Hepatitis E: Molecular Virology and Pathogenesis

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Hepatitis E virus is a single, positive-sense, capped and poly A tailed RNA virus classified under the family *Hepeviridae*. Enteric transmission, acute self-limiting hepatitis, frequent epidemic and sporadic occurrence, high mortality in affected pregnant are hallmarks of hepatitis E infection. Lack of an efficient culture system and resulting reductionist approaches for the study of replication and pathogenesis of HEV made it to be a less understood agent. Early studies on animal models, sub-genomic expression of open reading frames (ORF) and infectious cDNA clones have helped in elucidating the genome organization, important stages in HEV replication and pathogenesis. The genome contains three ORF's and three untranslated regions (UTR). The 5' distal ORF, *ORF1* is translated by host ribosomes in a cap dependent manner to form the non-structural polyprotein including the viral replicase. HEV replicates via a negative-sense RNA intermediate which helps in the formation of the positive-sense genomic RNA and a single bi-cistronic sub-genomic RNA. The 3' distal ORF's including the major structural protein pORF2 and the multifunctional host interacting protein pORF3 are translated from the sub-genomic RNA. Pathogenesis in HEV infections is not well articulated, and remains a concern due to the many aspects like host dependent and genotype specific variations. Animal HEV, zoonosis, chronicity in immunosuppressed patients, and rapid decompensation in affected chronic liver diseased patients warrants detailed investigation of the underlying pathogenesis. Recent advances about structure, entry, egress and functional characterization of *ORF1* domains has furthered our understanding about HEV. This article is an effort to review our present understanding about molecular biology and pathogenesis of HEV. (J CLIN EXP HEPATOL 2013;3:114–124)

Hepatitis E virus was first identified in 1983 as the agent responsible for the large scale waterborne epidemics of acute, self-limiting hepatitis in developing nations.¹ It was shown to be a naked 28–34 nm virus-like particle with spikes and inundations.^{1,2} Successful use of non-human primate models for HEV propagation helped in its isolation, cloning and sequencing and as presumed turned out to be an RNA virus.^{3,4} It had a ~7.2 kb single stranded genome with sequence homology to other positive-sense RNA viruses.^{4,5} Computer based genome annotation revealed three overlapping open reading frames *ORF1*, *ORF2* and *ORF3*.^{4,5} The longest ORF, *ORF1* formed the 5' distal end of the genome and coded for the non-structural replication proteins including *methyltransferase*, *protease*, *helicase*, and RNA dependent RNA polymerase (*RdRp*).

The 3' distal ORF, *ORF2* coded for an arginine rich protein, together with immunodominant sero-reactivity and agglutination against the expressed epitopes, it was predicted to be the major structural protein. A third ORF, *ORF3* coded for a small protein thought to be a minor structural protein.^{4–6}

Molecular characterization and replication model of HEV remained hypothetical till the use of strand-specific PCR and the detection of negative-sense RNA replicative intermediate.^{7,8} Further understanding of the HEV replication came from the development of infectious cDNA clone of HEV which helped in elucidating the importance of 5' end cap, *RdRp* activity and non-structural polyprotein processing.^{9,10} Mutational and strand-specific PCR analysis using the HEV replicon helped in delineating the bi-cistronic sub-genomic RNA based expression strategy for *ORF2* and *ORF3* proteins and the presence of an additional untranslated region (*UTR*) in the intergenic region with probable *cis* reactive element thought to function as sub-genomic promoter.^{11–14} Till date only one *cis*-acting element has been characterized, that in the 3' *UTR*, which was shown to form stable secondary structures and help in the localization of *RdRp*.¹⁵ Further the viral replicase was shown to localize to the endoplasmic reticulum membrane, which is the probable site of HEV replication.¹⁶

The mechanism of HEV pathogenesis especially at the molecular level is poorly understood due to lack of an

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Abbreviations: *RdRp*: RNA dependent RNA polymerase; *ORF*: open reading frames; *UTR*: untranslated region; *NTP*: nucleoside triphosphate; *ER*: endoplasmic reticulum; *EMSA*: electrophoretic mobility shift assay; *ELI-SPOT*: enzyme-linked immunosorbent spot; *DUB*: deubiquitinating

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in vitro cell culture system and an ideal practical animal model. However, a wide body of clinical, immunopathological and histopathological studies helped in identifying the stages of infection and the hallmark immune mediated disease outcome, similar to other viral hepatitis.^{17,18} HEV pathogenesis varies, from being asymptomatic or acute, self-limiting in low risk groups to causing chronic and/or fulminant hepatic failure in high risk groups; the modus operandi remains hypothetical. Increasing reports typify route, establishment and pathogenic outcome during hepatitis E infection to host factors and genotype involved.

The recent developments in understanding the structure, entry, egress, host interactions and functional characterization of the HEV proteins helped in further providing a better insight in understanding its replication and pathogenesis. This review provides an overview of the recent advances in the understanding of the molecular biology and pathogenesis of HEV.

GENERAL CLASSIFICATION

There are four major mammalian genotypes (1–4) and one avian HEV. HEV distribution shows a socioeconomic and genotypic basis to it, with the highest seroprevalence in countries affected with HEV genotypes 1 and 2 and poor sanitation. Genotype 1 and 2 strains are mainly associated with large waterborne anthroponotic epidemics and frequent sporadic acute hepatitis in these regions. Genotype 3 and 4 HEV strains are found both in human and other mammalian reservoirs (swine, wild boar, deer, mongooses, rabbit, and cattle) and are mostly subclinical and zoonotic, however, genotype 3 is increasingly being associated with chronic infections in immunosuppressed patients. Genotype 3 is found mainly in Europe, United States and Japan, and genotype 4 has been identified mainly in Asia. In these regions, the seroprevalence within the general population is 1–3% and higher in certain populations involved in animal husbandry or consuming undercooked meat. Hepatitis E-like viruses have been successfully isolated from chickens, wild rats and trout fish^{19–21} and are yet to be classified.

MOLECULAR VIROLOGY

Genome Organization

HEV genome contains a short 5' untranslated region (UTR) of 27 nucleotides, followed by largest ORF (*ORF1*) of 5079 bases that code for a viral non-structural polyprotein (pORF1) (Figure 1). A second ORF (*ORF2*) begins 38 nucleotides 3' of the termination of ORF1 consisting of 1980 nucleotides and codes for the major structural protein (pORF2). A 65-nucleotide 3' UTR is contiguous with the termination of ORF2 and is terminated by a 3' stretch of 150–200 or more adenosine residues. A third and smallest

ORF (*ORF3*), 345 nucleotides in length, begins 23 nucleotides 3' of the termination of ORF1 and extends into ORF2 and encodes for a phosphoprotein (pORF3).²² HEV forms a single 3' terminal, capped and polyadenylated ~2.2 kb bicistronic sub-genomic RNA which codes for both ORF3 and ORF2.¹⁴ The sub-genomic RNA start site was found to be from 5124 (FJ457024) and contains a very short untranslated region of 9 nucleotides (FJ457024) followed by the reading frames of *ORF3* and *ORF2*. *ORF3* overlaps *ORF2* with their start codons just 11 nucleotides apart (FJ457024).¹⁴

