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Review

Hepatitis E Virus Mutations: Functional and Clinical Relevance

Hoang van Tong [a,*], Nghiem Xuan Hoan [a], Bo Wang [b], Heiner Wedemeyer [c], C.-Thomas Bock [b,**,1], Thirumalaisamy P. Velavan [a,1]

[a] Institute of Tropical Medicine, University of Tübingen, Tübingen, Germany
[b] Department of Infectious Diseases, Robert Koch Institute, Berlin, Germany
[c] Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany

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ABSTRACT

Hepatitis E virus (HEV) infection is a major cause of acute hepatitis and affects more than 20 million individuals, with three million symptomatic cases and 56,000 recognized HEV-related deaths worldwide. HEV is endemic in developing countries and is gaining importance in developed countries, due to increased number of autochthone cases. Although HEV replication is controlled by the host immune system, viral factors (especially specific viral genotypes and mutants) can modulate HEV replication, infection and pathogenesis. Limited knowledge exists on the contribution of HEV genome variants towards pathogenesis, susceptibility and to therapeutic response. Nonsynonymous substitutions can modulate viral proteins structurally and thus dysregulate virus-host interactions. This review aims to compile knowledge and discuss recent advances on the casual role of HEV heterogeneity and its variants on viral morphogenesis, pathogenesis, clinical outcome and antiviral resistance.

Contents

Abbreviations: HEV, hepatitis E virus; ORF, open reading frame; MeT, methyltransferase; Y, Y-domain; PCP, papain-like cysteine protease; HVR, hypervariable region; X-domain, macro-domain; Hel, RNA helicase; RdRp, RNA-dependent RNA polymerase; PPR, polyproline region; CP, capsid protein; aa, amino acid; vgRNA, viral genomic RNA; sgRNA, subgenomic RNA; CRE, *cis*-reactive element.

* Correspondence to: H. van Tong, Institute of Tropical Medicine, University of Tübingen, Wilhelmstrasse 27, 72074 Tübingen, Germany.
** Correspondence to: C.-T. Bock, Department of Infectious Diseases, Division of Viral Gastroenteritis and Hepatitis Pathogens and Enteroviruses, Robert Koch Institute, Seestr. 10, D-13353 Berlin, Germany.
E-mail addresses: tong.van-hoang@uni-tuebingen.de (H. van Tong), bockc@rki.de (C.-T. Bock).
[1] CTB and TPV contributed equally and thus share last authorship.

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1. Introduction

Hepatitis E virus (HEV) infection is being increasingly recognized in medical research as HEV infection has reached industrialized countries. Although HEV was discovered in 1983 (Balayan et al., 1983) and subsequent experimental analyses were initiated since 1990/1991 on HEV isolates (Reyes et al., 1990), there exists a considerable lack of understanding and knowledge of transmission routes, life-cycle, pathogenesis, genome variability and viral evolution.

Substantial epidemics and sporadic outbreaks of hepatitis E occur in tropical and sub-tropical countries (e.g., in India, Uganda, Sudan, and Mexico), with up to tens of thousands affected (Dalton et al., 2013; Kamar et al., 2012). Approximately two billion people (one-third of the world population) live in areas endemic for HEV and are at risk (Perez-Gracia et al., 2013). HEV infections are less frequently documented in industrialized countries, as it is believed to be associated with travel to HEV-endemic countries. However, by the end of the millennium, the numbers of autochthone cases were rising exponentially. HEV infections in Western Europe have been reported (Dalton et al., 2013; Kamar et al., 2012; Pischke et al., 2014). Reasons for discrepancies of HEV presentation between developing and developed countries are diverse. The possible likelihood refers to the route of transmission and the different distribution of HEV genotypes (Pauli et al., 2015; Sayed et al., 2015). In developing countries, HEV infection is transmitted mainly as waterborne/fecal-oral due to poor hygiene conditions, whereas in developed countries HEV is transmitted mainly foodborne due to zoonotic transmission by consumption of undercooked meat and bowels (Mansuy et al., 2016). In this regard, HEV is unique, as the only hepatitis virus with an animal reservoir.

HEV variants are viral factors that are known to be associated with transmission dynamics and pathogenicity (Kamar et al., 2012, 2014a; Lee et al., 2016; Meng, 2011). HEV mutations occur under selective pressure imposed by the host immune system and by antivirals. HEV heterogeneity shall contribute towards HEV physiology, pathogenesis and transmission patterns (Lhomme et al., 2014a). In this review, we aim to compile knowledge and discuss recent advances on the casual role of HEV heterogeneity and its variants on viral morphogenesis, pathogenesis, clinical relevance and antiviral resistance.

2. Clinical Course and Pathogenesis of HEV Infection

Although the majority of HEV infections are asymptomatic, the clinical course of symptomatic infections includes acute and chronic hepatitis E, fulminant liver failure and extrahepatic symptoms (Debing et al., 2016a; Hoan et al., 2015). Acute hepatitis E is usually defined as a self-limiting disease and lasts approximately 8 weeks and the symptoms are typically unspecific and mostly indistinguishable from other types of acute viral hepatitis (Wedemeyer et al., 2012). HEV-RNA can be detected in both serum and stool before the onset of clinical symptoms and lasts less than a month after symptom onset in serum but may persist longer in the stool (Krain et al., 2014). A severe form of acute hepatitis (fulminant hepatic failure) has been observed in patients with pre-existing liver diseases and in pregnant women (Dalton et al., 2007; Navaneethan et al., 2008). The severity of HEV infection in pregnant women may be associated with the hormonal balance and immunologic complexity during pregnancy (Bose et al., 2011; Navaneethan et al., 2008). HEV replication occurring in the human placenta may lead to poor pregnant outcomes, including HEV transmission from mother to newborn and abortion (Bose et al., 2014; Navaneethan et al., 2008).

Chronic hepatitis E is defined by the persistence of HEV-RNA and/or *anti*-HEV IgM for more than six months with elevated alanine aminotransferase (ALT) levels. Chronic HEV infection has been reported primarily in immunocompromised individuals, in organ transplant recipients, patients under chemotherapy, and HIV-infected patients (Dalton et al., 2009; Kamar et al., 2008). Chronic hepatitis E has been associated with the development of fibrosis and/or cirrhosis in patients with solid-organ-transplantation (Kamar et al., 2008). Chronic HEV infection largely depends on the host immune responses, and thus the suppressed immunity in those specific groups of patients enables the virus to persist and establish chronic infection. The impairment of HEV-specific T-cell responses is likely associated with the development of chronic hepatitis E (Suneetha et al., 2012). However, rare cases of chronic and/or persistent HEV infection have also been reported in healthy, immunocompetent individuals (Gonzalez Tallon et al., 2011).

