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Molecular biology and pathogenesis of hepatitis E virus

Shahid Jameel

Hepatitis E virus (HEV) infection results in hepatitis E, an acute and self-limited disease. The virus is transmitted in a faecal–oral manner and is a major cause of viral hepatitis in much of the developing world, where it causes rampant sporadic infections and large epidemics. A curious feature of hepatitis E is the unusually high rates of mortality that are observed in pregnant women, in whom the disease is exacerbated by the development of fulminant liver disease. In the absence of viable in vitro propagation systems, several geographical isolates of HEV have been maintained in vivo in nonhuman primates and, subsequently, the viral genome has been cloned and sequenced. HEV has been classified provisionally into a separate family known as the HEV-like viruses, which has at least four recognised genotypes, but has only a single serotype. The viral genome is a positive-stranded (+)RNA of ~7.5 kb and encodes at least three proteins. Open reading frame 1 (*ORF1*) encodes the viral nonstructural polyprotein, which has domains that are homologous to some of the replication and processing enzymes found in other +RNA viruses. The HEV protein itself remains poorly characterised. The protein encoded by open reading frame 2 (*ORF2*) is the major HEV capsid protein, and the protein encoded by open reading frame 3 (*ORF3*) appears to be involved in virus–host interactions. Several questions related to the biology, epidemiology and pathogenesis of HEV remain unanswered; the progress of a few of these is reviewed here.

In the winter of 1955–56, a large epidemic of acute viral hepatitis, affecting 29 000 people, followed an incident of sewage contamination of drinking water in New Delhi, India. Although it was originally considered to be an epidemic of hepatitis A, retrospective testing of stored sera from these patients suggested that a novel infectious agent was responsible for the epidemic. This disease, which was initially described from the Indian subcontinent, was first called enteric non-A, non-B hepatitis (ET-NANBH). Since the early 1990s, following the identification and sequencing of its aetiological agent, the disease became known as hepatitis E and its agent as hepatitis E virus (HEV) (Ref. 1). The 'E' can stand for 'enteric' (in the gut), 'endemic' or 'epidemic', all of which are features that adequately describe the epidemiology of HEV. Fortuitously, 'E' also

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Accession information: (99)00127-1a.pdf (short code: txt001sjd); 6 December 1999
ISSN 1462-3994

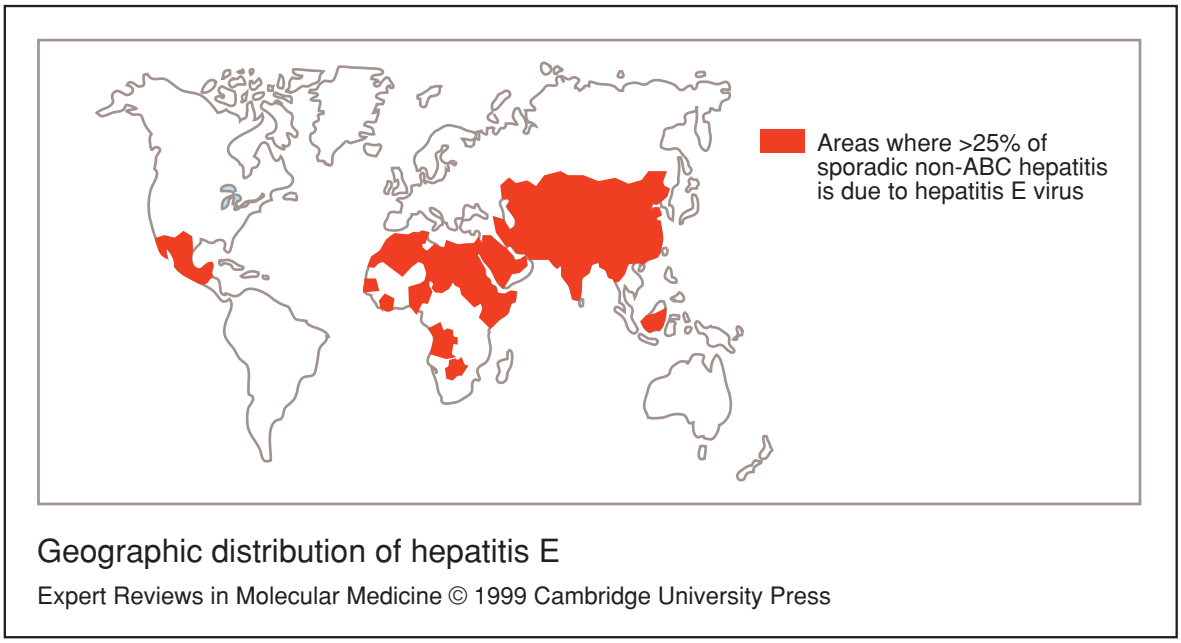

Figure 1. Geographic distribution of hepatitis E. Regions of the world where >25% of sporadic non-ABC hepatitis is due to hepatitis E virus (HEV) are shown in red. Adapted from the viral hepatitis slide set published by the US Centers of Disease Control and Prevention, Atlanta, GA, USA, at http://www.cdc.gov/ncidod/diseases/hepatitis/slideset **(fig001sjd)**.

makes sense alphabetically because HEV was the fifth infectious agent shown to be associated with hepatitis in humans, after the hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV) and the delta agent (HDV), a viroid that superinfects carriers of HBV.

Epidemiology of hepatitis E

HEV causes epidemics of viral hepatitis, often involving very large numbers of patients. Such epidemics have been reported from several countries on all continents, although most are in tropical and subtropical areas of the world (Ref. 2). Apart from the 1955–56 New Delhi epidemic, other notable epidemics have occurred: Kirgiz Republic, Soviet Union (1955–56, 10 000 cases); Kathmandu Valley, Nepal (1973–74, 10 000 cases); Mandalay, Myanmar (1976–77, 20 000 cases); Kashmir, India (1978–82, 52 000 cases); Xinjiang, China (1986–88, 120 000 cases); and Kanpur, India (1991, 79 000 cases). In addition to epidemic hepatitis, HEV causes rampant sporadic infections in endemic areas. Figure 1 shows the regions of the world where more than 25% of sporadic non-ABC hepatitis cases are due to HEV infection. In India, for example, ~30% of all sporadic viral hepatitis is due to infection by HEV.

HEV is transmitted primarily through the faecal–oral route in contaminated drinking water (Ref. 2). The disease is found most frequently in geographical regions and situations, such as refugee camps, where faecal contamination of the drinking water supply is frequent. Although they have only minor contributions, other routes of transmission cannot be ruled out. For example, vertical transmission in utero of HEV from infected mothers to their newborn has been documented (Ref. 3). The possibility of parenteral transmission has also been suggested (Ref. 4), especially in endemic areas. However, person-to-person transfer is minimal because household contacts of HEV-infected patients do not appear to be at an increased risk.

As expected, the seroprevalence of anti-HEV antibodies in endemic areas is significantly higher than in areas where HEV infection is rare. Acute hepatitis E has been reported in patients residing in developed countries such as Australia, France, Israel, The Netherlands, Spain, UK and USA. These are mainly associated with travel to endemic areas (Ref. 2); however, rare cases of acute hepatitis E have also been reported from these regions in the absence of travel to risk areas. HEV infection is also probably more prevalent in industrialised nations

Accession information: (99)00127-1a.pdf (short code: txt001sjd); 6 December 1999
ISSN 1462-3994 ©1999 Cambridge University Press

than previously appreciated; for example, fulminant HEV-associated hepatitis is found in Europe, where ~1.5% of healthy adults have anti-HEV antibodies (Ref. 5). In addition, in the USA, domesticated swine have been found to be infected with HEV, and sequence analysis has shown that swine-derived HEV is similar to a human-derived HEV from a USA isolate (Ref. 6).