The 5' end of both genomic and sub-genomic RNA is capped. The m⁷G cap was confirmed by reverse transcription-PCR (RT-PCR) assay based on the monoclonal antibody (MAb) to 2,2,7-trimethyl guanosine (m³G) in case of the genomic RNA and by RNA. Ligase-mediated rapid amplification of cDNA ends (RLM-RACE) in case of both genomic and sub-genomic RNA.^{11,23} The 5' UTR was predicted to form a hair-pin structure. Mapping of the RNA revealed that a 76-nucleotide region at the 5' end of the HEV genome was responsible for binding the ORF2 protein,²⁴ and this interaction may play a role in viral encapsidation. The 3' UTR of HEV has been shown to form cis-active stem-loop structures which localized the viral RNA dependent RNA polymerase in *in vitro* binding studies and henceforth implicated in the initiation of virus replication.^{10,15} The poly A tail at the 3' end is approximately 150–200 nucleotides long and is crucial for RdRp binding to the 3' UTR. An additional UTR was recently established with the elucidation of sub-genomic RNA start site downstream of the ORF1 stop site.^{11,12} This junction region with similarities to the junction sequence of rubella virus and alphavirus is thought to play a role in sub-genomic RNA formation. Similarly, a highly conserved region in the *ORF2* reading frame was found to form stable stem-loop structures (ISL1 and ISL2), and were found to effect pORF2 production *in vitro* when mutated silently.²⁵

Non-structural Proteins

ORF1 of HEV encodes a non-structural polyprotein with a molecular mass of ~186 kDa. Computer analysis suggested that the pORF1 contains several putative functional domains: a methyltransferase (56–240 aa); a Y domain (216–442 aa); a papain-like cysteine protease (433–592 aa); a proline-rich hinge domain (712–778 aa); a X domain (785–942 aa); an RNA helicase (960–1204 aa); and an RNA dependent RNA polymerase (1207–1693 aa).⁵ (Figure 1).

The enzymatic activity of methyltransferase was experimentally demonstrated for N-terminal fragment of HEV polyprotein, produced in insect cells using recombinant baculovirus vector which catalyzed both guanine-7-methyltransferase and guanylyl-transferase activities

Figure 1 Genome organization of hepatitis E virus.

required for capping of viral mRNAs.²⁶ Capping activity of HEV 5' UTR has been shown to be essential for infectivity *in vivo* and replication *in vitro* by using a HEV infectious cDNA clone and a HEV replicon expressing green fluorescent protein, respectively.^{10,27}

Conservation of the X domain, which has been exclusively found in association with viral papain-like proteases, suggests that HEV encoded protease bears similarity to proteases observed in other positive-strand RNA viruses like *alphaviruses* and RubV. The catalytic residues predicted by computer alignment for HEV protease were Cys483 and His590. The Cys483 residue is highly conserved among all HEV isolates, whereas His590 is not.²⁸ ORF1 polyprotein processing has been observed in transfection studies of infectious HEV cDNA clone.⁹ The exact cleavage sites of the ORF1 polyprotein are still not known. Another study demonstrating partial cleavage of the full-length 185 kDa ORF1 polyprotein had been shown. Two potential processed products of 107 kDa and 78 kDa were observed

in vivo following extended incubations (24–36 h).²⁸ However, site-specific mutagenesis of cysteine 483, failed to abolish this cleavage. Contradicting this study, no processing of the ORF1 polyprotein was observed into discrete units, either *in vitro* or in HepG2 cells²⁹ and Huh7 cells.³⁰ In a more recent analysis of HEV ORF1, two conserved LXGG cleavage sites for deubiquitinating (DUB) enzymes (viral or cellular) at 664 and 1205 were determined, and the DUB activity of PCP was determined *in vitro*.³¹ Although the LXGG site in ORF1 could not be cleaved by PCP, possibility of HEV polyprotein processing by cellular DUB enzymes cannot be ruled out. In a follow up study, use of proteasome inhibitors and later by over expressing ubiquitin, authors showed that an active ubiquitin-proteasome system is required for HEV replication.³²

In the positive-stranded RNA viruses a conserved domain of unknown function flanks the papain-like protease domain in the polyprotein. This domain is referred to as X domain (785–942 aa) and is present downstream of

a proline-rich hinge (PPR) domain (712–778 aa) that is thought to be important for the flexibility of the molecule and contains a hypervariable sequence (HVR) motif.⁵ The HVR deletions were shown to be tolerated if small, and markedly reduced replication when large. A completely deleted HVR avian HEV replicon, although was still replication competent in LMH cells, resulted in a loss of avian HEV infectivity in chickens.³³ Recent reports recognize HVR and PPR as recombination hotspots and involved in viral adaptation and evolution.^{34–37}

HEV RNA helicase shows similarity with the helicase of beet necrotic yellow vein virus and with that of rubella virus.⁵ HEV RNA helicase was shown to have two conserved motifs involved in purine nucleoside triphosphate (NTP)-binding activity (GVPGSGKS at amino acid position 975–982) and RNA- or DNA-dependent NTPase activity (DEAP at position 1029–1032).⁴ Recently, Karpe and Lole group have experimentally demonstrated the NTPase and RNA unwinding activities of the helicase domain.³⁸ HEV helicase also possesses gamma-phosphatase activity, which might help catalyze the first step in RNA cap formation.³⁹

The RNA dependent RNA polymerase of HEV was first characterized by Agrawal et al. The *Escherichia coli* expressed 63 kDa recombinant HEV RdRp was able to synthesize RNA *in vitro* using 3' end of HEV RNA with poly (A) stretch as the template.¹⁵ This showed the specific binding of HEV RdRp to the 3' end of the viral genome. Similar to rubella virus, the GDD motif (1550–1552 aa) in RdRp was reported to be important for HEV replication and mutations in this motif abolished replication altogether.²⁷ Co-localization studies showed that HEV replicase localize on endoplasmic reticulum membranes.¹⁶ A recent study also shows the localization of pORF1 to endoplasmic reticulum especially in the perinuclear region, implicating the possible use of modified early secretory pathway membranes for replication.³⁰

The ORF2 Encoded Structural Protein

ORF2, the second largest ORF approximately 2 kb in length which starts at nucleotide position 5147 and present at the 3' end of HEV genome, encodes the major viral capsid protein (pORF2) of 660 aa.⁴ ORF2 expression has been demonstrated during viral infection by the presence of antibodies in infected humans directed against epitopes present on pORF2.^{6,40,41}

The pORF2 expression in mammalian cells using plasmid based expression showed ~74 kDa–~88 kDa forms, corresponding to non-glycosylated and glycosylated forms respectively.⁴² By means of pulse-chase experiments, it has been shown that pORF2 is synthesized as a larger precursor of about 82 kDa which is co-translationally translocated into the endoplasmic reticulum (ER), where it is processed into the mature protein (pORF2), through signal sequence cleavage.⁴² This protein is then glycosylated (gpORF2) in

the ER at all three possible glycosylation sites, which were mapped by mutational analysis to asparagine residues (Asn137, Asn310 and Asn562). These three N-linked glycosylation sites are universally conserved in all isolates of human HEV.^{4,41,43,44} as well as in swine.⁴⁵ HEV suggesting functional significance for glycosylation. Torresi et al, has shown that although glycosylated-pORF2 represented majority of the total pORF2 and was largely membrane-associated, it was less stable than the non-glycosylated-pORF2, which accumulated in the cytosol.⁴⁶ Recently, Graff et al confirmed the N-linked glycosylation of pORF2 in replicon based expression system.⁴⁷ Further, mutations in the potential glycosylation sites of pORF2 inhibited the formation of infectious virus-like particles (VLPs) from transfected replicons, and these VLPs showed low infectivity in experimental macaques.⁴⁷ More recently, Yamada et al showed the intracellular expression and secretion of a ~83 kDa ORF2 protein in PLC/PRF/5 cells transfected with capped replicons of HEV genotype 3. The expression of ~83 kDa pORF2, larger than the predicted ~72 kDa, indirectly indicates the glycosylated form of pORF2. However, based on 3D-structure it has been found that glycosylation may interfere with capsid assembly or receptor binding.⁴⁸ These contradicting reports of glycosylation status of pORF2 are yet to arrive at consensus.

