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Youchun Wang *Editor*

Hepatitis E Virus

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Preface

Hepatitis E has long been regarded as an infectious disease that only affects developing countries. Since *Hepatitis E virus* genotype 3 was isolated from native patients in the USA in 1997 and the pig was confirmed as the animal reservoir for genotype 3, research into hepatitis E has advanced and significant progress has been made. Various strains of the *Hepatitis E virus* have been isolated from human populations and many other species, including pig, wild boar, mongoose, rabbit, camel, and chicken. Some strains exclusively infect human populations or animals, whereas others can infect both humans and animals. All the strains constitute one large family, the family Hepeviridae, which was first proposed in 2012. With the successful culture of *Hepatitis E virus* in cell lines, the study of the virus structure and its components has progressed. The HEV virions from cell culture and feces differ in structure and most of the ORF2 proteins may not be associated with HEV RNA. Reports that the *Hepatitis E virus* induces both chronic hepatitis and extrahepatic syndromes have also changed our understanding of the virus. The mechanism by which the *Hepatitis E virus* induces clinical disease and the appropriate treatment measures for patients have been widely studied in human and animal models. Several diagnostic tools, including immunological methods and nucleic acid tests, have become available, and *Hepatitis E virus* RNA, antigen, and antibodies can be detected with clinical laboratory tests. The detection of antigen in patient urine is a simple and useful measure for the diagnosis of the acute phase of hepatitis E. A prophylactic hepatitis E vaccine has also been approved for the prevention of this infection. Although enormous progress has been made in our understanding of *Hepatitis E virus*, many problems still exist and further global study is required. This book includes both basic knowledge and new research into the *Hepatitis E virus* and also discusses several problems that must be addressed in future studies.

I would like to thank all the authors for their excellent contributions. Although they are all very busy, they have still offered me great support. I would also like to thank Drs. Chenyan Zhao, Yansheng Geng, and Weijing Huang, who have provided

valuable assistance in reading and editing the manuscript. This work was supported by grants from the National Foundation of Natural Science (81171549 and 81371830), China. Without its support, this book would not have been published. Finally, I sincerely hope this book provides useful information to all its readers.

Beijing, China Youchun Wang

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About the Editor and Contributors

About the Editor

Youchun Wang M.D., Ph.D. was awarded a medical degree (MD) (part-time) in epidemiology from Peking Medical University in 1998 and a PhD in virology from University College London in 2000. He was trained in the quality control of a yeast-derived hepatitis B vaccine at Merck, Sharp, and Dohme Pty Ltd from October 1991 to February 1992. He received a postdoctoral grant from the Royal Society to support his work on a mutant analysis of the *Hepatitis B virus* at the Royal Free Hospital School of Medicine in the UK from May 1995 to May 1996. Between 1997 and 2000, he received a grant from the Wellcome Trust of the UK and spent 3–5 months each year working at the Royal Free Hospital School of Medicine, University College London, where he focused on research into the *Hepatitis E virus*. He also obtained several grants from the Ministry of Science and Technology, the National Foundation of Natural Science, and other institutions in China. He has mainly worked on the hepatitis viruses, human immunodeficiency virus, human papillomavirus, and other viruses. He has published more than 200 academic papers as the first or corresponding author, 60 of which were published in Science Citation Index journals. He has also held several positions in academic associations, including as the Vice Chairman of Medical Microbiology and Immunology in the Chinese Medical Association and Vice Chairman of Medical Virology in the Chinese Medical Association, Vice Chairman of the Chinese Laboratory Animal Association, Chairman of Beijing Medical Virology, and Vice Chairman of Virological Products of the Chinese Pharmacopoeia. He is now Deputy Director of the National Institutes for Food and Drug Control, China.

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Chapter 1
Hepatitis E Virus

Youchun Wang, Chenyan Zhao, Ying Qi, and Yansheng Geng

Abstract Since the sequence of hepatitis E virus (HEV) was determined from a patient with enterically transmitted non-A, non-B hepatitis in 1989, similar sequences have been isolated from many different animals, including pigs, wild boars, deer, rabbits, bats, rats, chicken, and trout. All of these sequences have the same genomic organization, which contains open reading frames (ORFs) 1, 2, and 3, although their genomic sequences are variable. Some have proposed that they be classified as new family, *Hepeviridae*, which would be further divided into different genera and species according to their sequence variability. The size of these virus particles generally ranged from 27 to 34 nm. However, HEV virions produced in cell culture differ in structure from the viruses found in feces. Those from cell culture have a lipid envelope and a little ORF3 on their surfaces, whereas the viruses isolated from feces lack lipid envelope and ORF3. Surprisingly, most of the secreted ORF2 protein from both these sources is not associated with HEV RNA.

Keywords Biology • Classification • Genome • Sequence • Structure

Abbreviations

ET-NANBH	Enterically transmitted non-A, non-B hepatitis
HAV	Hepatitis A virus
HBV	Hepatitis B virus
Hel	RNA helicase
HEV	Hepatitis E virus
MeT	Methyltransferase
NTR	Non-translating region

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ORF	Open reading frame
PCP	Papain-like cysteine protease
RdRp	RNA-dependent RNA polymerase
SISPA	Sequence-independent single-primer amplification
UTR	Untranslated region

1.1 The Discovery of ET-NANBH

The enterically transmitted, non-A, non-B hepatitis virus is named ET-NANB hepatitis virus. The first documented outbreak of ET-NANBH was in New Delhi, India, in 1957, when 29,000 cases of hepatitis were identified following the widespread fecal contamination of drinking water [49]. This outbreak was originally thought to be caused by hepatitis A virus (HAV); however, a retrospective serological analysis of serum samples from documented cases revealed that neither HAV nor hepatitis B virus (HBV) could be implicated as the etiological agent [26, 52]. Similar outbreaks of ET-NANBH were reported in Nepal [25], Burma [19], Pakistan [10], Mexico [7], and China [58]. Both HAV and ET-NANBH are transmitted enterically and can cause outbreaks due to inadequate sanitary conditions. However, epidemiological investigation revealed that two notable features differ between HAV and ET-NANBH. Unusually high mortality rates of approximately 20 % were observed in pregnant women infected with ET-NANBH [22], while significantly lower mortality rates were seen in HAV-infected pregnant women. The other notable feature of ET-NANBH is the relatively low incidence of clinical disease observed in case contacts. Only 2.4 % of household contacts of ET-NANBH patients developed clinical hepatitis, while about 10–20 % of household contacts of HAV patients developed clinical hepatitis in the same region [25]. The ET-NANBH virus was originally designated as hepatitis E virus at the international meeting on non-A, non-B hepatitis that was held in Tokyo on 27–30 September, 1989.

1.2 Molecular Cloning of the ET-NANBH Virus Genome

The first cloning source was bile obtained from cynomolgus macaques (cyno#121) infected with a third-passage Burma ET-NANBH isolate. A cDNA library was made from the RNA extracted from the cloning source, constructed in the lambda gt10 (λgt10) phage vector, and screened by hybridization to random-primed ^{32}P-labeled cDNA probes derived from either infected (cyno#121) or uninfected (cyno#126) bile. Several clones were identified through screening. By testing the inserts of clones, only the 1.3-kb cDNA, derived from clone ET1.1, detected a uniquely hybridizing band in DNA prepared from the ET-NANBH library. To exclude ET1.1 as a potential source of exogenous sequence contamination, primers

derived from the ET1.1 sequence were used in polymerase chain reaction (PCR) assays to investigate the various sources. All of the sources that were unrelated to ET-NANBH were negative. Meanwhile, sequence-independent single-primer amplification (SISPA) was also used to amplify the cDNA from infected or uninfected cynomolgus macaque bile [39, 40]. The amplified cDNA was separated by electrophoresis and hybridized with probes from the ET1.1 clone. Only the amplified cDNA from infected cynomolgus macaque bile was positive for hybridization. This indicated that ET1.1 was derived from the infected bile source and was not a cloning artifact. The oligonucleotides based on the end sequence of ET1.1 were used as a hybridization probe to rescreen the original bile-derived cDNA library. Another clone, BET6-1, containing an insert of approximately 2.6 kb was found, and comparative analysis between ET1.1 and BET6-1 revealed that the insert of ET1.1 was contained within clone BET6.1. The oligonucleotides from BET6-1 were then used as probes to screen the oligo(dT)-primed cDNA libraries. Another overlapping clone, BET1, was identified. Further study showed that a long poly(A) tail was located at the 3′ end of BET1. This finding indicates that the 3′ end of the viral genome was present in the clone BET1. The 5′ end of the viral genome was isolated from the cDNA library by primer extension using HEV sequence-specific primers. The final clone, BET-SP1, was identified as being located in the 5′ end of the viral genome. The resulting composite cDNA map spanned approximately 7.5 kb from the 5′ end of BET-SP1 to the 3′ end of clone BET1. In summary, the ET-NANBH virus has a polyadenylated, single-stranded RNA genome of approximately 7.5 kb [39, 45].

The second cloning approach was to use SISPA to construct cDNA libraries using stool samples from a case from a Mexican outbreak of ET-NANBH. Briefly, cDNA was synthesized using random primers. The blunt-ended cDNA was modified for SISPA by the ligation of oligonucleotide linkers, and the modified cDNA was then subjected to amplification with SISPA. The SIPSA PCR products were digested with the appropriate restriction enzymes for the restriction sites located in the linkers and were ligated into the λgt10 phage vector. The cDNA library was then immunoscreened with the convalescent serum from another well-documented case of ET-NANBH. Several overlapping, virus-specific clones were identified. The full-length sequence of the Mexican HEV isolate was reported, and it was variable as compared with the first reported ET-NANBH virus sequences [20]. This isolate was designated as HEV genotype 2.

The third cloning approach was to use an affinity capture method (anti-HEV immunoglobulin M [IgM]) in combination with reverse transcription (RT)-PCR [3]. Briefly, microcentrifuge tubes were coated with goat antihuman IgM. These tubes were incubated with anti-HEV-positive acute HEV patient sera to allow for capture of the anti-HEV IgM. The stool suspension was then incubated with these tubes to capture HEV particles. Next, the captured virus was disrupted, and the enclosed viral RNA was used as a template for RT-PCR. The sequence of each oligonucleotide primer was based on the Burmese HEV sequence. This method was found to be much more efficient than previously used approaches for cloning HEV RNA.

After that, many similar strains of HEV isolates from Pakistan, Nepali, India, China, and Africa were amplified using normal RT-PCR or long-range RT-PCR [6, 8, 15, 31, 46–48]. The partial genomes or full-length genomes of these HEV strains were sequenced and analyzed. The identity at the nucleic acid level among them was very high, over 90 %.

In 1997, primers near the 5′-end of open reading frame (ORF) 1 of the Mexican strain were used in RT-PCR to detect HEV RNA from the serum of a 62-year-old white man (US-1). The resulting PCR product was cloned and sequenced, and the entire genome of US-1 HEV has been extended using a gene-walking method that is dependent on RT-PCR. These PCR reactions, which used standard amplification or touchdown amplification, utilized three kinds of PCR primer pairs: (1) two HEV consensus primers, each based on a Mexican or Burmese isolate within conserved regions; (2) one HEV consensus primer and one US-1HEV-specific primer; and (3) two US-1 HEV-specific primers [42]. The resulting full-length sequence has only ~80 % identity to the reported genotypes 1 and 2, so it was designated as HEV genotype 3. Notably, US-1 HEV was isolated from an American patient who had never traveled abroad. Meng et al. was the first to discover that the majority of adult pigs in the United States are positive for anti-HEV immunoglobulin G (IgG). To identity the agent responsible for the anti-HEV IgG seropositivity in pigs, a novel virus was cloned and sequenced from piglets. The results confirmed that the novel virus has high identity to US-1 HEV [33], and this virus was designated as swine HEV.

Before 1999, when sera from patients with acute hepatitis in China were assessed as negative for hepatitis viruses A–E, most of this assessment was based on serology results, but HEV was excluded based only on the results from testing for anti-HEV IgG antibodies. One study later searched for HEV sequences in these patient samples by using RT-PCR based on degenerate primers designed within the conserved sequences of the HEV ORF1 and ORF2 regions and found that some HEV isolates were very similar to each other, but divergent from all other known HEV sequences (74–83 % nucleotide identity in ORF1 or ORF2). These results indicate that the sequences may belong to a novel genotype of HEV [50]. Further, the complete genomic sequence of a representative isolate of this novel genotype HEV was amplified directly from the stool of an acutely infected patient. Analysis of the entire sequence confirmed that these Chinese isolates belong to a novel genotype, designated as genotype 4 [51].

Payne et al. was the first to report HEV-related sequences isolated from chickens with big liver and spleen disease in Australia. These sequences shared approximately 62 % nucleotide sequence identity with human HEV [35]. The first full-length sequence of avian HEV was isolated from bile samples of chickens with hepatitis-splenomegaly syndrome in the United States. Its genomic organization is very similar to human HEV, but it shares approximately 60 % identity with human HEV at the nucleic acid level [18].

When the serum samples from the farmed rabbits in China were tested for anti-HEV antibody using enzyme immunoassays (EIAs) and for HEV RNA using nested RT-PCRs with ORF2 primers, 57.0 % (191/335) and 7.5 % (25/335) of them were positive for anti-HEV antibody and HEV RNA, respectively. The nucleotide

sequences isolated from these rabbits had 84–99 % identity to each other but less than 82 % identity to other HEV genotypes. Two representative full-length sequences were also determined, and they had less than 80 % identity to other HEV genotype full-length nucleotide sequences [57]. Koch's postulates were fulfilled when specific pathogen-free (SPF) rabbits were experimentally infected with rabbit HEV and the same viruses were also recovered from the experimentally infected rabbits [30]. This study further indicated that rabbits are more susceptible to rabbit HEV than to genotype 4 or genotype 1; only a small percentage of rabbits can be infected with genotype 4 HEV, and none can be infected by genotype 1 HEV. After that, more strains of rabbit HEV were isolated from the United States and France [9, 23].

The positivity of anti-HEV IgG was also detected in rats worldwide, with seroprevalence rates varying from 13 to 90 % [55]. Additionally, a HEV-related virus was isolated from rats. Its nucleotide sequence shares approximately 55.9 % identity with genotype 1–4 HEV [24]. Recently, novel HEV strains were also isolated from wild boars in Japan [41, 44], as well as from cutthroat trout [2], bats [11], and camels in the Middle East [53].

To date, HEV genomes have been isolated not only from humans but also from diverse animal species. In general, the genomic organization of these HEV isolates is similar, but their sequences are genetically variable.

1.3 Structure of the HEV Genome

HEV contains a single-stranded, positive-sense RNA genome of approximately 7.2 kb. Molecular analysis of the HEV genome indicates that it contains three separate ORFs. The 5′ non-translating region (NTR) is about 26 nucleotides in length. However, a 5′ NTR was not reported for the Mexican strain. The HEV-US2 strain has the longest 5′NTR among all the known HEV isolates, at 35 nucleotides in length. The 3′NTR of HEV is about 65 nucleotides in length and is variable among different isolates. The putative nonstructural ORF is located at the 5′-end, and the structural ORF is at the 3′-end of the genome. ORF1 begins at the 5′-end of the viral genome and extends 5079 bp before termination at nucleotide position (nt) 5107. Before an alternative strategy for the translation of ORFs 2 and 3 of genotype 4 HEV was identified, the second major ORF (ORF2) was thought to begin 41 nucleotides downstream of ORF1, and the third ORF (ORF3), which contains 369 nucleotides, was believed to overlap ORF1 by one nucleotide and overlap ORF2 by 328 nucleotides [5, 37]. One single nucleotide (U) was found to be inserted at nt 5159 for genotype 4 HEV [51], and this difference in sequence for genotype 4 HEV changed the translation of ORF3 to start at nt 5174 and end at nt 5509 (total length: 336 nucleotides). Genotype 4 HEV ORF3 starts 28 bases downstream of ORF1, unlike the ORF1 and ORF3 in other isolates, which reportedly overlap by only one base. The ORF3 polypeptide length is 112 amino acids for genotype 4, whereas it is considered to be 123 amino acids for other genotypes.

To determine whether or not the initiation strategy of the HEVORF3 for other genotypes is the same as that for genotype 4 HEV, Graff et al. and Huang et al. examined the ORF3 translation for genotypes 1 and 3 [16, 21]. An additional T residue was inserted after nt 5116 of an infectious cDNA clone of a genotype 1 strain, which mimicked the gene structure in this region of genotype 4 HEV strains. The ORF3 protein produced by the recombinant mutant contained only 114 amino acids, rather than the 123 amino acids predicted if initiation occurred at the first in-frame AUG of the genotype 1 wild-type parent. These results suggest that the first AUG codon previously assumed to serve as the initiation codon for ORF3 protein synthesis was not used for this purpose in the genotype 1 strain but that the third in-frame AUG codon was used instead, in the same manner as in genotype 4. Huang et al. [21] found that genotype 3 mutations with substitutions in the first in-frame AUG in the junction region or with the same T insertion at the corresponding position of HEV genotype 4 did not affect the virus infectivity or rescue. However, a single mutation at the third in-frame AUG completely abolished virus infectivity in vivo. These results indicate that the third in-frame AUG in the junction region is required for virus infection and is likely the authentic initiation site for ORF3. Thus, the initiation strategy of HEVORF3 for all genotypes is the same as that firstly reported for genotype 4.

Through computer analysis, several conserved domains were found within ORF1, which is about 1693 codons in length, including methyltransferase, Y domain papain-like cysteine protease, X domain, NRA helicase domain, and RNA-dependent RNA polymerase domain. The function of the X and Y domains is unknown. The papain-like cysteine protease might function to cleave the ORF1 product into smaller, functional proteins. The RNA helicase and RNA-dependent RNA polymerase may be involved in the replication of the viral RNA. A hypervariable region was found to be located from nt 2002 to nt 2424 within ORF1. It exhibited a high degree of sequence diversity between all reported HEV sequences [27].

ORF2 encodes a protein approximately 660 amino acids long that seems likely to be the HEV capsid protein. The deduced amino acid sequence of the protein contains three Asn-X-Ser/Thr(N-X-S/T) sequences in the Burmese strains and two such sequences in the Mexican isolates; they are potential N-linked glycosylation sites, suggesting that the protein product is likely to be a glycoprotein. A putative signal peptide sequence was identified at the extreme amino terminus of the ORF2 protein. This sequence probably directs the newly synthesized protein to the endoplasmic reticulum and may be important for capsid assembly and secretion [38].

ORF3 also encodes another protein, about 112 amino acids in length, which has an immunoreactive epitope. This protein seems to be phosphorylated at a serine residue [56]. The biological role of the ORF3-encoded protein has not yet been elucidated. However, most of the amino acids in the amino-terminal half of the protein are contained in two hydrophobic peptides that are separated by a short hydrophilic segment. The hydrophobic segment nearest the amino end has eight cysteine residues. The primary amino acid sequence suggests that this protein may be membrane associated [38].

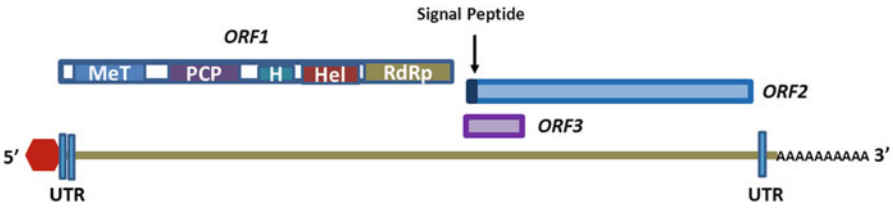


Fig. 1.1 HEV genome structure. A schematic of the genomic structure shared by all known isolates of HEV. *UTR* untranslated region, *ORF* open reading frame, *MeT* methyltransferase, *Pcp* papain-like cysteine protease, *Hel* RNA helicase, and *RdRp* RNA-dependent RNA polymerase

All of the reported HEV isolates have been shown to possess the basic genomic features described above and illustrated in Fig. 1.1, even though they may belong to different genotypes of HEV.

1.4 Classification of HEV

Before 2000, HEV was classified in the family *Caliciviridae* because the virion size, morphology, viral particle sedimentation coefficient, and buoyant density of HEV are all very similar to those of caliciviruses [45]. The *Caliciviridae* family of viruses is composed of non-enveloped viruses whose particles are round, 27–34 nm, and have a "ragged" outer edge that lacks a definite surface structure. The buoyant density of calicivirus particles is 1.33–1.411 g/cm^3. Additionally, calicivirus genomes are composed of single-stranded, positive-sense RNA that is polyadenylated at its 3′-terminus [54], and they have three ORFs. ORF1 encodes a nonstructural protein, ORF2 encodes a capsid protein, and ORF3 encodes a small protein, whose function is not clear. Between 2000 and 2004, HEV was removed from the *Caliciviridae* family into an "unassigned" classification status by the International Committee on Taxonomy of Viruses (ICTV) [17] because HEV lacks a phylogenetic relatedness with other members of the *Caliciviridae* family and because both the types of putative replicative enzymes used by HEV and the cap structure at the 5′ end of the viral genome are much different from those of other *Caliciviridae* family members.

Through HEV cDNA cloning and sequencing, comparative sequence analyses mainly based on the larger nonstructural protein were made between HEV and "alpha-like" viruses, such as alphavirus, rubivirus (RubV), and beet necrotic yellow vein virus (BNYVV). Three shared domains, encoding a putative methyltransferase, a putative RNA helicase, and a putative RNA polymerase, were found on the genomes of both HEV and "alpha-like" viruses. Four additional domains, including the Y domain, a putative papain-like protease, a proline-rich hinge domain, and the X domain, were also found in the genomes of both HEV and Rubella virus, and a

Table 1.1 Proposed classification of the family *Hepeviridae*

Genus	*Species*	Predominant host species	Genotype	Reference accession
Orthohepevirus	*Orthohepevirus* A	Human	HEV-1	M73218
		Human	HEV-2	M74506
		Human, pig, rabbit	HEV-3	AF082843
		Deer, mongoose	HEV-4	AJ272108
		Human, pig	HEV-5	AB573435
		Wild boar	HEV-6	AB602441
		Wild boar	HEV-7	KJ496143
		Camel		
	Orthohepevirus B	Chicken		
	Orthohepevirus C	Rat	HEV-C1	GU345042
		Ferret	HEV-C2	JN998606
	Orthohepevirus D	Bat		JQ001749
Piscihepevirus	*Piscihepevirus* A	Trout		HQ731075

Modified from Smith et al. [43]

highly conserved motif was observed in the RNA-dependent RNA polymerase. RNA helicase, a putative methyltransferase, and Y domain also showed significant similarity between HEV and "alpha-like" viruses. However, the virions of HEV are 27–34 nm, non-enveloped particles, whereas alphavirus, rubivirus, and BNYVV all have enveloped viral particles [27], indicating that HEV is clearly different from alphavirus, rubivirus, and BNRVV. The classification of HEV remained unsettled for several years, until 2004, when it was designated as the *Hepeviridae* family [12].

The HEV variants used to be classified into genotypes and subtypes within *Hepeviridae*, based on their degree of sequence relatedness to existing variants. As more sequences of HEV-related viruses were isolated from a wide range of mammalian species, as well as from chickens and trout, this classification system caused both controversy and confusion because it could not cover all of the new HEV strains isolated from difference species. A new proposal for reclassification of the family *Hepeviridae* was published recently [43]. This revised system contains three classification levels, namely, genus, species, and genotype. The family *Hepeviridae* is composed of *Orthohepevirus* and *Piscihepevirus* at the genus level, with *Orthohepevirus* into A–D at the species level and *Piscihepevirus* containing only one species, named *Piscihepevirus* A (Table 1.1; Fig. 1.2).

These demarcation criteria are based on phylogenetic analyses of HEV nucleotide and amino acid sequences. Maximum-likelihood trees were produced by using the program models and phylogeny in MEGA 6.0. A pairwise (p)-distance model was used to calculate the genetic distances of viral variants. Within species *Orthohepevirus* A, the phylogenetic analysis of the amino acid sequences of concatenated ORF1 and ORF2 (excluding the HVR) revealed seven branches. Variants derived from genotypes 1, 3, 4, and rabbit HEV show ranges of amino acid sequence

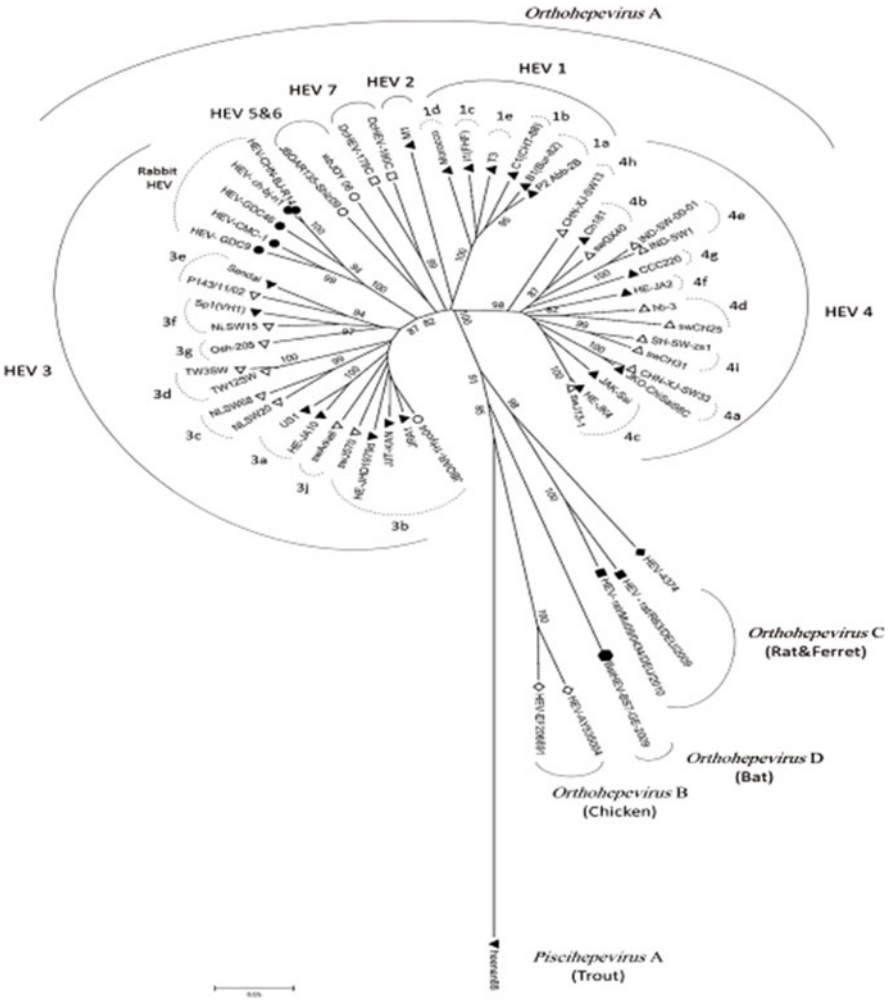


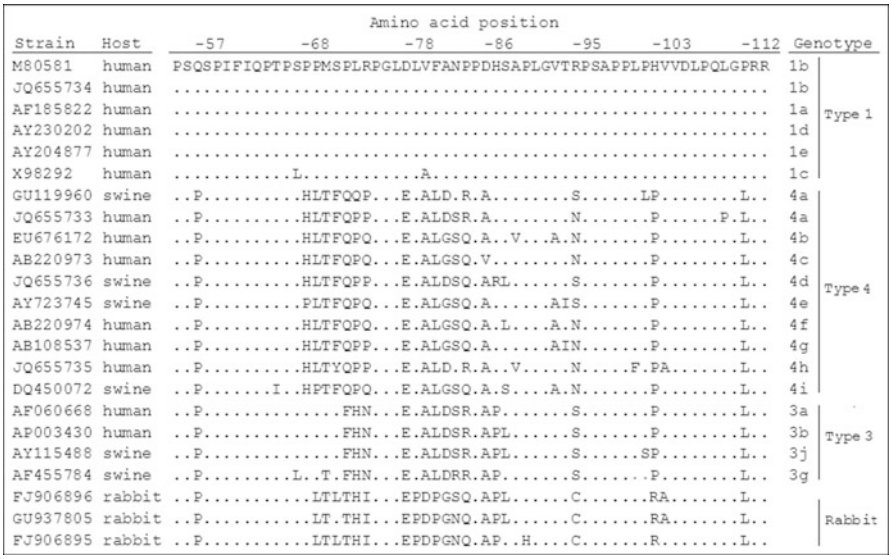
Fig. 1.2 Phylogenetic tree of the *Hepeviridae* family. The phylogenetic tree shown here is based on the 304 nucleotides at the 5′end of ORF2 of 56 isolates of the family *Hepeviridae* (*Orthohepevirus* A, B, C, D, and *Piscihepevirus* A). ▲: sequences from human, ▲: sequences from pig, ●: sequences from rabbit, ○: sequences from wild boar, □: sequences from camel, ◇: sequences from chicken, ◆: sequences from ferret, ■: sequences from rat, ⬣: sequences from bat, and ▼: sequences from trout

distances from each other with values of 0.004–0.041, 0–0.053, 0–0.053, and 0.012–0.081, respectively. Minimum distances between variants derived from rabbit and genotype 3 variants are lower (0.061) than those between them and other genotypes (0.108). Therefore, the rabbit HEV sequences were considered to belong to genotype 3. On this basis, an amino acid sequence p-distance of 0.088 could act as a threshold to demarcate intra- and inter-genotype distances. Using this criterion,

the two grouping variants isolated from wild boars would become genotype 5 and 6 by >0.10, and the variants derived from camels (differing from all other sequences by >0.095) would belong to genotype 7 [43].

To date, only *Orthohepevirus* A and C can be subdivided into genotypes: *Orthohepevirus* A has seven genotypes and C has two genotypes. All genotypes that can infect humans are located within *Orthohepevirus* A and have been designated as genotypes 1–4. Genotypes 1 and 2, which are mainly distributed in Asia, Africa, and North America, have been identified exclusively in humans and are mainly associated with large waterborne epidemics, although they can cause sporadic cases of hepatitis E. Genotypes 3 and 4, which seem to be mainly porcine strains but have also been found in other mammalian reservoirs (wild boar, deer, and mongooses), are mainly responsible for sporadic cases of hepatitis E in humans [32, 34]. Genotype 3 is predominantly distributed in western countries, while genotype 4 is mainly found in the Asia region. Rabbit HEV strains have been identified from farm rabbits in China, the United States, and France [9, 14, 23, 57]. The overall nucleotide similarity between rabbit HEV and genotypes 1–4 is about 77–79 % [57]. Although rabbit HEV is most closely related to genotype 3 HEV, with 79 % identity of its full-length nucleotide sequence, and it was provisionally assigned as genotype 3, rabbit HEV has different biological characteristics than genotype 3. No rabbit HEV has been found in swine populations, even in areas where pig farms are located in close proximity to rabbit farms. There has been no evidence of natural cross-species HEV transmission between rabbit and pigs [13]. It has been demonstrated that a quarter to half of pigs can be experimentally infected with rabbit HEV when inoculated intravenously; however, compared with pigs that have been experimentally infected with the genotype 3 swine HEV, the pigs infected with rabbit HEV have a delayed onset, a shorter duration of viremia and fecal virus shedding, and no detectable level of seroconversion to anti-HEV antibodies in the serum. These findings suggest that at least some rabbit HEV strains are able to infect pigs, but they do so less robustly than swine HEV strains [9]. The amino acid sequence of the ORF3 region is more highly variable than that of the other two ORFs. The specific sequence of rabbit HEV ORF3 (Fig. 1.3) indicates that it is different from that of genotype 3 [29]. Another unique feature of rabbit HEV is that all rabbit HEV ORF1s have an insertion of 93 nt in the X domain compared with HEV genotypes 1–4 [23].

HEV genotypes 1 and 2 differ from genotypes 3 and 4 in their transmission patterns. Genotypes 1 and 2 have been identified exclusively in humans and are mainly associated with large waterborne epidemics; genotypes 3 and 4 are recognized as zoonotic pathogens and are mainly responsible for sporadic cases of hepatitis E [14, 32, 34]. Thus, genotypes 3 and 4 have circulated in different animals, which may result in genetic variability. Consistent with this hypothesis, genotypes 3 and 4 present a greater range of nucleotide sequence differences at the subtype level than genotypes 1 and 2 [28]. The differences among the complete nucleotide sequences for genotypes 3 and 4 range from12.1 % to 18.0 %, while those for genotypes 1 and 2 only range from 6.2 to 11.0 %. Genotype 1 can be divided into subtypes 1a, 1b, 1c, 1d, and 1e; genotype 2 into subtypes 2a and 2b; genotype 3 into subtypes 3a, 3b,



Strain	Host	Amino acid position -57 -68 -78 -86 -95 -103 -112	Genotype
M80581	human	PSQSPIFIQPTPSPPMSPLRPGLDLVFANPPDHSAPLGVTRPSAPPLPHVVDLPQLGPRR	1b
JQ655734	human	.....	1b
AF185822	human	.....	1a
AY230202	human	.....	1d
AY204877	human	.....	1e
X98292	human	.....L.....A.....	1c
GU119960	swine	..P.....HLTFQQP...E.ALD.R.A.....S.....LP.....L..	4a
JQ655733	human	..P.....HLTFQPP...E.ALDSR.A.....N.....P.....P.L..	4a
EU676172	human	..P.....HLTFQPQ...E.ALGSQ.A..V...A.N.....P.....L..	4b
AB220973	human	..P.....HLTFQPQ...E.ALGSQ.V.....N.....P.....L..	4c
JQ655736	swine	..P.....HLTFQPP...E.ALDSQ.ARL.....S.....P.....L..	4d
AY723745	swine	..P.....PLTFQPQ...E.ALGSQ.A.....AIS.....P.....L..	4e
AB220974	human	..P.....HLTFQPQ...E.ALGSQ.A.L...A.N.....P.....L..	4f
AB108537	human	..P.....HLTFQPP...E.ALGSQ.A.....AIN.....P.....L..	4g
JQ655735	human	..P.....HLTYQPP...E.ALD.R.A..V.....N.....F.PA.....L..	4h
DQ450072	swine	..P.....I..HPTFQPQ...E.ALGSQ.A.S...A.N.....P.....L..	4i
AF060668	human	..P.....FHN...E.ALDSR.AP.....S.....P.....L..	3a
AP003430	human	..P.....FHN...E.ALDSR.APL.....S.....P.....L..	3b
AY115488	swine	..P.....FHN...E.ALDSR.APL.....S.....SP.....L..	3j
AF455784	swine	..P.....L..T.FHN...E.ALDRR.AP.....S.....P.....L..	3g
FJ906896	rabbit	..P.....LTLTHI...EPDPGSQ.APL.....C.....RA.....L..	
GU937805	rabbit	..P.....LT.THI...EPDPGNQ.APL.....C.....RA.....L..	Rabbit
FJ906895	rabbit	..P.....LTLTHI...EPDPGNQ.AP..H....C.....R.....L..	

Fig. 1.3 Comparison of the alignment of ORF3 sequences (amino acid residues 55–114) of representative strains of different genotypes. The HEV isolates are identified by GenBank accession Nos. The amino acid position in ORF3 protein is according to M80581. Cited from Ma et al. [29]

3c, 3d, 3e, 3f, 3g, 3h, 3i, and 3j; and genotype 4 into subtypes 4a, 4b, 4c, 4d, 4e, 4f, and 4 g [28].

