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World Journal of Virology (*World J Virol*, *WJV*, online ISSN 2220-3249, DOI: 10.5501) is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

WJV covers topics concerning arboviral infections, bronchiolitis, central nervous system viral diseases, DNA virus infections, encephalitis, eye infections, fatigue syndrome, hepatitis, meningitis, opportunistic infections, pneumonia, RNA virus infections, sexually transmitted diseases, skin diseases, slow virus diseases, tumor virus infections, viremia, zoonoses, and virology-related traditional medicine, and integrated Chinese and Western medicine. Priority publication will be given to articles concerning diagnosis and treatment of viral diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

We encourage authors to submit their manuscripts to *WJV*. We will give priority to manuscripts that are supported by major national and international foundations and those that are of great basic and clinical significance.

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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

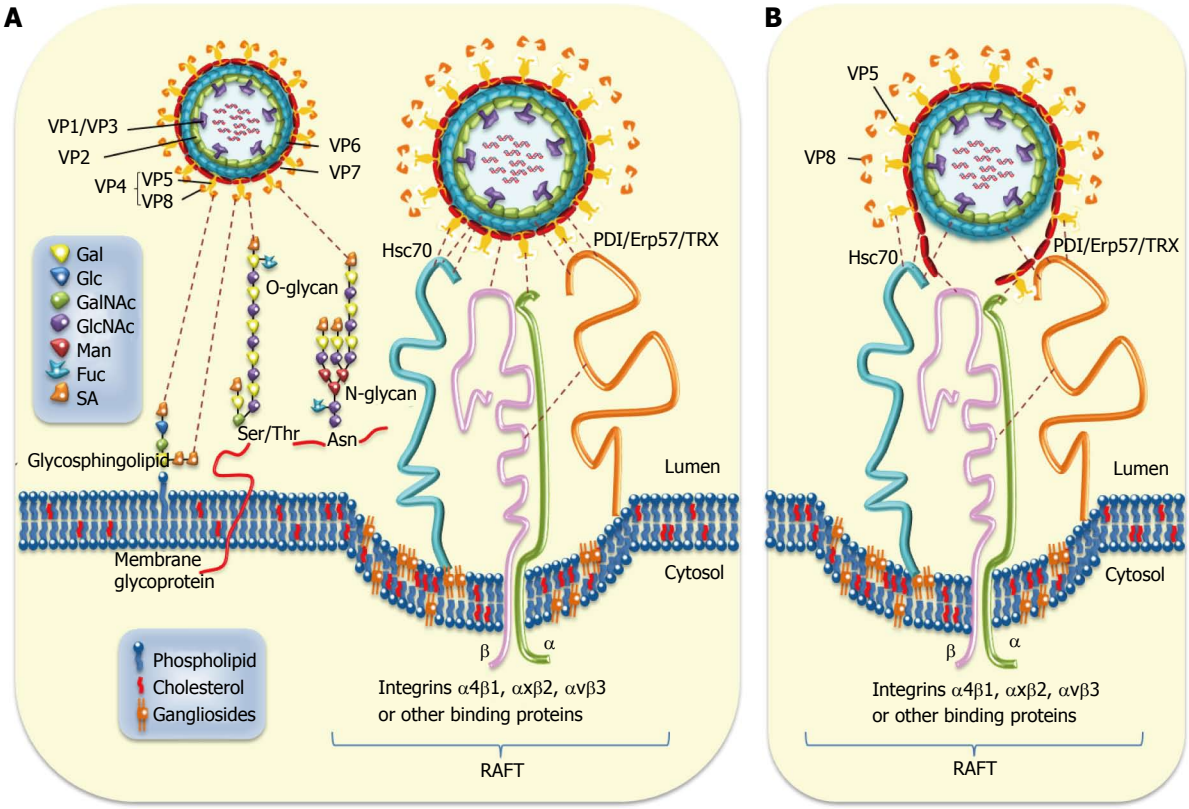

