

# World Journal of *Virology*

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## Hepatitis delta virus: A fascinating and neglected pathogen

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### Abstract

Hepatitis delta virus (HDV) is the etiologic agent of the most severe form of virus hepatitis in humans. Sharing some structural and functional properties with plant viroids, the HDV RNA contains a single open reading frame coding for the only virus protein, the Delta antigen. A number of unique features, including ribozyme activity, RNA editing, rolling-circle RNA replication, and redirection for a RNA template of host DNA-dependent RNA polymerase II, make this small pathogen an excellent model to study virus-cell interactions and RNA biology. Treatment options for chronic hepatitis Delta are scarce and ineffective. The disease burden is perhaps largely underestimated making the search for new, specific drugs, targets, and treatment strategies an important public health challenge. In this review we address the main features of virus structure, replication, and interaction with the host. Virus pathogenicity and current treatment options are discussed in the light of recent developments.

**Key words:** Hepatitis delta virus; Hepatitis B virus; RNA replication; Pathogenesis; Treatment

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**Core tip:** Hepatitis delta virus (HDV) is the etiologic agent of probably the most severe form of virus hepatitis. HDV replication and spread depends on the presence of hepatitis B virus which provides the envelope proteins coded exclusively by its own genome. About 20 million people are currently chronically infected with HDV and no specific therapy is still available. Here, we review the current knowledge on HDV biology, epidemiology, pathogenesis, and treatment. Future trends and perspectives are discussed in the light of recent developments on HDV biology and its interaction with the host.

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## INTRODUCTION

Over 35 years have passed since Rizzetto *et al.*<sup>[1]</sup> reported the discovery of what has been called Delta antigen in a patient with diagnosis of severe hepatitis B infection. Subsequent research on the nature of this antigen led to the identification, in 1980, of a new hepatotropic virus, hepatitis delta virus (HDV)<sup>[2,3]</sup>. This new infectious agent was later found to be a sub-viral agent dependent on the presence, in infected cells, of hepatitis B virus (HBV) to accomplish the replication cycle<sup>[3,4]</sup>. In nature, both viruses, HBV and HDV, share the same envelope proteins coded exclusively by the HBV genome<sup>[5,6]</sup>.

Today, the World Health Organization estimates that about 400 million people are chronically infected with HBV worldwide<sup>[7,8]</sup>, of which approximately 20 million are co-infected with HDV<sup>[9,10]</sup>. The Amazon basin and some central African and east European countries are among the regions with higher prevalence. However, there is still a considerable lack of information concerning a significant number of countries mostly situated in Africa, Asia, and Latin America (Figure 1). The geographic distribution of the so far identified eight HDV clades is also far from being uniform. Clade 1 may be found worldwide, in contrast with clade 3 which seems to be confined to the Amazon region (Figure 1). The most frequent outcome of the acute co-infection with HDV is virus clearance and patient's recovery. However, in up to 5% of the infected individuals a chronic form of HDV infection will develop<sup>[11]</sup>. In the case of super-infection, when a chronic HBV carrier gets super-infected with HDV, the outcome is distinct. About 70%-90% of super-infected individuals will become chronic carriers for both viruses, HBV and HDV<sup>[12]</sup>.

As compared to the individuals that are chronic carriers of HBV alone, HDV additionally increases the risk of hepatocellular carcinoma (HCC) and mortality threefold and twofold, respectively, in HDV/HBV carriers<sup>[13,14]</sup>. Currently, in clinical practice, there are no drugs used that directly and specifically target HDV. None of the currently approved anti-HBV drugs efficiently blocks HDV infection<sup>[7,9,14-17]</sup>.

All of the above, given additional HDV-inflicted liver pathogenesis, and inability to efficiently circumvent HDV infection by anti-HBV drugs, makes HDV a very serious pathogen, and it does call for additional attention to HDV and development of specific anti-HDV interventions.

HDV is mostly endemic in low income countries in which the budget for new, potentially expensive drugs is, of course, not the first priority. Accordingly, development of new treatment options based on specific drugs has not only proved to be difficult (the virus apparently does not code for any specific enzymatic activity that could be

targeted) but may also represent an uninteresting option for pharmaceutical companies, speaking from a strictly financial point of view.

Nevertheless, this small human pathogen bears a set of features that make it a formidable model to study fundamental aspects of host-pathogen interactions and RNA biology including mechanisms of transcription, replication, and genome evolution<sup>[18,19]</sup>. The small size and structure of the genome bearing only one open reading frame (ORF), which is edited by host enzymes, its ribozyme activity and still largely undeciphered mechanism of RNA-directed RNA replication, are prominent examples of the uniqueness of this human pathogen<sup>[19]</sup>.

In this review, we will address the specific features of HDV structure and replication, its interaction with host cells and HBV. Future perspectives of research based on recent important developments will be discussed.

## The virus and its replication

**The virus:** HDV is an enveloped spherical subviral agent about 36 nm in diameter<sup>[19]</sup>. The virus particle contains a ribonucleoprotein (RNP) core consisting of one copy of the RNA genome and approximately 200 copies of the only virus encoded protein, the Delta antigen (HDAg)<sup>[20]</sup>. The HDV envelope contains hepatitis B virus surface antigens (HBsAg), provided solely by HBV. In accordance, the two viruses share virtually indistinguishable envelopes<sup>[6]</sup>.

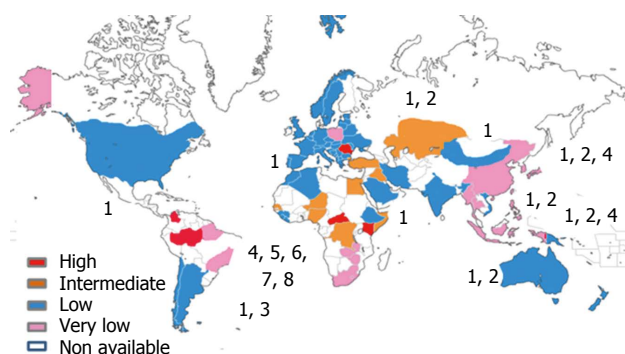
The virus genome is a circular single-stranded RNA molecule of around 1.7 kb and negative polarity<sup>[21,22]</sup>. A significant degree of internal base-pairing (about 70% of all nucleotides) is an important feature, with potential not yet unveiled functional implications, observed in this molecule<sup>[23,24]</sup>. This structure is similar to that described for plant viroids, albeit the latter have a smaller size and do not code for any protein (Table 1). On the contrary, the HDV genome displays one ORF which codes for the only viral protein, the Delta antigen<sup>[25-27]</sup>. This protein can be found in virions under two distinct forms: Small (S-HDAg, 195 aa) and large (L-HDAg; 213 or 214 aa, depending on the genotype). L-HDAg is synthesized mainly later in the replication cycle<sup>[28,29]</sup> as a consequence of an editing mechanism that takes place in the so-called anti-genome, an exact copy of the genome that arises as a replicative intermediate during RNA replication. The editing reaction is catalyzed by cellular adenosine deaminase 1 which converts an amber stop codon into a tryptophan codon (UGG) allowing a 57 nucleotide and consequently 19 aa extension of the ORF<sup>[30,31]</sup>.

Both L-HDAg and S-HDAg share the same functional domains with the exception of the L-HDAg-specific C-terminal extension, which bears an isoprenylation signal present in cysteine residue 211<sup>[32]</sup>. Farnesylation of this residue is reported to be crucial albeit not sufficient for interaction with HBsAg and subsequent virion packaging and release from the cells<sup>[33,34]</sup>. The common functional motifs are a nuclear localization signal (NLS; aa 66-75), a coiled-coil domain (aa 12-60), and a

**Table 1** Similarities and differences between hepatitis delta virus and plant viroids

| HDV (1700 nt)   | Pospiviroidae (200-400 nt)                                      | Avsunviroidae (200-400 nt)                                      |
|---|---|---|
| Circular ssRNA  | Circular ssRNA  | Circular ssRNA  |
| Extensive intramolecular base pairing                           | Extensive intramolecular base pairing                           | Extensive intramolecular base pairing                           |
| A DNA-directed RNA polymerase makes both plus and minus strands | A DNA-directed RNA polymerase makes both plus and minus strands | A DNA-directed RNA polymerase makes both plus and minus strands |
| Encodes for protein   | No proteins encoded   | No proteins encoded   |
| Virion maturation depends on a helper virus                     | Replication does not depend on the presence of a helper virus   | Replication does not depend on the presence of a helper virus   |
| Symmetric rolling circle RNA replication                        | Asymmetric rolling circle RNA replication                       | Symmetric rolling circle RNA replication                        |
| Replicates in the nucleus                                       | Replicates in the nucleus                                       | Replicates in chloroplasts                                      |
| Ribozyme activity   | No ribozyme activity  | Ribozyme activity   |

HDV: Hepatitis delta virus.



**Figure 1** Prevalence and geographical distribution of eight hepatitis delta virus clades in the world.

bipartite arginine-rich RNA binding domain (aa 97-107 and 136-146; ARM1 and ARM2, respectively)<sup>[35-37]</sup>. More recently, however, it was shown that mutation in the core arginines of both ARM1 and ARM2 did not impair the RNA-binding ability of a C-terminal HDAG-160 truncated form of HDAG<sup>[38]</sup>. The authors suggested that HDAG establishes numerous contacts with HDV RNA to assemble ribonucleoprotein complexes.

**Delta antigens:** Several properties have been assigned to S-HDAG but none related to any known enzymatic activity. Among the reported putative and observed functions are the promotion of nuclear import of HDV RNPs<sup>[39]</sup>, regulation of HDV RNA editing<sup>[40]</sup>, facilitation of ribozyme cleavage (chaperone)<sup>[41,42]</sup>, and facilitation of accumulation of processed RNA transcripts<sup>[43,44]</sup>. Both Delta antigens are post-translationally modified by host enzymes. Several post-translational modifications (PTM) have been described in HDAG and these include phosphorylation, methylation, acetylation, and sumoylation<sup>[45-48]</sup>. Phosphorylation occurs at multiple sites and can be mediated by different host kinases, dsRNA-activated protein kinase R, protein kinase C, and ERK1/2<sup>[49-51]</sup>. All these modifications may have distinct functional significance but it seems consensual that they are all involved in promoting virus RNA replication<sup>[52]</sup>.

Methylation of Arg 13 on S-HDAG by arginine methyltransferase I was reported and proposed to be important to enhance both genomic RNA and mRNA

synthesis<sup>[46]</sup>. Additionally, cellular p300 acetyltransferase was found to acetylate Lys72 on the NLS of S-HDAG<sup>[53]</sup>. Although speculative, this modification may have impact on the efficiency of nuclear import.

Finally, sumoylation was the most recent PTM to be reported on S-HDAG. It occurs at multiple lysine residues and is catalyzed by host small ubiquitin-related modifier isoform 1. Sumoylation was proposed to be important to promote genomic RNA and mRNA synthesis<sup>[48]</sup>.

Undoubtedly, these observations represent only a tiny part of the whole picture drawn by HDAGs inside the cell. In fact, Delta antigens can also be found as peptides of different smaller sizes in the nucleus of HDV replicating cells<sup>[54]</sup>. Do these additional smaller forms correspond to distinct functional features? The answer is still far from being clear as no evidence supporting this point of view have been reported. In addition, it has been shown that S-HDAG can form multimers in HDV replicating cells<sup>[20,55,56]</sup>. These multimers may play an important role in virus replication by facilitating the accumulation of virus RNAs. Moreover, it is known that HDAGs are basic proteins with an estimated overall + 12 charge<sup>[57]</sup>. Thus, it is not surprising that, at least *in vitro*, the protein can bind nonspecifically to several types of nucleic acids including dsDNA and several distinct RNAs<sup>[58]</sup>.

Furthermore, S-HDAG may also be involved in sequestering and manipulating host cell components to facilitate HDV replication. In this context, it is not surprising that the search for S-HDAG interacting proteins unveiled a considerable number of potential partners. First Cao *et al.*<sup>[59]</sup> used an immunoprecipitation followed by mass spectrometry approach being able to identify more than 100 host proteins in the assay. Later, Gowans *et al.*<sup>[60]</sup> performed a yeast two-hybrid screen using a human liver cDNA library and identified 30 host candidate proteins capable of specifically interacting with S-HDAG. Making use of RNA silencing strategies some of these candidate interactions were found to be of potential functional significance. However, the above mentioned strong positive charge of HDAGs compels one to be careful when analyzing the specificity and role of these interactions in the HDV replication cycle.

S-HDAg is predicted to be an intrinsically disordered protein, a property already assigned to several other virus and cellular proteins<sup>[61]</sup>. This feature may be responsible for the lack of success in all, to our knowledge at least in three different laboratories, attempts to crystalize and solve the 3D structure of the Delta antigen. These properties of the Delta antigen make the study of HDV biology much more complex than perhaps initially believed. However, as we shall discuss below, they are not the only most important ones.

**HDV replication:** HDV replication takes place in the nucleus of infected cells<sup>[60,62,63]</sup>. The study of the HDV replication has long been difficult due to the lack of an appropriate cell culture system capable of supporting all steps of the virus life cycle, from attachment to release from the cells. Primary human hepatocytes have been long the only cells known to support the complete life cycle of HDV<sup>[64]</sup>. These are expensive and not easy to cultivate. Thus, other approaches needed to be developed and a number of alternatives arose with time. Among them are the Hepa RG cell line and the stably transfected HEK-293 cells expressing S-HDAg under the control of a tetracycline inducible promoter<sup>[65,66]</sup>. Although not representing ideal models, they became important tools for HDV research. The recent identification of the sodium-taurocholate co-transporting polypeptide (NTCP, encoded by SLC10A1) as the bona fide receptor for HBV and HDV culminated a long run that included a number of tested hypothesis and putative isolations<sup>[67,68]</sup>. It represented an important breakthrough since it allowed engineering cell lines overexpressing it and consequently also supporting the initial steps of virus attachment and entry. So far, these human NTCP-expressing cell lines include human HepG2 and Huh7 as well as mouse Hepa1-6, AML-12, and primary mouse hepatocytes<sup>[69]</sup>.

After the uncoating of virus particles, HDV RNPs are transported to the nucleus, where RNA replication takes place<sup>[70]</sup>. The existing data indicates replication of the virus genome involves a double rolling-circle mechanism with formation of multimeric anti-genomic and genomic molecules<sup>[71]</sup>. These RNA multimers are cleaved at precise monomeric intervals by a ribozyme activity present in both genomic and antigenomic molecules<sup>[72,73]</sup>. The presence of ribozymes in HDV RNAs is a feature shared with the viroid family of Avsunviroidae<sup>[74]</sup> (Table 1).

Although it is well established that the presence of S-HDAg stimulates virus RNA accumulation, the precise role of this virus antigen in the mechanism of HDV RNA replication remains elusive. Controversy on which host polymerase or polymerases are involved in synthesis of genomes and antigenomes lasted, for a long time. Some groups claimed that both RNA pol I and pol II are involved in genome and antigenome synthesis, respectively<sup>[75]</sup>. Mainly, these evidences were obtained in *in vitro* assays using different inhibitory concentrations of  $\alpha$ -amanitin and on reports showing the presence of virus RNA in the nucleolus<sup>[75,76]</sup>. By

contrast, other groups, using different types of transcription inhibitors, actinomycin D, 5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole,  $\alpha$ -amanitin, provided data suggesting the involvement of solely RNA pol II<sup>[77]</sup>. Furthermore, the presence of virus RNA in the nucleolus could not be observed in the absence of Delta antigen suggesting that this presence lacks functional relevance<sup>[62,63]</sup>. In recent years, the use of immunoprecipitation and proteomic approaches, among others, led to the identification of several pol I, pol II, and pol III subunits as binding partners for HDV RNA<sup>[59]</sup>. These results need to be interpreted with care since the observed binding to HDV RNA could be a result of indirect interaction through other non-identified partners. However, independently of the host polymerase(s) involved in replication of virus RNAs a striking question is still hanging in the air: How does the virus redirect a host DNA-dependent RNA polymerase to use an RNA template? Here, the eventual participation of the S-HDAg, which as mentioned before displays a net positive charge and intrinsic disorder, may play a crucial role allowing the virus to overcome obstacles posed by the host environment for its replication.

The search for promoter sequences in virus RNA has also been followed by a few groups with inconclusive results. Yet, there is evidence from *in vivo* models supporting that mRNA synthesis initiates at nt 1630<sup>[78,79]</sup>. It may additionally be possible that multiple binding sequences for host RNA polymerases are present both in the virus genome and antigenome. This "nonspecific" binding could be a consequence of the RNA secondary structure bearing an extensive base-pairing with a number of predicted internal loops. Additionally, S-HDAg could also play an important role since it can bind nonspecifically to several nucleic acids, from dsDNA to ssRNA. It could be possible that S-HDAg plays a role as mediator between the virus RNA and a host RNA polymerase promoting its binding to several sequences in the genome and antigenome. Alternatively, S-HDAg could simply act as a chaperone, stabilizing RNA molecules and making them available for transcription. Assembly of HDV virions takes place in the cytoplasm. In this cellular compartment HBV-derived HBsAgs interact with HDV RNPs that are exported from the nucleus<sup>[80,81]</sup>. This interaction was shown to be mediated by L-HDAgs<sup>[82,83]</sup>. Tavanetz *et al.*<sup>[81]</sup> used heterokaryon assays to show that HDV RNPs shuttle between the nucleus and the cytoplasm. The authors claimed that nuclear import is mediated by an NLS located in Delta antigens (aa 66-75) and provided evidence that export to the cytoplasm is mediated by a cis-acting sequence in virus RNA<sup>[35]</sup>. However, Lee *et al.*<sup>[84]</sup> (2001) have shown a year before that aa 198-210 in L-HDAg were able to promote the export of a reporter protein. More recently, Freitas and Cunha used a well-established CAT reporter system to investigate a possible presence of nuclear export elements (NEEs) in HDV RNAs<sup>[85]</sup>. The authors showed that NEEs may be present in both genomic and antigenomic molecules and that nuclear export is, at

least in part, sensitive to leptomycin B, an inhibitor of the host CRM1-mediated export pathway. Whether a NES present in L-HDAg or a NEE in virus RNA are responsible for promoting HDV RNP export may be considered still controversial. Consequently, further research is mandatory to unequivocally answer this question.

**Clinical manifestations and therapy:** It is widely and for a longtime known that HDV infection is associated with a broad range of clinical manifestations, from asymptomatic to fulminant hepatitis. In the latter cases, mortality often reaches 80% of the affected individuals<sup>[86,87]</sup>.

Concomitant infection of HBV and HDV usually displays more severe symptoms when compared with a single HBV infection. Nevertheless, the most frequent outcome is virus clearance, a situation reported in about 95% of the cases<sup>[88]</sup>. In contrast, HDV super-infection of chronic HBV patients results in progression to chronicity in up to 80% of patients. Moreover, about 60%-70% of these patients will develop cirrhosis<sup>[89]</sup>. These patients usually progress more rapidly to cirrhosis, show increased liver decompensation, and eventually death when compared with those chronically infected with HBV alone<sup>[90,91]</sup>.

The factors influencing the distinct clinical course in coinfecting and superinfected patients are still poorly understood. In both cases the organism produces a strong anti-HDAg antibody response which is, unfortunately, unable to modulate the course of infection<sup>[92-94]</sup>. The majority of superinfected patients progresses to chronic disease independent of the presence of high titers of anti-HDV antibodies. Despite the limited number of studies there are evidences supporting a role of cytotoxic T cells in HDV infection including the destruction of infected hepatocytes<sup>[95]</sup>. In any case, immunology of HDV infection is perhaps one of the most poorly understood aspects of the disease.

From the histologic point of view there are no detectable differences between anomalies observed in the liver of HDV-infected patients and patients with other acute or chronic virus liver disease<sup>[96,97]</sup>. These anomalies mostly consist of hepatocellular necrosis and inflammation and may represent, at least in part, a consequence of the immune response of the host. Proteomic and systems biology approaches have more recently been used to investigate changes in protein expression patterns and metabolic pathways altered during HDV replication. Although the model systems used can hardly be considered ideal, the obtained results provided consistent evidence that HDV replication results in significant alterations in pyruvate and glycolysis metabolism<sup>[98-100]</sup>. Of note, these studies have shown that cancer was the most likely disease associated with HDV replication and provided evidence that the G2/M cell cycle checkpoint is altered as a consequence of the presence of the virus<sup>[100]</sup>. Definitely, these observations, of which a significant number of arise from proteomic

experiments and analysis, need to be interpreted and handled with care. In any case, it seems uncontroversial that further research on liver biopsies of infected patients may possibly help confirming these findings.

There is no efficient therapy for chronic HBV/HDV infection. Pegylated interferon- $\alpha$  (PEG-IFN- $\alpha$ ) is perhaps the most popular therapy and the one that has shown some antiviral activity against HDV<sup>[15,101]</sup>. However, the efficacy is limited - a temporary reduction in virus titers is usually observed in 15%-40% patients - and the need for prolonged administration often results in severe adverse effects<sup>[101,102]</sup>. These effects include fatigue, weight loss, and psychiatric disturbances. Ribavirine, lamivudine and other nucleotide analogues have also been tested but have shown a very limited, if any, efficacy<sup>[103-106]</sup>. The Hep-Net International hepatitis D intervention trial included 77 patients from Germany, Greece, and Turkey. In this study a PEG-IFN- $\alpha$ 2a therapy was compared with adefovir and a combination of PEG-IFN- $\alpha$ 2a and adefovir<sup>[107]</sup>. Adefovir showed a very limited efficacy and the combination therapy based on PEG-IFN- $\alpha$ 2a and adefovir was only superior in reducing HBsAg levels but not in HDV RNA<sup>[17]</sup>. In any case, HDV RNA relapses were often observed in a long-term follow-up (median time 4.5 years). The nucleoside analog entecavir, which showed antiviral efficacy in the woodchuck model of hepatitis B, was assayed in thirteen chronic hepatitis D patients for one year also proving to be ineffective<sup>[17]</sup>. It thus seems evident that current anti-HBV drugs are unable to efficiently circumvent HDV infection.

Today, it is usually recommended to treat chronic hepatitis D with PEG-IFN- $\alpha$  for at least one year if the patient tolerates the eventual adverse effects. However, in patients with advanced liver disease, liver transplantation may represent the only available option<sup>[108]</sup>. It is thus clear that current therapeutic options are unsatisfactory and there is an urgent need for more effective and specific anti-HDV drugs that will directly target HDV. Prenylation inhibitors may become an interesting and effective option and have been shown to be safe when used to treat neoplasias<sup>[109,110]</sup>. As discussed before, prenylation of L-HDAg is essential for interaction with HBsAg and virion assembly, and thus may be regarded as a potential target for therapeutic intervention.

Most recently, and as a consequence of the identification of NCTP as the host cell HDV receptor, inhibitors of viral entry have been tested and proposed as potential anti-viral drugs. Namely, Myrcludex B, a synthetic N-acylated preS1 lipopeptide and cyclosporine A were shown to inhibit virus entry by interfering with the receptor functions of NCTP, however, currently there is no data available regarding the performance of this drug in actual HDV-infected individuals<sup>[111,112]</sup>.

However, it is clear that a higher investment in research of fundamental aspects of HDV biology as well as of anti-HDV specific compounds is crucial in order to improve the quality of life and life expectancy of chronic HBV/HDV carriers.



### Recent trends in HDV research

In the past few years a number of interesting developments have occurred in the field of HDV research and its interaction with HBV.

Using super-infection with WHV-enveloped HDV of the woodchucks that were chronic carriers of WHV and already developed HCCs, it was found that HDV was able to infect fractions of the cells of WHV-induced HCCs. These results suggest that at least a certain percentage of HCC cells *in vivo* express functional WHV receptors and support the attachment, entry, trafficking, and complete replication cycle of HDV<sup>[113]</sup>. The data also opens new avenues of research that will further address the mechanisms of the relationship between established HCCs and ongoing virus infection.

A second study compared several types of HDV that differed only by the envelope proteins of HBV that coated the virions<sup>[114]</sup>. Twenty five different types of HBV envelope proteins that belonged to twenty five different HBV variants of nine genotypes A-I were analyzed. It was found that all nine HBV genotypes tested were able to support the production of infectious HDV virions that contained HDV genome of genotype I. Significant differences in infectivity were found for the envelope proteins of different HBV variants. The data generated strongly suggest that HBV envelope proteins facilitate not only attachment and entry, but also at least one additional immediate post-entry step of the HDV life cycle. In addition, testing of infectivity suggested that it cannot be concluded that the envelope proteins of HBV produced during chronic stage of HBV infection are mainly responsible for assembly of the virions with diminished infectivity. The study also suggested that correctly regulated disassembly of HDV RNP from the HBV envelope proteins after entry is critical for the overall infectivity of HDV particles<sup>[114]</sup>.

Finally, a third recent study demonstrated that infectious HDV virions can be assembled by the envelope proteins derived from the naturally integrated HBV DNA in the absence of ongoing HBV replication<sup>[115]</sup>. These findings suggest that HDV can possibly persist *in vivo* in the absence of HBV replication (or when HBV replication is suppressed by a drug), when functional HBV envelope proteins are supplied from integrated HBV DNA. Such a mechanism of HDV persistence was not explored previously. The results obtained explain, at least in part, inability of anti-HBV drugs to efficiently block HDV infection *in vivo*. Additionally, they also suggest that HDV can be actually a more independent and more significant pathogen than it is currently assumed<sup>[116]</sup>.

### Origin of the virus

As discussed earlier, HDV bears a number of characteristics similar to those found in plant viroids (Table 1).

These similarities may allow speculation on a possible HDV origin from the plant world. According to this hypothesis, HDV could have evolved to encode the Delta antigen thus providing an explanation for its larger genome when compared with viroids<sup>[117]</sup>. However, a

deeper analysis of this homology was evaluated as non-significant and this hypothesis seems to be, at least for the time being, ruled out.

One of the key features of HDV genomic and anti-genomic RNA molecules is their ribozyme activity. Ribozymes are considered to be characteristic of viroids. However, the two HDV ribozymes are not only structurally different from those of Avsunviroidae but also display similarities to several HDV-like ribozymes found in eukaryotes<sup>[74,116]</sup>. This finding rather supports the hypothesis of a human transcriptome origin of HDV.

We can thus conclude that the plant or animal origins of HDV are still questionable and highly speculative. But this is one of the many fascinating questions that still remain to be unveiled for this awkward and awesome virus.

## CONCLUSION

Almost 40 years after its discovery, HDV remains a challenge for clinicians and researchers. It is disconcerting simplicity, with a small RNA genome and a single protein, the Delta antigen, make it an excellent model not only for virologists but also for those interested in RNA and cell biology. The virus bears a number of unique features including a RNA-directed RNA replication mechanism of the genome catalyzed by host RNA polymerase II. Enzymatic activities were not identified in Delta antigens thus making difficult the identification of potential targets for specific and effective therapies. Development of such therapies is crucial to reduce the number of chronic patients progressing to cirrhosis and hepatocellular carcinoma. The burden of disease caused by HDV is most probably underestimated since there is a considerable lack of epidemiologic data from several countries where HBV is highly prevalent.

In conclusion, despite considerable progress made in HDV research a significant number of questions remain to be answered concerning fundamental aspects of its biology, pathogenesis, and interaction with the host. The next few years will hopefully bring to light new answers but also new exciting questions, helping understand this fascinating pathogen, and contributing to reducing morbidity and mortality among infected individuals.

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## Update on hepatitis B and C virus diagnosis

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and are transmitted by parenteral route, sexual and vertical transmission. One important measure to reduce the burden of these infections is the diagnosis of acute and chronic cases of HBV and HCV. In order to provide an effective diagnosis and monitoring of antiviral treatment, it is important to choose sensitive, rapid, inexpensive, and robust analytical methods. Primary diagnosis of HBV and HCV infection is made by using serological tests for detecting antigens and antibodies against these viruses. In order to confirm primary diagnosis, to quantify viral load, to determine genotypes and resistance mutants for antiviral treatment, qualitative and quantitative molecular tests are used. In this manuscript, we review the current serological and molecular methods for the diagnosis of hepatitis B and C.