Orthohepevirus A also includes variants that infect wild boars and camels. According to amino acid sequence p-distances (>0.10), the wild boar isolates would comprise genotypes 5 and 6, while the variants from camels, with p-distances greater than 0.095, would belong to genotype 7 [43].

1.5 Morphological Appearance and Physiochemical Properties of ET-NANB

The virus-like particles (VLP) from stool samples from the acute phase of ET-NANB hepatitis from Nepal, Burma, Pakistan, Somalia, and Mexico were observed using immune electron microscopy [10, 25]. The size of these virus particles ranged from 27 to 34 nm. The variability in the HEV particle size reported by various laboratories may be related to the proteolytic digestion of HEV in its passage through the gut, to its sensitivity to freeze-thawing, or to differences in the storage of stool preparations. The HEV particles have characteristic surface features, including indentations and projections.

When the virus particles ranging from 27 to 34 nm isolated from stools of sporadic ET-NANB hepatitis cases in former Soviet Central Asia were inoculated in a

human volunteer [1], the virus particles were subsequently recovered from the volunteer's acute phase stool and had the same size and morphology as the inoculated particles. That indicated that the virus particles are infectious. In another study, the acute phase or serum pools from cases of ET-NANB hepatitis in the former Soviet Union, Pakistan, Burma, Nepal, Somalia, Sudan, and Mexico were all shown to aggregate VLPs from a case of ET-NANB hepatitis in Tashkent, the former Soviet Union. In contrast, neither normal human sera nor one serum positive for antibody to HAV could aggregate these 27–34 nm VLPs from Tashkent [4]. The same results were also observed for VLPs from cases of ET-NANB hepatitis in Pakistan, Burma, and Mexico. These results indicate that these particles might be etiologically related to ET-NANB hepatitis.

Although the virus particles were observed in stool samples of patients, the detailed information about structure of the virus particles was not clear. Thus, the study on structure of the virus particles is important, especially on comparing the structure difference for different sources of HEV virus particles. When HEV from cell culture systems or from the feces of infected patients was purified by ultracentrifugation, the ORF2 protein mainly stayed in the top fractions for both of these sample types. This finding indicates that most of the secreted ORF2 protein is not associated with HEV RNA. Infectious virions from these two sources, feces and cell culture supernatant, both formed single virion bands, but their densities differed. The density of the virions from the cell culture supernatant changed to that of virions from feces when the lipid was removed with NP40. This indicates that, like HAV, HEV from cultured cells can hijack the host membrane to form an envelope. Several studies showed that the binding percentage of virions to anti-ORF3 antibody increased significantly when the lipids were removed from cell culture-derived virions. However, minimal binding to anti-ORF3 antibody was detected for virions from feces. This result indicates that infectious HEV virions produced in cell culture differ in structure from the virus found in feces. HEV infectious virions from cell culture have a lipid envelope containing little ORF3, and most of the ORF3 protein on these virions is protected by lipid, whereas the virions from feces have no lipid and more ORF3 (Fig. 1.4) [36].

HEV has been reported to be labile and will not tolerate exposure to high concentrations of salt. The computed sedimentation coefficient of HEV particles is approximately 183S, in contrast to157S for HAV. Additionally, the buoyant density of HEV particles is 1.29 g/cm^3 in KTar/Gly gradients. The HEV virus is extremely sensitive to freeze-thawing and spontaneously degrades when held at 4–8°Cfor periods of time exceeding 3–5 days.

1.6 Conclusions and Perspective

Because the host-range of HEV is very wide and several genotypes or subtypes within each species have been reported, the virulent, pathogenic, and higher prevalence genotypes or subtypes of HEV still need to be defined. Studies on the HEV

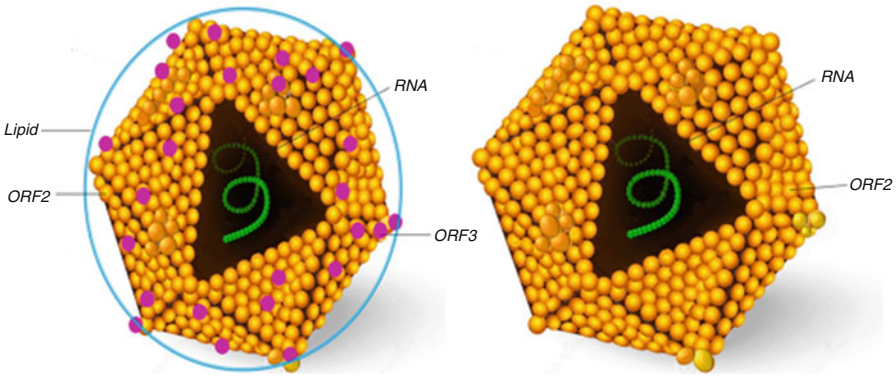


Fig. 1.4 Models of HEV from cell culture and feces. A model of HEV from cell culture is shown on the *left* and a model of HEV from feces is shown on the *right*

structure have indicated that the ORF2 protein and the viral RNA banded at different densities following ultracentrifugation. ORF3 protein and lipid are on the surface of HEV virus particles from the cell culture supernatant, but they are not present on HEV virus particles from feces. The impact of the structure differences among viruses from different sources on the diagnosis and prevention of HEV infection should be investigated.

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Chapter 2
Characteristics and Functions of HEV Proteins

Yan Zhou, Chenyan Zhao, Yabin Tian, Nan Xu, and Youchun Wang

Abstract Hepatitis E virus (HEV) is a non-enveloped virus containing a single-stranded, positive-sense RNA genome of 7.2 kb, which consists of a 5′ noncoding region, three open reading frames (ORFs), and a 3′ noncoding region. ORF1 is diverse between genotypes and encodes the nonstructural proteins, which include the enzymes needed for virus replication. In addition to its role in virus replication, the function of ORF1 is relevant to viral adaption in cultured cells and may also relate to virus infection and HEV pathogenicity. ORF2 protein is the capsid protein, which is about 660 amino acids in length. It not only protects the integrity of the viral genome but is also involved in many important physiological activities, such as virus assembly, infection, and host interaction. The main immune epitopes, especially neutralizing epitopes, are located on ORF2 protein, which is a candidate antigen for vaccine development. ORF3 protein is a phosphoprotein of 113 or 114 amino acids with a molecular weight of 13 kDa with multiple functions that can also induce strong immune reactivity.

Keywords Expression • Function • Protein • Pathogenicity • Replication • Adaption

Abbreviations

ASGR	Asialoglycoprotein receptor
CDK	Cyclin-dependent kinase

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EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ERK	Extracellularly regulated kinase
GST	Glutathione s-transferase
Hel	RNA helicase
HVR	Proline-rich hypervariable region
MAPK	Mitogen-activated protein kinases
MeT	Methyltransferase
ORF	Open reading frame
PCP	Papain-like cysteine protease
PLG	Plasminogen
RdRp	RNA-dependent RNA polymerase
STAT	Signal transducer and activator of transcription
VDAC	Voltage-dependent anion channel
VLP	Virus-like particle

Hepatitis E virus (HEV) contains three open reading frames (ORFs) and encodes three proteins, each of which has unique features and functions. Although HEV can be divided into many genotypes within the genus and species of family *Hepeviridae*, all HEV genotypes that can infect humans belongs to *Orthohepevirus* A and have been designated as genotypes 1–4. Thus, the three HEV proteins described in this chapter focus on genotypes 1–4 and the positions of nucleic acids and amino acids referred to those of genotype 1.

2.1 Characteristics and Functions of ORF1 Proteins

2.1.1 Structural Features of ORF1 Proteins

ORF1, which is 5082 bp long, is located on the 5′ terminus of the HEV genome and encodes a nonstructural polyprotein of 1693 amino acid residues. The functional domains of this polyprotein consist of methyltransferase (MeT), Y domain, papain-like cysteine protease (PCP), proline-rich hypervariable region (HVR or polyproline region, PPR), X domain (macro-domain), RNA helicase (Hel), and RNA-dependent RNA polymerase (RdRp) [36, 37] (Fig. 2.1).

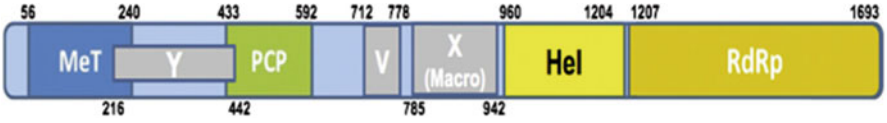

Fig. 2.1 HEV ORF1 protein domains. A schematic of the ORF1 protein domains: methyltransferase (MeT), Y domain, papain-like cysteine protease (PCP), proline-rich hypervariable region (V), X domain (macro), RNA helicase (Hel), and RNA-dependent RNA polymerase (RdRp) [2]

2.1.2 Expression of ORF1

Two products, N-78 kDa and C-107 kDa, were obtained when expressing ORF1 in mammalian cells by using recombinant vaccinia virus [66]. Expressing ORF1 in an *Escherichia coli* plasmid expression system or in HepG2 carcinoma cells yielded only an unprocessed polyprotein of 186 kDa, but no processed functional unit products [3, 70]. In contrast, in vitro transfection of HepG2 cells with infectious clones containing the whole HEV genome yielded different ORF1 processed products. The bands 35 kDa (MeT), 38 kDa (Hel), and 36 kDa (RdRp) were identified from these expression products by using anti-MeT, anti-Hel, and anti-RdRp antibodies [55]. Only a sole 191 kDa polyprotein was produced when the recombinant plasmid pTriEx-ORF1 is expressed in an in vitro transcription–translation system, but when this plasmid was transfected into S10-3 cells, an N-terminal product of 35 kDa and a C-terminal product of 78 kDa were detected by an immunoprecipitation assay [56]. When expressing ORF1 in a baculovirus–insect system in the form of fusion protein His6-ORF1-Flag, a polyprotein of 192 kDa was produced, and the number of processed short fragments that reacted with anti-His and anti-Flag antibodies increased overtime. This processing procedure could be inhibited by the cysteine proteinase inhibitor (E-64d) [68]. The 410–610 amino acid ORF1 fragment expressed in *E. coli* C43 showed disintegrating activity to nonstructural protein ORF1 and structural protein ORF2. The results of a mass spectrometry analysis indicate that ORF1 protein can be digested into N-terminal 35 kDa methyltransferase and C-terminal 35 kDa replicase by the expressed 410–610 amino acid ORF1 protein. The cleavage sites were G-15/I-16 and A-1364/V-1365, which confirmed the in vitro ORF1 protein disintegrating activity of PCP-like proteinase [53].

Presently, the difference in function among the ORF1 proteins expressed by different systems and the extent of the involvement of host proteinase in ORF1 expression are not clear. Additionally, the expression of nonstructural ORF1 proteins after HEV infection has not been reported. Furthermore, it is not completely clear whether or not the functional domains of ORF1 proteins are processed to produce entities having biochemical function. Further studies are needed to address these questions.

2.1.3 Virus Infection and Pathogenicity Relevant to ORF1

An investigation into the heterogeneity of the HEV ORF1 gene and the outcome of infection in solid-organ transplant patients during the hepatitis E acute phase found that the entropy and genetic distance of HEV sequences in chronic hepatitis E patients were higher than those in patients who cleared the virus. Specifically, the PPR and macro-domains of ORF1 were dramatically higher. The high genetic heterogeneity of the PPR and macro-domains may be associated with persistent infection of HEV virus in the acute period due to regulation of the host immune response by mutation [39].

The HVR domain may play a vital role in HEV pathogenicity as described in Chap. 5. Bu Q et al. [5] sequenced a strain of genotype 4 HEV that was collected from a patient with hepatic failure and compared it with other HEV genotype 4 isolates; they found that 12 amino acid residues in ORF1 and three amino acid residues in ORF2 were substituted. Moreover, a comparative analysis of the mutations present in the nucleic acid/amino acid sequences of ORF1 in genotypes 4 and 3 found mutations in 12 amino acid residues, with 11 mutations in the PCP domain and the remaining one in the RdRp domain [98]. Mishra N. et al. [45] compared amino acid sequences between strains of genotype 1 from patients with fulminant hepatic failure, as well as with the genotype 1 strains from acute virus hepatitis patients in the same subcontinent. Six identical substitutions in HEV strains of all fulminant patients occurred only in ORF1, namely, F179S, A317T, T735I, L1110F, V1120I, and F1439Y. These mutations were significantly associated with the fulminant hepatic failure caused by genotype 1. It was reported that [22] the nonsense mutation of U3148 in the Hel domain of ORF1 was associated with the severity of hepatitis E. Billam et al. [4] aligned the complete sequences of a nonpathogenic and a pathogenic poultry HEV strain and found that the highest number of mutations was in ORF1 with 41 mutated sites, whereas there were only ten mutated sites in other ORFs. These discoveries indicate that ORF1 may have relationship with the pathogenicity of HEV.

2.1.4 ORF1 and Virus Replication

Capped RNA transcripts of HEV cDNA clones were able to be transfected into Huh-7 cells where they successfully replicated. These transcripts showed infectivity and were also able to produce virions when inoculated intravenously into the swine. In contrast, uncapped RNA transcripts did not show these abilities [19]. Notably, the activities of methyltransferase and guanylyltransferase in the MET domain could be detected in the 110 kDa polyprotein expressed in baculovirus [44]. Additionally, the capping of genomic RNA could be confirmed by HEV 5′ RNA ligase-mediated rapid amplification of the cDNA ends, which selectively amplify capped RNAs [93]. The methyltransferase activities catalyze RNA capping, and the removal of the 5′ terminal γ-phosphorous group on the initial transcript by RNA triphosphatase is the key step of capping. Study showed that co-incubation of HEV helicase with 5′-[γ-^{32}P] RNA and 5′-[α-^{32}P] RNA released ^{32}P from 5′-[γ-^{32}P] RNA only, indicating the specificity of the helicase to a γ-β-triphosphate bond. These findings suggest that HEV RNA helicase might mediate the first step of 5′-terminal capping. RNA helicase is necessary for the genomic replication of positive-sense RNA viruses. HEV RNA helicase displays nucleotide triphosphatase activity and has an RNA-binding domain [29]. When the Hel domain on HEV ORF1 amino acid position (aa) 960–1204 was expressed in prokaryotic cells, the HEV RNA helicase was able to hydrolyze all rNTPs (ribonucleotide triphosphates), but showed lower hydrolysis activity against dNTPs (deoxyadenosine nucleoside triphosphates). This enzyme

has unwinding activity in the 5′ → 3′ direction to 5′-sticky double-stranded RNA only [28].

A recombinant HEV RdRp expressed in *E. coli* was able to bind to the 3′-terminal noncoding region of the HEV genome and used 3′-polyadenylated HEV RNA as a template to synthesize complementary strands [1]. A study of HEV infection in A549 cells and suckling pigs found that RNA interference to RdRp could effectively inhibit the replication of HEV [21]. Karpe et al. [30] found that an active ubiquitin–proteasome system was necessary for HEV replication and that this could be inhibited by a proteasome inhibitor. Notably, the overexpression of ubiquitin in proteasome inhibitor-treated cells partially reversed the inhibition of HEV replication.

The protein expressed by the PCP domain has de-ubiquitin enzymatic activity, and PCP may be involved in the replication of HEV via this enzymatic activity. Notably, the mutations of G816V and G817V in G815-G816-G817 of the X domain prevented virus replication. Additionally, the mutation N806A did not preclude RNA replication, whereas the mutations N809A and H812L resulted in a lack of live virus, indicating the involvement of X domain amino acid residues at the post-translational stage of HEV replication [56, 57].

2.1.5 ORF1 and Viral Adaption

Aided by biological software, Purdy [59] computerized and forecasted PPRs, informatics entropy, selective pressure, homoplastic density, intrinsically unstructured regions (IDRs), linear motifs, electrostatic surfaces, secondary structures, structure-based functions, and protein-binding sites of ORF1 PPR sequences from four HEV genotypes and found that the PPRs from four HEV genotypes were IDRs that all contained seven putative linear binding motifs for ligands. The structural analysis of the molecular functions of these motifs indicated that PPRs tended to bind to various ligands. The existence of nucleotide mutations in PPR was due to high frequencies of insertion and deletion. Although the mutation rate of PPR is the same as that of other ORF1 domains, PRR has a higher tolerance than the other ORF1 domains for substitution between its first and second codes. This high mixture led to more proline, glycine, serine, and threonine instead of histidine, phenylalanine, tryptophan, and tyrosine, indicating that these regions are typical proline-rich IDRs. Alignment analysis on PPR sequences from HEV strains of all genotypes found a common origin for animal strains and a higher tolerance to mutation in carboxyl moieties than in the remaining PPR domain amino acid residues. In contrast with other nonstructural polyproteins, the evolution of HEV PPR appears to have been shaped under selective pressure to use more proline and fewer aromatic amino acids, a ratio which favors the formation of an IDR structure. IDRs are able to bind to various ligands and have a regulatory effect on transcription and translation [100]. Therefore, PPR may play a key role in the ability of HEV to adapt to different circumstances.

Izopet et al. [23] compared the sequence of rabbit HEV with that of human HEV, and they found that one human HEV strain was very close to rabbit HEV. There was an insertion of 93 nucleotides in the ORF1 X domain of the human HEV strain and rabbit HEV strains. This study suggested that the host range of HEV had been expanding and that rabbit HEV may be transmitted among animals. By using recombination software RDP and SimPlot to analyze the intra-genotype and inter-genotype differences in the HEV genome, it was found that the recombinant fragments are non-randomly distributed in the HEV genome. The X domain, Hel, and RdRp were all hot spots with high recombination rates. These nonrandom distributions were due to the high adaption of recombination in this region as well as to the effects of natural selection [9].

2.2 ORF2 Protein

ORF2 protein, which is approximately 660 amino acids in length, is translated from a 2.2 kb subgenome into the capsid protein. There is a conserved stem-loop structure in *ORF2* that may be related to early-stage viral replication [13]. Moreover, it was reported that ORF2 could specifically bind to the HEV genome RNA at the 5′ end and plays an essential part in the HEV assembly process [71]. As the capsid protein, ORF2 not only protects the integrity of the viral genome but is also involved in many important physiological activities, such as virus assembly, infection, and host immunity. It has an N-terminal signal sequence by which it is co-translated into the endoplasmic reticulum (ER) where it is sequentially N-linked glycosylated [2]. Since the C-terminal of the ER signal sequence of ORF2 includes an arginine-rich domain, it is expected to play a key role in genomic RNA binding and HEV assembly. Moreover, the C-terminal 52 amino acid residues of ORF2 are also involved in HEV genome encapsidation and stabilization of the capsid particles [69].

2.2.1 Expression of HEV ORF2 Protein

Although the growth of HEV in cell culture has been reported [18, 32, 85], the quantities of natural HEV proteins that are produced in this manner are not sufficient for further study. The structural proteins of HEV have been expressed using various expression systems, including bacteria, mammalian cells, baculovirus, yeast, and vaccinia.

The full ORF2 [54], the carboxyl-terminal one-third of ORF2, and the carboxyl-terminal two-thirds of ORF2 were expressed in *E. coli* as fusion proteins with glutathione s-transferase (GST) or as trpE-HEV fusion proteins [60, 99]. Based on the primary structure of synthetic peptides possessing HEV-specific antigenic activity, mosaic proteins of HEV representing aa394–470, aa562–580, and aa631–660 of ORF2 and aa91–123 of ORF3 from the Burmese strain and the same regions from

the Mexican strain were designed and similarly expressed in *E. coli* as GST-fusion proteins [34, 35]; for each strain, only one recombinant protein band of HEV ORF2 or ORF3 was observed in the *E. coli* expression system, and this band had a molecular weight of the expected size of a protein lacking glycosylation and proteolytic posttranslational processing. Another group found that the ORF2 E239 peptide could be expressed in an *E. coli* system, and this expression successfully forms particles, which have been developed as an HEV vaccine [43].

Full ORF2 proteins have been expressed in COS-1, HepG2, and BHK-21 cells. Three forms of ORF2 protein were observed in normal transfected cells using both plasmid-based expression [24] and the Semliki Forest virus (SFV) vector [77] with molecular weights of 72–74 kDa, 79–82 kDa, and 84–88 kDa, even though the expected size for ORF2 protein is approximately 72 kDa. The glycosylation status of ORF2 was evaluated in experiments in which tunicamycin was used to inhibit glycosylation in transfected cells [24]. This experiment confirmed that the 74 kDa protein is the ORF2 protein without glycosylation. The two larger proteins were identified as glycosylated forms of ORF2 with different extents of glycosylation. Through pulse-chase analysis, tunicamycin inhibition, and endoglycosidase sensitivity, studies have found that ORF2 protein is likely co-translationally translocated via its N-terminal signal sequence into the ER. The protein may be glycosylated in the ER at asparagine residues at one or more sites. When the signal peptide sequence was deleted, the modified ORF2 construct was found to express only one form of protein, and no glycosylated forms were observed, even though all three of the potential glycosylation sites were located in the construct. These results suggest that the signal peptide sequence can direct the protein into the ER for glycosylation. An expression analysis of ORF2 also found that ORF2 protein is expressed intracellularly, as well as on the cell surface [91].

The main advantage of using baculovirus for expression is that, in most cases, proteins expressed in insect cells are processed in the same way they would be in mammalian cells, resulting in authentic, functional proteins. Additionally, the protein expression levels in insect cells may also be much higher than those in mammalian cells. The full ORF3 and ORF2 from the Burmese strain were expressed in Sf9 cells using a baculovirus-expressing vector [17]. ORF2 protein, with a molecular weight of approximately 70.9 kDa, was observed in cell pellets, and no recombinant proteins were identified in cell supernatants. In another study, the major protein band had an apparent molecular weight of 75 kDa [79]. Minor bands were also observed, and some of these bands had molecular weights of more than 75 kDa, possibly due to varying extents of glycosylation, while others had lower molecular weights, which may reflect proteolytic processing posttranslation. In a further study, three major bands were observed, including the complete structural protein at the earlier stages of recombinant baculovirus infection as well as two products of proteolytic cleavage (55 kDa and 63 kDa) at the later stages [80]. The 75 kDa protein is slightly larger than the predicted size for the entire ORF2, and this size difference could be due to glycosylation. An analysis of the other two proteins (55 kDa and 63 kDa) found that they were coterminal at the amino end and shared the same N-terminal Ala-112. The 63 kDa protein had an intact C-terminus, while the 55 kDa

protein was the product of an additional cleavage of 51 amino acids from the C-terminus [65].

The entire ORF2 was also expressed in two insect cell lines, Sf9 and Tn5. Three major proteins with molecular weights of 72, 58, and 50 kDa were found in the lysates of both cell types. All three of these proteins have reactivity to anti-HEV antibody-positive sera. They were tightly cell-associated and were not found in the culture supernatant. However, when the structural protein derived from ORF2 aa112–660 was expressed in the Tn5 cell line, a large amount of protein with a molecular weight of 50 kDa was produced and efficiently released into the culture medium [40]. Through electron microscopy, the 50 kDa protein was found to form empty viruslike particles (VLPs) in the culture medium. Li et al. later found that these HEV VLPs induced a strong immune response after their oral administration in mice and monkeys [41, 42]. They demonstrated that both ORF2 aa126–601 and aa112–608 can form T = 1 particles. When the RNA fragment was contained, ORF2 aa112–608 could also forma T = 3 particle. The average size of T = 1 particles was 27 nm and that of T = 3 particles was 41 nm [16, 84, 87].

When the peptide sequence of aa126–621 in HEV genotype 4 was expressed in insect cells, two forms were obtained in the cells, VLP and non-VLP. The two forms have obvious differences in their granularity, which was reflected in their behavior during ultracentrifugation, dynamic light scattering, and chromatographic analysis, as well as by their appearance during electron microscopy [61]. VLPs have a more uniform granularity than non-VLPs. Although VLPs and non-VLPs behaved identically on SDS-PAGE, different peptides were produced when they were digested with the same enzymes under the same conditions. The peptide mapping detected by using LC–MS/MS showed that they have different posttranslational modifications. Additionally, VLPs induced stronger immune responses than non-VLP. Thus, increasing the yield of VLPs in insect expression system is important.

The translation of HEV ORF2 is predicted to yield a 72 kDa protein including a putative signal sequence and potential sites of N-linked glycosylation [76]. When the full-length ORF2 of the Burmese, Pakistan, and Japanese strains were expressed in the baculovirus system, the predicted 72 kDa protein products were processed into a 50, 55, 58, or 62 kDa protein due to posttranslational processing [40, 92]. However, similar processing of HEV ORF2 protein was not observed in mammalian cells. When the full-length ORF2 was expressed in mammalian cells [24, 77], multiple ORF2-specific proteins with molecular weights estimated as 72–74, 79–84, and 84–88 kDa were detected. The smallest of these proteins with a molecular weight of 72–74 kDa correspond to the predicted size, and the larger proteins, 79–84 kDa and 84–88 kDa, reflect the super-glycosylation of the HEV ORF2 protein in mammalian cells [78]. Thus, different expression systems may result in different posttranslational modifications of the same protein.

2.2.2 The Host Proteins Involved in HEV Infection and Intercellular Transduction

Interactions between ORF2 and extracellular matrix proteins were detected in host cells. It was reported that once heparan sulfate was removed from the cell surface by heparinase or sodium chlorate treatment, the binding of HEV VLP to the Huh-7 cell surface was significantly reduced. Syndecan-1 plays an important role in the ORF2 binding process because it is a ubiquitous proteoglycan on the cell [26]. However, the interaction between ORF2 and heparan sulfate proteoglycan is a nonspecific adsorption, and the identity of an HEV-specific receptor remains unknown. Although HEV is a non-enveloped virus, it may use heparan sulfate proteoglycan as its cell adhesion receptor, similarly to enveloped viruses.

Recent studies found that some chaperones and cytoskeleton proteins take part in HEV intercellular transportation through their interaction with HEV capsid protein ORF2. During the protein synthesis process, chaperones recognize partially folded polypeptides, and they participate in peptide folding and help the protein assemble correctly. The heat shock proteins are the main subset of chaperones. Some heat shock proteins, such as heat shock protein 90 (HSP90), were demonstrated to take part in the early stage of HEV infection. The p239 VLPs formed from recombinant ORF2 proteins were used to investigate the cellular interactions. HSP90, Grp78/Bip, and α-tubulin were identified as binding ORF2 by matrix-assisted laser desorption/ionization time of flight mass spectrometry. Inhibition of HSP90 blocked the p239 transportation in HepG2 cells, but it did not affect p239 cellular entry. The specific HSP90 inhibitor also significantly obstructed the transportation of HEV. Together, these findings indicate that HSP90 plays a key role in HEV intercellular transportation [96].

Asialoglycoprotein receptor 2 (ASGR2) is a transmembrane glycoprotein that is highly expressed in the liver tissue. ASGR2 and ASGR1 can be composed of homologous or heterologous dimmers of ASGPR, which mediate the lysosomal-dependent degradation of various desialylated glycoproteins in hepatocytes. In recent years, ASGPR was found to play an important role in hepatitis A virus, hepatitis B virus, and hepatitis C virus infection, indicating that it may mediate hepatitis virus entry into cells [11, 67, 94]. Additionally, ASGPR is involved in and facilitates HEV infection by binding to ORF2, but the mechanism is still unclear [95].

2.2.3 ORF2 and the Endoplasmic Reticulum Stress Response (ERSR)

Numerous proteins are translated and modified on the ER. Some pathological changes can destroy the balance of intercellular protein translation and posttranslational modification, leading to the accumulation of incorrectly folded peptides and inappropriately modified proteins. The unfolded or misfolded proteins form many

aggregates, causing ER stress. John et al. found that overexpression of HEV ORF2 in cells induced ER stress by activating the amino acid response elements of the pro-apoptotic gene C/EBP homologous protein (CHOP), which is a pro-apoptotic gene. The ORF2 proteins activate the transcription of CHOP by increasing the phosphorylation level of eukaryotic initiation factor 2 alpha (eIF2α). However, the ERSR caused by ORF2 overexpression did not cause the expected apoptosis of host cells. At the same time, it was reported that the expression of heat shock proteins Hsp72, Hsp70B, and Hsp40 were upregulated in ORF2 overexpressing cells, which indicates the increased chaperones may help HEV-infected cells avoid apoptosis [25]. Furthermore, the results of a co-immunoprecipitation study demonstrated the protein–protein interaction between ORF2 and Hsp72 in vivo, which indicates that Hsp72 might facilitate the ORF2 folding process. Meanwhile, the nuclear accumulation of Hsp72 appeared in the cells that expressed HEV ORF2 [25].

Incorrectly folded peptides should degrade through the ER-associated degradation (ERAD) process, and, because of this process, the ER unloads much of the pressure caused by viral infection [86]. Whether HEV ORF2 was overexpressed by the transfection of recombinant VLPs or by that of an *ORF2* plasmid, only a fraction of ORF2 was located on the ER, and most of the rest of the ORF2 proteins were spread throughout the cytoplasm. It is possible that there is a connection between the ERSR and the retrotranslocation of ORF2 from the ER. Because ORF2 was co-translocated and N-linked glycosylated in the ER through its N-terminal signal sequence, it was initially believed that nearly all of the ORF2 proteins were located on the ER. If the N-linked glycosylation was blocked in host cells by tunicamycin or kifunensine, ORF2 cannot be glycosylated and retrotranslocation to the cytoplasm was subsequently inhibited. Then, the interaction between ORF2 and p97 was confirmed by immunoprecipitation, which found evidence of interaction with p97 by both the full-length ORF2 and KDEL-ORF2 (a mutagenesis by modifying the native C-terminal sequence of ORF2, KTREL, to KDEL). Additionally, GRP94, an ER stress-inducible chaperone, was found to be upregulated in ORF2 overexpressed cells, as was protein disulfide isomerase (PDI) [73].

Nuclear factor kappa B (NF-kB) plays an essential role in the host cell survival during infections by many different pathogens. The activation of NF-kB requires the phosphorylation and degradation of Ikβ to release the nuclear localization signal of the NF-kB dimmer. ORF2 blocks the ubiquitin–proteasome-mediated degradation of Ikβ, and, as a result, NF-kB activity is inhibited in HEV-infected human hematoma cells. In contrast, ORF2 showed direct interaction with a beta transducin repeat-containing protein (βTRCP), which is a member of the F-box protein family. Because of the competitive binding of ORF2, the associations of Ikβ with Cul1 and SKP1 were significantly reduced. As the HEV capsid protein, ORF2 plays a role in the survival of HEV-infected host cells, and it regulates the replication and amplification of the virus [74].

2.3 Structure and Function of ORF3 Protein

2.3.1 Molecular Structure of ORF3 Protein

ORF3 protein, a phosphoprotein of 113 or 114 amino acids with a molecular weight of 13 kDa, is a protein with multiple functions. Analysis of its structure found that ORF3 protein consists of two highly hydrophobic domains, D1 (aa7–23) and D2 (aa28–53), at its N-terminus and two proline-rich domains, P1 and P2 (Fig. 2.2). The D1 domain, which is rich in cysteine, is the binding site for microtubulin [27] and mitogen-activated protein kinase (MAPK) phosphatase [31] and also interacts with the cytoskeleton [90]. The D2 domain is a hydrophobic region that acts as a binding site for hemopexin. The PMS^{71}PLR in the P1 domain contains two overlapping potential phosphorylation kinase motifs, of which PMS^{71}P is a potential MAPK phosphorylation motif and S^{71}PLR is a potential cyclin-dependent kinase (CDK) phosphorylation motif. The serine S^{71} can be phosphorylated by MAPK in ORF3-transfected cells in vitro [38]. However, it remains unknown whether or not S^{71} is phosphorylated during HEV infection. The ORF3 antigenic epitope is found in the P2 domain [38], where there are two overlapping PXXP motifs found in many viruses, and associated with the signal transduction of cellular proteins. These motifs are binding sites for Src homology 3(SH3) containing structural domain and signal transduction molecules, and they play a key role in the virus release of HEV [31]. Interestingly, the PSAP motifs of genotype 3 and avian HEV ORF3 did not significantly impact virus infection but they played a role in the virion release of HEV [33, 49]. However, HEV infection and virus release were not detected in rhesus monkeys infected with ORF3–PXXP mutant strains [12]. These data indicate that PXXP plays a key role in virus release and budding, but these processes are also related to the virus genotype and the host species.

2.3.2 ORF3 Protein and Host Cell Survival

ORF3 protein is able to interact with some signal transduction molecules, impact the pathway response of host cells, and maintain host cell survival. Kar-Roy et al. [31] reported that ORF3 protein could activate external cellular regulatory kinase (ERK) of the MAPK family, which was not dependent on the traditional RAF/MEK pathway, but instead inhibited the phosphatase activity of Pyst1 by the binding of

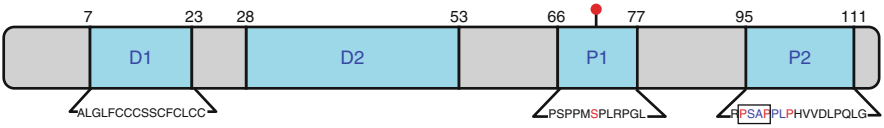

Fig. 2.2 HEV ORF3 protein domains. A schematic of the domains of HEV ORF3 [2]

ORF3 protein to Pyst1 [31]. Pyst1 is a member of the ERK-specific MAPK phosphatase family that can mediate the dephosphorylation of ERK, which inactivates ERK [48]. By binding its D1 region in the N-terminus to the central connection region of Pyst1, ORF3 blocks its phosphorylation, which prolongs the activated status of ERK and allows the continuous operation of the MAPK pathway and, therefore, potentially facilitates host cell survival and viral proliferation. ORF3 has also been shown to activate MAPK–JNK of the anti-apoptosis signaling pathway. Parvez et al. transfected HEV replicon RNA or ORF3 expression vectors into hepatocarcinoma cells to quantitatively test the phosphorylation level of JNK1/2. They found that the phosphorylation level of JNK1/2 increased by approximately 66 % in ORF3 vector-transfected cells, which is higher than the phosphorylation level of 54 % that was observed in the replicon RNA-transfected cells (HEV replication cells). The phosphorylation of JNK1/2 is favorable for both the maintenance of host cell survival and the persistent infection of HEV [58]. Chandra et al. [6] found that the subcellular location of ORF3 was in early and recycling endosomes, which delays the post-internalization activation of epidermal growth factor receptor (EGFR) to late endosomes/lysosomes, therefore potentially prolonging the EGF-mediated intracellular signal. Further study in this lab found that ORF3 protein delays the internalized degradation of activated hepatocyte growth factor receptor (c-MET). However, this function is not related to the localization of ORF3 in endosomes [7]. The mechanism responsible for the prolongation of the growth factor receptor-mediated signal transduction by ORF3 protein may be related to the competitive binding of Cbl to CIN85, which is an adaptor protein that mediates the regulation of many signal pathways, including the endocytosis of ligand-induced receptor tyrosine kinases (such as EGFR and c-MET) and lysosomal degradation. ORF3 binds to the CIN85 SH3 domain via its PXXP motif, competing with Cbl and thereby inhibiting lysosome degradation-activated EGFR or c-MET [7]. This can prolong the signal transduction of growth factor and facilitate host cell survival and HEV proliferation.