Truncated form of the HEV capsid protein (112–660 aa) expressed in insect cells could self-assemble to form virus-like particles (VLPs) of 23.7 nm diameter with *T* (triangulation number) = 1 icosahedral symmetry.^{49,50} The capsid protein expressed in insect cells including 14–608 aa form *T* = 3 VLP. The X-ray crystal structure of pORF2 revealed three functional domains, S (shell), M (middle), and P (protruding).^{48,51} The S domain is the most conserved region among HEV genotypes, and forms an icosahedral shell that serves as the base for arranging M and P domains. The P domain serves as the putative binding site for both neutralizing antibody and cellular receptor.⁵²

In addition to the baculovirus expressed recombinant pORF2, the recombinant ORF2 protein expressed in *E. coli* have also been shown to assemble into higher order structures. The p239 (368–606 aa), E2 (394–606 aa) and E2a (459–660 aa) proteins predominantly occur as homodimers under mildly dissociating conditions, which model the dominant antigenic determinants and neutralizing site of HEV.^{53–55} A recent study revealing the crystal structure of the E2s protein (455–602 aa) shows it to form a tight homodimer that is essential for HEV interaction with the host cell, and contains the HEV neutralizing antibody recognition site.⁵⁶ In a study, the yeast two-hybrid system and *in vitro* immobilization experiments showed that full-length pORF2 is capable of self-association, thus forming a homodimer. Any deletion in pORF2 results in loss of its dimerization potential except an N-terminal 127 amino acid deletion. This study suggested that the dimerization

property of pORF2 may not be amino acid sequence dependent but instead a complex formation of a specific tertiary structure that imparts pORF2 its property to self-associate.^{57,58}

ORF2 protein has been shown to contain RNA binding activity by which it can interact specifically with the 5' end of the HEV genome. Recently, Xing et al suggested that HEV virion assembly is not an independent event but is driven by RNA encapsidation.⁵⁹ A recent report showed ex-vivo RNA packaging by a baculovirus expressed ORF2 protein, which could infect cells *in vitro*.⁶⁰ A recent report also suggests the stabilization role of the ORF2 c-terminal 52 aa during encapsidation, as the deleted mutants have resulted in producing empty virus particles, even though it is known that c-terminal region is not essential for the capsid morphology.⁶¹

Regulatory functions of pORF2 during infection was recently postulated in reports showing increased expression of anti-apoptotic mediators⁶² and inhibition of NF-kappaB activity with heterologous expression of ORF2.⁶³

The ORF3 Encoded Phosphoprotein

pORF3 is a small protein of 123/122 amino acids encoded by the smallest ORF (ORF3) in the HEV genome. It has been expressed using various expression systems including *E. coli*,⁴¹ yeast *Pichia pastoris*⁶⁴ and mammalian cell lines.⁴² In animal cells pORF3 was expressed as a 13.5 kDa non-glycosylated protein that interacts with a cellular protein 3IP of about 18 kDa.⁴² However, two forms of pORF3, 13.5 and 28 kDa, were observed *in vitro*⁴² and in *E. coli* expression system⁴¹ suggesting that the 28-kDa species represents a highly stable dimeric form of pORF3. The (³²P) orthophosphate labeling experiment and mutation studies demonstrated this expressed protein as a phosphoprotein, phosphorylated at Ser-80 residue.⁶⁵ Interestingly, this serine residue is conserved among all HEV isolates except for the more divergent Mexican isolate.⁴⁴ On subcellular fractionation, pORF3 appears to associate with the cytoskeleton using one of its two large N-terminal hydrophobic domains D1 (7–23 aa) and D2 (28–53 aa).⁶⁵ The D1 domain contains a polycysteine stretch and is required for the pORF3 to associate with cytoskeleton⁶⁵ to bind microtubules⁶⁶ and to MAPK phosphatase.⁶⁷

C-terminal part of pORF3 contains two proline-rich domains P1 (66–77 aa) and P2 (95–111 aa). The P2 domain contains two overlapping PXXP motifs. *In vitro* binding studies have shown that pORF3 through its P2 domain binds to SH3 domains of many signaling proteins and a cytoskeletal protein. The SH3 binding domains, the phosphorylation signal, and the dimerization domains overlapping with each other suggest that self-association of pORF3 may play a regulatory function in the signal transduction pathway.⁶⁸ Several reports suggest pORF3 role in cell survival and proliferation signaling.^{69–74}

Co-localization and yeast two-hybrid system demonstrated that pORF3 protein interacts with pORF2 protein through a 25-amino acid region (residues 57–81) and this interaction is dependent upon the phosphorylation at pORF3 Ser-80. It was believed that Ser-80 is necessary for viral infectivity. Interestingly, pORF3 from the Mexican isolate contains leucine instead of serine at position 80 and fails to associate with pORF2. This suggests that either the pORF2–pORF3 interaction is not critical for virus assembly and infection. Graff et al, 2005 has shown that the ORF3-null mutant could not infect macaques consistent with its suggested role in capsid assembly. But, this explanation is not convincing as the interaction of pORF2 and pORF3 proteins *in vitro* absolutely required the phosphorylation of pORF3 protein at serine 80.⁷⁵ In agreement with the above findings, three of four macaques were unable to be infected with wild-type genomes in which Ser-80 was mutated to alanine: the fourth macaque mildly seroconverted to anti-HEV, suggesting limited viral replication had occurred. In contrast, an alternative mutant in which phosphorylation was prevented by mutating Ser-80 to leucine instead to alanine infected both macaques into which it was inoculated and the ORF3 region was amplified from the serum of both macaques at the time of peak viremia and was confirmed to have retained the leucine mutation. Therefore, phosphorylation of Ser-80 of pORF3 protein was not necessary for infection *in vivo*.⁷⁵

Several recent findings suggest that pORF3 has a definite role in virus egress and its association with virions. In a report, HEV particles from the infected human serum could be captured by anti-ORF3 monoclonal antibodies suggesting that pORF3 is involved in virion assembly.⁷⁶ In another study, immunocaptured PCR showed the similar results in the culture supernatant.⁷⁷ A recent study demonstrated that the release of hepatitis E virions from cultured Caco-2 and Huh7 cells depends on the presence of a functional ORF3 protein and the ORF3 proteins, in association with lipids, was found to coat the virion surface.⁷⁸ Another recent study showed that at least one of the two intact PSAP motifs within the P2 domain of pORF3 is required for the formation of membrane-associated HEV particles possessing pORF3 protein on their surface. This suggests that the PSAP motif plays an important role as a functional domain for HEV budding.⁷⁹ Similar role of the PSAP motif in virus release have been shown *in vivo* in chickens and not in chicken liver cell line expressing pORF2.⁸⁰

pORF3 was initially predicted to express as 123 aa long polypeptide. Recent reports suggest that both pORF2 and pORF3 proteins are translated from a single sub-genomic RNA which initiates from AUG at nt position 5131.^{11,14} This would result in a 114 aa long ORF3 protein that is 9 aa shorter at its 5' terminus than the earlier proposal.¹¹ Though numerous functions has been assigned to pORF3 from providing structural stability to viral particle

to creating an immunosuppressed environment around the infected hepatocytes, but still the debate exists for assigning a proper function to this small phosphorylated regulatory viral protein.

