-142 [PMID: 22329519 DOI: 10.2174/157016212799937245]

8 **Datta S**, Chatterjee S, Veer V, Chakravarty R. Molecular biology of the hepatitis B virus for clinicians. *J Clin Exp Hepatol* 2012; **2**: 353-365 [PMID: 25755457 DOI: 10.1016/j.jceh.2012.10.003]

9 **Féray C**, Zignego AL, Samuel D, Bismuth A, Reynes M, Tiollais P, Bismuth H, Brechot C. Persistent hepatitis B virus infection of mononuclear blood cells without concomitant liver infection. The liver transplantation model. *Transplantation* 1990; **49**: 1155-1158 [PMID: 2360255]

10 **Brind A**, Jiang J, Samuel D, Gigou M, Feray C, Bréchot C, Kremsdorf D. Evidence for selection of hepatitis B mutants after liver transplantation through peripheral blood mononuclear cell infection. *J Hepatol* 1997; **26**: 228-235 [PMID: 9059940 DOI: 10.1016/S0168-8278(97)80035-9]

11 **Coffin CS**, Osiowy C, Gao S, Nishikawa S, van der Meer F, van Marle G. Hepatitis B virus (HBV) variants fluctuate in paired plasma and peripheral blood mononuclear cells among patient cohorts during different chronic hepatitis B (CHB) disease phases. *J Viral Hepat* 2015; **22**: 416-426 [PMID: 25203736 DOI: 10.1111/jvh.12308]

12 **Datta S**. An overview of molecular epidemiology of hepatitis B virus (HBV) in India. *Virol J* 2008; **5**: 156 [PMID: 19099581 DOI: 10.1186/1743-422X-5-156]

13 **Banerjee A**, Chandra PK, Datta S, Biswas A, Bhattacharya P, Chakraborty S, Chakrabarti S, Bhattacharya SK, Chakravarty R. Frequency and significance of hepatitis B virus surface gene variant circulating among 'antiHBc only' individuals in Eastern India. *J Clin Virol* 2007; **40**: 312-317 [PMID: 17997353 DOI: 10.1016/j.jcv.2007.08.009]

14 **Banerjee A**, Kurbanov F, Datta S, Chandra PK, Tanaka Y, Mizokami M, Chakravarty R. Phylogenetic relatedness and genetic diversity of hepatitis B virus isolates in Eastern India. *J Med Virol* 2006; **78**: 1164-1174 [PMID: 16847957 DOI: 10.1002/jmv.20677]

15 **Kramvis A**. Genotypes and genetic variability of hepatitis B virus. *Intervirology* 2014; **57**: 141-150 [PMID: 25034481 DOI: 10.1159/000360947]

16 **Kidd-Ljunggren K**, Zuker M, Hofacker IL, Kidd AH. The hepatitis B virus pregenome: prediction of RNA structure and implications

for the emergence of deletions. *Intervirology* 2000; **43**: 154-164 [PMID: 11044809 DOI: 10.1159/000025041]

17 **Gruber AR**, Lorenz R, Bernhart SH, Neuböck R, Hofacker IL. The Vienna RNA websuite. *Nucleic Acids Res* 2008; **36**: W70-W74 [PMID: 18424795 DOI: 10.1093/nar/gkn188]

18 **Hofacker IL**. Vienna RNA secondary structure server. *Nucleic Acids Res* 2003; **31**: 3429-3431 [PMID: 12824340 DOI: 10.1093/nar/gkg599]

19 **Zuker M**, Stiegler P. Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. *Nucleic Acids Res* 1981; **9**: 133-148 [PMID: 6163133 DOI: 10.1093/nar/9.1.133]

20 **McCaskill JS**. The equilibrium partition function and base pair binding probabilities for RNA secondary structure. *Biopolymers* 1990; **29**: 1105-1119 [PMID: 1695107 DOI: 10.1002/bip.360290621]

21 **Ding Y**, Chan CY, Lawrence CE. RNA secondary structure prediction by centroids in a Boltzmann weighted ensemble. *RNA* 2005; **11**: 1157-1166 [PMID: 16043502 DOI: 10.1261/rna.2500605]

22 **Mathews DH**, Sabina J, Zuker M, Turner DH. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *J Mol Biol* 1999; **288**: 911-940 [PMID: 10329189 DOI: 10.1006/jmbi.1999.2700]

23 **Steffen P**, Voss B, Rehmsmeier M, Reeder J, Giegerich R. RNAshapes: an integrated RNA analysis package based on abstract shapes. *Bioinformatics* 2006; **22**: 500-503 [PMID: 16357029 DOI: 10.1093/bioinformatics/btk010]

24 **Mangold CM**, Streeck RE. Mutational analysis of the cysteine residues in the hepatitis B virus small envelope protein. *J Virol* 1993; **67**: 4588-4597 [PMID: 8392600]

25 **Caligiuri P**, Cerruti R, Icardi G, Bruzzone B. Overview of hepatitis B virus mutations and their implications in the management of infection. *World J Gastroenterol* 2016; **22**: 145-154 [PMID: 26755866 DOI: 10.3748/WJG.v22.i1.145]

26 **Vieira VC**, Soares MA. The role of cytidine deaminases on innate immune responses against human viral infections. *Biomed Res Int* 2013; **2013**: 683095 [PMID: 23865062 DOI: 10.1155/2013/683095]

27 **Mous K**, Jennes W, De Roo A, Pintelon I, Kestens L, Van Ostade X. Intracellular detection of differential APOBEC3G, TRIM5alpha, and LEDGF/p75 protein expression in peripheral blood by flow cytometry. *J Immunol Methods* 2011; **372**: 52-64 [PMID: 21784078 DOI: 10.1016/j.jim.2011.06.028]

28 **Janahi EM**, McGarvey MJ. The inhibition of hepatitis B virus by APOBEC cytidine deaminases. *J Viral Hepat* 2013; **20**: 821-828 [PMID: 24304451 DOI: 10.1111/jvh.12192]

29 **Jammeh S**, Thomas HC, Karayiannis P. Replicative competence of the T131I, K141E, and G145R surface variants of hepatitis B Virus. *J Infect Dis* 2007; **196**: 1010-1013 [PMID: 17763322 DOI: 10.1086/521198]

30 **Schilling R**, Ijaz S, Davidoff M, Lee JY, Locarnini S, Williams R, Naoumov NV. Endocytosis of hepatitis B immune globulin into hepatocytes inhibits the secretion of hepatitis B virus surface antigen and virions. *J Virol* 2003; **77**: 8882-8892 [PMID: 12885906 DOI: 10.1128/JVI.77.16.8882-8892.2003]

31 **Croagh CM**, Desmond PV, Bell SJ. Genotypes and viral variants in chronic hepatitis B: A review of epidemiology and clinical relevance. *World J Hepatol* 2015; **7**: 289-303 [PMID: 25848459 DOI: 10.4254/WJH.v7.i3.289]

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SYSTEMATIC REVIEWS

Geographic integration of hepatitis C virus: A global threat

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Author contributions: Daw MA designed the study, extracted the data, drafted and finalized the manuscript; El-Bouzedi AA, Ahmed MO, Dau AA, Agnan MM and Drah AM analyzed the data and contributed to the drafting of the data; all the authors read and approved the final manuscript.

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Data sharing statement: We confirm that the data is available from the corresponding author who will provide a permanent, citable and open access home for the dataset.

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Abstract

AIM

To assess hepatitis C virus (HCV) geographic integration, evaluate the spatial and temporal evolution of HCV worldwide and propose how to diminish its burden.

METHODS

A literature search of published articles was performed using PubMed, MEDLINE and other related databases up to December 2015. A critical data assessment and analysis regarding the epidemiological integration of HCV was carried out using the meta-analysis method.

RESULTS

The data indicated that HCV has been integrated immensely over time and through various geographical regions worldwide. The history of HCV goes back to 1535 but between 1935 and 1965 it exhibited a rapid, exponential spread. This integration is clearly seen in the geo-epidemiology and phylogeography of HCV. HCV integration can be mirrored either as intra-continental or trans-continental. Migration, drug trafficking and HCV co-infection, together with other potential risk factors, have acted as a vehicle for this integration. Evidence shows that the geographic integration of HCV has been important in the global and regional distribution of HCV.

CONCLUSION
HCV geographic integration is clearly evident and this should be reflected in the prevention and treatment of this ongoing pandemic.

Key words: Geo-epidemiology; Integration; Hepatitis C virus genotypes; Geography; Phylogeography

Core tip: Geographic integration of hepatitis C virus (HCV) is a newly described epidemiological phenomenon that is illustrated for the first time in this review article. The global burden of HCV infection has surpassed expectations and HCV genotypes are no longer restricted to certain countries or regions. All countries and their citizens are at a higher risk of HCV infection. HCV integration can be either intra-continental or trans-continental. Globalization, immigration and drug trafficking, in addition to the traditional HCV transmission factors, have acted as vectors for the geographical integration of HCV. International efforts and new strategies that go beyond borders should be combined to tackle this global threat.

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INTRODUCTION

Infection with hepatitis C virus (HCV) is a global public health threat that affects millions of individuals worldwide. In recent years, HCV has become one of the most important viruses. The global epidemic of HCV is distributed unevenly, with a high disease burden in low income regions and more than one-third of the estimated worldwide burden in the Western Pacific region. Approximately 32.2 million people have chronic HCV infection in Southeast Asia alone, Sub-Saharan Africa accounts for almost one-fifth of worldwide infections and over six million people are infected in Latin America[1].

Globally, about 27% of cirrhosis cases and 25% of hepatic cellular carcinoma cases are attributable to HCV[2]. Based on death certificate analyses, it has been estimated that there were about 3500 HCV-related deaths in France in 2001 and 15000 in the United States in 2007. In Egypt, there were an estimated 7379 HCV-related deaths in 1999 and the number is expected to more than double by 2020[3]. HCV-infected individuals have a 2.4 times higher risk of all-cause mortality compared to the non-infected population, 26.5 times the risk of liver-related mortality, and 1.8 times the risk of non-liver-related mortality[4].

Studies have reported an upsurge in the prevalence of HCV infection, particularly in developing countries and in some European regions. Southern provinces in Greece, Italy, France and Spain have reported higher levels of HCV infection (2%-7%) than in the North African nation of Libya[5]. Certain geographical spots in the Netherlands and Germany have a higher rate of HCV infection (7%) than other regions in the same countries[6].

The predisposing risk factors and modes of transmission of HCV have evolved in various ways in different parts of the world and this could have major implications for prevention programs[7]. However, many questions concerning the roles of risk factors and lifestyles that might be associated with the spread of HCV in different regions remain unanswered. Like some other important infectious diseases, HCV infection has been correlated with geographical, historical, social, economic and even political factors. Globalization and worldwide integration have added new epidemiological concepts that are clearly reflected in the prevalence of HCV worldwide. Neither HCV genotypes nor risk of exposure can be easily confined to certain regions or countries. Immigration, massive population displacement, unsettled conflicts and drug trafficking have aggravated the status of HCV infection and made it difficult to obtain a clear picture of HCV spread over the world. The objective of this review was to assess the worldwide geographical integration of HCV and its global evolution and to use the assessment to propose strategies to intervene in its spread.

MATERIALS AND METHODS

This study was conducted in four stages: (1) identification of the literature on HCV integration; (2) selection of relevant studies; (3) extraction of data; and (4) data sensitivity analysis. This review was designed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses statement[8].

Search strategy and literature review

Relevant studies were identified by searching PubMed, Scopus, Google Scholar and other databases using the following key terms: HCV, HCV integration, history, geography, epidemiology, phylogeography and evolutionary analysis. Some articles were also found by checking the lists of references in published papers. No language or time restrictions were applied.

Publication selection criteria

All identified abstracts were reviewed by two co-authors independently (Daw MA and El-Bouzedi AA or Ahmed MO/Agnan MM and Drah AM). They were considered eligible for full-text review if they provided accurate information on the geo-epidemiology of HCV and the distribution of genotypes. Data on evolutionary analyses were also included to estimate the dates of HCV origin and the temporal rates of virus spread from West Africa and China to Europe and North America.

Data abstraction and quality assessment

The data were independently abstracted by two co-authors (Daw MA with El-Bouzedi AA or Dau AA and Agnan MM or Drah AM). The studies were assessed using standardized data collection forms. The collected data include publication details, type of study and information on HCV transmission, the vectors involved, phylogenetic analysis, historical follow-up and evaluation criteria of the spread of HCV. If the evaluation results were controversial, a consensus among the authors was reached.

Data analysis

Data on the spatial spread of HCV was combined with the phylogenetic and epidemiological information to understand the dispersal of the virus worldwide. Furthermore, sensitivity analysis was conducted and the consistency of the data search results was evaluated.

Statistical analysis

A statistical review of the study was performed by a biomedical statistician.

RESULTS

Literature categorization and analysis of the publications

The publications were identified primarily by online searches. Unrelated studies were excluded based on the title or the abstract. The search results were categorized according to the historical, geographical, epidemiological and clinical parameters. Each of these fields was analyzed in the context of HCV integration.