**Key words:** Diagnostic methods; Genotypes; Hepatitis B virus; Molecular diagnostic techniques; Serological tests; Hepatitis C virus

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**Core tip:** Reliable methods for diagnosing hepatitis B virus (HBV) and hepatitis C virus (HCV) infection are essential to reduce the burden of these infections. Serological and molecular assays are used to identify acute and chronic cases of infection. In this article, the current knowledge about HBV and HCV diagnosis is updated and emphasized the characteristics of each techniques to be useful to most laboratory personnel.

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### Abstract

Viral hepatitis B and C virus (HBV and HCV) are responsible for the most of chronic liver disease worldwide

### INTRODUCTION

Hepatitis B virus (HBV) infection is caused by a virus that



shows a diameter of 42 nm and comprises an icosahedral capsid surrounded by a lipid envelope containing hepatitis B surface antigen (HBsAg)<sup>[1]</sup>. Viral capsid is formed by core protein and carries viral genome and polymerase<sup>[2]</sup>. HBV genome is composed by a circle DNA partially double stranded and has four open reading frames regions overlapped (ORFs): PreC/C that encodes for hepatitis B e Antigen (HBeAg) and core protein (HBcAg); P for polymerase (reverse transcriptase), S for surface proteins [three structures of HBsAg, small (S), middle (M) and large (L)], and X for a transcriptional transactivator protein<sup>[3,4]</sup>. Despite HBV has a DNA genome, it shows high mutation rates, similar to those observed in RNA viruses and retroviruses<sup>[5]</sup> what could be due to viral polymerase mistakes associated with the additional step of reverse transcription, necessary for genetic material replication<sup>[6]</sup>. HBV has been classified in 10 genotypes (A to J) with between approximately 4 and 8% intergroup nucleotide divergence across the complete genome and genotypes A-D, F, H, and I are classified further into subgenotypes<sup>[4,7]</sup>.

In 1989, hepatitis C virus (HCV) was described after extensive research conducted to find the cause of post-transfusion hepatitis<sup>[8]</sup>. In 1991, Choo *et al*<sup>[9]</sup> were able to identify complete HCV genomic sequence and thereafter, it was possible to conceive hybridization probes and primers to amplify viral genome by polymerase chain reaction (PCR)<sup>[10]</sup>. HCV particle is constituted by a spherical envelope which involves an icosahedral capsid. It possess a RNA genome with 9.5 kb and an ORF responsible for encoding viral polyprotein, constituted by structural (core, glycoproteins E1 and E2 and protein P7) and nonstructural (NS2, NS3, NS4a, NS4b, NS5a, NS5b-RNA polymerase) proteins. HCV presents high genome variability due to low proofreading of viral RNA polymerase. This variability allows virus classification into 7 genotypes (1 to 7), based on their genomic features<sup>[11]</sup>.

Worldwide HBV and HCV infection are the principal etiological agents of chronic liver disease<sup>[12-14]</sup>. Both viruses are transmitted by parenteral route, sexual and vertical transmission is more common for HBV infection compared to HCV<sup>[15,16]</sup>. Approximately 240 million people are HBV chronic patients while 150 million individuals present HCV infection in the world<sup>[13,14]</sup>.

HBV endemicity is classified as high, intermediate or low according HBsAg prevalence<sup>[12,17]</sup>. South East Asia, sub-Saharan Africa, China, Indonesia and Nigeria are considered high endemicity regions since HBV chronic infection can be present in more than 8% of the population<sup>[17]</sup>. Developed countries in Western Europe and North America are classified as low endemicity areas since chronic infection rates range from 0.5% to 2.0% of population. South America and Central countries, Eastern and Southern Europe and South West Asia are considered intermediate areas since HBV prevalence rates between 2% and 7% of population<sup>[12,17]</sup>.

HCV global prevalence is 1.6% corresponding to 115 millions of infected individuals and viremia prevalence is

1.1% corresponding to 80 million of cases<sup>[18]</sup>. Although HCV infection presents global distribution, different prevalences are observed according geographic area<sup>[18,19]</sup>. High anti-HCV prevalence is observed in Central Asia (5.4%), Eastern Europe (3.3%), the Midwest of North Africa region (3.1%) and Central and Western Sub-Saharan Africa (4.2% and 5.3%, respectively). Intermediate prevalence rates are found in southern sub-Saharan Africa (1.3%), Central Europe (1.3%), Australia (1.4%), Latin America (1%-1.25%). Low prevalence are observed in Oceania (0.1%), Caribbean (0.8%) and Western Europe (0.9%)<sup>[18]</sup>. Highest rates of chronic infection can be found in Egypt (22%), Pakistan (4.8%) and China (3.2%) where the main infection mode is the reuse of contaminated needles<sup>[13,14,20]</sup>.

One important measure to diminish the burden of HBV and HCV infection is the diagnosis of acute and chronic cases. In this article, we describe the current serological and molecular methods for HBV and HCV diagnosis.

## SEROLOGICAL ASSAYS FOR HBV DIAGNOSIS

Takahashi *et al*<sup>[1]</sup> described the Australia antigen in 1965 and after this was named HBsAg, while the Dane particle (complete hepatitis B virion) was subsequently identified in 1970. After these discoveries, the identification of HBV antigens and antibodies allowed: (1) clarify the natural history of the disease; (2) evaluate the clinical phases of infection; (3) monitor antiviral treatment; (4) identify infected individuals; and (5) monitor the efficacy of immunization<sup>[21-23]</sup>.

HBsAg is the first serological marker to appear indicating active infection. Chronic infection is characterized by the persistence of this marker for more than 6 mo<sup>[24]</sup>. Recently, HBsAg quantification has become important to monitor polyethylene glycol interferon treatment<sup>[25]</sup> since this assay could be used along to HBV DNA test to define the clinical phase of HBV infection and to evaluate the therapy<sup>[26]</sup>. To date, two chemiluminescent microparticle immunoassay (CMIA) (Architect HBsAg QT and Elecsys HBsAg II, Elecsys) could be used for HBsAg quantification showing good agreement<sup>[27]</sup>.

Anti-HBs is a neutralizing antibody, and its presence indicates immunity to HBV infection<sup>[28,29]</sup>. The simultaneous presence of anti-HBs and HBsAg has been documented in HBsAg positive patients<sup>[30,31]</sup>, probably due to the incapability of antibodies to neutralize the circulating virions. In this situation, these people are classified as carriers of HBV infection.

HBeAg marker indicates viral replication and risk of transmission of infection, and seroconversion of HBeAg to anti-HBe is associated with remission of liver disease<sup>[32]</sup>. However, some anti-HBe reactive subjects continue to have active viral replication and hepatic disease caused by mutations in the pre-core and core

**Table 1** Clinical significance of hepatitis B virus serological markers

| Marker       | Clinical significance  |
|--------------|--|
| HBsAg        | First marker to appear in course of infection<br>Appears one to 3 wk before the onset of symptoms<br>The permanence of this marker for more than 24 wk indicates chronicity  |
| Anti-HBc IgM | Marker of recent infection marker<br>Appears with the onset of symptoms and persists up to 32 wk after infection   |
| Anti-HBc IgG | This marker did not indicate immunity and it is not elicited by vaccination  |
| HBeAg        | This presence indicates prior contact with the virus<br>It appears before the onset of symptoms and indicates viral replication independent of disease phase (acute or chronic)  |
| Anti-HBe     | This presence indicates high infectivity<br>It appears after the disappearance of HBeAg<br>Their presence suggests reduction or absence of viral replication, except when infection is due to HBV strains with pre-core mutant (not producing the protein "e") |
| Anti-HBs     | It appears one to 3 mo after HBV vaccination or after recovery of HBV acute infection and indicates immunity to HBV infection  |

HBV: Hepatitis B virus; HBeAg: Hepatitis Be Antigen; HBsAg: Hepatitis B surface antigen; Anti-HBe: Antibodies against HBeAg; Anti-HBs: Antibodies against HBsAg.

region in the HBV genome, which reduces the production of HBeAg<sup>[24]</sup>.

The HBcAg is intracellular and for this reason is not detected in the serum of infected individuals. Antibodies against core protein (anti-HBc) appear shortly after HBsAg in acute infection and persist after acute phase indicating previous exposure. The IgM anti-HBc antibody is the first detected during acute infection, approximately 1 mo after the onset of HBsAg and disappears after 6 mo of infection. Anti-HBc IgG remains detectable in patients cured of hepatitis B and among chronic cases of HBV infection<sup>[28,33]</sup>.

Isolated Anti-HBc can be found in three situations: (1) during the window period of acute phase when the anti-HBc is predominantly IgM; (2) years after acute infection had finished and anti-HBs has diminished to undetectable levels; and (3) when the titer of HBsAg has decreased thereunder the detection level after many years of chronic HBV infection. In order to evaluate the presence of isolated anti-HBc, serum samples should be retested for anti-HBc, HBsAg, anti-HBe, and anti-HBs. If the sample still had an isolated anti-HBc positive result, this sample should be tested for IgM anti-HBc in order to eliminate the possibility of recent HBV infection. HBV DNA testing should be done in chronic liver disease patients to eliminate low-level chronic HBV infection<sup>[34,35]</sup>. The clinical significance of serum markers detected during the course of infection with HBV is disclosed in Table 1.

Specific serologic assays to detect HBV markers were developed around the 70s<sup>[36]</sup>. Various serological techniques could be employed, such as radioimmun-

oassay (RIA), enzyme immunoassay (EIA), Electrochemiluminescence immunoassay (ECLIA) and chemiluminescence immunoassay (CLIA), micro-particle enzyme immunoassay (MEIA), CMIA<sup>[36,37]</sup>.

RIA was the first technique used for HBV diagnosis where one of reactants is conjugated to radioisotopes<sup>[38,39]</sup>. This method present good sensitivity but high cost and risk to operator. In 1971, Engvall *et al.*<sup>[40]</sup> described a technique named EIA that is similar to RIA where enzymes are attached to one of the reactants in an immunoassay to allow detection through the development of color after the addition of a suitable substrate/chromogen. The colored product is monitored visually or by spectrophotometer where the amount of the substance measured is related to color intensity. The advantages of this technique include highly reproducible results, automation, and low cost<sup>[41]</sup>. An evaluation of 70 HBsAg kits showed that 17 HBsAg EIA kits present high analytical sensitivity 4 IU/mL, but reduced sensitivity for HBsAg was observed in samples containing genotypes/subtypes *D/ayw3*, *E/ayw4*, *F/adw4* and *S* gene mutants<sup>[42]</sup>.

MEIA is a method that uses very small microparticles in liquid suspension as solid-phase support. Particles are coated with related molecules specific for the measured material. MEIA is executed in less time than other immunological methods due to the presence of active surface area of microparticles what rises the kinetics study and decreases the incubation time. ECLIA uses molecules as conjugated, generate chemiluminescence like, luminol derivatives, nifrophenil oxalate derivatives or rutenium tri-bipyridyl with tripropylamine. The electrochemiluminescence occurs when is applied an electric current in an electrode platinum, creating an electric field that making all materials respond. This reaction hydrolyzes the chemiluminescent substrate, producing an unstable product which after stabilization generates emission of light photons (amplified) what is measured by a photomultiplier<sup>[43]</sup>.

Kim *et al.*<sup>[44]</sup> compared a radioimmunoassay, an ECLIA (Modular E170 analyzer, Roche Diagnostics, Mannheim, Germany) and CMIA (Architect i2000 analyzer, Abbott Diagnostics, Abbott Park, IL, United States) for HBV markers detection showing concordance rates among the three analyzers of 100%, 91.6%, 94.6%, and 82.2% for HBsAg, anti-HBs, HBeAg, and anti-HBe, respectively. High difference results among three immunoassays analyzers (Abbott AxSYM, Roche Modular Analytics E170, and Abbott Architect i2000) for HBV markers detection was observed in samples with low level of serum HBV markers<sup>[45]</sup>. Xu *et al.*<sup>[46]</sup> also showed that most weak positive results, determined by ECLIA, were negative determined by ELISA.

Huzly *et al.*<sup>[47]</sup> compared the performance of six different automated immunoassays (one MEIA and five chemiluminescence assays) and three EIAs for anti-HBs quantification. The assay specificity ranged between 96.8% and 100% and sensitivity ranged between 93.5% and 100%. There was no difference between anti-HBc-

positive and -negative individuals and, hemolysis or lipemia did not seem to influence the measurement. However, classical EIAs tend to detect lower anti-HBs levels than the automated systems.

EIA is the most widely technique used for detection of HBV serological markers. Although EIAs are sensitive and specific, they are time consuming, need for sophisticated equipment and trained technicians, continuous supply of electricity, and long turnaround time hampering the execution of these assays in field settings and during the household surveys<sup>[48]</sup>. Due to this situation, rapid point-of-care tests (RPOCTs) were developed for HBV diagnosis. These assays use particle agglutination, immunochromatography, immunodot or immunofiltration. The device contains a solid support (cellulose or nylon membranes, latex microparticles or plastic cards) where viral antigens or antibodies are fixed and results can be read in up to 10 min<sup>[49]</sup>. Rapid assays offer advantages of simplicity, low need for instrumentation, minimum training to execute the assay and performance at room temperature.

Since 1990s, several RPOCTs for HBsAg detection have been developed. The sensitivity of these assays varies from 43.5% to 100% while specificity varies from 95.8% to 100%<sup>[50-54]</sup>. A recent systematic review showed that the performance of rapid tests for HBsAg detection was higher in developed countries compared to developing countries what could be due to minimal heterogeneity observed in first than in later. RPOCTs presented analytical sensitivity of 4 IU/mL, but the performance of these assays is poor in seroconversion panels and among individuals infected by several HBV mutants. Thus these tests are not indicated in situations with low concentrations of HBsAg such as in healthy blood donors, general populations, patients recovering from acute HBV infection and those on antiviral therapy<sup>[49]</sup>. RPOCTs for detection of anti-HBc, anti-HBs, HBeAg, anti-HBe markers have demonstrated sensitivities of 85.5%, 64.2%, 80% and 82.8% and specificities higher than 95% for those assays<sup>[55,56]</sup>.

Actually, the evolution of development of nanoscience and nanotechnology has increased the development of immunosensors for HBV diagnosis. Immunosensors are solid-state affinity ligand-based biosensing apparatus that combine immunochemical reactions to proper transducers. Generally, an immunosensor comprises of a sensing element and a transducer. The sensing element is composed by means of the immobilization of antigens or antibodies, and the binding event is transformed into a measurable signal by the transducer<sup>[57]</sup>. The goal of immunosensor is to produce a signal proportional to the concentration of analyte<sup>[58-60]</sup>. Wang and collaborators<sup>[61]</sup> developed a gold nanorod based localized surface plasmon resonance biosensor that quantify HBsAg until 0.01 IU/mL. This limit of detection is about 40 times lower than the limit of detection of the EIA method. Other immunosensors for HBsAg detection uses magnetic nanoparticle and three dimensional carbon nanotube-conducting polymer network detecting 0.001 to 0.015

ng/mL of HBsAg<sup>[62,63]</sup>.

For HBV antigen or antibodies detection, it is necessary blood sample collection by venipuncture in order to obtain sera or plasma samples. However, venipuncture is difficult in some individuals like drug users, haemodialysis, obese and elder individuals. In addition, the transport of these samples from remote areas to laboratories could be difficult. This situation has led to development of methods for HBV diagnosis in alternative fluids, like saliva or dried blood spot (DBS) samples<sup>[64,65]</sup>.

EIA for HBsAg detection among saliva samples present sensitivities of 74.29% to 95.24% and specificities of 89.88% to 100%<sup>[66-70]</sup>. Assays for anti-HBs, total anti-HBc in saliva samples showed low sensitivity (< 15%) while assay for detecting IgM anti-HBc assay demonstrated sensitivity and specificity of 100%<sup>[71]</sup>.

Using DBS samples along to immunoassays, sensitivities varies from 78.6% to 98% for HBsAg detection, 90.5% to 97.1% for anti-HBc detection and 74.2% to 97.5% for anti-HBs detection. In all of these studies specificities varies from 88.6% to 100%<sup>[72-76]</sup>. HBV markers could be detected in DBS samples using EIA until 60 d of storage in room temperature<sup>[74]</sup>. HBV prevalence has been evaluated using DBS in specific groups at risk for infection, such as drug users, and endemic areas<sup>[77-79]</sup>.

Serological diagnostic tests for HBV have improved in sensitivity, time, need for trained personnel and cost over time. In laboratories with low infrastructure or in field situations, rapid tests could be useful since they can be storage at room temperature, results are available in few minutes and they do not need trained technicians. While in laboratories presenting good infrastructure and with high-demand of results, automated ELISA ECIAs, MEIAs, CLIAs and CMIAAs can be used due to its high sensitivity although trained technicians, sophisticated equipment, and continuous supply of electricity are necessary. The main characteristics, advantages, disadvantages and specific applications of HBV serological assays are disclosed in Table 2.

## MOLECULAR METHODS FOR HBV DIAGNOSIS

HBV DNA is detectable at the beginning of infection (1 mo after exposure to HBV) and increases to get a peak approximately 3 mo after the exposure to HBV reaching usually more than 10<sup>8</sup> copies/mL and then progressively decreases in chronic infection or disappears at the resolution of infection. In many chronic patients, when HBeAg seroconversion occurs, low levels of viral load persists (< 10<sup>4</sup> copies/mL)<sup>[80]</sup>.

HBV DNA qualitative and quantitative methods are useful to: (1) diagnose HBV replication in chronic infections; (2) evaluate the prognosis of the disease and follow the risk of progression to cirrhosis and hepatocellular carcinoma; (3) define the beginning of

**Table 2** Advantages and disadvantages characteristics of hepatitis B virus serological assays

| Technique                 | Advantages  | Disadvantage  | Commercial assays  | Ref.          |
|---------------------------|---|---|--|---------------|
| RIA                       | High sensitivity  | High cost<br>Risk to operator   | IRMA kit (North Institute of Biological Technology, Beijing, China)  | [38,39,44]    |
| EIA                       | Automation<br>High reproducible results<br>Low cost   | Time consuming, need sophisticated equipment and trained technicians, continuous supply of electricity, not suitable for field settings | ETI-AB-AUK-3 (DiaSorin)<br>Enzygnost anti-HBs (Dade Behring)<br>Monolisa anti-HBs (Bio-Rad Laboratories)   | [41,42,47,48] |
| MEIA                      | High sensitivity<br>Faster than other immunological methods   | Sophisticated equipment, trained technicians, continuous supply of electricity  | Abbott AxSYM AUSAB assay   | [47]          |
| ECLIA                     | High sensitivity<br>Results available in few minutes<br>Automation  | High cost, sophisticated equipment, trained technicians, continuous supply of electricity   | Modular E170 analyzer, Roche Diagnostics   | [43,44,46]    |
| CLIA                      | High sensitivity and specificity<br>Automation  | High cost, sophisticated equipment, trained technicians, continuous supply of electricity   | Advia Centaur anti-HBs<br>Vitros anti-HBs on the Vitros ECI Immunodiagnostic system (Ortho Clinical Diagnostics)<br>Roche Elecsys anti-HBs on the Modular System (Roche Diagnostics)<br>Liaison anti-HBs on the Liaison system (DiaSorin)<br>Abbott Architect anti-HBs assay on the Architect i2000 system (Abbott)  | [47]          |
| CMIA                      | High sensitivity and specificity<br>Automation  | High cost, sophisticated equipment, trained technicians, continuous supply of electricity   | Architect i2000 analyzer (Abbott Diagnostics)  | [44]          |
| Rapid point-of-care tests | Simplicity, do not need sophisticated equipments, minimum training to execute the assay, storage and performance at room temperature, results can be read in up to 10 min | Poor performance in seroconversion panels and among individuals infected by several HBV mutants   | Determine™ HBsAg (Abbott Laboratories)<br>Virucheck® HBsAg (Orchid Biomedical Systems)<br>Cypress HBsAg (Cypress Diagnostics)<br>Hexagon® HBsAg (Human)<br>Cortez Rapidtest (Cortez Diagnostics)<br>VIKIA HBsAg (Biomérieux)<br>Quick Profile™ (Lumiquick)<br>DRW-HBsAg v2.0 (Diagnostics for the Real World™)<br>AMRAD ICT<br>Binax<br>Advanced quality™ one step test (Intec Products) | [49-56]       |

HBV: Hepatitis B virus; RIA: Radioimmunoassay; EIA: Enzyme immunoassay; MEIA: Micro-particle enzyme immunoassay; ECLIA: Electrochemiluminescence immunoassay; CLIA: Chemiluminescence immunoassay; CMIA: Chemiluminescent microparticle immunoassay; HBsAg: Hepatitis B surface antigen; Anti-HBs: Antibodies against HBsAg.

antiviral treatment; and (4) monitor antiviral treatment and to identify resistance to nucleoside/nucleotide analogues drugs<sup>[81,82]</sup>.

Molecular assays with high-sensitivity are clearly important for the diagnosis of chronic hepatitis B without HBeAg detection in serum (core promoter, precore stop mutation), and occult HBV, where viral loads can be quite low. The principle of methods to detect and quantify HBV DNA is the signal amplification such as branched DNA technology (bDNA) and hybrid capture or target amplification such as PCR<sup>[83-85]</sup>.

In therapeutic monitoring of HBV, a more sensitive assay with a lower limit of detection of 10 IU/mL is recommended for early detection of viral reactivation. In addition, the assay employed should equally quantify all HBV genotypes<sup>[86]</sup>. A commercial PCR method to quantify HBV DNA is COBAS Amplicor HBV Monitor test (Roche Molecular Systems, Pleasanton, California, United States) that measure HBV DNA in serum samples, being considered reproducible, with a high degree of accuracy and precision in both intra-assay and inter-assay com-

parison<sup>[87]</sup>. This assay presented a limit of detection of 200 HBV DNA copies/mL and has the disadvantage of requiring dilution of samples with high viral load, which is not a practical solution for all laboratories<sup>[88]</sup>.

Methods of signal amplification, like bDNA, allow the direct quantification of HBV DNA in human serum or plasma. In this method, sample DNA is caught by a set of capture nucleotide probes fixed on a microtitulation well. Then, another set of target probes hybridize with both the target DNA and capture probes, and this complex is hybridized with multiple copies of alkaline phosphatase. After incubation with a chemiluminescent substrate, the luminometer measures the results as relative light units and the amount of HBV DNA in each sample is determined by comparison to a standard curve. VERSANT HBV 3.0 Assay (Siemens Healthcare, United States) is a commercial method that uses bDNA technology and presented a detection limit of 2000 HBV DNA IU/mL<sup>[83,89]</sup> (Table 3).

Cobas Amplicor HBV Monitor assay (PCR technology) is more sensitive than Quantiplex HBV DNA; Bayer



**Table 3** Performance characteristics of commercial methods for hepatitis B virus and hepatitis C virus detection and quantification

| Assay (manufacture)   | Method                               | Measurable range (IU/mL) | Limit of detection IU/mL (using WHO HBV standard) | Conversion factor (IU/mL to copies/mL) |
|---|--------------------------------------|--------------------------|---|--|
| <b>HBV</b>  |                                      |                          |   |  |
| Cobas ampliPrep/Cobas TaqMan HBV test v2.0 (Roche Diagnostics, California, United States) | Semi-automated qPCR                  | 20-1.7 × 10 <sup>7</sup> | 20  | 5.82                                   |
| Cobas TaqMan HBV test (for use with high pure system)                                     | Semi-automated real time PCR         | 29-1.1 × 10 <sup>7</sup> | 6   | 5.82                                   |
| Abbott real time HBV (Abbott Diagnostic, Chicago, United States)                          | Automated real time PCR              | 10-1 × 10 <sup>9</sup>   | 10  | 3.41                                   |
| Versant HBV 3.0 assay (Siemens Healthcare, United States)                                 | Branched DNA                         | 2000-1 × 10 <sup>8</sup> | 2000  | 5.6                                    |
| <b>HCV qualitative</b>  |                                      |                          |   |  |
| Cobas Amplicor HCV v2.0 (Roche)   | Semiautomated RT-PCR                 | 50                       | 50  | -                                      |
| Versant HCV RNA qualitative assay (TMA)   | Transcription-mediated amplification | < 9.6                    | 5-9.6   | -                                      |
| <b>Quantitative</b>   |                                      |                          |   |  |
| Versant HCV RNA 3.0 (Siemens)   | Branched DNA                         | 7.7 × 10 <sup>6</sup>    | 615   | 5.2                                    |
| Cobas Amplicor Monitor HCV v2.0 (Roche)   | Semiautomated RT-PCR                 | 5 × 10 <sup>5</sup>      | 600   | 2.7                                    |
| Real time HCV (Abbott)  | Real time PCR                        | 1 × 10 <sup>7</sup>      | 10  | 3.8                                    |
| Cobas AmpliPrep/Cobas TaqMan (Roche)  | Automated real time PCR              | 6.9 × 10 <sup>7</sup>    | 43  | 3                                      |
| High Pure/Cobas TaqMan (Roche)  | Semiautomated real time PCR          | 3.9 × 10 <sup>8</sup>    | 25  | 3                                      |

WHO: World Health Organization; HBV: Hepatitis B virus; HCV: Hepatitis C virus; PCR: Polymerase chain reaction; RT-PCR: Reverse transcriptase-PCR; qPCR: Quantitative PCR.

Diagnostics (bDNA technology) to detect HBV DNA among HBeAg negative samples and useful for monitoring the viral load during treatment in chronic HBV infection<sup>[90]</sup>. On the other hand, Cobas Amplicor HBV Monitor presented poor agreement to Digene Hybrid Capture 2 (HC2; Digene Corporation, Gaithersburg, Md.)<sup>[88]</sup>.

Currently, real time PCR has become the standard technique of choice to detect and quantify HBV DNA in clinical practice due to its capacity of detecting low viral loads (10-15 IU/mL) and having a broad dynamic range (upper range of quantification of 7-8 Log<sub>10</sub> IU/mL) as shown in Table 2. Moreover, they do not carry over contamination and can be fully automate<sup>[91]</sup>.

At this time, several real time PCR assays are commercially available, such as, Da-an real-time HBV DNA assay (Da-an Gene Co. Ltd, Sun Yat-Sen University, Guangdong, China), COBAS TaqMan HBV test (Roche Molecular Diagnostics, Pleasanton, CA, United States), Abbott RealTime HBV assay (Abbott Molecular, Des Plaines, IL, United States), Artus RealART HBV LC PCR kit (QIAGEN, Hamburg, Germany), AdvanSure HBV real-time PCR assay (LG Life Sciences, Ltd., Seoul, South Korea). Cobas Taqman HBV (Roche) can be used along to automated sample preparation, Cobas AmpliPrep system (CAP-CTM, Roche Molecular System, Pleasanton, CA)<sup>[92]</sup> and the Abbott HBV (Abbott Diagnostic, Chicago, IL) uses m2000rt amplification platform along to m2000sp device for sample preparation<sup>[93]</sup>. Characteristics of quantitative methods for HBV DNA are described on Table 3.

Kim *et al.*<sup>[94]</sup> showed good correlations among Abbott RealTime HBV Quantification Kit, the COBAS

TaqMan HBV Test, and the VERSANT bDNA 3.0 assay, AdvanSure HBV real-time PCR assay detecting HBV genotypes A-F and without cross reactivity with high HCV RNA levels or high protein concentrations. Qiu *et al.*<sup>[95]</sup> demonstrated good correlation among Abbott and Da-an assay for HBV DNA quantification. Morris *et al.*<sup>[93]</sup> observed good correlation among Cobas Taqman HBV Test and Abbott RealTime HBV for HBV DNA quantification, although Abbott assay presented wide dynamic range without additional dilution or repeating of HBV high titers. In house real time PCR molecular beacon based was builder up by Paraskevis *et al.*<sup>[96]</sup> showing good correlation to COBAS TaqMan HBV test.

HBV DNA detection and quantification are made using serum samples, but alternative fluids, like saliva, DBS samples have been studied as potential fluids for HBV molecular diagnosis<sup>[64,75,97-100]</sup>. Kidd-Ljunggren *et al.*<sup>[98]</sup> demonstrated that concentration of HBV DNA in saliva is 1000-fold lower than in serum, while Heiberg *et al.*<sup>[99]</sup> could quantify HBV DNA using Cobas TaqMan HBV Test (Roche Diagnostics) in saliva samples. Mohamed *et al.*<sup>[75]</sup> demonstrated sensitivity of 98% and specificity of 100% for the detection of HBV DNA using DBS and performed HBV genotyping and mutation detection among those samples with total concordance between the 10 paired DBS and plasma samples. Jardi *et al.*<sup>[101]</sup> also revealed no decrease in HBV DNA levels or integrity among DBS storage for 7 d at room temperature and 21 d at -20 °C.