HEV may also contribute to various extrahepatic manifestations, including pancreatitis, hematological disorders (thrombocytopenia and anemia), kidney disorders and neurological complications (Guillain-Barré syndrome and meningoencephalitis) (Singh and Gangappa, 2007; Thapa et al., 2009; Wedemeyer et al., 2012). The discovery of HEV quasispecies in serum and cerebrospinal fluid additionally suggest a possible role in neurological disorders and relate to the emergence of neurotropic HEV variants (Kamar et al., 2010). The extrahepatic manifestation mechanism can be explained by HEV replication in the extrahepatic tissues/organs and cause local tissue damage and inflammation. This is supported by recent findings, which showed HEV replication in the human placenta and neuronal-derived tissues (Bose et al., 2014; Drave et al., 2016). Other mechanisms such as cross-reactive immune responses, generation of immune complexes, and secondary infections have been proposed (Feng, 2016). However, the exact underlying mechanism of extrahepatic manifestations by HEV warrants further investigation.

3. HEV Biology and Molecular Virology

HEV is a small RNA, non-enveloped virus, 32–34 nm in diameter and belonging to the genus *Orthohepevirus* of the *Hepeviridae* family (Kamar et al., 2012). The HEV genome is a positive-sense single-stranded RNA molecule of 7.2 kb containing three open reading frames (ORF1, ORF2, and ORF3), 5′- and 3′-untranslated regions (UTRs), and a polyA-tract at the 3′-end (Kamar et al., 2012) (Fig. 1). ORF1 encodes the non-structural proteins and enzymes including methyltransferase (MeT), RNA helicase (Hel) and RNA-dependent RNA polymerase (RdRp) required for RNA replication. ORF2 expresses the capsid protein. ORF3 overlaps partially with ORF2 and encodes a multifunctional phosphoprotein that can modulate cellular signaling and is related to particle secretion (Parvez and Al-Dosari, 2015). A novel ORF4 of 158 amino acids within ORF1 has been described recently for HEV-1. ORF4 is involved in HEV replication by interacting with multiple viral proteins (helicase, RdRp and X) and host factors such as eEF1α1 (eukaryotic elongation factor 1 isoform-1) and β-tubulin (Nair et al., 2016). However, the presence and functional role of ORF4 in other HEV genotypes need to be explored. Additionally, two *cis*-reactive elements (CRE) located at the junction (between ORF1 and ORF3) and at the 3′-end of the ORF2 and 3′-UTR are essential for HEV replication and promoter activity for the subgenomic viral RNA (Emerson et al., 2001).

Although the HEV life-cycle relates to other ssRNA viruses, it warrants further investigation (Fig. 2). HEV attaches the cells via the interaction of ORF2 with attachment receptors such as heparan sulfate proteoglycans (HSPGs) and heat shock cognate protein 70 (HSC70) and enters the cells through dynamin-2, clathrin, membrane cholesterol and actin dependent endocytosis (Holla et al., 2015; Kalia et al., 2009). After entry, the virion uncoats and releases the viral RNA into the cytoplasm. The virus utilizes the host translation machinery to translate the ORF1 polyproteins which include viral enzymes. The viral genomes are replicated by the viral RNA helicase and RdRp, the ORF2 and ORF3 proteins are also translated from the viral subgenomic RNA (Debing et al., 2016a). The replication complex of HEV is likely positioned at the ER-Golgi intermediate compartment, where the viral proteins and positive single-stranded RNA could be localized (Perttila et al., 2013; Rehman et al., 2008). The assembly of RNA and ORF2 protein forms the progeny viral particles, which are then released from the host cells

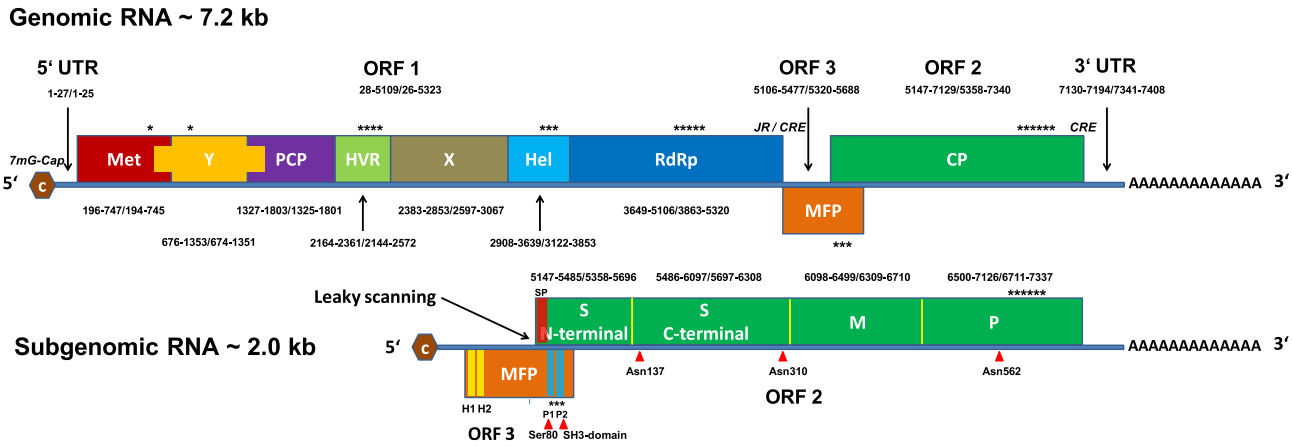

Fig. 1. Schematic description of the HEV genome and viral proteins. The figure shows linear, ssRNA (+) genome of ~7.2 kb HEV genome and corresponding viral proteins. The 5′-end is capped and the 3′-terminus is polyadenylated. ORF1 encodes the nonstructural polyprotein, including methyltransferase (Met), Y-domain (Y), papain-like cysteine protease (PCP), hypervariable region (HVR), macro-domain (X), RNA helicase (Hel) and RNA-dependent RNA polymerase (RdRp). ORF2 encodes the capsid protein (CP), containing S domain (S), M domain (M) and P domain (P). The glycosylation sites of ORF2 are indicated (Asn137, Asn310, and Asn562). ORF3 encodes a small multifunctional protein (MFP) including hydrophobic regions (D1, D2) and proline-rich regions (P1, P2). The phosphorylation site (Ser80) and the SH3-binding domain are indicated. ORF2 is translated by leaky scanning from the bicistronic ORF3-2 ~2.0 kb subgenomic RNA. JR is ORF2 and ORF3 overlapping/intergenic-junction region; CRE is *cis*-reactive element; SP is signal peptide. Nucleotide positions are relative to the HEV-1 Burmese strain (Acc. No. M73218)/HEV-3 47832 strain (Acc. No. KC618402). Asterisk (*) indicates the hot spot region for clinical mutations (adapted from (Cao and Meng, 2012; Chandra et al., 2008b).

through the endosomal sorting complexes required for transport (ESCRT) machinery. The interaction of the conserved PSAP motifs in the viral ORF3 protein with the tumor susceptibility gene 101 (*TSG101*) (a component of the ESCRT machinery) is likely essential for the maturation and egress of HEV (Nagashima et al., 2011a, 2011b) (Fig. 2).