History of HCV integration: Historical estimates have speculated about divergence time, distribution patterns and epidemic behaviors of HCV. Evolutionary analyses of the HCV genome have shed light on its epidemic history and transmission. Studies combining demographics with phylogeographic and molecular clock analyses have demonstrated the global dissemination of HCV[9,10]. The origin and evolution of HCV may date back to centuries ago in ancient China. An *et al*[11] recently estimated that the common ancestor of Chinese HCV variants (6 g and 6 w subtypes) isolated in Hainan Island dates back to between the sixth and ninth century. The authors speculated that the ancestors of a particular group of Austronesian-descended aborigines might have carried the earliest HCV-6 strains when they sailed to and settled in the Indochina peninsula in Southeast Asia, where HCV-6 is now indigenous.

Viral phylodynamic analysis indicates that HCV was disseminated in Africa before the rise of global travel and modern medicine[12]. It has been estimated that the most recent common ancestor of the CAR HCV-4 strains existed in the sixteenth century[13]. CAR HCV-4 strains spread rapidly and exponentially from 1935 to 1965, at about the same time as in Cameroon and Gabon. There is also epidemiological evidence of a wave of infection in Western countries during 1945-1965[14].

It has been suggested that colonization by European countries played a major role in the spread of these HCV genotypes, predominantly to the Americas but also to former colonial territories in Asia and Africa[14]. Global dissemination of HCV-2 seems to have been facilitated by the slave trade across the Atlantic and by colonization. During the intense period of slave trade (1700-1850), HCV-2 was disseminated from what is now Ghana/Benin to the Caribbean. HCV-2 also found its way from the Dutch colonies of Indonesia and Surinam to the Netherlands with the migration of Javanese workers to the Netherlands[14].

Epidemiological integration of HCV: The prevalence of HCV varies widely, from as low as 0.1% in certain Scandinavian countries to 23% in some African countries (Figure 1). The prevalence rate is classified as low (< 2.5%), intermediate (2.5%-10%) or high (> 10%). According to this classification, regions of low endemicity include North America, Europe, Australia and the Far East. Intermediate prevalence regions include some Mediterranean countries, the Middle East, Africa and South America. High prevalence countries are Egypt, Cameroon, Burundi, Rwanda, Gabon and Guinea in Africa, Bolivia in South America, and Mongolia in Asia[15]. However, 60% of all infected people are in Asia, particularly in its southern and eastern regions. China used to be considered a relatively high endemic area (average seroprevalence of HCV 3.2% in the general population)[16]. In Taiwan, the prevalence was estimated at 5.5%; it was 2.0%-14.2% in towns on the main island and higher (2.3%-26.4%) on the Penghu islands[17]. The overall prevalence in South Korea and Hong Kong is low (0.6%-1.1%) and is higher among females[18,19]. Japan has one of the highest endemic rates of HCV infection[20]. The prevalence rate of viral hepatitis in Southeast Asia is higher, where over 11 million people are estimated to have HCV. The prevalence of HCV infection among Malaysian adults has been estimated at 2.5%. Similar results have been reported from Indonesia, Cambodia, Thailand and the Philippines. However, it is less than 1% in Laos, Myanmar and Singapore, whereas the highest prevalence (> 6%) has been reported in Vietnam[21]. In India and Afghanistan, HCV prevalence ranged from 0.5%-1.5%. However, in Pakistan, about 6% of the population is suspected of having HCV infection and in some regions the estimated rate reached 31.9%[22-24].

In the Persian-Arab regions of West Asia, there are considerable regional differences in the prevalence of HCV. In Iran, the prevalence of anti-HCV antibodies in the general population ranges from 0.2% to 6.25% but the overall average is < 1%, which classifies the country as having a low frequency[25,26]. HCV prevalence was higher in Iraq (2.3%), Jordan (3.5%) and the Gaza strip (2.2%) but moderate in Lebanon (1%) and Syria (1%). There are no national studies on the prevalence HCV in the Arabian Peninsula and Yemen. HCV is considered

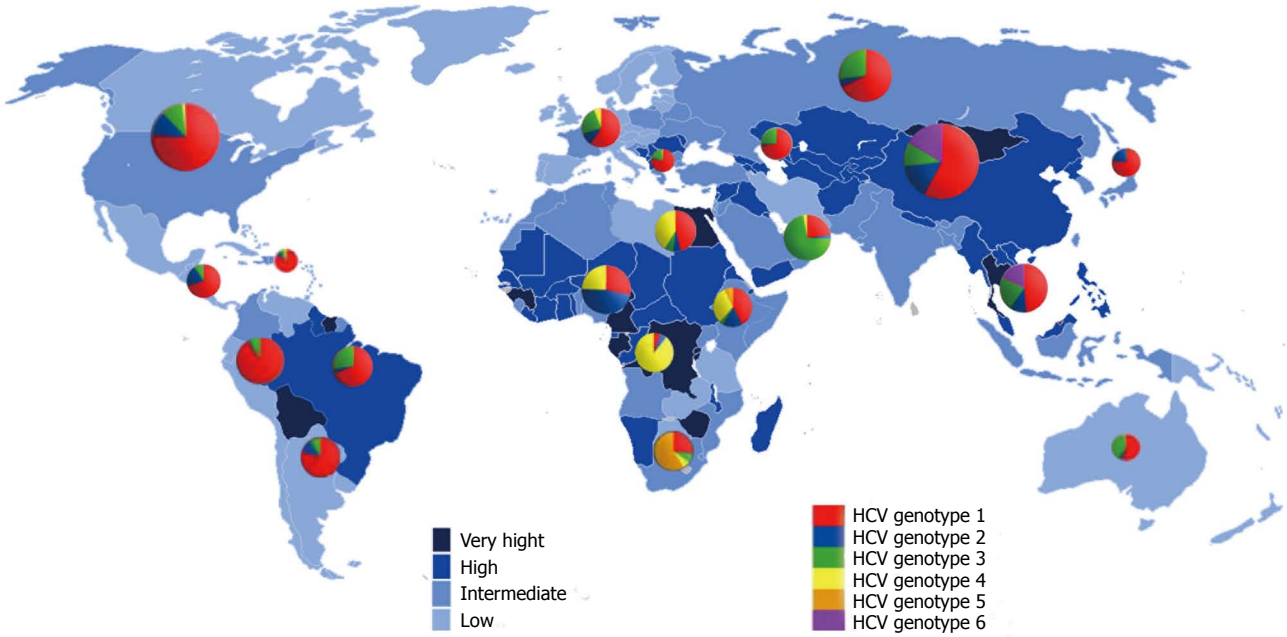

Figure 1 Overall prevalence of hepatitis C virus infections and the distribution of different hepatitis C virus genotypes worldwide. HCV: Hepatitis C virus.

endemic among the less populated countries, such as Qatar (6.3%), UAE (2.3%), Saudi Arabia (2%), Kuwait (1.8%) and Yemen (2.5%).

In Africa, > 28 million people have chronic HCV infection and future trends are difficult to predict[27]. Egypt has the highest prevalence in the world, with the prevalence rate estimated at 14.7% among people aged 15 to 59 years[1,28]. HCV has been well studied in the other North African countries, particularly in Libya. These countries are considered areas of low endemicity, with prevalence rates of 1%-1.5%[1,27]. In a recent study including 38 countries, seroprevalence was highest (after Egypt) in Central African countries, such as Gabon, Cameroon and Angola, and in some West African countries, such as Burkina Faso and Benin. The largest numbers of infected adults were in Nigeria, the Democratic Republic of Congo and Ethiopia[27].

In Latin America, it is estimated that 6.8 to 8.9 million adults are infected with HCV, of whom over 4 million are in Mexico and Brazil, which are the only South American countries that have carried out national population-based studies. The overall prevalence of HCV in Latin America is 1.5%; it varies from 0.1%-0.9% in Suriname, Chile, Peru, Venezuela, Panama and some other Latin American countries to 1.0%-3.4% in Brazil, Mexico and Argentina[29].

In Scandinavian countries, the spread of HCV infection started in the 1960s and peaked in the 1970s. Nosocomial and sexual transmission were minor routes and the main route was intravenous drug injection. The overall prevalence of HCV was 0.4%-0.6%, with higher rates among intravenous drug users and immigrants[30]. In central European countries, the prevalence of HCV was slightly higher than in the northern region. It varied from 0.6% in England, Luxemburg, Austria and Belgium to 1.5% in certain parts of Germany and Ireland[31]. High prevalence rates of HCV have been found in the southern European countries, particularly the southern regions of Spain, Portugal, Italy and Greece, which have the highest rates in Europe at 2.6%[32,33]. In Turkey, which is the European bridge to the oriental states, reported estimates range from 0.6% to 2.1%[34].

The HCV epidemic began later in Eastern European countries, particularly in the Czech Republic, Albania and Croatia. This delay is attributable to geographical barriers, limited immigration from neighboring endemic countries, and a delay in the increase in intravenous drug use[35]. The Czech Republic, Albania, Croatia, Estonia and Hungary are low-endemicity countries for HCV infection, where the prevalence rates range from 0.2% to 1%, but it was 1.4% in Latvia, Poland and Bulgaria. The highest prevalence was reported in Romania (3.3%), followed by Lithuania and Ukraine (2.3%). The Russian Federation and Baltic states have a high prevalence of HCV, ranging from 1.26% in the Republic of Belarus to 4.1% in some Russian states[36]. In the United States, Canada and Australia, the prevalence ranged from 0.61% to 1.8% and the number of people living with HCV is expected to continue to rise[37,38].

However, some studies indicated that there could be high rates of false positive results in HCV serological assays, as has been reported in Africa and China. In studies in Sub-Saharan Africa, such as in Uganda, Nigeria and the Republic of South Africa, the distinct variations in results were attributed to the wide variety of assays in use and to the different sample storage conditions[27,39]. Hence, future studies on HCV should give sufficient attention to testing strategies, sample handling and storage and include these details in the study reports.

Phylogenetic analysis of HCV genomes led to the

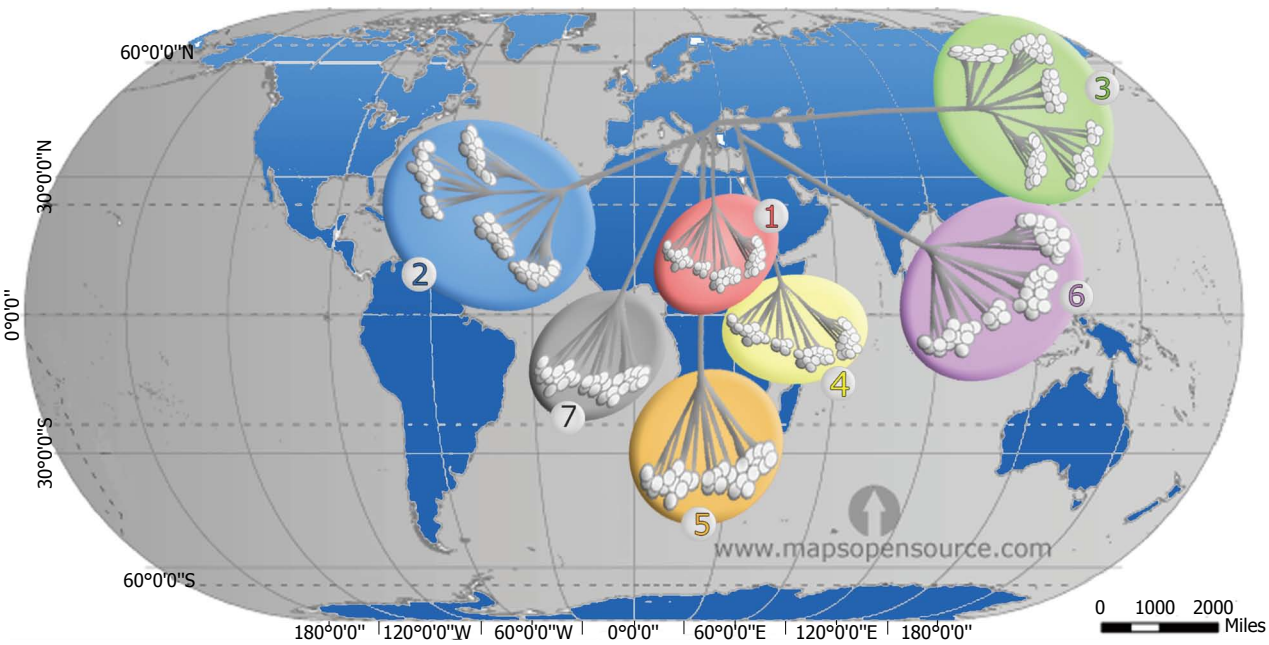

Figure 2 Geographical distributions of endemic pattern diversity of phylogenetic clades of hepatitis C virus (1-7).

development of a nomenclature for distinct virus types and subtypes, comprising seven recognized genotypes[1,7]. The genotypes differ at 30%-35% of nucleotide sites. The 7 genotypes are sub-classified into 67 subtypes plus 20 provisional subtypes. For strains of the same subtype, nucleotide differences are less than 15%[40,41]. However, while some genotypes are ubiquitous, others are found only in specific regions, where they exhibit a high diversity of subtypes (Figure 2). This distribution pattern and the antigenic and biological differences between the HCV types point to long periods of endemic infection during which there was no significant exchange with types from other regions[42].

