Molecular biology and pathogenesis of hepatitis E virus

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The hepatitis E virus (HEV) is a small RNA virus and the etiological agent for hepatitis E, a form of acute viral hepatitis. The virus has a feco-oral transmission cycle and is transmitted through environmental contamination, mainly through drinking water. Recent studies on the isolation of HEV-like viruses from animal species also suggest zoonotic transfer of the virus. The absence of small animal models of infection and efficient cell culture systems has precluded virological studies on the replication cycle and pathogenesis of HEV. A vaccine against HEV has undergone successful clinical testing and diagnostic tests are available. This review describes HEV epidemiology, clinical presentation, pathogenesis, molecular virology and the host response to HEV infection. The focus is on published literature in the past decade.

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1. Introduction

A large outbreak of acute viral hepatitis in New Delhi in 1955–56 (Vishwanathan 1957) was retrospectively found to be due to a unique agent called enteric non-A, non-B hepatitis. This agent was later named hepatitis E virus (HEV) and molecularly characterized following the cloning of its genome (Reyes *et al* 1990). Much information is now available on the epidemiology, virology, transmission and pathogenesis of HEV. A review with the same title was published almost a decade ago (Jameel 1999). While maintaining the earlier style, this review will highlight new developments in the field since then.

2. Epidemiology

HEV is the causative agent for hepatitis E, a major form of acute viral hepatitis. The disease is endemic in large parts of Asia, Africa and Latin America (figure 1) from where epidemic and sporadic disease has been reported (Panda and Jameel 1997; Jameel 1999). It is estimated that about 2 billion people live in areas endemic for HEV.

The common feature between almost all the epidemics is the contamination of water supplies with sewage, confirming the feco-oral route of transmission. The highest rates of infection occur in regions with poor sanitation and socio-economic status of the population. Minor modes of transmission in endemic areas could be vertical (Khuroo *et al* 1995) and through blood transfusions (Khuroo *et al* 2004). Person-to-person contact transmission is inefficient (Somani *et al* 2003).

Blood donors and healthy persons from some non-endemic areas also show high anti-HEV prevalence (Mast *et al* 1997; Thomas *et al* 1997; Meng 2000a, b) (figure 1). This could be due to zoonotic transmission. Besides the discovery of a swine HEV (Meng *et al* 1997), related viruses have been found in pigs (Clayson *et al* 1995; van der Poel *et al* 2001; Arankalle *et al* 2002; Huang *et al* 2002; Pei and Yoo 2002), deer (Tei *et al* 2003; van Cuyck *et al* 2005), and wild boar (Takahashi *et al* 2004). Direct transmission has been

Keywords. Hepatitis E; HEV; molecular virology; RNA virus; vaccine

Abbreviations used: ERK, Extracellularly regulated kinase; HEV, hepatitis E virus; IFNγ, interferon gamma; IgG, immunoglobulin G; ORF, open reading frame; PCP, papain-like cysteine protease; RdRp, RNA dependent RNA polymerase; UTRs, untranslated regions; TNFα, tumour necrosis factor alpha

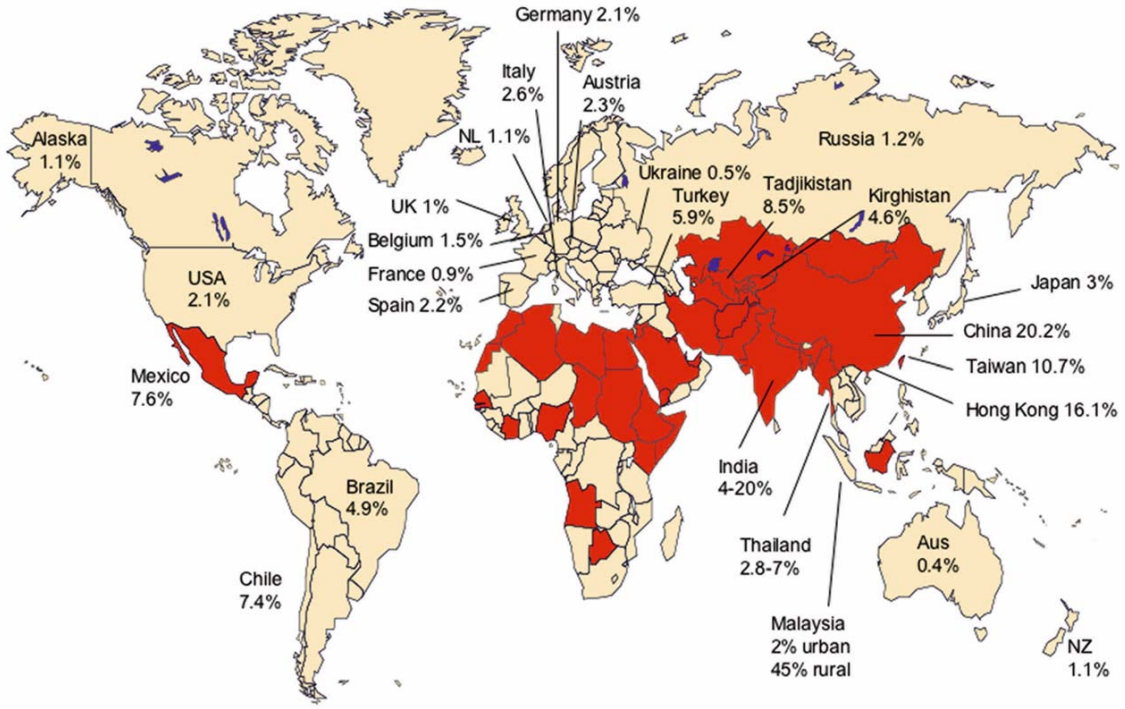

Figure 1. HEV endemic areas and global seroprevalence. Red areas on the map indicate regions of the world that are endemic for hepatitis E and where >25% of acute viral hepatitis is due to HEV. Superimposed on this map are seroprevalence rates of HEV from various countries, determined in independent studies.