Besides that, the detection of HBV DNA in plasma or serum is also important to determine the occult hepatitis B defined by detectable HBV DNA in peripheral blood or liver in the absence of HBsAg. Tests for occult



HBV detection are recommended in the following situations: (1) in cryptogenic liver disease, particularly when individual presented anti-HBc in serum; (2) prior to immunosuppression, due to the potential for hepatitis flares; and (3) in solid organ transplant donors whose only anti-HBc is detected in serum, due to the potential for transmission<sup>[83]</sup>.

Evidences are increasingly suggesting that the HBV genotyping is important for designing appropriate antiviral treatment and determining HBV disease progression. The disease progress faster in individuals infected by HBV genotype A than genotype D, subjects infected with genotype C progressed to end stage liver disease earlier than those infected by genotype B and higher mortality rates are observed in individuals infected with genotype F than those infected with genotype A or D<sup>[102]</sup>.

HBV genotyping can be determined using several methods: Reverse hybridization, restriction fragment-length polymorphism (RFLP), genotype-specific PCR assays, sequence analysis, microarray (DNChip), real time PCR and fluorescence polarization assay<sup>[102,103]</sup>. Among them, PCR-RFLP is widely used to genotype HBV since it is inexpensive and simple. Nevertheless, this technique is poor accurate to identify some genotypes<sup>[104]</sup>. A commercial method to genotype HBV is the INNO-LIPA® HBV Genotyping (Fujirebio Europe, Tokyo, Japan) based on reverse hybridization that shows high sensitivity and a detection limit of 700 copies of HBV<sup>[105,106]</sup>, but is relatively cost. INNO-LIPA can be completely automated if using the systems Auto-LiPA48 and AutoBlot 3000H and the LiRAS® for LiPA HBV to reading and interpretation of strips. INNO-LIPA® HBV Genotyping and direct sequencing presented the best results when genotyping methods of HBV were compared<sup>[107]</sup>.

To identify amino acid substitutions associated to antiviral resistance to treatment, direct sequence analysis and reverse hybridization methods are used. nowadays, early detection of HBV substitutions conferring resistance to nucleoside/nucleotide analogues could be useful to modify therapy in order to avoid HBV reactivation and hepatitis flare<sup>[108]</sup>. For this purpose, commercial assays are available, such as the Trugene HBV Genotyping Kit (Siemens Medical Solutions Diagnostics), which is based on direct sequence analysis of a portion of the reverse transcriptase domain of the HBV polymerase gene<sup>[109]</sup> and can also determine HBV genotype satisfactory<sup>[110]</sup>.

However, despite commercial methods can be used for mutational analysis in HBV DNA sequence, direct DNA sequencing is still the standard method cause yields accurate genotype assignments and also the method of choice for patients infected with recombinant genotypes and drug resistance mutations<sup>[110,111]</sup>.

In the past years, the diversity of HBV quasispecies and minority drug resistance mutations were estimated through cloning of individual amplicons followed by Sanger sequencing. However, the "next generation" ultradeep-sequencing allows direct sequencing of the

mixed population sample and relative quantification of individual mutations with extremely high coverage over a relatively short time frame<sup>[111-114]</sup>. The platforms of ultradeep sequencing include: 454 Sequencing (Roche Diagnostics), Illumina Sequencing (Illumina/Solexa) and Pyromark Sequencing (QIAGEN), SOLiD (Applied Biosystems/Life Technologies), Ion torrent (Life Technologies)<sup>[115]</sup>. All of them present the principle of sequencing by synthesis, that involves sequencing of a single strand of DNA through synthesis of the complementary strand, one base at a time, and the detection of the individual nucleotide incorporated at each step. Fluorescence or light is only emitted when the nucleotide solution complements the first unpaired base in the template DNA strand<sup>[116]</sup>. Because these signals are obtained by synthesizing new copies of DNA template, the results can be used for extremely reliable investigation of viral mutations<sup>[114]</sup>.

For the past twenty years fluorescence based quantitative PCR (qPCR) chemistries have revolutionized nucleic acid diagnostics and become the gold standard for viral load quantification. These methods are high sensitivity and could be used to evaluate the prognosis and risk of progression, to define the beginning of antiviral treatment and to monitor antiviral treatment but the major disadvantages is the high cost for commercial assays. Methods to determine HBV genotypes and mutations, like nucleotide sequencing have been developed and recently the arouse of third generation sequencing methods promises to reduce the lengthy manual handling times associated with current ultradeep-sequencing approaches, decreasing the generation of raw data, but increasing both throughput and read length in every facet of medical research.

The main characteristics, advantages, disadvantages and specific applications of HBV molecular assays are disclosed in Table 4.

## SEROLOGIC TESTS FOR HCV

The hepatitis C diagnosis is performed through the detection of antibodies anti-HCV in blood samples where a non-reactive test result indicates the absence of HCV infection. A positive result for anti-HCV detection or a suspected case of HCV exposure should be followed by HCV RNA test. Serological diagnosis for HCV infection are based on the detection of direct antibodies against viral antigens (non-structural and structural) in human serum or plasma. As the same as cited for HBV serological diagnosis and immunoassays (like EIA or ECLIA) are generally used for anti-HCV detection. EIA has the advantages of high sensitivity, fast processing, high reliability, ease of automation and relatively low cost<sup>[57,117]</sup>. This method has undergone some changes over time, seeking to improve their diagnostic capability and therefore increased sensitivity and specificity of the assay. Until now, three generations of EIA for detecting anti-HCV using recombinant proteins or synthetic peptides have been developed<sup>[57,117,118]</sup>.

**Table 4 Main characteristics of hepatitis B virus and hepatitis C molecular techniques**

| HBV and HCV molecular diagnosis               | Application   | Method                               | Advantages   | Disadvantages   | Ref.          |
|---|---|--------------------------------------|--|---|---------------|
| HBV DNA qualitative methods                   | Diagnose infection  | PCR                                  | Low cost; high sensitivity   | It only determines the presence or absence of HBV DNA   | [81,82,85]    |
|   | HBV occult cases identification   |                                      |  |   |               |
|   | Screening on blood donors   |                                      |  |   |               |
|   | Evaluate the prognosis and risk of progression  |                                      |  |   |               |
| HBV DNA quantitative methods                  | Define the beginning of antiviral treatment   | bDNA                                 | Wide dynamic range   | Low sensitivity to detect low HBV DNA levels  | [81,82]       |
|   | Monitor antiviral treatment   |                                      |  |   |               |
|   |   | Hybrid capture                       | More sensitive than bDNA; less labor-intensive   | Low sensitivity to detect low HBV DNA levels; individual probes are required  | [83]          |
|   |   | Real time PCR                        | Capacity of detecting low viral loads; broad dynamic range; do not carry over contamination; can be fully automated                | High cost   | [85,91]       |
| HBV DNA genotyping methods                    | Determination of HBV genotype   | RFLP                                 | Easily done; low cost; simple, rapid and suitable for large number of samples  | Low sensitivity for typing samples with low HBV DNA levels; poor accurate to determine some genotypes   | [85,104]      |
|   |   | Genotype specific PCR assays         | Automated systems; high sensitivity; easy to perform; suitable for detecting mixed genotype infections                             | High cost   | [107]         |
|   |   | Sequence analysis                    | Identification of patients infected with recombinant genotypes   | Technically demanded; time consuming  | [107]         |
|   |   | Direct DNA sequencing                | Accurate   | Technically demanded; time consuming; necessity of cloning for identification of mixed population   | [107,110,111] |
| HBV DNA aminoacid substitution identification | Identify antiviral resistance to treatment  |                                      |  |   |               |
|   |   | Commercial methods                   | Sequencing of mixed population, relative quantification of individual mutations with extremely high coverage                       | Differences between the statistical and biological/clinical relevance of HBV mutation maximal sequence read length and PCR amplification bias | [114]         |
|   |   |                                      |  |   |               |
|   |   |                                      |  |   |               |
| HCV RNA qualitative methods                   | To confirm chronic hepatitis C in patients with positive HCV antibodies   | RT-PCR                               | High sensitivity   | It only determines the presence or absence of HCV RNA   | [121,168]     |
|   | To identify virological response during, at the end or after antiviral therapy  |                                      | Equal sensitivity for all genotypes  |   |               |
|   | To screen blood donations for evidence of infection with HCV  |                                      |  |   |               |
|   |   | Transcription-mediated amplification | High sensitivity; amplifies viral RNA; more sensitivity for detection of genotype 1  | It only determines the presence or absence of HCV RNA   | [121,168]     |
| HCV RNA quantitative methods                  | To guide treatment decisions;   | bDNA                                 | Wide range of detection of HCV independent of HCV genotype (615 IU/mL to 8 million IU/mL)  | Low sensitivity to detect samples presenting low HCV RNA levels   | [172]         |
|   | To evaluate the prognosis;  |                                      |  |   |               |
|   | To monitor the antiviral efficacy of treatment  | qRT-PCR                              | Capacity of detecting low viral loads; broad dynamic range; not carry over contamination; can be fully automated                   | High cost   | [170-173]     |
|   |   |                                      |  |   |               |
| HCV RNA genotyping methods                    | HCV genotyping is mandatory for double antiviral treatment (interferon and ribavirin), since patients infected with genotypes 1 or 4 are treated for longer times than patients infected by genotypes 2 and 3 | RFLP                                 | Easily done; low cost; simple, rapid and suitable for large number of samples  | Low sensitivity for typing samples with low HCV RNA levels; Poor accurate to determine some genotypes   | [186,187]     |
|   |   |                                      |  |   |               |
|   |   | Probes                               | Easily done; low cost; useful to detect HCV genotypes and subtypes based on region 5'UTR and core and has a low limit of detection | Identify only subtypes 1a and 1b; discrepant results among subtypes when compared to sequence analysis of NS5B region                         | [180-184]     |
|   |   |                                      |  |   |               |

|   |  |                   |   |   |                   |
|---|--|-------------------|---|---|-------------------|
| HCV RNA aminoacid substitution identification | Identify antiviral resistance to treatment | qPCR              | Can be fully automated avoiding contamination; determines the viral genotype and subtypes 1a, 1b, 2a, 2b, 3, 4, 5 and 6   | High cost   | [186,187]         |
|   |  | Direct sequencing | Gold standard; identification of patients infected with recombinant genotypes   | Technically demanded; time consuming  | [120,182]         |
|   |  | Direct Sequencing | Identification of antiviral resistance in majority population   | Technically demanded; time consuming; necessity of cloning for identification of quasispecies | [188,189,193]     |
|   |  | Deep Sequencing   | Identification on resistant variants predominate in the HCV population; powerful tool for obtaining more profound insight into the dynamics of variants in the HCV quasispecies | Need for in-depth knowledge to analyze the results  | [112,113,195,196] |

PCR: Polymerase chain reaction; HCV: Hepatitis C virus; HBV: Hepatitis B virus; RFLP: Restriction fragment-length polymorphism; RT-PCR: Reverse transcriptase-PCR; qPCR: Quantitative PCR; RFLP: Restriction fragment-length polymorphism; bDNA: Branched DNA technology.

The first generation HCV EIA, which is no longer used, was created using recombinant protein derived from the NS4 region (C100-3 the polypeptide), with a sensitivity of 70%-80% and a poor specificity. The C-100 antibodies are developed approximately 16 wk after HCV infection<sup>[57,119-122]</sup>.

The second generation HCV EIA have included recombinant/synthetic antigens from non-structural NS3 and NS4 (c33c and C100-3) and core (c22-3) regions improving sensitivity to about 95% and diminishing the number of false-positive results. Anti-HCV can be detected nearly 10 wk after HCV infection<sup>[57,119,121,122]</sup>.

Third generation HCV EIA was developed using recombinant antigens from the core region, NS3, NS4 and NS5 regions of the viral genome. These assays allowed anti-HCV detection nearly 4 to 6 wk after infection with specificity and sensitivity greater than 99%<sup>[57,121,123-125]</sup>.

Up to now, there is no reliable and simple diagnostic marker currently available to detect early HCV infection. The avidity of an antibody may be an early and reliable marker of recent viral infection, since antibodies of low avidity are usually indicative of recent infection. Commercial immunoassays for anti-HCV antibody detection have been optimized to evaluate avidity in serum and DBS samples and "in-house" anti-HCV IgG avidity assay has been developed using seroconversion panels and serum samples from chronically infected individuals<sup>[126-129]</sup>.

Serum samples are necessary to investigate the presence of anti-HCV using EIA, but some studies have been conducted using alternative fluids like saliva and DBS. Anti-HCV assays using saliva and DBS samples showed sensitivity and specificity higher than 90% and DBS samples can be stored for a period of 117 d, at room temperature. Then, these samples could be useful tool to increase the access of diagnosis in remote areas or individuals with poor venous access<sup>[130-136]</sup>.

Anti-HCV detection is not useful to identify current or past infection, since this marker will be in serum for life after HCV exposure<sup>[125,137]</sup>. In this situation, HCV

RNA detection is generally recommended, but the cost and availability of this assay difficult the access of this method. Assays for detection of HCV core antigen (HCV Ag) or simultaneous detection of HCV Ag and anti-HCV antibodies (HCV AgAb) were developed. HCV Ag levels seems to correlate well with HCV RNA levels indicating its potential use as an inexpensive technique for diagnosing HCV acute cases<sup>[138-141]</sup>. Brandão *et al.*<sup>[133]</sup> optimized commercial methods for HCV Ag/Ab in DBS samples showing specificity of 99.71% for Monolisa™ HCV AgAb ULTRA and 95.95% for Murex HCV AgAb and sensitivity of 97.5% for both assays. Larrat *et al.*<sup>[142]</sup> tested Monolisa™ HCV AgAb ULTRA along to oral mucosal transudate demonstrated sensitivity of 71.7% and specificity of 94.3%. These methods were employed among haemodialysis and homosexual individuals showing good performance to early detection of hepatitis C virus infection during window period of HCV infection<sup>[143,144]</sup>.

Other immunoassays for anti-HCV detection include ECLIA, CMIA, CLIA, MEIA. In these assays, HCV antigens are presented on distinct solid phases (microwell, magnetic and paramagnetic particles) and anti-HCV at clinical sample is then identified using a conjugate antibody (anti-human IgG labeled with acridinium or horseradish peroxidase) that catalyzes the oxidation of a luminol, generating light. The system measures the light signal that is normalized in relation to cut-off value [signal/cut-off (S/CO)], or given as relative lights units<sup>[145,146]</sup>. Most of these assays used antigens from core, NS3, NS4 and NS5 of HCV. Sample volume varies from 10 to 40 microliters and reaction is executed from 18 to 58 min<sup>[147]</sup>.

To confirm positive or indeterminate results by immunoassays, recombinant immunoblot assays (RIBA) can be used as complementary test, especially in low prevalence settings. This test is highly specific due to the presence of recombinant proteins and synthetic peptides of envelope, NS3, NS4, NS5 regions of HCV on a membrane strip. HCV antibodies present in clinical samples should react to these proteins leading to the

appearance of cored dots at specific antigens in the strips. Interpretation may be visual or automated and a positive result is considered when two or more bands are visualized on the nitrocellulose strip, representing a specific antigen-antibody reaction. Indeterminate result is showed when only one band is visualized and negative results are obtained when no bands are observed. The major disadvantage of RIBA is the occurrence of indeterminate results, especially in those specimens with grey-zone results in the screening assays. Currently, this assay has been substituted as a confirmatory test by widely-used molecular techniques, which can additionally distinguish between active and resolved infections<sup>[121,147,148]</sup>.

Nowadays there have been emergent needs for developing highly accurate, rapid and cheap analytical tools. To achieve this goal, many attempts have been focused on the development of rapid point-of-care testing (POCT) such as lateral flow tests. These techniques are based on immunochromatographic assay, lateral flow tests (LFT) or test strips. The advantages of POCT are the time for execution, simplicity and cost-effective<sup>[149]</sup>. The principle of LFT is similar to ELISA, and the base substrate is nitrocellulose membrane in which the solid phase is immobilized capture binding protein, usually an antibody or antigen. Labels such as latex, colloidal gold, carbon, and recently up-converting phosphorus technology have been employed in LFT development<sup>[150,151]</sup>. POCT is useful for HCV detection in field situations, particularly among hard-to-reach, high-risk populations, such as drug users or individuals living in remote areas<sup>[152-154]</sup>. A good performance of POCTs compared to EIA or PCR results were observed with sensitivities and specificities above 90%<sup>[152-156]</sup>. A recent metanalysis compared seven POCT and OraQuick had the highest test sensitivity and specificity and showed better performance than a third generation enzyme immunoassay in seroconversion panels<sup>[156]</sup>. This assay could be used along to serum, whole blood and saliva samples what could increase the access of diagnosis in emergency situations<sup>[151,154]</sup>.

Biosensor technology has emerged as alternative technique with low detection limit, higher selectivity and sensitivity, and faster responses for anti-HCV detection. Biosensor employs specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles, or whole cells to detect chemical compounds, usually by means of electrical, thermal, or optical signals. The surface of bioreceptor contains a biological recognition element that interacts to analyte; then the transducer converts the recognition event into a measurable signal; finally, the output from the transducer is amplified, processed and displayed. The concentration of the analyte is proportional to the intensity of signal<sup>[57]</sup>. The main advantages of biosensors are offering a quantitative test for detection in cases with about 100 copies of hepatitis virus, in addition to automation, multiplexing analysis and throughput. Some biosensors available for anti-HCV detection include

surface plasmon resonance, piezoelectric biosensors, microcantilever based biosensors, electrochemical biosensor and apta-sensor<sup>[121,149]</sup>.

EIA is widely used for anti-HCV detection, and has the advantage of high sensitivity, fast processing, high reliability, ease of automation and relatively low cost<sup>[57,117]</sup>, but this technique did not identify if the infection is current or past, and therefore requires confirmation of the results by a more specific method, such as HCV RNA<sup>[125,137]</sup>. Other immunoassays for anti-HCV detection as MEIA, CMIA, ECLIA and CLIA have the advantage of being performed in less time, with less incubation period and easily automatable. While RIBA can be used as a complementary test to confirm positive or indeterminate by immunoassays. The advantage is that this test is highly specific, due to the presence of recombinant proteins and synthetic peptide regions of the envelope, NS3, NS4, NS5 of HCV. However, its drawback is the occurrence of indeterminate results. Therefore, this assay has been replaced by molecular testing to confirm results, so distinguishing active infection cured<sup>[48,121,147]</sup>.

Every day more, is continuing need to develop highly accurate, rapid and inexpensive analytical tools. Therefore, the immunochromatographic techniques, such as rapid diagnostic tests have been developed and widely used. Present as the short runtime advantages, simplicity and low cost<sup>[149]</sup> as well as being useful for detecting HCV in difficult access populations, high risk, such as injecting drug users or people living in remote areas<sup>[152-154]</sup>. The biosensor technology has emerged as an alternative technique with low anti-HCV detection limit, higher sensitivity and selectivity, and faster for the detection of anti-HCV responses. The main advantages of biosensors are automation, multiplexing analysis and fast processing<sup>[121,149]</sup>, but these assays are not widespread and present high costs. Information regarding different aspects of HCV assays is available on Table 5.

## MOLECULAR TECHNIQUES FOR DIAGNOSING HCV INFECTION

In hepatitis C infection, the molecular virological techniques play a key role both in diagnosis and in monitoring of HCV antiviral treatment. Due to the difficulty to grow HCV in cellular culture, molecular techniques were used to identify this virus, making HCV one of the first pathogens to be identified solely by molecular diagnostics<sup>[8]</sup>.

The hepatitis C viral genome can be detected in blood, by nucleic acid testing (NAT). The presence of HCV RNA is a marker for HCV viremia and is detected only in persons who are currently infected. Patients with anti-HCV detected should be evaluated for the presence of HCV RNA in their blood<sup>[157]</sup>. In this regard, the detection and quantification is useful in clinical practice to: (1) diagnose chronic HCV infection; (2) guide treatment decisions (identify patients who need antiviral therapy and offer them the most adapted genotype treatment);



**Table 5** Main Characteristics of serological assays for hepatitis C virus diagnosis

| Serological assays   |                                | Antigen (region of the genome)                                     | Assay/manufacturer   | Sensitivity | Specificity | Ref.          |
|----------------------|--------------------------------|--|--|-------------|-------------|---------------|
| EIA                  | 1 <sup>st</sup> generation EIA | c100-3 (NS3-NS4)   | HCV-Ac-EIE Salck   | 70%-80%     | 50%-70%     | [118,119]     |
|                      | 2 <sup>nd</sup> generation EIA | c100-3 (NS3-NS4), c33-c (NS3), c22-3 (core)                        | ORTHO HCV ELISA test system  | 92%-95%     |             | [118,119]     |
|                      | 3 <sup>rd</sup> generation EIA | c100-3 (NS3-NS4), c33-c (NS3), c22-3 (core), NS5                   | ORTHO HCV 3.0 ELISA (Ortho)/ETI-AB-HCVK Sorin                              | 95%-99%     | 99%         | [57,118]      |
|                      | 4 <sup>th</sup> generation EIA |  | Monolisa™ HCV AgAb ULTRA (BioRad)/HCV Murex AgAb (Abbott)                  | 100%        | 99.5%       | [117,133,144] |
| MEIA                 |                                | HCr43 (Fusion core e NS3), c200 (NS3 - NS4), c100-3 (NS3-NS4), NS5 | AxSYM® HCV 3.0 (Abbott)  | 100%        | 99.8%       | [117,118]     |
| ECLIA                |                                | Core, NS3 and NS4 proteins   | Elecsys anti-HCV assay (Roche)   | 100%        | 99.7%       | [117,118]     |
| CLIA                 |                                | [c22-3 (core), c200 (NS3 - NS4) and NS5]                           | ARCHITECT i4000 anti-HCV assay (Abbott); VITROS Eci anti-HCV assay (Ortho) | 99.5%       | 98.2%       | [145]         |
| CMIA                 |                                | HCr43 (core - NS3), c100-3 (NS3-NS4)                               | ARCHITECT® anti-HCV (Abbott)   | 99.1%       | 99.6%       | [146]         |
| RIBA                 | RIBA-1                         | 5-1-1 (NS4) e c100-3 (NS3-NS4)                                     |  | NP          | NP          | [118]         |
|                      | RIBA-2                         | 5-1-1 (NS4), c100-3 (NS3-NS4), c33-c (NS3), c22-3 (core)           | Chiron RIBA-2.0 RIBA-2   | NP          | NP          | [148]         |
|                      | RIBA-3                         | c100-3 (NS3-NS4), c33-c (NS3), c22-3 (core), NS5                   | Chiron RIBA HCV 3.0 SIA  | NP          | NP          | [118]         |
| Biosensor technology |                                | Core protein (p22 fusion protein), NS3, NS4 and NS5                | mBio Diagnostics® company  | NP          | NP          | [57,137]      |

NP: Not presented; EIA: Enzyme immunoassay; MEIA: Micro-particle enzyme immunoassay; ECLIA: Electrochemiluminescence immunoassay; CLIA: Chemiluminescence immunoassay; CMIA: Chemiluminescent microparticle immunoassay; RIBA: Recombinant immunoblot assays; HCV: Hepatitis C virus; ELISA: Enzyme-linked immunosorbent assay.

(3) monitor the antiviral efficacy of treatment; (4) identify amino acid substitutions responsible for direct acting antiviral drug (DAA) resistance; (5) to confirm the presence of HCV viremia in patients who are seronegative (anti-HCV non reactive) but immunocompromised such as HIV infected individuals; and (6) in babies who are born to HCV positive mothers- once antibody testing in babies can give false positive results up to 18 mo of age<sup>[121,158]</sup>.

Approximately 1 mo before the appearance of total anti-HCV antibodies HCV RNA can be already detected 1-3 wk after infection<sup>[81]</sup>. Molecular methods are useful to identify the stage of infection. A negative NAT result with positive serological test is usually indicative of a resolved infection or spontaneous resolution<sup>[159]</sup>, but low-level of viremia may occur during chronic infection, for these reasons a second NAT should be performed 6 to 12 mo later. A positive HCV NAT result indicates active infection independent of antibody test results. NAT are used before, during, and after antiviral treatment to indicate whether HCV is present or not, and to determine when and whether treatment should be stopped or continued<sup>[160]</sup>. In acute infections, the NAT result will become positive within 1 to 3 wk, several weeks earlier than serological tests, as in occupational exposures<sup>[120]</sup>. This way, the serological window present in HCV infection can be resolved using qualitative and quantitative nucleic acid testing, whereas these techniques have a wide dynamic range of detection, which is well chosen according to the clinical needs (upper range of quantification: 7-8 log<sub>10</sub> IU/mL)<sup>[161]</sup>.

HCV detection and quantification is made using sera samples, but saliva, DBS and platelets have been studied as alternative samples<sup>[162-165]</sup>. The detection of HCV RNA in these samples could be useful to increase the access of molecular diagnosis for HCV infection and to evaluate antiviral response in some groups, like haemodialysis, children, drug users. A systematic review demonstrated good correlation among HCV RNA quantification from DBS and whole plasma<sup>[166]</sup>, but low levels of HCV among saliva and platelets compared to sera samples<sup>[162,163]</sup>.

Qualitative HCV assays comprises viral RNA isolation, complementary DNA (cDNA) production, PCR amplification and detection of PCR amplicons<sup>[121]</sup>. A large amount of commercial and in-house PCR for HCV uses primers for amplification of 5' untranslated region (UTR) since this region has above 90% of sequence identity among distinct HCV genotypes, where several fragments are almost undistinguishable among distinct strains<sup>[121]</sup>. The 5'UTR is the first region to be transcribed and has secondary and tertiary structures that are largely conserved<sup>[167]</sup>. Furthermore, core and 3'UTR are also used in PCR for detection of HCV<sup>[121]</sup>.

Qualitative NAT are used as the first diagnosis of a suspected acute infection, to confirm chronic HCV infection in patients with antibodies anti-HCV positive, confirmation of virological response during, at the end or after antiviral therapy and to screen blood donations for evidence of infection with HCV<sup>[120,168]</sup>. These tests usually utilize conventional reverse transcriptase-PCR (RT-PCR) or transcription-mediated amplification (TMA).



In RT-PCR-based assays, HCV RNA is the source for production of a single-stranded complementary cDNA by reverse transcriptase. DNA polymerase amplifies cDNA into multiple double-stranded DNA copies. In TMA assay, viral RNA is isolated from clinical sample and two enzymes (T7 RNA polymerase and reverse transcriptase) amplify this RNA. Hybridization protection assay detects these amplicons by probe hybridization in which only hybridized probes stay chemiluminescent and are detected in a luminometer<sup>[168]</sup>.

Qualitative RT-PCR assays should detect 50 HCV RNA IU/mL or less with the same sensitivity for all genotypes. With the advent of more qPCR that has a lower limit of detection to as low as 30 copies/mL, qualitative assays were replaced especially in diagnostic laboratories<sup>[120,169]</sup>. These qualitative tests are still very common due to its higher sensitivity, but the main inconvenience is that it only verifies the absence or presence of HCV RNA<sup>[121,168]</sup>.

Some qualitative commercial assays include Cobas Amplicor HCV version 2.0 (Roche Molecular Diagnostics, Pleasanton, CA, United States) and Versant HCV RNA qualitative assay (Siemens Healthcare Diagnostics, Deerfield, IL, United States). Cobas Amplicor HCV is performed according to standard RT-PCR and 50 IU/mL is the limit of detection for all HCV genotypes, while Versant HCV qualitative assay employs TMA and presents analytical sensitivity of 10 IU/mL for most genotypes and 5.3 IU/mL for genotype 1<sup>[121]</sup>.

HCV RNA quantification can be accomplished by target or signal amplification, respectively quantitative RT-PCR (qRT-PCR) and branched deoxyribonucleic acid (bDNA) technology. Due to its good sensitivity (99%) and specificity (98%-99%), the classical techniques for viral genome detection and quantification are progressively being replaced by quantitative PCR<sup>[120,169]</sup>. When these tests are used to monitor viral load during treatment, it is critical to use the same assay before and during therapy<sup>[120,169]</sup>.