Figure 1 Rotavirus-cell surface interactions during entry. A: The rotavirus particle-associated proteins (VP1/2/3/4/5/6/7) that enclose the viral genome are represented. Cell surface molecules including sialic acid (SA), Hsc70, PDI, Erp57, thioredoxin (TRX), and integrins $\alpha4\beta1$, $\alpha x\beta2$, and $\alpha v\beta3$ 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 $\alpha2\beta1$ through VP5* DGE sequence. SA-dependent strains bind first through VP8* to SA before interacting with integrin $\alpha2\beta1$ through VP5*. A putative caveolae containing raft-associated cell surface receptors is depicted. The sequence of virion-cell interactions taking place after binding to $\alpha2\beta1$ 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 $\alpha4\beta1$, $\alpha x\beta2$ and $\alpha v\beta3$) 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 $\alpha v\beta3$) 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 $\alpha2\beta1$ could undergo conformational changes associated to its trimerization[10,116]. Integrin $\alpha2\beta1$ 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

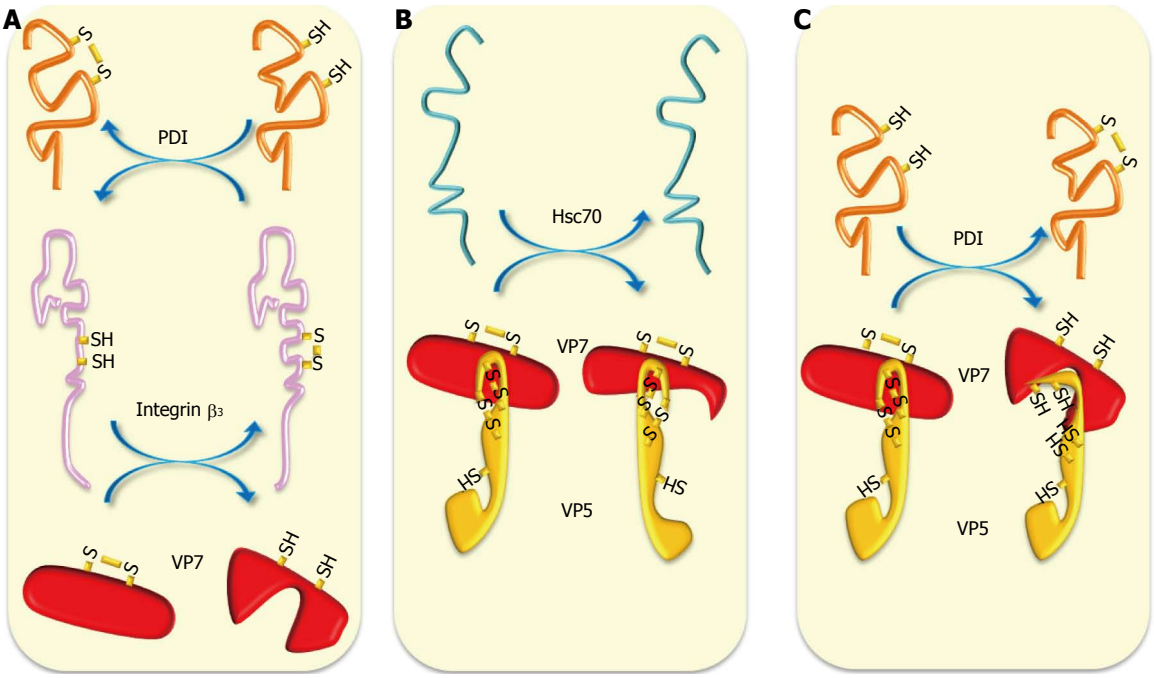

Figure 2 Schematic representation of chaperone and oxidoreduction activities during rotavirus entry. A: Cell surface reducing PDI has been shown to form complexes with integrins α2β1 and αvβ3 to generate free thiols in these integrins. Reducing integrin β3 can reduce thiol-disulfide bonds present in 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