reported from deer to humans as a result of eating uncooked meat (Tei *et al* 2003, 2004). Swine HEV was detected in raw pig livers (Yazaki *et al* 2003) and genotyping data indicate clustering of swine and human HEV strains from patients in the USA, Japan and other countries. Humans who consume contaminated pork products and are involved in the rearing of pigs are potentially at risk of HEV infection (Matsuda *et al* 2003; Tei *et al* 2004; Zheng *et al* 2006). Anti-HEV antibodies have been detected in many more animal species, including wild boar and deer and HEV genotype 3 was identified from a boar in Japan (Sonoda *et al* 2004). Swine HEV in India differs genetically from human HEV, indicating that pigs may not play an important role in the spread of human hepatitis E in endemic regions (Arankalle *et al* 2002; Shukla *et al* 2007).

High anti-HEV prevalence is reported in rodents from several geographic regions (Kabrane-Lazizi *et al* 1999; Arankalle *et al* 2001), and a strain of HEV has been genetically identified from wild rats (Tsarev *et al* 1998). Avian HEV was identified and molecularly characterized from chickens with hepatitis–splenomegaly syndrome in the USA (Haqshenas *et al* 2001). Like swine HEV, avian HEV is also related genetically and antigenically to human HEV (Huang *et al* 2004; Feagins *et al* 2007).

Seroprevalence rates are higher in endemic regions compared to areas where HEV infection is rare. Imported cases of hepatitis E have been seen in non-endemic regions such as Australia, France, Israel, The Netherlands, Spain, UK and USA; occasional cases with no recent history of travel are also observed. Anti-HEV seroprevalance of ~16% has been observed in southwest France; this could be responsible for active autochthonous transmission in this region (Mansuy *et al* 2008).

The zoonotic transmission of HEV is a serious issue in developed countries. Though a majority of infections are asymptomatic, sustained transmission can lead to the evolution of virulent strains in future (Ijaz *et al* 2005; Zheng *et al* 2006). HEV genotype 3 infections have become more common in the United Kingdom, like genotype 4 did in China in the past decade (Wang 2003; Ijaz *et al* 2005). Genotype 3 is widely distributed and evolution of virulent strains would have more far-reaching consequences (Zheng *et al* 2006).

3. Clinical presentation and pathogenesis

The HEV target population is young to middle aged adults, 15 to 40 years of age. The clinical symptoms are typical of acute viral hepatitis and include jaundice, malaise, anorexia, nausea, abdominal pain, fever and hepatomegaly; anicteric hepatitis is also observed (Smith 2001). The disease is self-limiting and no chronic sequelae have been reported in general. However, two recent reports present biochemical,

histological and genetic evidence of chronic HEV infection in transplant patients (Haagsma *et al* 2008; Kamar *et al* 2008). It would be interesting to test other immunosuppressed persons, such as those with HIV infection, for their ability to resolve acute hepatitis E.

Hepatitis E has a mortality rate of 0.2–1% in the general population. Increased morbidity and mortality is observed in chronic liver disease patients superinfected with HEV (Hamid *et al* 2002). A unique clinical feature is its increased incidence and severity in pregnant women, with mortality rates of 15–20% (Khuroo *et al* 1981). A role or endotoxin-mediated hepatocyte injury was proposed (Purcell and Ticehurst 1997; Jameel 1999), but the precise cellular/molecular mechanisms are not clear. A shift in the Th1/Th2 balance towards Th2 has been observed in pregnant women infected with HEV compared to non-pregnant women (Pal *et al* 2005), but how this influences the severity of HEV infection is not clear. Pregnant women with jaundice and acute viral hepatitis due to HEV showed higher mortality rates and worse obstetric and fetal outcomes than those with other types of viral hepatitis (Patra *et al* 2007). There were increased levels of estrogen, progesterone and βHCG in HEV-positive pregnant patients with fulminant hepatitis compared to HEV-negative patients and controls (Jilani *et al* 2007). Selective suppression of nuclear factor kappa B (NFκB) p65 in pregnant compared to non-pregnant fulminant hepatitis patients has also been proposed to cause liver degeneration, severe immunodeficiency and multi-organ failure (Prusty *et al* 2007).

4. Classification and Phylogeny

The International Committee for Taxonomy of Viruses has classified HEV as a *Hepevirus* in the family Hepeviridae (*http://www.ncbi.nlm.nih.gov/8threportICTV/*).

The HEV genomes of several geographically distinct isolates show a high degree of sequence conservation (Arankalle *et al* 1999). At least four phylogenetically distinct genotypes have been defined, which distribute by geographic regions (figure 2). Genotype 1 includes Asian and African HEV strains, genotype 2 includes the single Mexican HEV strain and few variants identified from endemic cases in African countries, genotype 3 includes human and swine HEV strains from industrialized countries, and genotype 4 includes human and swine HEV strains from Asia, particularly China, Taiwan and Japan. The avian HEV was proposed to belong to a new genotype 5 (Haqshenas *et al* 2001; Huang *et al* 2004), but this has not yet been confirmed.

A HEV genotype is dominant in a given geographic area, but not limited to it. For example, genotype 2 first identified in Mexico (Huang *et al* 1992) was later found on the African continent (Buisson *et al* 2000; Maila *et al* 2004; Nicand *et al* 2005). Recently, HEV genotype 1 was observed in Cuba in the Americas (Montalvo *et al* 2008). Swine HEV isolates belong to either genotype 3 or 4 (Hsieh *et al* 1999; Okamoto *et al* 2001; van der Poel *et al* 2001; Huang *et al* 2002; Takahashi *et al* 2003; Meng 2005; Feagins *et al* 2007), but recently genotype 1 was detected in a pig in Cambodia (Caron *et al* 2006).