The age-specific prevalence of anti-HEV antibodies has also been studied in endemic areas and compared with antibodies to HAV (Ref. 7). Whereas anti-HAV seroprevalence reaches >95% by 10 years of age in endemic areas, anti-HEV seroprevalence in an identical population slowly increases until the third decade of life. This pattern suggests a sporadic transmission of the virus that accumulates over age; this is consistent with the predominantly subclinical nature of infections, the short periods of infectious viraemia (virus in the bloodstream), and the consequently limited pool of HEV infection in the community. Lower prevalence rates of anti-HEV versus anti-HAV antibodies in the same population also suggests that, following infection, antibodies to HEV might disappear from circulation faster than antibodies to HAV, and that children might not mount such a brisk anti-HEV antibody response compared with that mounted by adults. However, these issues remain unresolved, owing mainly to the lack of sensitive and specific diagnostic systems that are suitable for use in endemic areas.

Pathogenesis and clinical spectrum of hepatitis E

Although the hepatitis viruses cause liver damage, none is directly cytopathic to hepatocytes (liver cells). Following acute liver injury, the clinical manifestations and outcome of viral hepatitis are actually determined by the host immune response. Because serological (antibody-based) assays for HEV have only recently become available, the pathogenesis of hepatitis E is not well understood. Following the entry of HEV into the host via the oral route, the primary site of replication is probably in the intestinal tract. It is still not clear how the virus reaches the liver, but it is presumably via the portal vein serving the liver. HEV replicates in the cytoplasm of hepatocytes (Ref. 8) and is released into the bile and bloodstream by mechanisms that are not understood.

The symptoms of hepatitis E are typical of acute viral hepatitis and the infection follows a natural history that is similar to that of hepatitis A. The incubation period (the time from infection to clinical symptoms) has been measured accurately only in a single case of transmission of HEV to a human volunteer, and was found to be 32 days (Ref. 4). Infectious viral particles are present in the bile and faeces during the late incubation phase of hepatitis E and they persist for a week or two following the onset of clinical disease. The virus is also present transiently in the bloodstream in the late incubation phase of hepatitis E, but it disappears just before the onset of clinical symptoms (Ref. 4). Anti-HEV antibodies of the IgA, IgG and IgM types appear during the course of disease. IgM antibodies are detectable in the acute phase and disappear in 3–6 months whereas, in various studies, IgG antibodies have been shown to persist for 2–13 years (Ref. 2). In ~10% of patients with HEV infection, protracted viraemia has been observed in the absence of anti-HEV antibodies (seroconversion) (Ref. 9). Seroconversion might be a critical marker for early clearance of the virus from the bloodstream, but this requires more extensive analysis. The correlation between clinical disease, viraemia and antibody response is shown in Figure 2.

In most hepatitis E outbreaks, the highest rates of clinically evident disease have been reported in young to middle-age adults; the lower disease rates in younger age groups might be the result of anicteric (i.e. without the elevation of serum bilirubin that is used as a marker of clinical jaundice) and/or subclinical HEV infection (Ref. 2). Hepatitis E is more severe than hepatitis A, with mortality rates in the range of 1–2%, compared with ~0.2% for hepatitis A.

Hepatitis E in pregnancy

One distinctive clinical feature of hepatitis E, compared with other forms of viral hepatitis, is its increased incidence and severity in pregnant women (Ref. 10), which results in up to 20% mortality. By contrast, none of the other recognised hepatitis viruses causes such severe hepatitis in pregnancy. Though the mechanism(s) is not known, a hypothesis has been put forward to explain the pathogenesis of fulminant hepatitis E in pregnancy (Ref. 11). This suggests that the liver sinusoidal cells, particularly the Kupffer cells, are damaged by HEV, which diminishes the ability of these cells to protect hepatocytes against endotoxins that originate from Gram-negative bacteria found in the intestinal tract. Hepatocytes can be injured directly by endotoxins or indirectly

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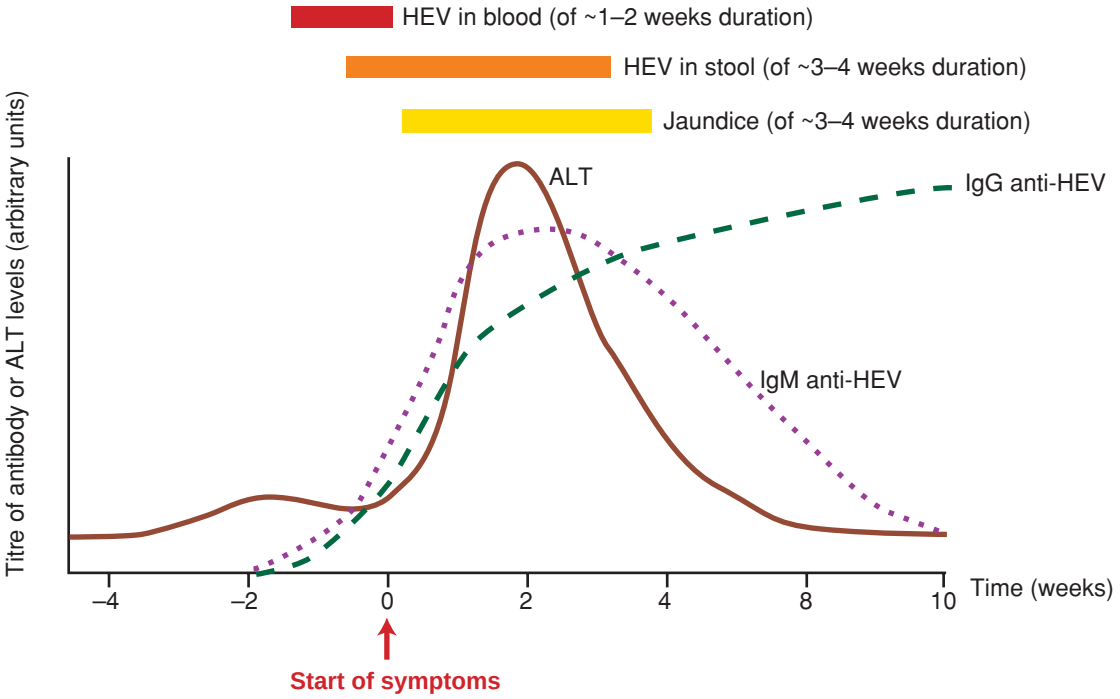

Time course of hepatitis E virus infection

Figure 2. Time course of hepatitis E virus infection. Biochemical markers (e.g. serum ALT levels) and symptomatic markers (e.g. jaundice) of viral hepatitis are correlated with detection of HEV RNA by RT-PCR in the bloodstream, or shedding of virus in stools, and the immune response is measured as anti-HEV IgM or IgG levels, detected by enzyme immunoassay on serum samples. Four to eight weeks after exposure to HEV, there is a rise in ALT and the appearance of jaundice. Immediately prior to the onset of clinical symptoms, HEV can be detected in the bloodstream for ~1–2 weeks and is shed in the stools for ~3–4 weeks. At the onset of clinical symptoms, HEV is lost from the bloodstream, but continues to be shed in stools. Anti-HEV IgM and IgG titres continue to increase in the asymptomatic phase. The anti-HEV IgM titre peaks during the symptomatic phase and declines thereafter to baseline values within 3–6 months of symptomatic disease. The anti-HEV IgG titre remains detectable for 2–13 years as determined in various studies. Abbreviations: ALT, alanine aminotransferase; HEV, hepatitis E virus; Ig, immunoglobulin; RT-PCR, reverse transcriptase polymerase chain reaction. Modified from the viral hepatitis slide set published by the US Centers of Disease Control and Prevention, Atlanta, GA, USA, at http://www.cdc.gov/ncidod/diseases/hepatitis/slideset **(fig002sjd)**.

by eicosanoids, which are 20-carbon chain (C_{20}) polyunsaturated fatty acids that cause platelet aggregation, inflammation, and other effects. Release of prostaglandins (a type of eicosanoid) can lead to chemotactic attraction of inflammatory neutrophils. This can result in swelling of the tissue by water accumulation (oedema) and arrest of bile flow (cholestasis). The enhanced sensitivity of pregnant women to such an endotoxin-mediated effect is well recognised and might explain the strikingly high mortality of hepatitis E in pregnancy (Ref. 11). However, the validity of this hypothesis and the precise cellular/molecular mechanisms underlying it have not been confirmed.