REPLICATION CYCLE

The proposed model for HEV replication is based on similarities and sequence homology to other well characterized positive-strand RNA viruses of alphavirus-like superfamily.⁸¹ According to the hypothesized model of HEV replication, when the virus genomic RNA enters into a permissive cell, the *ORF1* gets translated in a cap-mediated manner²³ to produce the non-structural polyprotein. It is not known whether this polyprotein gets processed to the functional domains or binds to the *cis*-acting elements in the 3' end of the virus to copy the genome into a negative-strand replicative intermediate. The HEV replication as in case of most positive-strand RNA viruses takes place via a negative-sense RNA intermediate.¹⁴ The replication mostly occurs in the cell cytoplasm Figure 2.¹⁴

The main target cells for hepatitis virus replication are the hepatocytes. Other sites of replication have also been suggested in the swine^{82,83} and in rhesus monkeys.⁸⁴ HEV antigens were detected in the hepatocytes of infected patients and experimentally infected animals.^{18,85} The presence of negative-strand HEV RNA replicative intermediates in the liver of infected rhesus monkeys constituted the first direct evidence of HEV replication in the liver.⁷ There is also evidence of extrahepatic replication of HEV

like the biliary epithelial cells in rhesus monkeys.⁸⁴ Extrahepatic sites of replication in HEV infected swine model has been reported which includes the small intestine, spleen, lymph nodes, colon and peripheral blood monocytes.^{82,83} In cynomolgus macaque model, HEV genomic RNA and two sub-genomic RNAs were detected.⁴ Whereas, in the rhesus macaque models both positive and negative-strand RNAs were detected in the liver, which is the primary site of HEV replication.⁷

Attachment and entry of HEV into cells however remained unknown till date, and being a non-enveloped virus it was thought to occur via a specific receptor on the host cell. Recent findings suggest that HEV attaches to the susceptible host cell by specific high affinity receptor and enters the cytoplasm by clathrin-mediated endocytosis.⁸⁶ No binding and uptake of the fluorescent-VLPs was observed on non-hepatic cell lines (HeLa and SiHa).⁸⁶ Similar specific receptor mediated attachment and entry was reported earlier by He et al, in 2008 using VLPs (p239) and a panel of susceptible cell lines (Huh7, HepG2, PLC/PRF/5 and A549 cells); a nonspecific cell line P815 was tested and found to be not susceptible to HEV. In a follow up study HSP90 was found to be associated in the HEV capsid (p239) intracellular transportation.⁸⁷ A recent report suggests the role of syndecans in HEV attachment to Huh7 cells by showing a marked reduction in the infectivity of heparinase treated cells.⁸⁸ However, the authenticity of these reports needs to be independently confirmed. And the involved receptor for HEV attachment and entry still remains a lacunae.

Figure 2 Proposed model of hepatitis E virus replication. Adapted from Expert Reviews in Molecular Medicine 1999 Cambridge University Press.

Upon entry the HEV genome is thought to undergo cap-mediated translation²³ to produce the non-structural polyprotein.⁹ Although capping of the replicons (from Sar-55 strain) was shown to be essential for infectivity,¹⁰ in a follow up study, *in vitro* transcripts (both capped and uncapped) from the same infectious cDNA clone could initiate virus replication in seven of the nine transfected primate cell cultures.²⁷ It is not clear whether pORF1 undergo initial processing in to the components or work as a single molecule. However in a report from Panda et al, 2000, transfected infectious full-length replicons revived from cDNA clone of an Indian HEV isolate (AF076239) helped in demonstrating processed components of the pORF1 polyprotein by metabolic pulse labeling. Post-translational processing of non-structural polyprotein could help to switch the synthesis of negative-sense RNA to positive-sense RNA, which also helps in maintaining a high ratio of positive to negative-sense RNA. Although such high ratio of positive to negative-sense RNA has been demonstrated in HEV,^{14,16,89} further study is necessary to understand the role and mechanism of pORF1 processing.

Replication complex assembly and membrane compartmentalization is an essential step in the replication of all positive-strand RNA viruses. It has been shown that for most positive-strand RNA viruses (both plant and animal), the RNA dependent RNA polymerase (RdRp/Replicase) associates with different membranous compartments/aggregates to carry out the viral replication. HEV replicase was found to localize to the membranes of endoplasmic reticulum (ER).¹⁶ It was observed that when the region from the nucleotide position 4449–5109 encoding a hydrophobic transmembrane domain between amino acid positions 1585–1601 was deleted there was loss of ER localization of RdRp. HEV is a positive-strand RNA virus similar to the alphavirus-like superfamily where, replication is initiated by the interaction of the replicase with the 3' end *cis*-acting elements and via the negative-sense RNA intermediate.¹⁵ *In vitro* analysis by Agrawal et al, 2001 showed that the 3' end of HEV genome (nucleotides 7084–7194 with a poly A tail) binds specifically to RdRp to initiate replication. *In vitro* Electrophoretic Mobility Shift Assay (EMSA) was used to analyze HEV positive-sense RNA-RdRp interaction and it was seen that deletion of nucleotides 7163–7194 from the 3' NCR and 3' poly (A) stretch of HEV positive-sense RNA completely abolished binding of RdRp. Recombinant RdRp catalyzed *in vitro* synthesis of complementary RNA and northern hybridization confirmed the specificity of the newly synthesized negative-sense RNA. This study illustrated that 3' NCR of nucleotides 7084–7194 with a poly A tail is crucial for HEV replication. This study also confirmed that the conserved GDD motif of RdRp was important for polymerase activity.

Nucleotide sequence analysis of the 3' non-coding region of the full-length HEV sequences in GenBank

identified sequences that had a high degree homology with little variations among Indian, Pakistani, Myanmar and Chinese isolates. Computer-assisted prediction of the thermodynamically stable structure of the 3' end of HEV RNA identified two stem-loop structures that were conserved among all viral isolates with minor difference in the Mexican isolate. This conserved region spans nucleotide position 7084–7194 in the Indian isolate of HEV. Pb2+ hydrolysis and RNase probing were used to confirm the predicted structure at the 3' end of HEV RNA. In another study it was observed that HEV replicons with 15- or more than 10-A residues replicate better than the ones with 5-A residues.⁹⁰ However, 5 A-residues are sufficient to generate a stem-loop structure at 3' NCR in both Indian and Pakistan strains of HEV and might serve as the prerequisite to HEV replication.