Globally, genotype 1 accounts for more HCV infections (46.2%) than any other single genotype. Subtypes 1a and 1b account for 90% of all genotype 1 strains, at a ratio of 1:2 respectively. Over one-third of infections with genotype 1 are in East Asia. The next most common genotype is 3, which accounts for 30.1% of cases and is found mainly in southern Asia and in regions of Scandinavia. Genotypes 2, 4 and 6 are responsible for most of the remaining cases of HCV worldwide (9.1%, 8.3% and 5.4% of cases, respectively)[43]. Genotype 2 is predominant in West Africa, genotype 4 in Central and North Africa, and genotype 6 in Southeast Asia. Genotype 5 is responsible for < 1% of all HCV cases worldwide and is found mainly in South Africa. The more recently identified genotype 7 was isolated from a Congolese immigrant in Canada[40,44].

In China, genotype 1 is predominant (69.6%) and type lb is more prevalent than 2a, whereas genotypes 3b and 6 are seen mainly in the southern provinces[45]. In India, the most prevalent genotypes are 3 and 1 (64% and 28% of HCV infections, respectively); genotype 1b is responsible for 16% of all infections, genotype 4a accounts for the remaining infections and the least prevalent is genotype 5 (< 1%)[46]. In Japan, 70% of infections are due to genotype 1b and 20% are caused by genotype 2a; the remaining infections are caused by genotype 2b. HCV genotype 6 is more geographically restricted than genotypes 1-3. It is found in parts of East Asia (South China, Hong Kong, Taiwan and Macao) and Southeast Asia (Singapore, Vietnam, Thailand, Indonesia and Burma). There is no information on HCV genotypes in the highly populated countries of Bangladesh, Malaysia and North Korea[47].

In most European countries, the most prevalent HCV subtype is 1b, although subtype 1a is more prevalent among patients co-infected with human immunodeficiency virus (HIV). In North America, parts of South America, United Kingdom, Scandinavia and Australia, 1a is the most prevalent subtype[42]. In Greece, Poland and the Netherlands, subtype 3 is responsible for 30% of all cases, and in Russia and the Baltic States, subtypes 1b and 3a share dominance[48]. It is noteworthy that the first HCV recombinant, RF2k/1b, was initially identified in Russia but since then has also been identified in Ireland, Estonia, Uzbekistan and Cyprus[49].

In Africa, genotypes 1, 2 and 4 appear to be endemic in regions of West and Central Africa and in the Middle East-North African region. Genotype 5 is more prevalent in southern and eastern Sub-Saharan Africa. Genotype 4 is the most frequent cause of chronic hepatitis C in the Middle East, North Africa and Sub-Saharan Africa[50]. In Egypt, 90% of all HCV infections are caused by type 4[51]. The emergent genotype 7, which originated from Central Africa, is phylogenetically very similar to genotype 2 variants. The greatest

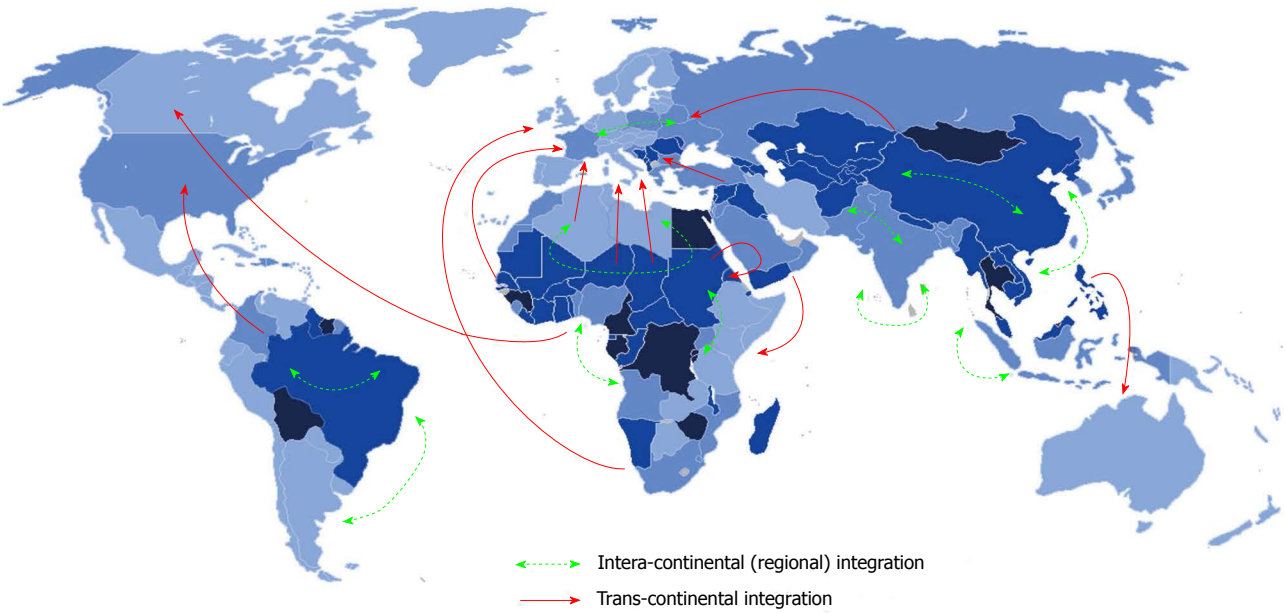

Figure 3 Plausible route of the integration of hepatitis C virus worldwide: The arrows indicate the probable route of spread (regional or transcontinental).

diversity of genotype 2 is observed in West Africa. It has been proposed that HCV genotype 2 originated from West Africa and then spread to the east[40]. Indeed, the finding of a new genotype further indicates the endemic nature of HCV in certain parts of Africa and again shows that HCV infection may have evolved from a common ancestor originating in this region of the world.

Geographic integration of HCV: The integration of HCV is influenced by host population size and density, the spatial distribution of the host, the frequency of contact between individuals, and other epidemiological factors. This is particularly evident in China and in many countries in Southeast Asia, as well as in Western Europe and Australia, possibly due to migration from Africa and/or Asia[45]. Endemic strains that are relatively rare have circulated for a long time in particular regions. Endemic strains of genotypes 1 and 2 are found mainly in West Africa, strains of genotype 3 in southern Asia, strains of genotype 4 in Central Africa and the Middle East, strains of genotypes 5 and 7 in southern Africa, and strains of genotype 6 in Southeast Asia. The global emergence of HCV has been either intra-continental or trans-continental (Figure 3). Nowadays, new strains of HCV have been reported in different regions and continents. They are due to surpass the commonly known strains resident in these places, particularly among high risk groups[52]. This evolving process of integration has played an important role in the spread of HCV and thus there is a need for developing specific strategies to combat the global spread of HCV infection.

Regional integration of HCV is seen in Africa and Asia. The endemic subtypes of HCV genotypes 1, 2 and 4, which are found mainly in geographically restricted areas of West Africa and Sub-Saharan Africa, are nowadays endemic in other parts of Africa. Egypt has the highest prevalence of HCV in the world (14.7%-32%) and has high rates of morbidity and mortality from chronic liver disease, cirrhosis and hepatocellular carcinoma. It is alarming that > 20% of Egyptian blood donors are seropositive for HCV. The dynamics of HCV in Egypt vary from one region to another. Desert areas have the lowest rates of anti-HCV positivity. The rates are higher in rural regions than in cities, and higher in the Nile Delta than in the Nile Valley[51]. Libya, neighboring Egypt, is considered an area of low endemicity for hepatitis C (1.2% average prevalence)[15]. The prevalence of HCV in Libya reaches its highest (1.6%) in the regions closer to Egypt and Sudan and lowest (0.2%) among other regions in the mid-coastal and western regions, where they resemble or are less than the rates in neighboring Tunisia[5]. The high prevalence rates in some parts of Libya could be due to its proximity to Sub-Saharan countries and the presence of large numbers of African immigrants. In the same way, the Albatnan region of Libya, which has a higher prevalence rate, borders Egypt, from where both legitimate and illegal workers come to Libya[5].

Most cases of HCV in Egypt are of genotype 4a. This strong homogeneity indicates epidemic spread of HCV. On the other hand, different genotypes were isolated from the Libyan population: Genotypes 1, 2, 3 and 4, as well as the newly emerged genotype 5. The prevalence of these genotypes in Libya varies from one region to another and is influenced by demographics and risk factors[53]. The dynamics of integration are applicable in Central Africa and East Africa, notably in Cameroon and Angola which have a high prevalence of HCV comparable to that in the Democratic Republic of Congo[54]. HCV seroprevalence was intermediate in the Horn of Africa: 2.7% in Ethiopia, 2.6% in Somalia and 0.3% in Djibouti[27]. In Southeast Asia, HCV genotype 6 integrated within northern countries, such

as Myanmar, Laos and Vietnam, while genotype 3 integrated in Thailand and Malaysia. In the island nations of Singapore, Indonesia and the Philippines, genotype 1 was the most prevalent. Similar integration dynamics have been observed in the Caribbean, India and the Baltic region[55].

The transcontinental integration of HCV is clearly mirrored between African and European countries, particularly around the Mediterranean regions of north African and southern European countries. The relatively high rates of HCV genotype 4 in southern Europe could be attributed to different factors: (1) the historic link between regions in southern Italy and Spain on the one hand and North Africa and the Middle East on the other hand; (2) the employment of multiple-use needles and glass syringes; and (3) the use of blood products that have not been tested for HCV. HCV genotype 4 seems to have recently spread from its endemic reservoir in Africa to southern Europe by immigrants. The prevalence rates of HCV type 4 have been rising in France, Italy, Greece and Spain[56]. In France, the prevalence of genotype 4 increased from 4% in 1990 to > 11% within one decade. In Europe, most patients infected with HCV genotype 4 are intravenous drug users or patients co-infected with HCV and HIV[57]. Recently, genotype 4 was shown to be the second most frequently detected genotype. One study identified genotype 4 in 23% of a large cohort of HIV-positive homosexual men from England, the Netherlands, France, Germany and Australia[58].

Another example of trans-continental integration is the existence of multiple types of "migrant clusters" of people who moved from West Africa to other regions of the world. These HCV types include the transfer of HCV-2e and 2f to Indonesia, HCV-2i to Morocco, France, Vietnam and Quebec, HCV-2j to Venezuela, HCV-2k to Martinique and France, HCV-2m to Vietnam, HCV-2r to Haiti and the Dominican Republic, and many unclassified type 2 lineages to Suriname[59]. The fact that genotype 2 is the most prevalent in West Africa, Europe, North America and parts of South America could reflect population dynamics resulting from the trans-Atlantic slave trade in the past and/or immigration, as illustrated by the identification of the new genotype 7 in Canada in an immigrant from Central Africa[40]. Figure 4 illustrates the integration dynamics of HCV genotypes all over the world. These trends clearly mirror the regional and global integration of HCV. Such a profile of interaction is associated with transmission and population dynamics of HCV and thus may reflect differences in when HCV infection occurred, which could influence the time of the peak burden of complications of HCV infection, such as cirrhosis and hepatocellular carcinoma. In view of these trends, regional differences in the prevalence of HCV genotypes and HCV epidemiology might have to be taken into consideration by tailoring prevention and treatment strategies to local needs.

Factors associated with HCV integration: HCV integration is a continuous dynamic phenomenon clearly influenced by population movements, demographic factors, clinical practice and personal behaviors in addition to the genetic entity of HCV. These global integration vectors play an important role in the spread of HCV worldwide. The outcomes of this integration coincide with the epidemiological evidence associated with immigration, trafficking and massive use of iatrogenic procedures and IDU, which escalated as early as the mid-twentieth century. The global prevalence of HCV is determined more by these social, behavioral and demographic factors than by genetic variation of the virus.

In 2013, 231 million people (3.2% of the world's population) migrated to new host nations. Migrants come mainly from developing countries in the south and migrate to the developed nations in North America and Western Europe. Migrants can be classified as immigrants, migrants and seasonal workers, refugees, asylum seekers, international students and others[60]. These newly emerged populations may suffer from infectious diseases usually more exotic or more prevalent in their own environment. These individuals come from regions in which HCV is endemic and thus pose a unique challenge to controlling the global prevalence of viral hepatitis[61]. A Canadian study from 2000 to 2007 compared immigrants with Canadian-born individuals and demonstrated high rates of HCV infection among immigrants. Compared to Canadian-born patients, immigrant patients were more likely to be female, non-white, older and to be infected with genotypes 4, 5 or 6[62]. United States studies on refugee populations found that the rates of HCV infection were up to 8% and only 1.8% among nationals[63].

One study estimated that 50% of HCV infections in the Netherlands are among immigrants, in whom the prevalence (2%) was tenfold higher than in the native population (0.2%)[61]. Likewise, the prevalence of chronic HCV in the United Kingdom among South Asians, and especially among migrants from Pakistan, may be as high as 2.7%, which is over fivefold higher than in the general population (0.5%)[64]. In Italy, the prevalence of HCV among Sub-Saharan refugees varied between 2.7% and 7.1%, while in Spain it was 12.5%, considerably higher than in the autochthonous population[65]. A recent study from Switzerland showed that the molecular epidemiology of HCV infection in low prevalence countries such as Switzerland is driven mainly by migration rather than by the distribution of virus genotypes in the native population[66].