Additionally, ORF3 protein also participates in regulating carbohydrate metabolism and the function of mitochondria. Moin et al. [46] found that in ORF3 protein-expressing cells, the expression of hexokinase and the oligomer form of voltage-dependent anion channel (VDAC) protein increased. VDAC is a type of ion channel pore protein located in the outer mitochondrial membrane that regulates the transportation of calcium ions and ATP-like solutes across this membrane. Mitochondria release pro-apoptotic proteins into the cytoplasm, which plays a key role in the apoptosis of mammalian cells. In ORF3 protein-expressing cells, the binding of additional hexokinase I to VDAC facilitates the oligomerization of VDAC, which closes the VDAC permeability of mitochondria, preventing the release of the pro-apoptotic protein cytochrome C and stopping the mitochondria from completing the apoptosis pathway. Moin et al. [47] established a cell line that stably expresses ORF3 protein and performed a proteome analysis on these cells; they found 89 upregulated proteins and 140 downregulated proteins compared with control cells. They also found that ORF3 protein enhances the expression of enzymes in the glycolysis pathway whose encoding genes are downstream response

genes of hypoxia-inducible factor (HIF)-1α. ORF3 protein promotes synthesis of the HIF-1α protein by activating the PI3K/Akt signal pathway, which increases the heteromerization of HIF-1α with HIF-1β after the former enters the nucleus and binds to HIF response element (HRE). The HIF-1 complex then recruits phosphorylated p300/CBP to the promoter of the target gene to induce gene expression. By activating ERK, ORF3 protein can increase the phosphorylation of p300/CBP and the transcription activity of HIF-1, therefore boosting the expression of enzymes in the glycolysis pathway and regulating the energy balance of HEV-infected cells.

2.3.3 ORF3 Protein and the Virus Replication Environment

Viruses take advantage of host cell mechanisms to maintain the phases of the virus life cycle after they infect hosts because escaping recognition by the host immune system during the early stage of virus infection is important for their survival. Viruses adopt several different strategies to escape the host immune responses or to establish an immunosuppressive environment. The interferon (IFN) system is an important component of the host antiviral response. The Janus kinase–signal transducer and activator of transcription (JAK-STAT) signal transduction pathway plays a critical role in the interferon-induced antiviral response. Dong et al. [10] found that the HEV ORF3 protein was able to bind to STAT1 and inhibit the IFN-α-induced phosphorylation of STAT1, resulting in an inhibition of the expression of the antiviral genes PKR (double-strand RNA activated protein kinase), 2,5-oligoadenylate synthetase (2,5-OAS), and myxovirus resistance protein A (MxA) and a subsequent escape from host attack. ORF3 can also downregulate the function of STAT3. After phosphorylation by JAK or other kinases, STAT3 forms a dimer or polymer, translocates from the cytoplasm to the nucleus, and triggers the gene expression of multiple acute-phase reaction proteins. Endocytosed receptors, such as EGFR, act as carriers to assist in the translocation of phosphorylated STAT3 into the nucleopore. Chandra et al. [6] found that ORF3 protein prolonged the inhibition of EGFR endocytosis, lowered the translocation efficiency of STAT3, decreased the level of phosphorylated STAT3 in the nucleus, and reduced the gene transcription of major host inflammatory response genes. In human lung epithelial cells, A549s, the HEV ORF3 protein of genotype 1 induces tumor necrosis factor-α (TNF-α) to inhibit the NF-kB signal pathway, which leads to a decreased level of inflammatory response gene expression and creates a favorable environment for virus replication [88]. An analysis of the gene expression in adenovirus-transfected hepatocarcinoma Huh-7 cells expressing ORF3 found that the expression of response gene hepatocyte nucleus factor 4 (HNF4) was downregulated. Further analysis showed that ORF3 protein increases HNF4 phosphorylation via ERK and Akt kinase and prevents HNF4 translocation to the nucleus, thus downregulating the liver-specific, HNF4-response gene expression and establishing a favorable environment for virus replication and proliferation [8].

Tyagi et al. [82, 83] found that ORF3 protein facilitates the secretion of α-microglobulin, which is an immunosuppressive molecule, and maintains an immunosuppressive environment in HEV-infected hepatocytes. Further study by Surjit et al. [72] found that ORF3 protein facilitated secretion of α-microglobulin is dependent on the PSAP motif in the ORF3 C-terminus. Via its PASP motif, ORF3 protein interacts with tumor-susceptible gene 101 protein (TSG101), which is a member of the endosome complex. ORF3 protein with a mutation in its PASP motif is not able to bind to TSG101 and loses its ability to facilitate α-microglobulin secretion. Further study found that ORF3 protein interacts with TSG101 and α-microglobulin simultaneously. A trimer of these three proteins can be precipitated by co-immunoprecipitation. ORF3 protein interacts with TSG101 protein, taking advantage of the cellular ESCRT (endosomal sorting complex required for transport) mechanism to facilitate the excretion of α-microglobulin, and protects HEV-infected cells. Acute-phase protein, fibrous protein β-chain, and hemopexin were demonstrated to interact with ORF3 protein via a yeast two-hybrid screen. Interaction between ORF3 protein and fibrous protein β-chain decreases excretion of the cellular fibrous protein β-chain. Meanwhile, in cells expressing ORF3 protein, the transcription of fibrous proteins α, β, and γ was reduced and hemopexin protects cells from hemoglobin-mediated oxidative damage during intravascular hemolysis [63, 64].

2.3.4 ORF3 Protein and Clinical Symptoms

Geng et al. [14] screened human liver proteins that were able to interact with the ORF3 protein of HEV genotype 1 by using the yeast two-hybrid technique, and 32 interacting proteins were screened out, of which 28 were new ORF3-interacting proteins. These interactions were validated by the co-immunoprecipitation method. The ORF3 protein of genotype 4 also interacts with these proteins. The results of a clustering analysis on the function of those proteins when they interacted with ORF3 showed that they were significantly involved in the biological pathways of coagulation and hemostasis. Zhou et al. [97] used a yeast two-hybrid technique to identify porcine liver plasminogen (PLG) and α2-antiplasmin (SERPINF2) as proteins that interact with the ORF3 of genotype 4 HEV, and they confirmed these interactions by co-immunoprecipitation and pulldown. PLG is an inactive precursor of plasmin, and it triggers the fibrinolytic process by activation. Plasmin degrades fibrous protein and fibrinogen, and it maintains the fluency of blood vessels and secretory ducts. SERPINF2, which is synthesized in the liver, is the major inhibitor of plasmin (plasminogen) and is also an inhibitor of fibrinolysis. In healthy individuals, the blood coagulation reaction system and the fibrinolysis system are correctively regulated, and the storage and elimination of fibrous protein are balanced to appropriately maintain the blood vessel system. Patients with HEV infection have clinical symptoms of blood coagulation disorders and hemorrhagic abortion in the late trimester of pregnancy. The balance between blood coagulation and fibrinolysis

may be broken following HEV infection, possibly as a result of the interaction between ORF3 protein and host proteins.

2.3.5 ORF3 Protein Is Associated with HEV Release

Studies show that the ORF3 protein is located on the surface of HEV and plays a key role in virus release. Yamada et al. [89] found that infectious cDNA clone pJE03-1760 F/wt with ORF3-deficient mutant can replicate effectively in PLC/PRF/5 and A549 cells. However, the number of viruses detected in the culture supernatant of cells infected with the ORF3-deficient mutant is only 1 % of that of cells infected with the wild-type infectious clone, indicating that ORF3 is highly important for virus release. Immunocapture polymerase chain reaction results show that the virus density of wild-type HEV in cell culture is lower than that of ORF3-defective HEV and that ORF3 protein located on the surface of HEV may bind to lipids. Interestingly, Takahashi et al. [75] found that anti-ORF3 antibodies can capture HEV virions from patient sera but not from feces, even though HEV density in sera is lower than that in feces. This finding supports the hypothesis that ORF3 combines with lipids and is located on the surface of virions. Qi and colleagues studied HEV from cell culture supernatant and feces shedding by ultracentrifugation, and they found that the surface of infectious HEV in cell culture consists of ORF3 protein and lipids, but that the lipids have no effect on virus adsorption and ORF3 protein mediates virus binding [62]. Emerson et al. [12] found that both replication and release of HEV genotype 1 in enterocyte Caco-2 and Huh-7 cells were dependent on having a functional ORF3 protein. By using HEV-infected rhesus monkeys, Graff et al. [15] affirmed that ORF3 protein is necessary for virus infection and that ORF3 protein interacts with lipids and is located on the surface of virion. Tyagi et al. [81] found that phosphorylated ORF3 protein on Ser^{71} interacts selectively with non-glycosylated ORF2 protein and may be involved in the assembly of the capsid protein. ORF2 protein is the HEV capsid protein, and it has RNA-binding activity that can specifically bind to the 5′ terminus of the HEV gnome [71], indicating that ORF2 protein plays a key role in virus assembly. ORF3 protein interacts with ORF2 protein, and ORF3 protein may participate in the formation of virions, but this idea still needs experimental confirmation.

Huang et al. [20] found that intact ORF3 is indispensable for HEV in vivo infection. Additionally, Yamada et al. [89] and Emerson et al. [12] reported that ORF3 protein is necessary for virus release and that ORF3 protein is located on the surface of released HEV virions. Nagashima et al. [49] found that an intact PSAP motif of the ORF3 P2 domain protein is necessary for the formation of membrane-associated HEV virions with ORF3 protein on their surface. Further study found that by interacting with the ORF3 protein PSAP motif, TSG101, Vps4A, and Vps4B have enzymatic activity that is involved in HEV release [50]. The surfaces of released virions contain lipids of the trans-Golgi network protein 2 (TGOLN2) from the trans-Golgi network [52]. HEV forms membrane-associated virions in the cytoplasm, buds in an

exosome-like manner, and releases virions via the multi-vesicular body (MVB) pathway [51].

2.4 Conclusion

HEV encodes three proteins, namely, ORF1, ORF2, and ORF3 proteins, with each protein having its own function. Generally, ORF1 encodes the nonstructural proteins that are mainly responsible for virus replication. ORF2 protein is the capsid protein, which contains most of the neutralizing epitopes. ORF3 protein is a short protein, which may have multiple functions. Although substantial progress on studying the functions of HEV proteins has been made, knowledge about some functions of these proteins is still limited. Recently, the interaction between HEV proteins and hosts or host cells has been investigated in several laboratories, and several host proteins have been found to interact with HEV proteins. However, their functions in HEV replication, pathogenicity, and overcoming species barriers are still not clear, and a HEV receptor has yet to be identified. Additionally, although many studies demonstrated that ORF3 protein may have multiple functions, its exact mechanism on HEV assembly and infection is not yet clear. Further studies are needed to address these issues.

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Chapter 3
Epidemiology of Hepatitis E

Yansheng Geng and Youchun Wang

Abstract Hepatitis E virus (HEV) is globally prevalent with relatively high percentages of anti-HEV immunoglobulin G-positive individuals in the populations of developing and developed countries. There are two distinct epidemiologic patterns of hepatitis E. In areas with high disease endemicity, primarily developing countries in Asia and Africa, this disease is caused mainly by genotype 1 or 2 HEV, both of which transmit predominantly through contaminated water and occur as either outbreaks or as sporadic cases of acute hepatitis. The acute hepatitis caused by either of these two genotypes has the highest attack rate in young adults, and the disease is particularly severe among pregnant women. In developed countries, sporadic cases of locally acquired genotype 3 or 4 HEV infection are observed. The reservoir of genotype 3 and 4 HEV is believed to be animals, such as pigs, with zoonotic transmission to humans. The affected persons are often elderly, and persistent infection has been well documented among immunosuppressed persons. A subunit vaccine has been shown to be effective in preventing clinical disease and has been licensed in China.

Keywords Hepatitis E virus • Genotype • Anti-HEV IgG • Hepatitis E • Outbreak • Prevalence • Reservoir

Abbreviations

ELISA	Enzyme-linked immunosorbent assay
HEV	Hepatitis E virus

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3.1 Introduction

Serological and molecular studies have shown that hepatitis E virus (HEV) is globally distributed and is the leading cause of enterically transmitted viral hepatitis illness worldwide. HEV infection has been reported in most countries of the world. Large annual epidemics are attributed to HEV in endemic areas, and sporadic cases are increasingly reported in developed countries. It is estimated that two billion people worldwide are infected with HEV every year with 14 million symptomatic cases and 300,000 deaths [87].

3.2 Worldwide HEV Serological Prevalence in the Human Population

Anti-HEV antibodies appear following HEV infection and can persist for several years [3, 4]. Thus, the detection of anti-HEV antibodies in blood from an individual is taken to indicate their prior exposure to HEV, and the prevalence of these antibodies may provide an epidemiological marker of the frequency of HEV exposure in a population. The prevalence of anti-HEV antibodies has been studied in various populations worldwide, and the results indicate that anti-HEV antibodies are present in persons living in all geographical areas. Tables 3.1, 3.2, 3.3, and 3.4 summarize the results reported from studies involving the collection of samples that are more or less representative of the general population of countries or regions from Asia, Europe, Africa, and the Americas.

Developing countries in Asia and Africa frequently displayed high HEV prevalence rates. In Asia, rates higher than 30 % were found among adults from India, Bangladesh, China, and Malaysia, while low rates (less than 10 % among adults) were consistently reported from Japan (Table 3.1). The anti-HEV-positive rates in most African countries are higher than 30 % (Table 3.2). In contrast, these rates are less than 20 % in most European countries (Table 3.3). The divergent anti-HEV immunoglobulin (Ig)G seroprevalence among different countries may roughly represent the geographical variations in the burden of hepatitis E infection. The prevalence of anti-HEV IgG is high in developing countries, reflecting the endemic nature of the disease in this setting.

HEV seroprevalence differs between rural and urban areas. In the general population, the positive rate of anti-HEV IgG was significantly higher in rural areas (41.7 %) than in urban areas (22.7 %) of China and in eastern Japan (5.6 %) than in western Japan (1.8 %) and varied greatly between different states/regions of the USA (range, 1.2–21.3 %) and Europe (range, 0.26–52.5 %) [31]. A difference in seroprevalence between rural and urban residents is also observed in Egypt, Mexico, and South Africa, with the seroprevalence being higher in rural areas [8, 110]. Conversely, one study in Gabon found that the HEV prevalence in urban areas (13.5 %) was over twice as high as that in rural areas (6.4 %) [20]. The population density,

Table 3.1 Prevalence of anti-HEV antibodies among the general population of Asia

Country	Number of samples	Anti-HEV rate (%)			References
		Children	Adults	Overall	
India	812	13–40	1677	15–73	[77]
Malaysia	134	40–50	43–67	44–50	[90]
China	3844	10–11	40–46	44	[66]
China	15,862	5.1–8.4		23.5	[53]
Bangladesh	1134	3.8	27.1–41.5	22.5	[64]
Korea	147			23.1	[84]
Korea	2450		5.9		[116]
Iran	510		46.3		[36]
Israel	729	0.5	1.1–37.5	10.6	[75]
Turkey	210	5.2–8.5			[11]
Japan	22,027		2.6–2.7		[97]
Hong Kong	450	6.8–8.0	18–60	28	[20]
Cambodia	868	5.8	21.2–35.3	18.4	[115]

Table 3.2 Prevalence of anti-HEV antibodies among the general population of Africa

Country	Number of samples	Anti-HEV rate (%)			References
		Children	Adults	Overall	
Zambia	300	16	42		[50]
Egypt	10,156	25.7–75.3	48.1–73.7	67.6	[37]
South Africa	767		5.8–19.1		[106]
Somalia	36			61.1	[18]

absence of sewer systems, consumption of bush meat, and presence of excreta from peri-domestic animals near habitations, all of which contribute to the precarious sanitary conditions in this area, might be risk factors for HEV spread.

In some countries, the seroprevalence of anti-HEV IgG has remained stable over time. The rate was approximately 5 % from 2007 to 2012 in Japan; approximately 3 % from 2000 to 2012 in Brazil, from 1999 to 2010 in Spain, and from 1994 to 2012 in Italy; and approximately 18 % from 1997 to 2013 in the USA, according to a review by [73]. In contrast, the anti-HEV IgG seroprevalence has increased over time in other countries: in Germany, from 5.5 % in 2010 to 15.5 % in 2013; in Greece, from 0.26 % in 1998 to 9.43 % in 2013; and in the Midi-Pyrénées region of France, from 16.6 % in 2008 to 52.5 % in 2011 [73]. Investigations in Pune, India [11], and Ankara, Turkey [12], showed no or minor increases in rates of HEV seropositive over time. However, a study from a tribal population of the Andaman and Nicobar Islands in India showed a significant rise in HEV seroprevalence, from 13 % in 1989 to 40 % in 1999 in children <15 years of age [79]. A few studies have addressed the issue of changing HEV seroprevalence over time in the same population, but the authors were unable to identify the specific factors responsible for this change [79].

Table 3.3 Prevalence of anti-HEV antibodies among the general population of Europe

Country	Number of samples	Anti-HEV rate (%)			References
		Children	Adults	Overall	
UK	710		3.9		[14]
Italy	236	8.7		6.3	[27]
Germany	4422		16.8		[36]
France	1031			34	[51]
The Netherlands	7270	0–0.3	1.4–6.4	1.9	[113]
Spain	2305	0.5	2.1	1.1	[39]
Spain	2529	4.6	7.3	6	[36]
England	1591	2–3	5–27	13	[50]
England	1140	2–3	5–25	13.5	[50]

Table 3.4 Prevalence of anti-HEV antibodies among the general population of the Americas

Country	Number of samples	Anti-HEV rate (%)			References
		Children	Adults	Overall	
USA	18,695	1–5	39–42	21	[64]
Canada	393	2.6	3.1	3	[75]
Mexico	273			36.3	[7]
Chile	100		17		[48]
Bolivia	226	2.4–8.7	6.3	4.0–24	[27]

The seroprevalence of HEV seems to be higher in pregnant women than in the general population in Ghana, 28.7 % [2] vs. 4.6 % [74], and also in Gabon, 14.2 % [20] vs. 0 % [90]. In most disease-endemic areas, anti-HEV antibodies have been detected in as many as 5 % of children less than 10 years of age, and this ratio increases to 10–40 % among adults older than 25 years of age. India, Malaysia, and Southern China displayed the highest rates among children (up to 20–50 %) (Table 3.1). Overall, there appears to be a gradual increase in the anti-HEV IgG seroprevalence as the age of individuals rises (Table 3.6 and Fig. 3.1).

The wide variation in the anti-HEV antibody seroprevalence among the populations of various countries or within the same country may also be partly due to differences in the HEV antibody detection assays used to assess the seroprevalence (Table 3.5). The various commercially available tests show important differences in sensitivity. Further, the sensitivity and specificity of a test depend upon the prevalence, as well as on the viral genotype present in the study population. In a population-based cohort study, 1025 randomly selected participants were enrolled from Matlab, Bangladesh (2004–2005), and were tested for anti-HEV antibodies using an in-house enzyme immunoassay developed by the Walter Reed Army Institute of Research (WRAIR). In 2014, the banked sera of 1009 of those participants were retested using the Wantai anti-HEV IgG enzyme-linked immunosorbent assay (ELISA). The WRAIR assay estimated the overall population seroprevalence as 26.6 % (95 % confidence interval [CI], 24.0–29.5), whereas the Wantai assay

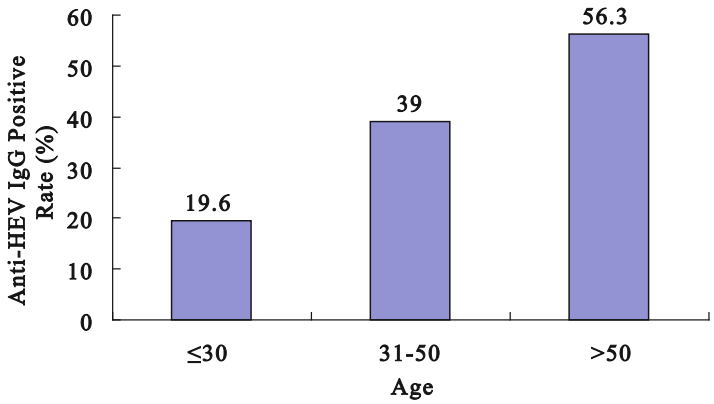

Fig. 3.1 Anti-HEV IgG distribution in the general population of rural Durango, Mexico, according to age [7]

Table 3.5 Results from selected studies reporting the prevalence of anti-HEV antibodies in the general population of the USA

No. of samples	Anti-HEV rate (%)	Year of investigation	Testing kit	References
8814	6	2009–2010	DS-EIA-ANTI-HEV-G (Saronno, Italy)	[28]
18,695	21	1988–1994	"In-house" EIA	[64]

Table 3.6 Anti-HEV IgG distribution in the general population of the Midi-Pyrénées area of France, according to age

Age (sample no.)	2–5 (215)	6–10 (104)	11–17 (137)	18–27 (127)	28–37 (106)	35–47 (184)	48–57 (116)	56–65 (40)
Percentage of anti-HEV IgG (%)	4.7	14.4	24.8	27.6	42.5	56	62.1	70

Data based on Kamar et al. [56]

produced a significantly higher estimated seroprevalence of 46.7 % (95 % CI, 43.5–49.8) ($p < 0.001$) [62]. Because these two tests produced nearly identical findings in those aged 5 years and below (n = 94) with a 98 % agreement between the tests [62], the different sensitivities of the two assays resulted in different seroprevalence. Thus, in the absence of standardized commercially available confirmatory assays, such as Western blots, differences in the seroprevalence rates between different populations must be interpreted with caution. Retesting populations with modern assays will be necessary to establish better population-level estimates of the HEV disease burden.

3.3 HEV Genotype Distribution Worldwide

In addition to humans, HEV infects many other wild and domestic animals, such as pigs, rabbits, rats, deer, mongoose, and chickens. The genomic sequences are different among HEV isolates from different hosts in different geographical areas. HEV is classified into the family *Hepeviridae*. A new proposed consensus for HEV classification [97] divides the *Hepeviridae* family in two genera: *Orthohepevirus* and *Piscihepevirus*. The latter currently includes only isolates from cutthroat trout. The genus *Orthohepevirus* is further subdivided into four species: *Orthohepevirus A*, with isolates from humans, pigs, wild boars, deer, mongoose, rabbits, and camels, and *Orthohepevirus B*, *C*, and *D*, with isolates from other mammals or from birds. *Orthohepevirus A* is additionally divided into at least seven genotypes (genotypes 1–7). Genotypes 1–4 are recognized to infect humans and can cause hepatitis E. Genotypes 1 and 2 are exclusively human HEV strains, whereas HEV genotypes 3 and 4 can also infect other animal species, particularly domestic pigs and wild boars. Each HEV genotype has a specific geographic distribution. The worldwide distribution of genotypes 1–4 is shown in Fig. 3.2.

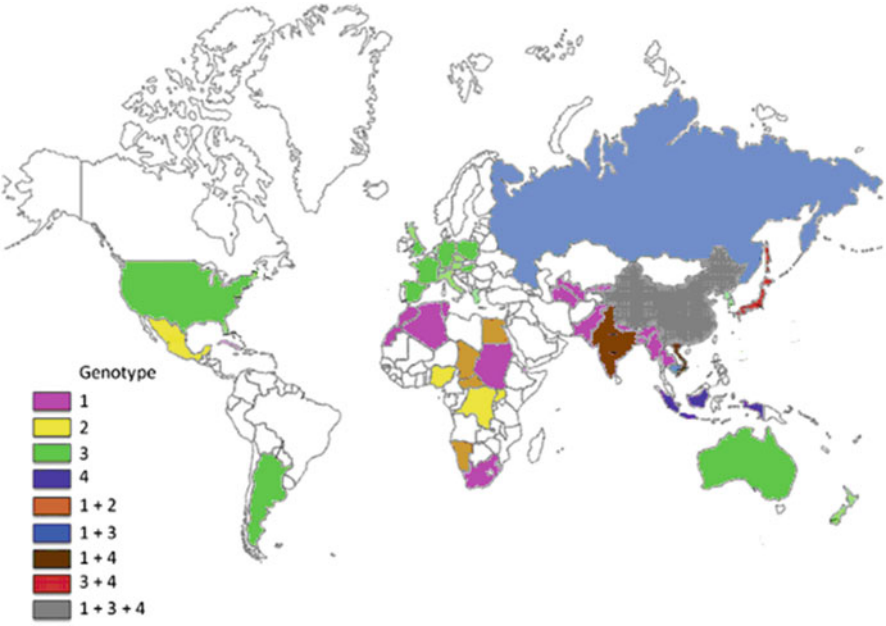

Fig. 3.2 Distribution of the HEV genotypes of viral isolates obtained from humans and animals (mainly pigs). The colors used for each country and the circle associated with it represent the predominant HEV genotypes of human and animal isolates, respectively, from that country (The figure is based on data from Okamoto [83] and Aggarwal [4])

3.3.1 Asia

The only HEV genotype that has been identified during the large waterborne outbreaks of HEV in India and in the neighboring South Asian countries, such as Nepal, Pakistan, and Bangladesh, is genotype 1. Additionally, HEV isolates from sporadic cases in these countries also belong to genotype 1. The nucleotide sequences of these isolates are highly homologous, indicating that genotype 1 is the dominant cause of hepatitis E disease in this region [98]. However, HEV isolates from pigs in India were identified as genotype 4 [13], indicating that different HEV genotypes can circulate in humans and pigs within the same country.

HEV strains isolated from the Xinjiang epidemic in 1988–1990, which were the first HEV strains analyzed in China, were assigned to genotype 1 [70]. Additionally, most of the HEV strains isolated prior to 2000 from regions across China also belong to genotype 1. However, genotype 4, which has been identified in most regions of China over the last 10 years, has overtaken genotype 1 in its frequency of isolation nationwide. Genotype 3 HEV strains have also been found in China and are thought to have been imported from Japan. Both genotypes 3 and 4 of HEV have been found in humans and pigs, and cross-species transmission of these two genotypes from pigs to humans may have occurred. Currently, HEV genotypes 1, 3, and 4 coexist in China, but genotype 4 is the predominant genotype in this country [70].

HEV genotypes 3 and 4 have been consistently dominant in East and Southeast Asian countries, such as Japan [58], Korea [54], and Singapore [102]. Hepatitis E shows a sporadic, non-endemic epidemiologic pattern in this area, and HEV strains of genotype 3 or 4 have been isolated from patients with autochthonous hepatitis E in these countries.

Large outbreaks and sporadic cases of hepatitis E have both been reported in Central Asia. The HEV strains isolated from outbreaks or sporadic cases in Turkmenistan were identified as belonging to genotype 1 [6]. In Kyrgyzstan, another Central Asian country, genotype 1 HEV strains were detected from patients during an outbreak, while the HEV strains detected from pigs in this country belong to HEV genotype 3 [71].

3.3.2 Africa

Data on the circulating HEV genotypes are available for nine African countries [61]. Based on these limited data, genotype 1 appears to be the most prevalent HEV genotype in Africa, as it was found in the Central African Republic [35], Sudan [82], Chad [82, 111], Egypt [15, 26], and Namibia [47]. Genotype 2 HEV, which was first reported in an outbreak in Mexico, was subsequently identified in patients in West Africa, Nigeria [18], Chad Nicand [35, 82], and Namibia [47, 72]. Genotype 3 HEV is rare in African countries and was found in one Egyptian child [57] and in one acute hepatitis patient in Mayotte (originally from France) [33]. Studies of

Ghanaians suggest that the anti-HEV antibody seroprevalence among pig handlers is over 34 % and that the predominant HEV genotypes in Ghana may be of zoonotic origin [1, 2].

Notably, the HEV genotype distribution can differ between neighboring countries, as was demonstrated by one study in Sudan and Chad reporting that genotype 1 HEV was more common in Sudan, while genotype 2 HEV was more common in Chad [82].

3.3.3 America

Genotype 3 HEV was first identified in human cases of locally acquired hepatitis E in the USA [65, 92]. However, the incident rates of hepatitis E are relatively low in the USA and in Canada. Only a few of the reported acute hepatitis E cases were acquired domestically. All of the HEV strains isolated from patients without a history of travel to a foreign county were identified as genotype 3 [30, 106, 118]. Genotype 3 HEV is also prevalent in pigs in North America.

In Latin America, molecular characterization studies identified the prototype strain for genotype 2 HEV (M74506) on the basis of characterization of a single strain, but subsequent studies found that some cases in this area were caused by genotype 3 HEV strains [32]. Epidemic outbreaks and sporadic cases of hepatitis E were also reported from Cuba; the 23 HEV strains recovered from two outbreaks and from 12 sporadic cases were all clustered within genotype 1 in phylogenetic trees [114]. In recent studies performed in Venezuela, the sequences of two strains of HEV genotype 1 and of one strain of HEV genotype 3 were detected in three sporadic cases. Genotype 3 HEV RNA was also found in samples from hepatitis E patients in Argentina, Brazil, Uruguay, and Southwest Bolivia [29, 76, 78]. These data confirm the co-circulation of HEV genotypes 1 and 3 in the Caribbean region. However, additional findings are still needed to confirm the presence of HEV genotype 2 in Mexico.

3.3.4 Europe

Since the 1990s, travel-associated imported cases of HEV infection have been reported in many European countries: in the UK from India and Saudi Arabia; in the Netherlands from Bangladesh, Somalia, and the Middle East; and in Sweden and Turkey [67]. The clinical features of these patients were similar to those in HEV-endemic countries, and all of the HEV strains linked to these imported infections belong to genotype 1. In contrast, most of the hepatitis E cases in European countries, such as the UK, Germany, Dutch, Spain, Sweden, Czech Republic, and France, in which the infection was acquired from within Europe, rather than from travel

outside of this area, are caused by HEV genotype 3 [9, 67]. Therefore, genotype 3 HEV is considered to be the autochthonous type in Europe.

In 2008, a genotype 4 HEV infection was reported in a German patient with no travel history [117]. A genotype 4 HEV strain was then detected in Belgian swine in 2011 [44], and the first autochthonous genotype 4 infection was reported in France [105], followed by two other cases in southern France associated with the consumption of uncooked pork liver sausage [23]. In 2011, 280 HEV RNA-positive infections were identified by the National Reference Centre for HAV and HEV, including nine infections due to HEV genotype 4 [53]. During 2011, five persons in the area of Lazio, Italy, were infected with a strain of HEV genotype 4 that showed high sequence homology with HEV isolates from swine in China [40]. These patients all lived in the same area and did not travel to disease-endemic areas, and epidemiologic information was unable to identify the transmission route. Strong sequence similarity (>96 %) was observed between the HEV isolates from human cases in northern and southern France and the strain isolated from swine in Belgium [16]. Overall, HEV genotypes 3 and 4 overlap in Europe, but genotype 3 seems to be more prevalent.

3.3.5 New Zealand and Australia

In New Zealand, HEV genotype 3 was isolated from four patients with unexplained hepatitis [25]. In Australia, there are few reported cases of locally acquired HEV; cases of hepatitis E are mainly travelers returning from disease-endemic countries [96]. Data from the Commonwealth Department of Health in Australia indicate that there were 378 reported HEV cases from 1999 to 2013, with an average of 25 cases per year, but the genotypes of the HEV strains responsible for these cases were not defined [96].

3.4 Epidemiologic Patterns of HEV Infection

HEV is considered hyperendemic in many developing countries, such as India, Bangladesh, Egypt, Mexico, and China. According to the Centers for Disease Control and Prevention, USA, hyperendemic countries carry a prevalence of 25 % of all non-A, non-B acute hepatitis cases or have experienced a major waterborne outbreak of hepatitis E; in contrast, HEV is considered endemic in places with a quantifiable prevalence of all reported non-A, non-B acute hepatitis that is less than 25 % [121]. Endemic countries and regions include much of Western Europe, the USA, New Zealand, many countries in South America, much of Asia, and the Middle East [121]. In these places, HEV infection is infrequent and occasionally occurs as sporadic cases [86].

Distinctly different epidemiologic patterns have been observed in the geographical regions where hepatitis E is hyperendemic compared with those in areas where

Table 3.7 Epidemiological features of hepatitis E in hyperendemic and endemic areas

Feature	Hyperendemic areas	Endemic areas
HEV genotypes	1 and 2	3 and 4
HEV reservoirs	Human	Animals
Transmission	Waterborne	Zoonotic
Outbreaks	Yes	No
Person-to-person spread	Very limited	No
Seasonality	Yes, outbreaks occur at times of flooding/monsoon	No, but relatively higher in spring in China

it is endemic. These two distinct patterns seem to correlate with the distribution of HEV genotypes, disease prevalence, frequencies of various transmission routes, affected population groups, and clinical characteristics of the disease (Table 3.7).

3.4.1 The Epidemiologic Patterns of Infection with HEV Genotypes 1 and 2

Hepatitis E in hyperendemic areas, located in tropical and subtropical regions with poor sanitation, has characteristic epidemiological features. Namely, it occurs as both an epidemic and a sporadic disease, affects a large part of the population, and is largely due to genotype 1 or genotype 2 HEV strains.

3.4.1.1 Reservoirs of HEV Genotypes 1 and 2 in Endemic Regions

In hyperendemic countries, hepatitis E is a waterborne infection caused mainly by genotype 1 or genotype 2 HEV. Because neither HEV genotype 1 nor HEV genotype 2 has ever been isolated from animals and both of these genotypes failed to infect pigs in experimental studies, zoonotic transmission appears unlikely to be responsible for the prevalence of HEV of these two genotypes. Their potential reservoir may be a continuously circulating pool of individuals with clinical or subclinical HEV infection. Punctuated by occasional dramatic outbreaks involving thousands or tens of thousands of cases, sporadic HEV cases occur throughout the year, and together these infections likely contribute to the HEV reservoir that is responsible for maintaining the disease in a given population [4].

The detection of HEV genotypes 1 and 2 in sewage indicates that it may play an important role as an environmental reservoir of HEV [49]. Studies of cynomolgus macaques experimentally infected with HEV found that infection in this model produces protracted viremia and prolonged fecal shedding of HEV [5, 46]. Similar fecal shedding of the virus by persons with subclinical HEV infection could lead to the continuous maintenance of a source of infection in a disease-endemic area. This

pool of infection could, in turn, lead to periodic contamination of drinking water supplies.

3.4.1.2 Outbreaks of Hepatitis E

Outbreaks of hepatitis E have been documented exclusively in resource-limited countries or in regions undergoing a humanitarian emergency, where there were overcrowding and limited access to potable water, proper sanitation, and hygiene. Hepatitis E outbreaks have been reported in Asia, the Middle East, North and West Africa, and Central America (Mexico), which are all considered to be hyperendemic areas of HEV infection. HEV outbreaks can generally be traced back to contaminated water sources and may affect several hundreds to several thousands of individuals. The occurrence and magnitude of outbreaks are strongly associated with the hygienic conditions and population density (Table 3.8).