Several tests are commercially available for HCV quantification and those assays employ competitive PCR (Cobas Amplicor HCV Monitor), bDNA technique (Versant HCV RNA), real time PCR (COBAS TaqMan assay and Abbott Real Time HCV test). To quantify HCV by Cobas Amplicor HCV Monitor 2.0, the target and internal standard is amplified in a single reaction tube. The initial quantity of HCV RNA is obtained by comparing the final amounts of both templates. The dynamic range of the Amplicor™ HCV 2.0 monitor assay is 500 to approximately 500000 IU/mL with a specificity of almost 100%, independent of the HCV genotype<sup>[170,171]</sup>. The Versant HCV quantitative test (Siemens Healthcare Diagnostics) is based on signal amplification by bDNA and present a range of detection of 615 IU/mL to 8 million IU/mL independent of HCV genotype<sup>[172]</sup>. Martins *et al.*<sup>[173]</sup> compared qualitative (in-house RT-nested PCR and COBAS AMPLICOR HCV Test v2.0 and TMA) and quantitative (COBAS AMPLICOR HCV Monitor Test v2.0 and bDNA) techniques for HCV quantification and detection, and TMA presented the highest rate (87.8%)

of HCV detection among qualitative tests being the most sensitive for HCV RNA detection over the early and late phases of HCV infection.

Real time PCR technology allows the direct monitoring of the PCR process due to the detection and amplification of the target nucleic acid at the same time. In order to detect and amplify DNA at the same time, a probe (oligonucleotides containing a quenching molecule and a fluorescent reporter molecule) binds to target cDNA between the two PCR primers and are degraded or released by DNA polymerase during DNA synthesis. When probe is degraded, it occurs the separation of reporter and quencher molecules, which leads to emission of an increased fluorescence signal from the reporter. The quantity of RNA in the starting (first cycle) sample is proportional to the fluorescence signal. Quantification in absolute numbers is obtained by the comparison of kinetics of the target amplification with the amplification kinetics of an internal control of a defined initial concentration<sup>[168]</sup>.

Cobas Taqman HCV present limit of detection and quantification of approximately 15 IU/mL among all HCV genotypes, and a linear amplification range of HCV RNA from approximately 15 to 10000000 IU/mL<sup>[174]</sup>. Abbott RealTime HCV assay has a lower detection limit of nearly 10 IU/mL, a specificity higher than 99.5% and a linear amplification range from 12 to 10000000 IU/mL independent of the hepatitis C genotype<sup>[175-177]</sup>.

Currently, there are leastwise seven genotypes and more than 80 subtypes of HCV<sup>[178,179]</sup>. Methods for HCV genotyping are direct sequence analysis, real time PCR, RFLP, and reverse hybridization technology. HCV genotyping is mandatory for double antiviral treatment (interferon and ribavirin), since patients infected with genotypes 1 or 4 are treated for longer times than patients infected by genotypes 2 and 3<sup>[180]</sup>. Nevertheless, with the accessibility of new and highly effective antiviral therapies, HCV genotyping will not be important in the future.

The first commercial assays for HCV genotyping evaluated exclusively the 5'UTR, which has a high ratio of misclassification particularly on the subtype level. Nowadays commercial tests analyze coding regions, especially non-structural protein NS5B and core protein.

TRUGENE-SIEMENS HCV 5'NC Genotyping Kit (Siemens Medical Solutions Diagnostics, Tarrytown, NY, United States) is useful to detect HCV genotypes and subtypes based on region 5'UTR and has a lower limit of detection of 5000 IU/mL<sup>[181,182]</sup>. Versant HCV Genotype 2.0 Assay (LiPA) uses oligonucleotide probes specific for the 5'UTR and core regions of the six HCV genotypes and has lower limit of detection of 3700 IU/mL<sup>[183]</sup>. LiPA is efficient for HCV genotyping, but some divergent results were observed when compared to sequence analysis of the NS5B region at the subtype level (sensitivity of 95.2% for subtype 1b and 96.1% for subtype 1a)<sup>[184]</sup>.

Real time PCR using TaqMan technology as Abbott Real time PCR HCV Assay (Abbott Diagnostics Europe,

Wiesbaden, Germany) determines the viral genotype of the samples for HCV genotypes 1a, 1b, 2a, 2b, 3, 4, 5 and 6 and has limit of detection of 6053 IU/mL<sup>[185]</sup>. On the other hand, in house methods like RFLP presented a limit of detection using the Probit test ranging from 51 to 3300 IU/mL<sup>[161]</sup>. RFLP is a useful technique for HCV genotyping in different groups since presents low cost compared to commercial methods<sup>[186,187]</sup>.

The direct sequencing (Sanger sequencing) is the gold standard for determining HCV genotypes and subtypes HCV. Nucleotide sequencing involves sequencing of one or more genes in the HCV genome (mainly the 5'UTR, core, E1, NS3 and NS5) and comparing these sequences to the established genotypes by computer analysis<sup>[120]</sup>. This provides the most complete information on the variations of the sequences analyzed. Furthermore, it is the most useful method for the study of viral genetic variability<sup>[182]</sup>.

The sequencing technique for HCV genotyping consists of PCR amplification of part of the viral genome, especially these regions: The 5'NC, NS5B and core regions. These regions present diversity for the discrimination of viral genotypes and subtypes and are sufficiently conserved for the development of reliable primers<sup>[188]</sup>. In addition to genotype determination, direct sequencing is also used for molecular epidemiological studies and to DAA resistance mutations<sup>[189-193]</sup>. In DAA treatment, the replication is intensively inhibited in drug-sensitive viral population, and the resistant variants gradually predominate in the HCV population<sup>[194]</sup>. This way, in a near future, the most appropriate treatment for HCV patients will be based on the analysis of the nucleotide or amino acid sequence<sup>[195]</sup>.

Actually, deep sequencing technologies is a promising approach to characterizing viral diversity, they have the ability to generate high throughput screening that provide exceptional resolution for studying the underlying genetic diversity of complex viral populations<sup>[112,113,196]</sup>. Currently Illumina deep sequencing technology (Illumina Inc. San Diego, CA) and PacBio sequencing technologies (Pacific Biosciences of California, Inc.) are the newest platforms in the market. In HCV studies, deep sequencing technologies are powerful tools for obtaining more profound insight into the dynamics of variants in the HCV quasispecies of human serum. It allows sequencing the complete genome in a short time and is able to generate much more information on the viral genome sequences in internal organs<sup>[195]</sup>.

Due to the rate of  $10^{-3}$  mutations per nucleotide, HCV results in high-circulating quasispecies in infected patients. Recently, it was observed that approximately 15.6% of samples from Pakistan did not match any genotype<sup>[197]</sup>. Ultra deep sequencing could be useful for identification of these genotypes what is important to determine the pattern of double antiviral therapy (interferon and ribavirin) that is a standard therapy in Pakistan and other countries with few resources.

HCV quantitative methods, like qPCR are useful to monitor antiviral therapy and could detect low viral

loads with broad dynamic range. These assays are fully automated and reduced contamination. However, commercial assays present high cost compared to in house qualitative methods. For HCV genotyping, the method most useful is nucleotide sequencing, principally ultra deep sequencing that could identify resistant variants predominate in the HCV population and give information about dynamics of HCV quasispecies. However, it is necessary in-depth knowledge to analyze the results.

The main characteristics, advantages, disadvantages and specific applications of HCV molecular assays are disclosed in Table 4.

## CONCLUSION

In this review, we attempted to give information regarding HCV and HBV serological and molecular methods available at clinical and research areas. Most of review articles regarding HBV and HCV diagnosis are relatively old<sup>[28,120,148,198]</sup> or discuss only one aspect of viral hepatitis diagnosis, for example, rapid tests for HBV<sup>[24]</sup>, rapid tests for HCV<sup>[156]</sup>, serological methods for HBV<sup>[49]</sup>, serological methods for HCV<sup>[117]</sup>, molecular methods for HBV<sup>[83]</sup> or molecular methods for HCV<sup>[120]</sup>. In the present review, both serological and molecular methods for HBV and HCV diagnosis were included and new methods such as biosensors and ultra deep sequencing were discussed giving new and updated information about this theme.

Diagnosis of HBV and HCV infection is a key tool to identify acute and chronic cases of infection in order to define preventive measures and to initiate antiviral treatment. Nowadays HBV vaccination and antiviral therapies for HBV and HCV infection have arisen drug resistant, vaccine and diagnosis escape variants that complicate diagnosis and treatment. In this situation, effective diagnosis presenting high sensitivity and specificity is crucial.

Each detection method presents advantages and limitations. EIAs are the most important serological assays used for HBV and HCV detection due to its simplicity, automation and convenience. Nevertheless, they can be time-consuming and expensive and rapid assays have been developed in order to overcome these disadvantages. Molecular techniques are useful to diagnose chronic infection; to identify HBV occult cases; to evaluate the prognosis of disease; to help in treatment decisions and monitor the antiviral treatment efficacy; and to identify resistance mutants to antiviral treatment. Molecular methods present higher specificity and sensitivity and larger dynamic range of detection compared to other diagnostic assays like serological assays. Nevertheless, these methods are relatively expensive and require special instruments and specialized techniques. The choice of each method should be done according advantages and disadvantages and the purpose of diagnosis.

In the near future, biosensors and biochips seems to be useful technologies for serological diagnosis of

HBV and HCV, principally due to real-time diagnosis and early intervention to reduce the burden of diseases. Alternative specimens and rapid assays can also be extremely useful for remote areas, low resource settings and health services with limited laboratory infrastructure. In respect to molecular HBV and HCV assays, digital PCR promises to resolve some of the deficiencies of qPCR by transforming the analog, exponential nature of PCR into a digital and linear signal. For DNA sequencing, ultra deep sequencing will be helpful for analysis of HBV and HCV mutants in order to study the dynamics of viral variants.

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## Hepatitis E virus infection: Epidemiology and treatment implications

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### Abstract

Hepatitis E virus (HEV) infection is now established as an emerging enteric viral hepatitis. Standard treatments in acute and chronic hepatitis E remain to be established. This study undertakes a review of the epidemiology, treatment implication and vaccine prevention from published literature. HEV infection is a worldwide public health problem and can cause acute and chronic hepatitis E. HEV genotypes 1 and 2 are primarily found in developing countries due to waterborne transmission, while the zoonotic potential of genotypes 3 and 4 affects mostly industrialized countries. An awareness of HEV transmission through blood donation, especially in the immunocompromised and solid organ transplant patients, merits an effective anti-viral therapy. There are currently no clear indications for the treatment of acute hepatitis E. Despite concerns for side effects, ribavirin monotherapy or in combination with pegylated

interferon alpha for at least 3 mo appeared to show significant efficacy in the treatment of chronic hepatitis E. However, there are no available treatment options for specific patient population groups, such as women who are pregnant. Vaccination and screening of HEV in blood donors are currently a global priority in managing infection. New strategies for the treatment and control of hepatitis E are required for both acute and chronic infections, such as prophylactic use of medications, controlling large outbreaks, and finding acceptable antiviral therapy for pregnant women and other patient groups for whom the current options of treatment are not viable.

**Key words:** Treatments; Blood donors; Adverse effects; Vaccination; Pegylated-interferon; Ribavirin; Hepatitis E

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**Core tip:** Hepatitis E virus (HEV) infection affects individuals in both industrialized and developing countries and can cause acute and chronic hepatitis E. HEV genotypes 1 and 2 are primarily found in developing countries due to waterborne transmission, while the zoonotic potential of genotypes 3 and 4 affects mostly industrialized countries. An awareness of HEV transmission through blood donation, especially in the immunocompromised and solid organ transplant patients, merits an effective anti-viral therapy. The current treatment for HEV infection involving ribavirin and pegylated interferon- $\alpha$  therapy has shown limited efficacy. Although not widely used, an HEV vaccine is available for immunization in China.

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## INTRODUCTION

Hepatitis E infection is now regarded as a major cause of fecal-orally transmitted non-A, non-B hepatitis. Hepatitis E virus (HEV) is a non-enveloped, single-stranded RNA virus that contains three open reading frames (ORFs) that encode structural and non-structural proteins<sup>[1]</sup>. There are 4 major HEV genotypes (1, 2, 3 and 4). Symptomatic HEV infections produce varying clinical presentations depending on the HEV genotypes. Fulminant hepatic failures are caused mainly by genotype 1, while chronic infections have been observed only with genotype 3 thus far<sup>[2]</sup>. Recent reports of sporadic cases from developed countries in Europe and in the United States resulted from HEV genotype 3, which is zoonotic<sup>[3]</sup>. In a 2015 survey on the seroprevalence and

risk factors for HEV infection among slaughterhouse workers in South Korea, the seropositive rate for HEV IgG was 33.5%<sup>[4]</sup>. Another report from Thailand in 2014 found that HEV strains identified from acute symptomatic hepatitis E infections were all genotype 3f, which was genetically closely related to a strain isolated in swine<sup>[5]</sup>.

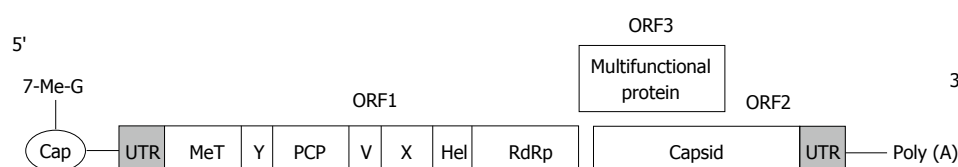
The clinical course of HEV infection among pregnant women is known to be more severe and often led to fulminant hepatic failure and death in up to 20%-25%, specifically in those living in developing countries<sup>[6]</sup>. The mortality rate has been found to be higher during the 2<sup>nd</sup> and the 3<sup>rd</sup> trimester<sup>[7]</sup>. However, underlying factors influencing high mortality during pregnancy are poorly understood.

Studies in chimpanzees to examine HEV and hepatitis C virus (HCV) infection revealed that HEV showed a lower frequency and a shorter duration of differentially expressed genes compared to HCV in intrahepatic transcriptome analysis. This suggests that HEV may be more susceptible to the innate immunity induced by interferon alpha<sup>[8]</sup>. However, HEV showed suppression of interferon alpha signaling *in vitro*<sup>[9]</sup>.

## MOLECULAR VIROLOGY OF HEV

HEV belongs to the family *Hepeviridae*<sup>[10]</sup>. It is a small non-enveloped virus 27-34 nm in diameter. The single-stranded positive-sense RNA genome of HEV is approximately 7.2 kb, 7-methylguanine capped at the 5' terminus, and polyadenylated at the 3' terminus<sup>[11]</sup>. Both ends of the genome consist of short 5' and 3' untranslated regions, which fold into stem-loop structures and are approximately 58 and 68 nucleotides, respectively<sup>[12]</sup>. The viral genome contains 3 open reading frames (ORF)<sup>[10,13]</sup>. ORF1 encodes a 1693 amino acid non-structural polyprotein including: Methyltransferase (MeT), which catalyses the capping of viral RNA; a papain like cysteine protease (PCP) with presumed post-translational protein processing; a helicase (Hel), which supports MeT by catalyzing the first step of RNA capping; an RNA-dependent RNA polymerase (RdRp) required for the synthesis of the genomic RNA; and several uncharacterized domains such as X or Macro, Y and V domain<sup>[1,12]</sup>. ORF2 located near the 3' end encodes 599 to 660 amino acids capsid protein<sup>[1,10]</sup>. It is involved in virion assembly, cell attachment, and immunogenicity. ORF3, which overlaps ORF2, encodes a protein of 114 amino acids shown to be associated with subcellular localization and virion morphogenesis<sup>[11,14]</sup> (Figure 1).

Replication steps in the HEV life cycle are not easily elucidated due to lack of a suitable cell culture system. However, proposed replication cycle commences with the viral attachment to the surface of target cells (hepatocyte) and binding to the unknown receptor(s). Next, the virus penetrates, uncoats, and releases the genomic RNA. Translation of the nonstructural proteins occurs in the cytoplasm. RdRp synthesizes the negative-sense intermediate RNA from the positive-sense genome, which subsequently acts as a template for



**Figure 1** Schematic representation of hepatitis E virus genome<sup>[1,12,14]</sup>. ORF: Open reading frame; UTR: Untranslated region; MeT: Methyltransferase; PCP: A papain like cysteine protease; Hel: Helicase.

the synthesis of subgenomic RNA and the full-length positive-sense transcripts. The subgenomic RNA is then translated into ORF2 and ORF3 proteins, which facilitates viral assembly and optimizes the host cell environment for viral replication<sup>[11,12]</sup>. The mechanism of viral egress from the host cells remains unclear<sup>[15,16]</sup>.

## IMMUNE RESPONSES IN HEV INFECTION

Investigation of host immune responses against virus infection are essential to understand host immunity and for vaccine development. Several studies have examined the host immune responses following HEV infection and the roles of immune components responsible for causing liver damage as a consequence of HEV infection. Although the mechanisms of hepatic injury enhanced by the host immune responses to HEV infection are still unclear<sup>[17-19]</sup>, we review here the available literatures on HEV infection and how it affects host immunity and induction of liver injury.

Once HEV enters the body, viral clearance involves recruitment of immune cells against infection. These cells recognize HEV in the early stages through pattern recognition receptors. HEV components are then detected by toll-like receptors and retinoic acid-inducible gene- I like receptors. Subsequent recruitment of adapter proteins MyD88 and TRIF mediate interferon regulatory transcription factor 3 (IRF3) and nuclear factor  $\kappa$ B (NF- $\kappa$ B) to produce type I interferons and pro-inflammatory cytokines vital to antiviral response<sup>[20]</sup>.

Type I interferons are cytokines, which play important roles in innate immunity against virus infection. Interferon-inducible genes are up-regulated in HEV infected PLC/PRF/5 cells. Replication of HEV in this cell type was inhibited when cells were treated with interferon (IFN)- $\alpha$ 2b<sup>[21]</sup>. A549 cells infected with HEV genotype 3 showed inhibition of IFN- $\alpha$  induced the signal transducer and activator of transcription 1 (STAT1) phosphorylation. Additionally, HEV ORF3 protein enhances IFN- $\beta$  production induced by poly (I:C), a double-stranded RNA analogue<sup>[22]</sup>. HEV ORF3 was also shown to bind STAT1 resulting in inhibition of STAT1 phosphorylation<sup>[9]</sup>. A study in hepatocyte cells demonstrated that HEV infection inhibited IFN- $\beta$  production induced by poly (I:C). Meanwhile, HEV ORF1 was identified as an IFN antagonist. It inhibited phosphorylation and ubiquitination of various proteins involving in interferon production such as IRF3, retinoic acid-inducible gene 1 and TANK-binding kinase 1<sup>[23]</sup>. ORF1 also activates the promoter activity of a chemokine,

chemokine (C-X-C motif) ligand 8<sup>[24]</sup>. These reports collectively demonstrated that HEV proteins are involved in the activation of host antiviral cytokines, although some viral components could also inhibit interferon signalling, which facilitate HEV evasion from the host innate immune defenses.

Natural killer (NK) cells and NKT cells are responsible for killing virus-infected cells. Comparison of the numbers and function of NK/NKT cells from HEV infected patients and healthy controls showed that the percentage of activated NK cells was higher in patients than in controls<sup>[18]</sup>. However, there was no difference in the target cell cytotoxicity of NK cells from both groups. A study of lymphocytes taken from liver biopsy demonstrated that CD56 cell counts were higher in HEV patients than in patients with hepatitis A, B and C virus infections<sup>[25]</sup>. This group also showed increased CD8<sup>+</sup> cells in liver failure cases caused by HEV and other viruses.

IFN- $\gamma$  is a cytokine responsible for activation of NK and T cell function. IFN- $\gamma$  expression was up-regulated in peripheral blood mononuclear cells (PBMCs) from HEV infected patients following HEV ORF2 stimulation<sup>[26]</sup>. In addition, PBMCs from acute viral hepatitis cases elicited higher responses to recombinant HEV ORF2 peptides than PBMCs from the liver failure group<sup>[27]</sup>. Studies in hepatocytes demonstrated that HEV ORF2 inhibited NF- $\kappa$ B activation<sup>[28]</sup>.

Besides the innate immune responses, the alteration of cell-mediated immune responses by HEV infection had also been reported. HEV infected patients had increased CD8<sup>+</sup> and CD4<sup>+</sup>CD8<sup>+</sup> cells compared with healthy individuals. In addition, proportion of IFN- $\gamma$  secreting cells in response to recombinant ORF2 and ORF3 were higher in patients than in controls. These data provided evidence of effector T cell responses induced by HEV components<sup>[29]</sup>.

The studies of clinical manifestations in patients with self-limited acute viral hepatitis and acute liver failure support the roles of immune responses in liver injury. HEV infected patients with acute liver failure had higher antibody titers and higher levels of cytokines such as tumor necrosis factor- $\alpha$ , IFN- $\gamma$ , interleukin-2 (IL-2) and IL-10. HEV RNA was detected in patients with self-limited acute hepatitis group but not in liver failure cases. This observation suggests that the immune responses rather than the virus are responsible for liver damage<sup>[30]</sup>.

For humoral immune response, the incubation period is around 15-60 d after infection<sup>[31]</sup>. IgM antibodies increase rapidly and then begin to subside after 3



mo<sup>[32,33]</sup>. Anti-HEV IgG antibodies start to increase when anti-HEV IgM is first detected and may persist for years<sup>[17,34,35]</sup>. Presently, the viral detection by serology tests has been developed for HEV. Examination of blood and stool of 10 patients with acute HEV infection can identify HEV RNA, but only for a short period<sup>[36]</sup>. Experimental infection in chimpanzee demonstrated that HEV RNA was detectable in blood around 22 d after inoculation. In human volunteer, HEV RNA could be detected in blood sooner than in feces<sup>[37]</sup>. Naturally acquired humoral immune response in the body not only increased rapidly after infection, but also afforded protect from infection during an outbreak<sup>[38]</sup>.

Regulatory T cells (Treg) and IL-10, components of the immune system which modulate immune response, were examined in acute hepatitis E patients, recovered individuals and healthy controls. Both percentage of Treg cells and IL-10 levels were higher in acute hepatitis patients than in recovered and healthy individual groups<sup>[39,40]</sup>, implicating a role for Treg in the immune response induction as a result of HEV infection.

The regulation of protection in the body involves a complex network of innate and adaptive immune response. Immune responses are shown to promote tissue injury in various infectious diseases. During viral infection, both innate and adaptive immune responses are elicited in order to eliminate pathogens. Even so, several viruses are able to evade eradication by the host immunity. Prolonged or hyper-immune responses to eradicate persistent infection, however, could result in tissue injury. Knowledge gained from HEV studies thus far have mostly relied on recombinant HEV proteins instead of viral infections due to the lack of an efficient cell culture system. Moreover, most HEV infections are self-limited. Future studies including patients presented with various degrees of clinical manifestations should allow better understanding of the immunity or immunopathology induced by HEV infection.

## EPIDEMIOLOGY OF HEV

HEV is a leading cause of hepatitis transmitted *via* fecal-oral route<sup>[41]</sup>. The first outbreak of HEV occurred in India around 1955-56, which affected as many as 29000 persons<sup>[42]</sup>. Other large outbreaks were reported in China, India, Somalia and Uganda<sup>[1]</sup>. Outbreaks were observed to occur after heavy rainfall and floods, which allowed contamination of human excreta with drinking water sources. Alternatively, outbreaks sometimes follow the dry summer season when water in the rivers or streams is reduced, resulting in increased concentration of fecal contamination in water<sup>[43]</sup>.

It is known that humans could be infected with all 4 HEV genotypes<sup>[44]</sup>. In developing countries where HEV is endemic (Indian subcontinent, Asia, Middle East and Africa)<sup>[11]</sup>, HEV genotypes 1 and 2 are common and restricted to human. In developed countries, these two genotypes were diagnosed only in persons who had recently traveled to highly endemic areas<sup>[45]</sup>. It is

estimated that 21.1 million people, or 71% of the world population, are infected with HEV genotypes 1 and 2. Furthermore, HEV infection results in approximately 3 million symptomatic acute cases each year and 70000 deaths worldwide<sup>[1,44,46]</sup>. Individuals could be infected with HEV genotypes 1 and 2 from drinking contaminated water<sup>[47]</sup>, and unfortunately people affected in large HEV outbreaks were found using water from a common source for cooking, drinking, and bathing. HEV RNA has also been detected in the sewage-contaminated water source. The limitation of inadequate public infrastructure may facilitate the spread of HEV infection, such as in refugee or military camps<sup>[43]</sup>. Areas affected by natural disasters such as earthquakes and monsoon storms are also at risk of HEV epidemic. Displaced populations with limited access to clean drinking water, lack of sanitary facilities, overburdened health-care infrastructure, and immunologically naive population lacking protective antibodies combined will increase the likelihood of HEV transmission<sup>[48]</sup>. Large outbreaks of HEV have occurred in Nepal, which in 2014 involved more than 10000 cases<sup>[49]</sup>.

HEV infection does not only affect developing countries. HEV genotypes 3 and 4 are autochthonous in several industrialized countries. Occasional foodborne outbreaks have occurred in Europe, North America, Japan and New Zealand from consuming undercooked meat contaminated with HEV<sup>[50-53]</sup>. HEV genotypes 3 and 4 are found in human and other animal species such as pig, wild boar, and shellfish<sup>[54-56]</sup>. Specifically, HEV genotype 3 is zoonotic in developed countries from reports in pig farmers, individuals who came into close contact with animal reservoir and who consumed raw meat or meat products from deer, wild boar, and pig. HEV RNA could also be detected in food products such as liver and sausage. Viral sequence extracted from uncooked meat or sausage were very similar (99.7%-100% identity) to sequences of virus recovered from HEV-infected patients. In addition, HEV genotype 4 infection could be detected in both human and swine in Eastern Asia and Europe<sup>[57,58]</sup>. These evidence supported foodborne zoonotic HEV transmission, which occurred from consuming infected meat<sup>[59-63]</sup>.

Several studies suggest that HEV genotypes 3 and 4 could be transmitted across multiple species. In animal models, pathogen-free pigs could be experimentally infected with human HEV genotypes 3 and 4. Furthermore, swine HEV genotypes 3 and 4 inoculated in rhesus monkey and chimpanzee resulted in seroconversion and virus shedding in feces. Anti-HEV IgG seroconversion in the pigs was observed within 2 wk. These experiments suggest that human HEV genotypes 3 and 4 could replicate in pigs and likely originated from swine<sup>[64]</sup>. In contrast, pigs inoculated with HEV genotypes 1 and 2 were not infected. This suggests that HEV genotypes 1 and 2 were host-restricted compared to genotypes 3 and 4<sup>[65]</sup>. Therefore, zoonotic transmission to humans is possible for HEV genotypes 3 and 4<sup>[65-67]</sup>. Other routes of transmission

**Table 1** Global prevalence of anti-hepatitis E virus IgG in different populations

| Regions              | Prevalence (%) | Ref.   |
|----------------------|----------------|--|
| Low-to-medium income |                |  |
| Kashmir region       | 49.6           | Khuroo <i>et al</i> <sup>[35]</sup>            |
| India                | 23.8-28.7      | Mathur <i>et al</i> <sup>[76]</sup>            |
| Myanmar              | 32.0           | Nakai <i>et al</i> <sup>[77]</sup>             |
| Egypt                | 67.7           | Stoszek <i>et al</i> <sup>[71]</sup>           |
| Bangladesh           | 22.5           | Labrique <i>et al</i> <sup>[78]</sup>          |
| China                | 19.7           | Dong <i>et al</i> <sup>[79]</sup>              |
| Mexico               | 36.6           | Alvarado-Esquivel <i>et al</i> <sup>[80]</sup> |
| Thailand             | 14.0           | Gonwong <i>et al</i> <sup>[75]</sup>           |
| Nigeria              | 42.7           | Junaid <i>et al</i> <sup>[81]</sup>            |
| Industrialized       |                |  |
| Germany              | 17.0           | Wenzel <i>et al</i> <sup>[82]</sup>            |
| United States        | 6.0            | Teshale <i>et al</i> <sup>[83]</sup>           |

including HEV-infected blood transfusion, mother-to-child, person-to-person, and sexual intercourse were also documented but relatively uncommon<sup>[68-70]</sup>.