The predominant HCV genotype in Middle Eastern and African countries is genotype 4. It is noteworthy that the prevalence of this genotype has been increasing in southern Europe, with region-specific increases in particular subtypes: 4a in Greece, 4d in Italy, 4c and 4d in Spain, and 4d in the Netherlands. The prevalence of genotype 4 among high-risk individuals in France increased from 15% of infections in 2003 to 22% in 2012. These changes in the trends of genotypes in these countries corresponds well with the increasing

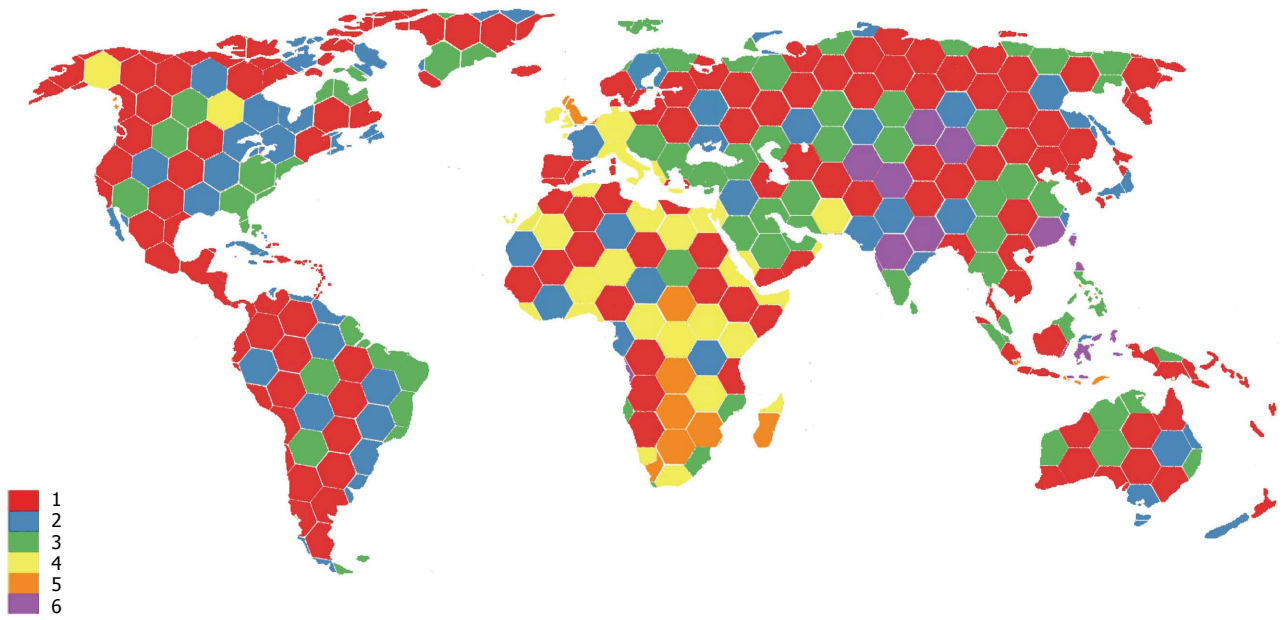

Figure 4 The integration complexity of hepatitis C virus worldwide: Virus genotypes mirroring the integration are shown in different colors.

numbers of migrants from Africa[64]. As the foreign-born populations expand, the integration dynamics of HCV will become globally imminent[67].

HCV transmission is closely associated with drug trafficking routes worldwide and HCV positivity is found among 15.6-98.7% of injection drug users. About 0.5% of the world's population injects drugs and of these about 6.8% are infected with HCV. Drug injection is the most common HCV transmission route and the main risk factor for acute and chronic hepatitis C (33.3% and 83.7%, respectively)[68].

The largest populations of people who inject drugs are found in China, Russia, the United States and Brazil, followed by Mexico, Pakistan and Thailand. People who inject drugs have a high seroprevalence of HCV in almost all European countries. This pattern is seen in Austria, Bulgaria, Cyprus, Greece and Romania and the rates are even higher in Latvia, Portugal, Turkey and Cyprus. The prevalence rate is < 30% only in the Czech Republic, Hungary and Slovenia. It is noteworthy that the rates of HCV seroprevalence have declined in Germany, France, the United Kingdom and Italy[69]. In most developing countries, transmission *via* drug injection is becoming more prevalent and replacing the iatrogenic and habitual transmission methods that have been reported for decades.

Intravenous drug use has become a predominant factor in the integration and transmission dynamics of HCV. This is clearly mirrored by the variations of the genotypes among these at-risk populations. The most commonly isolated genotypes worldwide from people who inject drugs are genotypes 1a and 3a. HCV subtype 3a is endemic in Southeast Asia and is spreading among intravenous drug users in the United States and Europe. An increase in the prevalence of genotypes 1a and 3a has been observed in Germany, France, Italy and Portugal. Mixed infections have been identified in some European countries: Italy (1b/3a), Germany (2a/3b) and Sweden (1a/1b)[70]. Eastern Europe (Russia and Estonia) and Central Asia have the largest drug epidemics globally, where the rapidly expanding HCV epidemic is associated with the injection of heroin and new synthetic or homemade drugs[71]. The frequency of genotype 3a is also rising in Eastern and Central European countries, as reported in Romania, Bulgaria, Poland and Serbia and Montenegro. It has been reported that people who use drugs in England are more likely to have genotype 3a in comparison to other risk groups, in whom genotype 1a is the most prevalent[71]. Thus, people who use drugs are important contributors to the spread of infections to the general population and drug trafficking is a key vector in the integration and diversification of HCV. This factor is boosted by other risk factors, such poverty, incarceration and HIV co-infection.

Worldwide, HCV and HIV are among the leading causes of death from infectious diseases[71]. Rates of co-infection with HCV and HIV range from 1.2% to 98.5% and co-infections are endemic, particularly in Asia and Africa[72]. In Europe and the United States, about one-fourth of HIV-infected individuals are co-infected with HCV. HCV infection outbreaks have been reported in HIV-positive men who have sex with men in North America, Europe and Asia. HCV co-infection reached up to 26.9% among heterosexuals compared with the HCV infection rate alone, which is reported to be 2.5%[1]. A cross-sectional study conducted between 2008 and 2010 in the Mazandaran province of Iran demonstrated that 33.8% of HIV-positive patients were co-infected with HCV and 25% were co-infected with both HBV and HCV[72]. The co-infection rates among intravenous drug users ranged between 58.2% and 91.6% and among commercial blood donors between 15.8% and

71.6%. These rates are higher than among those who become co-infected *via* sex (5.3%-20.0%). In Vietnam, between 89.8% and 98.5% of HIV-positive intravenous drug users are infected with HCV[73]. In China, 62.4% of HIV-infected individuals are seropositive for HCV. The co-infection rate is much lower in India (8.3%) and central, southeast and west African regions (4.9% to 8.5%) and much lower in North Africa (1.3%)[74].

Although HCV integration is clearly driven by migration, drug trafficking and HIV infection, there are many other additional factors: Health practices, hemodialysis, poverty, imprisonment and HBV/tuberculosis co-infections. A deeper understanding of HCV epidemiology should take into consideration all these factors. In developing countries, the routes of transmission of HCV are also found within medical care, such as the use of unsafe injections or improperly sterilized medical equipment, which are responsible for 40% of worldwide HCV infections, as well as the use of blood and blood products that have not been screened properly[1]. The prevalence of HCV infection in patients on maintenance hemodialysis reached 63% in the Arabian Peninsula (Kuwait, Saudi Arabia, Qatar and Yemen) and in China it was 41%. Moreover, hemodialysis patients who had blood transfusions were 5.65 times more likely to be infected with HCV than their counterparts who had no transfusions[1]. HCV co-infection among HBV-infected individuals ranged from 3% in Thailand to 22% in Japan, 23% in the United Kingdom and 30% in Spain[75]. HBV/HCV co-infection is more prevalent among the homeless, sexual assault victims and victims of intimate partner violence. Tuberculosis infection is also a major public health issue associated with HCV; it is responsible for more than a third of the opportunistic infections among drug users who are co-infected with HCV and HIV[75].

HCV infection can be acquired during travel for tourism or for medical treatment, particularly if the distant area is to the Indian subcontinent or Africa. Patients traveling abroad in general, and particularly if they are planning to have hemodialysis abroad, should be made aware of the risks and possibility of bringing new HCV genotypes to their homeland. Homosexuality is another prevailing factor for HCV transmission and outbreaks have been increasingly reported among men who have sex with men in Europe, Australia, Asia and the United States[76]. This is also emerging in developing countries, such as in the Arabian Peninsula, where such behavior is stigmatized. The spread of HCV may be influenced by habitual and social vulnerabilities between and within countries, urban and rural settings, and according to the burden of risk groups and economic status. This is clearly seen along the river Nile of Egypt and in China and Southeast Asian islands. Economic crises can influence HCV seroprevalence and the distribution of circulating genotypes. This was shown by outbreaks of HCV/HIV co-infections among intravenous drug users in Athens and Bucharest between 2011 and 2013, as both Greece and Bucharest have the highest unemployment rates among young people.

DISCUSSION

HCV is widely integrated, resulting in great heterogeneity both in the prevalence of infection and in the distribution of viral genotypes. This imposes a tremendous health burden globally in terms of morbidity and mortality. Hence, effective intervention requires a clear understanding of the dynamics of viral epidemics. Despite all scientific advances and the mounting knowledge of HCV, it remains "a hidden pandemic"[77]. HCV is considered to be endemic in developing countries, which have inadequate genotype data, and the largest populations of HCV-infected individuals are in Asia (accounting for 3.6% of the global population), followed by Africa (3.2% of the global population) and Latin America (1.4% of the global population)[15]. Most of these regions face many structural, cultural, societal and political obstacles in responding to this epidemic.

While preventive screening programs should be mandatory, 99 countries do not perform routine screening of blood donors for infectious agents that can be transmitted by transfusion. In other countries, the testing of blood donors for HIV, HBV and HCV is not consistent and attention is given mostly to HIV. Even where testing is done, there is preference for rapid assays with poor quality control and the result is lack of sensitivity. Testing based on nucleic acid is rare in countries with low or middle income due to lack of financial resources and skills. Even in Egypt, only 20% of the blood supply is tested by nucleic acid methods. Health authorities worldwide should give priority to this issue because any efforts to prevent or limit HCV transmission will not be effective unless the safety of blood and blood products is guaranteed. Moreover, most HCV infections are asymptomatic and in the absence of comprehensive, coordinated surveillance systems, the result is fragmented reporting and underestimation of the disease burden[1]. Improved surveillance is important not only for gaining a better understanding of the epidemiology of HCV but is also needed to identify population groups that should be targeted in prevention, testing and treatment programs.

Public healthcare systems, particularly in developing countries, should simplify service delivery to HCV-infected patients and specifically track progress to guarantee a high quality of services for both prevention and treatment[78]. People who inject drugs should have priority because prevalence estimates of HCV among this group is lacking in most countries. This is especially the case in countries with low or middle income, where data are scanty even for the general population[79]. Effective data collection and accurate reporting at the national level is the key in any program targeting HCV because it enables healthcare providers to implement policies targeting the populations that require the greatest attention.

Social and educational programs have to be promoted, particularly in countries that still ignore and stigmatize certain behaviors leading to HCV infection. All groups prone to HCV and associated co-infections should be included, whether they involve intravenous drug use, heroin sniffing or sexual promiscuity. Disseminating awareness of HCV infection in the general population can be beneficial in two ways. First, people who become aware exert pressure for provision of treatment. Second, promoting appropriate behavior helps to limit the risk of disease progression. Peer support has been proposed as one way to overcome these barriers. This is usually accompanied by screening as recommended by key United Nations agencies, such as the World Health Organization, United Nations Office on Drugs and Crime and Joint United Nations Program on HIV/AIDS. The high rates of HIV/HCV/TB co-infection in some settings indicate the need for another approach to increase access to HCV care, namely, to move towards an integrated policy resembling those used for HIV and tuberculosis[1,79].

A national action plan accompanied with national guidelines for the treatment of HCV infection should be advocated. Targeted populations should be provided with equal access to medical care, reliable supplies of medications and medical follow-up. Nowadays, it is possible to treat almost every person with HCV regardless of liver disease stage, viral genotype, past therapies and comorbidities. However, this approach imposes a heavy burden on the health system, particularly in developing countries where it is most needed. Research and operational projects supported by international funds should be established particularly in low-income countries[80].

Sensitivity and bias analysis

Sequential omission was performed for sensitivity analysis and we consider our data reliable. We observed no publication bias. The main limitation we noticed is the lack of specific data on certain aspects contributing to HCV integration, such as spatial and social factors associated with the global spread of HCV. Hence, further studies are needed to overcome such limitations.

Conclusion

From all that is described and discussed above, we conclude the following: HCV is an integrating dynamic threat and no country can be considered safe enough. Despite awareness of the spread of HCV infections, epidemiological data remain scarce. This study highlights the need for integrated cooperative actions at the local, regional and global levels if the spread and burden of hepatitis C virus are to be contained. Governments, the scientific community, industry and non-governmental organizations should develop a cooperation framework for combating HCV infection. Priority should be directed to help low or middle income countries to gain access to effective screening and medical care for HCV and associated infectious diseases.