The first retrospectively (serologically) confirmed HEV outbreak occurred in New Delhi, India, in 1955–1956 with more than 29,000 symptomatic jaundiced persons [115]. In India, HEV is responsible for large outbreaks, and the source of infection is usually a contaminated water supply. According to the surveillance across all Indian states conducted by the National Integrated Disease Surveillance Programme (IDSP) in India [63], 291 hepatitis outbreaks were reported to the IDSP during 2011–2013. Twenty-three (65.7 %) of the 35 states in India reported at least one hepatitis outbreak, and five states reported more than 20 outbreaks. Among the 163 reported outbreaks with known etiology, 78 (48 %) were caused by hepatitis E, and 19 (12 %) were caused by both hepatitis A and E. Additionally, contaminated drinking water was identified as the cause for 72 % (109 of 151) of the hepatitis A and E outbreaks and was implicated in 49 (38 %) of the 128 outbreaks for which laboratory confirmation was not available. More outbreaks were reported from rural

Table 3.8 Selected reported large outbreaks of hepatitis E

Location	Years	Cases	Transmission	References
India	1955–1968	29,300	Waterborne	[115]
Kashmir	1978–1979	>270	Waterborne	[60]
Mexico	1986	>200	Contaminated well water	[112]
Ethiopia	1988–1989	>750	After monsoon rains	[107]
India	1991	79,000	Contaminated river water	[81]
China	1991	119,000	Waterborne	[100]
Vietnam	1994	>300	After heavy rains	[24]
Pakistan	1993–1994	3827	Contaminated plant water	[89]
Nepal	1995	692	Contaminated drinking water	[22]
Sudan	2004	>2600	Safe water insufficient	[43]
Uganda	2008	>10,000	Substantial person to person	[104]
Bangladesh	2010	>62	Contaminated tap water	[45]
Sudan	2014–2015	>1117	Safe water insufficient	[17]

areas (199/291, 68 %) than from urban areas (92/291, 32 %). The large number of hepatitis A and E outbreaks in India might be explained in part by the lack of adequate sewage and sanitation systems; defecation in open fields, which can contaminate surface drinking water sources, remains a common practice in this region [63].

The first documented outbreak of HEV infection in Africa likely occurred in 1950 in Tunisia, and HEV outbreaks have been detected in 11 African countries since the 1980s [103]. In recent years, some hepatitis E outbreaks have been reported from areas in Africa with conflict, violence, and major human displacement [61, 103]. Several HEV outbreaks have occurred in refugee camps. From April 2014 to January 2015, a total of 1117 suspected cases of HEV, with 21 (1.9 %) deaths, were reported among refugees residing in the Gambella region [17]. The limited availability of facilities for safe drinking water or for the proper disposal of human feces in refugee camps appears to have been the main cause for the spread of HEV infection [17].

In North America, two outbreaks of hepatitis E took place in two Mexican villages in 1986–1987. In the village of Huitzila, 94 icteric cases were found among their 1157 residents; of these, two patients died. In Telixtac, 129 icteric cases were recorded among their 2194 inhabitants, with death reported in one patient [112]. Hepatitis E has not been reported since from Mexico; nevertheless, the country is considered hyperendemic for HEV [59].

Hepatitis E incidence in South Asia has been characterized by marked seasonality, with outbreaks occurring during the rainy or monsoon seasons. These epidemics have been documented in April and October in countries such as India, Bangladesh, and Nepal [22, 42, 63].

During outbreaks, HEV mainly targets young to middle-aged adults, generally 15–40 years of age, with a significantly lower seroprevalence in individuals <10 years old. Overall the attack rates during hepatitis E outbreaks range from 1 to 15 %, with males generally outnumbering females (male/female ratio = 2–3:1), suggesting that males are more likely to be affected by hepatitis E than females. In outbreaks in Pakistan and Nepal, the ratios of patients with mild anicteric symptoms to patients with severe jaundice were 4:1 and 3:1, respectively [99].

The clinical symptoms of HEV infection are typical of acute viral hepatitis and include jaundice, malaise, anorexia, nausea, abdominal pain, fever, and hepatomegaly; anicteric hepatitis is also observed [80]. The disease is self-limiting, and no chronic sequelae are generally reported. A unique clinical feature of HEV infection is its increased incidence and severity in pregnant women, with mortality rates of 15–20 % [60]. Pregnant women with jaundice and acute viral hepatitis due to HEV showed higher mortality rates and worse obstetric and fetal outcomes than those with other types of viral hepatitis [85].

3.4.1.3 Sporadic Hepatitis E in High-Endemic Regions

In HEV-endemic areas, epidemics of hepatitis E are more frequent and are usually separated by a few years. A periodicity of 5–10 years has been suggested for recurring HEV epidemics in India, China, and certain Central Asian republics of the

former Soviet Union. Cyclic outbreaks have been documented in the tropics of Asia and Africa. In hyperendemic regions, hepatitis E continues to occur between epidemics in the form of sporadic hepatitis, irrespective of the age group [4]. In India, although the peak incidence occurs during the rainy season, low levels of HEV infection continue through the winter [9]. In the high-endemic regions, the sporadic patients and those patients during hepatitis E outbreaks share several epidemiological and clinical characteristics, such as predominant affliction of adolescents and young adults, the association between pregnancy and severe disease, and clinical presentation as acute hepatitis.

3.4.2 Epidemiologic Pattern of HEV Infection in Industrialized Countries

In contrast to the larger epidemics and outbreaks of genotype 1 and genotype 2 HEV in developing countries, autochthonous hepatitis E in industrialized countries is considered prevalent but is only limited to sporadic cases caused by HEV genotype 3 or genotype 4. Pigs, and likely many other animals, are natural reservoirs of HEV genotypes 3 and 4, and most infections are related to zoonotic transmission (details in Chap. 6).

HEV genotypes 3 and 4 seem to be much less virulent in humans than HEV genotypes 1 and 2. In immunocompetent individuals, HEV infections are usually asymptomatic and have no consequences. A careful investigation performed on people involved in an outbreak of genotype 3 HEV infection associated with shellfish intake among the passengers of a cruise ship found 11 cases of acute hepatitis and 22 asymptomatic infections [91]. A large prospective vaccination study in China showed that fewer than 5 % of those exposed to genotype 4 HEV develop signs of acute hepatitis E [124]. Another study reported that, as in adults, hepatitis E caused by genotype 3 HEV is very rarely symptomatic in children [116]. Additionally, in agreement with the typical male-to-female infection rate for HEV, an analysis of sporadic cases of acute hepatitis due to genotype 3 HEV found that the likelihood of infection was significantly higher among men than among women [91].

In recent years, the number of reported HEV cases in many developed countries has risen sharply, whereas the detected prevalence of antibodies against HEV in serum has remained fairly constant [88]. This observation suggests that the increase in cases of hepatitis E reported to the Robert Koch Institute likely arises from an increased awareness, rather than an increased incidence, of this disease [88]. An increase in the number of HEV infections acquired in developed countries and the discovery of chronic hepatitis E in immunosuppressed individuals have dispelled the perception of hepatitis E as merely an acute tropical illness, thus lending new importance to this infectious disease.

3.4.3 *The Shifting Epidemiologic Pattern of Hepatitis E in China*

China is generally considered to be an HEV-endemic area. The extended waterborne outbreak of 1989 in Xinjiang Province, northwestern China, resulted in 120,000 cases. The outbreak was caused by HEV genotype 1 and affected mainly young adults. Since then, a couple of outbreaks of different scales caused by genotype 1 HEV have been reported in different geographical areas. However, since 2000, hepatitis E has mainly occurred as sporadic cases and occasional food-borne outbreaks, and no HEV outbreaks have been reported in recent years, suggesting a transition from a high-endemicity pattern to a low-endemicity pattern. Currently, the predominant circulating HEV genotype is genotype 4, with only occasional genotype 1 cases, and the sporadic cases of hepatitis E are more common in elderly men [41, 70]. This pattern of infection is similar to that seen in Europe with genotype 3 HEV.

The trend of diminishing numbers of HEV outbreaks is in accordance with the shift in the prevalence of HEV genotypes over the past 20 years that has been observed in China and some other countries. The reason for this shift toward genotype 4 HEV as the predominant genotype in China is unclear, but it might reflect the improvements in water supply and sanitary conditions in China over the past few decades, allowing zoonotically transmitted genotype 4 HEV to become dominant in the human population.

3.5 HEV Prevention and Control

As an enterically transmitted virus, HEV is primarily transmitted by the contamination of drinking water and undercooked meat products. Proper disposal of human feces, consumption of clean water, sanitary handling and proper cooking of meat products, and education about personal hygiene help prevent this disease.

Passive immunoprophylaxis with antibodies against HEV capsid was successful in treating hepatitis E in cynomolgus monkeys [108]. Although there are four HEV genotypes, all known HEV strains share common epitopes on their capsid genes, suggesting that they belong to a single serotype [93]. Therefore, a protective vaccine against a broad spectrum of HEV isolates should be possible. Various recombinant capsid proteins expressed in insect cells and *Escherichia coli* were reported to be successful recombinant vaccines conferring protection against both homologous and heterologous HEV strains [33, 69, 109]. Two subunit vaccines have been developed against HEV infection and have been shown to be highly protective against clinical hepatitis E in clinical trials [95, 124]. The first of these vaccines is a 56-kDa protein encoded by the ORF2 of a genotype 1 HEV strain that is expressed in insect cells. In a trial among 2000 volunteer Nepalese soldiers, three doses of 20 μg of the 56-kDa protein (at 0, 1, and 6 months) achieved 100 % seroconversion and a protective efficacy of up to 95.5 % (95 % CI, 85.6–98.6 %) during a 2-year follow-up [95].

The second vaccine, a 26-kDa protein encoded by the ORF2 of a genotype 1 HEV strain, is expressed in *E. coli* and occurs as viruslike particles of 23 nm in diameter [124]. In the phase II study and the phase III trial, this vaccine was found to be safe and immunogenic, and it conferred protection against HEV infection [122, 124]. This vaccine has been licensed with the commercial name of Hecolin for use in China since 2012. A study addressing the long-term efficacy of this vaccine was carried out over 4.5 years in which the efficacy, immunogenicity, and safety of the vaccine were evaluated in a vaccinated group of 56,302 participants in comparison with a control group of 56,302 participants. In this study, 60 cases of hepatitis E were identified, of which only seven belonged to the vaccinated group, revealing that the efficacy of this vaccine is 87 %. No issues concerning the safety of the vaccine were observed [123]. The use of the vaccine should be considered to mitigate or prevent outbreaks of hepatitis E infection, especially in high-risk groups, such as pregnant women.

3.6 Conclusion

Hepatitis E is highly endemic in several developing countries in Asia and Africa where contamination of water supplies and lack of adequate sanitation are frequent. In developed countries, autochthonous cases of HEV infection are sporadic, and the importance of animal reservoirs has become clear. The clinical and epidemiological characteristics of hepatitis E in hyperendemic and endemic countries are summarized as follows:

In Developing Countries

- Hepatitis E occurs as outbreaks or sporadic cases.
- Outbreaks are generally caused by genotype 1 or genotype 2 HEV strains through water contamination.
- The highest attack rate appears to be among individuals between 15 and 40 years of age.
- HEV infection is self-limited and has no chronic sequelae.
- Hepatitis E occurs more often in pregnant women than in nonpregnant women or men, and pregnant women with hepatitis E have a high mortality rate (10–25 %).

In Developed Countries

- Hepatitis E is mostly caused by HEV of genotype 3 or 4.
- The main routes of HEV transmission are probably zoonotic and food-borne; person-to-person transmission is rare.
- The estimated HEV infection incidence varies among different age groups, and the males of aged 50 years or older account for most of the total patients.
- Chronic HEV infections have been observed in immunocompromised individuals.

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Chapter 4
Hepatitis E as a Zoonosis

Frederik Widén

Abstract Hepatitis E (HE) virus infection is not limited to spread from human to human but also occurs between animals and more importantly as zoonotic spread from animals to humans. Genotyping of strains from hepatitis E virus-infected patients has revealed that these infections are not all caused by genotypes 1 or 2 but often by genotypes 3 or 4. Therefore, it is important to understand the striking difference between the spread of genotypes 1 and 2 in countries with poor sanitary standards and the spread of genotypes 3 and 4 in countries with good sanitary standards. The number of animal species known to be infected with HEV is expanding rapidly. The finding of HEV in new host species always raises the question regarding the zoonotic potential of these newfound strains. However, as new strains are found, the complexity increases.

Certain genotypes are known to have the ability of zoonotic spread from certain animal species and these animals may even constitute an infection reservoir. Some animal species may contribute to zoonotic infections albeit on a smaller scale, while others are believed to be of minor or no importance at all. This chapter reviews possible sources of zoonotic hepatitis E virus infection.

Keywords Hepatitis E virus • Zoonosis • Swine HEV • Rabbit HEV • Avian HEV

4.1 Introduction to Zoonotic HEV Infections

Large disease outbreaks of hepatitis E (HE) occur in countries with poor sanitary conditions as described elsewhere in this book. The disease is usually endemic in these countries and spread indirectly from person to person through contaminated water, food and toilets especially in refugee camps and other poor dwellings. It causes epidemics from time to time. However, it has been well documented that hepatitis E (HE) also occurs sporadically in persons living in countries with good

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sanitary standard. The previously widely accepted assumption, that all human infections in countries with good sanitary standards were acquired while travelling or living in countries with an endemic HEV situation, has been challenged for a long time and is not in line with current scientific evidence. It is known that a part of these infections are caused by genotype 1 (or 2) and occur in patients who recently travelled to endemic regions. However, another large part of these infections occurred in patients who did not travel to endemic regions during the calculated incubation time, or not at all travelled abroad, and that was not caused by genotypes 1 or 2 but rather by genotypes 3 or 4. The increased awareness of this situation has in turn led to more samples from patients being sequenced and genotyped. Thus it has been demonstrated that several strains causing disease in humans demonstrate a high degree of similarity to strains detected in animals and food of animal origin. The application of serological test for detection of HEV antibodies in the human population in industrialized countries has demonstrated a surprisingly high (1–53 %) antibody prevalence in several countries [17]. Furthermore, HEV RNA, mainly of genotype 3, has been detected in blood products from donations given by healthy humans. For example, in Germany, 1 out of 4500 and, in Sweden, 1 out of 8000 healthy blood donors had HEV RNA in the blood at the time of donation. Since these HEV-positive samples came from healthy blood donors and the viraemic stage is rather short, the number of humans that are infected during their lifetime can be expected to be much higher. All facts taken together have led to the conclusion that there is an autochthonous source of HEV present in industrialized countries that cause infections and disease in humans and cannot be disregarded [6, 20]. While HEV has been detected in many animal species, only HEV strains belonging to genotypes 1–4 are regarded as possibly zoonotic.

4.2 Introduction to HEV Infection in Animals

The list of animal species susceptible to infection with HEV has been expanding rapidly during the two last decades [29]. The list is now extensive and still continues to grow. The list is based on results from PCR amplification of nucleic acid (RNA) but can be made significantly longer if serological results are also taken into account. Such serological results are generally generated by ELISA. There are now a number of commercially available ELISAs that can be used for testing of animal sera for antibodies against HEV. However, several reports suggesting infection of animal species are based on a small number of serologically positive samples. If there are no previous experiences of analysing sera with the assay, the results should be interpreted with caution. Single serological results are difficult to evaluate because a confirmatory assay, a gold standard, is lacking. The commercially available ELISA assays for antibodies to HEV that have been developed all suffer from a lack of specificity, and different assays will not give identical results for a given selection of sera. This is not a unique feature of HEV ELISA assays as almost all assays will

show some disagreement when compared to each other. However, when applied to one or a few samples only and without prior experience of the assays compatibility with sera from the species, the disagreement becomes a critical factor. Therefore, reports of isolated, serologically positive individuals from animal species not previously reported as HEV hosts cannot be relied on and thus need to be confirmed by PCR amplification with sequencing, seroconversion or by other confirmatory assay.

While the genotypes 1 and 2 infect only humans and primates and are known to cause large disease outbreaks or endemic disease in humans in countries with poor sanitary conditions, the genotypes 3 and 4 are found in swine and wild boar and are able to infect humans. It has been demonstrated that that porcine HEV strains of genotype 3 can infect primates and that human genotype 3 strains can infect swine. Contrary to genotypes 1 and 2, these genotypes cause only sporadic cases of overt disease. Reports of disease, caused by genotypes 3 and 4, have been restricted to humans living in countries with good sanitary conditions. However, genotypes 3 and 4 may also circulate in countries where gt1 or 2 is present but is not observed due to the dominance of gt1 and 2. Furthermore, HEV of genotype 3 has also been found in various deer species, and infection of humans through consumption of contaminated deer meat has been demonstrated [42].

4.3 Taxonomical Considerations

The rapidly expanding number of proven HEV-positive animal species and the zoonotic potential has made a revision of the previously accepted taxonomy almost unavoidable, because too many species remain unassigned at the genus level in the former HEV taxonomy. The previously accepted taxonomy contained only one genus, *Hepevirus*, with one species, hepatitis E virus and four genotypes, and one unassigned genus containing one species, avian hepatitis E virus with several genotypes. The currently accepted taxonomy divides the *Hepeviridae* family in two genus, *Orthohepevirus* with four species and *Orthohepevirus* A–D and *Piscihepevirus* with only one species, *Piscihepevirus* A. The *Orthohepevirus* has been proposed to contain six or seven genotypes ([14] release).

4.4 Swine HEV

While it has been known since 1980 that a non-A or B hepatitis virus, later to be named hepatitis E, caused outbreaks of disease in humans [45], it was not until 1995 that HEV in swine was detected first in Nepal and subsequently also in the USA, and further studies revealed more than 90 % similarity between swine and human HEV strains [4, 24]. This was followed by similar reports from several non-endemic countries. The disease in humans was described in detail and proven by experimental infection in 1983. The finding and characterization of HEV in swine followed

studies in swine and primates showing that they could produce HEV antibodies naturally and that HEV RNA could be detected in swine faeces and sera and thus should have been infected with HEV or similar virus previously. This finding of HEV RNA and antibodies in swine raised concerns that HEV could be a zoonotic virus. Indeed, partial sequencing and comparison of the swine and human HEV genome demonstrated a high degree of similarity. However, more thorough studies demonstrated that the genomic sequence of swine HEV differed substantially from genotypes 1 and 2 in humans. Subsequently, two different genotypes, gt 3 and gt 4, have been identified in swine. A couple of years ago, two additional genotypes were suggested for wild boar due to their high divergence with known HEV sequences [40, 41]. Reports of seemingly food-related infections from Japan and elsewhere supported the assumption that HEV could be a zoonosis. However, the long incubation time, 3–8 weeks (average 40 days), creates difficulties to prove a causal link as in many cases the food items have since long been discarded when clinical symptoms first appear in the consumer. Several studies from Europe and Japan have now proven the link [2, 8, 11, 32, 42]. A high prevalence of gt 3 in swine and wild boar has been demonstrated in many parts of the world, and HEV is now regarded as a worldwide infection of swine. The gt 3 is completely dominating in European swine and can be found almost all over the world. Moreover, it is the only genotype detected, until now, in European wild boar and causes almost all autochthonous human HEV infections in Europe. Indeed, for several years only gt 3 was found in Europe and it was not until 2011 that gt 4 was detected in European swine [12] for the first time. Genotype 4 has been found in swine from Italy, Belgium and Denmark. The gt 4 is more frequent than gt 3 in China and is the predominant cause of hepatitis E in humans in China. In Chinese swine both gt3 and 4 can be found, but the gt 3 in China seem to cause only a few cases of human hepatitis E [39]. The gt 4 can also be found in swine in other Asian countries like Japan, India, Indonesia, Korea and Taiwan. At present it seems that in China the gt 4 causes almost all the autochthonous human infections detected so far, while in Japan it is either gt 3 or gt 4 that is the cause of autochthonous human infections. On the other hand, in Europe only a few autochthonous human infections caused by gt 4 have been identified. These cases were identified in France, Germany, Denmark, the United Kingdom, Italy and possibly also in Russia. It has been speculated that genotype 3 originated in Europe in the early nineteenth century reached Asia 100 years later and was spread from there to North America and the rest of the world. Genotype 4 is thought to have originated in Japan in the early nineteenth century.

In swine the infection route is, as in humans, faecal-oral, and the disease normally follows a subclinical course inducing mild to moderate lesions in the liver and regional lymph nodes. The litter size of the sow is not affected. The combined prevalence of markers for current (RNA) or past (antibodies) HEV infection is usually very high in swine as demonstrated by publications from several countries around the world. The peak of viraemia and virus excretion occurs at 2–4 months of age when the maternal antibodies have waned and piglets of different origin are mixed.

This is followed by seroconversion. With increasing age, the prevalence of virus-positive (RNA) swine goes down, while the prevalence of antibody positive goes up since the infection is self-limiting and antibodies protect against reinfection, at least for some time. However, some studies indicate that the immunity against reinfection is rather short and that swine may be infected several times during their life span [3]. The antibody prevalence goes up with age due to continuous exposure to infected swine excreting virus that remain stable in the environment for a long time. Infected swine start excreting virus approximately 1 week after infection, remain viraemic for 1–2 weeks and excrete virus for approximately 3 weeks. The virus can be detected in the liver for 4 weeks; however, other studies indicate that the virus could remain in the liver for 3 months. The virus has also been detected in muscle samples. Excretion in faeces is an important route of infection for swine but other routes, like urine, are also important. Furthermore, excretion of HEV genotype 3 for a period of more than 5 months has been demonstrated in a wild boar [38].

Given the normal path of HEV infection in swine, the risk for human exposure through pork products should be rather low at the time of slaughter. However, several studies have demonstrated that swine of slaughter age can still be virus positive. Indeed, in a study in the Netherlands, four out of 62 swine livers were positive for HEV at slaughter. In the Netherlands this could be extrapolated to roughly 1800 contaminated livers being consumed annually. This may be due to primary infection late in life, repeated infections due to poor immunity and prolonged virus persistence in organs as stated above.

What is stated above for HEV infection of swine generally also applies to wild boar since it is the same species. However, the epidemiology of HEV in wild boar may differ considerably from swine because of the differences between the natural habitat of wild boars and the rearing in swine farms [36, 37].

4.5 Rabbit HEV

Zhao et al. [49] reported the finding of HEV in farmed rabbits (*Oryctolagus cuniculus*) in China. This was followed by several reports of HEV detection from farmed rabbits in China [9], Mongolia, France [15] and the USA [5]. In France HEV was also detected in wild rabbits, and an HEV virus from a human with clinical symptoms, in France, demonstrated a high degree of similarity with rabbit HEV. The sequence identity with gt 1–4 varies between 73 and 79 %. Inoculation of pigs and cynomolgus monkeys with rabbit HEV demonstrated replication; increase of liver enzymes, indicating liver damage; and excretion of virus. It has now been accepted to place rabbit HEV in gt 3 as it forms a distant gt 3 subgroup ([14] release). These results demonstrate that rabbits may constitute a risk for zoonotic infection of humans. However, the prevalence of HEV infection in rabbits is not known.

4.6 Avian HEV

The disease known by two names, hepatitis-splenomegaly (HS) in North America and big liver and spleen disease (BLS) in Australia, is caused by avian hepatitis E virus (AHEV). It is present in, at least, three genotypes and was first described in Canada [34]. In 1999 [30] a virus that could be connected to the disease was detected and partially sequenced in the USA and Australia. Further studies revealed that the Australian and North American virus both are distantly related variants of AHEV and caused the same disease. Since AHEV share part of the sequence with swine HEV and human HEV, it was important to determine if AHEV could infect humans. Therefore, experiments with non-primate monkeys were performed. These experiments concluded that AHEV did not cause viraemia or seroconversion in rhesus macaques [13]. Further sequencing of AHEV showed that only 50 % of the sequence is shared with human and swine HEV and phylogenetical comparisons of the HEV family members indicate only a distant relationship. Thus, the three genotypes of AHEV are related distantly. However, AHEV is even more distantly related to HEV in swine and humans. Over 20 years of experience with AHEV without detection of avian HEV in humans gives a strong indication that AHEV does not infect and cause disease in humans. Even if not fully proven, these are good reasons to believe that AHEV is not a zoonotic virus.

4.7 Other Animal Species Infected by HEV

At present only strains belonging to genotypes 1–4 are regarded as zoonotic. However, the increasing number of HEV strains detected in several animal species makes the separation between zoonotic and non-zoonotic strains more difficult. The list of animal species known to be susceptible to HEV infection is long and continues to expand as new species are investigated. Currently, apart from the species mentioned above, the list contains primates (experimentally and naturally infected; cynomolgus monkeys, Japanese macaque (*Macaca fuscata*), etc. [22, 33, 47]), wild boar (*Sus scrofa*, [36, 44]), five bat species from three families ((Hipposideridae, Vespertilionidae and Phyllostomidae, [7]), Norwegian rat (*Rattus norvegicus*, [16]), black rat (*Rattus rattus* [25]), cotton rat (*Sigmodon hispidus*), greater bandicoot rat (*Bandicota indica*), other rat species (*Rattus* spp., [25])), Asian musk shrew (*Suncus murinus*, [10]), tree shrew [48], roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*, [26, 43]), mongoose (*Herpestes javanicus*, [27]), moose (*Alces alces*, [21]), sika deer (*Cervus nippon* [42], Rex rabbits (*Oryctolagus cuniculus*, [49]), ferret (*Mustela putorius*), farmed mink (*Neovison vison*, [18]), camel (*Camelus dromedarius*, [46]) and cutthroat trout (*Oncorhynchus clarkii*, [1]). Even more animal species have been implicated as potential host animals. These are, for example, Asian black bear (*Selenarctos thibetanus*), clouded leopard (*Neofelis nebulosa*), dog and cats (*Canis lupus*, [23]), fox (*Vulpes vulpes*), horse (*Equus caballus*, [35]),

cattle (*Bos taurus*), yak (*Bos grunniens*), goat (*Capra aegagrus*) and sheep (*Ovis aries*). However, these isolated findings remain to be confirmed by other studies using different methods or repeating the finding in other individuals. This is also the case with the reported findings of antibodies to HEV in goat, sheep, cat and dog as well as for the finding of HEV in fox. The HEV detected in fox was found in faeces and could therefore have originated from an animal that was eaten by the fox. Bioaccumulation in mussels has also been demonstrated (*Mytilus galloprovincialis*).

The wild boar belongs to the same species as pigs, so it is not at all surprising to find that it can also be infected by HEV genotype 3.

The HEV infecting mongoose also belongs to genotype 3 and can therefore be regarded as a zoonotic virus. However, no human cases caused by infection from mongoose have been described. However, humans and mongoose rarely come close to each other, and the risk for oral ingestion of infected material from a mongoose by a human seems quite remote. If mongoose also can be infected by genotype 4 remains to be shown.

The HEV found in bats forms a separate phylogenetic branch distinct from the known zoonotic hepeviruses. There are no known cases of humans becoming infected with HEV from bats. Therefore, bat hepevirus is currently not believed to be zoonotic. However, the same reasoning as for mongoose could be applied to bats. The prevalence of HEV in bats seems to be low, humans and bats rarely have contact, and oral ingestion, by humans, of infected material from bats is unlikely. However, fruit bats eating fruits from trees, for example, mango, may contaminate fruit pieces, with bat saliva or urine, subsequently falling down to the ground. Bats may also contaminate date palm sap, later to be ingested by humans as is suspected for Nipah transmission in Bangladesh [31]. For HEV transmission from bats to humans, this is just a hypothetical reasoning since the HEV virus in bats is so different from genotypes 1 to 4 and the risk for exposure probably is low.

Several species of rats can be infected with HEV but mainly by a variant that is distant to genotypes 1–4 and forms a separate branch in phylogenetic trees. This virus is not thought to be zoonotic. However, rats in the USA have also been infected by genotype 3 of HEV and could therefore constitute a zoonotic risk [19]. If rats also can be infected with genotype 4 is not known. Ferrets can be infected by HEV that cluster with HEV in rats. However, the phylogenetic distance between rat HEV and ferret HEV is larger than between genotypes 1 and 2 of humans. HEV found in mink is also related but distinct to ferret HEV as well as to rat HEV. Similar to HEV in rats (except genotype 3 variants found in the USA) and ferret, the distant relation to genotypes 1–4 and the clustering with rat and ferret HEV support the assumption that HEV from mink is not zoonotic.

Moose are frequently infected by HEV as has been demonstrated [21]. However, the HEV infecting moose is distantly related to HEV genotypes 1–4 and there are no indications that this virus should be zoonotic. It should be noted that moose is traditionally hunted and the meat is eaten but the liver is not. This may contribute to reducing the risk for human infections both in the short, infected material is not

eaten, and the long run since HEV in moose and genotype 4 with time are becoming even more distantly related.

Importantly, roe deer, red deer, sika deer and other deer species can be infected by HEV genotypes 3 or 4. The seroprevalence varies between 2 and 30 %, while the RNA prevalence is approximately 30 %. The zoonotic potential of HEV-infected deer has been documented. In Japan several members of a family became infected with HEV and fell sick after eating sika deer meat [42]. The HEV sequence recovered from the patients and from frozen meat was almost identical.

Hepatitis E virus has also been found in mussels. Mussels become contaminated with HEV through bioaccumulation while filtering large volumes of seawater. The risk is higher if the mussel cultivations are placed close to river mouths. Rivers may be contaminated by runoff water from pig farms or from sewage that is treated in water-cleaning plants that cannot stop HEV from passing through.

4.8 Conclusions

Research on zoonotic hepatitis E has taken big strides forward. The research field is still very dynamic with new hosts being found frequently. In little more than two decades, the number of known variants has increased from two infecting two animal species (poultry and humans, not counting experimental infections of monkeys) to over 20 infecting over 20 animal species. The hepeviruses are also very variable in genome sequence. While a large number of strains, encompassing genotypes 1–4, show a high degree of sequence similarity and a similar host pattern, other strains are significantly more different, compared to genotypes 1–4, like HEV in rats and HEV in moose. Avian HEV is only 50 % similar to genotypes 1–4 and cuttroat trout HEV is only very distantly related to other HEVs. On the other hand, rabbit HEV was before considered not to belong to genotypes 1–4, but it is now accepted that this virus belongs to genotype 3. This demonstrated how dynamic and complex is the HEV family. Furthermore, it demonstrates the difficulties in drawing a sharp line between zoonotic and non-zoonotic HEV strains.

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Chapter 5
Genetic Evolution of Hepatitis E Virus

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Abstract Comparative analysis of the genomic sequences of multiple hepatitis E virus (HEV) isolates has revealed extensive genomic diversity among them. Recently, a variety of genetically distinct HEV variants have also been isolated and identified from large numbers of animal species, including birds, rabbits, rats, ferrets, bats, cutthroat trout, and camels, among others. Furthermore, it has been reported that recombination in HEV genomes takes place in animals and in human patients. Also, chronic HEV infection in immunocompromised individuals has revealed the presence of viral strains carrying insertions from human genes. This paper reviews the current knowledge on the genomic variability and evolution of HEV.

Keywords Genetic evolution • Hepatitis E virus • Mutant • Open reading frame • Genotype

Abbreviations

HEV	Hepatitis E Virus
Hel	Helicase
MeT	Methyltransferase
ORF	Open reading frame
PCP	Papain-like cysteine protease
RdRP	RNA-dependent RNA polymerase
VLPs	Viruslike particles

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5.1 General Variation

Full-length genomic sequences from various genotypes and geographic isolates of hepatitis E virus (HEV) have become available since this virus was discovered in 1983 [8, 14, 37]. HEV is a small, nonenveloped virus with a single-stranded RNA genome of 7.2 kb. The genome contains three partially overlapping open reading frames (ORFs) that are bracketed by short 5′ and 3′ nontranslated regions. ORF1 encodes the nonstructural enzymatic activities required for viral replication, and ORF2 encodes the structural viral capsid that includes neutralizing epitopes. The function of ORF3 is unknown, but it appears to be necessary for cellular egress [13].

HEV has been recognized as a member of the *Hepevirus* genus of the Hepeviridae family [21]. However, a novel classification has recently been proposed that divides this family into two genera: *Orthohepevirus* (all mammalian and avian HEV isolates) and *Piscihepevirus* (cutthroat trout virus). At least four genotypes within *Orthohepevirus* A (isolates from human, pig, wild boar, deer, mongoose, rabbit, and camel) have been recognized to date [48]. Genotype 1 is a conserved genotype that is classifiable into five subtypes (1a, 1b, 1c, 1d, 1e), and these come from tropical and several subtropical countries in Asia and Africa. Genotype 2 sequences, encoded by viruses isolated from Mexico, Nigeria, and Chad, are limited in number but can be divided into two subtypes (2a, 2b). Genotypes 3 and 4 are extremely diverse and can be subdivided into ten and seven subtypes, respectively (3a, 3b, 3c, 3d, 3e, 3f, 3 g, 3 h, 3i, and 3j and 4a, 4b, 4c, 4d, 4e, 4f, and 4g). Geographically, genotype 3 is found almost worldwide (e.g., in Asia, Europe, Oceania, North and South America), whereas genotype 4 is found exclusively in Asia [29]. A virus with a 6.6 kb RNA genome and sharing 50 % nucleotide sequence identity with mammalian HEVs, called avian HEV (*Orthohepevirus* B), was isolated from chickens exhibiting hepatitis-splenomegaly syndrome [11, 15]. Recently, rat HEV and ferret HEV (*Orthohepevirus* C) have been isolated and characterized from Norwegian rats [19, 43]. Rat HEV shares a sequence identity of 55–59 % with HEV genotypes 1–4, while ferret HEV shares an identity of 72.3 % with rat HEV. In addition, novel HEV strains have been identified recently in bats (*Orthohepevirus* D), but the prevalence level seems low (0.18 %), and bat HEV apparently does not infect humans [7]. A unique HEV strain identified from farmed rabbits in China shares 74–79 % nucleotide sequence identity with existing HEV strains and 46 % identity with avian HEV [64]. Sequence analysis reveals 89.3–95.9 % identity scores with rabbit HEV strains from other sites in China, and the strains clustered into three distinct groups within HEV genotype 3 [18]. Finally, a virus that infects various trout species was isolated from cutthroat trout; it bears only 18–27 % sequence similarity with avian or mammalian HEVs and may be classified as a new genus, namely, *Piscihepevirus*, within the Hepeviridae family [2].

It is suspected that a high level of genomic intermixing of HEVs takes place in animals and in patients, and recombination in HEV genomes has been reported [53]. Compartmentalization of HEV quasispecies between serum and cerebrospinal fluid was observed in a kidney transplant patient with chronic hepatitis E [20].

Additionally, chronic HEV infections in immunocompromised individuals have revealed the presence of viral strains carrying insertions from human genes, specifically the S17 insertion, which confers an adaptive advantage in culture and expands the host range and tropism of the virus [46]. Also, some mutations in HEV ORF2 significantly influence the virulence and severity of hepatitis E, and in some cases, the mutation in ORF2 is involved in viral pathogenesis.