The kidneys of cynomolgus monkeys (*Macaca fascicularis*) infected intravenously with HEV were shown to develop acute tubular necrosis with focal haemorrhages, suggesting that HEV can replicate in monkey (and possibly also human) kidneys. By affecting this tissue, HEV might precipitate pregnancy-associated eclampsia, leading to increased mortality in pregnant women (Ref. 12). One feature observed in patients with eclampsia is disseminated

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ISSN 1462-3994 ©1999 Cambridge University Press

intravascular coagulation affecting the liver and kidneys. In pregnant women, a high incidence of disseminated intravascular coagulation associated with hepatitis E is well recognised. However, in experimental HEV infection of pregnant monkeys, no increased mortality has been observed, casting doubt on whether this is a good model for this aspect of human hepatitis E.

Liver histology of patients with hepatitis E reveals portal triaditis, cholestasis, lobular inflammation and degeneration of the liver to varying degrees, which are all suggestive of acute viral hepatitis. However, nearly half of the patients have distinctive morphological changes designated as cholestatic viral hepatitis. The discrepancy between the time of appearance of viral replication in the liver with the histopathological and biochemical changes suggests that HEV might not be directly cytopathic and its pathogenesis might be immunologically mediated. However, there is no direct evidence for, or against, this hypothesis (Ref. 11).

It is not known whether HEV causes other sequelae or extrahepatic manifestations. None has been recognised apart from the increased incidence of miscarriage, which has been reported in some, but not all, studies on fulminant hepatitis E during pregnancy.

Classification and phylogeny of HEV

To date, HEV has not been classified conclusively into any virus family. On the basis of morphological similarities to the Norwalk agent, it was placed first into the family *Calciviridae*, under a separate genus (Ref. 13); however, the genome organisations of HEV and the Norwalk agent are different. On the basis of homologous regions across the genome and the production of subgenomic RNA during HEV replication, it has also been suggested that HEV might be a non-enveloped alpha-like virus (Ref. 14). New recommendations of the International Committee on the Taxonomy of Viruses (http://www.ncbi.nlm.nih.gov/ICTV/) now place HEV into a separate family called HEV-like viruses. Analysis of the RNA helicase (Hel) and RNA-dependent RNA polymerase (RdRp) regions of HEV and other positive-stranded RNA (+RNA) viruses show that HEV forms a phylogenetically distinct group, closer to rubella virus (family *Togaviridae*) than to members of the family *Calciviridae* (Ref. 15). However, a conclusive classification of HEV awaits further knowledge of its expression and replication strategy and of the nature, processing and properties of its component proteins.

Different geographical isolates of HEV have been the focus of phylogenetic comparisons (Ref. 16). Although a single serotype of HEV is recognised worldwide, comparisons of the nucleotide sequences of regions within the nonstructural and structural ORFs of HEV show at least four phylogenetically distinct clades, which correspond to the geographical origin of the isolates (Fig. 3). These include two groups of isolates from Asia (Asia-1 and Asia-2), one from North Africa, and one from North America that also includes the recently described swine HEV (swine-1 isolate). It is interesting to note the phylogenetic clustering of swine HEV with the US-1 isolate that was obtained from a person with no record of travel to HEV-endemic areas. Such relatedness suggests a role for zoonotic (animal-acquired) reservoirs in the transmission of HEV. Because the extent of genetic variation observed in HEV genomes from various isolates is small, this geographical clade distribution might represent independent expansion of ancestral HEV isolates in different regions of the world.

Virus biology

Animal models and in vitro culture

Several nonhuman primates have been used for HEV transmission studies (Ref. 2). The most useful of these have been cynomolgus macaques and rhesus macaques (*Macaca mulatta*). Pigs (Ref. 6) and rats (Ref. 17) have also been reported to be susceptible to infection with HEV; however, unlike primates, experimentally infected pigs developed jaundice (a clinical sign of liver disease), suggesting that the disease was relatively severe. In experimentally infected rats, histopathological changes and/or viral antigens were observed in the duodenum, spleen, mesenteric lymph nodes (in the abdomen) and in peripheral blood mononuclear cells, suggesting that HEV replication in rats can occur in these tissues as well as in the liver (Ref. 17).

The replication of HEV in cell culture [in FRhK (rhesus kidney) cells] has been reported for an HEV isolate recovered in Russia (Ref. 18), and in 2BS diploid human embryonic lung cells (Ref. 19) and A549 human diploid cells (Ref. 20) for a Chinese HEV isolate. Recently, a tissue culture system has been developed to propagate HEV in vitro (Ref. 21); in this system, hepatocytes isolated from experimentally infected cynomolgus

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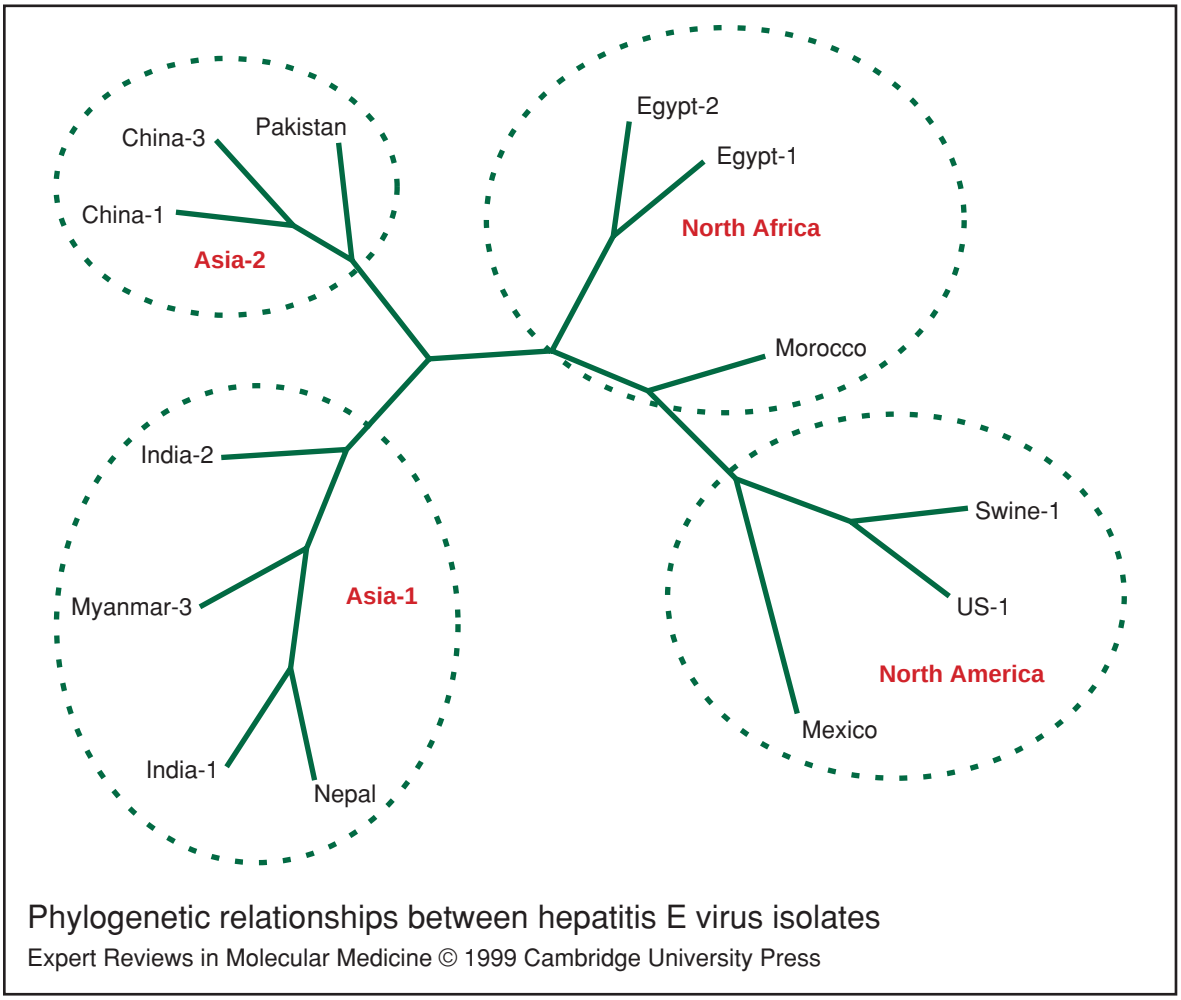
ISSN 1462-3994