Though there is inter- and intra-patient diversity of HEV, its relevance to viral pathogenesis is not clear (Grandadam *et al* 2004). All HEV genotypes show varying degrees of intra-genome diversity (Okamoto 2007). Some recent reports indicate an effect of genotype on viral transmission and disease severity. Outbreaks due to HEV genotype 1 and 2 are the result of efficient human-to-human feco-oral transmission. HEV strains of genotype 3 and 4 are maintained among animal species and occasionally infect humans probably due to inefficient cross-species transmission. This is supported by the recovery of HEV isolate HE-JA4 from a patient who was infected after ingestion of undercooked pig liver; the sequence was identical to the swine HEV isolate swJL145 (Okamoto 2007). Genotype 4 was recently shown to cause more severe disease than genotype 3 (Mizuo *et al* 2005), and higher viral loads were observed for genotype 4 in a co-infected patient (Takahashi *et al* 2002).

5. Molecular virology

5.1 *Animal models and* in vitro *culture*

HEV transmission studies have mostly been done in non-human primates such as cynomolgus, rhesus and owl monkeys, and chimpanzees (Uchida *et al* 1991; Ticehurst *et al* 1992; Vitral *et al* 1998; McCaustland *et al* 2000). These have provided important information regarding the biology and pathogenesis of HEV, and are indispensable tools for vaccine and drug testing (Kamili *et al* 2004; Purcell *et al* 2003). Experimental transmission studies have also been done in pigs, an established reservoir for HEV (Williams *et al* 2001).

There has been only limited success in generating suitable tissue culture replication systems for HEV. Early studies reported propagation of HEV in 2BS (Huang *et al* 1992), A549 (Huang *et al* 1995; Wei *et al* 2000) and FRhK (Kazachkov *et al* 1992) cells. Infection of primary cynomolgus hepatocytes and PLC/PRF/5 cells has been shown, but replication was inefficient (Tam *et al* 1996; Meng *et al* 1997). Recently, HEV genotype 3 from a high titer stool suspension was successfully passaged for multiple generations in PLC/PRF/5 cells (Tanaka *et al* 2007) and these cells were used to assess the infectivity of HEV shed in patients' stools (Takahashi *et al* 2007). The replication of HEV has been observed in cell lines transfected with

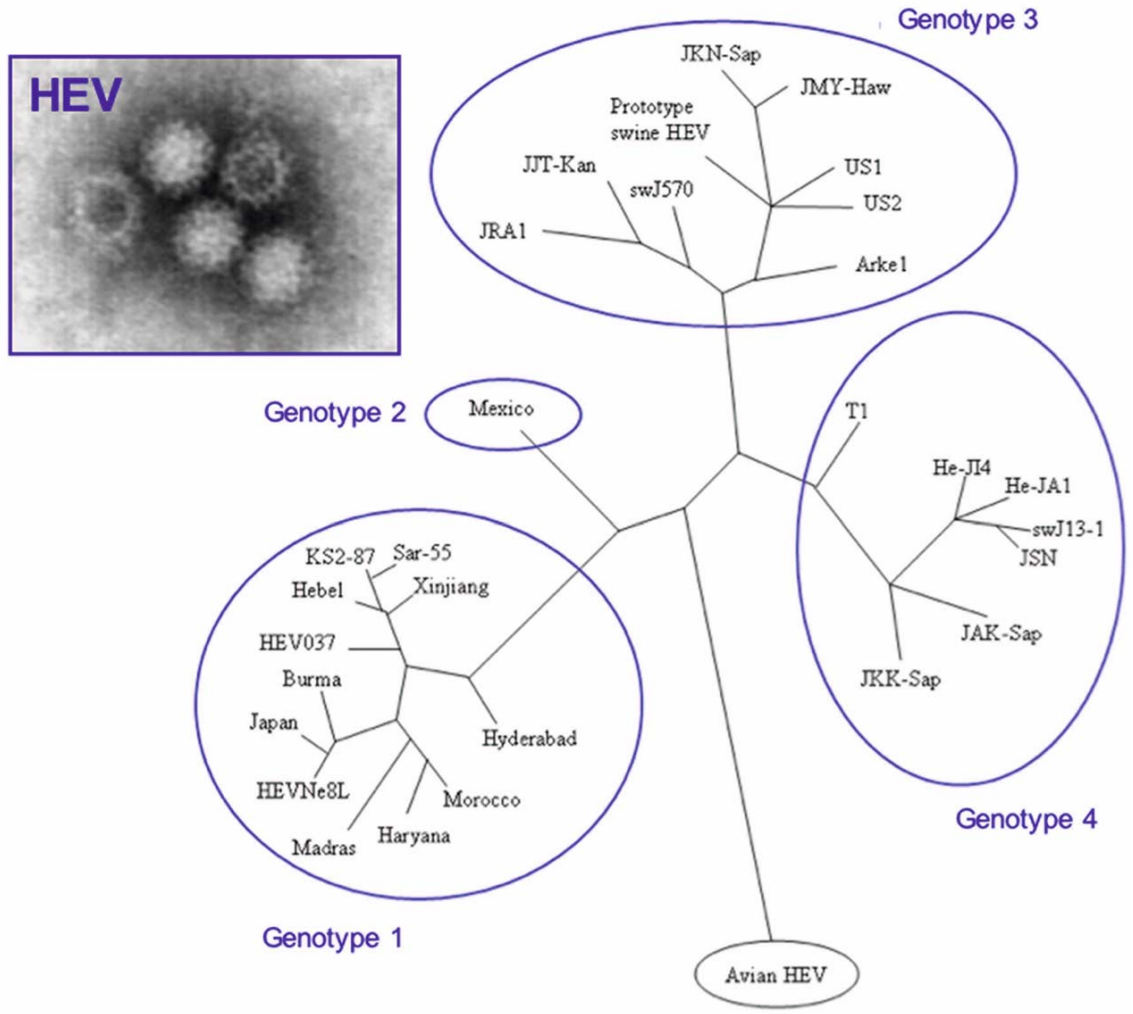

Figure 2. HEV and its genotypes. Electron micrograph showing HEV particles from the stool of a hepatitis E patient visualized after aggregation with anti-HEV positive serum and negative staining. A phylogenetic tree showing the distribution of human and swine HEV isolates in four distinct genotypes number 1 to 4 and an outlier group containing avian HEV.