In the rhesus macaque model, as mentioned above, both HEV positive and negative-strand RNAs could be demonstrated in the liver, whereas in serum and bile, where only mature virions are expected, only positive-strand viral RNA was detected.⁷ In a reporter replicon based study ~200-fold of positive over negative-strands (2.97×10^9 vs 1.4×10^7 viral copy number) with capped HEV RNA has been detected,⁸⁹ which also confirmed the asymmetrical mode of replication seen in viruses of alphavirus-like superfamily where, the genomic sense-strands are detected in relative abundance to the negative-strands. The negative-strand RNA intermediate, by analogy with alphaviruses, acts as a template for the synthesis of additional copies of the genomic positive-strand as well as sub-genomic positive-strand RNAs. The analogy to alphavirus replication is presumed because of the presence in the negative-strand replicative intermediate of HEV, a junction sequence which is similar to the Sindbis alphavirus.⁵ In Sindbis virus replication this sequence acts as a sub-genomic promoter for transcription of the structural region. The presence of one genomic (~7.5 kb) and two sub-genomic (~3.7 kb and ~2 kb) HEV RNAs has been shown in experimentally infected cynomolgus macaques⁴ and in cell culture.⁹¹ In another report northern blot analyses detected full-length replicon RNA and a single sub-genomic RNA of ~ 2.2 kb from HEV replicons containing the neomycin resistance gene expressed from ORFs 2 and 3 after transfection into Huh7 cells. No ~3.7 kb sub-genomic RNA was detected.¹¹ In a recent study a conserved double stem-loop RNA structure, which may be important for HEV replication, is identified in the junction region which might act as a sub-genomic promoter.⁹² The proposed sub-genomic positive-strand HEV RNA is translated into the structural proteins(s) to package the viral genome to form progeny virions. The confirmation of the formation of both pORF3 and pORF2 from the proposed bi-cistronic sub-genomic RNA has been recently reported,¹⁴ pORF2 was shown to interact with the 5' end of

the genome and help in encapsidation of the genome.^{24,59,60} The role of pORF3 during virion egress has recently been proposed.⁷⁷ Reports of the presence of pORF3 in the virions from culture derived HEV and in serum further suggest the role of ORF3 in egress and packaging.⁹³ However, a complete delineation of the proposed replication strategy is still awaited.

PATHOGENESIS

The pathogenesis of hepatitis E is poorly understood. Entry of the virus into the host is believed to be primarily by the oral route via contaminated water or food. The primary site of HEV replication has not been identified and is believed that virus first replicate in the intestinal tract from where it reaches to the liver presumably via the portal vein. It then replicates in the cytoplasm of hepatocytes and released into the bile and the blood by an unknown mechanism. The pathogenesis of HEV infection and particularly the severe forms of it remains obscure. The prevalence of HEV infection in young adults (15–40 years) could be due to immune enhancement mechanisms, while the primary immune stimuli result from 'silent' infection occurring during childhood. Alternatively, it can be assumed that the immunity acquired in an early age is not stable and vanishes in 10–20 years. One can also speculate that the putative cell receptors to HEV appear late in life as a result of continued maturation of hepatocytes and other liver cells.

A generalized clinical course of natural hepatitis E infection was proposed after the human volunteer studies.^{1,94} Shedding of virus in feces at the end of the first week or beginning of the second week after inoculation has been considered as the first sign of infection. HEV RNA by RT-PCR can be detected in feces and sera from most patients within 6 days after exposure to the virus^{95,96} and disappears with the increase in ALT levels and appearance of anti-HEV antibodies. However, protracted viremia ranging from 4 to 16 weeks has also been reported in approximately 10% of the patients who did not show seroconversion.^{94,97,98} The IgM anti-HEV antibodies are detectable as early as 1–4 days after onset of disease^{99,100} and decline within six months, whereas IgG anti-HEV antibody is detected during the peak of alanine aminotransferase (ALT) value and can persist for several months to years^{101–103} and may play an important role against HEV infection.

The elevation of aminotransferases simultaneously with the histopathological changes in the liver generally correspond to the presence of anti-HEV in the serum and with decreasing levels of viral antigen in hepatocytes. This indicated that the liver injury during HEV infection might be immune mediated. In addition, the detection of infiltrating lymphocytes in the liver with a cytotoxic/suppressor immunophenotype favored the idea.

The delayed appearance of anti-HEV until after pathologic changes occurred suggests that antibody is not necessary for initiating the pathologic process. More marked pathological changes after increase in anti-HEV level was supportive of the idea that initial injury is independent of antibody. It was presumed that antibody or other component of the host immune response is mediating the clearance of virus and infected cells thereby causing more prominent histopathologic changes.¹⁰⁴ This idea was further supported by the presence of infiltrating lymphocytes of a cytotoxic/suppressor immunophenotype suggesting a cell-mediated immune mechanism for hepatocytes damage during HEV infection. The findings of several recent studies favored the proposal of HEV pathogenesis to be immune mediated. Wu et al, (2008); studied the HEV specific T-cell response together with IgM anti-HEV antibodies in acute hepatitis E patients by enzyme-linked immunosorbent spot (ELISPOT) assay and enzyme-linked immunosorbent assay respectively.¹⁰⁵ A strong HEV specific cellular immune responses occurred against capsid protein, HEV 239 (368–606) that decreased along with the decreasing IgM anti-HEV antibody titer and normalization of liver function. The decrease in HEV specific T-cell responses with convalescence suggests the involvement of T cell responses in the pathogenesis of acute hepatitis E and recovery. A recent comparative study to examine the association of viral load and host immune status in HEV induced acute viral hepatitis and FHF cases reported the absence of HEV RNA, higher anti-HEV-IgM and IgG antibody titers with significantly higher levels of both Th1 (IFN-gamma, IL-2 and TNF-alpha) and Th2 (IL-10) cytokines in FHF patients and suggested that status of host immune response plays a role in outcome.¹⁰⁶ These findings suggest a definitive role of HEV specific T cells in immunopathogenesis of hepatitis E infection. But the nature of specific T-cell responses during HEV infection was unclear. Prabhu et al (2010), more specifically demonstrated the involvement of activated CD8⁺ T-cells containing granzymes in acute liver failure cases of HEV infection.¹⁸ The absence of Treg cells in HEV infected liver tissues suggests that no regulation of immune mediated killing during HEV infection might be associated with severity of the disease.¹⁸ Tripathy et al (2011) studied the frequency of peripheral Treg cells (CD4⁺CD25⁺Foxp3⁺ and CD4⁺CD25⁺Foxp3⁺) in acute hepatitis E patients by flow cytometry and HEV-specific cytokines/chemokines quantitation and compared it with recovered individuals and healthy controls. The median percentage of CD4⁺CD25⁺Foxp3⁺ (True Treg) and CD4⁺CD25⁺Foxp3⁺T (Effector Treg) cells in acute hepatitis E patients were significantly higher compared to controls and recovered individuals. The level of IL-10, a signature cytokine of Tregs, was also elevated in the similar manner. The elevation in frequencies of Treg cells and rise in IL-10 cytokine suggests the role of these cells in infection and recovery of hepatitis E.¹⁰⁷

SUMMARY

HEV still remains a grossly understudied pathogen, the lack of a robust *in vitro* propagation system made investigators to overexpress individual proteins to understand their role in HEV replication and pathogenesis, however the significance of these data in the context of the actual virus infection is largely unknown. Significant advances, starting with the development of infectious cDNA clones, three dimensional structure determination, and development of small animal models, and the recently developed culture systems show promise in understanding the life cycle of HEV. Further studies and new experimental designs are necessary to understand the molecular mechanisms of HEV replication and pathogenesis.

CONFLICTS OF INTEREST

All authors have none to declare.