4. Genetic Variability of HEV and Clinical Implication

Seven HEV genotypes are recognized within the *Orthohepevirus A* species based on the phylogeny of entire viral genomes (HEV-1 to HEV-7) (Smith et al., 2016). Four HEV genotypes (HEV-1 to HEV-4) are well recognized as human pathogens while HEV-5 and HEV-6 are identified only in animals so far (wild boars) (Smith et al., 2014). Recently, the camelid HEV-7 has been reported to infect human and cause chronic hepatitis E, as observed in a liver transplanted patient (Lee et al., 2016). The human pathogenic prototype strains include HEV-1, HEV-2, HEV-3, HEV-4, and of HEV-7 (Kamar et al., 2012, 2014a; Meng, 2011). Four major human pathogenic HEV genotypes have been further classified into 24 sub-genotypes including five HEV-1 sub-genotypes (1a to 1e), two HEV-2 sub-genotypes (2a and 2b), ten HEV-3 sub-genotypes (3a to 3j) and seven HEV-4 sub-genotypes (4a to 4g) (Lu et al., 2006). However, the sub-classification of HEV genotypes is controversial, because of missing valid data and an inadequate number of reference strains available for the various sub-genotypes.

The HEV genotypes have different reservoirs, and distinct distribution and transmission patterns. HEV-1 is frequently distributed in Asia, HEV-2 in Africa and Mexico, HEV-7 in the Middle East, whereas HEV3 and HEV4 are distributed throughout the world (Kamar et al., 2012; Lee et al., 2016; Rasche et al., 2016). HEV-1 and HEV-2 exclusively infect humans, whereas HEV-3 and HEV-4 are more permissive with a wider host range (especially swine, wild boar, rabbits, deer, and humans) (Kamar et al., 2012, 2014a; Meng, 2011). HEV-7 can infect both camels and humans (Lee et al., 2016). Zoonotic HEV has potential to cause fatal disease in infected patients and may become more transmissible among humans (Aggarwal and Jameel, 2011). HEV-1 and HEV-2 are mainly transmitted through the waterborne/fecal-oral route and are responsible for large waterborne outbreaks and epidemics in developing countries, whereas HEV-3, HEV-4 and HEV-7 are associated with zoonotic transmission and cause sporadic infections in developed countries (Kamar et al., 2012, 2014a; Lee et al., 2016; Meng, 2011).

The HEV genotypes are believed to be associated with the clinical course of the symptomatic infections. HEV-1 and HEV-2 mainly contribute to severe acute hepatitis but not towards chronic HEV infection (Aggarwal and Jameel, 2011; Krain et al., 2014). Infections with HEV-3, HEV-4 and HEV-7 do not only cause acute hepatitis but can also lead to chronic hepatitis in immunocompromised patients (Geng et al., 2014; Geng et al., 2016; Lee et al., 2016; Rivero-Juarez et al., 2015). Patients infected with HEV-4 had more severe outcomes compared to those infected with HEV-3 (Mizuo et al., 2005). Infection with HEV-1 but not with HEV-3 and HEV-4 was associated with severe forms of liver disease and complications in pregnant women (Krain et al., 2014; Kumar et al., 2004). In addition, infection with HEV-3 has been shown to cause fulminant liver failure in patients with pre-existing liver diseases (Dalton et al., 2007; Peron et al., 2007). These data indicate that HEV variability contributes significantly to the pathogenesis and severity of HEV infection.

HEV heterogeneity of the polyproline region (PPR) and macro domain (X-domain) have been characterized in eight immunocompromised patients with HEV persistence and six with resolving infections. The diverse complexity of nucleotides and amino acids (aa) in both the PPR and macro domain of ORF1 is higher in patients with chronic HEV infections than those with resolving infections (Lhomme et al., 2014a). Selection pressure of the host immune response during acute infection may be a possible reason for this diverse complexity. This suggests that the genetic heterogeneity enables the virus to better adapt to the host and persist longer, and thus establish chronicity.

5. Recombination of HEV

Recombination events of HEV occur within seven defined HEV genotypes and also between human and HEV strains (Smith et al., 2014). Recombination events were frequently distributed in the X- and helicase domains of ORF1 (Wang et al., 2010). Notably, HEV strain with ORF1 rearrangement (derived from a chronically infected patient) can efficiently adapt in cell culture (Johne et al., 2014). Insertion and deletions were reported in the hypervariable domain (polyproline region; HVR/PPR) of ORF1. A 171-nucleotide insertion encoding a 58 aa fragment of the human *RPS17* gene (ribosomal protein S17) (Shukla et al., 2011) and a 174 bp insertion of *RPS17* (detected in a chronically infected HEV patient) were associated with an increased HEV replication in hepatoma cells (Kenney and Meng, 2015; Shukla et