transcripts of infectious cDNA clones and with a replicon derived from it (Panda *et al* 2000; Emerson *et al* 2004). Monkeys inoculated with culture media or lysates of HEV replicon-transfected cells developed infection, but viral titers were low. Some species barrier for HEV replication might exist since replicons did not function in non-primate cell lines.

5.2 *Genome organization*

HEV is a spherical, non-enveloped virus of about 27–34 nm (Krawczynski *et al* 2000) (figure 2). The viral genome is a single-stranded, positive-sense 5′-capped RNA of ~7.2 kb. It consists of short 5′ and 3′ untranslated regions (UTRs), and three partially overlapping open reading frames (ORF), called ORF1, ORF2 and ORF3 (Tam *et al* 1991) (figure 3). The expression kinetics of the viral proteins is not clear, but their expression during infection is confirmed by presence of antibodies in infected humans and experimental animals (Khudyakov *et al* 1994; Panda *et al* 1995). The UTRs and a conserved 58-nucleotide region within ORF1 (figure 3A) are likely to fold into conserved stem-loop and hairpin structures (Tam *et al* 1996), and together with an alphavirus junction homologous sequence (figure 3A), are postulated to be important for HEV RNA replication (Purdy *et al* 1993). Earlier results from experimentally infected monkeys suggested the generation of two subgenomic RNAs (Tam *et al* 1996) but this has now been questioned. Graff *et al* (2006) recently proposed that the ORF2 and ORF3 proteins are translated from closely spaced AUG codons on a bicistronic subgenomic RNA. While this rationalizes the reading frame differences observed in HEV genotype 4, it remains to be confirmed in an experimental model.

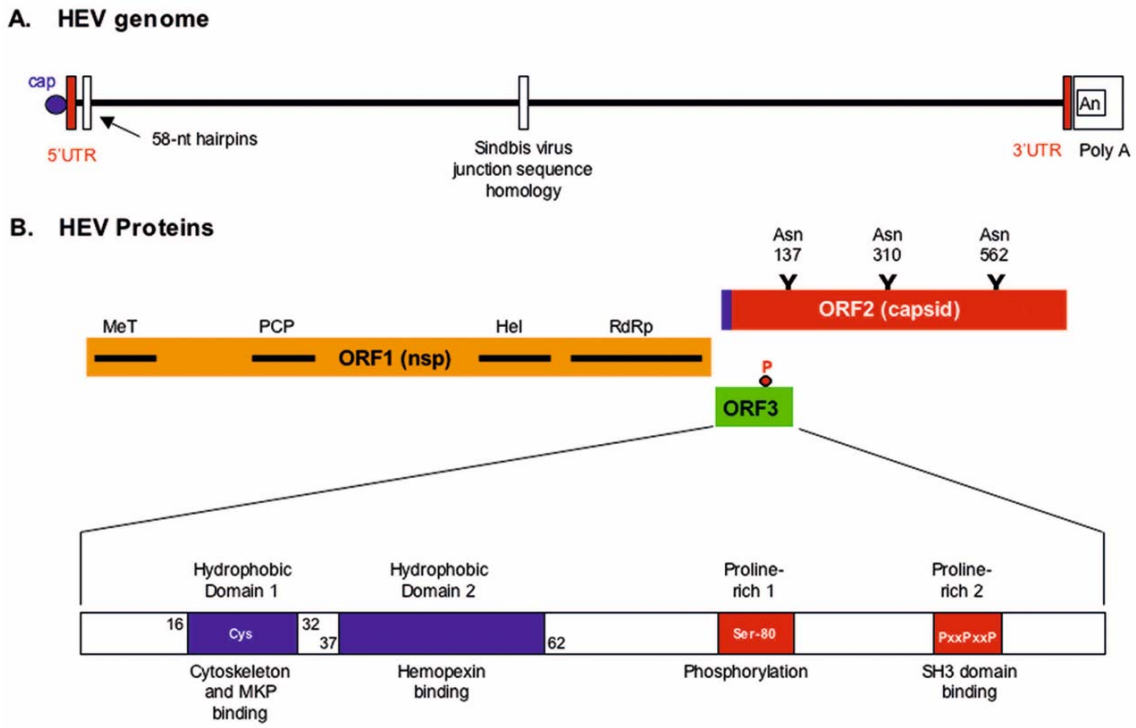

Figure 3. Genome organization and proteins of HEV. (**A**) The ~7.2 kb positive strand RNA genome of HEV is capped at the 5′ end and polyadenylated at the 3′ end. It contains short stretches of untranslated regions (UTR) at both ends (red box). Other structural features proposed to be important for replication are also indicated. (**B**) The three open reading frames (ORFs) are shown. ORF1 encodes the nonstructural polyprotein (nsp) that contains various functional units – methyltransferase (MeT), papain-like cysteine protease (PCP), RNA helicase (Hel) and RNA dependent RNA polymerase (RdRp). ORF2 encodes the viral capsid protein; the N-terminal signal sequence (blue box) and glycosylation sites are indicated. ORF3 encodes a small regulatory phosphoprotein. Details of the ORF3 proteins are shown, including two N-terminal hydrophobic domains (blue boxes) and two C-terminal proline-rich regions (red boxes). Functions discovered for these domains are indicated below the illustration.