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COMMENTS

Background

Hepatitis C virus (HCV) infection is a major public health threat and its geo-epidemiology varies widely worldwide and over time. HCV genotypes and the risk of infection can be easily transmitted to any country and cannot be restricted to certain regions. Therefore, new evaluations of the epidemiology of HCV should be considered.

Research frontiers

Geographical integration is an epidemiological phenomenon that highlights the spread of HCV globally. Globalization, immigration and drug trafficking are important driving forces in this integration.

Innovations and breakthroughs

Worldwide HCV integration is a newly described phenomenon that can be either intra-continental or trans-continental.

Applications

Geographical integration should be taken into consideration in both prevention and treatment policies for HCV. National and international strategies should be designed on the basis of accurate analysis of HCV epidemiology.

Terminology

Intra-continental (regional) integration: The dynamic spread and dissemination of HCV genotypes within a certain region or continent is clearly evident in Africa and Asia; Trans-continental integration: The dynamic spread of HCV from one continent to another. This is clearly evident in the Mediterranean basin (Africa-Europe), Africa-America.

Peer-review

This is a very well-conceived, lucid, informative mini review that should be shared with the scientific community. The readers will gain valuable insight into the evolution of HCV at the global level.

REFERENCES

1 **Daw MA**, Dau AA. Hepatitis C virus in Arab world: a state of concern. *ScientificWorldJournal* 2012; **2012**: 719494 [PMID: 22629189]

2 **Tanaka K**, Hirohata T, Koga S, Sugimachi K, Kanematsu T, Ohryohji F, Nawata H, Ishibashi H, Maeda Y, Kiyokawa H. Hepatitis C and hepatitis B in the etiology of hepatocellular carcinoma in the Japanese population. *Cancer Res* 1991; **51**: 2842-2847 [PMID: 1851661]

3 **Deuffic-Burban S**, Mohamed MK, Larouze B, Carrat F, Valleron AJ. Expected increase in hepatitis C-related mortality in Egypt due to pre-2000 infections. *J Hepatol* 2006; **44**: 455-461 [PMID: 16310281 DOI: 10.1016/j.jhep.2005.08.008]

4 **Daw MA**, Dau AA, Agnan MM. Influence of healthcare-associated factors on the efficacy of hepatitis C therapy. *ScientificWorldJournal* 2012; **2012**: 580216 [PMID: 23346018]

5 **Daw MA**, El-Bouzedi A. Prevalence of hepatitis B and hepatitis C infection in Libya: results from a national population based survey. *BMC Infect Dis* 2014; **14**: 17 [PMID: 24405790 DOI:

10.1186/1471-2334-14-17]
6 **Kauhl B**, Heil J, Hoebe CJ, Schweikart J, Krafft T, Dukers-Muijrers NH. The Spatial Distribution of Hepatitis C Virus Infections and Associated Determinants--An Application of a Geographically Weighted Poisson Regression for Evidence-Based Screening Interventions in Hotspots. *PLoS One* 2015; **10**: e0135656 [PMID: 26352611 DOI: 10.1371/journal.pone.0135656]
7 **Wedemeyer H**, Duberg AS, Buti M, Rosenberg WM, Frankova S, Esmat G, Örmeci N, Van Vlierberghe H, Gschwantler M, Akarca U, Aleman S, Balık I, Berg T, Bihl F, Bilodeau M, Blasco AJ, Brandão Mello CE, Bruggmann P, Calinas F, Calleja JL, Cheinquer H, Christensen PB, Clausen M, Coelho HS, Cornberg M, Cramp ME, Dore GJ, Doss W, El-Sayed MH, Ergör G, Estes C, Falconer K, Félix J, Ferraz ML, Ferreira PR, García-Samaniego J, Gerstoft J, Giria JA, Gonçales FL, Guimarães Pessôa M, Hézode C, Hindman SJ, Hofer H, Husa P, Idilman R, Kåberg M, Kaita KD, Kautz A, Kaymakoglu S, Krajden M, Krarup H, Laleman W, Lavanchy D, Lázaro P, Marinho RT, Marotta P, Mauss S, Mendes Correa MC, Moreno C, Müllhaupt B, Myers RP, Nemecek V, Øvrehus AL, Parkes J, Peltekian KM, Ramji A, Razavi H, Reis N, Roberts SK, Roudot-Thoraval F, Ryder SD, Sarmento-Castro R, Sarrazin C, Semela D, Sherman M, Shiha GE, Sperl J, Stärkel P, Stauber RE, Thompson AJ, Urbanek P, Van Damme P, van Thiel I, Vandijck D, Vogel W, Waked I, Weis N, Wiegand J, Yosry A, Zekry A, Negro F, Sievert W, Gower E. Strategies to manage hepatitis C virus (HCV) disease burden. *J Viral Hepat* 2014; **21** Suppl 1: 60-89 [PMID: 24713006 DOI: 10.1111/jvh.12249]
8 **Moher D**, Liberati A, Tetzlaff J, Altman DG. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *J Clin Epidemiol* 2009; **62**: 1006-1012 [PMID: 19631508 DOI: 10.1016/j.jclinepi.2009.06.005]
9 **Choo QL**, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 1989; **244**: 359-362 [PMID: 2523562 DOI: 10.1126/science.2523562]
10 **Hajarizadeh B**, Grebely J, Dore GJ. Epidemiology and natural history of HCV infection. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 553-562 [PMID: 23817321 DOI: 10.1038/nrgastro.2013.107]
11 **An Y**, Wu T, Wang M, Lu L, Li C, Zhou Y, Fu Y, Chen G. Conservation in China of a novel group of HCV variants dating to six centuries ago. *Virology* 2014; **464-465**: 21-25 [PMID: 25043585 DOI: 10.1016/j.virol.2014.06.011]
12 **Volz EM**, Koelle K, Bedford T. Viral phylodynamics. *PLoS Comput Biol* 2013; **9**: e1002947 [PMID: 23555203 DOI: 10.1371/journal.pcbi.1002947]
13 **Njouom R**, Nerrienet E, Dubois M, Lachenal G, Rousset D, Vessière A, Ayouba A, Pasquier C, Pouillot R. The hepatitis C virus epidemic in Cameroon: genetic evidence for rapid transmission between 1920 and 1960. *Infect Genet Evol* 2007; **7**: 361-367 [PMID: 17137845 DOI: 10.1016/j.meegid.2006.10.003]
14 **Markov PV**, van de Laar TJ, Thomas XV, Aronson SJ, Weegink CJ, van den Berk GE, Prins M, Pybus OG, Schinkel J. Colonial history and contemporary transmission shape the genetic diversity of hepatitis C virus genotype 2 in Amsterdam. *J Virol* 2012; **86**: 7677-7687 [PMID: 22573865 DOI: 10.1128/JVI.06910-11]
15 Transmission of hepatitis C virus. *Ann Intern Med* 1990; **113**: 411-412 [PMID: 2116749 DOI: 10.7326/0003-4819-113-5-411]
16 **Xia GL**, Liu CB, Cao HL, Bi SL, Zhan MY, Su CA, Nan JH, Qi XQ. Prevalence of hepatitis B and C virus infections in the general Chinese population. Results from a nationwide cross-sectional seroepidemiologic study of hepatitis A, B, C, D, and E virus infections in China, 1992. *International Hepatology Communications* 1996; **5**: 62-73 [DOI: 10.1016/S0928-4346(96)82012-3]
17 **Sun CA**, Chen HC, Lu CF, You SL, Mau YC, Ho MS, Lin SH, Chen CJ. Transmission of hepatitis C virus in Taiwan: prevalence and risk factors based on a nationwide survey. *J Med Virol* 1999; **59**: 290-296 [PMID: 10502258 DOI: 10.1002/(SICI)1096-9071(199911)59:]
18 **Kim YS**, Pai CH, Chi HS, Kim DW, Min YI, Ahn YO. Prevalence of hepatitis C virus antibody among Korean adults. *J Korean Med Sci* 1992; **7**: 333-336 [PMID: 1284374 DOI: 10.3346/jkms.1992.7.4.333]
19 **Leung N**, Chu C, Tam JS. Viral hepatitis C in Hong Kong. *Intervirology* 2006; **49**: 23-27 [PMID: 16166785 DOI: 10.1159/000087259]
20 **Nishioka K**, Watanabe J, Furuta S, Tanaka E, Iino S, Suzuki H, Tsuji T, Yano M, Kuo G, Choo QL. A high prevalence of antibody to the hepatitis C virus in patients with hepatocellular carcinoma in Japan. *Cancer* 1991; **67**: 429-433 [PMID: 1845946 DOI: 10.1002/1097-0142(19910115)67]
21 **Nguyen VT**, McLaws ML, Dore GJ. Prevalence and risk factors for hepatitis C infection in rural north Vietnam. *Hepatol Int* 2007; **1**: 387-393 [PMID: 19669334 DOI: 10.1007/s12072-007-9008-3]
22 **Chowdhury A**, Santra A, Chaudhuri S, Dhali GK, Chaudhuri S, Maity SG, Naik TN, Bhattacharya SK, Mazumder DN. Hepatitis C virus infection in the general population: a community-based study in West Bengal, India. *Hepatology* 2003; **37**: 802-809 [PMID: 12668973 DOI: 10.1053/jhep.2003.50157]
23 **Raja NS**, Janjua KA. Epidemiology of hepatitis C virus infection in Pakistan. *J Microbiol Immunol Infect* 2008; **41**: 4-8 [PMID: 18327420]
24 **Rajabali A**, Moin O, Ansari AS, Khanani MR, Ali SH. Communicable disease among displaced Afghans: refuge without shelter. *Nat Rev Microbiol* 2009; **7**: 609-614 [PMID: 19609262 DOI: 10.1038/nrmicro2176]
25 **Alavian SM**, Adibi P, Zali MR. Hepatitis C virus in Iran: Epidemiology of an emerging infection. *Arch Iranian Med* 2005; **8**: 84-90
26 **Merat S**, Rezvan H, Nouraie M, Jafari E, Abolghasemi H, Radmard AR, Zaer-rezaii H, Amini-Kafiabad S, Maghsudlu M, Pourshams A, Malekzadeh R, Esmaili S. Seroprevalence of hepatitis C virus: the first population-based study from Iran. *Int J Infect Dis* 2010; **14** Suppl 3: e113-e116 [PMID: 20362479 DOI: 10.1016/j.ijid.2009.11.032]
27 **Riou J**, Aït Ahmed M, Blake A, Vozlinsky S, Brichler S, Eholié S, Boëlle PY, Fontanet A. Hepatitis C virus seroprevalence in adults in Africa: a systematic review and meta-analysis. *J Viral Hepat* 2016; **23**: 244-255 [PMID: 26477881 DOI: 10.1111/jvh.12481]
28 **Daw MA**, Elkaber MA, Drah AM, Werfalli MM, Mihat AA, Siala IM. Prevalence of hepatitis C virus antibodies among different populations of relative and attributable risk. *Saudi Med J* 2002; **23**: 1356-1360 [PMID: 12506296]
29 **Kershenobich D**, Razavi HA, Sánchez-Avila JF, Bessone F, Coelho HS, Dagher L, Gonçales FL, Quiroz JF, Rodriguez-Perez F, Rosado B, Wallace C, Negro F, Silva M. Trends and projections of hepatitis C virus epidemiology in Latin America. *Liver Int* 2011; **31** Suppl 2: 18-29 [PMID: 21651701 DOI: 10.1111/j.1478-3231.2011.02538.x]
30 **Fitzsimons D**, François G, Alpers K, Radun D, Hallauer J, Jilg W, Gerlich W, Rombo L, Blystad H, Nøkleby H, van Damme P. Prevention of viral hepatitis in the Nordic countries and Germany. *Scand J Infect Dis* 2005; **37**: 549-560 [PMID: 16099768 DOI: 10.1080/00365540510043284]
31 **Bosetti C**, Levi F, Boffetta P, Lucchini F, Negri E, La Vecchia C. Trends in mortality from hepatocellular carcinoma in Europe, 1980-2004. *Hepatology* 2008; **48**: 137-145 [PMID: 18537177 DOI: 10.1002/hep.22312]
32 **Guadagnino V**, Stroffolini T, Rapicetta M, Costantino A, Kondili LA, Menniti-Ippolito F, Caroleo B, Costa C, Griffo G, Loiacono L, Pisani V, Focà A, Piazza M. Prevalence, risk factors, and genotype distribution of hepatitis C virus infection in the general population: a community-based survey in southern Italy. *Hepatology* 1997; **26**: 1006-1011 [PMID: 9328327 DOI: 10.1002/hep.510260431]
33 **Sánchez-Quijano A**, Abad MA, Torronteras R, Rey C, Pineda JA, Leal M, Macias J, Lissen E. Unexpected high prevalence of hepatitis C virus genotype 4 in Southern Spain. *J Hepatol* 1997; **27**: 25-29 [PMID: 9252069 DOI: 10.1016/S0168-8278(97)80275-9]
34 **Dursun M**, Ozekinci T, Ertem M, Saka G, Yilmaz S, Canoruc F, Celenk S, Celik M, Paşa S, Aydin K. Prevalence of Hepatitis C in adults in the south-eastern region of Anatolia: a community-based study. *Hepatol Res* 2004; **29**: 75-80 [PMID: 15163428 DOI: 10.1016/j.hepres.2004.02.012]
35 **Naoumov NV**. Hepatitis C virus infection in Eastern Europe. *J Hepatol* 1999; **31** Suppl 1: 84-87 [PMID: 10622566 DOI: 10.1016/