Seroprevalence of anti-HEV IgG varies in different parts of the world. In developing countries, positive anti-HEV IgG serology might reach as high as 70%<sup>[71]</sup>. Being male, having pet animals or frequent contact with animal reservoirs, residing in the endemic area, or consuming liver or other organ meats were highly associated with positive HEV serology. Veterinary and slaughterhouse workers also have high seropositive rates for anti-HEV IgG compared to individuals with no occupational exposure to swine<sup>[72,73]</sup>. In swine-dense areas, individuals were more likely to be HEV-seropositive compared to those living in areas with few pig farms<sup>[74,75]</sup>. The seroprevalence of anti-HEV IgG antibodies is shown in Table 1.

## CLINICAL MANIFESTATIONS AND DIAGNOSTIC CRITERIA

HEV infection produces wide-ranging clinical manifestations. Most cases of HEV-related acute viral hepatitis resolve within 1 to 2 mo. However, HEV sometimes leads to acute liver failure, chronic infection, or extrahepatic symptoms<sup>[17]</sup>. An acute hepatitis from HEV is indistinguishable from other forms of acute viral hepatitis and is usually asymptomatic or self-limiting in most individuals<sup>[34]</sup>. However, acute hepatitis E infection can be severe and prolonged among the immunocompromised and even healthy individuals<sup>[84]</sup>. Severe acute hepatitis E infections have been described in pregnant women, elderly men, and persons with pre-existing chronic liver disease<sup>[85-87]</sup>. Among people with the chronic liver disease, acute hepatitis E can adversely lead to acute-on-chronic liver failure<sup>[88]</sup>. Acute HEV infection with pronounced symptoms resulted in higher mortality rates of 1%-4% and up to 11%, compared to the mortality rates of acute HAV (1%) or HBV (1.5%) infection based on CDC viral hepatitis surveillance data (2010)<sup>[84]</sup>. Extrahepatic manifestations including pancreatitis, arthritis, aplastic anemia, and neurologic

complications have all been reported<sup>[11,89,90]</sup>.

Hepatitis E, especially genotype 3, is known to cause chronic infection among the immunocompromised, especially solid organ recipients. Recently, however, there are reports of chronic hepatitis E infection in elderly immunocompetent patients<sup>[91,92]</sup>. Diagnosis of acute hepatitis E infection is established in patients with clinically relevant symptoms of acute hepatitis with positive level of anti-HEV IgM or a level of anti-HEV IgG that is twice the baseline concomitant with detectable HEV RNA in the serum and/or stool<sup>[93]</sup>. Chronic hepatitis E is defined by the persistent increase in liver enzyme levels and polymerase chain reaction-detectable HEV in the serum and/or stool over 6 mo<sup>[94]</sup>.

## TREATMENT OF ACUTE HEPATITIS E

Currently, there is no indicated treatment of acute hepatitis E. Nevertheless, treatment with ribavirin showed significant clinical improvements by reducing the symptomatic period (Table 2). Considering the high mortality caused by acute hepatitis E in acute-on-chronic liver failure, ribavirin therapy provides significant benefits for those with poor prognosis or at high risk of fulminant liver failure such as underlying chronic liver disease. Moreover, a high percentage of immunosuppressed individuals [such as human immunodeficiency virus (HIV)-positive individuals or recipients of organ transplant] who eventually develop chronic hepatitis E necessitate early and effective treatment of acute hepatitis E<sup>[84,88,95-98]</sup>.

## TREATMENT OF CHRONIC HEPATITIS E

In a case-control study of solid-organ transplant recipients performed in France, 22 out of 38 individuals who tested positive for HEV genotype 3 (58%) developed chronic infection. Compared to the control group of 148 individuals who had no markers for HEV infection, one independent factor associated with HEV infection was the consumption of game meat (68% vs 47%, OR = 2.32)<sup>[3]</sup>. Moreover, a retrospective analysis of data from Europe and the United States found that among 85 recipients of solid organ transplants who had HEV infection, 56 patients (65.9%) developed chronic hepatitis E<sup>[94]</sup>. The main factor associated with developing chronicity assessed by multivariate analysis in this study was tacrolimus use for immune-suppression (OR = 1.87; 95%CI: 1.49-1.97). The reduction of immunosuppressive drugs, however, enabled HEV clearance in one-third of the individuals.

When reduction of immunosuppression is impossible or when clearing HEV by immunosuppression could not be achieved, 2 alternative therapies for chronic hepatitis E may be pursued: (1) Ribavirin monotherapy (dose 29-1200 mg/d, for 1-18 mo)<sup>[96,99,100]</sup>; and (2) Pegylated (Peg)-IFN- $\alpha$  for 3-12 mo<sup>[101,102]</sup>.

It was reported that among 59 patients with chronic HEV after solid organ transplantation, HEV clearance was observed in 95% of the patients at the end of

**Table 2** Recent evidences on the outcome of therapies against acute hepatitis E infection

| Ref.  | Type of study                     | Patient profile  | HEV genotype | Ribavirin regimen                            | Results  |
|---|-----------------------------------|--|--------------|--|--|
| Gerolami <i>et al</i> <sup>[84]</sup>         | Case report                       | 61-year-old man, 7 d after admission<br>ALT 4565 IU/L  | 3            | 1200 mg/d for 21 d                           | At day 21 of treatment, ALT normalized, RNA almost undetectable  |
| Péron <i>et al</i> <sup>[88]</sup>            | Case report                       | 79-year-old man with chronic liver disease, acute kidney failure                                     | 3f           | 200 mg/d for 3 mo                            | Serum HEV RNA negative at 1 mo therapy, stopped dialysis at 2 mo   |
|   |                                   | A patient with chronic liver disease   | 3f           | 1000 mg/d for 10 d                           | Viral load 4.07 log copies/mL declined to 2.54 log copies/mL at day 6, Hgb 12.6 g/dL declined to 11.6 g/dL at day 6 of treatment |
| Del Bello <i>et al</i> <sup>[95]</sup>        | Case report                       | 65-year-old man, liver transplant recipient Guillain-Barré syndrome with severe necrotizing myositis | 3f           | 400 mg/d adapted to GFR (40 mL/min) for 3 mo | HEV RNA undetectable by day 15, progressive recovery of mobility   |
| Pischke <i>et al</i> <sup>[96]</sup>          | Case from prospective case series | 42-year-old woman had traveled to Eritrea and acquired severe acute hepatitis E                      | 1e           | For 6 wk (dose: Undefined)                   | Rapidly improved liver function and cleared HEV  |
| Robbins <i>et al</i> <sup>[97]</sup>          | Case report                       | 39-year-old man HIV (+) CD4 51/mm <sup>3</sup> prothrombin index 45%                                 | 3c           | 1200 mg/d (15 mg/kg per day) for 12 wk       | Gradual normalization of LFT-HEV RNA decreased to < 100 copies/mL at 1 mo of treatment   |
| Riveiro-Barciela <i>et al</i> <sup>[98]</sup> | Case report                       | 68-year-old man with Waldenström's macroglobulinemia   | 3f           | 800 mg/d for 12 wk                           | Achieved SVR after 12 wk; no ribavirin-related side effect reported  |

LFT: Liver function test; ALT: Alanine transaminase; Hgb: Haemoglobin; GFR: Glomerular filtration rate; SVR: Sustained virological response; HIV: Human immunodeficiency virus; HEV: Hepatitis E virus.

ribavirin therapy and that 78% of the patients achieved sustained virologic response (defined by undetectable serum HEV RNA at least 6 mo after cessation of therapy). These patients received ribavirin for a median of 3 mo. Longer treatment duration allowed 4 patients who had recurrence to achieve sustained virologic response<sup>[100]</sup>. The mechanism that ribavirin acts against HEV is not clearly understood<sup>[103]</sup>. However, a recent study suggests that ribavirin exerts an antiviral effect against HEV by depleting intracellular guanosine 5'-triphosphate pools<sup>[104]</sup>. Further research on this mechanism as well as other possible mechanisms of action of ribavirin is to be revealed. Meanwhile, overall data showed that ribavirin provided therapeutic effect in the treatment of chronic hepatitis E with the only significant adverse effect being anemia (Table 3).

Treatment with Peg-IFN- $\alpha$  has been reported (Table 4). The duration of therapy ranged from 3 mo to 1 year. Most of the cases are from solid organ transplant recipients, all of them showed the favourable outcome in liver enzyme levels as well as viral RNA suppression. However, 2 out of 6 transplanted patients developed acute allograft rejection after 3-mo Peg-IFN therapy<sup>[101,109]</sup>.

The slight synergistic effect for ribavirin combined with Peg-IFN- $\alpha$  was observed in a recent study *in vitro*<sup>[104]</sup>. Successful combination therapy had also been reported for a chronic HEV infection in an HIV-positive patient<sup>[110]</sup>. Decreasing the ribavirin dosage may help reduce anemia and other treatment-associated side effects<sup>[111]</sup>.

from infection or vaccination can protect individuals from symptomatic hepatitis E. In a large cohort study, the risk of infection was highest among the baseline seronegative placebo group participants (2.04%). The risks of HEV infection in population with pre-existing immunity or vaccine-induced immunity were significantly reduced to 0.52% and 0.30%, respectively<sup>[112]</sup>. Two recombinant hepatitis E vaccines developed from HEV genotype 1, by Glaxo SmithKline and Xiamen Innovax Biotech, have had short-term efficacy in clinical trials<sup>[113,114]</sup>. The latter vaccine, commercially available as Hecolin, has been in use in China since 2012. Its long-term efficacy was 86.8% during the 4.5-year follow-up period<sup>[93]</sup>. Estimated long-term persistence of anti-HEV IgG from hepatitis E vaccine is predicted to be from 8 years to nearly life-long based on mathematical assumptions<sup>[115]</sup>. The only currently licensed hepatitis E vaccine (Hecolin) is approved for use in China in those aged 16-65 years available in prefilled syringe for intramuscular injection at 0, 1, and 6 mo. Expansion of vaccine coverage to other HEV endemic country is necessary and might significant decrease burden of the disease<sup>[85]</sup>. In addition to improved personal hygiene, sanitation, and health education, vaccination might play a crucial role in the future prevention and control of HEV infection.

## HEV IN BLOOD DONORS: CLINICAL IMPLICATIONS

Experiments involving the transfusion of blood plasma from anti-HEV IgM positive and anti-HEV IgG negative blood donors to rhesus monkey demonstrated that the

## HEPATITIS E VACCINE

Current data demonstrated that immunity acquired

**Table 3 Treatment of chronic hepatitis E virus with ribavirin regimen**

| Ref.                                   | Type of study                          | Patient profile   | Ribavirin regimen   | Result   | Adverse effects   |
|--|--|---|---|--|---|
| Kamar <i>et al</i> <sup>[99]</sup>     | Prospective case series                | 6 kidney transplant recipients, HEV RNA (+) for median of 36.5 mo                                       | 600-800 mg/d for 3 mo adapted to GFR, Hgb                                     | SVR in 4/6 patients; relapse in 2/6; AST, ALT normalized all   | Anemia led to blood transfusion and RBV dose reduction in 2/6 patients                              |
| Mallet <i>et al</i> <sup>[105]</sup>   | Case report                            | A kidney and pancreas transplanted man, a women with idiopathic CD4 <sup>+</sup> T lymphocytopenia      | 12 mg/kg daily for 12 wk  | Both cleared HEV after 4 wk of treatment and remained undetectable, LFT normalized                             | Anemia in 1 <sup>st</sup> patient led to Ribavirin dose reduction to 200 mg/d                       |
| Pischke <i>et al</i> <sup>[106]</sup>  | Prospective case series                | Organ transplant recipients 11 subjects   | 600-1000 mg/d for 5 mo, dose reduction according to Hgb or anemia             | 9/11 showed SVR  | Anemia, the mean Hgb decline was 3.4 g/dL (range 0-7.9 g/dL)  |
| Neukam <i>et al</i> <sup>[106]</sup>   | Case report                            | 2 HIV (+) male with liver cirrhosis with severe immunosuppression                                       | Oral ribavirin 1200 mg/d (case 1) 1000 mg/d (case 2) for 24 wk                | LFT normalized-Liver stiffness improved HEV RNA was detected after the end of treatment in both patients       | -   |
| Giordani <i>et al</i> <sup>[107]</sup> | Case report                            | 60-year-old man with lymphocytic leukemia   | 1000 mg/d in 2 doses (400 and 600 mg), for 3 mo                               | HEV cleared and sustained over 6 mo after therapy  | Mild anemia (Hgb 10.5 mg/dL)  |
| Kamar <i>et al</i> <sup>[100]</sup>    | Retrospective, multicentre case series | 37 kidney, 10 liver, 5 heart, 5 kidneys and pancreas, and 2 lung transplant recipients with chronic HEV | Median dose of 600 mg/d (range 29-1200), for a median of 3 mo (range 1-18 mo) | At the end of the therapy, 95% cleared HEV, 18% recurred after cessation of therapy is stopped, 78% showed SVR | Anemia required dose reduction (29%); use of erythropoietin (54%); required blood transfusion (12%) |

LFT: Liver function test; AST: Aspartate aminotransferase; ALT: Alanine transaminase; SVR: Sustained virological response; Hgb: Haemoglobin; GFR: Glomerular filtration rate; HEV: Hepatitis E virus; HIV: Human immunodeficiency virus.

**Table 4 Treatment of chronic hepatitis E virus with pegylated interferon- $\alpha$  therapy**

| Ref.                                  | Patient profile  | Peg-IFN- $\alpha$ regimen   | Result   | Adverse effects   |
|---------------------------------------|--|---|--|---|
| Kamar <i>et al</i> <sup>[101]</sup>   | 29-year-old man with liver transplantation   | Peg-IFN- $\alpha$ -2a for 12 wk (135 $\mu$ g/wk)                              | Liver enzyme levels decreased. HEV RNA levels remained undetectable until week 12  | At week 12, signs of acute humoral rejection in liver biopsy          |
|                                       | 26-year-old man with liver transplantation   | Peg-IFN- $\alpha$ -2a for 12 wk (135 $\mu$ g/wk)                              | HEV RNA levels undetectable by week 12; liver enzyme levels normalized by week 12  |   |
|                                       | 58-year-old man with liver transplantation liver cirrhosis from chronic HEV infection  | Peg-IFN- $\alpha$ -2a for 12 wk (135 $\mu$ g/wk)                              | HEV RNA was redetected 2 wk after completion of treatment; Liver enzyme levels normalized by 3 mo of therapy   |   |
| Haagsma <i>et al</i> <sup>[102]</sup> | 37-year-old woman with liver transplantation   | Peg-IFN- $\alpha$ -2b for 52 wk (80 $\mu$ g/wk declined to 60 $\mu$ g/wk)     | Serum HEV RNA sustained undetectable during 3 mo follow-up; serum liver enzyme became normalized   | Leukopenia  |
|                                       | 59-year-old man with liver transplantation   | Peg-IFN- $\alpha$ -2b 150 $\mu$ g/wk, dose reduction due to leukopenia        | HEV viral load and aminotransferases declined, but Peg-IFN discontinued from lack of further efficacy, HEV RNA level undetectable at 4 wk after the discontinuation of Peg-IFN and aminotransferase normalized |   |
| Alric <i>et al</i> <sup>[108]</sup>   | 57-year-old man with hairy cell leukemia   | Discontinued at week 16, Peg-IFN- $\alpha$ -2b 1 $\mu$ g/kg per week for 3 mo | Achieved a complete virologic response by week 4   |   |
| Kamar <i>et al</i> <sup>[109]</sup>   | 24-year-old man with kidney transplantation, kidney failure from chronic HEV infection | 3-mo Peg-IFN- $\alpha$ -2a 135 $\mu$ g/wk                                     | Serum RNA undetectable after 5 mo, SVR for 6 mo after treatment  | Acute rejection of the kidney allograft by month 3 of Peg-IFN therapy |

Peg-IFN: Pegylated interferon; SVR: Sustained virological response; HEV: Hepatitis E virus.

virus was transmissible<sup>[116]</sup>. Therefore, many developed countries are now focused on studying the prevalence of viral transmission from blood donation<sup>[117-124]</sup> (Table 5). The demand for pathogen-free blood and blood components is highly needed for hospital patients and individuals requiring continuous blood transfusion (*e.g.*, thalassemia patients). Thus, these patients are at-risk for being infected with HEV from donated blood. The development of screening methods for detecting

HEV in donated blood involves both serology test and nucleic acid test. Novel techniques are being developed to increase the efficiency and sensitivity to identify HEV rapidly even at low viral concentration<sup>[125-127]</sup>. Many countries are aware of the necessity to screen the blood supply for HEV among blood donors and have begun to implement diagnostic tests for HEV. For example, Japan has started monitoring for HEV by comparing the increase in the alanine aminotransferase as a biomarker



**Table 5** Incidence of detectable hepatitis E virus in blood donors (hepatitis E virus-RNA)

| Year of study | Countries       | Technique used for detection               | No. of tests | Ratio of positive detections | Ref.                                  |
|---------------|-----------------|--|--------------|------------------------------|---------------------------------------|
| 2005          | China           | Real-time fluorescence RT-PCR              | 10741        | 1:1094                       | Ren <i>et al</i> <sup>[117]</sup>     |
| 2011          | England         | PCR  | 42000        | 1:7000                       | Ijaz <i>et al</i> <sup>[118]</sup>    |
| 2011          | German          | Real-time RT-PCR                           | 18100        | 1:4525                       | Baylis <i>et al</i> <sup>[119]</sup>  |
| 2011          | Sweden          | Real-time RT-PCR                           | 95835        | 1:7986                       | Baylis <i>et al</i> <sup>[119]</sup>  |
| 2011          | United States   | Real-time RT-PCR                           | 51075        | None detected                | Baylis <i>et al</i> <sup>[119]</sup>  |
| 2011          | German          | Real-time RT-PCR                           | 16125        | 1:1241                       | Vollmer <i>et al</i> <sup>[120]</sup> |
| 2011-2012     | The Netherlands | Real-time PCR                              | 45415        | 1:2672                       | Slot <i>et al</i> <sup>[121]</sup>    |
| 2012-2013     | England         | RT-PCR                                     | 225000       | 1:2848                       | Hewitt <i>et al</i> <sup>[122]</sup>  |
| 2012          | France          | RT-PCR                                     | 53234        | 1:2218                       | Gallian <i>et al</i> <sup>[123]</sup> |
| 2013          | Spain           | Transcription-mediated amplification assay | 9998         | 1:3333                       | Sauleda <i>et al</i> <sup>[124]</sup> |

RT-PCR: Reverse transcription-polymerase chain reaction.

in the surveillance of HEV<sup>[128]</sup>. Germany is looking for a new approach to detecting HEV to find alternative ways to blood screening test<sup>[120]</sup>. Other countries have also implemented screening test but only focus on the suspected cases<sup>[129]</sup>. In Thailand, there were two reports on the incidence of HEV among blood donors. First, a study in 1996 reported the prevalence of HEV transmission among adults in different parts of Thailand, in which the studied population also included blood donors. The study found 9%-22% positive rates for anti-HEV IgG<sup>[130]</sup>. Another study found HEV seroprevalence around 8.7% among blood donors in 4 Northern provinces of Thailand<sup>[131]</sup>, which was similar to those in other countries (Table 5). Thus, HEV poses a significant public health problem especially in blood donation even if the prevalence and virulence of the disease are lower than other infections<sup>[132]</sup>.

## CHALLENGES IN THE TREATMENT OF HEPATITIS E

Although the recent reports of treatment of HEV infection are showing beneficial outcomes, there are still areas to be overcome in the treatment of HEV infection. First of all, as described by previous articles, there are known severe side effects of current regimen with Peg-IFN- $\alpha$  and ribavirin. For Peg-IFN- $\alpha$ , the severe side effects include influenza-like symptoms<sup>[133]</sup> and acute rejection of allografts for solid organ transplant recipients<sup>[101]</sup>. For ribavirin monotherapy, it has the side effect of severe hemolytic anemia, sometimes resulting in treatment failures probably caused by dose reduction<sup>[100,111]</sup>. Furthermore, both ribavirin and Peg-IFN- $\alpha$  cannot be administered in pregnancy. Also, ribavirin use requires close monitoring of hemoglobin levels and other hematological parameters that make it difficult to apply in developing countries<sup>[111]</sup>.

There has been a case report showing resolution of acute liver injury cause by hepatitis E with steroid use that was initially intended for immunosuppression<sup>[134]</sup>. Also, there have been reports of fulminant hepatic failure from HEV in women taking oral contraceptives<sup>[135]</sup>. Known that immunosuppression can cause persistent infection of hepatitis E, the relation between steroid hormone use

and the clinical course of hepatitis E is not clear as the immune pathogenesis of hepatitis E infection itself being not explained thoroughly<sup>[2]</sup>. The various manifestations of hepatitis E according to the potential immune and hormonal status including pregnancy need to be explored precisely in their relation and the pathogenesis for the future direction of developing treatment regimen of hepatitis E. Larger research for establishing appropriate standard treatment as well as supportive treatment and steroid use are still in need in order to minimize limitations and side effects of the current administration of ribavirin and Peg-IFN- $\alpha$  monotherapy, with appropriate vaccination for the high-risk populations for controlling epidemics in resource-limited settings, and for pregnant women living in developing countries where the acute infections are threatening extremely great number of women and new-borns.

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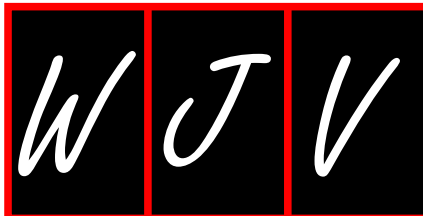


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## Human immunodeficiency virus/acquired immune deficiency syndrome: Using drug from mathematical perceptive

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### Abstract

Entry of acquired immune deficiency syndrome virus into the host immune cell involves the participation of various components of host and viral cell unit. These components may be categorized as attachment of the viral surface envelope protein subunit, *gp120*, to the

CD4<sup>+</sup> receptor and chemokine coreceptors, *CCR5* and *CXCR4*, present on T cell surface. The viral fusion protein, *gp41*, the second cleaved subunit of Env undergoes reconfiguration and the membrane fusion reaction itself. Since the CD4<sup>+</sup> T cell population is actively involved; the ultimate outcome of human immunodeficiency virus infection is total collapse of the host immune system. Mathematical modeling of the stages in viral membrane protein-host cell receptor-coreceptor interaction and the effect of antibody vaccine on the viral entry into the susceptible host cell has been carried out using as impulsive differential equations. We have studied the effect of antibody vaccination and determined analytically the threshold value of drug dosage and dosing interval for optimum levels of infection. We have also investigated the effect of perfect adherence of drug dose on the immune cell count in extreme cases and observed that systematic drug dosage of the immune cells leads to longer and improved lives.

**Key words:** Human immunodeficiency virus; Acquired immune deficiency syndrome; Antibody vaccine; Perfect drug adherence; Impulsive differential equation

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**Core tip:** Use of single-cell antibody-cloning techniques uncover naturally arising, broad and potent human immunodeficiency virus (HIV) neutralizing antibodies. These antibodies can protect against infection and suppress new HIV infection. This antibody vaccination gives new ideas about the fight against the HIV infection. From the analytical study of the effect of antibody vaccination we found the threshold value of drug dosage and dosing interval for optimum levels of infection. We have also investigated the effect of perfect adherence of drug dose on the immune cell count in extreme cases and observed that systematic drug dosage of the immune cells leads to longer and improved lives.

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## INTRODUCTION

Over the last two decades there has been extensive research on the area of human immunodeficiency virus (HIV) infection invading the human immune system. According to the World Health Organisation (WHO), almost 75 million people have already been infected with the HIV virus and about 36 million people have died of HIV. Globally, 35.3 million people were living with HIV at the end of 2012. An estimated 0.8% of adults aged 15-49 years worldwide are living with HIV, although the burden of the epidemic continues to vary considerably between countries and regions. Sub-Saharan Africa remains most severely affected, with nearly 1 in every 20 adults living with HIV and accounting for 71% of the people living with HIV worldwide. CD4<sup>+</sup> T lymphocyte count is the only way to discover the disease progression monitoring during anti retroviral treatment (ART). From the premature days of infection, CD4<sup>+</sup> T-lymphocyte cells have been acknowledged as most important for HIV disease progression<sup>[1]</sup>. A healthy human adult has about 1000 CD4<sup>+</sup> T cells per microliter of blood<sup>[1]</sup>, and when the number of CD4<sup>+</sup> T cells is reduced below 200/ $\mu$ L, as HIV infected patient considered as acquired immune deficiency syndrome (AIDS) patients<sup>[2,3]</sup>. The infection process of the human cells by the human immunodeficiency virus (HIV) is very complicated process. When HIV invades into the body, it targets the immune cells, mainly the CD4<sup>+</sup> T cells. HIV virus can easily hit into CD4<sup>+</sup> T cells by a binding process between the envelope proteins (*gp41/gp120*) on the surface of HIV with both the CD4 receptor and the chemokine coreceptor. The entry process is initiated by binding of *gp120*, a cleaved subunit of viral surface envelope protein, *Env*, to the CD4<sup>+</sup> receptor present on the T cell surface. However, this binding will be futile unless a conformational change is induced in *gp120* resulting in exposure of a coreceptor binding site and enabling it to bind to as N-terminus of chemokine coreceptor, namely *CCR5* and *CXCR4*, when a heterotrimeric complex of *gp120* - *CD4*-coreceptor is formed. Basically, the chemokines are small soluble paracrine signaling molecules involved in trafficking and recruitment of leukocytes to sites of injury and inflammation. Chemokines and their receptors contribute significantly to disease progression in HIV-afflicted patients. Co-receptor availability and expression determine host susceptibility to infection. These coreceptors are determinants of viral tropism. After that, viral fusion occurs in the target cell membrane and the genetic material viral RNA gets entry into the CD4<sup>+</sup> T cell. This genetic material has a reverse transcriptase enzyme.

By the reverse transcription process the RNA genome is reverse-transcribed to a DNA copy, and thus the cell becomes infected. This provirus can enter into the host cell genome where it can stay in an actively infected state or a latently infected state.

Virus specific antibodies are created by a complex differentiation pathway that includes B cell proliferation, isotype switching, germinal center formation and affinity maturation<sup>[4]</sup>. As a result, the presence of specific antibody secreting plasma cells and antibody production tends to continue long after the infection. In this outlook we consider B cell responses by assuming that antibodies are produced in response to free virus and that antibody production diminishes at a certain rate. But the antibody response diminishes during the course of infection and thus the immune system cannot fight against the HIV.

A recent vaccine experiment shows the use of single-cell antibody-cloning techniques that uncovered naturally arising, broad and potent HIV neutralizing antibodies (bNAbs)<sup>[5]</sup>. It is observed experimentally that these antibodies can defend against infection and suppress HIV infection in animals. The invention of this antibody vaccination gives new ideas about the fight against the HIV infection. The most contemporary approach to study the drug dynamics is determined by use of impulsive differential equations. Perfect or imperfect drug adherence and drug holidays can make easy the development of resistance. In recent years the effects of perfect adherence to antiretroviral therapy have been studied by impulsive differential equations<sup>[4,6-10]</sup>. Using this method, the dosing period and threshold values of dosage can be obtained more precisely. Also the effect of maximal acceptable drug holidays can be found by using impulsive differential equations<sup>[4]</sup>. Impulsive differential equations result if drug effect as well as that of the metabolites are assumed to decay with time in an exponential manner during each cycle and are assumed to change instantaneously at dosing times,  $t_i$  for different drug doses and can result in either implicit or explicit models<sup>[6-8]</sup>.

This article is arranged in the following manner: In the first section, we formulate the basic mathematical model on the basis of antibody responses against the virus and applying impulsive differential equations, we show how antibody responses on the human immune system. Analytical and numerical studies have been performed in the next sections. Lastly, we discuss the implication of the results which were found in the earlier sections.