5.3 *HEV proteins*

5.3.1 *The ORF1 protein:* The ORF1 of HEV encodes a large nonstructural protein with several putative functional motifs and domains such as methyltransferase, papain-like cysteine protease (PCP), RNA helicase and RNA dependent RNA polymerase (RdRp) (figure 3B). *In vitro* expression of the HEV ORF1 produced a polyprotein (Ansari *et al* 2000; Ropp *et al* 2000) that was processed into two products following extended incubation (Ropp *et al* 2000). When expressed in insect cells, ORF1 was processed and this was partially blocked by a cell permeable cysteine protease inhibitor (Sehgal *et al* 2006), but the viral or cellular nature of the protease remained unclear. The presence of methyltransferase motifs in ORF1 suggested HEV to have a capped RNA genome. A 5′-methylguanosine residue in the HEV genome was shown to be essential for infectivity and replication (Emerson *et al* 2001, 2004; Zhang *et al* 2001). The GDD motif in RdRp was reported to be important for HEV replication (Agrawal *et al* 2001; Emerson *et al* 2001). Two predicted stem-loop (SL) structures at the 3′ NCR and the polyA tract were necessary for RdRp binding during HEV genome replication (Agrawal *et al* 2001; Emerson *et al* 2001). Except for the methyltransferase (Magden *et al* 2001), none of the other putative components of ORF1 have been expressed, purified and biochemically characterized.

5.3.2 *The ORF2 protein:* The ORF2 of HEV encodes its capsid protein (pORF2) of 660 amino acids and is proposed to encapsidate the viral RNA genome (Purdy *et al* 1993). The ORF2 protein enters the endoplasmic reticulum (ER) (Zafrullah *et al* 1999), but a fraction retrotranslocates to the cytoplasm to trigger a stress pathway (Surjit *et al* 2007). Replicon-based expression of pORF2 recently confirmed its N-linked glycosylation (Graff *et al* 2008) (figure 3B). Mutations in the pORF2 glycosylation sites prevented the formation of infectious virus particles and had low infectivity in macaques (Graff *et al* 2008). In insect cells recombinant pORF2 expressed as a 56 kDa protein that lacked 111 N-terminal and 53 C-terminal residues, and self-assembled into virus-like particles (VLPs) (Robinson *et al* 1998).

The structure of a self-assembled VLP was solved by cryo-electron microscopy and showed the capsid to be

dominated by dimers (Xing *et al* 1999). Self-association and homo-dimerization of pORF2 has been also demonstrated through yeast two-hybrid analysis (Tyagi *et al* 2001). The ORF2 protein also bound the 76-nucleotide (nt) region at the 5′ end of the HEV genome in agreement of its capsid encapsidation function. The RNA binding activity of pORF2 was lost when deletions were made beyond the N-terminal 111 amino acids (Surjit *et al* 2004).

The size of the ORF2 protein in the virus particle and its glycosylation status are issues that are not clear. Whether this protein has any nonstructural functions as well is also not understood at this time.

5.3.3 *The ORF3 protein:* The ORF3 of HEV encodes a small protein (pORF3) of 123 amino acids. Recently it was proposed to be translated from a bicistronic subgenomic RNA and to be 9 amino acids shorter at its N-terminus (Graff *et al* 2006). While ORF3 was dispensable for replication *in vitro* (Emerson *et al* 2006), it is required for infection in monkeys inoculated with HEV genomic RNA (Graff *et al* 2005).

Expression of pORF3 in mammalian cells showed it to interact with various cellular proteins. Through domain 1 (figure 3B) it colocalized with the cytoskeleton (Zafrullah *et al* 1997) and bound a MAP kinase phosphatase (Kar-Roy *et al* 2004). Domain 2 was responsible for its interaction with hemopexin, an acute-phase plasma glycoprotein (Ratra *et al* 2008). The P1 region contains the phosphorylated serine residue that is conserved in all HEV strains except the Mexican isolate and the P2 region contains a PxxPxxP motif that binds several proteins containing src-homology 3 (SH3) domains (Korkaya *et al* 2001).

The ORF3 protein is likely to regulate the host cell environment through its interaction with various intracellular pathways (figure 4). It activates the extracellularly regulated kinase (ERK) by binding and inhibiting its cognate phosphatase (Kar-Roy *et al* 2004). Prolonged activation of ERK would generate a survival and proliferative signal (figure 4A). Higher levels of hexokinase and oligomeric voltage-dependent anion channel (VDAC) were found in ORF3-expressing cells, which displayed attenuated mitochondrial death signalling (Moin *et al* 2007) (figure 4A). The ORF3 protein might act as an adaptor to link intracellular transduction pathways (Pawson 1995), and this might promote HEV replication and assembly.

We recently found that pORF3 localized to early and recycling endosomes, and delayed post-internalization trafficking of epidermal growth factor receptor (EGFR). This is likely to prolong endomembrane signalling and promote cell survival (Chandra *et al* 2008) (figure 4B). Another effect of this is reduced nuclear translocation of pSTAT3 and attenuation of the acute phase response (Chandra *et al* 2008). Thus, pORF3 might reduce the host inflammatory response, further creating an environment favourable for viral replication (figure 4B).

The alpha-1-microglobulin and bikunin precursor protein (AMBP) and its constituents α1-microglobulin and bikunin were also identified as pORF3 binding partners (Tyagi *et al* 2004; 2005). There was increased secretion of α1-microglobulin from ORF3-expressing cells (Surjit *et al* 2006). Since α1-microglobulin is immunosuppressive, this is proposed to protect virus-infected cells (figure 4C).