5.2 ORF1 Variation

Different genotypes have variable ORF1 gene lengths. Genotypes 1 and 2 have similar lengths for ORF1; genotypes 3 and 4 also have similar ORF1 lengths, but more sequence variation, and are longer than those of genotypes 1 and 2. According to an analysis of the HEV sequences from the 5′ end of the ORF1 region from GenBank (http://www.ncbi.nlm.nih.gov/genbank/), genotype 1 is divided into five subtypes; genotype 2 is represented by a single isolate, whereas genotypes 3 and 4 can be divided into eight and four subtypes, respectively [29]. A comparison of the 5′ ends of ORF1 sequences generated results for separation of different levels. The nucleotide differences for genotypes 1 and 2 were 19.1 % at the genotype level, 5.6–8.5 % at the subtype level, and 0.5–4.5 % at the isolate level. The nucleotide differences for genotypes 3 and 4 were 18.8–23.6 % at the genotype level, 11.5–20.0 % at the subtype level, and 5.1–11.8 % at the isolate level [29]. The homology of the ORF1 proteins among these genotypes is 80–86 %, with higher homology between genotypes 1 and 2 than between genotypes 3 and 4. An amino acid sequence alignment of ORF1 proteins from genotypes 1, 3, and 4 showed that MeT and the Y domain are highly conserved with few mutations, while PCP, macro domain, Hel, and RdRP contain more mutations. However, the key functional domain sites in these genotypes are conserved (i.e., the six cysteine residues in PCP, C457, C459, C471, C472, C481, and C483; G815-G816-G817 in the macro domain; the seven motifs in Hel, I, GVPGSGKSRS; Ia, VVVPTREL; II, GRRVVIDEAP; III, HLLGDPNQ; IV, THRCPA; V, TVHEAQGATYTETTI; and VI, VALTRHTEK; and K981 and D1029–E1030 in RdRP). A comparison of the ORF1 sequences from rabbit HEV strains with reference ORF1 sequences from HEV genotypes 1–4 shows that rabbit HEV is characterized by a 93-nt insertion in the X domain of ORF1, which does not exist in HEV genotypes 1–4, but is present in all rabbit strains isolated worldwide [6, 17, 64].

The hypervariable region (HVR) is most variable in mammalian HEVs [15]. The size differences among the different HEV genomes are confined mainly to the HVR of ORF1 [15, 40, 41, 51]. HVR sequences from genotypes 3 and 4 are longer in length and higher in mutation frequency than those of genotypes 1 and 2. Genotypes 3 and 4 share a conserved motif immediately preceding their HVRs [(T/V) SGFSS(D/C)FSP] that lacks any homology to genotype 1 or 2 sequences at the equivalent position. Amino acid substitutions in the HVR region of different HEV genotypes are shown in Table 5.1.

Table 5.1 Conserved amino acids in the HVR region of various HEV genotypes

Genotype	Amino acid and sites
Genotype 1	707E, 709D, 710A, 714P, 717–720(PDLG), 723–725 (SEP), 729S, 731A, 737A, 741P, 753A, 757–760 (EPA), 769–771 (AIT), 773Q, 776R, 777H
Genotype 2	720G, 759P
Genotype 3	708–712 (SGFSS), 714 F, 716P, 717P, 736P, 792R
Genotype 4	708S, 709G, 711–716 (SSCFSP), 778P, 792R

The amino acid positions shown are in accordance with M73218 (genotype 1), M74506 (genotype 2), AF060669 (genotype 3), and KC492825 (genotype 4). Cited from Yang et al. [60]

5.2.1 Common HVR Properties Between Genotypes

The nucleotide and amino acid sequences of the HVR vary in the different genotypes. Nevertheless, there are some general properties shared by them. For genotypes 1, 3, and 4, a relative excess of proline (mean values of 24–30 % compared with 7–8 % for ORF1 as a whole) and serine (mean values of 11–14 % compared with 6 % for ORF1) can be seen in the amino acid composition of the HVR. Similarly, there is an excess of proline and serine in the HVR sequences for genotype 2, for the divergent viruses isolated from wild boar, for the rabbit HEVs, and even for the more distantly related rat and avian HEV genomes. Also, a consistent deficiency in leucine, arginine, and tyrosine residues exists in all the HVRs. Furthermore, the presence of Src homology 3 (SH3)-binding motifs such as PxxP is another characteristic of the HVR among HEV genotypes [41]. There is an excess of PxxP motifs in all genotype 1 HVR sequences and most genotype 4 HVR sequences, while there are fewer PxxP motifs than expected in the single genotype 2 HVR and on average for genotype 3 sequences, whether or not they contain an internal duplication (0.93 for insert, 0.87 for non-insert). In contrast, no excess PxxP motifs exist in the rabbit HEVs or in the divergent HEV isolates from wild boar. It has been reported that any polypeptide with the same proline composition of the HEV HVR could have a similar number of PxxP motifs [47].

5.2.2 Divergence and Variation in the HVR from Various HEV Genotypes

The HVR (amino acids 711–798) exhibits variations in size and amino acid deletions or insertions in the different HEV genotypes. The inter-genotypic amino acid sequence identity in the HVR among HEV isolates from different genotypes differs by as much as 71 %, whereas the intra-genotypic amino acid sequence identity scores among isolates differ by 31 % among genotype 1 isolates, 41 % among genotype 3 isolates, 46 % among genotype 4 isolates, and 30 % between the only two available avian HEV isolates [40]. It has been reported that all strains of zoonotic origin (genotypes 3 and 4) contain seven conserved amino acids 708S, 709G, 711S,

712S, 714 F, 716P, and 792P, while all the strains isolated from humans (genotypes 1 and 2) have only two conserved amino acids (720G, 759P). However, it is not clear if the amino acids in the genotype 2 HVR are conserved since only one strain of genotype 2 HEV has been isolated to date [60].

HVR nucleotide sequences of genotype 1 are relatively similar to each other and collinear with no insertions or deletions.

By comparison, more diversity in both amino acid residues and in length was observed in the 153 HVR sequences from genotype 3 viruses. Even so, these variants are related to each other by nucleotide substitution, deletion, and duplication [47]. An 87-nucleotide insertion, which appears to be a duplication of the HVR, was observed in a group of 33 genotype 3f isolates (30 human isolates from southern France and three pig isolates from northern Spain). Additionally, further 20 genotype 3 HVR sequences contained insertions of 12, 15, or 18 nucleotides, and these were rich in pyrimidines, particularly cytosine, but their origin is not obvious. Also, deletions of 3 or 4 nucleotides have occurred in some HEV HVR sequences. Notably, the divergent sequences of HVR from different HEV genotypes might result from the incorporation and mutation of different host sequences. Two unusual HVR sequences isolated from chronically infected patients contained insertions of human-derived sequences containing 5–8 % proline [35, 46]. In one case, a variant containing an in-frame insertion of 171 nucleotides derived from the human ribosomal protein S17 was selected in the course of serial passages in HepG2/C3A cells. In the second case, the HVR had a 117-nucleotide in-frame insertion derived from the human ribosomal protein S19 gene [35]. Additionally, the HEV HVR sequences from rabbits differed from each other at 31–38 of the 69 amino acid positions and were not aligned with other genotype 3 sequences, although they belong to genotype 3. Similarly, there was considerable amino acid sequence divergence among genotype 4 HVR sequences. However, amino acid substitutions were the most common type of genetic diversity in these genotype 4 HVR sequences.

In general, these studies have revealed extensive sequence variation in HVR and its involvement in the divergence and evolution of HEV isolates.

5.2.3 The Relationship Between HVR Variation and HVR Characteristics

The existence of extensive inter- and intra-genotypic sequence variation in the HVRs of HEV genomes indicates that this region may not be essential for virus replication. It is also reported that HEV infectivity can tolerate small deletions in the HVR and that amino acid residues in this region are dispensable for virus infectivity [40]. However, contrary to this proposal, the HVR was shown to be capable of interacting with viral and host factors to modulate the efficiency of HEV replication [41]. Moreover, the high genetic heterogeneity of the HVR and the macro domain at the acute phase of an HEV infection might be associated with viral persistence [26].

In addition, the Kernow-C1 and LBPR000379 strains contain insertions of human-derived sequences, which were previously reported to be capable of efficient replication during cell culture [46].

The discovery of insertions and deletions (indels) in the HEV genotype 3 HVR led to the assumption that the evolution of the HVR was too complex to model because of the difficulty of reconstructing its indel history [42]. In conclusion, the analysis of HEV HVR variation suggests that the HVR is intrinsically disordered and may regulate transcription and translation and may also be important for virus replication.

5.3 ORF2 Variation

ORF2, which is located at the 3′ end of the genome, begins 38 nucleotides 3′ of the termination site in ORF1 and consists of 1980 nucleotides. It encodes the viral capsid protein, which contains three domains: S (residues 118–313), P1 (residues 314–453), and P2 (residues 454–606) [3]. ORF2 encodes a capsid protein of 72 kDa (660 amino acids). It is suitable for serological diagnosis of HEV and is a candidate for a vaccine against HEV infection, because it is immune dominant and highly conserved among HEV species and also induces long-lived immunity. When expressed, the full-length capsid protein (72 kDa) is not a suitable diagnostic target, because the important epitopes are relatively hydrophobic, insoluble, and therefore masked. However, truncated forms of the capsid protein are considered diagnostic antigens [39].

The ORF2 protein is the most highly conserved among proteins encoded by ORFs in HEV. The genotypes that infect humans currently are HEV-1, HEV-2, HEV-3, and HEV-4. HEV-1 shares 92–93 %, 90–91 %, and 89–92 % amino acid sequence identities with HEV-2, HEV-3, and HEV-4, respectively. Also, HEV-2 shares 89–90 % and 88–89 % sequence identities with HEV-3 and HEV-4. The amino acid sequence identity between HEV-3 and HEV-4 is 90–94 % [64]. It can be seen that the sequence identities for HEV-1 and HEV-2, and for HEV-3 and HEV-4, are higher, which may bear a relationship with their infection characteristics.

5.3.1 Epitope Analysis of ORF2 Protein

The HEV ORF2 capsid protein is immunogenic; both linear and conformational epitopes for neutralizing antibodies have been located to residues 578–607, 452–617, and 458–607 in the C-terminal portion of ORF2 [33, 45], respectively. Recently, crystal structure analysis and cryoelectron microscopy were used to identify the antigenic domain in HEV [57, 59]. The results of the Yamashita et al. [59] study suggest that the MAB1323 neutralizing mAb clone recognizes the peripheral region

of the apical surface, specifically residues S487, S488, T489, P491, N562, and T564, and the mAb clone MAB272 recognizes the horizontal region of the protruding (P) domain above the M domain at the threefold axis, specifically residues D496, T497, G591, and P592. Additionally, the neutralizing mAb MAB8C11 clone recognizes the groove region, specifically residues E479, Y485, D496, R512, K534, H577, and R578, and the neutralizing mAb MAB8G12 clone recognizes the dimerization region, specifically residues E549, K554, and G591 [10]. All of these mapping studies place the neutralizing epitopes in the P domain of the ORF2 protein. However, the epitopes of the neutralizing mAb MAB12A10 clone are D430 and L433, which are located in the M domain [10].

Epitopes in the various HEV genotypes differ. The major epitope of HEV genotype 4 lies within amino acids 477–613, and both Leu477 and Leu613 are critical in forming this neutralization epitope [63]. In contrast, the major epitope of HEV genotype 1 lies within amino acids 459–602 [28]. Differences also exist in the amino acid positions of the epitopes: HEV genotype 1 contains Ser497 and Ala575, whereas HEV genotype 4 contains Thr497 and Pro575. A study of HEV mutants found that amino acid 497 is essential for differentiating genotypes 1 and 4 [10]. However, HEV genotypes 1 and 4 have the same neutralizing epitopes, with conserved residues, Glu549, Lys554, and Gly591 [10]. In general, most immune epitopes are located at the N-terminal of the ORF2 protein, but one prominent epitope defined in the ORF2-encoded structural protein (amino acids 613–666) expressed by the recombinant λgt11 406.3-2 clone was not identified in a peptide mapping study [1]. The absence of reactivity to peptides synthesized in this region of ORF2 may be caused by an absence of the proper conformation required for recognition of this epitope. Therefore, it is very important to map the conformational epitopes of HEV to enable future development of diagnostic kits and preventative vaccines.

5.3.2 Mutations in Potential Glycosylation Sites

Panda and Varma [38] have shown that pORF2 is synthesized as a large precursor of about 82 kDa and then co-translationally translocated to the endoplasmic reticulum (ER), where it is processed into the 72 kDa mature protein (pORF2), through signal sequence cleavage. This protein is then glycosylated in the ER at three possible glycosylation sites, Asn137, Asn310, and Asn562 [38]. These three N-linked glycosylation sites are universally conserved in all isolates of human HEV and in swine [34]. Mutations in the potential glycosylation sites of pORF2 inhibited the formation of infectious viruslike particles (VLPs) from transfected replicons, and these VLPs showed low infectivity in experimental macaques [38]. In another study, the yeast two-hybrid system and in vitro immobilization experiments showed that full-length pORF2 is capable of self-association, by forming a homodimer [4].

5.3.3 Mutations in the Capsid Protein

The ORF2 capsid protein is involved in virion assembly, immunogenicity, and host cell receptor binding [49]. Partial ORF2 nucleotide sequences have been predicted to be well suited for phylogenetic classification of HEV. Studies investigating humoral responses against HEV have reported prominent antibody responses against this and other linear or conformational epitopes of ORF2 [33]. Thus, recombinant ORF2 protein has been used as a vaccine candidate.

ORF2 encodes the structure protein of the virion; thus, its sequence is relatively conserved, except for avian HEV. Avian HEV shares about 46–48 % nucleotide identity in ORF2 with human and swine HEV [15]. Evolution of ORF2 is driven by nucleotide insertions and substitutions, and it is subject to positive selection [47]. However, some mutations in HEV ORF2 significantly influence the virulence and severity of hepatitis E infections, and in some cases, mutations in ORF2 are involved in disease pathogenesis. Recently, Cordoba et al. showed that three amino acid mutations (F51L, T59A, and S390L) in the HEV ORF2 capsid protein result in viral attenuation. A total of 60 pigs were intrahepatically inoculated with in vitro-transcribed full-length capped RNA transcripts from the infectious clones of each single mutant; these three mutations significantly reduced viremia, delayed the onset time of viremia, shortened the duration of fecal virus shedding and viremia, and reduced the viral loads in the liver, bile, and intestinal contents [5]. Recent research studies have shown that a few nucleotide substitutions in HEV genomes are associated with fulminant hepatitis and disease severity. Some of these nucleotide substitutions belong to ORF2. Through partial sequencing of the ORF2 capsid protein genes of HEV genomes from patients with acute liver failure, including pregnant women in northern India, Borkakoti et al. indicted that P259S, in the capsid gene, might be associated with the poor outcomes in the patients [3]. Likewise, in a comparison of 28 full-length nucleotide sequences from HEV genotype 4, it was suggested that the substitution of C at nucleotide 5907 (C5907) was most closely associated with fulminant hepatitis (fulminant hepatitis, 100 %; acute hepatitis, 39.1 %; $p = 0.0204$) [3]. Also, C5906, a silent substitution located in the capsid gene, does not change the amino acid. In this research, the author also proposed that C5907 together with U3148, which is located in the RNA helicase domain, may be associated with high HEV load ($\geq$10^5) and the severity of hepatitis E infections [16]. Wen et al. [56] have recently revealed a significant immunogenicity difference between two HEV vaccines. In the Wen et al. [56] study, p239-induced IgGs reacted more strongly against p166W01 and p166Mex than against p166US and p166Chn in mice and humans. By contrast, p179-induced IgGs showed a stronger reactivity against p166US and p166Chn than against p166W01 and p166Mex [56]. This difference has also been shown in monkeys. Xu et al. [58] indicated that specific substitutions at position 562 have a more measurable effect on the activity of the HEV-neutralizing epitope than dimerization or glycosylation of the structural protein. Furthermore, the secretion of monomers fully immunoreactive may call into question the importance of dimerization for effective presentation of HEV neutralization epitopes.

5.4 ORF3 Variation

pORF3 is a small 113–114 amino acid protein encoded by the smallest ORF (ORF3) in the HEV genome. The ORF3 protein contains two large N-terminal hydrophobic domains, D1 (amino acids 7–23) and D2 (amino acids 28–53), and two proline-rich domains, P1 (amino acids 66–77) and P2 (amino acids 95–111) (Fig. 5.1). The D1 domain is very cysteine rich and is required for the ORF3 protein to associate with the cytoskeleton, to bind microtubules, and for inhibition of the MAPK phosphatase, resulting in the activation of cellular ERK [22]. The ORF3 protein binds hemopexin through its D2 domain, and this interaction is proposed to affect cellular iron homeostasis [44]. The P1 domain contains a PMSP motif in which Ser-71 is phosphorylated by MAPK. The P2 domain has two overlapping PXXP motifs, which can bind to SH3 domains. This plays an important role in signal transduction pathways, thereby promoting cell survival [25].

5.4.1 Divergence of ORF3 Genes and Proteins from Various HEV Genotypes

The ORF3 of HEV was initially predicted to express a protein of 123 amino acids. It was suggested recently that the ORF3 protein is translated from a bicistronic subgenomic RNA from an AUG codon at position 5131 (in the SAR-55 isolate), and this would result in a 114 amino acid protein that is nine amino acids shorter at its N-terminus than what was proposed for it earlier [9]. The amino acid sequence identities of strains within genotypes 1, 3, 4, and rabbit HEV were 96.5–99.6 %, 92–100 %, 83–100 %, and 91.1–95.5 %, respectively, while the identities of strains between genotypes ranged from 72–79 % to 83–86 % [64] (Table. 5.2). The ORF3 amino acid sequence is highly conservative within the same genotypes.

The results of comparative homology analyses on nucleotide and amino acid sequences show that ORF3 homology is high between genotypes 1 and 2 and

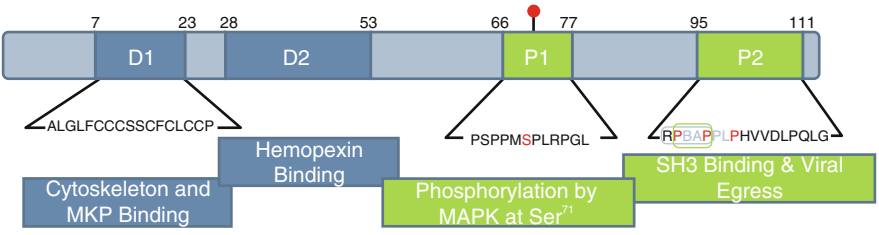

Fig. 5.1 Details of the ORF3 proteins, such as the hydrophobic domains (D1 and D2) and two proline-rich domains (P1 and P2). In mammalian cells, the ORF3 protein is phosphorylated at serine 71 (*red dot*) by cellular MAPK (Cited from Ahmad et al. [1])

Table 5.2 Amino acid identity (%) of ORF3 from different HEV genotypes

	HEV genotype	Type 1	Type 3	Type 4	Rabbit HEV
ORF3	Type 1	95.6–100			
	Type 3	77.0–84.1	92.9–100		
	Type 4	70.5–81.3	75.0–86.7	83.3–100	
	Rabbit HEV	75.6–80.0	82.3–86.7	72.6–83.9	91.1–95.5

Fig. 5.2 Alignment analysis of ORF3 amino acid sequences. An alignment analysis of representative ORF3 protein sequences from HEV genotypes 1, 3, and 4 and from rabbit HEV (Note: */·, conserved amino acids; colored sites, identical amino acids; HEV-1, genotype 1 HEV; HEV-3, genotype 3 HEV; HEV-R, rabbit HEV; HEV-4, genotype 4 HEV)

between genotypes 3 and 4. Genotypes 1 and 2 infect humans only, whereas genotypes 3 and 4 infect both humans and pigs. It remains unknown whether or not homology divergence in the ORF3 sequences has an impact on the host species of HEV. An alignment analysis of the ORF3 amino acid sequences from genotypes 1, 3, 4, and rabbit HEV, which are all epidemic in China, found that divergence in ORF3 amino acid sequences is genotype specific. A different alignment analysis of ORF3 amino acid sequences from various genotypes found that the N-terminal sequences varied. Genotype 3 and rabbit HEV have identical sequences, which differ from the sequences of HEV genotypes 1 and 4. The D1 domain is mostly conserved without obvious changes in its sequence. However, a mutation between valine and alanine was found in the D2 domain from different genotypes. The P1 domain is more variable than the other domains; indeed, serine at site 71 from HEV genotypes 1 and 3 is replaced by other amino acids in genotype 4 and rabbit HEV. It is not yet known whether or not mutations in the phosphorylation site impact function. The two overlapping PXXP motifs in the P2 domain are conserved, but the sequences within the P1 and P2 regions from different genotypes are variable. The ORF3 amino acid sequence is conserved among strains from the same HEV genotype (Fig. 5.2).

5.4.2 *Immunogenicity and Antigenic Epitopes in the ORF3 Protein*

On subcellular fractionation, pORF3 appears to associate with the cytoskeleton using one of its two large N-terminal hydrophobic domains, D1 and D2 [62]. Furthermore, the phosphorylated ORF3 protein interacts with the non-glycosylated capsid protein via a 25 amino acid region in the ORF3 protein. This ORF2–ORF3 interaction depends on phosphorylation of the ORF3 protein at the Ser80 residue [52]. The ORF2–ORF3 interaction plays an important role in a variety of gene-specific antigenicities.

HEV membrane envelopment as well as the nature and the composition of the membrane are yet to be further defined, but ORF3 protein appears to be at the heart of particle secretion and, possibly, particle formation [52]. Importantly, ORF3 protein is present on the secreted membrane of the wrapped virion, as demonstrated by HEV particle capture by ORF3 antibodies in culture supernatant and serum but not in feces [50]. It has been reported that rhesus monkeys vaccinated with HEV ORF3 protein were endowed with some prevention from virus infection; this suggests that antibodies to the ORF3 protein may provide some neutralization effects. Ma et al. [30] vaccinated rhesus monkeys with HEV ORF3 protein and found that this provided some protection against HEV infection. This result confirmed that antibodies against ORF3 protein provide a neutralization affect.

Panda et al. [36] expressed ORF2 and ORF3 of the Indian HEV strain in prokaryotic cells and used immunoblotting to assay the antibody levels of individuals from a highly epidemic HEV region. They found that the levels of anti-HEV ORF3 IgM were significantly higher during the acute phase of HEV infection than those of anti-HEV ORF2 IgM. Wang et al. [55] expressed HEV genotype 4 ORF3 and used it to measure the anti-HEV IgM and IgA levels in the serum from an HEV-infected patient; the assay was able to differentiate between the early acute phase and acute phase of HEV infection. The ORF3 protein of the China HEV strain expressed by Li et al. [27] was able to react with sera from most patients that were tested as well as sera from experimentally infected monkeys within 17–100 days of infection with HEV. However, the reactivity to this protein waned over time. The antigenic reactivity of ORF3 shows an obvious association with the course of HEV disease and plays some role in the acute-phase reaction.

Herremans et al. [12] tested 16 genotype 3-related and eight genotype 1-related sera from HEV patients and found that ORF3 antigens had clear genotype specificity. Khudyakov et al. [23] systemically compared the antigenicity of the ORF3 proteins of the Burmese HEV strain (genotype 1) and Mexican strain (genotype 2) and also found that ORF3 protein has genotype specificity. The C-terminal regions of these two strains contain some antigenic sites, and the homology of their amino acid sequences is 71 %; this difference in their amino acid sequences leads to the variety observed in their antigenicity [24]. Yarbough et al. [61] found that the recombinant ORF3 antigen of the Burmese HEV strain did not react with sera from monkeys infected by the Mexican HEV strain and vice versa. Synthesized polypeptides based

on the amino acid sequence 91–123 of the Mexican HEV strain and the corresponding sequence of the Burmese HEV strain also did not have antibody cross-reactivity. This genotype specificity may relate to the distribution of antigenic epitopes and conformation dependence among the different HEV genotypes. The antigenic sites in the Burmese HEV strain showed more conformation dependence than those of the Mexican HEV strain. The major sites for immune reaction to the Mexican HEV strain are located in the 95–101 amino acid region, whereas there is only one antigenic site in the 112–117 amino acid region of the Burmese HEV strain. Through phage display, Wang et al. [54] found that the continuous amino acid sequence (VDLP) at the C terminus of ORF3 is an important antigenic epitope in the ORF3 protein; notably, the three upstream proline residues, P99, P102, and P103, are extremely important for the reactivity of this sequence [54]. Ma et al. found reactivity of the C terminus of HEV ORF3 with anti-HEV IgM [31] and also found that ORF3 consists of genotype-specific antigens. The reactivity of the ORF3 protein of HEV genotype 1 with serum from an HEV genotype 1-infected monkey is stronger than that with serum from an HEV genotype 4-infected monkey. Similarly, the reactivity of ORF3 protein from HEV genotype 4 with serum from an HEV genotype 4-infected monkey is stronger than that with serum from an HEV genotype 1-infected monkey. Therefore, the antigen-antibody reaction of ORF3 protein with homotypic virus-induced serum is stronger than that with heterotypic virus-induced serum [32].

5.5 Conclusion

Divergence of ORF1 from various HEV genotypes is confined mainly to the HVR in this virus. It has been reported that quasispecies heterogeneity in the ORF1 regions encoding the HVR and the macro domain may facilitate HEV persistence. By comparison, some mutations in HEV ORF2 have a significant influence on the virulence and severity of hepatitis E infections, and in some cases, mutation of ORF2 is involved in disease pathogenesis.

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Chapter 6
Transmission of Hepatitis E Virus

Yansheng Geng and Youchun Wang

Abstract Transmission of hepatitis E virus (HEV) occurs predominantly by the fecal–oral route. Large epidemics of hepatitis E in the developing countries of Asia and Africa are waterborne and spread through contaminated drinking water. The reservoir of HEV in developed countries is believed to be in animals with zoonotic transmission to humans, possibly through direct contact or the consumption of undercooked contaminated meat. HEV transmission through blood and vertical transmission have also been reported.

Keywords Hepatitis E virus • Foodborne • Transfusion • Transmission • Zoonotic • Waterborne

Abbreviations

HEV	Hepatitis E Virus
HBV	Hepatitis B Virus
FHF	Fulminant hepatic failure

6.1 Introduction

Hepatitis E is generally transmitted by the fecal–oral route. In highly endemic areas, where genotypes 1 and 2 are the prevalent HEV genotypes, contaminated water and contaminated food are the main sources of HEV infections. Fecal shedding of HEV by humans with clinical or subclinical infection maintains a circulating pool of

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infectious individuals who contaminate water supplies, thus maintaining the disease in endemic populations.

In industrialized countries, the importance of animal reservoirs has become clear, and hepatitis E is regarded as a zoonotic disease. Pigs are the main reservoirs of genotype 3 and genotype 4 HEV. The transmission of these two genotypes through the consumption of contaminated meat or direct contact with animals has been confirmed as the main cause of sporadic hepatitis E in developed countries.

In developing countries, where HEV genotype 1 or 2 is prevalent:

- *Waterborne transmission*
  - Primarily contamination of water supplies
- *Person-to-person transmission*
- *Vertical transmission*
- *Transmission via blood transfusion*

In developed and developing countries, where genotype 3 or 4 is prevalent:

- *Zoonotic transmission*
  - Consumption of undercooked meat and close contact with animals
- *Transmission via blood transfusion*
- *Organ transplantation transmission*

6.2 Waterborne Transmission of HEV

The fecal–oral route is the primary and most well-documented mode of HEV transmission. In the hyperendemic areas of developing countries, fecal–oral transmission usually occurs through the consumption of contaminated drinking water [2, 98, 129]. Most outbreaks of hepatitis E can be traced to the contamination of drinking water supplies with human feces; therefore, these outbreaks are known as waterborne outbreaks.

6.2.1 Waterborne Transmission of HEV Genotypes 1 and 2

Hepatitis E outbreaks linked to contaminated water supplies have been widely documented. The earliest well-documented report of this disease is a large epidemic of waterborne hepatitis that occurred in New Delhi, India, in 1955 [129]. Since then, in the last century, large waterborne epidemics and small outbreaks have been reported in India, Pakistan, China, Vietnam, Bangladesh, Mexico, Sudan, and some other countries in Asia, Africa, and Latin America. During this time period, in Southeast Asia, the disposal of human excreta into rivers was common, and water

from the same rivers was used for drinking, cooking, and personal hygiene at downstream locations. Fecal contamination of water was also a main source of hepatitis E outbreaks across African countries [41, 49, 51, 57]. The outbreaks often followed heavy rainfall and floods, which facilitate the mixing of human excreta with drinking water sources. During the dry summer months when the water flow in rivers and streams is reduced, the concentration of fecal contaminants consequently increases, which can also lead to HEV outbreaks. These waterborne outbreaks have been exclusively associated with strains of HEV belonging to genotypes 1 or 2.

The HEV reservoir in endemic regions is persons with sporadic or subclinical HEV infection. HEV-infected persons excrete HEV in their feces [107]. In a city of North India, 79 of 192 (41 %) sewage specimens collected were positive for HEV RNA, and the seasonal pattern of HEV RNA positivity was as follows: winter months (November–February), 28 of 61 (46 %); summer months (March–June), 36 of 66 (55 %); and monsoon months (July–October), 15 of 65 (23 %) [61]. The large proportion of sewage specimens in each of these periods that contain human HEV RNA suggests that HEV infection and fecal viral excretion are common in HEV-endemic regions throughout the year, even during non-epidemic periods. The HEV in this human-origin sewage may subsequently contaminate the drinking water supplies.

The high positive rate of HEV RNA in sewage may also indicate that an environmental reservoir of the virus plays an important role in HEV outbreaks. In India, anti-HEV IgG positivity was significantly higher among sewage workers (83/147, 56.5 %) than among control subjects (19 %) [128]. An increased anti-HEV antibody-positive rate has also been reported in Turkish farmers who used untreated wastewater for agriculture [23]. Increased rates of human HEV infection in certain countries in Southeast Asia are associated with utilizing untreated river water for everyday tasks, such as bathing, drinking, and disposal of waste products [138]. Environmental catastrophes and annual flooding are also associated with elevated HEV attack rates, especially in regions where river, pond, or well water use is prevalent [138].

6.2.2 Waterborne Transmission of HEV Genotypes 3 and 4

Epidemic HEV outbreaks have never been documented in developed industrialized countries where safe water supply has been established. However, the waterborne route of infection may also be important for HEV genotypes 3 and 4. HEV strains of these two genotypes are known to be transmitted through zoonosis, mainly via the consumption of uncooked or undercooked infected pork or game meat [93]. Additionally, the use of HEV-containing pig manure or of water contaminated with animal waste for land application and crop field irrigation may lead to the contamination of produce or of shellfish through the runoff of the HEV-contaminated water into rivers and coastal waters, which can eventually cause disease among consumers.

6.2.2.1 Surface Water Contamination and Transmission of HEV

Surface waters may be contaminated by sewage overflows or discharge of insufficiently treated sewage water. In the USA, genotype 3 HEV RNA was detected in swine manure collected from pig manure storage facilities in farms, and, most importantly, the HEV detected in the pig manure slurry remained infectious when inoculated into naïve pigs [68]. Several studies have reported the detection of HEV genotypes 3 and 4 in sewage waters, indicating that contamination of aquatic environments through this route is possible [59, 61, 87, 109]. HEV has been detected in wastewater derived from gut processing at slaughterhouses and in pig slurry stores [112]. Furthermore, epidemiological evidence strongly suggests that HEV can persist in environmental waters and in soil [100].

Consequently, swine manure land application and runoffs could be a source of contamination for water in nearby wells, rivers, ponds, or costal water that is subsequently used for irrigation and drinking. In Italy, genotype 3 HEV was detected in sewage and river samples, suggesting that surface water can be a potential source of HEV exposure [59]. In Slovenia, genotype 3 HEV was recovered from 20 % of fecal samples in local pig farms, and 3.3 % of the surface water samples were positive for HEV RNA [122]. HEV genotype 3 was detected in 17 % of samples collected from the Meuse River [112], which runs from France through Belgium into the Netherlands and is used for both recreational purposes and drinking water. Thus, water from this river is a potential source for exposure to HEV.

The quality of surface water directly affects the populations utilizing that source for drinking water, and intensive farming practices lead to higher detection rates of viruses within these sources. Typical irrigation practices increase the potential for human exposure to pathogens [138]. In Canada, genotype 3 HEV detected from field-grown strawberries shared 99 % nucleotide sequence identity with local swine HEV strains [21, 130]. A recent investigation conducted in three European countries demonstrated that HEV was found in 4.8 % of lettuce sampled at the primary production level and in 3.2 % of samples at the point of sale, as well as in 5 % of irrigation water samples [71].

6.2.2.2 Coastal Water Contamination and Transmission of HEV

In developed countries, seawater has been also shown to contain infectious HEV strains that are closely related to the strains circulating in swine [62]. Bivalve molluscan shellfish are known to concentrate viral particles during the filtration they perform as part of their feeding process. A large variety of human enteric viruses have been detected in different shellfish species during either environmental studies or outbreak investigations. Genotype 3 HEV has been reported in shellfish collected in Japan, Korea, Italy, and the UK [28, 35, 83, 121]. In China, genotype 4 HEV strains were found in contaminated shellfish from the Bohai Gulf rim [48].

Seafood contaminated with viruses poses the risk of causing human infection through ingestion. Mussels, cockles, and oysters are typically eaten raw or only slightly cooked, and HEV remains infectious at temperatures up to 60 °C [40], suggesting that raw, rare-

cooked, or slightly steamed seafood may pose the risk of transmitting HEV to consumers. HEV has been documented as a causative agent for foodborne illness associated with the consumption of contaminated shellfish [34, 113].

6.3 Zoonotic Risks and Foodborne Transmission of HEV

6.3.1 Known and Potential Animal Reservoirs of HEV

Anti-HEV antibodies have been detected in pigs both in the developing and developed countries suggesting that HEV is enzootic in pigs worldwide. In 1997, swine HEV was first identified from pigs in the USA [94]. Since then, genotype 3 or genotype 4 HEV strains were isolated from swine in various geographic regions of the world. Pigs are recognized as the most important reservoirs of genotype 3 and genotype 4 HEV [92, 103]. Direct and indirect evidence of HEV transmission from pigs to humans has been reported. Hepatitis E infection is now considered as a zoonotic disease.

In addition to pigs, a high prevalence of genotype 3 or 4 HEV in wild boar has been detected in Japan, Germany, and many other European countries [1, 22, 114]. Genotype 3 HEV also has been isolated from deer, mongoose, and rabbits [97, 99, 127, 139], indicating that these animals are susceptible to HEV infection.

Other putative HEV genotypes also have been genetically identified from wild boards [124, 125], ferrets [105], bats [37], rats [65], camels [134], chickens [53], and cutthroat trout [6]. However, these HEV variants have not been found in humans. The complete range of animal species that may act as reservoirs for HEV is unknown.

Recently Smith et al. proposed a taxonomic scheme in which the family *Hepeviridae* is divided into the genera *Orthohepevirus* (all mammalian and avian HEV isolates) and *Piscihepevirus* (cutthroat trout virus) [116]. Species within the genus *Orthohepevirus* are designated *Orthohepevirus* A–D. Of the four genotypes that infect humans (genotype 1–4) within species *Orthohepevirus* A, genotype 3 and genotype 4 are enzootic and infect both humans and animals.

6.3.2 Zoonotic HEV Infection Through Direct Contacts with Infected Animals

The zoonotic transmission caused by genotype 3 and 4 HEV strains has been documented. Direct contact with HEV-infected animals is a possible route of transmission of HEV. Seroprevalence studies show that humans exposed to animals, particular pigs, are more likely than the general population to be anti-HEV IgG positive. Farmers, veterinarians, and workers contacting with animals comprise the highly at-risk, exposed group for HEV infection [24, 46, 93, 110]. In Germany, an increased risk of HEV infection in humans occupationally exposed to pigs was demonstrated [77]. Krumbholz et al. tested 106 sera obtained from slaughterers,

meat inspectors, pig farmers, and veterinarians for the presence of HEV-specific antibodies comparing with 116 sera obtained from age- and gender-matched blood donors and found 28 % (28.3 %, 30/106) of the swine-exposed humans and 15.5 % (18/116) of the blood donors without contact to pigs were anti-HEV IgG positive ($P < 0.05$). The slaughterhouse workers have a 1.5–3.5 times higher risk for morbidity than other workers who had not had any occupational contact with animals [78]. In the USA, swine veterinarians were 1.5–3.5 times more likely to be anti-HEV positive than normal blood donors, and also individuals from traditionally major swine states are more likely to be seropositive than those from traditionally non-swine states [92, 138]. Taken together, occupational contact with infected swine is a risk factor for zoonotic HEV transmission in humans and pig farmers, and swine veterinarians are at increased risk of zoonotic HEV infection.