REFERENCES

- Balayan MS, Andjaparidze AG, Savinskaya SS, et al. Evidence for a virus in non-A, non-B hepatitis transmitted via the fecal-oral route. *Intervirology*. 1983;20:23–31.
- Panda SK, Datta R, Kaur J, Zuckerman AJ, Nayak NC. Enterically transmitted non-A, non-B hepatitis: recovery of virus-like particles from an epidemic in south Delhi and transmission studies in rhesus monkeys. *Hepatology*. 1989;10:466–472.
- Reyes GR, Purdy MA, Kim JP, et al. Isolation of a cDNA from the virus responsible for enterically transmitted non-A, non-B hepatitis. *Science*. 1990;247:1335–1339.
- Tam AW, Smith MM, Guerra ME, et al. Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. *Virology*. 1991;185:120–131.
- Koonin EV, Gorbalenya AE, Purdy MA, Rozanov MN, Reyes GR, Bradley DW. Computer-assisted assignment of functional domains in the nonstructural polyprotein of hepatitis E virus: delineation of an additional group of positive-strand RNA plant and animal viruses. *Proc Natl Acad Sci U S A*. 1992;89:8259–8263.
- Yarborough PO, Tam AW, Fry KE, et al. Hepatitis E virus: identification of type-common epitopes. *J Virol*. 1991;65:5790–5797.
- Nanda SK, Panda SK, Durgapal H, Jameel S. Detection of the negative strand of hepatitis E virus RNA in the livers of experimentally infected rhesus monkeys: evidence for viral replication. *J Med Virol*. 1994;42:237–240.
- Tam AW, White R, Reed E, et al. In vitro propagation and production of hepatitis E virus from in vivo-infected primary macaque hepatocytes. *Virology*. 1996;215:1–9.
- Panda SK, Ansari IH, Durgapal H, Agrawal S, Jameel S. The in vitro-synthesized RNA from a cDNA clone of hepatitis E virus is infectious. *J Virol*. 2000;74:2430–2437.
- Emerson SU, Zhang M, Meng XJ, et al. Recombinant hepatitis E virus genomes infectious for primates: importance of capping and discovery of a cis-reactive element. *Proc Natl Acad Sci U S A*. 2001;98:15270–15275.
- Graff J, Torian U, Nguyen H, Emerson SU. A bicistronic subgenomic mRNA encodes both the ORF2 and ORF3 proteins of hepatitis E virus. *J Virol*. 2006;80:5919–5926.
- Huang YW, Opriessnig T, Halbur PG, Meng XJ. Initiation at the third in-frame AUG codon of open reading frame 3 of the hepatitis E virus is essential for viral infectivity in vivo. *J Virol*. 2007;81:3018–3026.
- Ichiyama K, Yamada K, Tanaka T, et al. Determination of the 5'-terminal sequence of subgenomic RNA of hepatitis E virus strains in cultured cells. *Arch Virol*. 2009;154:1945–1951.
- Varma SP, Kumar A, Kapur N, Durgapal H, Acharya SK, Panda SK. Hepatitis E virus replication involves alternating negative- and positive-sense RNA synthesis. *J Gen Virol*. 2011;92:572–581.
- Agrawal S, Gupta D, Panda SK. The 3' end of hepatitis E virus (HEV) genome binds specifically to the viral RNA-dependent RNA polymerase (RdRp). *Virology*. 2001;282:87–101.
- Rehman S, Kapur N, Durgapal H, Panda SK. Subcellular localization of hepatitis E virus (HEV) replicase. *Virology*. 2008;370:77–92.
- Wedemeyer H, Rybczynska J, Pischke S, Krawczynski K. Immunopathogenesis of hepatitis E virus infection. *Semin Liver Dis*. 2013;33:71–78.
- Prabhu SB, Gupta P, Durgapal H, et al. Study of cellular immune response against hepatitis E virus (HEV). *J Viral Hepat*. 2011;18:587–594.
- Marek A, Bilic I, Prokofieva I, Hess M. Phylogenetic analysis of avian hepatitis E virus samples from European and Australian chicken flocks supports the existence of a different genus within the Hepeviridae comprising at least three different genotypes. *Vet Microbiol*. 2010;145:54–61.
- Zhang W, Shen Q, Hua X, Cui L. Novel hepatitis E virus genotype in Norway rats. *Germany Emerg Infect Dis*. 2011;17:1981–1983.
- Batts W, Yun S, Hedrick R, Winton J. A novel member of the family Hepeviridae from cutthroat trout (*Oncorhynchus clarkii*). *Virus Res*. 2011;158:116–123.
- Panda SK, Thakral D, Rehman S. Hepatitis E virus. *Rev Med Virol*. 2007;17:151–180.
- Kabrane-Lazizi Y, Meng XJ, Purcell RH, Emerson SU. Evidence that the genomic RNA of hepatitis E virus is capped. *J Virol*. 1999;73:8848–8850.
- Surjit M, Jameel S, Lal SK. The ORF2 protein of hepatitis E virus binds the 5' region of viral RNA. *J Virol*. 2004;78:320–328.
- Emerson SU, Nguyen HT, Torian U, Mather K, Firth AE. An essential RNA element resides in a central region of hepatitis E virus ORF2. *J Gen Virol*. 2013;March 20. [Epub ahead of print].
- Magden J, Takeda N, Li T, et al. Virus-specific mRNA capping enzyme encoded by hepatitis E virus. *J Virol*. 2001;75:6249–6255.
- Emerson SU, Nguyen H, Graff J, Stephany DA, Brockington A, Purcell RH. In vitro replication of hepatitis E virus (HEV) genomes and of an HEV replicon expressing green fluorescent protein. *J Virol*. 2004;78:4838–4846.
- Ropp SL, Tam AW, Beames B, Purdy M, Frey TK. Expression of the hepatitis E virus ORF1. *Arch Virol*. 2000;145:1321–1337.
- Ansari IH, Nanda SK, Durgapal H, et al. Cloning, sequencing, and expression of the hepatitis E virus (HEV) nonstructural open reading frame 1 (ORF1). *J Med Virol*. 2000;60:275–283.
- Perttila J, Spuul P, Ahola T. Early secretory pathway localization and lack of processing for hepatitis E virus replication protein pORF1. *J Gen Virol*. 2013;94:807–816.
- Karpe YA, Lole KS. Deubiquitination activity associated with hepatitis E virus putative papain-like cysteine protease. *J Gen Virol*. 2011;92:2088–2092.
- Karpe YA, Meng XJ. Hepatitis E virus replication requires an active ubiquitin-proteasome system. *J Virol*. 2012;86:5948–5952.
- Pudupakam RS, Kenney SP, Cordoba L, et al. Mutational analysis of the hypervariable region of hepatitis E virus reveals its involvement in the efficiency of viral RNA replication. *J Virol*. 2011;85:10031–10040.
- Purdy MA, Lara J, Khudyakov YE. The hepatitis E virus polyproline region is involved in viral adaptation. *PLoS One*. 2012;7:e35974.
- Purdy MA. Evolution of the hepatitis E virus polyproline region: order from disorder. *J Virol*. 2012;86:10186–10193.