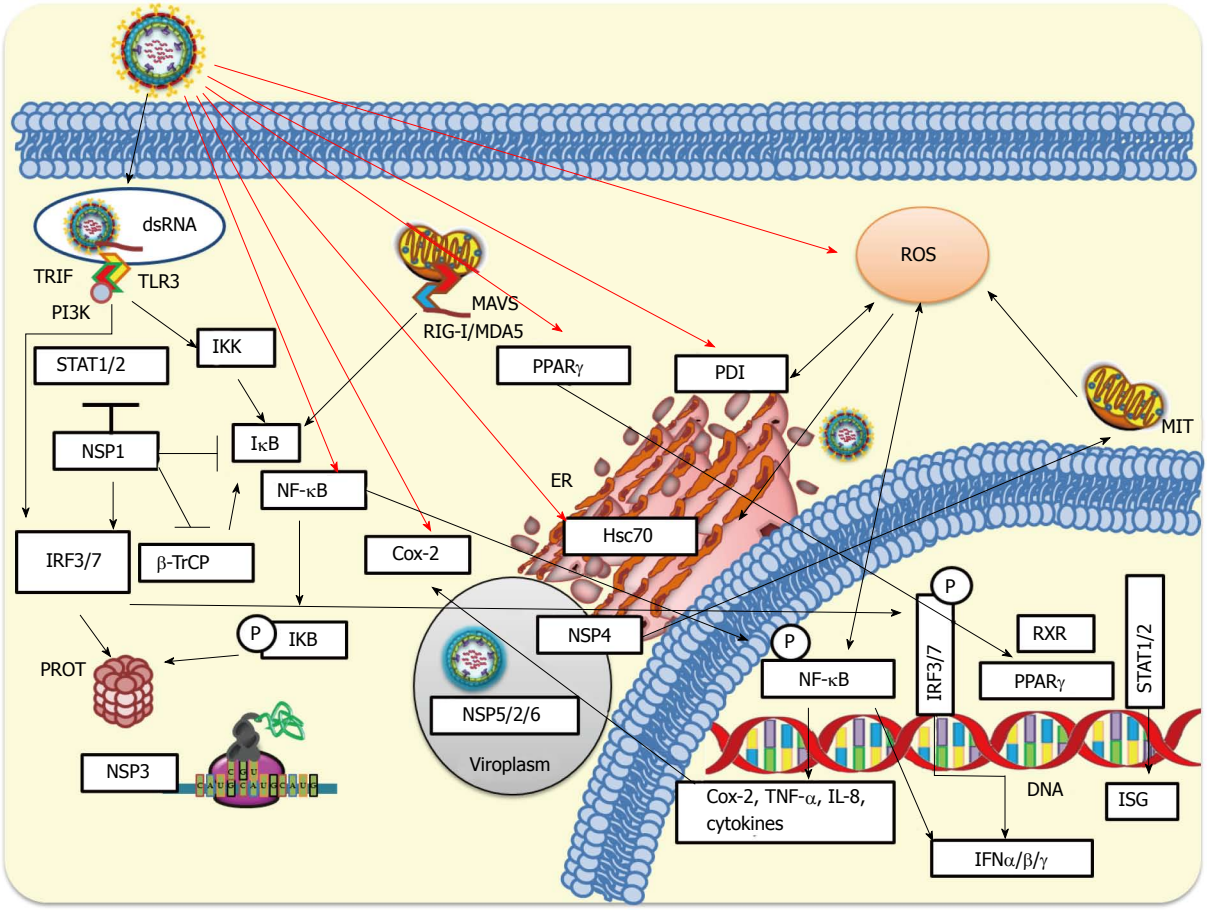

Figure 3 Cellular innate response to rotavirus infection. During rotavirus internalization viral nucleic acid may be exposed and recognized by either Toll-like receptors (TLR3) or intracellular RIG-I-like receptors (RLRs). Activated RLRs can bind and activate mitochondrial antiviral-signaling protein (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,