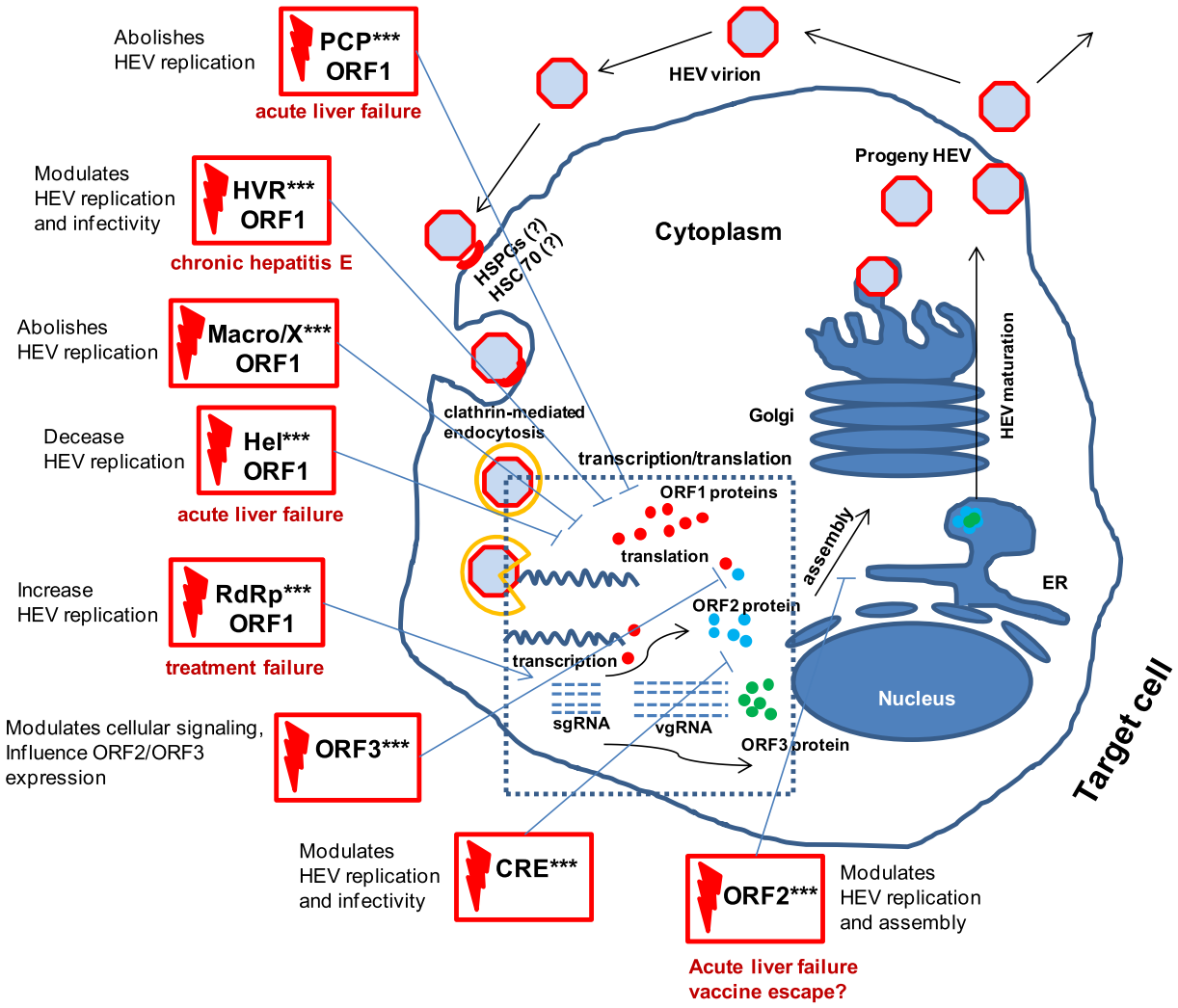

Fig. 2. Effect of mutations on HEV replication cycle and clinical significance. Schematic description of the HEV replication cycle and effect of mutations occurring in the HEV genome (red boxes; domain and region are indicated) on the transcriptional/translational machinery (blue dotted box) of HEV. Asterisks (***) indicate mutations described in the text. The possible effect of mutations on HEV replication is denoted at the side, described clinical outcome below the red boxes.

al., 2011). A recent study characterized the PPR of the HEV genome in 27 immunocompromised patients with HEV persistence and 32 with resolving infections. Of the 27 strains isolated from patients with HEV persistence, the recombination occurred in three HEV strains over the infection period (Lhomme et al., 2014b). Recombination events increase the likelihood of genetic variability and thus diverse pathogenesis with a prospective potential for chronification of HEV infection (Nguyen et al., 2012). In addition, two fragments of human origin (inter-α-trypsin inhibitor-*ITI*, and tyrosine aminotransferase-*TAT*) were found to be inserted in the PPR. Those inserted fragments enhanced the HEV replication, probably by providing a new potential regulatory site (Lhomme et al., 2014b).

6. HEV Mutations and Their Functional Role

RNA viruses exhibit high genetic variability by rapid evolution with estimated mutation rate ranging from 10^{-6} to 10^{-4} substitutions per nucleotide per strand copying (Sanjuan et al., 2010). The HEV mutation rates were estimated indirectly from clinical isolates as 1.5 base substitutions per site per year and were similar to those reported for hepatitis C viruses (Takahashi et al., 2004). Mutations can occur frequently over the entire HEV genome during propagation and consecutive passages for adaptation to cell culture (Lorenzo et al., 2008). High variability and frequent selection of mutations in the HEV genome is due to the transcription process. The viral RdRp, which lacks the proof-reading ability of DNA polymerases, likely increases the variations in the HEV genome. On the other hand, the selection pressure imposed by antiviral drugs and host immune responses may additionally contribute to increased HEV variability (Lhomme et al., 2014a). Although a quasispecies is linked to mutations, not all mutations in the viral genome shall generate viable virus quasispecies (Lauring and Andino, 2010).

6.1. Mutations in the ORF1 Region

ORF1 encodes several non-structural proteins required for HEV replication and protein processing including MeT, papain-like cysteine protease (PCP), Hel, and PdRp activities (Fig. 1). ORF1 also contains several functional domains namely the Y-domain, HVR and macro domain (X-domain), which show homologies to other positive-stranded RNA viruses (Cao and Meng, 2012; Koonin et al., 1992). MeT is responsible for capping the 5′-end of the viral pregenomic RNA, which is critical for viral infection (Emerson et al., 2001). In the capping process, Hel is involved in phosphatase activity that catalyzes the initial cap formation. The Hel-domain also possesses RNA duplex unwinding activities (Karpe and Lole, 2010a, 2010b).

Although the PCP has been predicted within the ORF1 polyprotein by a computer-assisted analysis (Koonin et al., 1992), the function of the protease activity is unclear. By constructing a series of HEV replicons harboring numerous mutations in the PCP region and followed by in vitro analyses, nine aa substitutions (H443L, C457A, C459A, C471A, C472A, C481A, C483A, H497L and H590L) were associated with complete suppression of HEV replication (Parvez, 2013). A catalytic dyad (C434–H443) and bivalent metal-binding motifs (C457–H458–C459 and C481–C483) were essential for HEV protease structural-integrity (Parvez and Khan, 2014). These results indicate that protease activity is essential for HEV replication and mutations in the PCP region may affect HEV protease activity by modifying the enzyme structure (Table 1).

The HVR domain overlaps PPR between the PCP and X-domain (Macro-D) and contributes to viral replication efficiency and adaptation (Pudupakam et al., 2009, 2011; Purdy et al., 2012). HVR varies in length among HEV strains and genotypes, shows sequence heterogeneities, and can tolerate small deletions and insertions (Pudupakam et al., 2009). The variation in length of the HVR domain is associated with HEV attenuation (Pudupakam et al., 2009). Consistently, the deletions in the N-terminal and central regions of the HVR domain have significant effect while deletion in the C-terminal region has a relatively lesser impact on the replication efficiency. Furthermore, complete HVR deletion of the avian HEV eliminates virus infectivity, but not viral replication in vivo (Pudupakam et al., 2011). These findings indicate that the HVR domain is not essential for viral replication, but has a role in HEV infectivity. The HVR domain may involve virus entry and assembly by interacting with other viral and host factors. A 282 bp-insertion (duplicated 258 bp HVR-derived and 24 bp RdRp-derived fragments) in the HVR domain of an isolate from patient with chronic hepatitis E was associated with increased viral replication. Particularly, the 24 bp RdRp-derived insertion contributed to viral replication (Debing et al., 2016b). Therefore, the deletion/insertion or recombination events occurring in HVR may be associated with HEV pathogenesis and thus clinical outcome.

The X-domain is involved in ADP-ribose metabolism and posttranslational modifications and is homologous to other pathogens (Holla et al., 2013). However, X-domain function in HEV physiology is poorly characterized. The macro domain could recruit poly (ADP-ribose)-modified cellular factors and might have an impact on HEV replication (Egloff et al., 2006). A highly conserved 'glycine-triad' comprised of three aa substitutions G815V, G816V and G817V in the downstream X-domain was identified and two of those (G816V and G817V) resulted in the complete suppression of HEV replication (Parvez, 2013). Six HEV replicons harboring the aa substitutions N806A, N809A, H812L, G815A, G816A and G817A were constructed to characterize the functional role of the X-domain. The results revealed that the mutations N809A, H812L, G816A/V and G817A/V lead to a complete abrogation of HEV replication (Parvez, 2015a). These findings indicate the critical role of X-domain in regulation of HEV physiology and mutations in this domain completely damage HEV replication (Table 1).