Two broad roles are thus predicted for pORF3 in HEV pathogenesis (figure 4). The first is promotion of cell survival through ERK activation, prolonged endomembrane signaling and attenuation of the intrinsic death pathway (figure 4A, B). The second is to downregulate innate host responses through reduced expression of acute phase proteins and increased secretion of α1-microglobulin (figure 4C).

5.4 *The HEV replication cycle*

5.4.1 *Viral receptor and entry:* Little is known about the cellular receptors for HEV or its entry process. A recent study showed that a truncated peptide p239 spanning aa 368-606 of pORF2 formed 23 nm particles that bind and penetrate HepG2, Huh-7, PLC/PRF5 and A549 cells (He *et al* 2008) and prevent further infection of these cells. The cell surface molecules that bind HEV or its capsid protein are not known.

5.4.2 *Model of HEV replication:* A model for HEV replication and gene expression was proposed based on similarities and sequence homology to better characterized positive strand RNA viruses (Reyes *et al* 1993; Jameel 1999). This is shown in figure 5. Following entry into a permissive cell (step 1), the viral genomic RNA is uncoated (step 2) and translated in the cytosol of infected cells to produce the ORF1-encoded nonstructural polyprotein (nsP) (step 3). Cleavage of the ORF1 nsP is achieved by cellular proteases, possibly with help from the viral PCP. The viral replicase (RdRP) replicates the genomic positive strand into the negative strand replicative intermediates (step 4A). These serve as template for the synthesis of additional copies of the genomic positive strands as well as subgenomic positive strands (step 4B). This is akin to alphaviruses and a region homologous to alphavirus junction sequences is proposed to serve as the subgenomic promoter. The subgenomic RNA can then be translated into the structural protein(s) (step 5). The capsid proteins package the viral genome to assemble progeny virions (step 6) that exit the cell through an undefined pathway. Direct experimental confirmation of this replication scheme is still awaited but several findings increase our belief in this model. ln experimentally infected rhesus monkeys (Nanda *et al* 1994) and pigs (Meng *et al* 1998), HEV positive and negative strand RNAs are observed in the liver. Since *in vitro* transcripts of full-length cDNA clones are infectious for nonhuman primates and

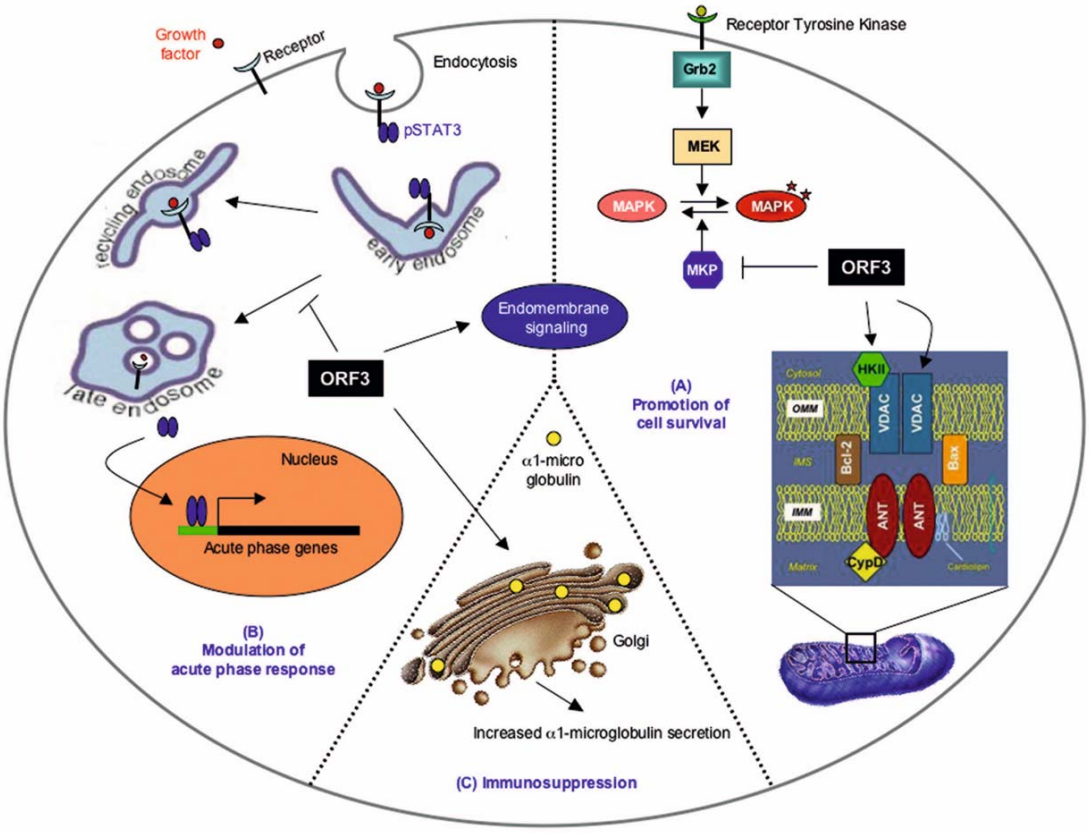

Figure 4. Role of the ORF3 protein in HEV pathogenesis. Published data show three broad functions for the ORF3 protein. (**A**) *Promotion of cell survival.* The ORF3 protein activates MAP kinase by binding and inactivating its cognate phosphatase (MKP). Additionally, it upregulates and promotes homo-oligomerization of the outer mitochondrial membrane porin, VDAC, and increases hexokinase levels, thus reducing mitochondrial depolarization and inhibiting intrinsic cell death. (**B**) *Modulation of the acute phase response.* The ORF3 protein localizes to early and recycling endosomes, and inhibits the movement of activated growth factor receptors to late endosomes. This prolongs endomembrane growth factor signaling and contributes to cell survival. Through this mechanism, pORF3 also reduces the nuclear transport of pSTAT3, a critical transcription factor for the expression of acute phase response genes. (**C**) *Immunosuppression.* The ORF3 protein promotes the secretion of α1-microglobulin, an immunosuppressive protein that could act in the immediate vicinity of the infected cell.

pigs, the subgenomic RNAs are not required to initiate an infection, and must be synthesized as part of the replication process. Replicons have shown mixed results with respect to detection of negative-stranded replicative intermediates (Panda *et al* 2000; Emerson *et al* 2004).