Renou et al. in 2007 reported a possible case of HEV transmission from a pet pig to its human owner. In this case, isolation of virus with related HEV sequences from the patient and his pet pig suggests that the most likely route of transmission was from pig to human. The multitude of novel strains of HEV in wildlife and other domestic animal species suggest direct contact with pets and animals, and fieldwork may also have potential risks for zoonotic HEV infection [110].

6.3.3 Animal-Derived Foodborne Transmission of HEV

In addition to the liver tissues, HEV RNA has been detected from the stomach, kidney, salivary glands, tonsils, lungs, and multiple muscle masses of pigs, chickens, and rabbits when inoculated intravenously [17, 52, 133]. Due to the high rate of HEV infection in pigs, pork and other swine meats are inevitably contaminated by HEV. It has been shown that commercial pork or pig liver purchased from local grocery stores as food in Japan, the USA, Germany, the Netherlands, the UK, Italy, Spain, and the Czech Republic was contaminated by HEV [14, 15, 18, 19, 32, 42, 131, 137] and that HEV from some contaminated commercial pig livers was demonstrated to be infectious [42]. Cross-contamination can occur during swine slaughtering, and in fact slaughterhouse tools (knives) and surfaces (belt and floor) were found positive for HEV RNA [33]. Pig meat is more likely a vehicle of infection for consumers and eating raw and uncooked meet has high risks for HEV infection.

Sequences of HEV strictly related genetically to those recovered from human cases were detected in samples of raw smoked liver sausages (i.e., figatellu) during a case–control study carried out in Corsica, France, in 2010 [27]. In other studies in France and Italy, infectious HEV virions were also detected from pork liver sausage, suggesting that the consumption of these products containing pork liver may be a risk factor for HEV infection in humans [14, 32]. The thermal stability of HEV has been investigated. HEV remains viable after heating to 56 °C for 1 h [40], and cooking temperatures of 71 °C for 20 min are required to fully inactivate the virus [5]. HEV remains infectious at up to 60 °C, suggesting the possibility of HEV transmission by consumption of raw and slightly steamed contaminated food [138].

There is clear evidence linking the onset of hepatitis E to the consumption of contaminated food items. In Japan, four hepatitis E cases have been linked directly to eating raw deer meat [127], and several cases of acute hepatitis E have been epidemiologically linked to eating undercooked pork liver or wild boar meat [90, 137]. Hepatitis E cases associated with meat consumption were reported in the following years also in Europe [27, 31, 132].

6.4 Blood-borne Transmission

The rapidly growing number of seroprevalence studies published over the past 20 years shows the ever-increasing interest in HEV among the transfusion medicine community. The high rate of asymptomatic HEV infections worldwide has raised concern of infection via blood donation.

6.4.1 Seroprevalence and Incidence of HEV Infection in Blood Donors

Many studies have examined the prevalence of HEV in blood donors in different countries (Tables 6.1 and 6.2). In the USA, 1939 blood donors had a prevalence of IgG of 18.8 % (95 % CI: 17. 0–20.5 %) and 0.4 % for IgM; prevalence ranged from 3.4 % in those between 18 and 35 years old to 42.2 % in those >65 years old [136]. Even higher IgG seroprevalence rates of 52.5 % have been documented in Southwest France [86], linked to the consumption of locally produced pork products containing undercooked pork. The high prevalence of anti-HEV IgG among volunteer

Table 6.1 HEV IgG antibody seroprevalence in blood donors

Country	Positive rate (%)	ELISA Kit	References
USA	18.8	Wantai	[136]
Japan	3.7	In house	[123]
France	52.5	Wantai	[86]
Denmark	19.8	Wantai	[56]
England	16.2	Wantai	[12]
England	15.8	/	[30]
Iran	11.5	Biokit, Spain	[4]
China	32.6	Wantai	[50]
Switzerland	4.9	MP Biomedicals	[69]
Saudi Arabia	18.7	Bioelisa, Barcelona	[64]
Germany	6.8	MIKROGEN GmbH	[66]
Central Iran	14.3	Dia. Pro Diagnostic BioProbes	[38]
Spain	19.96	Wantai	[115]
Austria	13.6	Wantai	[44]

Table 6.2 Prevalences of anti-HEV IgG, anti-HEV IgM, and HEV RNA in blood donors

Region	Donation no.	Anti-HEV IgG	Anti-HEV IgM	HEV RNA+	References
China	44,816	14608(32.8)	420(0.94)	6(0.07)	[50]
China	816	172(21.1)	4(0.5)	0	[85]
Spain	10,741	2945(27.5)	109(1.02)	4	[115]
France	53,234	22	22	22	[47]
Dutch	45,415	17	17	17	[120]
UK	43,560	3	3	3	[25]
Brazil	300	30(10)	1	0	[101]

blood donors in both non-developed countries (endemic and hyperendemic) and developed countries indicates past subclinical infection and thus confirms that exposure to HEV is common in blood donor populations (Table 6.2). The data reported on blood donor populations substantially confirm the great difference between developed countries (non-endemic) and seem to reflect the prevalence found in the respective general populations. The common finding of an age-dependent increase in seroprevalence suggests that many infections occur in middle age and thus during the period of blood donation activity.

Viremia in individuals infected with HEV is usually of a short duration with a brief incubation period followed by a symptomatic phase. There is increasing evidence that asymptomatic infections constitute majority of the infected individuals. The ratio of symptomatic to asymptomatic cases ranged from 1:2 to 1:13 in the developing countries [80]. In recent years, studies have shown asymptomatic viremia in blood donors which is suggestive of ongoing subclinical infection. The rate of HEV RNA-positive donations was reported to be 1:7986 in Sweden and 1:4525 in Germany [8]. In another study, out of 23,500 donors, 35 (0.14 %) were found per year to have detectable HEV RNA [66]. RNA of genotype 3 HEV can be recovered from Dutch blood donors at rates of as high as 1:3000 with sequences closely related to patients and pigs in the area [120]. In France, in the western part of which a high incidence and prevalence of HEV has been reported, a rough estimate of the incidence of viremic donations was one HEV-positive sample to 2218 blood donations [47]. HEV is obviously a blood-borne pathogen and the presence of HEV RNA in blood donors is not a rare event (Tables 6.2 and 6.3). Thus, there is a potential risk for transmission of HEV through blood.

The risk for HEV transmission may become substantial if blood products are pooled. Point seven percent of plasma mini-pools from English donors contained HEV RNA [60]. Up to 10 % of plasma fractionation pools tested HEV RNA positive in a global investigation, while HEV RNA concentrations were rather low ($\leq$1000 copies/ml) in all of these contaminated plasma pools [8, 9]. In contrast, no HEV RNA was present in the ready-for-use coagulation factor concentrates derived from eight different manufacturers in another investigation [96]. The low viral loads found in pools may explain why HEV RNA was not detectable in any final preparations of plasma-derived coagulation factors, which undergo further processing steps after initial cryoprecipitation [96]. Although so far there are no reports about the transmission of HEV through plasma derivatives, further and larger studies are needed to exactly assess the risk of HEV transmission by blood products.

Table 6.3 The prevalence of HEV RNA in blood donors

Country	Donation no.	Donation no. with HEV RNA+	Positive rate (%)	References
England	225,000	79	0.004	[55]
Australia	58,915	7	0.01	[44]
Spain	9998	3	0.03	[115]
France	53,234	22	0.045	[47]
Dutch	45,415	17	0.037	[120]
UK	43,560	3	0.007	[25]

6.4.2 Transfusion-Acquired Hepatitis E Cases

In a retrospective study, markers of acute HEV infection (IgM anti-HEV and HEV RNA) were detected in a significantly higher number of multiple transfused patients (13 of 145) compared to controls (two of 250), suggesting a potential risk of HEV transmission through blood transfusion.

In 2004, the first case of a clinically manifested HEV infection after transfusion of 23 blood products in Japan was described. A nucleic acid amplification technique (NAT) investigation of archive samples and sequence analysis of the NAT products revealed that the HEV infection could be linked to a fresh frozen plasma: the HEV RNA detected in the donor showed complete identity for two distinct regions of HEV genome compared to those detected in the recipient [89].

A transfusion-transmitted HEV infection through a red blood cell unit was reported from the UK. While in the transfusion recipient the infection was asymptomatic apart from a mild jaundice and an elevation of liver enzymes, the donor became ill from an acute HEV infection, and the illness of the donor and diagnosis of HEV infection led to the investigation of the recipient [20]. In the following year, another case of a child who suffered from transfusion-transmitted HEV infection after administration of a red blood cell unit was reported in France [26]. In both cases, from the UK and France, sequence homology in donor and recipient suggested a correlation between the transfusion and the HEV infection by genotype 3 of the recipients.

Another case of transfusion-transmitted HEV infection was reported from Japan. A retrospective investigation revealed that the donor of a platelet concentrate became infected through consumption of grilled pork 23 days before donation. Subsequently, the infection had been transmitted to the recipient by transfusion [87]. In France, [54] reported two cases of HEV transmission by two units of intercept-treated plasma originating from the same donor. Such novel HEV transmission through intercept-treated fresh frozen plasma establishes resistance of HEV to intercept pathogen reduction technology.

The transfusion transmission of hepatitis E can occur both in endemic areas and non-endemic areas as autochthonous hepatitis E has been increasingly reported in developed countries where the hepatitis E virus (HEV) is not prevalent. At least ten hepatitis E cases transmitted by transfusion of platelets, red cells, and even intercept-treated fresh frozen plasma were verified by sequence homology analysis of the transfusion-related disease (Table 6.4). A recently retrospective study showed that

Table 6.4 Molecularly confirmed cases of transfusion-transmitted HEV infection

Recipient	HEV genotype	Blood supply content	Countries	References
/	4	Fresh frozen plasma	Japan	[89]
/	3	Fresh frozen plasma	UK	[20]
T-cell lymphoma	3	Red blood cell	Japan	[126]
Rhabdoid tumor underwent chemotherapy	3	Red blood cell	France	[26]
/	4	Platelet	Japan	[87]
Kidney transplantation	3	Intercept-treated plasma	France	[54]
Thrombotic thrombocytopenic purpura		Plasma	Canada	[3]
Underwent re-thoracotomy for hemostasis	3	Platelet	Japan	[91]
Immunocompromised and immunocompetent	3	Platelets	Germany	[58]
Leukemia	3	Frozen plasma, red cell concentrates, and platelet concentrates	Japan	[45]

79 (0.04 %) of 225,000 blood donations in England contained HEV RNA, 43 of these blood products had already been transfused before the study results became known, and 18 of the 43 recipients (42 %, 18/43) developed signs of hepatitis E [55].

The risk of HEV transmission by plasma products is currently estimated to be low since steps have been introduced for most of the products (except for solvent-/detergent-treated plasma) that are considered to be at least partly effective in deactivating or removing HEV.

6.4.3 The Consequences of Transfusion-Transmitted HEV Infection

Since sporadic cases of transfusion-related HEV infection have been reported and confirmed by molecular linkage between donor and recipient, the issue at hand is not whether HEV can be transmitted by transfusion, but rather how often and with what consequences?

Currently, 75 % of blood or blood components used in the UK are given to immunosuppressed patients [10]. Throughout the prospective follow-up of the patient, the viral kinetics, chronological anti-HEV antibody level changes, and disease progression during the entire course of HEV infection from transfusion until the end of viremia were analyzed in some studies. Since 2012, five of 367 consecutive liver transplant recipients (1.4 %) acquired chronic hepatitis E through blood

transfusion and subsequently developed persistent liver graft damage [43]. Treatment of chronic hepatitis E infection in liver transplant recipients is decreasing immunosuppression and ribavirin. In these patients, eradication of hepatitis E is not always obtained by antiviral drugs, and substantial liver damage might persist, even after viral clearance.

Increasing immunosuppression prolongs viremia and delays seroconversion. Although eight of 12 viremic recipients underwent seroconversion, coinciding in some with a biochemical transaminitis, seroconversion does not necessarily bring about clearance and can still be followed by long-term viremia (patients 13 and 15) [55]. At the other end of this range, four heavily immunosuppressed patients either did not produce anti-HEV or had very delayed seroconversion and exhibited prolonged viremia as described previously in recipients of solid-organ transplants. The natural course of transfusion-associated hepatitis E is not known, but in high-risk recipients like pregnant females, patients with pre-existing chronic liver disease, and immunocompromised patients, it is supposed to be associated with considerable morbidity and mortality.

6.4.4 Hepatitis E Screening for Blood Donations: An Urgent Need?

Over the last two decades, much attention has been given to the prevention of transfusion-transmitted viral infections such as HIV-1 and HIV-2, human T-cell lymphotropic virus (HTLV) I and II, hepatitis C virus (HCV), hepatitis B virus (HBV), and West Nile virus (WNV). Today, donor evaluation, laboratory screening tests, and pathogen inactivation procedures are considered crucial tools to reduce the risk of transfusion-transmitted infections, but do not completely eliminate all risks [16]. Blood donor screening and deferral procedures can minimize the possibility of transmitting an infectious agent from a unit of donated blood to the recipient of that unit, as well as ensuring the welfare of the donor himself. Laboratory tests performed on the unit of blood collected for the presence of markers of infectious disease are the most frequently used methods for donor screening. These tests are performed because donors may be unaware that they are asymptomatic carriers of an infectious agent or may be unwilling to identify themselves as a member of a high-risk group. Donor deferral is the temporary or permanent rejection of a donor, based on the results of the screening measures listed above.

Transfusion-transmitted hepatitis E has been reported following transfusion of blood components in both endemic regions and also in non- or low-endemic areas. Seventy-five percent of blood or blood components used in the UK are given to immunosuppressed patients [10], indicating that intercurrent immunosuppression is common in blood component recipients. The immunocompromised can delay viral clearance and lead to viral persistence in patients with solid-organ transplant [67] and HIV infection [63]. These findings have raised the question of whether blood

products and transplants should always be tested for HEV. The medical threat of hepatitis E is obviously not comparable to that of HIV. However, the transfusion-associated risk of HEV and the risk of transmission for HEV at present are far higher than for viruses such as HIV. The potential clinical results of blood-borne HEV infection should not be downplayed; in particular, the risk of serious complications and death exists. Thus, some experts proposed that systematic screening of blood components for markers of hepatitis E infection should be implemented in areas where HEV is endemic (e.g., the European Union), based on HEV RNA detection [43, 104]. Donor testing for HEV genome by means of PCR or antibody detection (IgM and/or IgA as early infection markers) is possible in principle. Because serological testing is poorly sensitive, hepatitis E nucleic acid testing was suggested to be considered [43]. A recent proposal to amend the European pharmacopoeia monograph 1646 – human plasma (pooled and treated for virus inactivation) – would see the introduction of HEV NAT (nucleic acid amplification technique) [7, 36]. Therefore, HEV NAT screening for blood products in high endemic areas is a meaningful consideration for the near future.

6.5 Vertical Transmission of HEV

Hepatitis E is usually a self-limiting disease, with a low rate of fulminant hepatic failure (FHF). However, when this infection occurs in pregnant women, the consequences are disastrous. High mortality, up to 20–30 %, was observed in HEV-infected pregnant women [73]. HEV infections during pregnancy also lead to congenital defects, spontaneous abortion, and even death. Additionally, mother-to-infant transmission of HEV from infected pregnant women has been reported.

6.5.1 The Incidence of HEV Infection in Pregnant Women

In developing countries, during epidemics of hepatitis E, the disease has increased incidence and severity in pregnant women. A prospective field study carried out during an epidemic of hepatitis in India showed that the incidence of the disease in pregnant women was higher than it was in nonpregnant women of childbearing age or in men [77]. Of the 208 pregnant women, 36 (17.3 %) developed viral hepatitis, as compared with 71 of the 3350 nonpregnant women (2.1 %) and 107 of the 3822 men (2.8 %). The incidences of viral hepatitis in the first, second, and third trimesters were 8.8, 19.4, and 18.6 %, respectively. Furthermore, FHF developed in eight (22.2 %) of the pregnant women with viral hepatitis, while it only occurred in three (2.8 %) of the men and did not occur at all in the nonpregnant women in that study. The significantly increased incidence of fulminant hepatitis in pregnant women was indicative of the greater severity of hepatitis during pregnancy. The increased susceptibility to fulminant hepatitis was observed exclusively in the last trimester.

In another prospective study in Saudi Arabia [73], over a 3-year period, 76 pregnant women and 337 nonpregnant women of childbearing age with sporadic acute viral hepatitis were investigated. Among them, 65 (85.5 %) pregnant women and 140 (41.5 %) nonpregnant women had hepatitis E. The proportion of pregnant women in the HEV group was 31.7 %, while it was only 5.3 % in the non-HEV group. The high HEV seroprevalence, particularly at the onset of the pregnancy, in women who reported no history of liver disease confirms that most HEV infections are subclinical or unrecognized.

Geographic discrepancies in the frequency and severity of clinical hepatitis E in pregnant and postpartum women persist. In Egypt, where the prevalence of anti-HEV antibodies in rural communities is very high, one study examined a cohort of 2428 pregnant women and found that the anti-HEV antibody prevalence was 84.3 % [72]. However, a history of jaundice and liver disease was rare in this population and was not increased in those with anti-HEV antibodies. These results confirm that Egypt is highly endemic for HEV and demonstrate that almost all women of childbearing age in these communities had prior HEV exposure without a history of liver disease. The reasons for the lack of clinical hepatitis remain unclear but could be the result of early childhood HEV exposures that produced long-lasting immunity and/or modified the subsequent responses to HEV exposure [72].

In developed countries, the anti-HEV antibody seroprevalence rates reported in pregnant women may not be higher than those in other populations in the same areas. An overall anti-HEV IgG prevalence of 7.74 % was observed among 315 pregnant women in the south of France, and no anti-HEV IgG seroconversion or anti-HEV IgM detection was observed during pregnancy [109]. Anti-HEV IgM was detected at a rate of 0.67 % during the first trimester of pregnancy in a Spanish cohort [84], but no clinical symptoms and normal aminotransferase levels were reported in these women, suggesting that they had silent forms of HEV infection. Thus, in developed countries, HEV infection is a rare occurrence during pregnancy, even in regions of Western countries with high anti-HEV antibody seroprevalence rates.

6.5.2 The Incidence of Vertical HEV Transmission

A landmark study published by Khuroo et al [74] was the first to document the vertical transmission of HEV using serologic and molecular methods. Of eight infants whose mothers had serologic evidence of an HEV infection preceding delivery, six had evidence of hepatitis E infection. Five of these infants had detectable HEV RNA levels in the cord blood, and all these infants had elevated alanine aminotransferase levels at birth. The presence of serum viremia and anti-HEV IgM antibodies and the persistence of hepatitis for several weeks in two of the surviving infants could not be explained solely by contamination of the cord blood with maternal blood and suggest that vertically transmitted HEV had occurred [74].

Vertical transmission of hepatitis E virus from infected mothers to their infants has since been documented in many endemic areas. There is a very high incidence rate of vertical transmission in HEV-infected pregnant women. The documented rates of vertical HEV transmission range from 30 % [29, 79], through 50 % [119], 70 % [74], and 79 % [76], to up to 100 % [81] in various small case series. These high transmission rates signify the importance of the vertical transmission of HEV infection.

Notably, to date, the vertical transmission of HEV from mother to fetus has only been associated with genotype 1 HEV infections, and there have been no reports of the vertical transmission of HEV genotype 3 or 4 infections. However, vertical transmission was demonstrated in pregnant rabbits that were experimentally infected with rabbit HEV, suggesting the potential ability of genotype 3 HEV to undergo mother-to-fetus transmission [135]. Moreover, in the same study, vertical transmission was found to be associated with the replication of HEV in the placenta, as indicated by the presence of HEV RNA and antigen in the placenta from HEV-infected pregnant rabbits [135].

6.5.3 The Outcome of HEV-Infected Babies

HEV infection is known to cause severe liver disease in pregnant women. Additionally, pregnant women with acute viral hepatitis caused by HEV infection had worse fetal outcomes than did pregnant women with acute viral hepatitis caused by infection with other hepatitis viruses [102]. There is a very high risk of preterm delivery with poor neonatal survival rates in HEV-infected pregnant women [72, 102]. In a study from India, it was observed that about half of the studied pregnant women with hepatitis E had intrauterine fetal death and stillbirth [118]. During a HEV outbreak in Sudan in 2010–2011, among the 39 pregnant women with HEV infection, there were 14 intrauterine deaths and nine premature deliveries [108]. In two separate studies from India, 15–50 % of live-born infants of mothers with HEV infection died within 1 week of birth [102, 106]. Studies suggest that the severity of HEV infection in mother and baby may be related to each other, and fetal disease influenced the course of maternal HEV infection. Vertical transmission has been associated with neonatal HEV infection that can present with jaundice at birth (Table 6.5) and can cause death within the first 48 h, usually due to severe hypothermia, hypoglycemia, and FHF [76, 81].

A small percentage of the babies born to HEV-infected mothers with active disease were either preterm or had anicteric hepatitis (Table 6.5). The clinical course of mother-to-fetus HEV infection in surviving neonates is self-limiting with a short-lasting viremia. In a study by Khuroo et al., among the five HEV RNA-positive babies who survived, HEV RNA was not detectable by 4 weeks of birth in three babies, by 8 weeks of birth in one baby, and by 32 weeks of birth in one baby [76]. The clinical profiles of the infected neonates varied and included either elevated

Table 6.5 Vertical transmission of HEV from infected mothers to neonates and its consequences

Country	Consequences of vertical hepatitis E transmission	Frequency of transmission from affected mother to neonate	References
Saudi Arabia	Acute viral hepatitis with complete recovery; limited early neonatal deaths	100 %	[81]
India	Preterm birth	33 %	[79]
Egypt	Respiratory distress syndrome preterm birth sepsis	33 %	[39]
	Hepatosplenomegaly		
India	Icteric hepatitis, anicteric hepatitis, and neonatal death	79 %	[76]
	All surviving babies had self-limiting disease and none had prolonged viremia		
India	Icteric neonatal hepatitis, non-icteric neonatal hepatitis, hypothermia, and hypoglycemia and died within 24 h; massive hepatic necrosis	79 %	[76]
India	Intrauterine fetal death	/	[102]
	Stillbirth		
	Preterm		
India	Intrauterine fetal death	/	[108]
	Preterm		
India	Intrauterine fetal death	/	[118]
	Preterm		
	Stillbirth		
Ghana	Icteric at birth	/	[19]

liver enzyme levels alone, elevated bilirubin levels alone, or elevated bilirubin levels with increased liver enzyme levels. Evidence of severe necrosis in liver tissue samples from neonatal autopsies suggests that some babies, like their mothers, experience FHF as a result of HEV infection.

Neither persistent viremia nor a prolonged clinical course has been observed in neonates infected with HEV via vertical transmission [76], in contrast with the clinical course of hepatitis B virus (HBV) infection in neonates who acquired this virus by perinatal transmission, which may be persistent for a lifelong period [11]. This may be related to the different ways in which these two viruses cause hepatic injury. The liver injury caused by HEV is likely related to direct cytopathogenic changes in liver cells [76]. In contrast, HBV is a not a cytopathogenic virus, and liver injury during HBV infection is caused by the host immune response to the pathogen [11].

HEV infection is commonly transmitted from mother to child via intrauterine or perinatal routes. However, it remains unclear whether or not HEV is transmissible via breast milk. Furthermore, there is currently no information about the influences of asymptomatic HEV infection during pregnancy.

6.6 HEV Transmission Through Organ Transplantation

HEV transmission via grafted organ has been reported but is uncommon. One case of occult HEV transmission in a liver allograft was described by Schlosser et al. in Germany [117]. This HEV infection occurred in a 73-year-old male patient after receiving a HEV-infected liver from a donor with HEV infection. In the recipient, anti-HEV IgG and IgM and HEV RNA were detected in the first tested serum sample obtained 150 days after liver transplantation. Because earlier samples after the liver transplantation were not available, it was not possible to determine when the infected markers initially presented. The donor had tested negative for HEV RNA and anti-HEV antibodies shortly before donation, but, surprisingly, high concentrations of HEV RNA were detected retrospectively in the liver tissue of the donor. Thus, this report not only substantiates the ability of HEV to be transmitted via liver transplantation, but it also reveals that HEV can persist in liver tissue without detectable serological evidence of HEV infection.

The above case seems rather coincidental in that HEV was present in the grafted liver but was not detected in the blood of the donor. However, this situation has also been described in other studies. A HEV prevalence study in wild boar showed that the overall anti-HEV antibody seroprevalence was approximately 25–30 % even though HEV RNA was detected in up to 68 % of the animals, indicating that a significant proportion of viremic animals do not show an anti-HEV antibody response [1]. Furthermore, HEV RNA was exclusively detected in the bile in a significant proportion of boars, supporting the existence of an occult HEV carrier state [1]. Legrand-Abranevel et al. recently reported a high ratio of HEV infections in solid-organ transplant recipients. However, none of those patients tested positive for anti-HEV antibodies before receiving their transplantation, suggesting a greater likelihood of de novo infections than of HEV reactivations [82].

In an attempt to prevent donor-derived infections following transplantation, organ and tissue donors are evaluated to identify those that might be more likely to harbor transmissible pathogens. Many centers performing liver transplantation perform thorough chart histories to screen potential donors and test for the occurrence of communicable diseases. Donor testing includes serologic assays to detect antibodies against HIV, HBV, and HCV, among others, and specific PCR assays for nucleic acids when applicable [13]. However, it is important to note that organ transplants are not routinely tested for HEV infection. Now that HEV transmission through solid-organ transplantation has been confirmed, there may be cause for the routine screening of organ donors and recipients for HEV. However, difficulties in performing the assays to detect HEV antibodies will make the screening of potential donors an imperfect process. In this situation, there is an urgent need for the development of commercially available high-throughput HEV diagnostic assays.

Stem cells may represent another source of risk for HEV infection. Recently, a stem cell donor has been identified who was clinically healthy at the time of evaluation but suffered from acute hepatitis E at the time of leukopheresis [70].

6.7 Conclusion

Waterborne transmission of HEV in highly endemic regions of developing countries with poor sanitation and zoonotic transmission, especially foodborne transmission through the consumption of raw or undercooked meat of HEV-infected animals in industrialized countries, accounts for most HEV transmissions. Occasionally, the exact mode of HEV transmission remains controversial, and sources of viral infection are often not well identified, particularly in sporadic cases of acute hepatitis E. Recent investigations reinforce the idea that there are three additional routes of HEV transmission: blood-borne transmission, organ transplant transmission, and vertical transmission from mother to child.

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Chapter 7
Immunobiology and Host Response to HEV

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Abstract *Hepatitis E virus* (HEV) causes acute self-limiting hepatitis in most cases and chronic infection in rare circumstances. It is believed to be noncytopathic, so immunologically mediated events should play important roles in its pathogenesis and infection outcomes. The anti-HEV antibody response was clarified when the major antigenic determinants on the ORF2 polypeptide were determined, which are located in its C-terminal portion. This subregion also forms the conformational neutralization epitopes. Robust anti-HEV immunoglobulin M (IgM) and IgG responses usually develop 3–4 weeks after infection in experimentally infected nonhuman primates. In humans, potent specific IgM and IgG responses occur in the very early phase of the disease and are critical in eliminating the virus, in concert with the innate and adaptive T-cell immune responses. They are also very valuable in the diagnosis of acute hepatitis E, when patients are tested for both anti-HEV IgM and IgG. The long-term persistence and protection of anti-HEV IgG provide the basis for estimating the prevalence of HEV infection and for the development of a hepatitis E vaccine. Although HEV has four genotypes, all the viral strains are considered to belong to a single serotype. It is becoming increasingly clear that the innate and adaptive T-cell immune responses play critical roles in the clearance of the virus. Potent and multispecific $CD4^+$ and $CD8^+$ T-cell responses to the ORF2 protein occur in patients with acute hepatitis E, and weaker HEV-specific $CD4^+$ and $CD8^+$ T-cell responses appear to be associated with chronic hepatitis E in immunocompromised individuals.

Abbreviations

Aa	Amino acids
Anti-HEV	Antibodies directed against HEV
ELISA	Enzyme-linked immunosorbent assay
HEV	*Hepatitis E virus*

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IFN	Interferon
IL	Interleukin
ISG	Interferon-stimulated gene
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells	Natural killer cells
NKT cells	Natural killer T cells
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cell
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha

Hepatitis E virus (HEV) is a nonenveloped, single-stranded positive-sense RNA virus with a genome of approximately 7.2 kb. There are three open reading frames (ORFs) in the viral genome: ORF1 encodes a nonstructural polyprotein of 1693 amino acids (aa); ORF2 encodes a 76-kDa protein of 660 aa, which is the principal (and probably only) structural protein of the nucleocapsid; and ORF3 encodes a polypeptide of 123 aa with various assumed functions. Infection with HEV may be asymptomatic or cause acute self-limiting hepatitis. However, the virus can sometimes cause acute fulminant hepatitis, particularly in pregnant women, with a 20–30 % mortality rate in the third trimester. In rare cases, it may lead to chronic infection in immunocompromised individuals. The pathogenesis of HEV infection appears to be predominantly mediated by the host immune responses, which may clear the virus and provide specific immunity to HEV.

7.1 Innate Immune Responses to HEV

The innate immune system is the host's first line of defense against various pathogens, including viruses. The system also plays important roles in priming the adaptive immune responses. The processes involved in the activation of the innate immune responses to viral infections have not yet been fully clarified. The early antiviral events usually consist of three major mechanisms: (1) production of type I interferon (IFN); (2) destruction of infected cells by natural killer (NK) cells; and (3) production of various pro- and anti-inflammatory cytokines and chemokines, which may directly clear the virus and promote the maturation and recruitment of the adaptive immune responses [31]. The innate immune responses to HEV infection are not well studied, mainly because it is difficult to identify patients in the very early asymptomatic phase and because there is no efficient HEV cell culture system. Despite these obstacles, the data acquired from animal studies, cell culture, and

humans provide us with a scenario of the innate immune responses induced during HEV infection.

7.1.1 Induction of Apoptosis

Generally, higher eukaryotic cells can use their innate ability to undergo programmed cell death (apoptosis) to terminate viral replication after viral infection. This also appears to be the case in HEV infection. Apoptotic hepatocytes are frequently detected in acute hepatitis E [56], and the HEV ORF2 protein may activate the proapoptotic gene *CHOP* and the antiapoptotic heat shock proteins with a mammalian cell expression system [39]. The apoptosis of renal epithelial cells has recently been observed during the acute phase of HEV infection in a Mongolian gerbil model infected with swine HEV [78]. This appears to be consistent with the finding that HEV is detectable in the urine of patients with acute hepatitis E [28].

7.1.2 Innate Sensing by Toll-Like Receptors (TLRs)

TLRs are a class of proteins that play essential roles in the innate immune response to various pathogens, including viruses. TLRs can recognize specific conserved viral features and then initiate pro-inflammatory signaling pathways. The levels of TLR4, 7, and 8 are significantly higher in patients in the acute phase of hepatitis E than in control subjects, whereas the levels of TLR2 and 3 are similar to those in controls. After viral clearance in the convalescent phase, the levels of these TLRs become similar to those of healthy subjects, except that the level of TLR2 declines [8]. An independent research group compared the expression levels of TLR2, 3, 4, 7, and 8 in patients with acute hepatitis E and those with HEV-related acute liver failure. When the peripheral blood mononuclear cells (PBMCs) were not stimulated, the expression levels of TLR2, 3, and 4 were significantly higher in the acute hepatitis E patients than in the patients with acute liver failure. However, after the PBMCs were stimulated by the ORF2 protein (aa 452–617), the expression of the *TLR2*, *TLR3*, and *TLR7* genes increased nearly 3-, 100-, and 10-fold, respectively, in patients with acute liver failure, whereas their expression did not change significantly in patients with acute hepatitis E. The interferon γ (IFN-γ) levels in the culture supernatants of stimulated PBMCs from patients with acute hepatitis E (292.3 ± 88 pg/ml) were significantly higher than those in PBMCs from patients with acute liver failure (3.24 ± 0.8 pg/ml) or healthy controls (0.7 ± 0.29 pg/ml). The levels of tumor necrosis factor alpha (TNF-α), interleukin 10 (IL-10), and transforming growth factor beta (TGF-β) were all significantly higher in patients with acute hepatitis E than in those with acute liver failure and were higher in these patients than in healthy controls. In patients with acute hepatitis E, the expression of *TLR3* in PBMCs correlated positively ($r^2 = 0.896$) with the level of IFN-γ in the PBMC

culture supernatant [55]. It has also been shown that in HEV-infected pregnant women with acute liver failure, the expression of TLR3, TLR9, the downstream signaling molecule MYD88, IFN regulatory factor 3 (IRF3), and IRF7 is significantly downregulated compared with their expression in HEV-infected pregnant women without acute liver failure or in non-HEV-infected pregnant women with acute liver failure [74]. These results suggest that inadequate triggers of the innate immune responses contribute to the development of severe hepatitis E or even acute liver failure. Therefore, it is becoming apparent that HEV may be subject to innate sensing by TLRs.

7.1.3 Activation of NK Cells

NK cells are a type of cytotoxic lymphocyte and play a critical role in the innate immune system. NK cells act as important sentinels of the immune system, initiating defense responses to certain viral infections. In patients with acute hepatitis E, the proportion of NK cells among their PBMCs appeared to be somewhat lower than that in healthy individuals (8.9 % [2.4–47.0] vs 11.2 % [2.6–35.4], $P < 0.05$). However, the proportion of NK cells with activation markers was greater than that in healthy controls (43.5 % [11.2–58.6] vs 15.5 % (3.0–55.8], $P < 0.05$) and returned to normal after clinical and biochemical recovery [79]. Similarly, the proportion of NK cells was lower in the peripheral blood of solid organ transplant patients with acute hepatitis E than in that of transplant patients without HEV infection (133 [41–390] vs 214 [56–1140], $P = 0.08$), although the difference was not statistically significant, possibly because the number of patients tested was small (23 and 32, respectively) [1]. This reduction in the total NK cells in the peripheral circulation may be attributable to the relocation of these cells to the liver, because statistically significantly increased numbers of $CD56^+$ NK cells have been observed in the livers of recently deceased patients with HEV-related acute liver failure [69]. Although NK cells may play important roles in the clearance of the virus, they may also contribute to the liver injury associated with acute HEV infection.