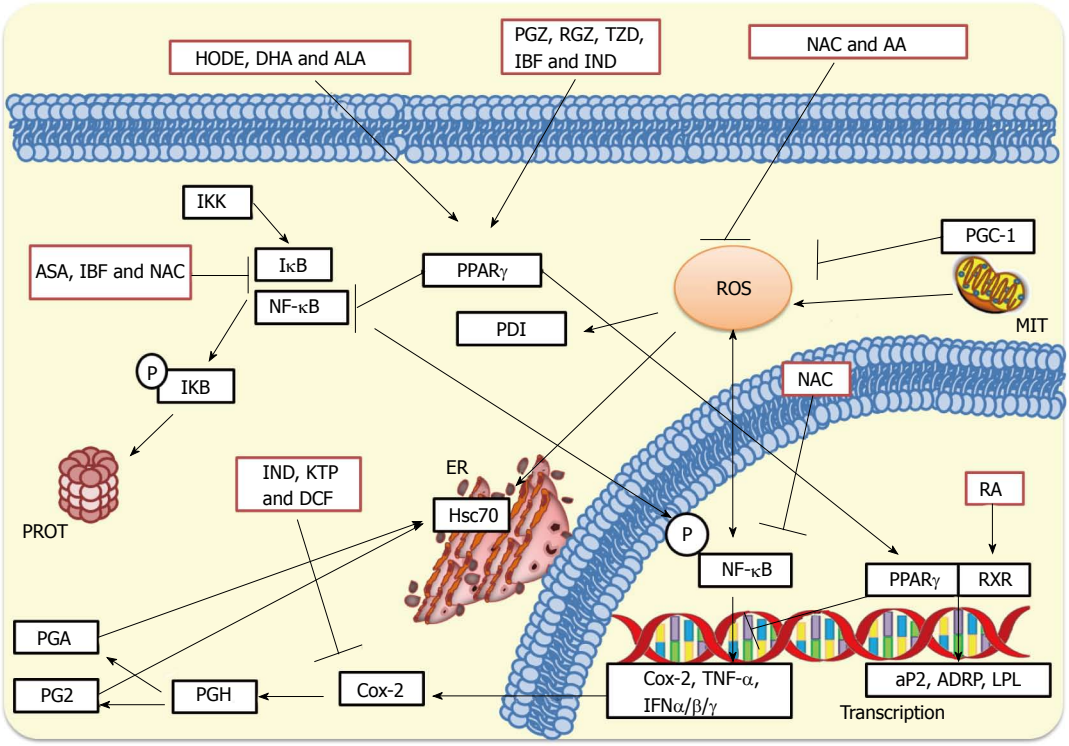

Figure 4 Inhibition of rotavirus infection by treatment with antioxidants, nonsteroidal antiinflammatory drugs and peroxisome proliferator-activated receptor gamma agonists. NAC and AA can inhibit the production of ROS, whereas NAC can also affect IκB preventing the cytoplasmic activation of NF-κB. NAC 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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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.

Cummins NW, Badley AD, Kasten MJ, Sampath R, Temesgen Z, Whitaker JA, Wilson JW, Yao JD, Zeuli J, Rizza SA. Twenty years of human immunodeficiency virus care at the Mayo Clinic: Past, present and future. *World J Virol* 2016; 5(2): 63-67 Available from: URL: http://www.wjgnet.com/2220-3249/full/v5/i2/63.htm DOI: http://dx.doi.org/10.5501/wjv.v5.i2.63

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,

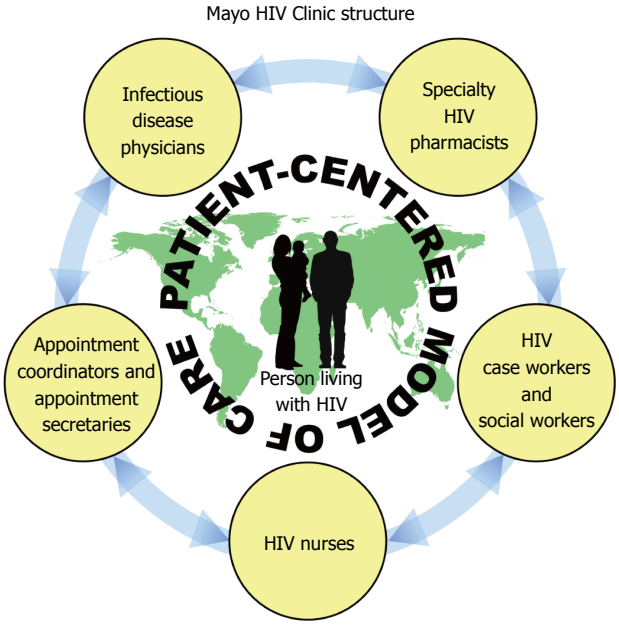

Figure 1 The Mayo human immunodeficiency virus Clinic structure. The Mayo human immunodeficiency virus (HIV) Clinic provides a model of multidisciplinary, patient-centered care.