Viral helicase activity is critical for HEV replication and specific mutations in the Hel region can stop helicase activity (Karpe and Lole, 2010a, 2010b). The amino acid substitutions in the Hel domain (L1110F and V1120I) are frequently detected in HEV-1 isolates derived from patients with fulminant hepatic failure (Devhare et al., 2014). These distinct mutants were shown to influence ATPase activity but not the RNA duplex unwinding activity of the helicase enzyme. Notably, HEV mutant replicons with the single mutation (L1110F or V1120I) and the double mutation (L1110F/V1120I) showed a significant decrease in viral replication in comparison to wild-type HEV (Devhare et al., 2014). In addition, artificial deletions in the Hel domain (motifs Ia and III) significantly impaired ATPase and unwinding activities of the helicase enzyme (Mhaindarkar et al., 2014). These findings indicate that the negative regulation of helicase activity by these non-synonymous substitutions (L1110F or V1120I) and deletions (motifs Ia and III) in the Hel domain are associated with reduced HEV replication (Table 1).

The RdRp domain contains eight conserved motifs that are closely homologous to RdRps of other positive-stranded RNA viruses such as hepatitis C virus (Agrawal et al., 2001). The RdRp replicates the HEV genome through an anti-genomic RNA intermediate and the RdRp activity could be localized to the endoplasmic reticulum (Rehman et al., 2008). Several HEV mutations namely Y1320H, K1383N, D1384G, K1398R, V1479I, Y1587F and G1634R have been identified in patient-derived HEV isolates. These mutations were demonstrated in vitro to associate with HEV replication fitness (Debing et al., 2014, 2016b; Todt et al., 2016). The findings indicate that the mutations occurring in the RdRp domain can affect the HEV replication by modulating the RdRp activity (Table 1).

6.2. Mutations in the ORF2 Region

ORF2 encodes the viral capsid protein, which assembles after glycosylation and encapsidation of the viral genomic RNA into the infectious viral particles (Jameel et al., 1996). The capsid protein is immunogenic since neutralizing antibodies effectively target conformational epitopes at the P-domain (Zhou et al., 2005) (Fig. 1). Besides structural properties, HEV capsid protein is involved in host cell interaction by a potential ER localization signal (Jameel et al., 1996). ORF2 contributes to virus-host interaction as ORF2 revealed modulatory effects on eIF2a, ATF-4, Hsp72, NFkB, and activation of the CHOP promoter (John et al., 2011; Surjit et al., 2012). Interactions of capsid proteins with host factors (Grp78/BiP, α-tubulin, and Hsp90) are necessary for virus attachment, uptake, and trafficking (Zheng et al., 2010). The C-terminal 52 aa (C52aa) domain of the capsid protein is required to promote accurate encapsidation and stabilize encapsidated viral particles (Shiota et al., 2013).

Three mutations (T5338C, A5362G, and C6356T) resulted in aa changes (F51L, T59A, and S390L, respectively) and a deleterious point mutation A756 resulted in a downstream frame-shift of the *ORF1* gene have been identified in ORF2 (Huang et al., 2005). These mutations occur naturally under selective immune pressure and may influence the viral protein function thus contribute to a decreased HEV replication and infectivity (Huang et al., 2005). Although ORF2 production was not significantly affected by these mutations (F51L, T59A, and S390L), the F51L mutation partially contributed to virus attenuation and the T59A and S390L mutations resulted in a more drastic HEV attenuation (Cordoba et al., 2011). In this regard, the F51L and T59A mutations may affect viral genomic RNA packaging, and the S390L mutation may prevent the interaction between virus and host cell receptor by changing the structure of antigenic epitopes (Cordoba et al., 2011).

The non-synonymous substitutions at aa-positions 137, 310 and 311 (especially Asn to Gln) prevent glycosylation of corresponding sites in the glycosylation motif of HEV capsid protein. These mutations do not significantly affect either the viral replication or capsid protein synthesis. However, they eliminate HEV infectivity by preventing the formation of HEV particles. Although the mutation N562Q does not stop HEV morphogenesis, it rather affects the dimerization of the ORF2 protein and the infectivity of the newly synthesized HEV particles (Graff et al., 2008). The substitutions N562Q/D/P/Y were further verified to evolve in glycosylation, dimerization and especially in the activity of neutralizing epitopes of the capsid protein (Xu et al., 2016). Recently described substitutions in ORF2 L477T and L613T (HEV-4) and V606A (HEV-1) were associated with HEV immunoreactivity by affecting the neutralization epitope (Liang et al., 2010; Zhang et al., 2008). These findings indicate that the ORF2 protein structure is critical for HEV replication, infectivity, and immunoreactivity (Table 1). The ORF2 non-synonymous substitutions resulting in an alteration of epitope structure may facilitate HEV to adapt and/or escape successfully the host immune response, which can lead to chronicity (Todt et al., 2016). Under host immune pressure, HEV mutations in ORF2 may

Table 1
Artificial HEV mutations and their functional significance in the physiological activity and infectivity.