36. Chen X, Zhang Q, He C, et al. Recombination and natural selection in hepatitis E virus genotypes. *J Med Virol.* 2012;84:1396–1407.
37. Shukla P, Nguyen HT, Torian U, et al. Cross-species infections of cultured cells by hepatitis E virus and discovery of an infectious virus-host recombinant. *Proc Natl Acad Sci U S A.* 2011;108:2438–2443.
38. Karpe YA, Lole KS. NTPase and 5' to 3' RNA duplex-unwinding activities of the hepatitis E virus helicase domain. *J Virol.* 2010;84:3595–3602.
39. Karpe YA, Lole KS. RNA 5'-triphosphatase activity of the hepatitis E virus helicase domain. *J Virol.* 2010;84:9637–9641.
40. Khudyakov YE, Khudyakova NS, Fields HA, et al. Epitope mapping in proteins of hepatitis E virus. *Virology.* 1993;194:89–96.
41. Panda SK, Nanda SK, Zafrullah M, Ansari IH, Ozdener MH, Jameel S. An Indian strain of hepatitis E virus (HEV): cloning, sequence, and expression of structural region and antibody responses in sera from individuals from an area of high-level HEV endemicity. *J Clin Microbiol.* 1995;33:2653–2659.
42. Jameel S, Zafrullah M, Ozdener MH, Panda SK. Expression in animal cells and characterization of the hepatitis E virus structural proteins. *J Virol.* 1996;70:207–216.
43. Aye TT, Uchida T, Ma X, et al. Sequence comparison of the capsid region of hepatitis E viruses isolated from Myanmar and China. *Microbiol Immunol.* 1992;36:615–621.
44. Huang CC, Nguyen D, Fernandez J, et al. Molecular cloning and sequencing of the Mexico isolate of hepatitis E virus (HEV). *Virology.* 1992;191:550–558.
45. Meng XJ, Purcell RH, Halbur PG, et al. A novel virus in swine is closely related to the human hepatitis E virus. *Proc Natl Acad Sci U S A.* 1997;94:9860–9865.
46. Torresi J, Li F, Locamini SA, Anderson DA. Only the non-glycosylated fraction of hepatitis E virus capsid (open reading frame 2) protein is stable in mammalian cells. *J Gen Virol.* 1999;80(Pt 5):1185–1188.
47. Graff J, Zhou YH, Torian U, et al. Mutations within potential glycosylation sites in the capsid protein of hepatitis E virus prevent the formation of infectious virus particles. *J Virol.* 2008;82:1185–1194.
48. Yamashita T, Mori Y, Miyazaki N, et al. Biological and immunological characteristics of hepatitis E virus-like particles based on the crystal structure. *Proc Natl Acad Sci U S A.* 2009;106:12986–12991.
49. Li TC, Yamakawa Y, Suzuki K, et al. Expression and self-assembly of empty virus-like particles of hepatitis E virus. *J Virol.* 1997;71:7207–7213.
50. Xing L, Kato K, Li T, et al. Recombinant hepatitis E capsid protein self-assembles into a dual-domain T = 1 particle presenting native virus epitopes. *Virology.* 1999;265:35–45.
51. Guu TS, Liu Z, Ye Q, et al. Structure of the hepatitis E virus-like particle suggests mechanisms for virus assembly and receptor binding. *Proc Natl Acad Sci U S A.* 2009;106:12992–12997.
52. He S, Miao J, Zheng Z, et al. Putative receptor-binding sites of hepatitis E virus. *J Gen Virol.* 2008;89:245–249.
53. Zhang H, Dai X, Shan X, Meng J. The Leu477 and Leu613 of ORF2-encoded protein are critical in forming neutralization antigenic epitope of hepatitis E virus genotype 4. *Cell Mol Immunol.* 2008;5:447–456.
54. Zhang JZ, Ng MH, Xia NS, et al. Conformational antigenic determinants generated by interactions between a bacterially expressed recombinant peptide of the hepatitis E virus structural protein. *J Med Virol.* 2001;64:125–132.
55. Li SW, Zhang J, Li YM, et al. A bacterially expressed particulate hepatitis E vaccine: antigenicity, immunogenicity and protectivity on primates. *Vaccine.* 2005;23:2893–2901.
56. Li S, Tang X, Seetharaman J, et al. Dimerization of hepatitis E virus capsid protein E2s domain is essential for virus-host interaction. *PLoS Pathog.* 2009;5:e1000537.
57. Tyagi S, Jameel S, Lal SK. The full-length and N-terminal deletion of ORF2 protein of hepatitis E virus can dimerize. *Biochem Biophys Res Commun.* 2001;286:214–221.
58. Tyagi S, Jameel S, Lal SK. A yeast two-hybrid study on self-association of the ORF2 protein of hepatitis E virus. *Biochem Biophys Res Commun.* 2001;284:614–621.
59. Xing L, Li TC, Mayazaki N, et al. Structure of hepatitis E virion-sized particle reveals an RNA-dependent viral assembly pathway. *J Biol Chem.* 2010;285:33175–33183.
60. Parvez MK, Purcell RH, Emerson SU. Hepatitis E virus ORF2 protein over-expressed by baculovirus in hepatoma cells, efficiently encapsidates and transmits the viral RNA to naive cells. *Virol J.* 2011;8:159.
61. Shiota T, Li TC, Yoshizaki S, Kato T, Wakita T, Ishii K. Hepatitis E virus capsid C-terminal region is essential for the viral life-cycle: implication in viral genome encapsidation and particle stabilization. *J Virol.* 2013;87(10):6031–6036.
62. John L, Thomas S, Herchenroder O, Putzer BM, Schaefer S. Hepatitis E virus ORF2 protein activates the pro-apoptotic gene CHOP and anti-apoptotic heat shock proteins. *PLoS One.* 2011;6:e25378.
63. Surjit M, Varshney B, Lal SK. The ORF2 glycoprotein of hepatitis E virus inhibits cellular NF-kappaB activity by blocking ubiquitination mediated proteasomal degradation of IkappaBalpha in human hepatoma cells. *BMC Biochem.* 2012;13:7.
64. Lal SK, Tulasiram P, Jameel S. Expression and characterization of the hepatitis E virus ORF3 protein in the methylotrophic yeast, *Pichia pastoris*. *Gene.* 1997;190:63–67.
65. Zafrullah M, Ozdener MH, Panda SK, Jameel S. The ORF3 protein of hepatitis E virus is a phosphoprotein that associates with the cytoskeleton. *J Virol.* 1997;71:9045–9053.
66. Kannan H, Fan S, Patel D, Bossis I, Zhang YJ. The hepatitis E virus open reading frame 3 product interacts with microtubules and interferes with their dynamics. *J Virol.* 2009;83:6375–6382.
67. Kar-Roy A, Korkaya H, Oberoi R, Lal SK, Jameel S. The hepatitis E virus open reading frame 3 protein activates ERK through binding and inhibition of the MAPK phosphatase. *J Biol Chem.* 2004;279:28345–28357.
68. Tyagi S, Jameel S, Lal SK. Self-association and mapping of the interaction domain of hepatitis E virus ORF3 protein. *J Virol.* 2001;75:2493–2498.
69. Moin SM, Panteva M, Jameel S. The hepatitis E virus Orf3 protein protects cells from mitochondrial depolarization and death. *J Biol Chem.* 2007;282:21124–21133.
70. Moin SM, Chandra V, Arya R, Jameel S. The hepatitis E virus ORF3 protein stabilizes HIF-1alpha and enhances HIF-1-mediated transcriptional activity through p300/CBP. *Cell Microbiol.* 2009;11:1409–1421.
71. Chandra V, Kar-Roy A, Kumari S, Mayor S, Jameel S. The hepatitis E virus ORF3 protein modulates epidermal growth factor receptor trafficking, STAT3 translocation, and the acute-phase response. *J Virol.* 2008;82:7100–7110.
72. Tyagi S, Surjit M, Roy AK, Jameel S, Lal SK. The ORF3 protein of hepatitis E virus interacts with liver-specific alpha1-microglobulin and its precursor alpha1-microglobulin/bikunin precursor (AMB) and expedites their export from the hepatocyte. *J Biol Chem.* 2004;279:29308–29319.
73. Tyagi S, Surjit M, Lal SK. The 41-amino-acid C-terminal region of the hepatitis E virus ORF3 protein interacts with bikunin, a kunitz-type serine protease inhibitor. *J Virol.* 2005;79:12081–12087.
74. Surjit M, Oberoi R, Kumar R, Lal SK. Enhanced alpha1 microglobulin secretion from hepatitis E virus ORF3-expressing human hepatoma cells is mediated by the tumor susceptibility gene 101. *J Biol Chem.* 2006;281:8135–8142.
75. Graff J, Nguyen H, Yu C, et al. The open reading frame 3 gene of hepatitis E virus contains a cis-reactive element and encodes