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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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

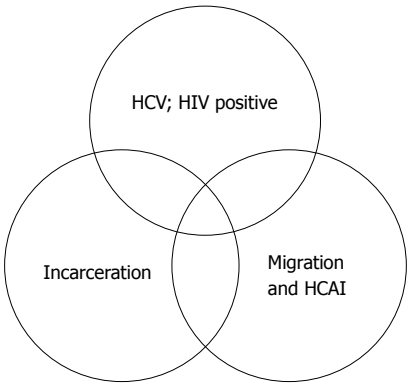

Figure 1 Global risk factors for co-infection with hepatitis C virus and human T lymphotropic virus-1/2 in people who inject drugs. Adapted from Roger and Castro, 2014[15]. In this model, each circle represents a risk factor of hepatitis C virus (HCV)/human T lymphotropic virus-1/2 co-infection in people 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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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%)

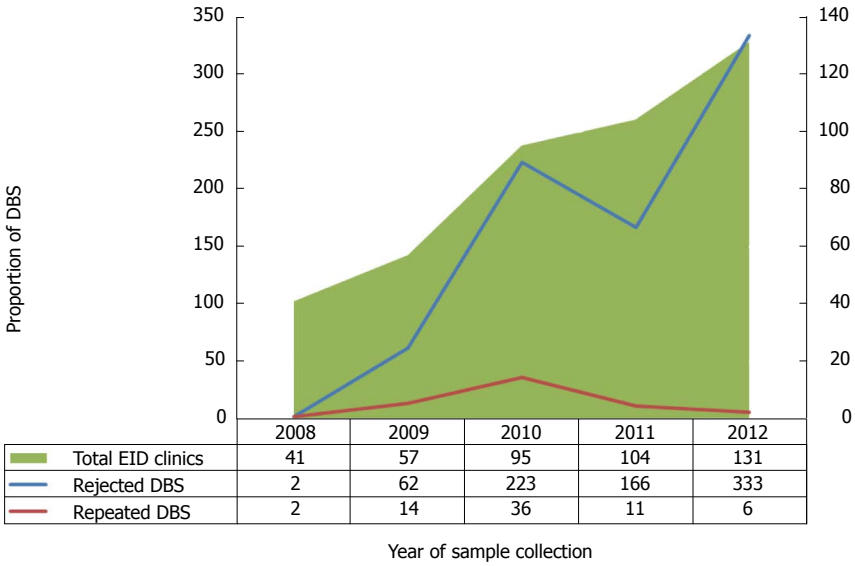

Figure 1 Annual early infant diagnosis collected at clinics *vs* proportion of rejected and repeated samples. EID: Early infant diagnosis; DBS: Dried blood spots.

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.

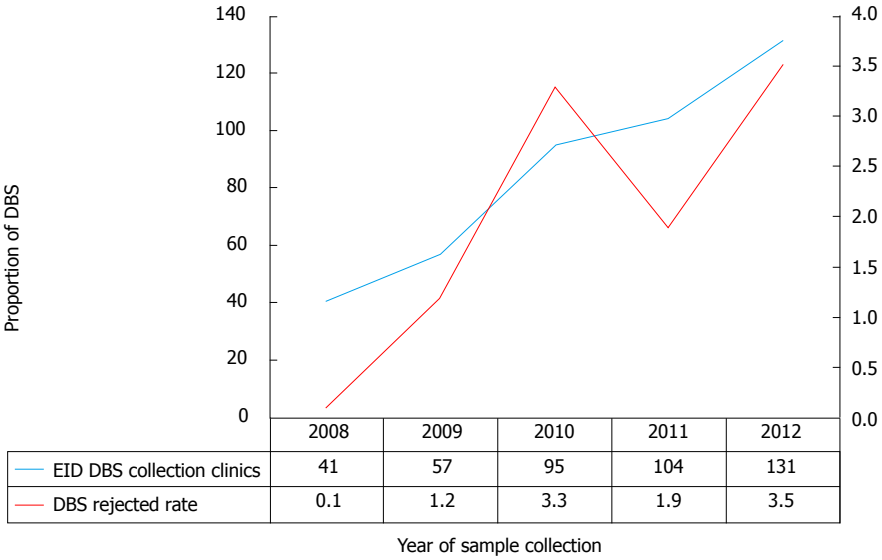

Figure 2 Annual total early infant diagnosis collected at clinics *vs* sample rejection rate. EID: Early infant diagnosis; DBS: Dried blood spots.

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.

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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.

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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 Σ 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.

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