7.1.4 Alteration of NF-κB Activity

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a protein complex that functions broadly in the regulation of transcription. NF-κB plays key roles in the cellular responses to various stimuli, including viral infection. The DNA-binding activity of NF-κB appeared to be higher in both the PBMCs and postmortem liver tissues of HEV-infected pregnant patients with fulminant hepatic failure than in those of nonpregnant women or pregnant women with acute hepatitis E but without fulminant hepatic failure. This increased binding activity might be attributable to a higher level of subunit p50 expression, but not subunit p65

expression, because p65 is not present in the NF-κB transactivation complex [70]. When human hepatoma cells were cotransfected with either HEV ORF2 or ORF3 and a reporter vector with the IL-2 receptor (*IL2R*) promoter region containing an NF-κB element cloned upstream from the chloramphenicol acetyl transferase coding sequence (NF-κB–CAT), the ORF2 protein, but not the ORF3 protein, inhibited NF-κB activity [82]. By contrast, when human lung epithelial cells (A549) were transfected with plasmids containing ORF3, other investigators found that the ORF3-encoded protein of genotype 1 HEV activated NF-κB through the unfolded protein response in an early stage of transfection of plasmid and then inhibited TNF-α-induced NF-κB signaling in a late phase [91]. Because these data were mainly derived from cells expressing HEV proteins, studies based on the liver tissues and lymphocytes of patients or experimentally infected animals are required to validate the hypothesis that the activity of NF-κB is altered in the natural infection.

7.1.5 Alteration of Other Factors in the Innate Immune Responses

Microarray analyses of serial liver biopsies from experimentally HEV-infected chimpanzees showed that a number of immune-associated genes are activated by HEV infection, including three well-known IFN-γ-induced chemokine genes, *CXCL9*, *CXCL10*, and *CXCL11*, and IFN-stimulated genes (ISGs) 15 and 20 [93]. The chemokines, CXCL9, CXCL10, and CXCL11, and ISG15 and ISG20 are involved in neutrophil and T-cell trafficking. ISG15, in particular, can attract and activate neutrophils. This is consistent with the pathological findings in the livers of patients with hepatitis E, in whom the inflammation in the portal tracts is usually characterized by numerous neutrophils [56, 68].

When the replication of HEV in A549 lung tumor cells was examined, HEV was found to suppress IFN-α signaling by regulating STAT1 phosphorylation [21]. This is consistent with the undetectable expression of types I, II, and III IFN in a microarray analysis [93]. However, the innate immune response may be activated by the differential expression of genes involved in the RIG-I and JAK/STAT pathways and by the expression of genes known to be induced by type I IFN, because multiple pathways are involved in the induction of IFN-induced genes [93].

An in vitro study showed that HEV replication in hepatoma cells can inhibit poly(I · C) (double-stranded RNA)-induced IFN-β expression [64, 65]. Two domains of the HEV ORF1 protein, the X domain (macro domain) and the papain-like cysteine protease domain, appear to be elements that are critical for this inhibition. The overexpression of the X domain in HEK293T cells inhibited the poly(I · C)-induced phosphorylation of IRF3, an important transcription factor involved in the induction of IFN. Furthermore, the replication of an HEV replicon in hepatoma cells also impaired the phosphorylation of IRF3. By contrast, the same research group found that the HEV ORF3 protein enhances the induction of IFN because IFN-β mRNA

levels were upregulated in stably ORF3-expressing HeLa cells treated with poly(I · C) [64, 65]. The culture of HEV in the Alexander hepatoma cell line, PLC/PRF/5, also upregulated the expression of four IFN-inducible genes, *IFI27*, *IFI6*, *MX1*, and *CMPK2* [95]. Whether the HEV-associated inhibition or induction of IFN observed in vitro reflects the scenario in vivo remains to be confirmed.

In summary, the findings discussed above on the innate immune responses to HEV infection were mainly derived from observations of the peripheral blood or cultured cell systems. However, the intrahepatic innate immune responses in naturally infected humans remain to be studied.

7.2 Specific Antibody Responses to HEV

No natural virions secreted by infected humans or experimentally infected animals or derived from in vitro cell culture systems are yet available for developing serological assays to detect antibodies directed against HEV (anti-HEV). Therefore, the diagnostic reagents for anti-HEV include recombinant polypeptides or synthetic peptides of HEV. Precisely defining and understanding the antibody responses to HEV infection require the identification of adequate immunoreactive subregions on the HEV virions.

7.2.1 Major Antigenic Determinants

7.2.1.1 Identification of the Immunodominant Region on HEV

In an initial study, in which the linear B-cell epitopes on the three ORF proteins of HEV were mapped with the Geysen pin method, 12 linear epitopes were identified in the ORF1 protein, three in the ORF2 protein, and one in the ORF3 protein. Most of the epitopes defined in ORF1 were clustered in the putative RNA-dependent RNA polymerase gene region, near the carboxyl terminus [42]. Similar studies by other investigators that mapped the linear B-cell epitopes in the ORF2 and ORF3 proteins, based on a series of synthetic peptides, showed that four of five peptides from the ORF3 protein and four of 12 peptides from the ORF2 protein specifically reacted with antibodies in the sera of HEV-infected patients [43]. Using a set of 11 synthetic peptides containing regions of the polypeptide encoded by the ORF2 protein, the same study group found two immunodominant regions: one region was located at aa 546–580 of ORF2 and the other at aa 394–470 [44]. In contrast, investigators from Genelabs Technologies Inc. (Redwood City, CA, USA), who first identified HEV [72], reported that the last 42 aa at the C-terminus of ORF2 and the 33 aa at the C-terminus of the ORF3 protein appeared to be immunodominant epitopes [92]. However, synthetic peptides, whether derived from ORF2 or ORF3, were

least useful in serological tests for detecting anti-HEV, with low sensitivity and low specificity [57].

Before 1997, serological assays based on recombinant or synthetic antigens detected anti-HEV seropositivity more frequently in patients with suspected hepatitis E, but the overall seroprevalence of anti-HEV IgG in normal human populations in endemic countries was unexpectedly low, ranging from 2.8 to 20.2 %, and a low but constant anti-HEV seropositivity (0.4–2.6 %) was also observed in nonendemic developed countries [10]. In 1997, investigators from Johns Hopkins University, Genelabs Technologies Inc. and the National Institutes of Health reported that enzyme immunoassays based on a 55-kDa antigen (aa 112–607) of ORF2 and a 62-kDa ORF2 polypeptide detected rates of anti-HEV as high as 23.0 % (68 of 295), 15.9 % (47 of 295), and 21.3 % (64/300) in injecting drug users, homosexual men, and blood donors, respectively, in Baltimore, MD [87]. To compare the regional differences in the rates of anti-HEV positivity, they also tested blood donors from Sacramento, CA, and New York, NY, for anti-HEV and found positivity rates of 13.7 % (29/211) ($P > 0.05$) and 31.0 % (93/300) ($P < 0.001$), respectively [87]. These data demonstrate that the prevalence of anti-HEV in blood donors from nonendemic regions was unexpectedly much higher than previously reported [10]. Although the authors considered that the anti-HEV detected in that study did not necessarily indicate prior HEV infection at that time, subsequent studies have demonstrated that the ORF2 antigens used in their assays contain immunodominant conformational neutralization B-cell epitopes [102, 103]. Numerous studies have since demonstrated that the prevalence of anti-HEV is much higher in nonendemic regions than previously estimated (please see Chap. 3 Epidemiology of hepatitis E).

With a PCR-based seroneutralization assay in cell culture, Meng et al. [62] demonstrated that a polypeptide (pB166) covering aa 452–617 of the ORF2 protein contains conformational neutralization epitopes against HEV, based on immune sera collected from mice separately immunized with 51 overlapping synthetic 30-mer peptides spanning aa 221–660 of the ORF2 protein and 31 overlapping recombinant polypeptides of different sizes covering the full-length ORF2 protein (aa 1–660) [61]. With chimpanzee neutralizing monoclonal antibodies directed against HEV and antisera collected from experimentally HEV-infected chimpanzees in the convalescent phase, Zhou et al. demonstrated that aa 459–607 of the ORF2 protein contains neutralization epitopes [102], which is consistent with the findings of Meng et al. [61]. Subsequent investigations showed that the neutralization region in the ORF2 protein forms the immunodominant antigenic determinants in naturally infected human and in experimentally infected nonhuman primates [103]. Therefore, the critical and sufficient immunoreactive region of HEV is located in the C-terminal portion of the ORF2 protein, in a subregion spanning aa 459–607. Figure 7.1 shows a diagram of the immunodominant region of the ORF2 protein.

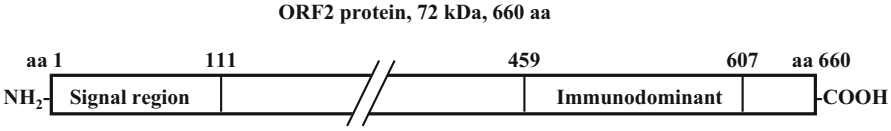

Fig. 7.1 Diagrammatic illustration of ORF2 protein. The region spanning amino acids (aa) 1–111 is assumed to contain a typical signal sequence. The region from aa 459 to aa 607 forms the major antigenic determinant, which also contains the conformational, neutralization epitopes

7.2.1.2 Critical Role of Correct Folding of the Immunodominant Region in Detecting Anti-HEV Antibodies

Because no natural HEV virions are available for the development of diagnostic reagents to detect anti-HEV, recombinant HEV antigens produced in various expression systems must effectively mimic the natural folding of the antigens so that they can be used to efficiently detect anti-HEV. Using an *Escherichia coli* expression system, Li et al. prepared two recombinant ORF2 polypeptides that included the carboxyl terminal one-third of the ORF2 protein (ORF2.1) and approximately two-thirds of the carboxyl terminal of the ORF2 protein (ORF2.2) and used them in a Western blotting analysis to detect anti-HEV in blood samples collected from patients in the acute and convalescent phases of hepatitis E and from experimentally HEV-infected monkeys. Polypeptide ORF2.1 detected anti-HEV in both acute- and convalescent-phase serum specimens. However, polypeptide ORF2.2 mainly detected anti-HEV in the acute-phase samples and did not detect antibodies in the convalescent samples, even though ORF2.2 contained all the residues of ORF2.1 [50]. A further study suggested that in the full-length ORF2 protein expressed in *E. coli*, the carboxyl terminal epitopes are masked [49, 54]. In contrast, a polypeptide of ORF2 (aa 112–606) produced in insect cells may form viral-like particles, with antigenicity similar to that of authentic HEV virions [49, 54]. A further study showed that this recombinant ORF2 polypeptide self-assembles into a dual-domain T = 1 particle presenting native virus epitopes [90]. A candidate hepatitis E vaccine composed of aa 112–607 of the ORF2 protein, produced in insect cells, was also effective in the prevention of hepatitis E in nonhuman primates [71] and in humans [77], indicating that the insect-cell-expressed polypeptide that includes aa 112–607 of ORF2 forms a conformational structure similar to that of the natural virion. This polypeptide first detected the unexpectedly high prevalence of anti-HEV in various populations of US citizens [87]. These data demonstrate that the polypeptide that includes aa 112–607 of ORF2 produced in insect cells can fold in a way highly similar to that in natural virions.

Whereas the carboxyl terminal epitopes of the full-length ORF2 protein expressed in *E. coli* may be masked [49, 54], a polypeptide including aa 112–607 of the ORF2 protein expressed in *E. coli* also appears to fold incorrectly, because it detected anti-HEV much less sensitively than did the same polypeptide produced in insect cells (Fig. 7.2). Therefore, it is very likely that the full-length (or nearly full-length)

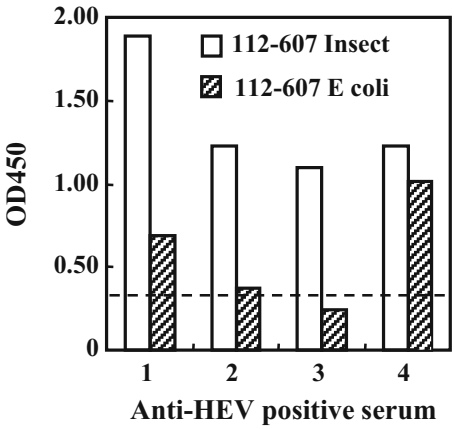

Fig. 7.2 Varied reactivity of anti-HEV to polypeptides with the same amino acid compositions but expressed in different systems. Equal amounts of a polypeptide containing amino acids 112–607 of ORF2 (genotype 1) expressed in insect cells (*open bar*) or in *E. coli* (*hatched bar*) were used in the indirect enzyme-linked immunosorbent assay. 1–4 on the X axis represent the sera (200-fold diluted) collected from monkeys experimentally infected with HEV genotypes 1–4, respectively

ORF2 protein expressed in *E. coli* cannot fold correctly, so the epitopes within the dominant antigenic site are not in the correct conformation for recognition by anti-HEV.

Fortunately, the C-terminal portion including the minimal subregion (aa 459–607) of the ORF2 protein folds correctly when it is expressed in *E. coli*. A polypeptide spanning aa 458–607 of the ORF2 protein produced in *E. coli* detected anti-HEV as efficiently as the polypeptide spanning aa 112–607 of ORF2 produced in insect cells in both naturally infected humans and experimentally infected nonhuman primates [102, 103] (Fig. 7.3). Numerous studies have also shown that monkeys immunized with C-terminal polypeptides containing the minimal subregion (aa 459–607) of the ORF2 protein are protected against HEV challenge [6, 36, 41, 51–53, 89, 99, 100]. Recently, a clinical trial demonstrated that a hepatitis E vaccine composed of aa 368–606 of the ORF2 protein produced in *E. coli* effectively prevented hepatitis E [104]. The recombinant *E. coli* vaccine was shown to be effective for at least 4.5 years [97]. Together, these data demonstrate that the C-terminal part of the ORF2 protein, including the minimal subregion (aa 459–607), expressed in *E. coli* folds correctly, producing a structure highly similar to that of the natural virion, and is suitable for the development of diagnostic reagents that detect anti-HEV. Currently, the assays used to detect anti-HEV, including commercially available and in-house assays, appear to be based on the correctly folded C-terminal immunodominant regions of the ORF2 protein [9, 32, 35, 47, 66].

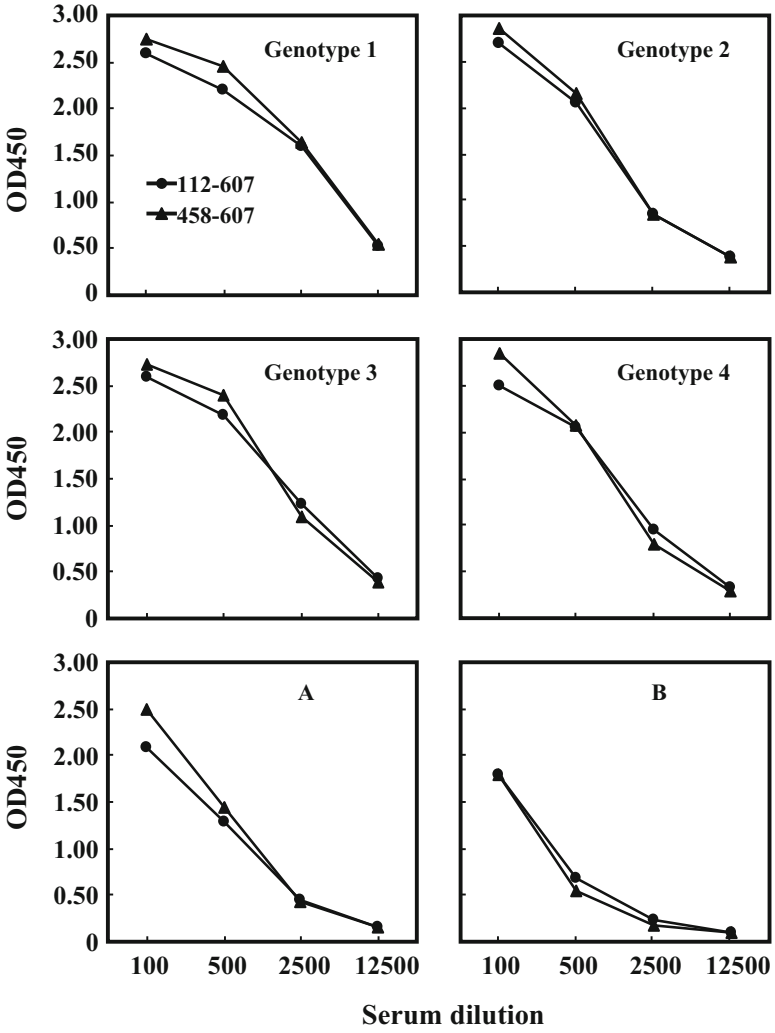

Fig. 7.3 Titration of anti-HEV IgG detected with indirect ELISA based on different ORF2 peptides. Polypeptide 112–607 (amino acids 112–607) was expressed in insect cells and polypeptide 458–607 (amino acids 458–607) was expressed in *E. coli*. Both polypeptide sequences were derived from HEV genotype 1 (Sar-55). Genotypes 1–4 represent four monkeys experimentally infected with HEV genotype 1–4, respectively. A and B display two persons who showed positive anti-HEV IgG without hepatitis history, respectively

7.2.1.3 Essential Role of Dimerization of the Immunodominant Region in Detecting Anti-HEV Antibodies

As the major (and probably only) capsid protein of HEV, the ORF2 protein should potentially self-associate to form oligomers. The full-length ORF2 protein expressed in mammalian cells and examined with sodium dodecyl sulfate polyacrylamide gel

electrophoresis (SDS-PAGE) displayed both dimeric and monomeric forms when unheated, but only the monomeric form when the sample was heated before electrophoresis [38]. When expressed in bacteria, the ORF2 polypeptides still formed dimers when truncated up to residue 459 from the N-terminus or up to residue 602 from the C-terminus [51, 52]. In vitro transcription and translation experiments also suggested that this same region (aa 459–602) is responsible for dimerization. When seven polypeptides from different regions of the ORF2 protein were compared with SDS-PAGE without prior heating, the polypeptides containing aa 459–602 formed dimers, whereas those lacking some or all of aa 459–602 only migrated as monomers (Fig. 7.4). These experiments suggest that the dimerization of the ORF2 protein requires the minimal region spanning aa 459–602. A mutational analysis [51, 52] localized the site of the dimeric interaction to a cluster of hydrophobic residues (Ala597, Val598, Ala599, Leu601, and Ala602), but other residues may also be involved, because (1) residues Ala597, Val598, and Ala599 of the ORF2 protein are not highly conserved among different HEV strains; and (2) an unheated ORF2 polypeptide containing a Asn-to-Gln mutation at aa 562 did not migrate as dimers on SDS-PAGE [30].

The immunoreactivity of the dominant antigenic determinants in the C-terminal portion of the ORF2 protein depends upon the dimeric form of the polypeptide. Both mouse monoclonal antibodies produced by immunizing mice with C-terminal ORF2 polypeptides and convalescent human sera reacted with the dimeric forms of the ORF2 polypeptides but reacted very poorly with the monomeric form [51, 52]. When the minimal polypeptide (aa 459–607) containing the immunodominant antigenic determinants of the ORF2 protein was used in a Western blotting analysis (Fig. 7.5b), human serum samples collected from patients with acute- or convalescent-phase hepatitis E, and from subclinically infected individuals, all reacted with the dimeric form of the ORF2 protein, but not with the monomeric form [67]. These samples also showed strong reactivity in an enzyme-linked immunosorbent assay (ELISA) based on the immunodominant polypeptide (aa 459–607 of ORF2), which formed a dimer, but no or poor reactivity in an ELISA based on the truncated polypeptide, which did not form a dimer (Fig. 7.5c). These results strongly indicate that, to accurately define the anti-HEV responses to HEV infection, the ORF2 polypeptide used in assays should be present in the dimeric form or in higher-order oligomeric structures.

7.2.2 Antibody Responses to Immunodominant Antigenic Determinants

7.2.2.1 Anti-HEV IgM and IgG Responses in Experimentally Infected Animal Models

Nonhuman primates, including monkeys and chimpanzees, experimentally infected with HEV have been used extensively as animal models to help us understand HEV infection, including aspects of the clinical course and immune responses, and in

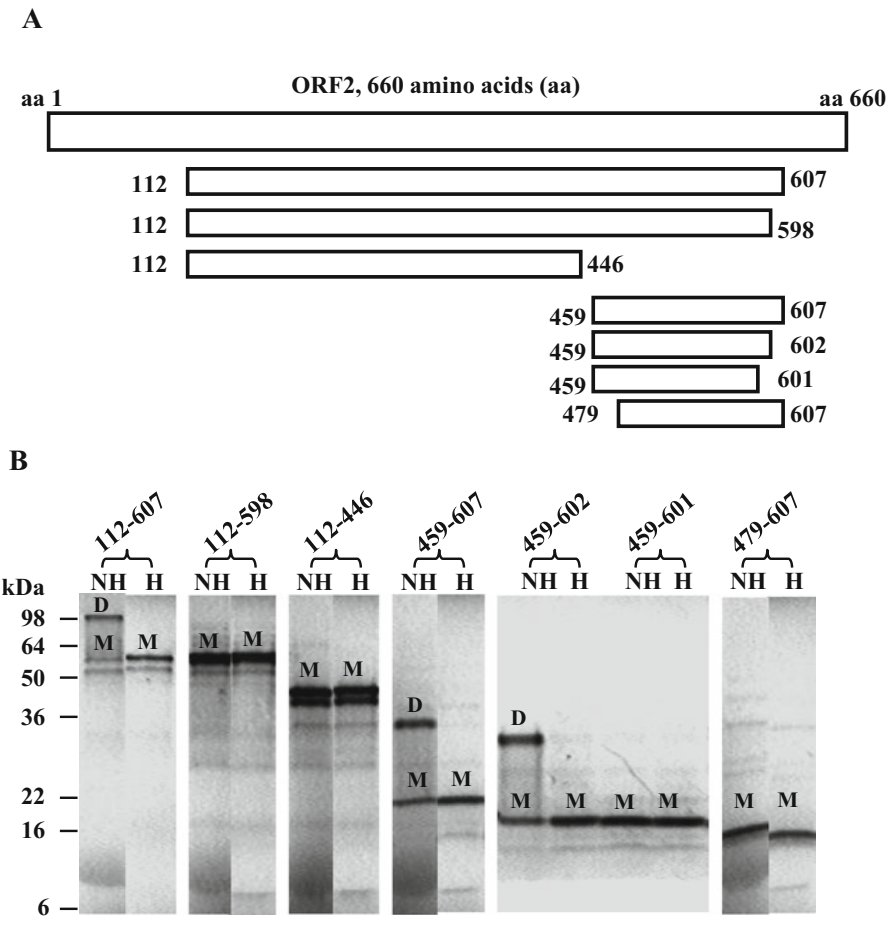

Fig. 7.4 Dimeric interaction of the ORF2 polypeptides characterized by SDS-PAGE. (**A**) Diagram of terminally truncated polypeptide ORF2 protein. The numbers beside each bar indicate the first and last amino acid (aa) of each polypeptide. (**B**) SDS-PAGE of each ORF2 polypeptide with heating (*H*) or without heating (*NH*) prior to electrophoresis. ^{35}S-labeled, truncated ORF2 polypeptides were synthesized by in vitro translation and detected by SDS-PAGE and autoradiography. When the polypeptides were not heated (*NH*), the polypeptides containing aa 459–602 display both dimeric (*D*) and monomeric (*M*) forms, while other polypeptides show monomeric (*M*) form only. All polypeptides only present monomeric form when they were heated before electrophoresis (*H*)

preclinical vaccine trials. These animal models have generated a considerable literature and made important contributions to our understanding of various aspects of HEV infection (please see Chap. 9 HEV animal models).

Experimentally infected nonhuman primates mimic the infection in humans, so the immune responses observed in these animals should be similar to those that occur in humans. In chimpanzees experimentally infected with HEV, a specific anti-HEV IgM response occurred as early as 3–4 weeks postinfection, and specific IgG

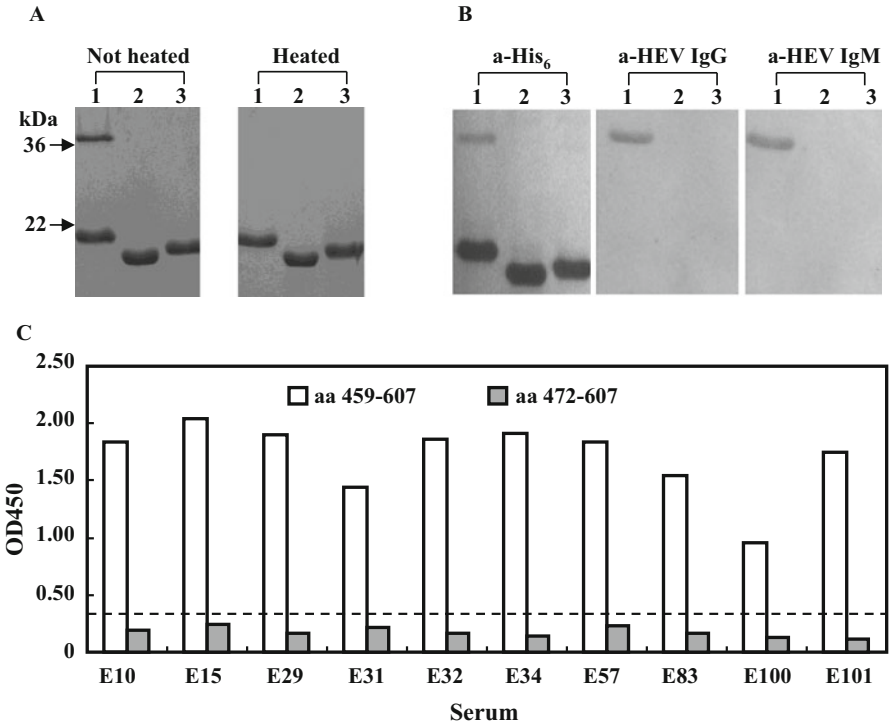

Fig. 7.5 Essential role of dimerization of immunodominant region in detecting anti-HEV. Lanes 1–3 indicate the His_6-tagged polypeptides covering amino acids 459–607, 472–607, and 459–594 in ORF2, respectively. (**A**) SDS-PAGE of purified polypeptides. The polypeptide in lane 1 presents both dimer and monomer when it was not heated and only monomer when it was heated. Two other polypeptides (lanes 2 and 3) only show monomer regardless of heated or not heated. (**B**) Reactivity of anti-His_6 or anti-HEV to the His_6-tagged ORF2 polypeptides analyzed by Western blot analysis. All three polypeptides were not heated in SDS-PAGE. The membrane was incubated with peroxidase-labeled anti-His_6, or with the serum from a hepatitis E patient, followed by probing with peroxidase-labeled antihuman IgG and IgM. (**C**) Reactivity of anti-HEV to an ORF2 polypeptide (aa 459–607) that can form dimers and non- or poor reactivity of anti-HEV to a truncated polypeptide (aa 472–607) that cannot form dimers determined by ELISA

developed at the same time or 1 week after the development of specific IgM, when specific antibodies were detected with an ELISA based on an insect-cell-expressed full-length or nearly full-length ORF2 polypeptide [88, 94]. Similarly, in monkeys experimentally infected with HEV, specific IgM and IgG immune responses to HEV developed as early as 3–4 weeks postinfection, at the same time or slightly delayed specific IgG response, when the specific antibodies were detected with an ELISA based on the insect-cell-expressed ORF2 polypeptides described above [88, 94] or on bacterially expressed C-terminal ORF2 polypeptides [36, 96]. In contrast, when specific antibodies were detected with an assay based on polypeptides that did not contain the dominant antigenic determinants [96], the development of anti-HEV in these animals appeared to be delayed by several days to 2 weeks. These results

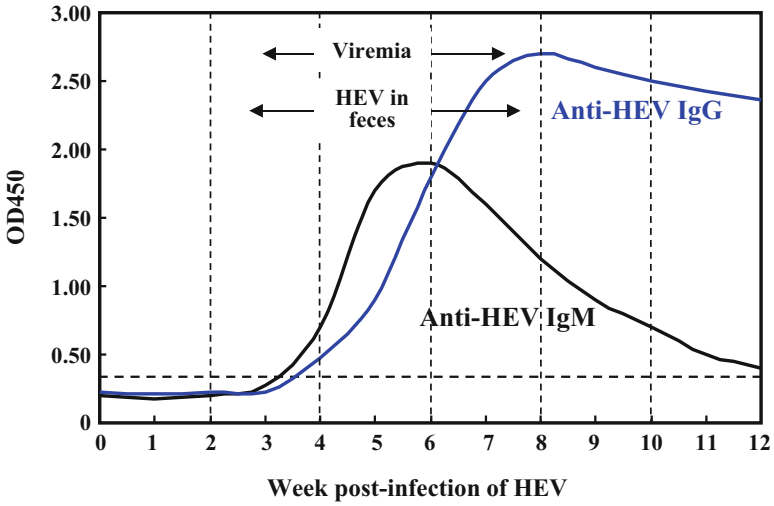

Fig. 7.6 Profile of anti-HEV IgM and IgG responses to HEV in experimentally infected monkeys

demonstrate that it is critical to use the appropriate antigens in the antibody assays if the anti-HEV responses are to be precisely defined. Typical anti-HEV responses in experimentally infected nonhuman primates, measured with assays based on the immunodominant polypeptides, are shown in Fig. 7.6. Like the general kinetics of specific IgM and IgG antibodies against other pathogens, anti-HEV IgM disappeared after several weeks in the experimentally HEV-infected animals, whereas anti-HEV IgG persisted for at least the periods of the studies, which ranged from 15 to 86 weeks [36, 88, 94, 96]. It is reasonable to infer that anti-HEV IgG persists longer in these animals than the observation periods investigated.

7.2.2.2 Anti-HEV IgM and IgG Responses in Naturally Infected Humans

Unlike in the experimental infection of animals, it is hard to define the exact time that humans are exposed to the virus in the natural infection process. However, two studies involving human volunteers have provided useful information that clarifies various aspects of acute hepatitis E [11, 16]. Anti-HEV IgG first became detectable by ELISA (Diagnostic Biotechnology test kit, Singapore) on day 41 after the ingestion of HEV and persisted for at least 2 years [16].

Although HEV is usually transmitted by the fecal–oral route, it may also be transmitted by blood transfusion [34, 86]. Cases of transfusion-acquired hepatitis E provide a way to calculate the accurate interval between intravenous HEV exposure and the detectability of anti-HEV. Anti-HEV antibodies usually developed within 4–14 weeks in immunocompetent individuals after transfusion with contaminated blood products [34, 46, 58, 59]. Therefore, the period from exposure to the develop-

ment of anti-HEV in humans with transfusion-acquired hepatitis E is generally similar to that in experimentally infected nonhuman primates. Under unusual circumstances, including in immunocompromised humans, the anti-HEV antibody responses may be delayed for several months [26, 34].

The observation of longitudinal samples from patients with hepatitis E, transmitted naturally via the fecal–oral route, has shown that anti-HEV IgM levels are usually highest when the patients are first tested. The actual peak IgM level may be missed because of the time lag between the onset of illness and laboratory testing. The IgM levels decline slightly within the following 2 weeks and then decrease rapidly over the next several weeks [75]. Anti-HEV IgM becomes undetectable after 3 months in most patients. Koshy et al. reported that anti-HEV IgM was detectable in 44 % of patients at 1 month but in no patients 3, 6, and 12 months after disease onset [48]. In a cohort of 48 patients with acute hepatitis E who were positive for anti-HEV IgM during the first week of illness, only 11 (18.3 %) remained IgM positive during the seventh week [14]. However, another report showed that around 25 % of patients in a cohort of 62 Nepalese adults with acute hepatitis E had detectable levels of anti-HEV IgM even after 14 months [63], although this might be attributable to the different cutoff values used in the assay.

In experimentally infected monkeys, anti-HEV IgG becomes detectable almost at the same time as anti-HEV IgM or 1 week thereafter, and IgG levels usually peak at 8–10 weeks postinfection [94, 103]. Seriwatana et al. reported that anti-HEV IgG levels were the highest in naturally infected humans at the first test, a median of 8 days after the onset of illness [75]. However, our observations of longitudinal serum samples from 21 symptomatic patients with acute hepatitis E showed that all patients were positive for both anti-HEV IgM and IgG at the first test (1–3 weeks after illness onset) and 13 patients showed increasing anti-HEV IgG titers during weeks 1–4 after the first test (2–6 weeks after onset) (unpublished data). These findings suggest that when symptomatic patients first visit their physicians, the vast majority, if not all, of them should already have developed a strong antibody responses to HEV and should be positive for both anti-HEV IgG and IgM. This has important implications for the diagnosis of acute hepatitis E. When a symptomatic patient is negative for anti-HEV IgG, regardless of his/her anti-HEV IgM status, the patient is less likely to suffer from hepatitis E.

7.2.2.3 Persistence of Anti-HEV IgG

Like IgG antibodies directed against other pathogens, anti-HEV IgG may persist for long periods of time, for at least several years to decades. Based on experimentally infected monkeys observed for 7 years, Arankalle et al. estimated that it would take 3.15–44.9 years (19.4 ± 11.6) for HEV IgG to decline to a titer of 1:100 and 6.9–84.3 years (35.4 ± 21.3) for it to decline to a titer of 1:50 [4]. When the anti-HEV IgG titers were followed up for 5 years in 37 patients infected during epidemics, IgG was estimated to persist for up to 80 years and an estimate based on two patients with sporadic hepatitis E was similar [13]. We followed 36 anti-HEV-IgG-positive

(subclinically infected) pregnant women with no history of hepatitis for 7–12 months after delivery, and their IgG levels showed little variation [101]. Of 55 anti-HEV-IgG-positive individuals with subclinical infections, 37 (67.3 %) remained IgG positive after 6 years, and those who converted to anti-HEV IgG negative still had relatively high value of optical densities at 450 nm wavelength in the ELISA [32]. These findings indicate that the anti-HEV IgG induced by subclinical infections may also persist for long periods of time. Other studies have also shown that the anti-HEV IgG induced by a hepatitis E vaccine persisted for at least 4.5 years [97] and that vaccine-induced anti-HEV IgG may persist at detectable levels for between 8 years and nearly a lifelong time [17].

7.2.2.4 Anti-HEV IgA Response

In addition to the specific IgM and IgG responses to HEV infection, the specific IgA (anti-HEV IgA) response to HEV infection has been also studied. The profile of the anti-HEV IgA response after natural infection is similar to that of the anti-HEV IgM response. Although it is assumed that the detection of anti-HEV IgA alone or together with anti-HEV IgM is useful for a serological diagnosis with increased specificity [84], several studies have shown that the positive rate of anti-HEV IgA in suspected hepatitis E patients or unknown hepatitis patients was lower than that of anti-HEV IgM [15, 22, 33, 98]. Therefore, the detection of anti-HEV IgA is of limited value for the serological diagnosis of acute hepatitis E.

7.2.2.5 Cross-Reactivity and Cross Protection of Anti-HEV Antibodies to Different HEV Genotypes

There are four human HEV genotypes, genotypes 1–4. However, there is thought to be only one serotype of HEV, and all human HEV isolates are considered to be serologically related. Two ELISAs, in which an insect-cell-expressed polypeptide (aa 112–607 of ORF2) of genotype 1 (Sar-55) or 3 (Meng strain of swine HEV) was used as the solid antigen, showed 98 % agreement in detecting anti-HEV IgG in serial serum samples from two chimpanzees and six rhesus monkeys experimentally infected with HEV, 93 % agreement in measuring anti-HEV in 792 swine sera, and 99 % agreement in testing 882 human sera, demonstrating that the antigens derived from human and swine HEV contain the same immunodominant epitopes [23]. A comparison of two assays based on recombinant ORF2 polypeptides derived from genotype 1 and genotype 4, respectively, also displayed 98.7 % concordance in detecting anti-HEV IgM and 97.7 % concordance in measuring anti-HEV IgG [5], suggesting that the epitopes of these two genotypes are highly similar. The polypeptide including aa 458–607 of ORF2 (genotype 1, Sar-55) detected anti-HEV IgG in a similar pattern in monkeys experimentally infected with HEV of all four genotypes (Fig. 7.3; [102]). These results show that anti-HEV IgG against one genotype cross-reacts with any other HEV genotype.