Phylogenetic relationships between hepatitis E virus isolates

Figure 3. Phylogenetic relationships between hepatitis E virus isolates. Nucleotide sequences encoding the structural regions [open reading frame 2 (*ORF2*) and *ORF3*] of selected hepatitis E virus (HEV) isolates from different geographic locales were subjected to phylogenetic analyses (S. Jameel, unpublished). The sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/). The Asia-1 clade includes India-1 (AF076239), India-2 (X99441), Nepal (AF051830) and Myanmar-3 (M73218) isolates; the Asia-2 clade includes China-1 (D11092), China-3 (L25547) and Pakistan (M80581) isolates; the North Africa clade includes Egypt-1 (AF051352), Egypt-2 (AF051351) and Morocco (AF065061) isolates; and the North America clade includes Mexico (M74506), US-1 (AF060668) and Swine-1 (AF082843) isolates. The 2024-nucleotide sequences were first aligned using Clustal in the McVector v6.0 software package (Oxford Molecular Group, UK). Phylogenetic analyses were then carried out with algorithms in the PHYLIP v3.5 package (http://evolution.genetics.washington.edu/phylip.html), as follows: the aligned sequences were subjected to 5000 rounds of bootstrapping with SEQBOOT; the bootstrapped replicates were then subjected to either DNA parsimony analysis with DNAPARS or DNA distance analysis using DNADIST and NEIGHBOR; and unrooted consensus trees were drawn using CONSENSE and DRAWTREE with the Mexico sequence as the outgroup. The illustration shows a DNA parsimony tree; the DNA distance tree also showed identical relationships between various isolates. Results from other phylogenetic analyses using shorter stretches of nucleotide sequences from various isolates of HEV also demonstrate similar relationships (Ref. 16) **(fig003sjd)**.

macaques can be maintained in long-term culture and can produce HEV, albeit at low levels; this production has been confirmed by immunoelectron microscopy (Ref. 21). Although cytopathic effects of HEV infection have been reported, HEV replication appears to be very inefficient in this system because it can only be detected by reverse transcriptase polymerase chain reaction (RT-PCR). This disappointing replication compromises the utility of the present

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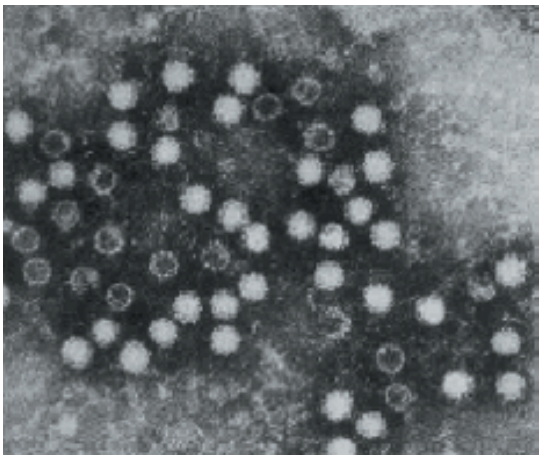

Hepatitis E virus

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Figure 4. Hepatitis E virus. An immunoelectron micrograph of hepatitis E virus (HEV) from the stool of a patient acutely infected with the Burmese isolate. The picture shows 27–34 nm particles aggregated with antibodies present in the serum of a patient infected with HEV. Obtained from the viral hepatitis slide set published by the US Centers of Disease Control and Prevention, Atlanta, GA, USA, at http://www.cdc.gov/ncidod/diseases/hepatitis/slideset **(fig004sjd)**.

in vitro propagation systems for studies on viral biology and for the eventual generation of sufficient quantities of virus for vaccine studies (e.g. the development of attenuated vaccine strains of HEV).

Genome organization of HEV

HEV is a spherical, nonenveloped, RNA virus that is approximately 32–34 nm in diameter (Fig. 4). The viral genome has been cloned and sequenced from several geographically distinct HEV isolates and shows a high degree of sequence conservation, both of nucleotides and of amino acids (Refs 22, 23, 24, 25, 26). The HEV genome is a +RNA of ~7.5 kb, spanning a coding region that includes three open reading frames (ORFs) (Ref. 22) (Fig. 5). Of these, *ORF1* (of ~5 kb) is predicted to encode the viral nonstructural polyprotein, *ORF2* (of ~2 kb) encodes the viral major capsid protein, and *ORF3*, the smallest one, encodes a small protein of undefined function. All three ORFs are expressed during viral infection as shown by the finding that antibodies directed against epitopes that are present on the proteins encoded by the translated ORFs are found in infected humans and experimental animals (Refs 26, 27). The viral RNA also contains short 5' and 3'-untranslated regions (UTRs) of 26 and 68 nucleotides, respectively (Ref. 21). These have the potential to fold into conserved stem–loop and hairpin structures. Such secondary structures are also found within a conserved 58-nucleotide region within *ORF1* (Ref. 21). These regions, together with a region showing homology to the junction sequence of Sindbis virus (Fig. 5), are postulated to be important for HEV RNA replication (Ref. 14).

Replication and expression of the HEV genome

A proposed model

A general model of HEV replication and gene expression has been proposed, based on similarities and sequence homology to other more completely characterised +RNA viruses (Ref. 28). As shown schematically in Figure 6, following entry into a permissive cell, the viral genomic RNA is translated in the cytosol of infected cells to produce the nonstructural *ORF1*-encoded polyprotein (nsP). Because it contains the viral replicase, nsP is postulated to replicate the genomic positive strand into the negative strand replicative intermediate. This intermediate, in analogy with alphaviruses, is postulated to act as a template for the synthesis of additional copies of the genomic positive strand as well as a subgenomic positive strand. The analogy to alphavirus replication is presumed because of the presence in HEV negative-strand RNA of a sequence stretch that is similar to the junction sequence found in the RNA replicative intermediate of the Sindbis alphavirus. During alphavirus RNA replication, this junction sequence acts as a subgenomic promoter for transcription of the structural region mRNA. The proposed subgenomic positive-strand HEV RNA can then be translated into the structural protein(s) at late stages of viral replication. The structural (capsid) protein then probably packages the viral genome to form progeny virions. However, direct experimental confirmation of this replication scheme is still awaited.