S0168-8278(99)80380-8]
36 **Maksyutov RA**, Gavrilova EV, Maksyutov AZ, Kanev AN. Genotyping of hepatitis B and C virus Russian isolates for reference serum panel construction. *J Med Virol* 2015; **87**: 1192-1198 [PMID: 25758235 DOI: 10.1002/jmv.24170]
37 **Edlin BR**, Eckhardt BJ, Shu MA, Holmberg SD, Swan T. Toward a more accurate estimate of the prevalence of hepatitis C in the United States. *Hepatology* 2015; **62**: 1353-1363 [PMID: 26171595 DOI: 10.1002/hep.27978]
38 **Zou S**, Tepper M, Giulivi A. Current status of hepatitis C in Canada. *Can J Public Health* 2000; **91** Suppl 1: S10-S15, S10-S15 [PMID: 11059123]
39 **Cui Y**, Jia J. Update on epidemiology of hepatitis B and C in China. *J Gastroenterol Hepatol* 2013; **28** Suppl 1: 7-10 [PMID: 23855289 DOI: 10.1111/jgh.12220]
40 **Murphy DG**, Sablon E, Chamberland J, Fournier E, Dandavino R, Tremblay CL. Hepatitis C virus genotype 7, a new genotype originating from central Africa. *J Clin Microbiol* 2015; **53**: 967-972 [PMID: 25520447 DOI: 10.1128/JCM.02831-14]
41 **Smith DB**, Bukh J, Kuiken C, Muerhoff AS, Rice CM, Stapleton JT, Simmonds P. Expanded classification of hepatitis C virus into 7 genotypes and 67 subtypes: updated criteria and genotype assignment web resource. *Hepatology* 2014; **59**: 318-327 [PMID: 24115039 DOI: 10.1002/hep.26744]
42 **Dusheiko G**, Schmilovitz-Weiss H, Brown D, McOmish F, Yap PL, Sherlock S, McIntyre N, Simmonds P. Hepatitis C virus genotypes: an investigation of type-specific differences in geographic origin and disease. *Hepatology* 1994; **19**: 13-18 [PMID: 8276349 DOI: 10.1002/hep.1840190104]
43 **Pybus OG**, Barnes E, Taggart R, Lemey P, Markov PV, Rasachak B, Syhavong B, Phetsouvanah R, Sheridan I, Humphreys IS, Lu L, Newton PN, Klenerman P. Genetic history of hepatitis C virus in East Asia. *J Virol* 2009; **83**: 1071-1082 [PMID: 18971279 DOI: 10.1128/JVI.01501-08]
44 **Jeannel D**, Fretz C, Traore Y, Kohdjo N, Bigot A, Pê Gamy E, Jourdan G, Kourouma K, Maertens G, Fumoux F, Fournel JJ, Stuyver L. Evidence for high genetic diversity and long-term endemicity of hepatitis C virus genotypes 1 and 2 in West Africa. *J Med Virol* 1998; **55**: 92-97 [PMID: 9598927 DOI: 10.1002/(SICI)1096-9071(199806)55:]
45 **Zhou Y**, Wang X, Mao Q, Fan Y, Zhu Y, Zhang X, Lan L, Jiang L, Tan W. Changes in modes of hepatitis C infection acquisition and genotypes in southwest China. *J Clin Virol* 2009; **46**: 230-233 [PMID: 19729342 DOI: 10.1016/j.jcv.2009.08.003]
46 **Narahari S**, Juwle A, Basak S, Saranath D. Prevalence and geographic distribution of Hepatitis C Virus genotypes in Indian patient cohort. *Infect Genet Evol* 2009; **9**: 643-645 [PMID: 19460332 DOI: 10.1016/j.meegid.2009.04.001]
47 **Bennett H**, Waser N, Johnston K, Kao JH, Lim YS, Duan ZP, Lee YJ, Wei L, Chen CJ, Sievert W, Yuan Y, Li H. A review of the burden of hepatitis C virus infection in China, Japan, South Korea and Taiwan. *Hepatol Int* 2015; **9**: 378-390 [PMID: 26071238 DOI: 10.1007/s12072-015-9629-x]
48 **Olinger CM**, Lazouskaya NV, Eremin VF, Muller CP. Multiple genotypes and subtypes of hepatitis B and C viruses in Belarus: similarities with Russia and western European influences. *Clin Microbiol Infect* 2008; **14**: 575-581 [PMID: 18373690 DOI: 10.1111/j.1469-0691.2008.01988.x]
49 **Karchava M**, Waldenström J, Parker M, Hallack R, Sharvadze L, Gatserelia L, Chkhartishvili N, Dvali N, Dzigua L, Dolmazashvili E, Norder H, Tsertsvadze T. High incidence of the hepatitis C virus recombinant 2k/1b in Georgia: Recommendations for testing and treatment. *Hepatol Res* 2015; **45**: 1292-1298 [PMID: 25689487 DOI: 10.1111/hepr.12505]
50 **Iles JC**, Raghwani J, Harrison GL, Pepin J, Djoko CF, Tamoufe U, LeBreton M, Schneider BS, Fair JN, Tshala FM, Kayembe PK, Muyembe JJ, Edidi-Basepeo S, Wolfe ND, Simmonds P, Klenerman P, Pybus OG. Phylogeography and epidemic history of hepatitis C virus genotype 4 in Africa. *Virology* 2014; **464-465**: 233-243 [PMID: 25105489 DOI: 10.1016/j.virol.2014.07.006]
51 **Medhat A**, Shehata M, Magder LS, Mikhail N, Abdel-Baki L, Nafeh M, Abdel-Hamid M, Strickland GT, Fix AD. Hepatitis c in a community in Upper Egypt: risk factors for infection. *Am J Trop Med Hyg* 2002; **66**: 633-638 [PMID: 12201604]
52 **Ciccozzi M**, Lo Presti A, Ciccaglione AR, Zehender G, Ciotti M. Phylogeny and phylodinamic of Hepatitis C in Italy. *BMC Infect Dis* 2012; **12** Suppl 2: S5 [PMID: 23173700 DOI: 10.1186/1471-2334-12-S2-S5]
53 **Daw MA**, El-Bouzedi A, Dau AA. Geographic distribution of HCV genotypes in Libya and analysis of risk factors involved in their transmission. *BMC Res Notes* 2015; **8**: 367 [PMID: 26293137 DOI: 10.1186/s13104-015-1310-x]
54 **Njouom R**, Caron M, Besson G, Ndong-Atome GR, Makuwa M, Pouillot R, Nkoghé D, Leroy E, Kazanji M. Phylogeography, risk factors and genetic history of hepatitis C virus in Gabon, central Africa. *PLoS One* 2012; **7**: e42002 [PMID: 22870274 DOI: 10.1371/journal.pone.0042002]
55 **Chao DT**, Abe K, Nguyen MH. Systematic review: epidemiology of hepatitis C genotype 6 and its management. *Aliment Pharmacol Ther* 2011; **34**: 286-296 [PMID: 21623850 DOI: 10.1111/j.1365-2036.2011.04714.x]
56 **Chiaramonte M**, Pupo A, Menegon T, Baldo V, Malatesta R, Trivello R. HBV and HCV infection among non-European Union immigrants in North-East Italy. *Epidemiol Infect* 1998; **121**: 179-183 [PMID: 9747770 DOI: 10.1017/S0950268898001034]
57 **Peters L**, Klein MB. Epidemiology of hepatitis C virus in HIV-infected patients. *Curr Opin HIV AIDS* 2015; **10**: 297-302 [PMID: 26248117 DOI: 10.1097/COH.0000000000000183]
58 **Roman F**, Hawotte K, Struck D, Ternes AM, Servais JY, Arendt V, Hoffman P, Hemmer R, Staub T, Seguin-Devaux C, Schmit JC. Hepatitis C virus genotypes distribution and transmission risk factors in Luxembourg from 1991 to 2006. *World J Gastroenterol* 2008; **14**: 1237-1243 [PMID: 18300350 DOI: 10.3748/wjg.14.1237]
59 **Tokita H**, Okamoto H, Iizuka H, Kishimoto J, Tsuda F, Lesmana LA, Miyakawa Y, Mayumi M. Hepatitis C virus variants from Jakarta, Indonesia classifiable into novel genotypes in the second (2e and 2f), tenth (10a) and eleventh (11a) genetic groups. *J Gen Virol* 1996; **77** (Pt 2): 293-301 [PMID: 8627233 DOI: 10.1099/0022-1317-77-2-293]
60 **Greenaway C**, Thu Ma A, Kloda LA, Klein M, Cnossen S, Schwarzer G, Shrier I. The Seroprevalence of Hepatitis C Antibodies in Immigrants and Refugees from Intermediate and High Endemic Countries: A Systematic Review and Meta-Analysis. *PLoS One* 2015; **10**: e0141715 [PMID: 26558905 DOI: 10.1371/journal.pone.0141715]
61 **Manzardo C**, Treviño B, Gómez i Prat J, Cabezos J, Monguí E, Clavería I, Luis Del Val J, Zabaleta E, Zarzuela F, Navarro R. Communicable diseases in the immigrant population attended to in a tropical medicine unit: epidemiological aspects and public health issues. *Travel Med Infect Dis* 2008; **6**: 4-11 [PMID: 18342267 DOI: 10.1016/j.tmaid.2007.11.002]
62 **Gushulak BD**, MacPherson DW. Globalization of infectious diseases: the impact of migration. *Clin Infect Dis* 2004; **38**: 1742-1748 [PMID: 15227621 DOI: 10.1086/421268]
63 **Redditt VJ**, Janakiram P, Graziano D, Rashid M. Health status of newly arrived refugees in Toronto, Ont: Part 1: infectious diseases. *Can Fam Physician* 2015; **61**: e303-e309 [PMID: 26175381]
64 **Owiti JA**, Greenhalgh T, Sweeney L, Foster GR, Bhui KS. Illness perceptions and explanatory models of viral hepatitis B & amp; C among immigrants and refugees: a narrative systematic review. *BMC Public Health* 2015; **15**: 151 [PMID: 25886390 DOI: 10.1186/s12889-015-1476-0]
65 **García Comas L**, Ordobás Gavín M, Sanz Moreno JC, Ramos Blázquez B, Gutiérrez Rodríguez A, Astray Mochales J, Moreno Guillén S. Prevalence of hepatitis C antibodies in the population aged 16-80 years in the Community of Madrid 2008-2009. *J Med Virol* 2015; **87**: 1697-1701 [PMID: 25989026 DOI: 10.1002/jmv.24219]
66 **Hirzel C**, Wandeler G, Owczarek M, Gorgievski-Hrisoho M, Dufour JF, Semmo N, Zürcher S. Molecular epidemiology of hepatitis B virus infection in Switzerland: a retrospective cohort study. *BMC Infect Dis* 2015; **15**: 483 [PMID: 26518625 DOI: 10.1186/s12879-015-1234-z]
67 **Wandeler G**, Dufour JF, Bruggmann P, Rauch A. Hepatitis C: a

changing epidemic. *Swiss Med Wkly* 2015; **145**: w14093 [PMID: 25658972 DOI: 10.4414/smw.2015.14093]

68 **Matheï C**, Buntinx F, van Damme P. Seroprevalence of hepatitis C markers among intravenous drug users in western European countries: a systematic review. *J Viral Hepat* 2002; **9**: 157-173 [PMID: 12010503 DOI: 10.1046/j.1365-2893.2002.00339.x]

69 **Pierce RD**, Hegle J, Sabin K, Agustian E, Johnston LG, Mills S, Todd CS. Strategic information is everyone's business: perspectives from an international stakeholder meeting to enhance strategic information data along the HIV Cascade for people who inject drugs. *Harm Reduct J* 2015; **12**: 41 [PMID: 26471018 DOI: 10.1186/s12954-015-0073-y]

70 **Ruta S**, Cernescu C. Injecting drug use: A vector for the introduction of new hepatitis C virus genotypes. *World J Gastroenterol* 2015; **21**: 10811-10823 [PMID: 26478672 DOI: 10.3748/wjg.v21.i38.10811]

71 **Platt L**, Bobrova N, Rhodes T, Uusküla A, Parry JV, Rüütel K, Talu A, Abel K, Rajaleid K, Judd A. High HIV prevalence among injecting drug users in Estonia: implications for understanding the risk environment. *AIDS* 2006; **20**: 2120-2123 [PMID: 17053361 DOI: 10.1097/01.aids.0000247586.23696.20]