6. Host immune response, detection and prophylaxis of hepatitis E

Studies on experimentally infected macaques first defined the clinical and serological course of HEV infection. In those studies, serum anti-HEV immunoglobulin G (IgG) appeared around 3-4 weeks post-inoculation at the peak of ALT elevation. A human volunteer study showed anti-HEV IgM to peak in the symptomatic period and then decline to baseline within 3-6 months of illness. Serum anti-HEV IgG levels continued to rise during the symptomatic phase and were detectable in the convalescent phase for 2 years. In other studies anti-HEV IgG persisted for up to 13 years. These have been reviewed earlier (Jameel 1999; Mushahwar 2008).

Cellular immune responses to acute HEV infection are poorly characterized. Srivastava *et al* (2007) observed expansion of CD4+ cells in patients compared to controls. However, the proportions of CD4+/CD69+ and CD8+/CD69+ cells producing interferon gamma (IFNγ), tumour necrosis factor alpha (TNFα) and interleukin-4 (IL-4) remained unchanged following *in vitro* stimulation with

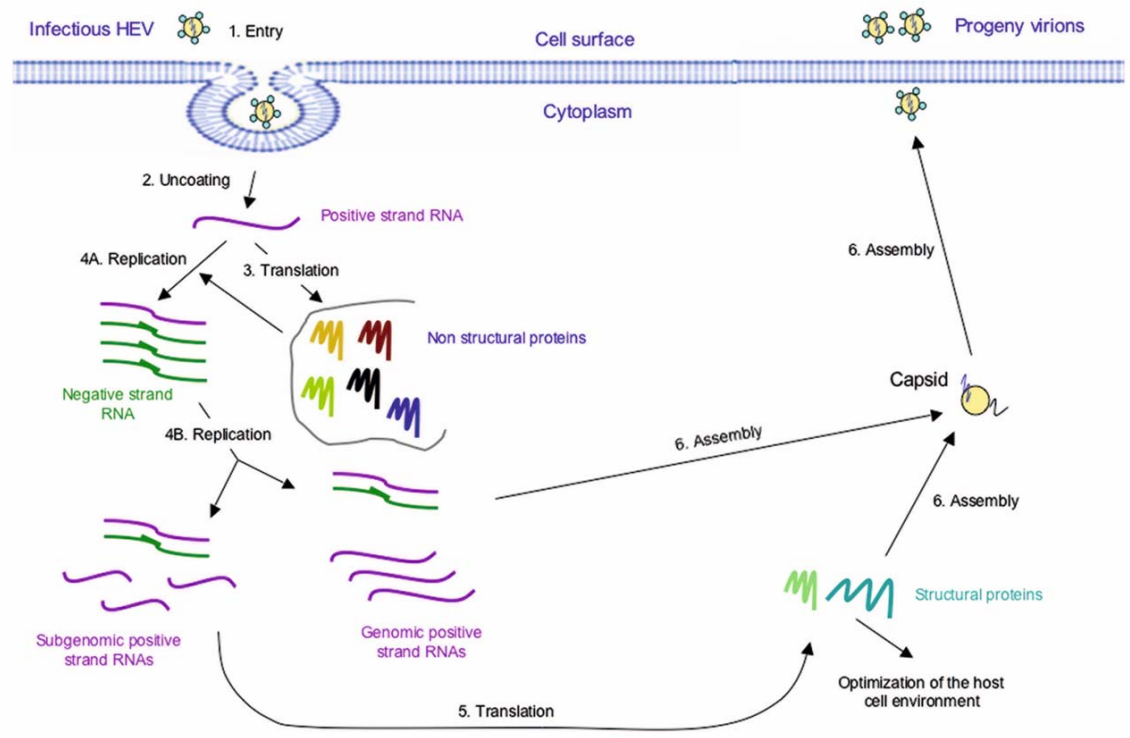

Figure 5. Proposed replication cycle of HEV. The virus enters the target cell (1) and uncoats (2) to release the HEV genomic RNA through uncharacterized processes. The genomic RNA is translated in the cytoplasm into nonstructural proteins (3). The replicase thus synthesized replicates the positive strand genomic RNA into negative strand RNA intermediates (4A) and back (4B). This is the genome amplification step. Additionally, the positive strand subgenomic RNA is also synthesized that is translated into structural proteins (5). The capsid protein packages the genomic RNA to assemble new virions (6) that are then released from the cell through an uncharacterized mechanism.

pORF2. A decrease in IFNγ and TNFα producing T cells in hepatitis E patients following polyclonal activation with PMA and ionomycin suggested an inherent T cell activation defect in HEV-infected individuals. The limited immune reactivity observed in the peripheral compartment may also result from the migration and sequestration of immune cells to the liver. Aggarwal *et al* (2007) characterized proliferative responses from patients and controls using overlapping ORF2 and ORF3 peptides. The mononuclear cells from patients showed increased proliferation compared to controls, with peptide pools corresponding to amino acids 73-156, 289-372, 361-444 and 505-588 of the ORF2 protein associated with significant proliferation. The lymphocyte proliferation with ORF2 peptide pool 289-372 was found to be associated with the presence of HLA-DRB1 allele 010X.