Evidence for the model

In experimentally infected cynomolgus macaques, the presence of one HEV genomic (~7.5 kb) and

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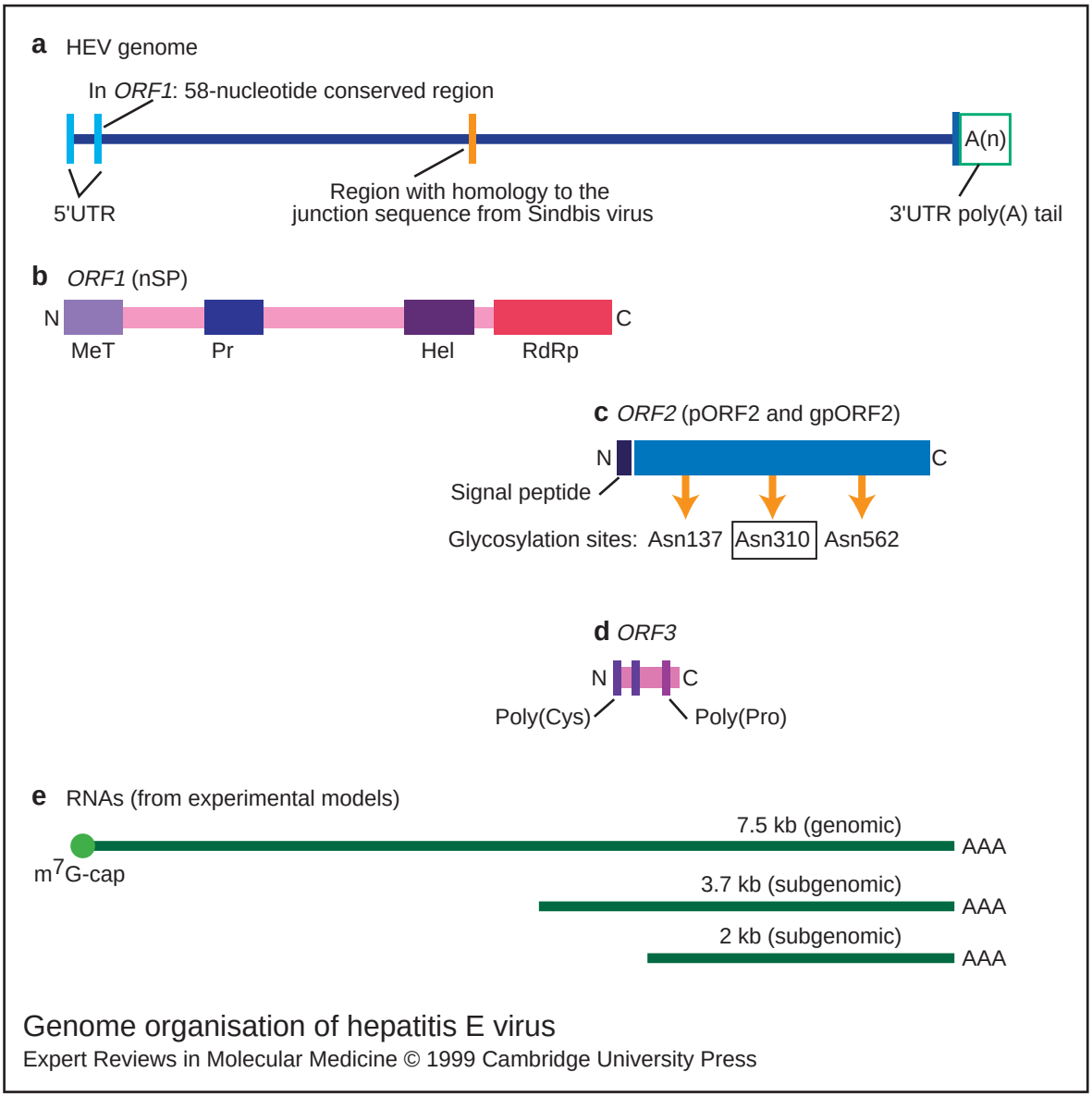

Figure 5. Genome organisation of hepatitis E virus (*see next page for legend*) **(fig005sjd)**.

two subgenomic (~3.7 kb and ~2 kb) RNAs has been shown (Ref. 21) (Fig. 5). In the rhesus macaque model, HEV positive- and negative-strand RNA have been demonstrated in the liver, which is the primary site of viral replication in this animal model. In the serum and bile, where only mature virions are expected, only positive-strand viral RNA was found (Ref. 29). This strongly indicates that, at least in this model, the HEV genomic RNA replicates through a negative-strand RNA intermediate, as proposed above. It has been shown recently (Ref. 30) that the genomic RNA of HEV is capped; that is, it carries a covalently attached 7-methylguanosine (m^7G) nucleotide. Such an addition, together with a short 5'UTR is compatible with cap-mediated translation of HEV genomic RNA and not through an internal ribosome entry site (IRES)-mediated mechanism observed in some other positive-strand RNA viruses, such as enteroviruses (poliovirus, HAV, etc.) and HCV. The 5' and 3' ends of HEV RNA exhibit conserved secondary structures, which are presumed to be involved in viral RNA replication, acting as binding sites for the replicase and host factors. An understanding of these RNA–protein interactions will be crucial

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ISSN 1462-3994 ©1999 Cambridge University Press

Figure 5. Genome organisation of hepatitis E virus. (a) The hepatitis E virus (HEV) genome is a ~7.5 kb polyadenylated RNA. At its 5' and 3' termini, the viral RNA carries two short (26- and 68-nucleotide) untranslated regions (UTRs) that form conserved stem–loop structures. A 58-nucleotide conserved region within *ORF1* can also fold into two hairpin loops. Within *ORF1*, the viral RNA also carries a sequence stretch with homology to the junction sequences found in Sindbis virus (an alphavirus). It is positive-sense and includes three open reading frames (ORFs): (b) *ORF1* (~5 kb), encoding a putative nonstructural polyprotein (nsP) that includes domains found in viral methyltransferases (MeT), papain-like cysteine proteases (Pr), viral RNA helicases (Hel) and viral RNA-dependent RNA polymerases (RdRp); (c) *ORF2* (~2 kb), encoding the major viral capsid protein (pORF2 and its glycosylated form gpORF2), carrying a signal peptide at its N-terminal end and three N-linked glycosylation sites [Asn137, Asn310 and Asn562, of which Asn310 (boxed) appears to be the major site of N-linked glycan addition]; (d) *ORF3*, encoding a small protein (pORF3) with two hydrophobic domains in its N-terminal half, which includes a polycysteine [Poly(Cys)] region, and proline-rich [Poly(Pro)] sequences in its C-terminal half. pORF3 is phosphorylated at a single (Ser80) amino acid by the cellular mitogen-activated protein kinase (MAPK), associates with the cytoskeleton through its N-terminal end and with proteins carrying the *src*-homology 3 (SH3) motifs through its C-terminal end. (e) Three HEV-specific RNAs have been found in the livers of experimentally infected monkeys: a genomic ~7.5 kb [covalently capped with a 7-methylguanosine (m^7G) nucleotide] and two subgenomic ~3.7 kb and ~2 kb species. This indicates a replication scheme similar to alphaviruses in which the structural genes are translated from subgenomic RNAs generated late in viral replication, but unlike other positive-stranded RNA viruses such as poliovirus or hepatitis C virus in which the genomic RNA is directly translated into the complete viral polyprotein **(fig005sjd)**.

for understanding replication of the HEV genome and therapeutic strategies aimed at preventing it.