## THE MODEL

In this research article, we have modified the explicit mathematical model as proposed by Roy *et al.*<sup>[11]</sup>, considering the perfect adherence behavior of CTL vaccination in HIV infected patients. Here  $G_1$  represents the concentration of viral *Env* subunit, *gp120 in vivo*, and  $C_{D4}$  denotes the concentration of *CD4* receptor on T cell surface. Let  $C_1$  be the concentration of the dimeric complex of *gp120* and *CD4* receptor,  $G_2$  be the concentration of viral fusion protein, *gp41*, and  $C_{CR}$  be the concentration of the chemokine



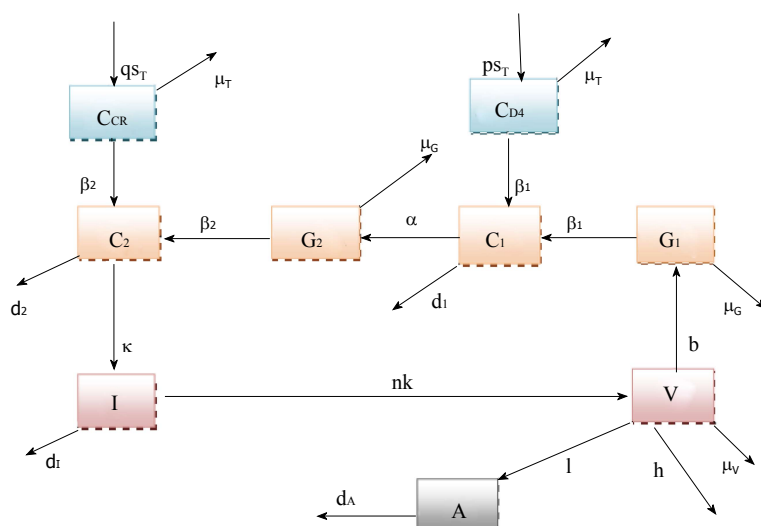


Figure 1 Schematic explanation for the model (1) showing the effect of high drug levels.

coreceptor on  $CD4^+$  cells. Also,  $C_2$  be the concentration of the combination of  $gp120$  and  $gp120 - CD4$  chemokine coreceptor ternary complex,  $I$  denote the concentration of infected  $CD4^+$  T cells, and  $V$  denote the concentration of HIV virus. The model is represented in Figure 1. Here  $A$  stands for concentration of antibody response. We suppose that antibody responds proportionally to the production of free virus particles at a rate  $hVA$ . In presence of antibody vaccination through perfect adherence, the model is given by:

$$\begin{aligned}
 dG_1/dt &= bV - \mu_G G_1 - \beta_1 G_1 C_{D4} \\
 dC_{D4}/dt &= pS_T - \mu_T C_{D4} - \beta_1 G_1 C_{D4} \\
 dC_1/dt &= \beta_1 G_1 C_{D4} - d_1 C_1 \\
 dG_2/dt &= \alpha C_1 - \mu_G G_2 - \beta_2 G_2 C_{CR} \\
 dC_{CR}/dt &= qS_T - \mu_T C_{CR} - \beta_2 G_2 C_{CR} \\
 dC_2/dt &= \beta_2 G_2 C_{CR} - d_2 C_2 \\
 dI/dt &= kC_2 - d_I I \\
 dV/dt &= nI - \mu_V V - hVA \\
 dA/dt &= lVA - d_A A \quad \text{for } t \neq t_k \quad (1)
 \end{aligned}$$

$$\Delta A \equiv A(t_k^+) - A(t_k^-) = \bar{A} \quad \text{for } t = t_k \quad (2)$$

In the system (1),  $b$  represents the multiplication capacity of  $gp120$  in response to virus. The parameter  $a$  represents the successful exposure of  $gp41$ , since it is exposed only after attachment between  $gp120$  and the  $CD4$  receptor is complete. The parameter  $\mu$  denotes the decay of  $gp120$  and  $gp41$ , and  $\beta_1$  denotes the bonding force between  $gp120$  and the  $CD4$  receptor. Further  $\beta_2$  denotes the bonding force between the dimeric complex of  $gp120$  and  $CD4$  receptor and also the chemokine coreceptor. The source of susceptible  $CD4^+$  T cells is represented by  $s_T$ , and  $p$  and  $q$  denote the number of  $CD4$  receptors and chemokine coreceptors on one  $CD4^+$  T cell respectively. The death rate of healthy  $CD4^+$  T cell  $\mu_T$  and the death rate of infected  $CD4^+$  T cells is  $d_I$ . The dissociation rate of  $C_1$  and  $C_2$  are  $d_1$  and  $d_2$  respectively and for the sake of simplicity, it is assumed that after dissociation of  $C_1$  and  $C_2$ , they will not return

to their respective components. Here  $n$  represents the number of virus particles that are produced by one infected  $CD4^+$  T cell. The clearance rate of free HIV virus is represented as  $\mu_V$ . Antibody responses by  $A$  are produced at a rate  $l$  and  $d_A$  indicates the death rate of antibody responses. The concentration of antibodies (bNAbs) is represented by  $A(t)$  in plasma. The drug dose,  $A$ , that is taken at each impulse time  $t_j$  ( $j = 1, 2, 3, \dots$ ) is kept constant. Since vaccination may be taken at either regular or irregular intervals, we have considered. The impulse time  $t_j$  to be fixed. The system (1) together with the system (2) represent the dynamics with vaccine. The vaccination interval  $\tau = t_{j+1} - t_j$  is fixed. Here we have only analyzed the models (1) - (2).

## ANALYSIS OF THE MODEL

### In absence of drug

To study the model (1) together with (2), we first analyze the model in absence of drug. When drug is not administered to the system, we have observed that there exist two equilibrium point: (1) The disease free equilibrium point  $\bar{E}$ ; and (2) The endemic equilibrium point  $E^*$ .

**The disease free state:** In absence of drug, there may exist disease free equilibrium point  $\bar{E}$  which is given by  $\bar{E}(0, pS_T/\mu_T, 0, 0, qS_T/\mu_T, 0, 0, 0, 0, 0)$  (3)

Now for the system (1) and (2) the Jacobian matrix is  $J = [J_1 | J_2]$ , where

$$J_1 = \begin{bmatrix}
 -(\mu_G + \beta_1 C_{D4}) & -\beta_1 G_1 & 0 & 0 & 0 \\
 -\beta_1 C_{D4} & -(\mu_T + \beta_1 G_1) & 0 & 0 & 0 \\
 \beta_1 C_{D4} & \beta_1 G_1 & -d_1 & 0 & 0 \\
 0 & 0 & \alpha & -(\mu_G + \beta_2 C_{CR}) & -\beta_2 G_2 \\
 0 & 0 & 0 & -\beta_2 C_{CR} & -(\mu_T + \beta_2 G_2) \\
 0 & 0 & 0 & \beta_2 C_{CR} & \beta_2 G_2 \\
 0 & 0 & 0 & 0 & 0 \\
 0 & 0 & 0 & 0 & 0 \\
 0 & 0 & 0 & 0 & 0
 \end{bmatrix}$$

and

$$J_2 = \begin{bmatrix} 0 & 0 & b & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ -d_2 & 0 & 0 & 0 \\ k & -d_1 & 0 & 0 \\ 0 & nd_1 & -(\mu_V + hA) & -hV \\ 0 & 0 & 0 & -d_A \end{bmatrix}$$

The characteristic equation for the disease free equilibrium  $\bar{E}$  is

$$(\Lambda + d_A)(\Lambda + \mu_T)^2 G(\Lambda) = 0. \quad (4)$$

where

$$G(\Lambda) = \begin{bmatrix} \Lambda + \mu_G + \beta_1 \bar{C}_{D4} & 0 & 0 & 0 & 0 & -b \\ -\beta_1 \bar{C}_{D4} & \Lambda + d_1 & 0 & 0 & 0 & 0 \\ 0 & -\alpha & \Lambda + \mu_G + \beta_2 \bar{C}_{CR} & 0 & 0 & 0 \\ 0 & 0 & -\beta_2 \bar{C}_{CR} & \Lambda + d_2 & 0 & 0 \\ 0 & 0 & 0 & -k & \Lambda + d_1 & 0 \\ 0 & 0 & 0 & 0 & -nd_1 & \Lambda + \mu_V \end{bmatrix}$$

we get,

$$G(\Lambda) = \Lambda^6 + \xi_1 \Lambda^5 + \xi_2 \Lambda^4 + \xi_3 \Lambda^3 + \xi_4 \Lambda^2 + \xi_5 \Lambda + \xi_6 = 0, \quad (5)$$

where,

$$\begin{aligned} \xi_1 &= d_1 + d_2 + d_1 + 2\mu_G + \mu_V + \beta_1 \bar{C}_{D4} + \beta_2 \bar{C}_{CR} > 0, \\ \xi_2 &= (\mu_G + \beta_1 \bar{C}_{D4})(\mu_G + \beta_2 \bar{C}_{CR}) + (d_1 + d_2 + d_1 + \mu_V) \times (2\mu_G + \beta_1 \bar{C}_{D4} + \beta_2 \bar{C}_{CR}) + (d_1 + d_2)(\mu_V + d_1) + \mu_V d_1 + d_1 d_2 > 0, \\ \xi_3 &= (\mu_G + \beta_1 \bar{C}_{D4})(\mu_G + \beta_2 \bar{C}_{CR})(d_1 + d_2 + d_1 + \mu_V) + (2\mu_G + \beta_1 \bar{C}_{D4} + \beta_2 \bar{C}_{CR}) [(d_1 + d_2)(\mu_V + d_1) + (d_1 \mu_V + d_1 d_2)] + [d_1 d_2 (\mu_V + d_1) + d_1 \mu_V (d_1 + d_2)] > 0, \\ \xi_4 &= (2\mu_G + \beta_1 \bar{C}_{D4} + \beta_2 \bar{C}_{CR}) [d_1 d_2 (\mu_V + d_1) + d_1 \mu_V \times (d_1 + d_2)] + (\mu_G + \beta_1 \bar{C}_{D4})(\mu_G + \beta_2 \bar{C}_{CR}) \times [(d_1 + d_2)(\mu_V + d_1) + (d_1 \mu_V + d_1 d_2)] + d_1 d_2 d_1 \mu_V > 0, \\ \xi_5 &= (\mu_G + \beta_1 \bar{C}_{D4})(\mu_G + \beta_2 \bar{C}_{CR}) [d_1 d_2 (\mu_V + d_1) + d_1 \mu_V (d_1 + d_2)] + d_1 d_2 d_1 \mu_V (2\mu_G + \beta_1 \bar{C}_{D4} + \beta_2 \bar{C}_{CR}) > 0, \\ \xi_6 &= d_1 d_2 d_1 \mu_V (\mu_G + \beta_1 \bar{C}_{D4})(\mu_G + \beta_2 \bar{C}_{CR}) - nkab\beta_1\beta_2 d_1 \bar{C}_{D4} \bar{C}_{CR}. \end{aligned} \quad (6)$$

For  $\xi_6 > 0$  there exist no positive roots and all roots are negative. Hence, the basic reproduction  $R_0$  is,

$$R_0 = \frac{nkab\beta_1\beta_2 pqs_T^2}{d_1 d_2 \mu_V (\mu_G + \beta_1 \bar{C}_{D4})(\mu_G + \beta_2 \bar{C}_{CR})}$$

**Remark:** At disease free equilibrium point  $\bar{E}$ , the system is locally stable if the basic reproduction number  $R_0 < 1$  and the system is unstable when  $R_0 > 1$ .

**The endemic state:** There exists another equilibrium in the form of endemic equilibrium ( $E^*$ ),

$$(G^*_1, C^*_{D4}, C^*_1, C^*_2, C^*_{CR}, C^*_2, I^*, V^*, A^*) \quad (7)$$

where,

$$C^*_{D4} = \frac{ps_T}{\mu_T + \beta_1 G^*_1}, \quad C^*_1 = \frac{ps_T \beta_1 G^*_1}{d_1 (\mu_T + \beta_1 G^*_1)},$$

$$V^* = \frac{d_A}{1}, \quad I^* = \frac{kG^*_2}{d_1},$$

$$C^*_{CR} = \frac{qs_T}{\mu_T + \beta_2 G^*_2}, \quad A^* = \frac{(nd_1 I^* - \mu_V V^*)}{hV^*},$$

$$C^*_2 = \frac{\beta_2 G^*_2 C^*_{CR}}{d_2},$$

$$G^*_1 = \frac{-\zeta_2 + \sqrt{\zeta_2^2 + 4\zeta_1\zeta_3}}{2\zeta_1},$$

$$G^*_2 = \frac{-\eta_2 + \sqrt{\eta_2^2 + 4\eta_1\eta_3}}{2\eta_1}, \quad (8)$$

and,

$$\zeta_1 = \beta_1 \mu_G, \zeta_2 = \iota(ps_T \beta_1 + \mu_G \mu_T) - bd_A \beta_1, \zeta_3 = bd_A \mu_T, \eta_1 = \mu_G \beta_2, \eta_2 = (qs_T \beta_2 + \mu_G \mu_T - \alpha \beta_2 C^*_1), \eta_3 = \alpha \mu_T C^*_1 \quad (9)$$

The endemic state with antibody response exist if the concentration of the combination of *gp41* and *gp120-CD4*-chemokine coreceptor ternary complex ( $C_2$ ) satisfy the condition

$$C^*_2 > \frac{\mu_V d_A}{nkl} \quad \text{and} \quad R_0 > 1$$

In this article, our main aim is to justify the effect of antibody vaccination mathematically. So we have not carried out the stability analysis for the endemic state. However we have derived the existence condition for the endemic state. Moreover we have carried out the numerical illustration for the endemic state and verified that the endemic state exist when  $R_0 > 1$ .

## THE SYSTEM WITH PERFECT ADHERENCE

To study the impulsive system it is assumed that the vaccine is taken at regular intervals with length  $\tau = t_{k+1} - t_k$ . For a impulsive cycle  $t_k \leq t \leq t_{k+1}$ , the solution is

$$A(t) = A(t_k^+) e^{-d_A(t-t_k)} \quad t_k < t \leq t_{k+1}$$

$$\geq A(t_k^+) e^{-d_A(t-t_k)} \quad (10)$$

Suppose that  $A(t_k^-)$  denotes the value immediately before the impulse and  $A(t_k^+)$  is the value immediately after. Calculating the least value of the concentration of  $A(t)$  for the perfect adherence with fixed interval length ( $\tau > 0$ ) we get

$$A(t) = A(t_k^+) e^{-d_A(t-t_k)} \quad (11)$$

This is the required concentration of antibody response to control the virus.

If  $A(0) = \bar{A}$ , then we have

$$A(t_1^+) = \bar{A}$$

$$A(t_2^-) = \bar{A} e^{-d_A \tau}$$

$$A(t_2^+) = \bar{A}(1 + e^{-d_A \tau})$$

$$A(t_3^-) = \bar{A}(1 + e^{-d_A \tau}) e^{-d_A \tau}$$

$$A(t_3^+) = \bar{A}(1 + e^{-d_A \tau} + e^{-2d_A \tau})$$

.

.

.

$$A(t_p^+) = \bar{A}(1 + e^{-d_A \tau} + e^{-2d_A \tau} + e^{-3d_A \tau} + \dots + e^{-(p-1)d_A \tau})$$

$$= \bar{A} \frac{1 - e^{-pd_A \tau}}{1 - e^{-d_A \tau}} \quad (12)$$

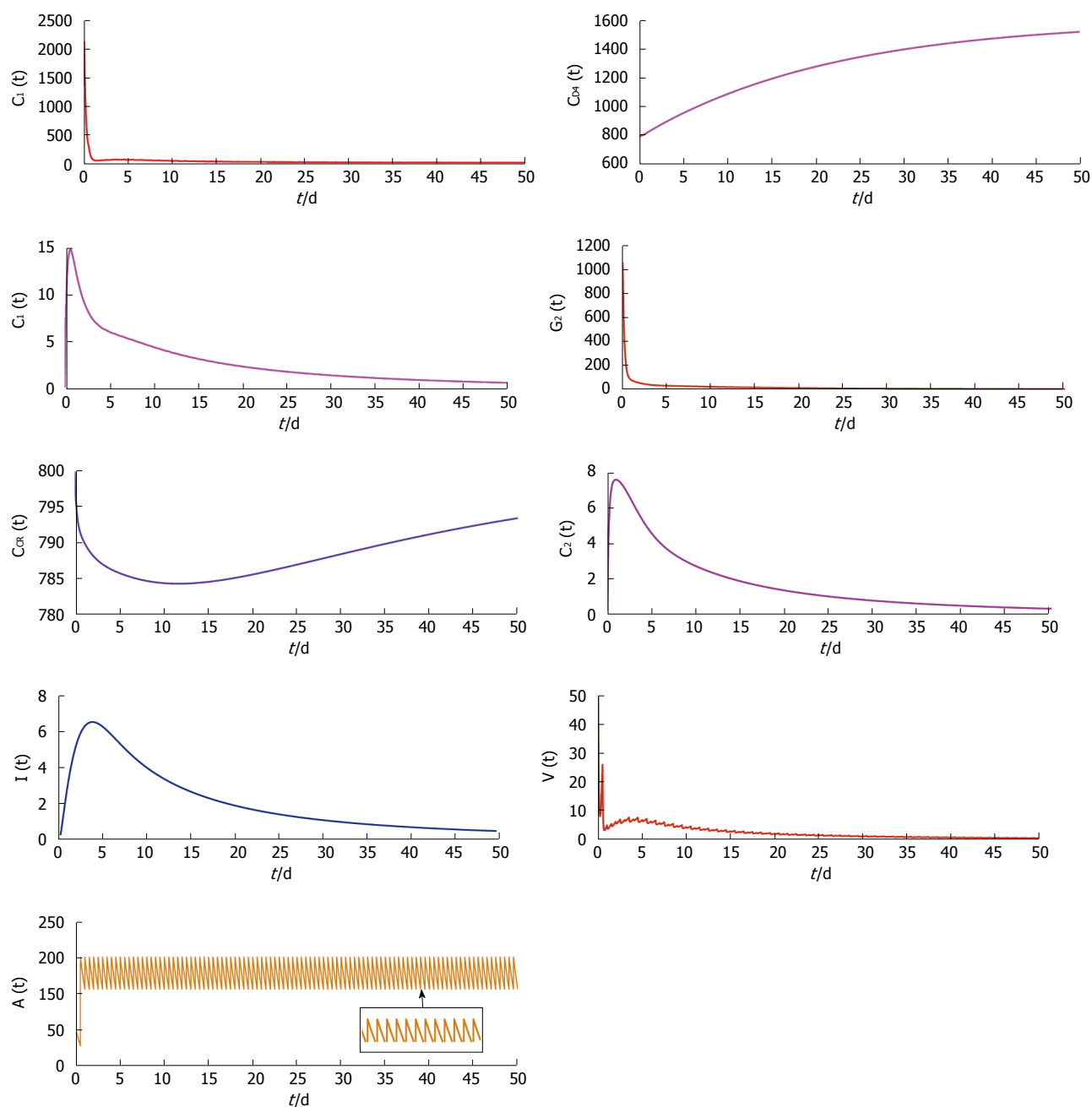


Figure 2 Trajectories showing the concentration changes with time of the model variable under restrained antibody vaccination with  $\tau = 0.5$  and  $\bar{A} = 100$ .

Hence,

$$\lim_{p \rightarrow \infty} A(t_p^+) = \frac{\bar{A}}{1 - e^{-d_A \tau}} \quad (13)$$

The periodic end points of the trajectories are

$$\frac{\bar{A}}{1 - e^{-d_A \tau}} \text{ and } \frac{\bar{A}e^{-d_A \tau}}{1 - e^{-d_A \tau}}$$

For perfect vaccination, the antibody response after the  $n^{\text{th}}$  vaccination is

$$A(t_n^+) = \frac{\bar{A}}{1 - e^{-d_A \tau}}$$

For perfect adherence, to control the virus and avoid resistance, the minimum value ( $A^*$ ) of the periodic orbit

must satisfy:

$$A^* < \frac{\bar{A}e^{-d_A \tau}}{1 - e^{-d_A \tau}} \quad (14)$$

Which implies that

$$\tau = \frac{1}{d_A} \ln\left(\frac{A^* + \bar{A}}{A^*}\right) = \tau_{\max} \quad (15)$$

**Remark:** If we can restrict the dosing interval of  $\tau$  satisfying the condition  $0 \leq \tau < \tau_{\max}$  (for fixed vaccination) then the disease can be controlled. However, if  $\tau > \tau_{\max}$ , the disease progression continues, even if drug is administered at fixed intervals. Thus maintenance of optimum dosage regimen is essential in order to control

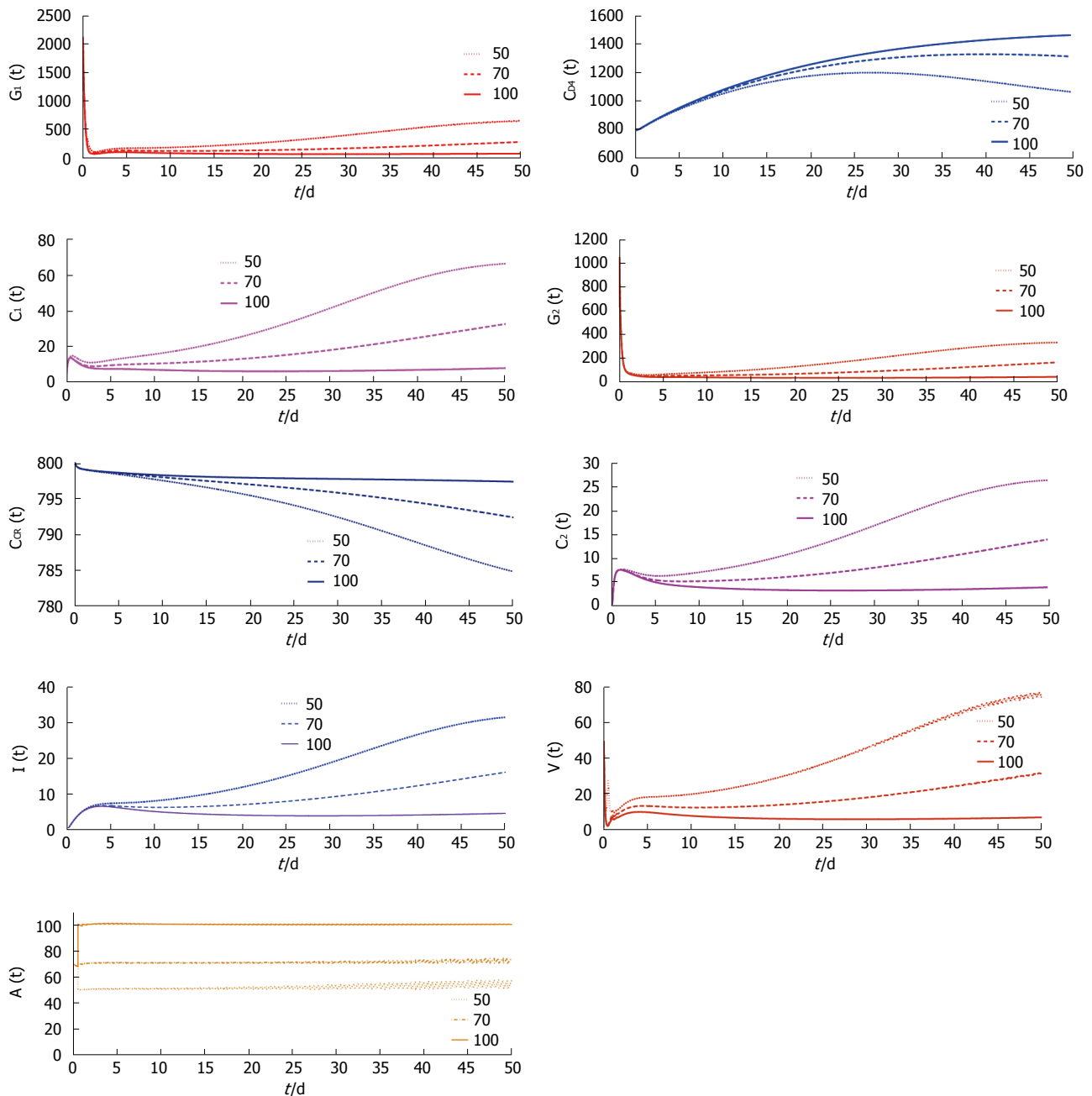


Figure 3 Trajectories showing the concentration changes with time of the model variable under different antibody vaccination concentration with  $\tau = 0.5$ .

the disease effectively.

## NUMERICAL SIMULATION

In our numerical illustration, we have described the perfect drug adherence of antibody vaccination. All the parameters are taken from Table 1. We have assumed the initial condition as  $G_1(0) = 2100$ ,  $C_{D4}(0) = 800$ ,  $C_1(0) = 0$ ,  $G_2(0) = 1050$ ,  $C_{CR}(0) = 800$ ,  $C_2(0) = 0$ ,  $I(0) = 0$ ,  $V(0) = 50$ ,  $A(0) = 100$  and the unit of the concentration is  $\text{mm}^{-3}$ .

In Figure 2 we observe that in the presence of vaccination ( $\bar{A} = 100$ ) with frequent dosing interval (*i.e.*,  $\tau = 0.5$ ), the virus population and infected CD4<sup>+</sup> T

cells population reduces. From this illustration, it can be predicted that proper antibody vaccination can restrict the infection process in HIV transmission. Now if the dosage of vaccination is not sufficient, then the effect of vaccination cannot be observed on the virus population and infected cell population.

In Figure 3, it is clearly observed that in presence of low vaccination (*i.e.*, for  $\bar{A} = 50$  or  $\bar{A} = 70$ ), the disease transmission process persists. Only adequate increment of the dosage of vaccination can restrict the disease progression. From these two figures we can predict that sufficient dosage of vaccination with frequent dosing interval can restrict the disease progression.

From Figures 4 and 5 we try to find out the effect of



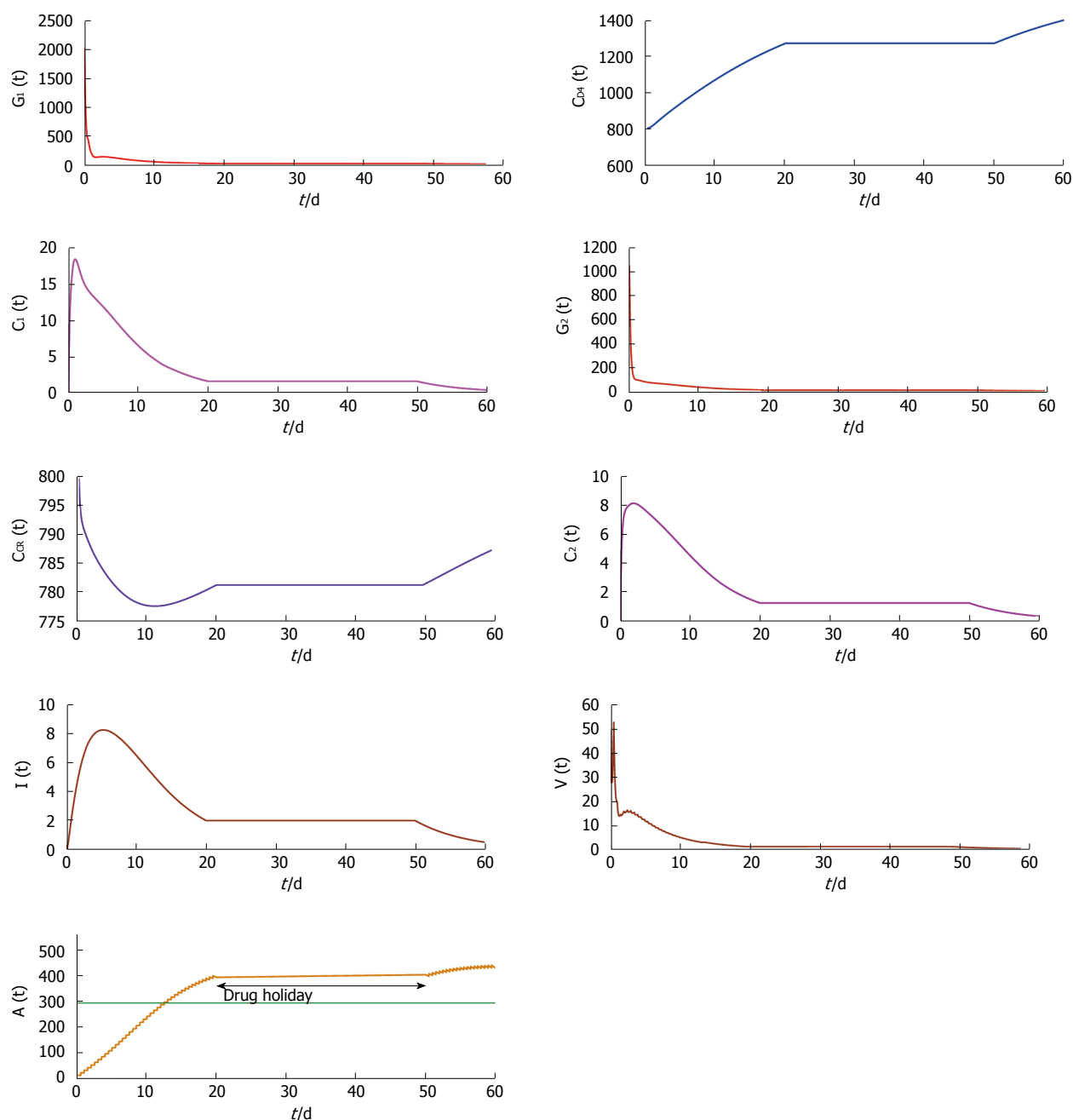


Figure 4 Trajectories showing the concentration changes with time of the model variable under restrained antibody vaccination with  $\tau = 0.5$  along with drug holidays for a period of 30 d.

drug holidays. In Figure 4, we restrict the drug holidays 30 d, whereas in Figure 5 the drug holidays is 10 d only. It is observed that when the drug holiday is 30 d the virus population attains its minimum value after 55 d. But if we restrict the drug holidays 10 d, the virus population attains its minimum value after 35 d. We also observe that for a 10 d drug holiday the  $C_{D4}$  receptor and  $C_{CR}$  receptor attains its maximum concentration within 40 d. Whereas  $C_D$  receptor and  $C_{CR}$  receptor attains its maximum concentration within 60 d for 30 d drug holidays. Thus most unpleasant circumstances occur in case of long-term effects of the drug holidays.