Substitution/mutation	Amino acid change	Domain/region	HEV genotype	Functional significance	References
NA	H443L; C457A; C459A; C471A; C472A; C481A; C483A; H497L; H590L	PCP/OFR1	HEV-1	Completely abolish HEV replication by modifying the enzyme structure	Parvez (2013); Parvez and Khan (2014)
Insertion/deletion	NA	HVR/ORF1	Human HEV-1 avian HEV swine HEV-3	Associated with HEV attenuation	Pudupakam et al. (2009)
Complete deletion	NA	HVR/ORF1	avian HEV human HEV	Abolish HEV infectivity but not influence HEV replication	Pudupakam et al. (2011)
Insertion/deletion of a 24 bp RdRp-derived fragment	NA	HVR/ORF1	human HEV-3	Decrease HEV replication	Debing et al. (2016b)
NA	N809A; H812L; G816A/V; G817A/V	X/OFR1	HEV-1	Completely abolish HEV replication	Parvez (2013); Parvez (2015a)
NA	L1110F; V1120I	Hel/ORF1	Human HEV-1	Decrease HEV replication by affecting the ATPase activity but not the RNA duplex unwinding activity of helicase enzyme	Devhare et al. (2014)
Deletion	NA	Hel/ORF1	NA	Decrease HEV replication impairing the ATPase and unwinding activities of helicase enzyme	Mhaindarkar et al. (2014)
NA	K1383N	RdRp/ORF1	HEV-3	Reduces viral replication and increases ribavirin sensitivity	Debing et al. (2016b)
NA	Y1320H; G1634R/K	RdRp/ORF1	HEV-1, HEV-3	Increased efficiency of viral replication and infectivity	Debing et al. (2016b); Debing et al. (2014); Todt et al., 2016
T5338C	F51L	ORF2	Swine HEV	Decrease HEV replication and infectivity by affecting viral genomic RNA packaging	Peron et al. (2016); Perttila et al. (2013)
A5362G	T59A	ORF2	Swine HEV	Decrease HEV replication and infectivity by affecting viral genomic RNA packaging	Huang et al. (2005); Cordoba et al. (2011)
C6356T	S390L	ORF2	Swine HEV	Decrease HEV replication and infectivity by preventing host virus interaction	Peron et al. (2016); Perttila et al. (2013)
NA	N137Q; N310Q; N311Q	ORF2	NA	Prevent glycosylation of capsid protein and formation of HEV particles	Graff et al. (2008)
NA	N562Q/D/P/Y	ORF2	NA	Affect the dimerization of ORF2 protein and HEV infectivity	Graff et al. (2008)
NA	L477T; L613T	ORF2	HEV-4	Influence immunoreactivity of HEV by affecting the neutralization epitope	Zhang et al. (2008); Liang et al. (2010)
NA	V606A	ORF2	HEV-1		
A5145C; A5178C; A5190C; G5676T; T5690G	NA	ORF2–ORF3	HEV-2	Abolish the ORF2 production (but not ORF3)	Graff et al. (2005a)
CGC5148–5150AGA	NA	ORF2–ORF3	HEV-2	Abolish ORF3 production (but not ORF2)	Graff et al. (2005a)
A5108Δ; T5109C; C5112U; TCT5116–5118AGC; T5121C	NA	ORF2–ORF3	HEV-2	Abolish both production of both ORF2 and ORF3	Graff et al. (2005a)
NA	S80A (V66G)	ORF3 (ORF2)	NA	May affect the regulatory role of ORF3 protein in HEV assembly, influence ORF2–ORF3 interaction	Tyagi et al. (2002); Graff et al. (2005a)
G5101U; U5100C; C5117G; U5118G	NA	CRE/ORF3	HEV-2	Affect HEV replication and infectivity by modifying the CRE structure	Parvez (2015b)
G6574C; C6570G; G7106T/A; G7097A; C7144A	NA	CRE/ORF2	HEV-3	Affect HEV replication and infectivity by modifying the CRE structure	Emerson et al. (2001); Emerson et al. (2013); Graff et al. (2005b)

NA: not applicable.

involve in modulation of HEV immunoreactivity, which is related to outcomes and progression of liver disease (Suneetha et al., 2012).

6.3. Mutations in the ORF3 Region

The ORF3 encodes a small phosphoprotein of 114 amino acids (HEV-1, -2 and -4) or 113 amino acids (HEV-3) that is translated from the 2.2 kb sgRNA. ORF3 is essential to promote cell survival and proliferation, modulation of the immune responses in the acute phase, and in immune-suppression (Cao and Meng, 2012). The ORF3 protein is required for regulation of HEV replication and infectivity and is a multifunctional, pleiotropic protein, and interacts with host cellular signaling (Chandra et al., 2008a; Moin et al., 2009). Therefore, ORF3 expression may have a pivotal effect on HEV pathogenicity. ORF3 interacts with ORF2 in a phosphorylation-dependent manner and the 25-aa region (residues 57–81), especially the phosphorylation at the position S80 of ORF3, is responsible for the ORF2–ORF3 interaction (Tyagi et al., 2002). The mutation S80A (but not S80L, which does not change aa in ORF2) is associated with abolishment of HEV infectivity. However, ORF3 phosphorylation at position S80 is not required for viral replication and infectivity (Graff et al., 2005a). Due to the overlapping of ORF2 and ORF3, the ORF3 S80A mutation also corresponds to the V66G aa substitution in ORF2 (Graff et al., 2005a). The S80A mutation therefore may affect the ORF3 regulation during HEV assembly by either an unknown mechanism or by influencing the ORF2–ORF3 interaction through modifying the structure of both ORF2 and ORF3. In addition, two conserved PSAP motifs have been identified to residues aa 86–89 and aa 95–98 of ORF3. Mutations in these motifs were associated with decreased HEV replication (Nagashima et al., 2011a) (Table 1).

Mutations in the ORF3 and ORF2–ORF3 overlapping regions are associated with the production of ORF2 and ORF3 proteins (Graff et al., 2005a). The mutations A5145C, A5178C, A5190C, G5676T and T5690G stop the ORF2 expression (but not ORF3) while the mutation CGC5148–5150AGA eliminates ORF3 production (but not ORF2). The mutations A5108Δ, T5109C, C5112U, TCT5116–5118AGC and T5121C damage the expression of both ORF2 and ORF3. These mutations eliminating ORF3 production are associated with HEV infectivity in an animal model system (Graff et al., 2005a) (Table 1). Furthermore, the interaction of ORF3 protein with various host factors deactivates the ORF3 protein function that may subsequently eliminate viral replication and infectivity (Moin et al., 2007, 2009). The highly dynamic activity of ORF3 protein in interaction with host factors may lead to stimulation and enhancement of the host immune response that enables the host to clear the virus more rapidly. This may provide an explanation for the self-limitation and a short period of HEV persistence during clinical course. In addition, mutations in the ORF3 protein may modulate its binding capacity to the host proteins that results in a complexity of immune response and clinical outcomes. However, more studies are required to clarify this hypothesis.

6.4. Mutations in the Junction and cis-Reactive Elements (CRE)

The junction region located between the end of ORF1 and beginning of ORF3 was predicted to contain a highly conserved stem-loop (SL) structure (Cao et al., 2010) (Fig. 1). A point mutation at the third in-frame AUG in the junction region, which is the exact start position for ORF3 translation, completely stops virus infectivity (Huang et al., 2007). An increased number of mutations in the loop, especially at the AGA motif, is associated with reduced HEV replication, and mutation on the stem of the sub-genome start sequence leads to an inhibition of HEV replication (Cao et al., 2010). The sequence and structure of the junction region play an important role in HEV replication while mutations occurring at the third in-frame AUG in the junction region may inhibit the ORF3 production. However, the precise mechanism of how the junction region influences the HEV replication is unclear.

Two CREs have been identified in HEV; the first CRE is located at the 3′-end of ORF2 (Emerson et al., 2001) and the second CRE is located in the ORF1–ORF3 intergenic junction region of the HEV genome, which may be essential for HEV replication and important for ORF2 and ORF3 expressions (Graff et al., 2005a) (Fig. 1). The mutations identified in the first CRE (G5101U, U5100C, C5117G, U5118G), which form the "lower-stem" structure of CRE, affect HEV replication and are associated with reduced HEV infectivity (Parvez, 2015b). A functional RNA element with two highly conserved stem-loop structures (ISL1 and ISL2) within ORF2 and 19 silent mutations were identified to disrupt the stem-loop structures and abolish capsid protein synthesis (Emerson et al., 2013). The mutations G6574C, C6570G, G7106T/A, G7097A and C7144A are also associated with HEV attenuation by disrupting the predicted stem-loop structures of HEV-RNA molecule (Emerson et al., 2001, 2013; Graff et al., 2005b) (Table 1).