HEV-encoded proteins

The HEV genome encodes at least three different polypeptides – the products of *ORF1*, *ORF2* and *ORF3*. Several reports describe the expression of *ORF2*- and *ORF3*-encoded proteins in prokaryotic as well as eukaryotic systems; however, experimental expression of the *ORF1*-encoded protein has so far not been reported. The functional analysis of the *ORF1*-encoded polyprotein or its constituent domains is either lacking or extremely limited in its scope, but would be useful information because it includes putative enzyme activities that would be important for HEV genome replication and expression.

The nonstructural ORF1-*encoded protein*

ORF1, the largest of HEV ORFs, is postulated to encode the viral nonstructural proteins. This is suggested from homology of the translation product of *ORF1* with protein motifs that are found in other positive-stranded RNA viruses (Ref. 14). The postulated regions include a viral methyltransferase (MeT), a papain-like cysteine protease (Pr), the Hel helicase and RdRp polymerase (Fig. 5). The presence of a m^7G cap at the 5' end of the HEV genomic RNA (Ref. 30) confirms a functional role for the viral methyltransferase. It is not yet clear whether the *ORF1*-encoded polyprotein itself (1693 amino acids) contains multiple biochemical activities, or whether it undergoes cis- or trans-processing to release biochemically distinct individual units. It will be important to know this, because when the HEV protease and RdRp are biochemically characterised, they might be potential targets for developing antiviral therapeutic agents.

The ORF2-*encoded protein*

ORF2 encodes the major viral capsid protein (pORF2), which has 660 amino acids. In animal cells, it is expressed in a ~74 kDa form (pORF2) and a ~88 kDa glycosylated form (gpORF2) (Ref. 31). Results with various experimentally generated mutant forms of the protein show that it carries an N-terminal signal sequence (Fig. 5), which translocates the pORF2 across the endoplasmic reticulum (ER) (Ref. 32). The pORF2 is glycosylated (to gpORF2) in the ER by the addition of N-linked high mannose sugar residues, and the ER also appears to be the major site of accumulation of gpORF2 (Ref. 32). There are three glycosylation sites on pORF2: asparagine (Asn) residues at amino acid positions 137, 310 and 562 (Fig. 5). Whereas all three sites are glycosylated, Asn310 appears to be the major site of N-glycan addition (Ref. 32). The *ORF2*-encoded protein can also been seen on the cell surface; while transit of pORF2 into the ER is required for cell-surface expression, glycosylation is not required (Ref. 32). Recently, it has been suggested that gpORF2 is an unstable form of the protein (Ref. 33). Although pORF2, the nonglycosylated

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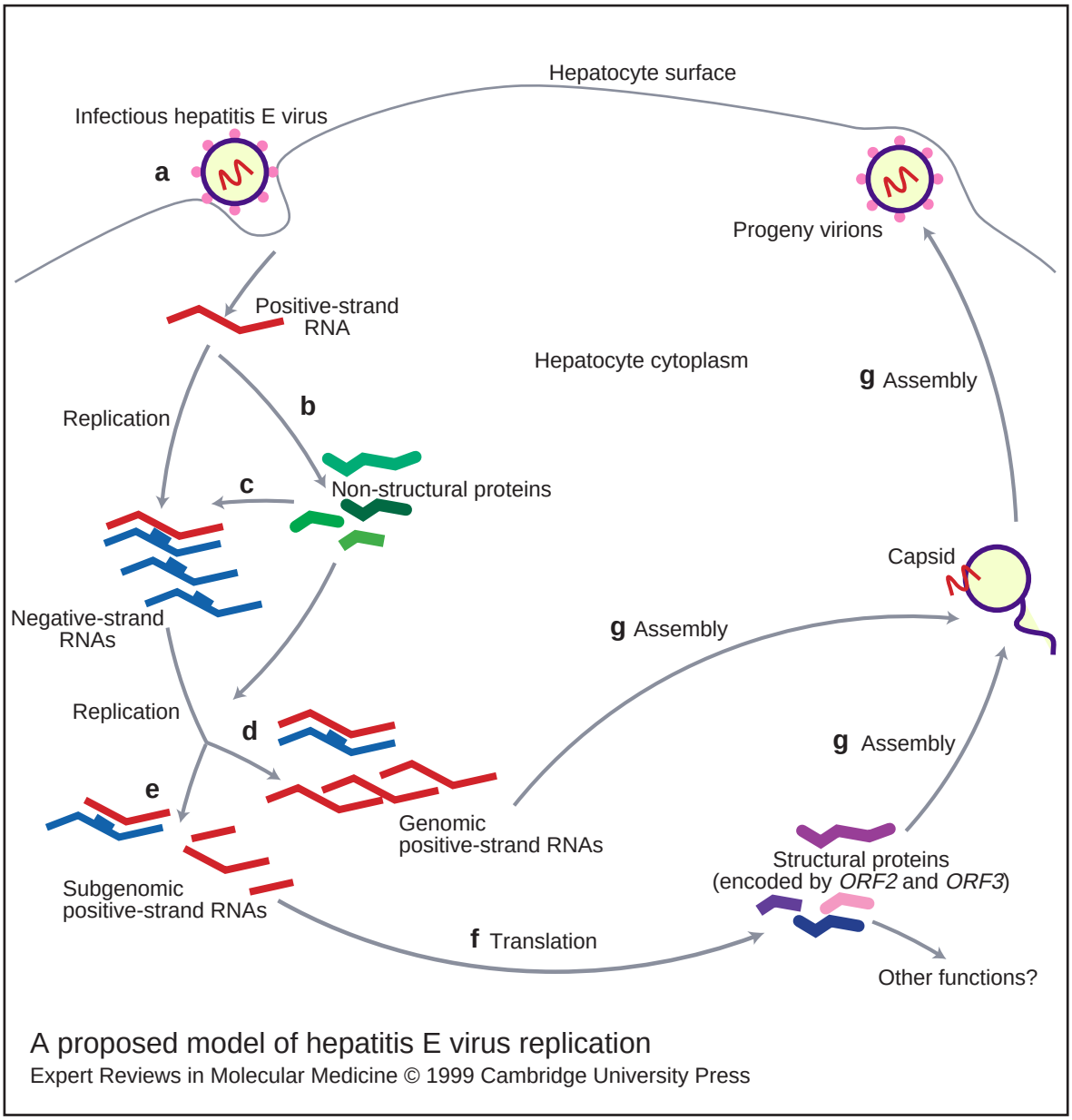

Figure 6. A proposed model of hepatitis E virus replication. This model is based on domain homologies between hepatitis E virus (HEV) and other positive-stranded RNA viruses. (a) Following attachment to an as-yet-uncharacterised receptor on the surface of hepatocytes, HEV is internalised and uncoated in the cytoplasm by unknown mechanisms. (b) The genomic positive-strand RNA is translated into nsP, the nonstructural polyprotein encoded by *ORF1*, which can be processed into individual functional units that might include methyltransferase, protease, helicase and replicase activities. (c) The replicase so generated can use the positive-strand RNA as a template to synthesise the negative-strand replicative intermediates. Because of homology to alphaviral junction sequences (shown as a boxed region on the negative-strand RNA), it is proposed that two classes of positive-strand RNA species, genomic (d) and subgenomic (e), are synthesised from the negative-strand RNA intermediates. (f) The subgenomic RNAs are translated into pORF2 viral structural proteins encoded by *ORF2* (and possibly pORF3 encoded by *ORF3*). (g) The structural protein subunits assemble into a capsid that includes the genomic positive-strand RNA to form progeny virions, which can infect other cells or be shed as infectious virus. The *ORF2*- and *ORF3*-encoded proteins might also provide other functions. Experimental proof for most of the steps in this model is awaited. Modified from a diagram in Ref. 2 **(fig006sjd)**.

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ISSN 1462-3994 ©1999 Cambridge University Press

protein, is proposed to take part in capsid assembly, the role of gpORF2 in the cell is not yet clear.