- a protein required for infection of macaques. *J Virol.* 2005;79:6680–6689.
76. Takahashi M, Yamada K, Hoshino Y, et al. Monoclonal antibodies raised against the ORF3 protein of hepatitis E virus (HEV) can capture HEV particles in culture supernatant and serum but not those in feces. *Arch Virol.* 2008;153:1703–1713.
77. Yamada K, Takahashi M, Hoshino Y, et al. ORF3 protein of hepatitis E virus is essential for virion release from infected cells. *J Gen Virol.* 2009;90:1880–1891.
78. Emerson SU, Nguyen HT, Torian U, Burke D, Engle R, Purcell RH. Release of genotype 1 hepatitis E virus from cultured hepatoma and polarized intestinal cells depends on open reading frame 3 protein and requires an intact PXXP motif. *J Virol.* 2010;84:9059–9069.
79. Nagashima S, Takahashi M, Jirintai T, et al. A PSAP motif in the ORF3 protein of hepatitis E virus is necessary for virion release from infected cells. *J Gen Virol.* 2011;92:269–278.
80. Kenney SP, Pudupakam RS, Huang YW, Pierson FW, LeRoith T, Meng XJ. The PSAP motif within the ORF3 protein of an avian strain of the hepatitis E virus is not critical for viral infectivity in vivo but plays a role in virus release. *J Virol.* 2012;86:5637–5646.
81. Reyes GR, Huang CC, Tam AW, Purdy MA. Molecular organization and replication of hepatitis E virus (HEV). *Arch Virol Suppl.* 1993;7:15–25.
82. Williams TP, Kasornrondkua C, Halbur PG, et al. Evidence of extra-hepatic sites of replication of the hepatitis E virus in a swine model. *J Clin Microbiol.* 2001;39:3040–3046.
83. de Deus N, Seminati C, Pina S, Mateu E, Martin M, Segales J. Detection of hepatitis E virus in liver, mesenteric lymph node, serum, bile and faeces of naturally infected pigs affected by different pathological conditions. *Vet Microbiol.* 2007;119:105–114.
84. Kawai HF, Koji T, Iida F, Kaneko S, Kobayashi K, Nakane PK. Shift of hepatitis E virus RNA from hepatocytes to biliary epithelial cells during acute infection of rhesus monkey. *J Viral Hepat.* 1999;6:287–297.
85. Krawczynski K, Bradley DW. Enterically transmitted non-A, non-B hepatitis: identification of virus-associated antigen in experimentally infected cynomolgus macaques. *J Infect Dis.* 1989;159:1042–1049.
86. Kapur N, Thakral D, Durgapal H, Panda SK. Hepatitis E virus enters liver cells through receptor-dependent clathrin-mediated endocytosis. *J Viral Hepat.* 2012;19:436–448.
87. Zheng ZZ, Miao J, Zhao M, et al. Role of heat-shock protein 90 in hepatitis E virus capsid trafficking. *J Gen Virol.* 2010;91:1728–1736.
88. Kalia M, Chandra V, Rahman SA, Sehgal D, Jameel S. Heparan sulfate proteoglycans are required for cellular binding of the hepatitis E virus ORF2 capsid protein and for viral infection. *J Virol.* 2009;83:12714–12724.
89. Thakral D, Nayak B, Rehman S, Durgapal H, Panda SK. Replication of a recombinant hepatitis E virus genome tagged with reporter genes and generation of a short-term cell line producing viral RNA and proteins. *J Gen Virol.* 2005;86:1189–1200.
90. Graff J, Nguyen H, Kasornrondkua C, et al. In vitro and in vivo mutational analysis of the 3'-terminal regions of hepatitis E virus genomes and replicons. *J Virol.* 2005;79:1017–1026.
91. Xia X, Huang R, Li D. Studies on the subgenomic RNAs of hepatitis E virus. *Wei Sheng Wu Xue Bao.* 2000;40:622–627.
92. Cao D, Huang YW, Meng XJ. The nucleotides on the stem-loop RNA structure in the junction region of the hepatitis E virus genome are critical for virus replication. *J Virol.* 2010;84:13040–13044.
93. Okamoto H. Hepatitis E virus cell culture models. *Virus Res.* 2011;161:65–77.
94. Chauhan A, Jameel S, Dilawari JB, Chawla YK, Kaur U, Ganguly NK. Hepatitis E virus transmission to a volunteer. *Lancet.* 1993;341:149–150.
95. Tokita H, Harada H, Gotanda Y, Takahashi M, Nishizawa T, Okamoto H. Molecular and serological characterization of sporadic acute hepatitis E in a Japanese patient infected with a genotype III hepatitis E virus in 1993. *J Gen Virol.* 2003;84:421–427.
96. Takahashi M, Kusakai S, Mizuo H, et al. Simultaneous detection of immunoglobulin A (IgA) and IgM antibodies against hepatitis E virus (HEV) is highly specific for diagnosis of acute HEV infection. *J Clin Microbiol.* 2005;43:49–56.
97. Khuroo MS, Rustgi VK, Dawson GJ, et al. Spectrum of hepatitis E virus infection in India. *J Med Virol.* 1994;43:281–286.
98. Nanda SK, Ansari IH, Acharya SK, Jameel S, Panda SK. Protracted viremia during acute sporadic hepatitis E virus infection. *Gastroenterology.* 1995;108:225–230.
99. Ke WM, Tan D, Li JG, et al. Consecutive evaluation of immunoglobulin M and G antibodies against hepatitis E virus. *J Gastroenterol.* 1996;31:818–822.
100. Favorov MO, Khudyakov YE, Mast EE, et al. IgM and IgG antibodies to hepatitis E virus (HEV) detected by an enzyme immunoassay based on an HEV-specific artificial recombinant mosaic protein. *J Med Virol.* 1996;50:50–58.
101. Favorov MO, Fields HA, Purdy MA, et al. Serologic identification of hepatitis E virus infections in epidemic and endemic settings. *J Med Virol.* 1992;36:246–250.
102. Dawson GJ, Mushahwar IK, Chau KH, Gitnick GL. Detection of long-lasting antibody to hepatitis E virus in a US traveller to Pakistan. *Lancet.* 1992;340:426–427.
103. Khuroo MS, Kamili S, Dar MY, Moecklii R, Jameel S. Hepatitis E and long-term antibody status. *Lancet.* 1993;341:1355.
104. Longer CF, Denny SL, Caudill JD, et al. Experimental hepatitis E: pathogenesis in cynomolgus macaques (*Macaca fascicularis*). *J Infect Dis.* 1993;168:602–609.
105. Wu T, Zhang J, Su ZJ, et al. Specific cellular immune response in hepatitis E patients. *Intervirology.* 2008;51:322–327.
106. Saravanabalaji S, Tripathy AS, Dhoot RR, Chadha MS, Kakrani AL, Arankalle VA. Viral load, antibody titers and recombinant open reading frame 2 protein-induced TH1/TH2 cytokines and cellular immune responses in self-limiting and fulminant hepatitis E. *Intervirology.* 2009;52:78–85.
107. Tripathy AS, Das R, Rathod SB, Gurav YK, Arankalle VA. Peripheral T regulatory cells and cytokines in hepatitis E infection. *Eur J Clin Microbiol Infect Dis.* 2012;31:179–184.