The detection of HEV infection is based on serological and nucleic acid tests. The former detect serum antibodies against HEV and the latter detect (and quantitate) HEV RNA in serum, bile and/or feces. Early tests for anti-HEV antibodies developed in various laboratories using synthetic peptides or recombinant proteins were employed in seroprevalence studies, and showed a wide variation in sensitivity (Mast *et al* 1998). Commercial IgG or IgM anti-HEV tests are now available from Genelabs Diagnostics, Singapore and Abbott Labs, Germany. An acute HEV infection is generally positive for both IgM and IgG anti-HEV, while only the latter is positive for past infection. Thus, in an endemic area, the IgM anti-HEV test is of value in deciding acute infection while the IgG test has more value in seroprevalence studies. Recently an ELISA for detecting putative neutralizing antibody responses to HEV genotypes 1 to 4 has been developed (Zhou *et al* 2004), which may be useful in future trials of candidate HEV vaccines. Serum viremia for HEV was shown to be positive by reverse transcriptase polymerase chain reaction (RT-PCR) before ALT elevation and to last for about one week to one month (Chauhan *et al* 1993). Various in-house assays for HEV RNA detection based have been described (reviewed in Mushahwar *et al* 2008) that are used to confirm an ongoing HEV infection. Robust, sensitive and rapid assays for HEV detection are required, not just for confirming an acute HEV infection, but to also detect the levels of virus contamination in water and food. Recently, Gyarmati *et al* (2007) developed two sensitive assays to detect HEV across genotypes from multiple sources.

Since there is no robust system to grow HEV in culture, inactivated or live attenuated vaccines are not feasible. However, several observations suggest that recombinant subunit vaccines will be possible (Aggarwal and Jameel

2008) and are likely to protect against all four genotypes of HEV as they share a common serotype. A subunit vaccine based on a truncated ORF2 protein produced in insect cells using recombinant baculoviruses (Robinson *et al* 1998) showed efficacy in a pre-clinical monkey challenge model (Tsarev *et al* 1994, 1997). This vaccine was also clinically tested in humans and showed ~95% efficacy (Shrestha *et al* 2007). There is no information yet on the licensure and marketing of this hepatitis E vaccine. Various other hepatitis E vaccine candidates are in different stages of development and testing (reviewed in Aggarwal and Jameel 2008).

7. Old mysteries and new challenges

The earlier review (Jameel 1999) had highlighted some outstanding questions in hepatitis E. A decade later, it is time to review progress on those and highlight important issues for the future.

7.1 *How is HEV maintained in the community during inter-epidemic periods?*

It is quite clear now from serological and genetic studies that multiple animal species harbour HEV or HEV-like viruses. Swine HEV (Meng *et al* 1997) and avian HEV (Haqshenas *et al* 2001) have been genetically characterized and direct transmission of HEV to humans from contaminated boar and deer meat has been demonstrated (Tei *et al* 2003, 2004; Takahashi *et al* 2004). Various studies suggest that zoonotic transmission may be the major mode of infection in non-endemic areas, whereas humans continue to be the major source of HEV in endemic areas.

7.2 *How does HEV cause disease in endemic areas despite patients with hepatitis E having anti-HEV antibodies?*

The most logical explanation for this observation is that titers of anti-HEV neutralizing antibodies decline rapidly. As a result, while people in endemic areas still carry anti-HEV antibodies from a previous infection, these offer no protection against new infection. Using the neutralizing antibody ELISA (Zhou *et al* 2004), it should now be possible to track anti-HEV neutralizing antibodies in persons living in endemic areas and to study the dynamics of this response following an outbreak.

7.3 *What are the pathogenic mechanisms in HEV infection?*

The central epidemiological question in hepatitis E is its increased severity in pregnancy. There are still no concrete answers. Pregnant monkeys experimentally infected with HEV do not show this differential effect and studies in human patients have not adequately addressed this issue. Much of what we know about the potential role of HEV proteins in pathogenesis is based on their over-expression in cultured cells, which itself can be misleading. There has been an increased understanding of the role of pORF3 in HEV pathogenesis, but almost all the information is derived from over-expression studies. The importance of this protein in HEV pathogenesis is however reinforced by the lack of experimental infection in monkeys by an ORF3-null virus (Graff *et al* 2005).

7.4 *Research on therapeutic interventions in hepatitis E*

This is an ignored area of hepatitis E research. Potential targets on HEV include the nonstructural proteins and the 5′ and 3′ UTRs that are critical for replication of the HEV genome. The nonstructural proteins are poorly characterized, with only the viral methyltransferase studied with any biochemical rigor (Magden *et al* 2001). Other potential targets are the putative papain-like cysteine protease, RNA helicase and replicase encoded by HEV. A report on the use of ribozymes to inhibit HEV RNA replication is available (Sriram *et al* 2003). A logical approach would be to focus on biochemical and structural characterization of the HEV nonstructural proteins and to develop *in vitro* and cell-based assays to screen for potential inhibitors. Since drugs targeted against proteases, helicases and replicases are already in clinical use, structure-based modeling can be employed to search existing databases (Chong and Sullivan 2007).

7.5 *Research on better disease management*

Hepatitis E is a self-limiting disease that attracts little active management in endemic areas. Thus, fulminant cases are poorly managed and have high rates of mortality. Since the host response is believed to determine the progression and outcome of hepatitis E, it makes sense to characterize biomarkers of disease progression. Research on the discovery and validation of biomarkers in the plasma and urine of hepatitis E patients and their association with disease severity would be important. Such surrogate markers might lead to tests that could identify patients with increased susceptibility to develop severe disease. Once identified, these patients can be clinically managed and this would reduce mortality.

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