72 **Babamahmoodi F**, Heidari Gorji MA, Mahdi Nasehi M, Delavarian L. The prevalence rate of hepatitis B and hepatitis C co-infection in HIV positive patients in Mazandaran province, Iran. *Med Glas* (Zenica) 2012; **9**: 299-303 [PMID: 22926367]

73 **Zhang L**, Celentano DD, Le Minh N, Latkin CA, Mehta SH, Frangakis C, Ha TV, Mo TT, Sripaipan T, Davis WW, Quan VM, Go VF. Prevalence and correlates of HCV monoinfection and HIV and HCV coinfection among persons who inject drugs in Vietnam. *Eur J Gastroenterol Hepatol* 2015; **27**: 550-556 [PMID: 25769097 DOI: 10.1097/MEG.0000000000000321]

74 **Solomon SS**, Srikrishnan AK, Mehta SH, Vasudevan CK, Murugavel KG, Thamburaj E, Anand S, Kumar MS, Latkin C, Solomon S, Celentano DD. High prevalence of HIV, HIV/hepatitis C virus coinfection, and risk behaviors among injection drug users in Chennai, India: a cause for concern. *J Acquir Immune Defic Syndr* 2008; **49**: 327-332 [PMID: 18845962 DOI: 10.1097/QAI.0b013e3181831e85]

75 **Gao X**, Cui Q, Shi X, Su J, Peng Z, Chen X, Lei N, Ding K, Wang L, Yu R, Wang N. Prevalence and trend of hepatitis C virus infection among blood donors in Chinese mainland: a systematic review and meta-analysis. *BMC Infect Dis* 2011; **11**: 88 [PMID: 21477324 DOI: 10.1186/1471-2334-11-88]

76 **Götz HM**, van Doornum G, Niesters HG, den Hollander JG, Thio HB, de Zwart O. A cluster of acute hepatitis C virus infection among men who have sex with men--results from contact tracing and public health implications. *AIDS* 2005; **19**: 969-974 [PMID: 15905679 DOI: 10.1097/01.aids.0000171412.61360.f8]

77 **Dalgard O**, Mauss S. No strategy to meet the HCV epidemic. *BMC Infect Dis* 2014; **14** Suppl 6: S2 [PMID: 25253032 DOI: 10.1186/1471-2334-14-S6-S2]

78 **Daw MA**, Shabash A, El-Bouzedi A, Dau AA, Habas M. Modelling the prevalence of hepatitis C virus amongst blood donors in Libya: An investigation of providing a preventive strategy. *World J Virol* 2016; **5**: 14-22 [PMID: 26870670 DOI: 10.5501/wjv.v5.i1.14]

79 **Harris M**, Albers E, Swan T. The promise of treatment as prevention for hepatitis C: Meeting the needs of people who inject drugs? *Int J Drug Policy* 2015; **26**: 963-969 [PMID: 26143385 DOI: 10.1016/j.drugpo.2015.05.005]

80 **Daw MA, El-Bouzedi A, Ahmed MO, Dau AA, Agnan MM.** Epidemiology of Hepatitis C Virus and Genotype Distribution in Immigrants Crossing to Europe from North and Sub-Saharan Africa. *Travel Med Infect Dis* 2016; **14**: 517-526[PMID: 27502972 DOI: 10.1016/j.tmaid.2016.05.020]

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CASE REPORT

Spread of human immunodeficiency virus 1 among men who have sex with men is emerging as a genuine social concern and affecting the general populace - case reports from Eastern India

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Author contributions: Chatterjee A, Sarkar A and Ansari S designed the report; Siddhanta S, Banerjee S and Sarkar R collected the patients' clinical samples; Chatterjee A and Chakraborty N analyzed the data and wrote the paper.

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Abstract

Human immunodeficiency virus (HIV) infection among men who have sex with men (MSM) has increased to a drastic proportion throughout India in the last couple of years due to a lack of productive identification and management framework. In apprehension of social disgrace these men attempt to live a normal hetero conjugal life and, in the process, act as a bridge in spreading the virus to their women partners. In this case report we have highlighted two cases which clearly distinguished the adequacy of HIV treatment among MSM when they are diagnosed during early or late phases of infection. An intensive and ample counseling to comprehend the psychology and sexual behavior of these men was found to be critically important in both the cases. Our study, which is actually the first of its kind, recorded and documented evidence of HIV infected MSM from Eastern India and renders a ray of hope among this marginally isolated group to comprehend the challenges and health risks faced by the MSM population. It also provides a format for the medical practitioners here in managing and treating related cases.

Key words: Human immunodeficiency virus; Men who have sex with men; Tuberculosis; Human cytomegalovirus

Core tip: The role of men having sex with men (MSMs) in the transmission and spread of the human immunodeficiency virus infection among the general population has been an active area of debate for the last few years. This case report highlights the grave weight of this issue from an Indian standpoint and describes the health risks and related treatment procedures concerning these men. Another major point touched by this article concerns those MSMs who in fear of social stigma and try to live a normal hetero conjugal life and in the process act as a bridge in spreading the virus to their women partners.

Chatterjee A, Sarkar A, Ansari S, Siddhanta S, Banerjee S, Sarkar R, Chakraborty N. Spread of human immunodeficiency virus 1 among men who have sex with men is emerging as a genuine social concern and affecting the general populace - case reports from Eastern India. *World J Virol* 2016; 5(4): 183-188 Available from: URL: http://www.wjgnet.com/2220-3249/full/v5/i4/183.htm DOI: http://dx.doi.org/10.5501/wjv.v5.i4.183

INTRODUCTION

Human immunodeficiency virus (HIV) infection among men who have sex with men (MSM) has been expanding drastically around the globe, especially in Asia. This worldwide pattern is being found in India, with the current evaluated HIV predominance among MSM extending somewhere around 7% and 16.5%[1]. This is an major cause of concern in light of recent HIV counteractive endeavours that have been drastically extended across the country, bringing up second thoughts about whether extra measures are required to capture the spread of HIV in this populace. In Mumbai, 12% of MSM looking for deliberate testing and medical advice were found to be HIV seropositive, while 18% of the MSM screened in Andhra Pradesh were observed to be infected[2]. In another study, it was discovered that close to 8% of the reported MSM's were seropositive[3].

Despite the fact that discoveries from the Independent Impact Assessment Study demonstrate that the National AIDS Control Program (NACP) has been consistently trying to end the HIV scourge in India over the period 2007-2012, current intervention measures for HIV transmission among MSM include single estimation modalities and thus fail to resolve the delicate problems associated with this socially marginalized group in India[4]. To deal with this issue, there is a requirement for far-reaching, multi-layered approaches that effectively counter the HIV aversion among these men. The idea concerning the sexual character of MSM in India can be fluctuating and fluid. Since people may change their self-perception with time and behaviours might be situational, crediting particular behaviours ascribed to these men is difficult and constraining. In the majority of these people, same-sex behaviour does not block having sex with women or taking part in conventional marriages. Thus the expression "MSM" is not used to depict someone's sexual identity but to identify his characteristic behavior.

An essential inconvenience in getting the related MSM information is that homosexuality is concealed in silence to a vast degree in India both on account of social standards and on the grounds that it is unlawful. Homosexuality in India was formally classified by lawful code Section 377 which, until recently, made sexual relations between two men a criminal offence. Endeavours are progressing to attempt legalization of homosexuality in India; however these have not been successful yet. In light of the fact that numerous MSM in India don't transparently share information on their sexual behaviour, this has brought about meager information about their sex conduct and its setting. Without this clear knowledge, it is hard to arrange successful MSM-related HIV counteractive action. With regards to this disproportionally abnormal state of HIV risk, it is critical to comprehend the socio-behavioural components that may worsen sexual danger among this population. MSM in India encounter different types of social and legal discrimination. It is this pervasive narrow-mindedness of society alongside the social pressure for men to take part in heterosexual marital relations that have driven numerous MSM to marry and have children. Numerous MSM participate in unprotected anal and vaginal sex with multiple male and female sexual partners. MSM in India may play a "connecting" role in the spread of HIV among the general population. Other studies have additionally discussed that disgrace and stigma contribute to the development of one's negative self-images and low self-regard, depression, expanded sexual risk behavior and/or diminished utilization of HIV prevention services. The silent riddle connected with institutional disgrace and separation may generate ideal conditions for the drastic acceleration of the AIDS epidemic. The social stigma among these people may arise from healthcare providers, employers and other administrative workers. These difficulties create genuine obstacles against successful HIV services procurement; segregation can hinder access to HIV and sexual healthcare administrations and relative prevention programs. A comprehensive understanding of the issues around disgrace and discrimination would help MSM cross the obstructions connected with disgrace concerning sexual risk, disclosure issues and access to human health services.

CASE REPORT

Case 1

A 40-year-old man suffering from severe diarrhoea, fever, drastic weight loss and nausea was admitted to the Department of General Medicine, Calcutta Medical College and Hospital, Eastern India. His HIV serostatus was ascertained by an ELISA (HIV ELISA, Rapid test)

and Western blot as recommended by the National Aids Control Organization (NACO), Ministry of Health and Family Welfare, Government of India. The fourth generation HIV detection test performed with the patient's blood confirmed severe HIV infection. HIV blood viral load was found to be > 1 × 10^5 copies/mL. $CD4^+$ T-cell count was 32 and $CD8^+$ T cell count was 516 (Ratio-0.06). The man was confirmed with a diagnosis of AIDS, the final stage of HIV infection. Upon rigorous interrogation the man confirmed that he was primarily diagnosed as HIV seropositive at another government hospital "a few years back" (no documentation) but he did not think of complying with the physician's advice or to follow up and lived a "normal" life. A thorough risk review and rigorous counselling revealed that the patient was homosexual and had sexual contacts (unprotected anal intercourse) with multiple men (probably the cause of HIV infection). But due to the fear of social stigma he never revealed his sexual preference or HIV status to anyone and continued to live a "straight" life with his wife as a heterosexual man. His wife was also diagnosed with HIV seropositivity.

Upon admission, the patient showed high fever with a significant evening rise, severe headache, rapid seizures, and drastic weight loss. The patient admitted to suffering from nausea, difficulty breathing, seizures and vomiting for quite a considerable time span. He also had a dry cough with mild expectoration and mild chest pain. The patient was mildly febrile with the presence of mild pallor, slightly anaemic, and had low blood pressure (106/58 mmHg) with a pulse rate of 110 beats/min. Clinical examination revealed acute lymphadenopathy.

On detailed examination, the man was found to have severe syphilis with chronic genital ulceration, acute pulmonary tuberculosis and human cytomegalovirus (HCMV) retinitis. HCMV IgM was detected in the patient's blood, followed by confirmation of HCMV DNA by PCR detection. Real-time PCR estimated the viral load of HCMV to be 5.9 × 10^6 DNA copies/mL of serum. Indirect ophthalmoscopic examination revealed the presence of retinal haemorrhage with a hardened peripheral lesion, characteristic of HCMV retinitis. Cerebrospinal fluid culture, Mantoux test and sputum culture confirmed the presence of *Mycobacterium tuberculosis*. Test for cryptococcal capsular antigen turned out to be negative. Head CT scan of the patient revealed abnormal enhancement of the basal cisternae. Chest X-ray and bronchoscopy showed bilateral lung infiltration with non-specific diffused interstitial pneumonitis.

No clear evidence of cerebral palsy was observed at neurological presentation but slight neck rigidity was ascertained. A complete blood profile analysis of the patient was recorded and is provided in Table 1. The patient showed severe cachexia and very poor nutritional index with a BMI of only 15.8.

After confirmation, the patient was immediately put under highly active anti retroviral therapy (HAART) therapy (zidovudine, lamivudine and nevirapine) for 2 mo. After 4 wk of treatment and a limited $CD4^+$ count increase (CD4-54), the patient was immediately started on a combination based antitubercular drug (ATD) therapy as Directly Observed Therapy Short-course (DOTS - Cat 1 regimen) along with dexamethasone as corticosteroid therapy (0.4 mg/kg per day) and pyridoxine 40 mg/d on a planned 4 wk regime. Along with the ATD, the patient was also administered valganciclovir tablet 450 mg once daily as maintenance therapy against HCMV infection. For treating genital syphilis the patient was given an intramuscular injection of benzathine penicillin G (2.4 million units) once daily for 2 wk. But even after another 2 wk treatment, the patient failed to show any significant improvements with regard to his health conditions. He suddenly developed severe respiratory distress, spasms and his fever relapsed. He also complained of a gradual dimness of vision and total visual blurring. He was put under ventilation and immediate respiratory support. After few days he succumbed to the veracity of the infections and died due to multi-organ failure.

Table 1 A detailed clinical blood profile analysis of the two patients before start of treatment

Factors	Patient 1	Patient 2
CD4 count	32	232
CD4:CD8 ratio	0.06	0.25
HIV viral load	> 1 × 10^5 copies/mL	50000 copies/mL
Haemoglobin	9.1 g%	9.5 g%
TC	7100 cells/mm^3	7000 cells/mm^3
Neutrophils	48%	52%
Lymphocyte	34%	24%
Eosinophil	12%	2%
Monocyte	12%	2%
Basophil	2%	1%
Platelets	270000/mL	200000/mL
Blood sugar (fasting)	117 mg/dL	109 mg/dL
Urea	48 mg/dL	38 mg/dL
Creatinine	1.1 mg/dL	2.1 mg/dL
Bilirubin	0.6 mg/dL	1.2 mg/dL
SGOT	129 IU/L	119 IU/L
SGPT	54 IU/L	47 IU/L
Alkaline phosphatase	225 IU/L	185 IU/L
Albumin	3.9 g/dL	2.9 g/dL
Globulin	3.7 g/dL	3.5 g/dL

SGOT: Serum glutamic-oxaloacetic transaminase; SGPT: Serum glutamic-pyruvate transaminase.