The *ORF2*-encoded protein has been characterised using various expression systems, including: (1) in *Escherichia coli*; (2) in insect cells using baculoviral vectors; and (3) in animal cells using expression plasmids, recombinant vaccinia viruses and alphaviruses. However, the results from infection of insect cells with recombinant baculoviruses appear to be unique because multiple forms of the protein (of ~72 kDa, ~55–60 kDa and ~52–55 kDa) were expressed. It has been observed that whereas the larger forms of *ORF2*-encoded proteins are cell associated, the ~52–55 kDa form is secreted in the forms of self-aggregated virus-like particles (VLPs) (Refs 34, 35, 36). Protein microsequencing has shown that the ~52–55 kDa form of *ORF2*-encoded protein lacks 111 amino acids at its N-terminal end and 51 amino acids at its C-terminal end. However, neither these multiple forms of the *ORF2*-encoded protein nor secretion of VLPs in the culture media have been observed following expression in animal cells (S. Jameel, unpublished). The unique results on *ORF2* expression with baculoviral vectors are possibly the result of intracellular processing by a protease that is found only in insect, but not in vertebrate, cells. Even in insect cells, VLP formation and secretion appear to be more efficient in cells from the insect *Trichopulsia ni* (Tn5 cell line) compared with cells from the insect *Spodoptera frugiperda* (Sf9 cell line) (Ref. 34). Either way, these observations are interesting and might shed some light on pORF2 expression and capsid formation. Furthermore, the *ORF2*-encoded protein that was over-expressed in insect cells is a promising candidate for a recombinant subunit vaccine for hepatitis E (discussed later).

The ORF3*-encoded protein*

HEV *ORF3* encodes pORF3, a small protein of 123 amino acids with an undefined function. When expressed in animal cells, pORF3 is a phosphoprotein that is modified at a single serine residue (Ser80) by the cellular mitogen-activated protein kinase (MAPK) (Ref. 37). On subcellular fractionation, pORF3 appears to associate with the cytoskeleton using one of its N-terminal hydrophobic domains (Ref. 37), which contains a polycysteine stretch (Fig. 5). The C-terminal half of pORF3 is rich in proline residues [Poly(Pro) on Fig. 5] and contains two stretches with homology to the polyproline helices that are present in proteins that bind the *src*-homology 3 (SH3) domains found in protein tyrosine kinases (PTKs) and some cytoskeletal proteins. Preliminary results show that purified pORF3 binds to glutathione-*S*-transferase (GST) fusion proteins containing SH3 domains from several different proteins (H. Korkaya and S. Jameel, unpublished). SH3 domains are found in proteins involved in signal transduction pathways. Together with other interaction domains, these are used to make critical protein–protein contacts essential for signal transduction. The phosphorylation of pORF3 by MAPK also suggests a possible role of this viral protein in signal transduction in HEV-infected cells. Its association with the cytoskeleton using the N-terminal end of pORF3, and a possible binding of SH3-containing targets to its C-terminal end, suggests that pORF3 might act as an adaptor protein. Adaptor proteins are known to play critical roles in the intracellular transduction of extracellular signals (Ref. 38). For example, through its interactions, pORF3 might recruit PTKs to the cytoskeleton. The phosphorylation of cytoskeletal proteins plays an important regulatory role in many cellular processes and it is possible that such interactions might also favour the replication of HEV RNA or the assembly of progeny HEV virions. A role for an HEV protein in the transduction of cytokine or hormonal signals would provide further molecular clues to the observed high mortality associated with HEV infection during pregnancy.

Outstanding questions in hepatitis E

Much has been written about the epidemiology and pathogenesis of hepatitis E, and several questions remain unanswered. However, some progress has been made recently in answering a few of these, as reviewed below.

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

Sporadic infections can maintain HEV in the population during inter-epidemic periods. Rampant sporadic infections occur in endemic areas and protracted viraemia has been observed in ~10% of the cases with acute disease (Ref. 9). In addition, subclinical infections are prevalent in endemic areas, which would contribute to the pool of HEV in the community. The presence of HEV (based on RT-PCR amplification of viral RNA) has been demonstrated in raw and treated wastewater (Ref. 39,) and large-scale

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ISSN 1462-3994

contamination of drinking water supplies often occur in endemic areas.

The possibility of nonhuman reservoirs of HEV has also been suggested. Serological evidence for HEV infection has been obtained in wild-caught rodents, nonhuman primates, domesticated swine, and chicken farmed for eating and eggs (Ref. 40). The presence of anti-HEV antibodies in swine has been positively correlated to proximity of the swine to human jaundice cases (Ref. 41). Definitive evidence for zoonotic reservoirs of HEV comes from the cloning of a swine HEV variant (Ref. 6) and two human HEV variants (US-1 and US-2) from patients in the USA who had no history of travel to an HEV-endemic area (Ref. 42). As shown above, the swine-1 and US-1 strains of HEV cluster together on a phylogenetic tree, away from other sporadic and epidemic strains of HEV that were recovered from humans living in endemic areas (Fig. 3). In addition, swine HEV has been shown to be infectious to nonhuman primates, and the US-2 strain of HEV was capable of infecting (otherwise pathogen-free) swine. These results suggest a strong possibility that domestic animals can be carriers of HEV and that transmission of HEV between animals and humans might be frequent.

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

Many people living in endemic areas have circulating antibodies to HEV: using commercially available enzyme immunoassays (EIAs), various studies have shown seroprevalence rates in endemic areas of IgG anti-HEV in the range of ~20% (Ref. 2). However, using purified recombinant HEV proteins in a western blot assay, IgG anti-pORF2 has been detected in greater than 80% of voluntary blood donors in India (Ref. 26). Despite the presence of antibodies to the viral capsid protein, how does HEV cause infection? It is possible that protective neutralising antibodies either do not develop or are only short-lived. No studies are available in which immunodominant epitopes (motifs on the protein that most often induce antibodies or bind antibodies) have been differentially identified in protected versus susceptible individuals. In addition, little information, if any, exists on the immune correlates of protection in hepatitis E. For example, the contribution of humoral (antibody-mediated) and cellular (T-cell-mediated) immunity to protection against hepatitis E is not known. Much work is required in this area.

Immunoelectron microscopy has shown that HEV isolated from one part of the world can be recognised by sera of hepatitis E patients from a geographically different locale (Ref. 43). Experimental transmission of HEV to macaques has further demonstrated that the sera of hepatitis E patients from one locale can neutralise the HEV isolate from another region (Ref. 44). These results indicate that only a single serotype of HEV exists globally. In view of this, the occurrence of sporadic infections and repeated epidemics in endemic areas is baffling. Is it possible that different variants of HEV might be responsible for repeat infections in the same people in an endemic zone? This seems unlikely based on the sequencing of many HEV isolates from different parts of the world, which suggests a stable (conserved) viral genome. However, it would be interesting to characterise a large number of HEV strains from sporadic and epidemic cases in a particular endemic area to see if they change with time or are different in more/less-severe infections. Such an approach would highlight minor genetic changes that might be responsible for this disease phenotype.

What are the pathogenetic mechanisms in HEV infection?

Hepatocyte death is the hallmark of all viral hepatitis. In both hepatitis B and hepatitis C, viral proteins have been found to modulate programmed cell death (apoptosis) of the liver cell (Refs 45, 46). In the case of HEV, this area remains largely unexplored. However histopathological examination of biopsy material from hepatitis E patients does show apoptotic bodies (Councilman bodies), and electron microscopic examination shows ballooning of the hepatocyte along with distension of the cellular and subcellular membranes, which are both signs of apoptosis. Electron microscopy has revealed apoptotic features in HepG2 (transformed human liver) and COS-1 (transformed monkey kidney) cells expressing the HEV *ORF2*-encoded capsid protein. Furthermore, it has not been possible to develop stable cell lines constitutively expressing the capsid protein (S. Jameel, unpublished), which suggests that this protein alone might be toxic or induce cell death.