Figure 6 shows that phase plane plotted against

dosing interval and antibody responses. From this figure it is clearly observed that the antibody responses attain its maximum value if the drug dosing interval is frequent. Also the antibody responses reduce for the outsized dosing interval.

## CONCLUSION

Antibody vaccination in AIDS therapy has been studied as a new policy. Here vaccination is delivered to the host system in an impulsive mode to reactivate the antibody response. Dosage of vaccination and dosing interval has been effectively studied by this present mathematical

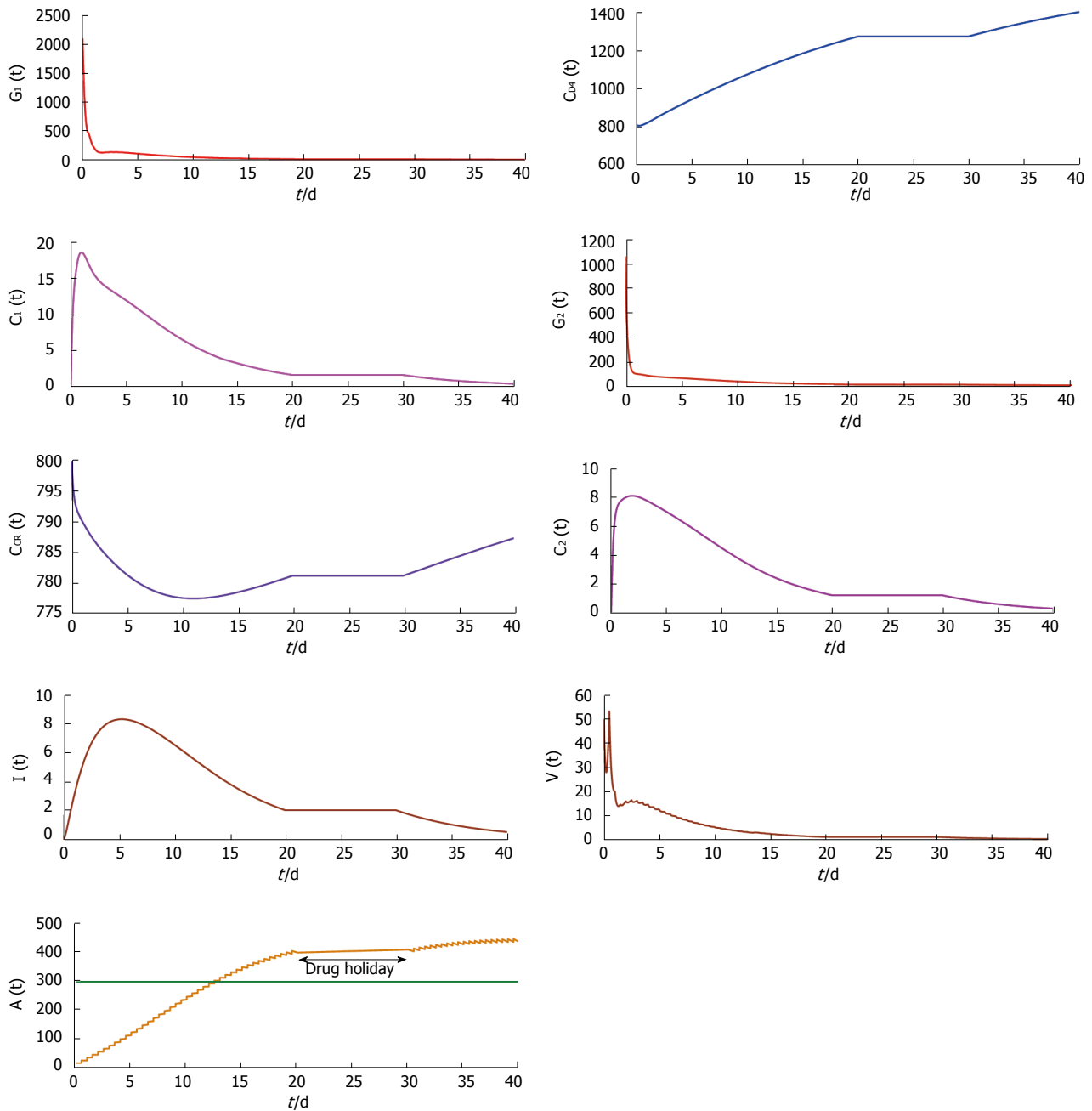


Figure 5 Trajectories showing the concentration changes with time of the model variable under restrained antibody vaccination with  $\tau = 0.5$  along with drug holidays for a period of 10 d.

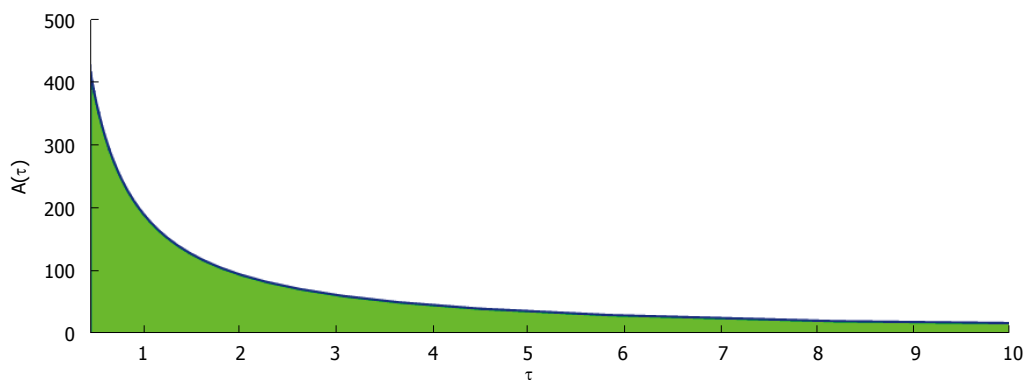


Figure 6 Phase plane showing the concentration of antibody responses against fixed dosing interval.

**Table 1** List of parameters for system (1) - (2)

| Parameter | Value (units)                   | Ref. |
|-----------|---------------------------------|------|
| $b$       | 42 (per day)                    | [6]  |
| $\beta_1$ | $10^{-5}$ (/mm <sup>3</sup> /d) | [6]  |
| $\mu_C$   | 5 (per day)                     | [9]  |
| $p$       | 1 (per day)                     | [6]  |
| $S_T$     | 80 (/mm <sup>3</sup> /d)        | [9]  |
| $\mu_T$   | 0.05 (per day)                  | [6]  |
| $d_1$     | 0.4 (per day)                   | [6]  |
| $\alpha$  | 25 (per day)                    | [6]  |
| $\beta_2$ | $10^{-5}$ (/mm <sup>3</sup> /d) | [6]  |
| $q$       | 0.6 (per day)                   | [6]  |
| $d_2$     | 0.4 (per day)                   | [6]  |
| $\kappa$  | 0.6 (per day)                   | [6]  |
| $d_1$     | 0.5 (per day)                   | [9]  |
| $\delta$  | 0.01 (per day)                  | [7]  |
| $n$       | 540                             | [6]  |
| $\mu_V$   | 3 (per day)                     | [7]  |
| $\iota$   | 0.01 (per day)                  | [12] |
| $d_A$     | 0.5 (per day)                   | [7]  |

model. It has been observed that when basic reproduction ratio lies below one, we expect the system attain its disease free state. However, at  $R_0 > 1$ , the system switches to endemic equilibrium. These conjectures have been supported by the results of numerical simulations.

It has been observed that the length of the dosing interval and the drug dose play a very decisive role in maintaining stable disease free equilibrium. From analytical as well as numerical finding it has been observed that the antibody responses attain its maximum value if the drug dosing interval is frequent. But the antibody responses reduce for the large dosing interval. Also we have observed that the drug holiday plays a pivotal role during the treatment schedule. From numerical findings we can predict that extensive drug holidays is unsafe for the treatment. Analytically and numerically, it has been observed that viral entry into the host cell is also inhibited with uninfected host CD4<sup>+</sup> T cell

population remaining unaffected. This happens because the antibody vaccination when administered following the best possible antibody responses can act against the free virus to neutralize free virus particles. This particular situation keeps the infected cell population at a very low level.

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Basic Study

## Rapid genotyping of human rotavirus using SYBR green real-time reverse transcription-polymerase chain reaction with melting curve analysis

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**Author contributions:** Tong Y performed the experiments, analyzed the data and wrote the manuscript; Lee BE contributed data and sample selections for the study and revised the manuscript; Pang XL designed the research and revised the manuscript.

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**Conflict-of-interest statement:** None.

**Data sharing statement:** Technical appendix, clinical dataset is available from the corresponding author at [xiao-li.pang@albertahealthservices.ca](mailto:xiao-li.pang@albertahealthservices.ca).

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### Abstract

**AIM:** To develop a real-time reverse transcription-polymerase chain reaction (RT-PCR) assay to genotype rotavirus (G and P) in Alberta from January 2012 to June 2013.

**METHODS:** We developed and validated a different approach to perform rotavirus G and P genotyping using a two-step SYBR green RT-PCR (rt-gPCR) by selecting genotype-specific primers of published conventional RT nested PCR (cnRT-PCR) assay and optimizing the amplification conditions. cDNA was first synthesized from total RNA with SuperScript™ II reverse transcriptase kit followed by amplification step using monoplex SYBR green real-time PCR. After the PCR reaction, melting curve analysis was used to determine specific genotype. Sixteen samples previously genotyped using cnRT-PCR were tested using the new assay and the genotyping results were compared as sensitivity analysis. Assay specificity was evaluated by testing other gastroenteritis viruses with the new assay. The amplicon size of each available genotype was determined by gel-electrophoresis and DNA sequences were obtained using Sanger-sequencing method. After validation and optimization, the new assay was used to genotype 122 pediatric clinical stool samples previously tested positive for rotavirus using electron microscopy between January



2012 and June 2013.

**RESULTS:** The new rt-gPCR assay was validated and optimized. The assay detected G1 to G4, G9, G12 and P[4] and P[8] that were available as positive controls in our laboratory. A single and clear peak of melting curve was generated for each of specific G and P genotypes with a  $T_m$  ranging from 80 °C to 82 °C. The sensitivity of rt-gPCR was comparable to cnRT-PCR with 100% correlation of the 16 samples with known G and P genotypes. No cross reaction was found with other gastroenteritis viruses. Using the new rt-gPCR assay, genotypes were obtained for 121 of the 122 pediatric clinical samples tested positive for rotavirus: G1P[8] (42.6%), G2P[4] (4.9%), G3P[8] (10.7%), G9P[8] (10.7%), G9P[4] (6.6%), G12P[8] (23.0%), and unknown GP[8] (0.8%). For the first time, G12 rotavirus strains were found in Alberta and G12 was the second most common genotype during the study period. Gel electrophoresis of all the genotypes showed expected amplicon size for each genotype. The sequence data of the two G12 samples along with other genotypes were blasted in NCBI BLAST or analyzed with Rota C Genotyping tool (<http://rotac.regatools.be/>). All genotyping results were confirmed to be correct.

**CONCLUSION:** rt-gPCR is a useful tool for the genotyping and characterization of rotavirus. Monitoring of rotavirus genotypes is important for the identification of emerging strains and ongoing evaluation of rotavirus vaccination programs.

**Key words:** Rotavirus A; Melting temperature; Real-time polymerase chain reaction; SYBR green; Genotyping

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**Core tip:** Genotyping rotavirus is essential for monitoring strain shifts in rotavirus surveillance and vaccine evaluation. Current conventional semi-nested real-time reverse transcription-polymerase chain reaction (RT-PCR), the most commonly used rotavirus genotyping assay is a labor-intensive, complex multi-step procedure and has long turn around-time. The newly developed SYBR Green real time RT-PCR assay is simple, fast and has comparable sensitivity and specificity as conventional semi-nested RT-PCR. This new assay was used to genotype clinical samples which tested positive for rotavirus from January 2012 to June 2013 and new emerging G12 strains were identified in Alberta, Canada.

Tong Y, Lee BE, Pang XL. Rapid genotyping of human rotavirus using SYBR green real-time reverse transcription-polymerase chain reaction with melting curve analysis. *World J Virol* 2015; 4(4): 365-371 Available from: URL: <http://www.wjgnet.com/2220-3249/full/v4/i4/365.htm> DOI: <http://dx.doi.org/10.5501/wjv.v4.i4.365>

## INTRODUCTION

Rotavirus group A is a major cause of severe acute gastroenteritis in children worldwide, with most children contracting the infection by five years of age<sup>[1]</sup>. Rotavirus vaccines are most efficacious in protecting children from severe rotavirus gastroenteritis, especially in areas with very low or low child and adult mortality<sup>[2]</sup>. Two effective rotavirus vaccines, RotaTaq® (Merck and Co., Inc.), a bovine-human reassortant vaccine covering the G1-G4 genotypes along with P[8] and Rotarix® (GlaxoSmithKline, Inc.), a monovalent attenuated human G1P[8] vaccine, were recommended by the WHO in 2009 for the routine immunization of infant globally<sup>[3]</sup>.

The viral genome consists of 11 double-stranded RNA segments, which encode six structure proteins (VP1-4, VP6 and VP7) and six non-structure proteins (NSP1-6). The traditional classification of rotavirus using serotyping has mostly been replaced with G and P genotyping that are based on the diversity of VP7 and VP4 gene sequences, respectively. Twenty-seven G genotypes (G1-G27) and 35 P genotypes (P[1]-P[35]) have been described and six G (G1-4, G9 and G12) and three P (P[4], P[6], and P[8]) genotypes predominate globally with some regional differences<sup>[4,5]</sup>. Introduction of vaccines or natural evolution of rotavirus may alter antigenic properties of circulating strains in the regions. The antigenic drift between G and P genotypes could result in a decrease of the effectiveness of vaccines against infection in the near future<sup>[6,7]</sup>. Therefore, understanding the presence and distribution of G and P genotypes and monitoring the emerging and recombination of rotavirus genotypes are very important prior to and after the introduction of rotavirus vaccines.

Various molecular methods have been developed for rotavirus genotyping. Conventional reverse transcriptase (RT) and nested polymerase chain reaction (cnRT-PCR) broadly used since the 90s<sup>[8-10]</sup> have been improved with more sensitive and specific primers<sup>[11,12]</sup>. These improved cnRT-PCR methods recognize a broad cluster of various rotavirus G and P genotypes with good specificity. However, there are several drawbacks of these type of assays including: (1) multi-step maneuvers and the requirement of two runs of PCR reactions and gel electrophoresis; (2) labor intensive protocols and a long turn-around time for final results; (3) difficulty with result interpretations due to non-specific amplicons or multiple amplicons in samples with co-infections; and (4) risk of cross contamination of PCR end-products because of the need for open tube maneuvers. With the 2010 recommendation of use of rotavirus vaccine for healthy infants in Canada<sup>[13]</sup>, a rapid and accurate genotyping assay is needed for the evaluation of the vaccine programs in many provinces.

Recently, real time RT-PCR assays in a closed tube system with their rapid turn around-time, sensitivity and specificity and low risk of cross contamination have been implemented in both research and diagnostic

**Table 1** Polymerase chain reaction primers used for G and P typing of human rotavirus by reverse transcriptase polymerase chain reaction

| Primer | Sequence (5'-3')   | Amplicon size (bp) | Direction | Ref.   |
|--------|--|--------------------|-----------|--|
| G-type |  |                    |           |  |
| VP7-R  | AAC TTG CCA CCA TTT TTT CC                                     |                    | Antisense | Iturriza-Gómara <i>et al</i> <sup>[10]</sup> |
| G1     | CAA GTA CTC AAA TCA ATG ATG G                                  | 618                | Sense     | Gouvea <i>et al</i> <sup>[8]</sup>           |
| G2     | CAA TGA TAT TAA CAC ATT TTC TGT G                              | 521                | Sense     | Gouvea <i>et al</i> <sup>[8]</sup>           |
| G3     | ACG AAC TCA ACA CGA GAG G                                      | 682                | Sense     | Iturriza-Gómara <i>et al</i> <sup>[10]</sup> |
| G4     | CGT TTC TGG TGA GGA GTT G                                      | 452                | Sense     | Gouvea <i>et al</i> <sup>[8]</sup>           |
| G8     | TTR1 TCG CAC CAT TTG TAA TT                                    | 756                | Sense     | Aladin <i>et al</i> <sup>[12]</sup>          |
| G9     | CTT GAT GTG ACT AY <sup>1</sup> A AAT AC                       | 179                | Sense     | Iturriza-Gómara <i>et al</i> <sup>[10]</sup> |
| G10    | ATG TCA GAC TAC AR <sup>2</sup> A TAC TGG                      | 266                | Sense     | Gouvea <i>et al</i> <sup>[8]</sup>           |
| G12    | GGT TAT GTA ATC CGA TGG CG                                     | 396                | Sense     | Aladin <i>et al</i> <sup>[12]</sup>          |
| P-type |  |                    |           |  |
| VP4-F  | TAT GCT CCA GIN <sup>3</sup> AAT TGG                           |                    | Sense     | Simmonds <i>et al</i> <sup>[11]</sup>        |
| P[4]   | CTA TTG TTA GAG GTT AGA GTC                                    | 362                | Antisense | Gentsch <i>et al</i> <sup>[9]</sup>          |
| P[6]   | TGT TGA TTA GTT GGA TTC AA                                     | 146                | Antisense | Gentsch <i>et al</i> <sup>[9]</sup>          |
| P[8]   | TCT ACT GGR <sup>2</sup> TTR <sup>2</sup> ACN <sup>3</sup> TGC | 224                | Antisense | Iturriza-Gómara <i>et al</i> <sup>[10]</sup> |
| P[9]   | TGA GAC ATG CAA TTG GAC  | 270                | Antisense | Gentsch <i>et al</i> <sup>[9]</sup>          |
| P[10]  | ATC ATA GTT AGT AGT CGG  | 462                | Antisense | Gentsch <i>et al</i> <sup>[9]</sup>          |
| P[11]  | GTA AAC ATC CAG AAT GTG  | 191                | Antisense | Iturriza-Gómara <i>et al</i> <sup>[10]</sup> |

<sup>1</sup>Y: C or T; <sup>2</sup>R: A or G; <sup>3</sup>N: A, G, C or T.

laboratories for the detection of rotavirus in stool samples associated with gastroenteritis<sup>[14-16]</sup>. To date, rotavirus genotyping using TaqMan real time PCR assay has been reported<sup>[17]</sup>. The protocol could successfully genotype G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] with only two multiplex reactions, however the other genotypes and combinations were still unable to be typed. Melting curve analysis uses the melting temperature of double-stranded PCR products to determine the identity of the PCR products and also can detect the presence of nonspecific PCR products or primer-dimers. Melting curve analysis is commonly used in SYBR green RT-PCR (rt-gPCR) to determine PCR products and omits the need for gel electrophoresis. SYBR green real-time PCR with melting curve analysis has been used to type Dengue virus as a genotyping tool<sup>[18]</sup>. This study is to develop and validate a simple and rapid rotavirus genotyping assay using SYBR Green RT-PCR to detect a broad range of rotavirus strains and to use the new assay to characterize the rotavirus strains circulated in Alberta from January 2012 to June 2013.

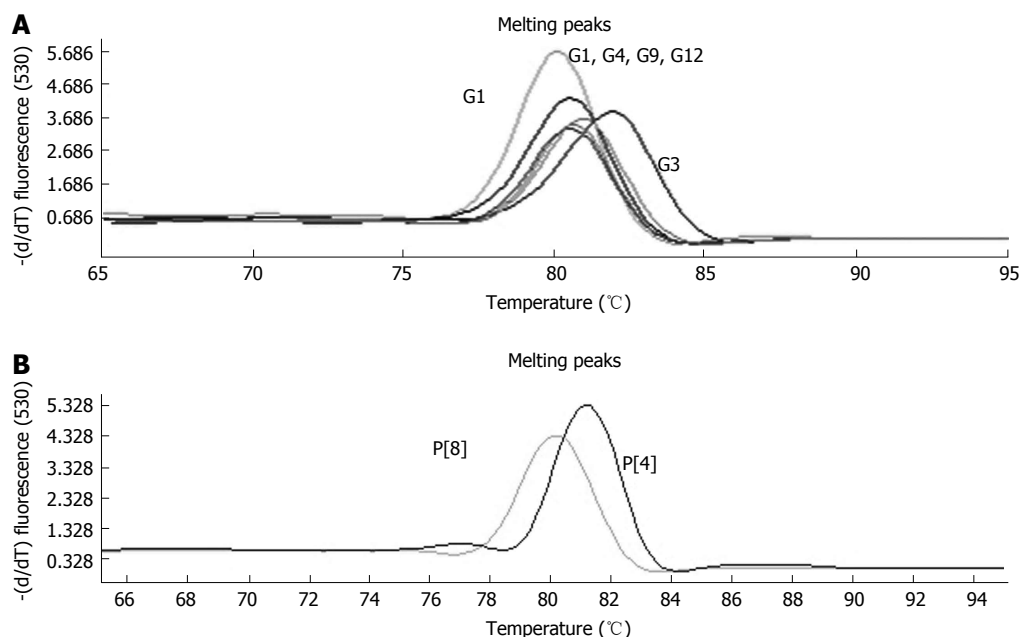
## MATERIALS AND METHODS

### Development of rt-gPCR assay

**Sample preparation, RNA extraction and RT reaction:** Archived rotavirus positive pediatric stool samples ( $n = 16$ ) obtained from the Provincial Laboratory for Public Health (ProvLab) and previously genotyped by the cnRT-PCR<sup>[10]</sup> were used for the development and validation of the rt-gPCR assay. Viral RNA was extracted from 200  $\mu$ L of 10% stool suspension (10% W/V with PBS buffer) using MagaZorb® total RNA Mini-Prep kit (Promega, Madison, United States) on an automaton extractor, KingFisher™ mL Magnetic Particle Processors, (Thermo Scientific, Mississauga, Canada) according to manufacturer's instructions. Using SuperScript™

II Reverse Transcriptase kit and random primer (Life technologies, Ontario, Canada), cDNA was synthesized from 5  $\mu$ L RNA at 42 °C for 1 h, 75 °C for 15 min after heating 5 min at 97 °C and stored at -20 °C before use.

**rt-gPCR:** A group of published primers used in cnRT-PCR for genotyping rotavirus G types (G1-4, G8, G9-10) and P types (P[4], P[6], P[8], P[9], P[10], and P[11]) were selected for the new assay development<sup>[8-10]</sup> (Table 1). Another two sets of primers for genotyping G8, and G12 which were not previously used in our laboratory was also added in the new rt-gPCR assay<sup>[12]</sup>. 5  $\mu$ L of 1:10 diluted cDNA was applied to 20  $\mu$ L of reaction mixture containing 2  $\mu$ L of LightCycler® FastStart DNA Master SYBR Green I (Roche Diagnostics, Quebec, Canada), 4 mmol/L MgCl<sub>2</sub>, 0.2  $\mu$ mol/L each of VP7-R and specific G-typing primers or 0.4  $\mu$ mol/L each of VP4-F and specific P-typing primers. The rt-gPCR was performed using LightCycler® 1.0 (Roche Diagnostics, Quebec, Canada) with four-step experimental run protocol: (1) denaturation program (10 min at 95 °C); (2) 45 cycles of amplification program (10 s at 95 °C; 10 s at 53 °C (P-typing), 57 °C (G-typing), and 25 s of extension at 72 °C); (3) melting curve program (0 s at 95 °C, 120 s at 65 °C and 0 s at 95 °C with ramp rate at 0.1 °C per second); and (4) cooling program down to 40 °C. Since different genotype specific primers would yield amplicons of different size with various GC content percentages, the temperature ( $T_m$ ) and melting curve of the amplicon of specific rotavirus G or P genotype would be different. Thus melting  $T_m$  profiles were used to identify specific rotavirus G or P genotypes in our design. For data analysis, the  $T_m$ , fluorescence ( $d[F1]/dT$ ) under the melting curve window was selected as the parameters for evaluation. A sample would be assigned to a specific genotype when the reaction  $T_m$  matched with known genotype controls, and the fluorescence  $d[F1]/dT$  was



**Figure 1** Melting curve analysis of different genotypes using reverse transcriptase polymerase chain reaction (A: G-typing: G1, G2, G4, G9, G12, G3; B: P-typing: P[8], P[4]). The peak of curve in X-axis is the melting temperature of each genotype DNA fragment; Y-axis indicates the SYB green fluorescence density.

above 1.0. The cycle threshold (Ct) of amplification curve was used to provide a relative quantification. Positive controls of specific G and P genotypes were included in each rt-gPCR run as reference genotype and for quality control.

**Assay sensitivity and specificity:** The sensitivity of rt-gPCR assay was compared with cnRT-PCR using ten-fold serial dilutions from neat (undiluted) to  $10^{-6}$  of samples with known G and P genotypes. Other common gastroenteritis viruses including norovirus, sapovirus, adenovirus, and astrovirus were also tested using the G and P primers to determine the specificity of the rt-gPCR assay.

**DNA sequencing:** Six G-types including G1 to G4, G9, G12 and two P-types P[4], P[8] detected by the rt-gPCR assay were sequenced with modification as described previously<sup>[19]</sup>. Briefly, the positive PCR products were run in 2.0% agarose gel and purified with QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) then sequenced using 3730 Genetic Analyzer (Applied biosystems, Foster City, United States) at University of Alberta.

### Genotyping clinical samples

ProVLab provides routine diagnostic testing of stool samples submitted for testing of gastroenteritis viruses using electronic microscope (EM). A total of 122 stool samples with rotavirus identified by EM between January 5, 2012 and June 8, 2013 were genotyped using the validated rt-gPCR assay. The purpose was to determine the performance of the assay for rapid genotyping of rotavirus in the clinical setting.

### Data analysis

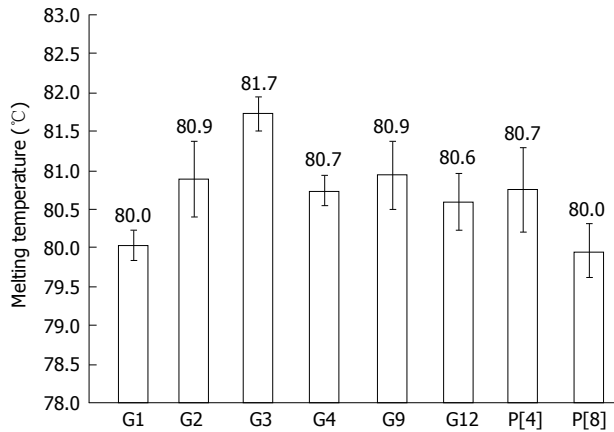
SD for the  $T_m$  of each genotype was calculated to show the variations in  $T_m$ . The sequence data of amplicons of G1 to G4, G9, G12, P[4] and P[8] were blasted in NCBI or analyzed with Rota C Genotyping tool (<http://rotac.regatools.be/>).