Case 2

A 21-year-old man suffering from fever and flu-like symptoms along with severe diarrhoea and abdominal cramping was admitted to the Department of General Medicine, Calcutta Medical College and Hospital, Eastern India. His HIV serostatus was found to be positive by performing HIV ELISA, Rapid test and Western Blot as recommended by the NACO, Ministry of Health and Family Welfare, Government of India. The fourth generation HIV detection test performed with the patient's blood confirmed HIV seropositivity. HIV blood viral load was found to be 50000 copies/mL. $CD4^+$ T-cell count was 232 and $CD8^+$ T cell count was

920 (ratio-0.25). The man was diagnosed with acute or latent HIV infection. Upon rigorous counselling the patient admitted to being a homosexual and involved in unprotected anal sex with multiple male partners for quite a few years. He had never been sexually involved with any women. Due to the fear of social isolation, he never revealed his sexual preference to anyone. On detailed examination, he was found to be infected with genital syphilis and oral ulceration. Upon admission, the patient showed high fever, severe headache, seizures, vomiting and severe diarrhoea. He also had a dry cough with no expectoration at all but complained of a mild pain on the right side of the chest. The patient was slightly anaemic, and had low blood pressure (115/68 mmHg) with a pulse rate of 92 beats/min.

No evidence of cerebral palsy or any other neurological involvement was ascertained. There was no sign of lymphadenopathy. A complete blood profile analysis of the patient was recorded and provided in Table 1. The patient showed poor nutritional index. Liver function was found to be highly deranged with elevated levels of both SGOT and SGPT. Hepatic cholestasis was adequately observed by USG of the abdomen.

After confirmed HIV diagnosis the patient was started on HAART (zidovudine, lamivudine and nevirapine). Nevivir (200 mg) and lamistar were administered daily for 8 wk. Septran DS and feronia were given orally daily for 2 wk. After 2 mo the patient showed significant signs of improvement with much-relaxed breathing and no abdominal cramping. Cholestasis was found to be resolving gradually. $CD4^+$ cell count increased to almost double (CD 4-454) and HIV load in the patient's blood decreased uniformly (< 10000 copies/mL). As he responded actively without any genotoxic side effects towards the treatment, the anti-retroviral therapy was carried on and he was kept under observation. The doctors clearly made him understand the implications of latent HIV infection and transmission and also discussed the importance of regular medication to keep the disease under control.

DISCUSSION

In the latest United Nations General Millennium development agenda on HIV/AIDS-Goal 6, it has been reported that the percentage of individuals living with HIV/AIDS globally has diminished by 40% up till the end of 2013. However, in a study corresponding to the MSM population in India who had undergone HIV testing in the past 12 mo at different survey locations across the nation, variable results ranging from 3%-67% were observed[5]. In 2009, 46.3% of MSM in Tamil Nadu had tested positive for HIV while the HIV pervasiveness in a study from Mumbai was 12.5% with 14% of these men reporting STD side effects[6]. Only 68% of the positively tested MSM returned to gather their test reports. The above information demonstrates the inability and failure of the health councils of India to legitimately comprehend the requirements and problems of this socially marginalized group as well as the dereliction in garnering their trust[7]. HIV infection among MSM has been increasing in an exponential manner throughout India in the last few years due to the absence of efficient identification and productive management systems[8]. Because of the United Nations sustainable developmental goals (Goal 3), a much higher percentage of HIV-infected people are receiving antiretroviral therapy now.

In trepidation of social stigma, these MSMs attempt to render a normal hetero marital life and in the process act as a bridge in spreading the virus to their women partners. In this case report, we have highlighted two cases which obviously distinguishes the adequacy of HIV treatment among MSM when they are diagnosed during early or late phases of infection. A thorough and ample counseling to understand the psychology and sexual behaviour of these men were found to be vital in both cases. With the advent of highly active antiretroviral therapy (HAART), which is a customized combination of different classes of retroviral medications that a physician prescribes based on patient's viral load, the particular strain of the virus, the $CD4^+$ cell count, and disease symptoms etc treating HIV has become much easier. There is a partial recuperation of the host immune framework portrayed by a significant rise in the number of $CD4^+$ T lymphocytes and this leads to a decrease in AIDS-related mortality.

Early identification and appropriate treatment are of most extreme significance to battle HIV infection. Be that as it may, in a developing nation like India, subjects having a place with a lower financial strata with no or poor educational foundation and no knowledge of HIV/AIDS, are mostly diagnosed late over the span of the disease, just like the instance of the first patient whose HIV status was recognized surprisingly when his $CD4^+$ T-cell count had gone down too low. The vast majority of the patients (like in our case) intentionally ignore the doctor's advice and do not follow up routinely. Thus due to the absence of updated information, awareness and the fear of transcendent social disgrace, a large fraction of subjects are diagnosed very late. Many of them remain reluctant to approach a doctor about their condition and look for medical guidance and consideration[9]. The most imperative reason for mortality in the case of patients who have low $CD4^+$ T-cell number (or have developed AIDS) is the advancement of a few end organ diseases (EODs). These EODs are caused by different opportunist infections (OIs) which have been left untreated as an after effect of late HIV diagnosis inciting a progressive failure of the immune framework[10].

This case report ideally documents the medical conditions and disease transmission history of two HIV-1 infected homosexual men from Eastern India. The first patient was diagnosed very late with his CD4 T cell count plunging to as low as 32. Due to his own negligence and in fear of social stigma he never disclosed his sexual preference or HIV status with anyone. Despite having

multiple male sexual partners he continued to live with his wife and had sexual relations with her. As a matter of fact, he transmitted the virus to his wife. When he was admitted to the hospital he was suffering from multiple infections (both bacterial and viral), lying almost at the verge of death. HAART would have helped him if he had sought restorative medical help earlier.

Whether the cause be the narrow mindset of society with respect to homosexuals, misconstruing the graveness of the issue by the patient or insufficient knowledge among the people about HIV, ultimately the truth is that the clinical improvement of the subject could have been accomplished only if he had been treated earlier. The second patient, a young homosexual man, somehow comprehended the gravity of the circumstances and got himself diagnosed in a timely manner with complete support from his family. He was lucky enough that the HAART responded actively for him. He survived and hopefully will be cautious enough not to transmit the virus to anyone else. To address this serious issue the government should immediately develop a nationwide programme to screen all MSM for HIV and enroll the positive HIV cases for intense treatment.

Our study, which is actually a documented evidence of HIV-infected MSM from Eastern India, provides a ray of hope among this marginally isolated group in India to understand the difficulties and health risks faced by the MSM population and provides a format for medical practitioners in dealing with and treating related cases.

COMMENTS

Case characteristics

Case 1: A 40-year-old male suffering from severe diarrhoea, fever, drastic weight loss and nausea was admitted for treatment; Case 2: A 21-year-old man suffering from fever and flu-like symptoms along with severe diarrhoea and abdominal cramping was admitted for treatment.

Clinical diagnosis

Case 1: The patient showed high fever with a significant evening rise, severe headache, rapid seizures, drastic weight loss, nausea, difficulty breathing and vomiting. He also had a dry cough with mild expectoration and mild chest pain. The patient was mildly febrile with presence of mild pallor, slightly anaemic, and had low blood pressure (106/58 mmHg) with a pulse rate of 110 beats/min. Clinical examination revealed acute lymphadenopathy; Case 2: The patient showed high fever, severe headache, seizures, vomiting and severe diarrhea. He also had a dry cough with no expectoration at all but complained of a mild pain on the right side of the chest. The patient was slightly anaemic, and had low blood pressure (115/68 mmHg) with a pulse rate of 92 beats/min.

Differential diagnosis

Both patients were diagnosed with severe human immunodeficiency virus (HIV) infection. The first patient was in the final stage characterized by acquired immune deficiency syndrome, infected by other opportunist pathogens like syphilis, tuberculosis and human cytomegalovirus (HCMV). The second patient was in an early stage of HIV seropositivityand mainly suffering from bacterial lung infection.

Laboratory diagnosis

All labs were within normal limits.

Pathological diagnosis

HIV1 infection was diagnosed in both the patients using standard protocol by PCR, ELISA, 4TH generation test suggested by National Aids Control Organization. HCMV and *M. tuberculosis* were identified by ELISA, culture and PCR.

Treatment

Highly active anti retroviral therapy was given to both the patients. Valganciclovir was given to treat HCMV infection. DOTS was administered to treat tuberculosis.

Related reports

The role of men having sex with men (MSMs) in the transmission and spread of the HIV infection among the general population has been an active area of debate for the last few years. The main aim of this study is to share the actual scenario in an economically poor resource setting concerning homosexual men who, in fear of social stigma, try to live a normal hetero conjugal life and in the process act as a bridge in spreading the virus to their women partners.

Term explanation

MSM are highly prone to develop HIV infection due to unprotected sex, but in fear of social boycott suppress their identity thereby act as vectors to spread the disease among other individuals.

Experiences and lessons

In this article, authors have discussed two cases which clearly distinguished the adequacy of HIV treatment among MSM when they are diagnosed during early or late phases of infection. Thorough counseling to understand the psychology and sexual behavior of these men was found to be very important in both the cases. In fear of social stigma these men try to render a normal hetero conjugal life and in the process act as a bridge in spreading the virus to their women partners.

Peer-review

The manuscript is well written and well presented. In this article the authors presented 2 cases, one MSM who did not take HIV infection seriously and died. The second case was a young MSM, starting treatment led to an improvement in the patient's condition. The paper is suited well for publication in the journal.

REFERENCES

1 **Jaffe HW**, Valdiserri RO, De Cock KM. The reemerging HIV/AIDS epidemic in men who have sex with men. *JAMA* 2007; **298**: 2412-2414 [PMID: 18042919 DOI: 10.1001/jama.298.20.2412]

2 **Thomas B**, Mimiaga MJ, Menon S, Chandrasekaran V, Murugesan P, Swaminathan S, Mayer KH, Safren SA. Unseen and unheard: predictors of sexual risk behavior and HIV infection among men who have sex with men in Chennai, India. *AIDS Educ Prev* 2009; **21**: 372-383 [PMID: 19670971 DOI: 10.1521/aeap.2009.21.4.372]

3 **Setia MS**, Brassard P, Jerajani HR, Bharat S, Gogate A, Kumta S, Row-Kavi A, Anand V, Boivin JF. Men who have sex with men in India: a systematic review of the literature. *J LGBT Health Res* 2008; **4**: 51-70 [PMID: 19856739 DOI: 10.1080/15574090902913727]

4 **Baral S**, Sifakis F, Cleghorn F, Beyrer C. Elevated risk for HIV infection among men who have sex with men in low- and middle-income countries 2000-2006: a systematic review. *PLoS Med* 2007; **4**: e339 [PMID: 18052602 DOI: 10.1371/journal.pmed.0040339]

5 Emerging Gay Identities in India - Implications for Sexual Health: Report on a conference held in Bombay, organised by Humsafar Trust and sponsored by Naz Project, 1994

6 **Mishra RM**, Dube M, Sahu D, Saggurti N, Pandey A. Changing epidemiology of HIV in Mumbai: an application of the Asian epidemic model. *Glob J Health Sci* 2012; **4**: 100-112 [PMID: 22980382 DOI: 10.5539/gjhs.v4n5p100]

7 **Dandona L**, Dandona R, Gutierrez JP, Kumar GA, McPherson S, Bertozzi SM. Sex behaviour of men who have sex with men and risk of HIV in Andhra Pradesh, India. *AIDS* 2005; **19**: 611-619 [PMID: 15802980 DOI: 10.1097/01.aids.0000163938.01188.e4]

8 **Go VF**, Srikrishnan AK, Sivaram S, Murugavel GK, Galai N, Johnson SC, Sripaipan T, Solomon S, Celentano DD. High HIV prevalence and risk behaviors in men who have sex with men in

Chennai, India. *J Acquir Immune Defic Syndr* 2004; **35**: 314-319 [PMID: 15076248 DOI: 10.1097/00126334-200403010-00014]

9 **Gupta A**, Mehta S, Godbole SV, Sahay S, Walshe L, Reynolds SJ, Ghate M, Gangakhedkar RR, Divekar AD, Risbud AR, Mehendale SM, Bollinger RC. Same-sex behavior and high rates of HIV among men attending sexually transmitted infection clinics in Pune, India (1993-2002). *J Acquir Immune Defic Syndr* 2006; **43**: 483-490 [PMID: 17019372 DOI: 10.1097/01.qai.0000243097.27029.b7]

10 National AIDS Control Orgainsation, Department of AIDS Control, Ministry of Health and Family Welfare, Government of India. HIV Sentinel Surveillance 2010-2011: A Technical Brief. New Delhi: Government of India, 2011

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