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ISSN 1462-3994

HEV *ORF2* encodes two forms of the major capsid protein: a glycosylated and a nonglycosylated form. These proteins also localise to different parts of the cell – some go to the cell surface, while the rest remain inside the cell (Ref. 31). The relationship between these two forms of *ORF2*-encoded proteins and their functional significance are not clear. It is possible that, whereas the nonglycosylated intracellular form might be involved in capsid assembly, the glycosylated cell-surface form of the protein might be involved in apoptotic signalling. Its phosphorylation by MAPK (Ref. 37) and the observed association with proteins containing SH3 domains (H. Korkaya and S. Jameel, unpublished) suggests that the HEV *ORF3*-encoded protein might be involved in signal transduction. In the context of liver cell apoptosis, cytokine-mediated or hormonal death signals at the cell surface might be transduced through pORF3. Apoptosis induced by late viral proteins would aid in the dissemination of HEV from an infected cell to neighbouring hepatocytes, with only minimal inflammatory and immune responses. Such an effect of viral proteins on cellular pathways would also explain the mortality that is associated with HEV-induced fulminant liver failure, particularly in pregnant women.

Detection of HEV and prophylaxis of hepatitis E

Diagnostic tests for HEV

Only one serological test to diagnose HEV infection is commercially available (Genelabs Technologies, Singapore). However, several diagnostic tests are available in research laboratories, including: (1) EIAs and western blot assays to detect anti-HEV IgM and IgG in serum (Ref. 47); (2) PCR tests to detect HEV RNA in sera and stools; and (3) immunofluorescent antibody-blocking assays to detect antibody to HEV antigen in the serum and in liver biopsies. However, the sensitivity and specificity of these tests have not been determined independently using a good panel of anti-HEV positive and negative sera.

Vaccine approaches

No products are currently available to prevent hepatitis E. Passive immunization using immunoglobulins prepared from plasma collected from HEV-infected persons in non-HEV-endemic areas is not effective in preventing clinical disease during hepatitis E outbreaks, and the efficacy of immunoglobulins prepared from plasma collected in HEV-endemic areas is also unclear. In studies with prototype anti-HEV vaccines in animals, vaccine-induced antibody could attenuate HEV infection but did not prevent virus excretion in the stools of infected immunised animals.

For viral pathogens that are difficult to culture and therefore not easily amenable to the development of live attenuated strains, a promising approach is to develop subunit vaccines. A subunit vaccine consists of a part of the virus, typically a protein capable of generating a protective immune response in immunised persons. Recombinant DNA technology is now routinely used to generate large amounts of purified viral proteins to be used as subunit vaccines. For HEV, the most promising subunit vaccine candidate so far appears to be the *ORF2*-encoded protein when expressed in insect cells using recombinant baculoviruses. Two such candidates were developed simultaneously at the National Institutes of Health (Bethesda, MD, USA) and at Genelabs Technologies (Redwood City, CA, USA) (Refs 35, 36). After pilot-scale production at SmithKline Beecham (Belgium), both vaccine candidates have undergone feasibility testing in experimental animals and have shown promise (Ref. 48). The NIH vaccine candidate has also been subjected to a Phase I trial in US volunteers and has been shown to be safe and immunogenic. A similar trial of this candidate vaccine in Nepal, an area endemic for hepatitis E, is planned to start in the year 2000. Recently, the products of N-terminally truncated *ORF2* were shown to form empty virus-like particles (VLPs) (Ref. 34). These VLPs retain native virus epitopes and appear to be a good vaccine candidate (Ref. 49).

Alternative strategies for developing anti-HEV vaccines are also being tried in research laboratories. A naked DNA immunisation approach in which *ORF2* was injected as an expression plasmid directly into muscle resulted in moderate anti-pORF2 titres in mice (Ref. 50). Within days of *ORF2* plasmid DNA injection, the subsequent injection of genes encoding either of the immunomodulatory cytokines interleukin 2 (IL-2) or granulocyte–monocyte colony-stimulating factor (GM-CSF) resulted in higher anti-pORF2 titres in mice (R. Tuteja and S. Jameel, unpublished). Naked DNA immunization with *ORF2* expression vectors has also been tried in macaques, with

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ISSN 1462-3994

promising results (S. Kamili and K. Krawczynski, unpublished). Other strategies such as the expression of *ORF2* in bacille Calmette–Guérin (BCG) recombinant mycobacteria or in transgenic plant expression systems are also being tried. If successful, these might lead to oral or edible vaccines to prevent enteric infection by HEV.

Therapeutic approaches to hepatitis E infection

No therapeutic compounds against hepatitis E are currently available; the only treatments are supportive in nature. Possible drug targets include the HEV Pr and RdRp enzymes, on which even the basic biochemical information is not yet available. Such information will be critical for developing assays to screen libraries of natural or synthetic molecules to search for compounds with anti-HEV activity. The HEV RNA 5' and 3' ends appear to interact with viral and cellular proteins and are crucial for its replication; strategies designed to block these interactions, for example with antisense oligonucleotides, ribozymes or small molecules, might be of therapeutic value. No information is available as to whether any of these approaches are currently being employed.

HEV, or ET-NANBH virus as it was called then, was recognised as a distinct entity in the early 1980s. It took almost 10 years to develop suitable animal models and obtain basic epidemiological information. This directly led to cloning of the viral genome in 1990 (Ref. 51) and its designation as HEV. Along with the molecular cloning of the HCV genome just a year earlier, this heralded a new age in molecular virology, in which the genomes were cloned without first isolating or propagating the viruses in culture. The initial cloning of the genome of the Burma isolate of HEV (Ref. 51) led to the subsequent cloning of genomes from other geographically distinct isolates and the development of a diagnostic test. These developments have further increased our knowledge of the epidemiology of hepatitis E. However, the pace of research on hepatitis E has been slow for a number of reasons. Hepatitis E is not a significant health problem in countries that have the technological capabilities and the funds to carry out front-line biological research. Furthermore, HEV causes an acute, self-limiting infection with no associated chronicity, unlike two of its more illustrious cousins, HBV and HCV. However, new findings that hepatitis E might have zoonotic reservoirs and indigenous pockets of infection in industrialised nations, and the identification of risk groups displaying high mortality such as pregnant women, are likely to provide impetus to hepatitis E research. This would be especially welcome in the direction of anti-HEV vaccines and therapeutics. Limited information available on the molecular biology of HEV shows it to be an interesting virus that might serve as a good model to study virus–host interactions at the molecular level. Perhaps this will also stimulate research.

Acknowledgements and funding

I thank Dr Tatsuo Miyamura, National Institute of Infectious Diseases, Tokyo, for a critical review of this manuscript, and my colleagues Dr Vijay Kumar, Dr Sunil Lal and Dr Kanury V.S. Rao for their suggestions. The HEV work in my laboratory is supported by internal funds from the International Centre for Genetic Engineering and Biotechnology (ICGEB) and these are gratefully acknowledged.

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Features associated with this article

Schematic figures

Figure 1. Geographic distribution of hepatitis E (fig001sjd).
Figure 2. Time course of hepatitis E virus infection (fig002sjd).
Figure 3. Phylogenetic relationships between hepatitis E virus isolates (fig003sjd).
Figure 4. Hepatitis E virus (fig004sjd).
Figure 5. Genome organisation of hepatitis E virus (fig005sjd).
Figure 6. A proposed model of hepatitis E virus replication (fig006sjd).

Further reading, resources and contacts

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