## RESULTS

### Development of rt-gPCR assay

All 16 positive control samples previously genotyped by cnRT-PCR were confirmed using the rt-gPCR assay with 100% concordant results. Using the rt-gPCR assay, a single and clear peak of melting curve was yielded for each of specific G and P genotypes with a  $T_m$  ranging from 80 °C to 82 °C (Figure 1). More than 30 of the G1, G3, G9, P[8] and more than 20 of the G2, G4, G12, P[4] replicates and/or different samples at different runs were used to calculate to mean  $T_m$  and SD. Each genotype  $T_m$  showed very small variation during different PCR runs among different samples. The mean  $T_m$  (°C  $\pm$  SD) for each of the genotypes were: G1 at 80.0 °C  $\pm$  0.20, G2 at 80.9 °C  $\pm$  0.49, G3 at 81.7 °C  $\pm$  0.22, G4 at 80.7 °C  $\pm$  0.20, G9 at 80.9 °C  $\pm$  0.45, G12 at 80.6 °C  $\pm$  0.37, P[4] at 80.7 °C  $\pm$  0.50, and P[8] at 80.0 °C  $\pm$  0.34 (Figure 2). Multiplex rt-gPCR could not be performed to simultaneously detect all G or P genotypes because the  $T_m$  generated from each genotype was very close. A lower  $T_m$  peak (< 77 °C) indicating primer-dimer was observed in the amplification reactions. All rt-gPCR genotype products showed expected amplicon sizes in the gel electrophoresis (Figure 3).

In the sensitivity comparison by serial ten-fold dilution of positive samples, both rt-gPCR and cnRT-



**Figure 2** Melting temperatures of different rotavirus genotypes using reverse transcriptase polymerase chain reaction. The mean  $T_m$  ( $^{\circ}\text{C} \pm \text{SD}$ ) for each genotype (G1 to G4, G9, G12, P[4] and P[8]) were calculated from more than 20 replicates of different samples at different polymerase chain reaction runs.

PCR detected G2, G3, G4, G9, G12, P[8] at  $10^{-5}$  dilution. cnRT-PCR detected G1 and P[4] at  $10^{-6}$  dilution while rt-gPCR detected at  $10^{-5}$  dilution. For the specificity test, no cross-reaction with other gastroenteritis viruses including norovirus, sapovirus, adenovirus, and astrovirus was observed using the rt-gPCR assay.

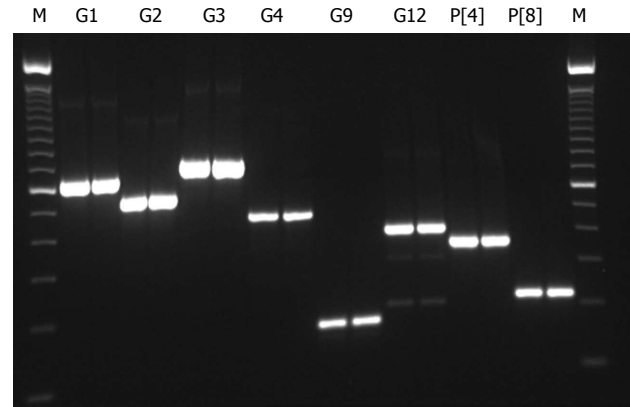
The assay was optimized after testing a range of annealing temperatures and primer concentrations. A non-specific amplification was observed for G types at low temperatures ( $53^{\circ}\text{C}$ ) but not the P types. The issue with non-specific amplifications was resolved by increasing the annealing temperature to  $57^{\circ}\text{C}$  and decreasing the concentration of primers to  $0.2 \mu\text{mol/L}$ .

### DNA sequence analysis

With Rota C Genotyping tool or sequence analysis using NCBI BLAST, all genotyping results generated using rt-gPCR were confirmed to be correct. Two of the G12 positive samples yielded clear reading of 329bp and 335bp respectively had 100% nucleotide identity using NCBI BLAST but could not be directly analyzed using the Rota C Genotyping tool because of the minimal requirement of 500 bp by the tool. The genotyping results of these two samples were further confirmed by analyzing the hits generated using NCBI BLAST with the Rota C tool. By blasting the sequence of these samples in NCBI, the first ten hits showed genotype G12 with 100% nucleotide identity. One of the hits [Rotavirus A strain RVA/Human-wt/ZWE/MRC-DPRU1858/2011/G12P[8] segment 9 capsid glycoprotein VP7 (VP7) gene, complete cds, Genbank: KP753228.1] was further analyzed by the Rota C tool and was confirmed to be G12.

### Clinical sample genotyping

Of the 122 stool samples tested positive for rotavirus by EM, 121 (99%) were characterized by G and/or P genotypes using the rt-gPCR assay except for one sample (1%) that was not typeable. During the study, five



**Figure 3** Agarose gel electrophoresis of different rotavirus genotype using reverse transcriptase polymerase chain reaction. Amplicon sizes for different genotypes (G1: 618 bp; G2: 521 bp; G3: 682 bp; G4: 452 bp; G9: 179 bp; G12: 396 bp; P[4]: 362 bp; P[8]: 224 bp. M: 100 bp DNA ladder (life technologies).

G-types were identified, G1, G2, G3, G9 and G12 while only two P types, P[4] and P[8] were found circulating in the province. The predominant G/P combination was G1P[8] at 42.6% ( $n = 52$ ), followed by G12P[8] (23.0%,  $n = 28$ ), G3P[8] (10.7%,  $n = 13$ ), G9P[8] (10.7%,  $n = 13$ ), G9P[4] (6.6%,  $n = 8$ ), and G2P[4] (4.9%,  $n = 6$ ). One sample was typed as P[8] but rt-gPCR for G genotyping did not yield any result. The only untypeable EM positive sample was retested using our in-house rotavirus RT-PCR assay<sup>[16]</sup> and was found to have a very high Ct of 39 indicating low viral load and possible sample degradation. No mixed infection of different genotypes was found.

## DISCUSSION

We developed a new rt-gPCR assay for genotyping rotavirus and compared the results to the cnRT-PCR assay. The rt-gPCR assay has the same specificity as the cnRT-PCR assay. The sensitivity of rt-gPCR was comparable to cnRT-PCR with 100% correlation of known G and P genotypes samples. The only difference between the two assays was the detection of G1 and P[4] at  $10^{-6}$  dilution only by cnRT-PCR, which was an expected result as nested PCR could be more sensitive. More importantly, this degree of difference in sensitivity is not significant for clinical applications as high viral load of rotavirus is usually excreted in acute gastroenteritis<sup>[15]</sup>. Due to the availability of genotypes in our laboratory, the most common G (G1 to G4, G9, G12) and P (P[4] and P[8]) types were validated.

While the rt-qPCR assay has many advantages, including a simpler protocol, shorter turn around-time and lower risk of cross contamination, the multiple multiplex real-time PCR reactions required to genotype respective G and P types have high reagents cost. For cost-saving, a laboratory can design a stepwise testing algorithm to first test for the more common G and P types to reduce reagent and labor costs. Based on our local data and other reported rotavirus genotypes in Canada, we would suggest: For P typing, first test



for P[8] and if P[8] is negative, then test for P[4]; for G typing, first test for G1 to be followed by G12 and G9, then G3, G2 +/- G4. This stepwise strategy would cover the range of G and P genotypes for most clinical samples.

The first G12 strain was identified in the Philippines in 1987 followed by reports of sporadic detection in other countries<sup>[20-22]</sup>. G12 is currently recognized as a globally emerging rotavirus genotype that appears to be spreading more rapidly in recent years<sup>[23]</sup>. Predominant of G12 was reported in Nepal in 2011, Cameroon in 2010/11 and rotavirus outbreak associated with G12 was found in United States in 2006-07<sup>[24-26]</sup>. In our study, G12 was detected as the second most prevalent genotype using rt-gPCR in Alberta. G12 could be detected by the new rt-gPCR assay but would be missed by the cnRT-PCR assay. We still believe that G12 was an emerging rotavirus genotype in 2012 as only 1.4% of EM rotavirus positive samples were untypeable from previous nine years in Alberta (data not shown). No G12 has been previously reported in Canada<sup>[27]</sup>. To our knowledge, this is the first report of G12 genotype detected in Canada. In addition, G9P[4] which was rarely detected in previous years increased dramatically in 2012. These findings emphasize the importance of ongoing surveillance for circulating rotavirus genotypes. In conclusion, the newly developed rt-gPCR assay with optimized primer selection enhanced the detection of broader genotypes which makes this assay a useful tool for the characterization and monitoring of strain shifts in rotavirus surveillance and the evaluation of vaccination program.

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## COMMENTS

### Background

Genotyping rotavirus is very important for the characterization and monitoring of strain shifts in rotavirus surveillance for the evaluation of vaccination program. The most commonly used rotavirus genotyping assay is a conventional nested reverse transcriptase (RT) polymerase chain reaction (cnRT-PCR) which has been used and revised for more than 30 years. The labor-intensive, complex multi-step procedure and high potential contamination risk due to nested PCR format make this method falling behind current demand.

### Research frontiers

An accurate, easy-to-perform and rapid rotavirus genotyping tool is needed. Several published studies developed new genotyping PCR assays using multiplex Taqman real time PCR assay to replace conventional nested PCR but has limited sensitivity and can detect only a few G or P types.

### Innovations and breakthroughs

Using SYBR Green based RT-PCR with melting curve analysis and melting temperature ( $T_m$ ) to genotype rotavirus is simple, fast and provides accurate and

broad identifications of genotypes. Gel electrophoresis required by traditional conventional nested PCR genotyping assay is not needed and eliminates the risk of contamination from handling post-PCR products. The new assay showed similar sensitivity and specificity as the conventional nested RT-PCR.

## Applications

The new assay identified common genotypes circulating in Alberta, Canada as well as the emergence of rotavirus G12 in 2012-2013. G12 has never been reported in Canada. During the study, the most predominant rotavirus genotypes were: G1P[8] (42.6%), G12P[8] (23.0%), followed by G3P[8] (10.7%), G9P[8] (10.7%), G9P[4] (6.6%), and G2P[4] (4.9%). These new findings support the importance of ongoing surveillance and characterization of rotavirus genotypes.

## Terminology

Rotavirus G-typing is genotyping viral gene sequences diversity which encoding structure protein VP7; Rotavirus P-typing is genotyping viral gene sequences diversity which encoding structure protein VP4; SYBR green is a commonly used fluorescent DNA binding dye, binds all double-stranded DNA and detection is monitored by measuring the increase in fluorescence throughout the cycle. SYBR Green master mixes are designed for quantitative real-time PCR using a set of two PCR primers that flank the target region; Melting curve analysis is the temperature-dependent dissociation between two DNA-strands can be measured using a DNA-intercalating fluorophore such as SYBR green. Melting  $T_m$  can be used for determination of the identity of the target.

## Peer-review

The article describes a new assay for genotyping the human rotavirus. The results presented seem pretty convincing and conclusions are valid.

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Retrospective Study

# Prevalence of adenovirus and rotavirus infection in immunocompromised patients with acute gastroenteritis in Portugal

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## Abstract

**AIM:** To characterize the prevalence of rotavirus (RV) and adenovirus (Adv) infections in immunocompromised patients with acute gastroenteritis.

**METHODS:** The presence of RV and Adv (serotypes 40 and 41) was evaluated in 509 stool samples obtained between January 2009 and December 2010 from 200 immunocompromised patients (83 females and 117 males; median age 21 years old, range 0-72). The diagnosis of infection was performed as a routine procedure and the presence of RV and Adv (serotypes 40 and 41) was determined by immunochromatography using the RIDA® Quick Rota-Adeno-Kombi kit (r-Biopharm, Darmstadt, Germany). The data analysis and description of seasonal frequencies were performed using computer software IBM® SPSS® (Statistical Package for Social Sciences) Statistics version 20.0 for Mac. The frequencies of infection were compared into different age and gender groups by  $\chi^2$  test.

**RESULTS:** The study revealed 12.4% Adv positive samples and 0.8% RV positive samples, which correspond to a prevalence of 6.5% and 1.5%, respectively. Adv was more frequent between October 2009 and April 2010, while RV was identified in April 2010 and July 2010. The stool analysis revealed that from the 509 samples, 63 (12.4%) were positive for Adv and 4 (0.8%) positive for RV, which by resuming the information

of each patient, lead to an overall prevalence of AdV and RV of 6.5% (13/200 patients) and 1.5% (3/200 patients), respectively. The stratification of the analysis regarding age groups showed a tendency to an increased prevalence of infection in paediatric patients between 0-10 years old. Considering the seasonal distribution of these infections, our study revealed that AdV infection was more frequent between October 2009 and April 2010, while RV infection was characterized by two distinct peaks (April 2010 and July 2010).

**CONCLUSION:** The overall prevalence of AdV and RV infection in immunocompromised patients with acute gastroenteritis was 8% and AdV was the most prevalent agent.

**Key words:** Viral gastroenteritis; Adenovirus; Rotavirus; Immunocompromised host; Stool samples

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**Core tip:** Acute gastroenteritis has been associated with significant rates of morbidity and mortality among immunocompromised patients. Rotavirus (RV) and adenovirus (AdV) are described as common agents of viral gastroenteritis causing acute diarrhoea. This is the first study in Portugal to characterize the prevalence and seasonal features of RV and AdV infections in immunocompromised patients with acute gastroenteritis. Results revealed 12.4% AdV positive samples and 0.8% RV positive samples, which correspond to a prevalence of 6.5% and 1.5%, respectively. Our results also demonstrate the importance of to add more screening methods for other emergent enteric viruses, in order to avoid the morbidity and mortality of the immunocompromised patients.

Ribeiro J, Ferreira D, Arrabalde C, Almeida S, Baldaque I, Sousa H. Prevalence of adenovirus and rotavirus infection in immunocompromised patients with acute gastroenteritis in Portugal. *World J Virol* 2015; 4(4): 372-376 Available from: URL: <http://www.wjgnet.com/2220-3249/full/v4/i4/372.htm> DOI: <http://dx.doi.org/10.5501/wjv.v4.i4.372>

## INTRODUCTION

Acute gastroenteritis, one of the main causes of morbidity and mortality in the world, is a consequence of microbial infections, which in industrialized countries are mainly caused by viruses, such as rotavirus (RV), enteric adenovirus (AdV), astrovirus and human calicivirus<sup>[1-3]</sup>.

RV is the most common cause of severe diarrhoea in children under 5 years of age, adults in close contact with infected children, hospitalised patients and the elderly<sup>[3,4]</sup>. Data from United States reveal that RV infection is responsible for approximately 3 million cases of acute diarrhoea in children each year<sup>[5]</sup>. RV infection is associated with high rates of morbidity throughout

the world and high rates of mortality in developing countries, where gastroenteritis caused by RV account for more than 800000 deaths per year due to poor nutrition and lack of health care<sup>[6]</sup>. In the majority of countries, RV infections occur dispersed throughout the year, nevertheless in temperate climates is characterized by a peak during the winter months<sup>[1-3]</sup>.

AdV, especially serotypes 40 and 41 (enteric AdV), are frequently related with high morbidity and mortality in immunocompromised patients with the most susceptible groups to be children and patients submitted to transplantation<sup>[7-9]</sup>. Enteric AdV have been related with acute diarrhoea in variable frequency (ranging from 1.4% to 10%), depending on the geographic location and type of patients<sup>[10,11]</sup>. AdV is known for its large distribution worldwide and the majority of the studies refer it to be equally distributed during all the seasons of the year<sup>[8,9]</sup>.

Immunocompromised patients constitute an important group for prevention of gastrointestinal infections<sup>[7,12,13]</sup>. In fact, the incidence of infection-related post-transplant diarrhoea has been reported to be up to 40%, with viruses being the most common pathogens<sup>[2,3,12]</sup>. Considering the importance of gastroenteritis prevention in immunocompromised individuals, the diagnosis of AdV and RV at early stages of the disease is extremely important, in order to reduce morbidity and mortality.

The aim of this study was to characterize the prevalence of RV and AdV infection in immunocompromised patients with acute gastroenteritis from the North region of Portugal treated at the Portuguese Institute of Oncology of Porto.

## MATERIALS AND METHODS

### Population

This study was performed as a cross-sectorial retrospective hospital-based case study with 509 stool samples obtained between January 2009 and December 2010 from immunocompromised patients diagnosed with acute diarrhoea at Portuguese Institute of Oncology of Porto (IPO Porto). Samples were obtained from 200 immunocompromised patients with different haematological malignancies (median age 21 years old, range 0-72): 83 female (median age 15 years old, range 0-65) and 117 male (median age 39 years old, range 0-72).

### RV/AdV detection

The diagnosis of infection was performed as a routine procedure at the Virology Service of IPO Porto. The stool specimens were tested as soon as possible after collection and the presence of RV and AdV (serotypes 40 and 41) was determined by immunochromatography using the RIDA® Quick Rota-Adeno-Kombi kit (r-Biopharm, Darmstadt, Germany) according to manufacturer instructions. The faecal samples were diluted in the dilution buffer supplied with the kit. This is a ready-to-use test based on a nitrocellulose membrane sensitized with



**Table 1** Adenovirus and rotavirus results discriminated by year *n* (%)

|                             | AdV positive | RV positive | Negative   |
|-----------------------------|--------------|-------------|------------|
| Total ( <i>n</i> = 509)     | 63 (12.4)    | 4 (0.8)     | 442 (86.8) |
| Year 2009 ( <i>n</i> = 189) | 24 (12.7)    | -           | 165 (87.3) |
| Year 2010 ( <i>n</i> = 320) | 39 (12.2)    | 4 (1.3)     | 277 (86.5) |

AdV: Adenovirus; RV: Rotavirus.

antibodies directed against RV and AdV (test lines).

### Statistical analysis

Data analysis and description of seasonal frequencies were performed using computer software IBM® SPSS® (Statistical Package for Social Sciences) Statistics version 20.0 for Mac. The frequencies of infection were compared into different age and gender groups by  $\chi^2$  test.

## RESULTS

In total, 509 stool samples were tested and it was possible to detect 63 (12.4%) positive samples for AdV and 4 (0.8%) positive samples for RV (Table 1). Comparing the number of cases during the period between January 2009 and December 2010, it was possible to observe a substantial increase in 2010, however the infection rates were similar.

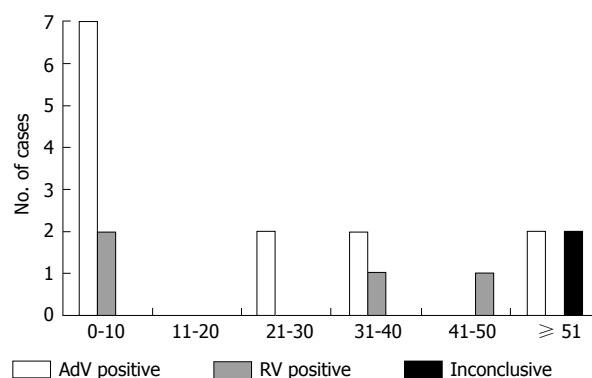
Considering only patients-related data, where we have combined the results of all samples obtained from each individual, the overall frequency of AdV and RV infection was 6.5% (13/200 patients) and 1.5% (3/200 patients), respectively. The prevalence of infection was characterized with stratification of individuals according to age groups and genre and the results showed a tendency to an increased prevalence of infection in paediatric patients between 0-10 years old (Figure 1 and Table 2).

Considering the seasonal distribution of these infections (Figure 2), our study revealed that AdV infection was more frequent between October 2009 and April 2010, while RV infection was characterized by two distinct peaks (April 2010 and July 2010).

## DISCUSSION

Immunosuppression treatments lead patients to be more susceptible to opportunistic infections, either bacterial or viral. Many studies have shown that RV and AdV are the most frequent pathogenic virus during acute diarrhoea in immunocompromised patients<sup>[3,7,9,13]</sup>. The overall distribution worldwide has been described in literature<sup>[5,14]</sup>, although little is known about the epidemiology of these viruses in Portugal, and therefore we aimed to add epidemiological information of the prevalence of these infections in immunocompromised patients with haematological diseases treated at IPO Porto.

Firstly, it is important to refer that our results should



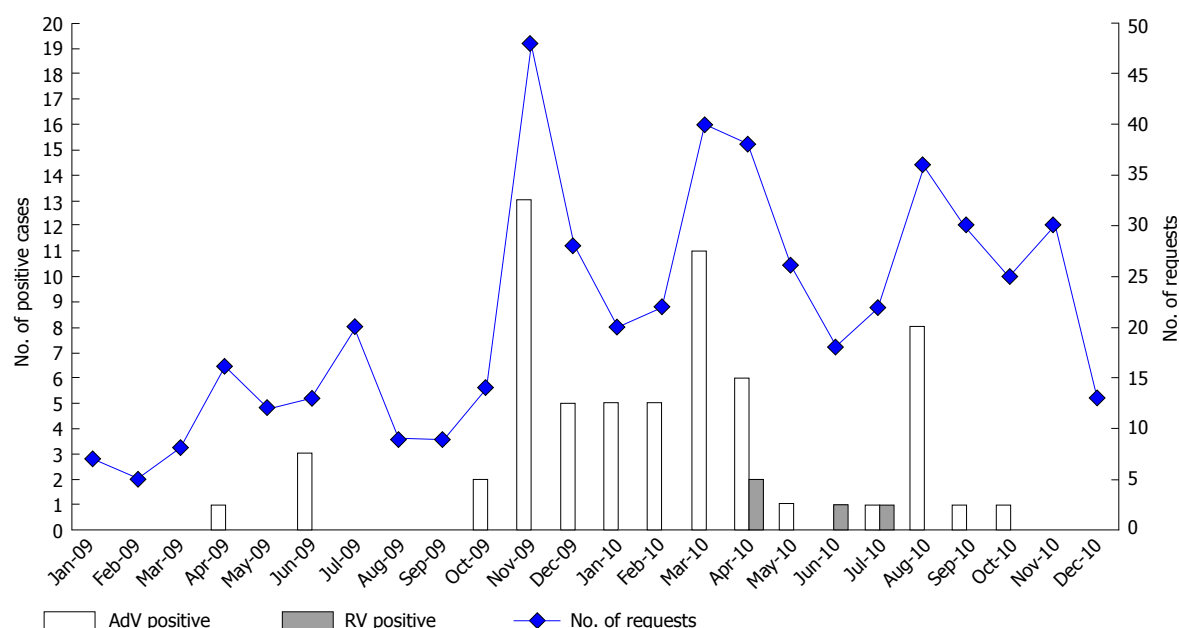
**Figure 1** Age-associated distribution of adenovirus and rotavirus prevalence. AdV: Adenovirus; RV: Rotavirus.

be discussed considering the limitations of the diagnostic test. This test is used to screen only infections by RV and enteric AdV, which could limit the identification of positive cases since the range of viruses involved in acute gastroenteritis is larger. Nevertheless, the frequencies that we have obtained were considerably low when compared with the number samples. These results might be explained by the fact that other virus such as Norovirus, or even bacteria, could be the cause of the acute gastroenteritis<sup>[15-18]</sup>. Moreover, acute diarrhoea is sometimes overestimated since chemotherapeutic agents and radiotherapy may also promote it<sup>[19]</sup>. In addition, there are no reports of these viruses in Portuguese immunocompromised patients, thus the fact that results are distinct from other studies remain to be further clarified<sup>[20]</sup>.

Recent data indicate that enteric AdV infection in immunocompromised patients remains constant along the year<sup>[3,8]</sup>, and the RV infection is more prevalent during the winter and beginning of the spring<sup>[2-4]</sup>. These features were not observed in our population, and in fact, our study showed that enteric AdV was more prevalent between November 2009 and April 2010, with a seasonal peak in August 2010. Between November 2010 and December 2010 there were no positive cases, but the number of suspected samples was also lower than in the same period the year before.

In contrast with literature, RV incidence was very low, with only four positive cases to be identified, and therefore no seasonal prevalence was estimated<sup>[2,3]</sup>. However, it is possible to observe that positive cases were identified in April and June/July months. These data are in agreement with previous studies performed in Portugal, which describe a higher prevalence of RV infection in paediatric population<sup>[21,22]</sup>. Rodrigues *et al.*<sup>[22]</sup> refers that the prevalence of RV presents clear differences between years and that in 2010 there were two distinct peaks with significant RV activity occurring much later and lasting into July.

To the best of our knowledge, this is the first study to characterize the prevalence of RV and enteric AdV in immunocompromised patients from Portugal. Despite the lower incidence of both viral infections, this study



**Figure 2** Seasonal distribution of adenovirus and rotavirus infection throughout the study period, taking into account the number of requests. AdV: Adenovirus; RV: Rotavirus.

**Table 2** Characterization adenovirus and rotavirus infection by age group and genre *n* (%)

|                         | AdV      |            |         | RV       |            |         |
|-------------------------|----------|------------|---------|----------|------------|---------|
|                         | Positive | Negative   | IC      | Positive | Negative   | IC      |
| Total ( <i>n</i> = 200) | 13 (6.5) | 185 (92.5) | 2 (1.0) | 3 (1.5)  | 196 (98.0) | 1 (0.5) |
| Age group               |          |            |         |          |            |         |
| 0-10 ( <i>n</i> = 60)   | 7 (11.7) | 53 (88.3)  | -       | 2 (3.3)  | 58 (96.7)  | -       |
| 11-20 ( <i>n</i> = 21)  | -        | 21 (100.0) | -       | -        | 21 (100.0) | -       |
| 21-30 ( <i>n</i> = 19)  | 2 (10.5) | 17 (89.5)  | -       | -        | 19 (100.0) | -       |
| 31-40 ( <i>n</i> = 19)  | 2 (10.5) | 17 (89.5)  | -       | 1 (5.3)  | 18 (94.7)  | -       |
| 41-50 ( <i>n</i> = 19)  | -        | 18 (94.7)  | 1 (5.3) | -        | 19 (100.0) | -       |
| ≥ 51 ( <i>n</i> = 62)   | 2 (3.2)  | 59 (95.2)  | 1 (1.6) | -        | 61 (98.4)  | 1 (1.6) |
| Genre                   |          |            |         |          |            |         |
| Male ( <i>n</i> = 117)  | 9 (7.7)  | 107 (91.4) | 1 (0.9) | 1 (0.9)  | 115 (98.2) | 1 (0.9) |
| Female ( <i>n</i> = 83) | 4 (4.8)  | 78 (94.0)  | 1 (1.2) | 2 (2.4)  | 81 (97.6)  | -       |

AdV: Adenovirus; RV: Rotavirus; IC: Inconclusive.

emphasizes the importance of vigilance and prevention of viral infections in the gastrointestinal tract. Moreover, as the results reveal a lower incidence of RV and enteric AdV, it is extremely important to add more screening methods for other emergent enteric viruses, in order to avoid the morbidity and mortality of the immunocompromised patients.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Acute gastroenteritis is one of the main causes of morbidity and mortality in

the world, especially for immunocompromised patients. Immunosuppression treatments lead patients to be more susceptible to opportunistic infections, either bacterial or viral. Many studies have shown that rotavirus (RV) and adenovirus (AdV) are the most frequent pathogenic virus during acute diarrhoea in immunocompromised patients.

### Research frontiers

Characterization of RV and AdV prevalence and seasonal distribution in immunocompromised patients with acute gastroenteritis.

### Innovations and breakthroughs

The overall prevalence of AdV and RV infection in immunocompromised patients with acute gastroenteritis was 8%. AdV was the most prevalent with 6.5% (13/200 patients) followed by RV with a prevalence of 1.5% (3/200 patients). Results revealed a lower prevalence of RV and enteric AdV than expected.

### Applications

The lower incidence of RV and enteric AdV observed in the authors' study pointed that it is extremely important to add more screening methods for other

emergent enteric viruses, in order to avoid the morbidity and mortality of the immunocompromised patients.

## Terminology

This study provides the first update on the prevalence of RV and AdV infection as agents of acute gastroenteritis in immunocompromised patients.

## Peer-review

This is an interesting study on the cause of diarrhea by viral agents. The study is well-designed and the manuscript is well-written.

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