7. HEV Mutations and Clinical Relevance

An in-frame deletion of 246 bp in ORF3 of Indian HEV strain isolated from clinical samples has been reported, however, the functional and clinical relevance of this deletion were unknown (Ray et al., 1992). An analysis of 22 HEV-4 full-length sequences from patients with fulminant and acute hepatitis found that the substitutions at the positions C1816 and U3148 were significantly associated with fulminant hepatitis (Inoue et al., 2006). However, only the mutation U3148 was confirmed to be associated with fulminant hepatitis by an additional analysis of 16 HEV-4 isolates. Further analysis of 86 HEV isolates showed that the U3148 variant revealed a stronger association with fulminant hepatitis in comparison to other variants (C3148 or G3148), and was associated with lower prothrombin activity (Inoue et al., 2006). A comparable study extended the results using 28 HEV-4 full-length sequences (from fulminant and acute hepatitis) and identified the C5907 variant most significantly associated with fulminant hepatitis (Inoue et al., 2009). An additive effect of both U3148 and C5907 mutations on fulminant hepatitis development was confirmed by further analysis of full-length sequences of 28 HEV-4 and 11 HEV-3 isolates as well as 35 partial sequences (Inoue et al., 2009). However, the mechanism of how U3148 and C5907 mutations influence hepatitis E progression is unresolved as these mutations are silent substitutions not changing the aa (Table 2).

The ORF1 mutation V1213A corresponding to the aa substitution V239A in the Hel domain was found in all patients with more severe hepatitis but not in the patient with mild clinical course indicating that the V239A mutation can be associated with increased virulence (Takahashi et al., 2009). Notably, the V239A substitution in the Hel domain may enhance helicase activity and subsequently increase HEV replication (Ahola et al., 2000). Six aa changes in HEV-1 ORF1 including T563C (aa F179S), G977A (A317T), C2232T (T735I), T3355C (L1110F), G3386A (V1120I), and T4344A (F1439Y), were identified to be significantly associated with fulminant hepatic failure (Mishra et al., 2013). A total of 22 nucleotide substitutions were identified in the ORF1 region of 55 HEV sequences obtained from patients with acute viral hepatitis and those with acute liver failure. Most of these mutations including two non-synonymous substitutions C4476G (C1483W) and A4616C (N1530T) in the RdRp were found only in the HEV sequences from acute liver failure patients (Borkakoti et al., 2016). The mutations C1483W and N1530T were significantly associated with high viral load, abnormal prothrombin time, high bilirubin, and high mortality (Borkakoti et al., 2016). The association of HEV mutations in ORF2 with disease severity was investigated. Six substitutions including C5927T, C5933T, T6014C, C6032T, G6098A, C6104T, and a novel amino acid mutation P259S in ORF2 were identified to be significantly associated with fulminant liver failure (Borkakoti et al., 2014). These results indicate that non-synonymous substitutions can be associated with virulence and may affect viral replication (mutations in the RdRp) and especially enhance host immune response via modifying the antigen epitopes (non-silent mutations in ORF2 region) (Table 2).

Table 2
HEV mutations detected in patient-derived isolates and their clinical relevance.

Nucleotide substitution	Amino acid change	Domain/region	HEV genotype	Associated clinical manifestation	Mechanism	Reference
C1816	no	ORF1	Clinical isolates HEV-4	Fulminant hepatitis failure	Unknown	Inoue et al. (2006); Inoue et al. (2009)
U3148	no	ORF1				
C5907	no	ORF2				
186 bp insertion	no	HVR/ORF1	Clinical isolates HEV-3	Chronic hepatitis	Unknown	Johne et al. (2014)
90 bp insertion	no	HVR/ORF1	Clinical isolates HEV-3	Unknown	Unknown	Legrand-Abravanel et al. (2009)
171 bp insertion	no	HVR/ORF1	Clinical isolates HEV-3	Chronic hepatitis	Efficient growth in cell culture	Shukla et al. (2011)
174 bp insertion	no	HVR/ORF1	Clinical isolates HEV-3	Chronic hepatitis	Cross-species infections	Shukla et al. (2011)
117 bp insertion	no	HVR/ORF1	Clinical isolates HEV-3	Chronic hepatitis	Growth advantage in cell culture	Nguyen et al. (2012)
a 282 bp-insertion	no	HVR/ORF1	Clinical isolates HEV-3	Ribavirin treatment failure	Unknown	Debing et al. (2016b)
NA	V239A	Hel/ORF1	HEV-3	More severe hepatitis	Enhance the helicase activity	Takahashi et al. (2009)
T563C	F179S	MeT/ORF1	Clinical isolates HEV-1	Fulminant hepatic failure	Unknown	Mishra et al. (2013)
G977A	A317T	Y/ORF1				
C2232T	T735I	HVR/ORF1				
T3355C	L1110F	Hel/ORF1				
G3386A	V1120I	Hel/ORF1				
T4344A	F1439Y	RdRp/ORF1				
C4476G; A4616C	C1483W; N1530T	RdRp/ORF1	Clinical isolates HEV-1	Fulminant hepatic failure	Maybe enhance HEV replication?	Borkakoti et al. (2016)
C5927T	NA	ORF2	Clinical isolates HEV-1	Fulminant hepatic failure	Unknown	Borkakoti et al. (2014)
C5933T	NA	ORF2				
T6014C	NA	ORF2				
C6032T	NA	ORF2				
G6098A	NA	ORF2				
C6104T	NA	ORF2				
NA	P259S	ORF2				
NA	Y1320H; G1634R/K	RdRp/ORF1	Clinical isolates HEV-3	Ribavirin treatment failure	Increased efficiency of viral replication and infectivity	Debing et al. (2016b); Debing et al. (2014); Todt et al. (2016)
NA	K1383N	RdRp/ORF1	Clinical isolates HEV-3	Ribavirin treatment failure	Unknown	Debing et al. (2016b); Todt et al. (2016)
NA	D1384G; K1398R; V1479I; Y1587F	RdRp/ORF1	Clinical isolates HEV-3	Ribavirin treatment failure	Unknown	Todt et al. (2016)
246 bp deletion	NA	ORF3	Clinical strain	Unknown	Unknown	Ray et al. (1992)

NA: not applicable.

The abundance of mutations in the HEV genome from patient isolates is probably due to selective immune pressure. These mutations enable the virus to better adapt and modulate the host immune responses that lead to severity of complications.

8. HEV Mutations and Vaccination

Prevention of HEV infection in endemic areas is based on the implementation of appropriate hygiene and sanitary measures to avoid fecal-oral transmission. In regions where HEV infection is sporadic, the consumption of raw food should be avoided. HEV infection can be prevented with effective HEV vaccines and ORF2 is widely used as a target for vaccine development (Zhang et al., 2015). Mutations in ORF2 may lead to a failure of an adaptive cellular immune response in vaccinated individuals. Therefore, mutations occurring in ORF2 sensitively affecting the ORF2 protein structure is one of the challenges for the protective efficiency of HEV vaccine programs. On the other hand, a number of mutations resulting in HEV attenuation (e.g., F51L, T59A, S390L, N562Q/D/P/Y, L477T, L613T and HVR deletion) may provide a basis for the development of live-attenuated vaccines against HEV (Cordoba et al., 2011; Pudupakam et al., 2009; Zhang et al., 2008). A strategy for viral attenuation and vaccine development has been proposed based on mutating the conserved active site lysine residue to arginine of the viral RdRp (Weeks et al., 2012). Therefore, HEV mutations in the RdRp domain and deletions in the HVR domain have repercussions for the development of live, attenuated HEV vaccines.

9. Clinical Relevance of HEV Mutations in Antiviral Therapy

Although no HEV-specific treatment options show significant antiviral activity, the effectiveness of PEG-interferon-α in combination with ribavirin, and ribavirin alone for HEV infection have been recently documented (Kamar et al., 2014b; Peron et al., 2016; Wedemeyer et al., 2012). However, ribavirin treatment failure was reported in patients with chronic hepatitis E (Debing et al., 2014, 2016b; Gisa et al., 2015; Lhomme et al., 2015; Todt et al., 2016) (Table 2). The entire HEV sequences before, during and after treatment courses were compared and a nucleotide substitution (G > A) resulting in a G1634R mutation in the C-terminal region of the HEV-3 RdRp was identified (Debing et al., 2014). Although showing no effect on ribavirin resistance, the variants 1634R and 1634K significantly contribute to an increased efficiency of viral replication and infectivity compared to the wild-type G1634 (Debing et al., 2014). Comparable results confirmed a similar role for HEV-1 (Debing et al., 2014). This result was further supported by a clinical observation that plasma HEV-RNA levels were significantly increased in patients infected with 1634R mutant compared to non-1634R mutant viruses (Lhomme et al., 2015). These findings suggest that G1634R/K may not be a direct antiviral resistance mutation but partially involved in ribavirin treatment failure by enhancing HEV replication. By analyzing 63 HEV sequences from solid-organ transplant patients with chronic hepatitis E, the prevalence of the G1634R mutation was shown to be higher in non-sustained virologic response (SVR) compared to SVR patients (Lhomme et al., 2015). This observation is supported by evidence that the proportion of the G1634R mutation was rapidly increasing in patients with ribavirin treatment failure (Parvez, 2013; Xu et al., 2016). Antiviral resistance mutations (G1634R/K) can occur in HEV genome under ribavirin treatment, since ribavirin has been recently demonstrated to cause mutations in the HEV genome as well as in other RNA viruses (Debing et al., 2016b). Ribavirin therapy can lead to an increased HEV variability (ORF1, ORF2 and ORF3) over time, especially in the RdRp domain (Todt et al., 2016). However, the presence of the 1634R mutation neither leads to absolute ribavirin resistance nor influences the response to re-treatment with ribavirin (Galante et al., 2015; Lhomme et al., 2015). This result is in line with a finding showing that the ribavirin treatment failure is not directly caused by the G1634R mutation (Debing et al., 2014).

Besides the described G1634R mutation, two other substitutions (Y1320H, K1383N) in the RdRp domain, as well as two mutations (A723V, A647T) and a 282 bp-insertion (a duplicated 258 bp HVR-derived and a 24 bp RdRp-derived fragments) in the HVR were identified. Notably, the frequency of these substitutions (Y1320H, K1383N, G1634R and A723V) increased during the course of ribavirin treatment (Debing et al., 2016b). The G1634R and Y1320H mutations enhanced viral replication but did not affect ribavirin susceptibility, whereas the K1383N mutation abrogated viral replication and was associated with increased ribavirin sensitivity by affecting the binding activity of the RdRp domain to guanosine-5′-triphosphate (GTP) (Debing et al., 2016b). Although Y1320H and G1634R/K can compensate for the harmful effect on viral replication caused by the K1383N mutation (Debing et al., 2016b), the interaction among these mutations and their functional role is yet to be understood. The A723V mutation had no effect on viral replication whereas the 282 bp-insertion in the HVR significantly increases viral replication. However, the artificial insertion and deletion of the 24 bp RdRp-derived fragment reduced viral replication compared to wild-type HEV, suggesting a role of the insertion and also other unknown factors (Debing et al., 2016b). In addition, four additional substitutions (D1384G, K1398R, V1479I and Y1587F) together with the known mutations K1383N and G1634R in ORF1 were identified. Of those, the mutation G1634R could be detected in low frequencies before ribavirin therapy (Todt et al., 2016). These additional mutations (D1384G, K1398R, V1479I and Y1587F) were associated with increased ribavirin sensitivity, and with higher HEV replication (Todt et al., 2016).

The recent findings clearly demonstrate the mutagenic effect of ribavirin on the HEV genome, which can lead to an emergence of distinct viral populations (Debing et al., 2014, 2016b; Gisa et al., 2015; Todt et al., 2016). These distinct viral populations resulted from ribavirin treatment failure may cause a more complicated clinical outcome, extrahepatic manifestations and may have different transmission patterns. Development of a new antiviral therapy or/and combination with an alternative therapeutic option (eg. PegIFNα, Sofosbuvir) may help to increase the efficiency of treatment course and to reduce the treatment failure risk as well as to avoid the emergence of the viral populations associated with drug resistance and fulminant liver failure. Investigating potential HEV mutations related to resistance to new antiviral therapies are recommended. In clinical practice, systematic examination of HEV genome variants by next-generation sequencing should be considered for any clinical relevance, which may associate with treatment failure, chronic and fulminant infections to predict therapy outcomes and in the progression of liver diseases.

10. Conclusions and Perspectives

Although HEV infection is largely controlled by host immune responses, viral factors including HEV genetic variability associate with the clinical course, host adaption, and antiviral resistances. Different HEV genotypes exhibit a selective host range with unique transmission patterns and pathogenesis. Deletion/insertion, recombination and substitutions occurring in the HEV genome can influence HEV replication and virus-host interaction, and be subsequently associated to pathogenesis (Table 1 and Fig. 2). Under host immune pressure, clinically relevant non-synonymous and silent mutations occurring throughout the entire HEV genome may be associated with severe forms of the disease and potentially *anti*-viral resistances (Table 2 and Fig. 2). Ribavirin treatment failure is associated with the RdRp mutations Y1320H, K1383N, D1384G, K1398R, V1479I, Y1587F and G1634R. The mutations Y1320H and G1634R contribute to decreased susceptibility to antiviral drugs by enhancing HEV replication and infectivity, whereas the other mutations (eg. K1383N) likely reduce viral replication and increases ribavirin sensitivity. These mutations may affect the efficiency of viral RdRp activity; however, the precise role of these identified mutations remains unclear. Except for the drug resistance-related HEV mutations in the RdRp domain, most mutations

in other regions found in clinical isolates do not corroborate with results from artificial mutations in functional studies, thus suggesting the nature of mutational complexity. Further studies will help to elucidate the possible contribution of HEV variants in HEV physiology, pathogenesis and clinical relevance.

Conflict of interest

The authors declare that there are no conflicts of interest.

Author's contributions

HVT, NXH, BW, CTB and TPV collected, studied, analyzed and discussed the literature. HVT, HW, CTB and TPV wrote the review.

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