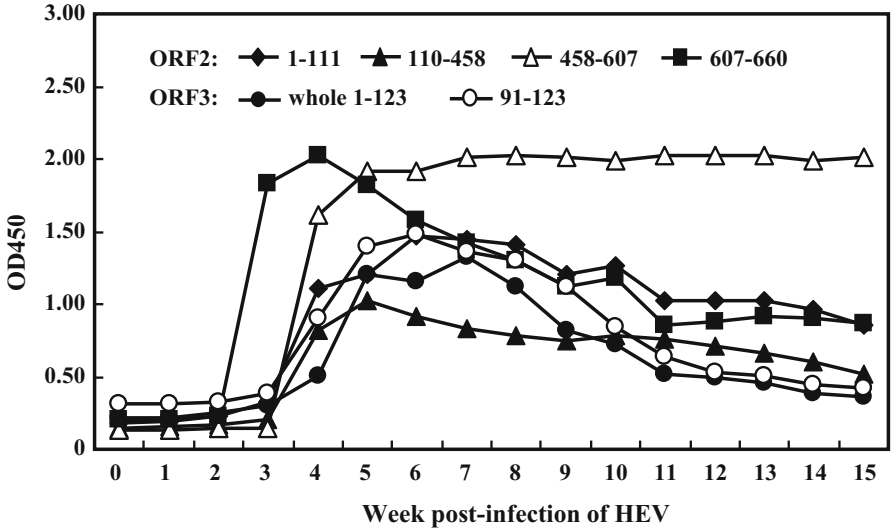

Fig. 7.7 Time course of anti-HEV responses to different subregions of ORF2 and ORF3 (genotype 1) in monkeys experimentally infected with HEV genotype 1. The sera were diluted 100-fold in 5 % skim milk and then tested for anti-HEV with ELISA based on various polypeptides indicated in the figure. The numbers represent the first and last amino acid of each polypeptide, respectively

Monkeys immunized with the ORF2 protein of HEV genotype 4 were protected against challenge with a genotype 1 virus [36]. Pigs that had recovered from infection with HEV genotype 3 were protected against subsequent challenge with HEV genotype 4 [73]. A hepatitis E vaccine composed of the ORF2 protein of HEV genotype 1 was effective in a clinical trial conducted in Jiangsu Province, China [104], where the epidemic HEV isolate is genotype 4, because all 210 viral isolates collected from HEV RNA-positive patients in the period from January 1, 2001 to April 30, 2011 were genotype 4 [18]. These data indicate that individuals immunized with a vaccine derived from genotype 1 may be protected against infection by genotype 4. Therefore, the IgG antibody responses to the immunodominant antigenic determinants of HEV ORF2 are cross protective against different human HEV genotypes.

7.2.2.6 Antibody Responses to Other Antigenic Determinants

In addition to the anti-HEV antibody responses defined by the immunodominant polypeptide derived from HEV ORF2, the antibody responses to other regions of the ORF2 protein and to the ORF3 protein have also been studied in experimentally infected monkeys [103]. As shown in Fig. 7.7, the anti-HEV responses directed against ORF2 polypeptides containing aa 1–111, 110–458, and aa 607–660 were much weaker than the response to the immunodominant polypeptide (aa 458–607 of

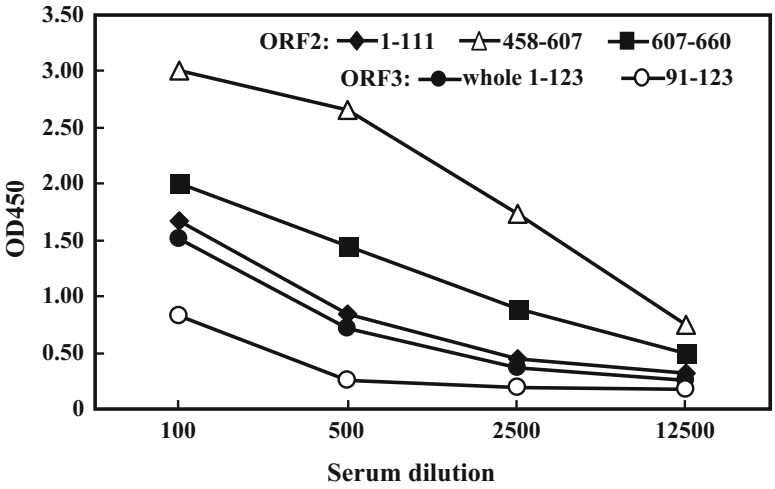

Fig. 7.8 Titration of anti-HEV IgG in a patient with acute hepatitis E. The serum was serially diluted in 5 % skim milk and then tested for anti-HEV with ELISA based on various polypeptides indicated in the figure. The numbers indicate the first and last amino acid of each polypeptide, respectively

ORF2 protein), and the antibody responses appeared to turn over much more rapidly. Similarly, the antibody responses to the ORF3 polypeptides were also much weaker and were maintained for a relatively short period of time.

The anti-HEV antibody responses to the abovementioned polypeptides were also relatively weak in naturally HEV-infected humans, as shown in Fig. 7.8. In an outbreak of hepatitis E among school children, all 19 (100 %) children tested were positive for anti-HEV IgM to the ORF2 protein, whereas only 11 (57.9 %) and 10 (52.6 %) children were positive for IgM to the ORF1 and ORF3 proteins, respectively [7]. Together with the findings in experimentally infected animals, this demonstrates that the ORF1 protein, the ORF2 protein regions other than aa 459–607, and the ORF3 protein do not contain immunodominant antigenic determinants. Therefore, assays used to detect anti-HEV that are based on these polypeptides cannot accurately reflect the true anti-HEV immune responses. This may explain why the prevalence of anti-HEV in earlier times was much lower than expected, because the first assay used to detect anti-HEV was based on the 44 amino acid residues located at the C-terminus of the ORF2 protein and the last 33 residues at the C-terminus of the ORF3 protein [20], both of which contain no immunodominant sites for HEV. A later study confirmed that the assays based on synthetic peptides (regardless of whether they were derived from the ORF2 or ORF3 protein) that contained no immunodominant region were inefficient in detecting anti-HEV [57]. This may also explain why in earlier times, it was postulated that anti-HEV IgG did not persist for a long period and disappeared soon after acute infection [10].

7.2.2.7 Rare Events in the Antibody Responses to HEV Infection

In rare cases, symptomatic patients with hepatitis E may be negative for both anti-HEV IgM and IgG but positive for serum HEV RNA [12]. It is unclear whether this negativity for anti-HEV IgM and IgG was true or was caused by low sensitivity assays because the reagents used in the assays did not contain the immunodominant polypeptide [12]. However, serum HEV RNA has been detected in the absence of anti-HEV IgM and IgG in asymptomatic blood donors and normal individuals, although the rates were very low (0.01–0.035 %) [24, 34]. However, those HEV RNA-positive donors had seroconverted to anti-HEV IgM and/or IgG in follow-up tests after 2–11 weeks [24]. Therefore, those serum HEV RNA-positive donors without serological markers were actually in the incubation period.

Anti-HEV IgM usually disappears after several months. However, under rare circumstances, anti-HEV IgM may last for several years after the virus is cleared. A woman who was positive for anti-HEV IgM and IgG during midterm pregnancy remained positive for both anti-HEV IgM and IgG in the following 6 years postpartum, whereas she was negative for HEV RNA during both the pregnancy and the postpartum period. IgG and IgM positivity was demonstrated with two ELISAs and Western blotting [32], so they were unlikely to represent false positive results. By contrast, some patients with acute hepatitis E are negative for anti-HEV IgM [85].

7.2.2.8 Significance of Anti-HEV Responses

The early development of anti-HEV IgM and IgG responses has important clinical implications for the diagnosis of the disease, the elimination of the virus, and the prevention of reinfection. Because its period of persistence is short, anti-HEV IgM has been used as a serological marker of acute infection. When symptomatic patients with hepatitis E first visit a physician, usually several days after the onset of the disease, more than 95 % are positive for both anti-HEV IgM and IgG [12, 75]. This indicates that the specific IgG antibody response to HEV infection may develop as early as the late incubation period or in a very early phase of the disease. This is significant in defining an acute infection, together with the detection of anti-HEV IgM. When an individual is anti-HEV IgM positive but anti-HEV IgG negative, IgM positivity may be true or false, so a follow-up test after 1–2 weeks is critical. If anti-HEV IgG becomes positive, the IgM positivity in the first test is true; if anti-HEV IgG is still negative, the IgM positivity is very probably false. Because the false positive rate is relatively high when based on the detection of specific IgM, testing for anti-HEV IgM alone may sometimes leads to misdiagnosis [2, 25, 45, 60]. Therefore, the simultaneous detection of anti-HEV IgM and IgG, and retesting after 1–2 weeks if necessary, will allow the more accurate and reliable diagnosis of acute infection.

Because anti-HEV IgG antibodies have potent neutralizing activity, the early development of a specific IgG response plays an important role in eliminating HEV, in concert with the innate immune and adaptive cellular immune responses. Because

the neutralization epitopes on the HEV ORF2 protein are also the immunodominant antigenic determinants [103] and anti-HEV IgG persists for a long time, the IgG response, induced by either natural infection or vaccination, may protect against subsequent exposure to HEV.

7.3 Adaptive T-Cell Immune Response to HEV

Compared with the extensive study of the specific antibody immune responses to HEV infection, the specific cellular immune response directed against HEV has been investigated in less detail in both experimentally infected animals and naturally infected humans. However, recent efforts to understand the cellular immune response to HEV infection have provided important insights in this complex process.

Potent and multispecific $CD4^+$ and $CD8^+$ T-cell responses generally play critical roles in the clearance of viral infections. $CD4^+$ T cells efficiently produce cytokines, which are required for the development of effector $CD8^+$ T cells and antibody production by B cells. This general rule may also be true in acute hepatitis E. The development of potent anti-HEV antibody responses during the early course of HEV infection suggests that specific T cells are activated to facilitate the production of specific antibodies. Several research groups have analyzed the HEV-specific cellular immune responses of PBMCs from patients with hepatitis E to identify potential T-cell epitopes in the ORF2 and ORF3 proteins with lymphocyte proliferation assays. Overall, specific T-cell responses to the ORF2 protein have been demonstrated in most reported studies, whereas the detection of specific T-cell responses to the ORF3 protein appear to vary across different studies.

As a common feature, the proportions of monocytes and macrophages in the peripheral blood of patients with acute hepatitis E are higher than those in healthy controls [74]. Srivastava et al. showed that, compared with healthy controls who were not exposed to HEV (anti-HEV IgG negative), patients with acute hepatitis E only had an increased proportion of $CD4^+$ cells (30.1 % [18.0–37.0 %] vs 35.4 % [range, 21.4–51.7 %], respectively, $P < 0.01$) but a similar proportion of $CD8^+$ cells (31.1 % [18.4–58.9 %] vs 30.1 % [15.5–38.7 %], respectively, $P > 0.05$) in their peripheral blood [80]. However, Husain et al. reported that the proportion of total $CD8^+$ cells was higher in patients with acute hepatitis E than in healthy controls (47.4 vs 37.7 %, respectively, $P < 0.01$) and the proportion of total $CD4^+$ cells in the patients was also increased but not statistically significantly (49.2 vs 38.9 %, respectively, $P > 0.05$) [37]. Although these studies did not analyze the HEV-specific $CD4^+$ and $CD8^+$ cells, it is reasonable to assume that a proportion of these cells would be specific for HEV.

It has been reported that when PBMCs from acute hepatitis E patients were stimulated with a recombinant ORF2 protein (55 kDa) expressed in insect cells, the total $CD4^+$ population was expanded, but the proportions of $CD4^+/CD69^+$ and $CD8^+/CD69^+$ cells producing helper T-cell type 1 cytokines (IFN-γ and TNF-α) or helper

T-cell type 2 cytokine (IL-4) were unchanged [80]. This expansion of $CD4^+$ cells was considered to have resulted from an increase in natural killer T (NKT) cells, because the IFN-γ levels in the supernatants and the IFN-γ mRNA levels in ORF2-stimulated PBMCs were elevated, whereas the levels of IL-2 and TNF-α remained unchanged [80]. These data suggest that there is no detectable ORF2-specific immune activation of $CD4^+$ or $CD8^+$ cells in the circulations of patients with acute hepatitis E. The increased production of IFN-γ, without detectable specific $CD8^+$ cell responses, indicates that nonspecific innate mechanisms (NK or NKT cells) are involved in the pathogenesis of hepatitis E and the clearance of HEV. However, the lack of detectable HEV-specific cytokine-producing $CD8^+$ cells in the peripheral circulation does not rule out the participation of specific cytotoxic T cells, because the immune response predominantly occurs in the liver and that study only used expressed ORF2 protein, rather than synthetic ORF2 peptides, to stimulate the PBMCs in the assays [80]. Furthermore, the liver contains a large number of $CD8^+$ cells. The failure to detect specific $CD8^+$ cells in the circulation does not necessarily indicate that there is no specific $CD8^+$ cell response in the liver.

Other research groups have performed similar studies using ORF2 polypeptides or synthetic ORF2 peptides. Taherkhani et al. cultured PBMCs from recovered hepatitis E patients and uninfected individuals (controls) in the presence of the truncated ORF2 protein (aa 112–660). The IFN-γ levels in the supernatants of the cultured PBMCs from the recovered patients were much higher than those in the supernatants from the controls (143.40 ± 52.33 vs 17.12 ± 6.93, respectively, $P < 0.001$). Consistent with this finding, IFN-γ ELISPOT responses after ORF2 protein stimulation in the HEV-recovered and control groups were 65.92 ± 58.99 and 4.39 ± 5.51 spot-forming units per 10^5 cells, respectively ($P < 0.001$) [83]. Using chimpanzees as an animal model, Shata et al. demonstrated that the median number of HEV-specific IFN-γ-secreting cells in HEV-infected chimpanzees in the presence of IL-7 and IL-15 was much higher than that in the uninfected chimpanzees (206.5 vs 12 per 10^6 PBMCs, respectively, $P < 0.001$). Similarly, the median number of HEV-specific IFN-γ-secreting cells in anti-HEV-positive subjects after stimulation with a synthetic ORF2 peptide was significantly higher than in the anti-HEV-negative controls (373 vs 1 per 10^6 PBMCs, respectively $P = 0.001$) [76]. These results demonstrate the presence of specific T-cell responses to HEV infection in both experimentally infected chimpanzees and naturally infected humans.

Because there are four genotypes of HEV, the cross genotype-specific T-cell responses in patients with acute hepatitis E were investigated [29]. The PBMCs of patients infected with either genotype 3 or genotype 1 were cultured separately in the presence of 15-mer five-residue-overlapping peptides covering the full length of the ORF2 protein or the ORF3 protein derived from genotype 1 and 3, respectively. Broad functional HEV-specific $CD4^+$ and $CD8^+$ T-cell responses were detected in the patients. The proliferation of $CD8^+$ T cells was predominant in the response to overlapping peptides spanning aa 131–265 of the ORF2 protein, whereas the proliferation of $CD4^+$ T cells showed diversified. Interestingly, the HEV-specific $CD8^+$ T cells predominantly produced only IFN-γ, whereas the $CD4^+$ T cells produced both IFN-γ and TNF. The memory HEV-specific T-cell responses persisted for at least

>1.5 years after infection. Similar to results reported previously [3], the responses to the ORF3 peptides were weaker than those to the ORF2 peptides. The findings not only demonstrate that the specific T-cell response to HEV genotype 3 is generally consistent with the results for endemic regions where HEV genotype 1 is dominant [3, 37] but also that the T-cell responses to HEV are cross genotype specific.

It has recently been demonstrated that HEV infection is chronic in immunocompromised individuals [19, 27, 40]. The specific T-cell responses in organ transplant recipients infected with HEV were investigated [81]. Compared with recovered healthy controls, the recovered patients who had also undergone organ transplantation had less potent and multispecific HEV-specific T-cell responses. Moreover, a small proportion of organ transplant patients with chronic hepatitis E had much weaker HEV-specific $CD4^+$ and $CD8^+$ T-cell responses. When the chronically infected patients recovered, their specific T-cell responses became detectable. Furthermore, the HEV-specific T-cell responses were restored in vitro by blocking the PD-1 or CTLA-4 pathways. Strong and significant levels of IFN-γ were also observed in both the $CD4^+$ and $CD8^+$ T cells of seropositive healthy subjects (anti-HEV IgG positive) in response to some ORF2 peptides, whereas the intracellular IFN-γ responses were much weaker in the transplanted patients with chronic or resolved HEV infections [81]. These findings suggest that chronic HEV infection is associated with impaired HEV-specific T-cell responses and that enhancing the adaptive cellular immunity against HEV might prevent persistent HEV infections. In other words, the recovery from HEV infection appears to correlate with the specific T-cell responses.

To identify potential T-cell epitopes in the ORF2 and ORF3 proteins, several groups have analyzed the HEV-specific cellular immune responses of PBMCs from patients with hepatitis E using lymphocyte proliferation assays. Aggarwal et al. mapped $CD4^+$ T-cell epitopes in the ORF2 and ORF3 proteins using 20-mer synthetic ORF2 and ORF3 peptides that overlapped by 12 amino acids and found that patients with acute hepatitis E mounted a proliferative T-cell immune response to the ORF2 protein. The $CD4^+$ T-cell epitopes are located in the regions covering aa 73–156, 289–444, and 505–588 of the ORF2 protein, but no consistent pattern of reactivity to individual peptides was observed. There was no significant lymphoproliferative response to synthetic ORF3 peptides [3]. The frequencies of IFN-γ-secreting cells among PBMCs after stimulation with ORF2 or the 15-mer synthetic peptide pool (aa 181–249, 301–369, 361–429, and 421–489 of the ORF2 protein) were significantly higher than in the controls. The frequencies of IFN-γ-secreting cells in response to stimulation with the ORF3 protein, but not in response to stimulation with any of the 15-mer overlapping peptides covering ORF3, were significantly higher than those in the controls [37]. Although HEV-specific $CD4^+$ and $CD8^+$ cell responses have been clearly demonstrated in the circulations and livers of patients with hepatitis E, T-cell epitope mapping showed no distinct dominant domains in the ORF2 or ORF3 protein [3, 37, 76, 80, 81]. Therefore, the T-cell responses appear to be multispecific to the ORF2 and ORF3 proteins.

7.4 Conclusion

Comprehensive studies of the antibody immune responses to HEV infection in experimentally infected nonhuman primates and naturally infected humans, based on the correct identification of the major antigenic determinants of the ORF2 protein, have clarified the profiles of the anti-HEV IgM and IgG responses to acute HEV infection. Anti-HEV IgM and IgG develop in the very early phase of the disease, and IgM usually disappears within 1–3 months, whereas IgG persists for a long time. Anti-HEV IgG has neutralizing activity. Recent investigations have shown that the activation of the innate immune responses and the development of multispecific $CD4^+$ and $CD8^+$ T-cell responses to ORF2 play critical roles in the clearance of the virus.

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Chapter 8
HEV Cell Culture

Feng Zhang and Youchun Wang

Abstract Cell culture is an important research method in virology. Although many attempts were tried to culture HEV in cells, only two cell culture systems were considered to have high enough efficient for usage. Concentration of virus stocks, host cells, and medium components affect the culture efficient, and the genetic mutations during HEV passage were found to be associated with the increased virulence in cell culture. As an alternative method for traditional cell culture, the infectious cDNA clones were constructed. The viral thermal stability, factors that impact the host range, posttranslation of viral proteins and function of different viral protein were studied using the infectious cDNA clones. The studies on progeny virus showed that the virus secreted from host cells have an envelope, and its formation was associated with pORF3. This result explained the phenomenon that virus could infect hosts cells in the presence of anti-HEV antibodies.

Keywords Cell culture • Passage • Cytopathetic effect • Tissue culture infective dose

Abbreviations

Asn	Asparagine
CPE	Cytopathetic effect
DMEM	Dulbecco's modified eagle medium
FFU	Fluorescence Forming Unit
HEV	Hepatitis E virus
Hel	Helicase

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HIV	Human immunodeficiency virus
HVR	Hypervariable region
JR	Joint region
mAb	Monoclonal antibody
MEM	Minimum essential medium
Met	Methyl-transferase
M199	Medium 199
NCRs	Non-coding regions
ORF	Open reading frame
Pro	Proline
pORFn	ORFn protein
RdRp	RNA dependent RNA polymerase
Ser	Serine
TCID	Tissue culture infective dose
Thr	Threonine
WB	Western blot

8.1 Introduction

HEV cell cultures were tried in primary hepatocytes and passaged cell lines [2, 15–17, 23, 27, 46] using different HEV strains for decades. However, no stability culture systems had been developed, and features and performance of these cultures were not deeply studied, and therefore, they did not get a further application. Recently, two HEV cell culture systems were developed, which were considered to have enough efficiency for usage, including the culture system in PLC/PRF/5 and A549 cells established by Tanaka et al. and the culture system in HepG2/C3A cells established by Shukla et al. In 2007, Tanaka et al. proved that HEV 3 could be cultured efficiently in PLC/PRF/5 (a hepatoma cell) and A549 cells (a lung cancer cell), and subsequent studies showed that HEV 4 could also be cultured in these two cells [47, 48]. Subsequently, in 2011, Shukla et al. proved that HEV 3 could be cultured in HepG2/C3A cells (a hepatoma cell) [38]. Although other researchers proved that IBRS-2 cells could support HEV 3 replication in vitro, but efficiency of this culture was not fully described and no further relevant articles of these culture were published [53].

While HEV cell culture can imitate HEV infection in vivo, but difficulties in viral genome engineering limited its usage in function studies of viral genes. To overcome this obstacle, HEV infectious cDNA clones were developed to generate genome modified progeny virus. By studies on the phenotype changing of genome modified progeny virus in cell culture, functions of viral genes and proteins could be inferred. Therefore, in HEV research, cell cultures and infectious cDNA clones are complementary, and these two systems will be discussed jointly in this review.

8.2 HEV Cell Culture

8.2.1 Overview of HEV Cell Culture

In HEV cell culture systems established by Tanaka et al. and Shukla et al., the positive conversion time and concentration of progeny virus were positive associated with the virus amounts inoculated. While 8.6×10^5 copies/well of JE03-1760F strain were inoculated with PLC/PRF/5 cells, progeny virus could be detected 12 days after inoculation, and the highest progeny virus concentration in supernatant reached about 10^8 copies/ml. When virus inoculum concentration decreased to 2.4×10^4 copies/well, positive conversion time prolonged to 24 days, and the highest progeny virus concentration was about 10^4 copies/ml [47]. In the studies of Shukla et al., positive conversion time for Kernow-C1 strain-inoculated HepG2/C3A was 8–9 days after inoculation [38].

With passage number increasing, HEV gradually adapted cell culture and virulence enhanced. In studies of Shukla et al., 1 FFU of passage 6 corresponded to 4.5×10^2 copies of HEV RNA, while it was 1.5×10^4 for primary virus. In the same time, the highest HEV RNA concentration in supernatant increased from 1.0×10^7 copies/ml for primary virus to about 5×10^8 copies/ml for passage 6. There was a ~30-folds increment in virulence. But this phenomenon was not observed in the studies of Tanaka et al. [38, 47]; it will be discussed in following chapters.

There was a controversy for the CPEs of HEV in cell culture. In the culture of 87A and G93 strains in 2BS cells, CPE of "cell rounding, expansion and fragmentation" was observed by Huang et al.[15, 17]. And in studies of Zhang et al., CPEs, that described as "destruction of cell monolayer, cell aggregation, rounding and finally dissolution," emerged after 8–12 passages in IBRS-2 cells and 22–24 passages in A549 cells. CPEs enhanced with increasing passages [53]. However, Tanaka et al. and Shukla et al. reported no significant CPEs. As of June 2012, Tanaka passed HEV 3 and 4 in cell culture for 53 and 33 passages, respectively, and no significant CPEs were observed [32]; the results were same as our study [54].

8.2.2 Sources of Primary Virus Stocks

As a starting material, virus stock was one of the key factors in determining whether the virus can be cultured in cells successfully. Except HEV 2, HEV 1, 3, and 4 had been reported to be cultured successfully. Primary virus stocks for initial culture in these reports have certain particularity [15, 38, 47]. In 1986, 87A strain of genotype 1 was cultured by Huang et al. in 2BS cells. 87A strain was isolated from feces of an acute viral hepatitis patient from Xinjiang province of China during a pandemic HEV outbreak. From the epidemiological characteristics of the virus, this HEV strain should have a high virulence to cause a pandemic outbreak. And the virus

concentration in patient feces from pandemic outbreak should be higher than the sporadic cases. Limited by conditions, Huang et al. didn't analyze the HEV RNA concentration of virus stock. But in the discussion, the author thought that using virus stock from acute phase feces was a key factor for successful culture. In reports of Tanaka et al., HE-JF5/15F strain of genotype 4 and JE03-1760F strain of genotype 3 could be cultured in vitro. The sources of these two HEV strains had particularity too. HE-JF5/15F strain was obtained from a 58-year-old patient with fulminate hepatitis, and HEV RNA concentration in virus stock reached 1.3×10^7 copies/ml [48]. JE03-1760F strain was from a 67-year-old HEV infection patient with chronic renal failure requiring regular dialysis, and the HEV RNA concentration reached 2.0×10^7 copies/ml. In this case, 121 days after onset of symptom, HEV could still be detected in feces, while time for positivity of HEV RNA in feces rarely exceed 1 month after symptom onset [41]. Compared with JE03-1760F, Kernow-C1 strains (HEV 3) cultured by Shukla et al. in 2011 reached 10^{10} copies/g (feces), it was extracted from a patient that HIV-1 and HEV coinfected for 2 years [5]. In above successful culture reports, there were two common things: the primary virus stocks were from patients with severe diseases or impaired immunity, and the HEV RNA concentration of virus stocks were very high.

High concentrations of virus stocks could contain a certain population of variants, which increased the possibility of getting an HEV strain that could be cultured in vitro. In cell culture, Shukla et al. sequenced and compared the genomic RNA of primary and passage 6 of Kernow-C1 strain. In the sixth passage, it was found that an insertion of 58 amino acid from swine S17 ribosomal protein occurred in ORF1 HVR domain. Swine S17 ribosomal protein is a highly conserved protein, indicated that the insertion occurred in the natural course of infection [38]. In primary virus, this insertion mutation could also be detected, but not dominant. Researchers amplified HVR genes of various passages, sequenced a certain number of amplification clones, and calculated the proportion of insertion mutated clones in each passage. From primary to passage 6, the proportions of insertion mutant clones were 0/120, 2/11, 9/10, 8/8, 8/10, 8/8, and 10/11, respectively. This result showed that the mutant virus had replication advantage and got a population advantage by several passages in cell culture [39]. Therefore, the concentrations and the proportions of variants contained in the virus stocks is an important factor that affected the success of HEV cell culture.

Then, the next question is what is the concentration that needed to establish an infection? Takahashi et al. cultured HEV from deer liver or swine liver sold as food, results showed that when the HEV RNA concentration of inoculums was lower than 2.0×10^4 copies/well, infection could not be established [44]. With a same result, Shukla et al. titered primary Kernow-C1 strains, One FFU corresponded a viral copy number of about 1.5×10^4 [38]. Calculated from above results, an HEV inoculums greater than $\sim 1.5 \times 10^4$ copies/well was needed to establish infection in cell culture.

8.2.3 Host Cells

Selection of host cells is another key factor for the success of virus cell culture. HEV can replicate in various organs and tissues besides liver, such as lymph node, spleen, intestine, placenta, etc. [3, 49]. Theoretically, many cell lines can support HEV culture. In earlier studies, 2BS (human embryonic lung diploid cells), A549, and PLC/PRF/5 cells can support HEV cell culture.

As a zoonotic pathogen, host range of HEV 3 is quite extensive. Shukla et al. reported absorption results in various cell lines of Kernow-C1 stain (HEV 3). Results showed that HEV could absorb and penetrate HepG2/C3A (human hepatoma cells), Huh-7 (human hepatoma cells), PLC/PRF/5 (human hepatoma cells), Caco-2 (human colon cancer cells), A549 (human lung cancer cells), three pig kidney cells, deer liver cells, chicken liver cells, monkey, cow, mouse, deer, chicken, cat, dog, and rabbit cells (the author did not provide specific name). The author did not state whether HEV can replicate and produce progeny virus in these cells (except for HepG2/C3A), but the sensitivity to HEV was different in different cells, as FFU formed by Kernow-C1 strain in HepG2/C3A cells were about 7.5-fold higher than PLC/PRF/5, A549, and Caco-2 cells [38]. From the author's other literatures, it can be summarized that at least HepG2/C3A, Huh-7, and Caco-2 cells could support HEV 3 replication [9]. In another literature, Tanaka et al. attempted to culture JE03-1760F strains (HEV 3) in 21 cell lines, including HepG2, HuH7, PLC/PRF/5, and A549 cells. Results showed that JE03-1760F strain could only replicate in PLC/PRF/5 and A549 cells [47]. The contradictory results might be caused by two reasons: the different host cell ranges of different strains, and/or the differences in cell line phenotypes under different culture conditions. The mechanism needs further study.

HEV 1 can only infect human, but in vitro cell culture studies showed that HEV 1 could infect nonhuman cells. Emerson et al. transfected 11 cell lines with HEV 1 infectious cDNA clones, including Huh-7, Caco-2, HepG2/C3A, PLC/PRF/5, BRL3A (rat hepatoma cells), Hepa 1-6(human hepatoma cells), PK (porcine kidney cells), ST (swine testis cells), A549, HS27 (human skin fibroblasts) and Vero (monkey kidney cells). Expression of pORF2 and pORF3 was detected in all above cells, but only PLC/PRF/5 and Huh-7 cells could produce infectious progeny virus [7]. Infecting HepG2, LLC-PK1 (porcine kidney cells) and deer liver cells with HEV 1 and 3, respectively. 3 days after infection, fluorescent stained cells could be found in HEV 1 or 3 infected HepG2 and LLC-PK1 cells. But there was difference in infection efficiency. HEV 1 had a higher infection efficiency in HepG2 than LLC-PK1, while HEV 3 showed a contrary result [38]. This phenomenon might be a reflection of different host ranges of different HEV genotypes.

Even the different individuals of the same cell strains, their sensitivity to HEV infection was different. S10-3, a subclone of Huh-7 established by Emerson et al., showed an infection ratio of 50 %, while the ratio was 10 % in Huh-7 [7, 9]. This phenomenon showed a possibility to improve cell culture efficiency by subcloning low susceptible cell lines.

8.2.4 Impact of Medium Components on HEV Culture

Medium components can affect the sensitivity of cells to HEV infection. In 1992, Huang et al. developed HEV 1 cell culture in 2BS with MEM containing 30 mM $MgCl_2$. The author did not describe the influence of $MgCl_2$ on HEV culture, but in the discussion, the author thought the addition of $MgCl_2$ was one of the key factors for his successful HEV cell culture [15]. Additionally, author replaced MEM with DMEM/M199 equally mixed medium and got a successful cell culture in A549, 2BS, and LLC-MK2 [17]. Subsequently, DMEM/M199 medium containing $MgCl_2$ was used as maintaining medium for PLC/PRF/5 and A549 in the HEV cell culture studies of Tanaka et al. [47]. Above two authors did not explain reasons for using of DMEM/M199 mixed medium. In the results of our experiments, it was showed that using of DMEM/M199 medium could advance the time point of progeny virus that appears in culture supernatant than MEM [54]. The impact of medium components on HEV cell culture might be associated with the different status and gene expressions profiles of cells caused by medium.

8.3 Genetic Mutation During HEV Passage

Adaptive mutations of HEV genome RNA can occur during passage in cell culture, and these mutations cause HEV more suitable for infection and replication in cell culture conditions. Studies on different passages of JE03-1760F strain showed that higher passages displayed a faster progeny virus production and higher RNA concentration in culture supernatant than lower passages. The progeny virus could be detected in supernatant at 12–16 days after inoculation for passages 0–5 (passage 1 was 36 days after inoculation), while it was 6–10 days for passages 6–10. Correspondingly, RNA concentration in supernatant raised about tenfolds higher for passages 6–10 than passages 0–5. For infectivity test, cells were infected with primary, passage 5, and passage 13 at the concentration of 3.0×10^3, 1.0×10^3, 3.0×10^2, and 1.0×10^2 copies/well, respectively. Positive ratios for primary HEV inoculated wells were 4/5, 0/5, 0/5, 0/5, while these ratios for passage 5 and passage 13 were 5/5, 5/5, 1/5, 0/5 and 5/5, 5/5, 5/5, 1/5. Correspondingly, when primary virus was passaged to passage 13, the RNA concentration of one TCID50 decreased from 2×10^3 copies to 1.4×10^2 copies. All above descriptions showed that HEV infectivity increased during passages. Compared with primary virus, progeny virus of primary and passages 5, 10, and 13 contained 1, 9, 18, and 19 nucleotide mutations, respectively. An U-A mutation on 22 nt was presented in progeny virus of primary and passages 5 and 10, while a reverse mutation occurred in passage 13. These results indicated that the mutations occurred in the passage process acted as a screening factor on primary virus [26].

Shukla et al. evaluated the effect of mutations during passage on pORF2 expression efficiency using infectious cDNA clone. Depending on the location, the mutations were divided into 3 groups, including 3′-UTR and the 3′-terminal of ORF2, 3′-terminal of ORF1 and ORF2/ORF3 overlapping area, and 5′-terminal of ORF1. Three groups were introduced into infectious HEV cDNA clones, respectively. Compared with the wild-type clones, pORF2 expression efficiency of these three mutant clones elevated, which indicated that these mutant clones were more suitable for replication in cell culture [39].

8.4 HEV Infectious cDNA Clone

In the early stage of HEV studies, HEV was hard to culture in vitro, and researchers performed genetic studies on HEV by constructing of HEV infectious cDNA clones (hereinafter referred to as "infectious clone" or "clone"). Briefly, the complete cDNA of HEV genome RNA with T7 promoter at 5′ end and restriction endonuclease site for linage at 3′ was amplified and connected into a plasmid. In application, the full HEV genome RNA transcript was used to transfect cells or intrahepatic injection to get progeny virus (Fig. 8.1).

By observing the expression of viral proteins or infection symptom of animals, the function of HEV genes and proteins were analyzed. Combined with cell culture systems, the progeny virus produced by infectious clones could be cultured, and further information could be acquired [12, 19, 28, 29, 33]. There was a great advantage for infectious clones in genome engineering. Using infectious clones, researchers constructed a variety of mutant HEV virus for viral protein function studies, viral genome structure studies and neutralization assays. These infectious clones played an important role in virology studies of HEV. In the following chapters, it will be introduced in combination with HEV cell culture systems.

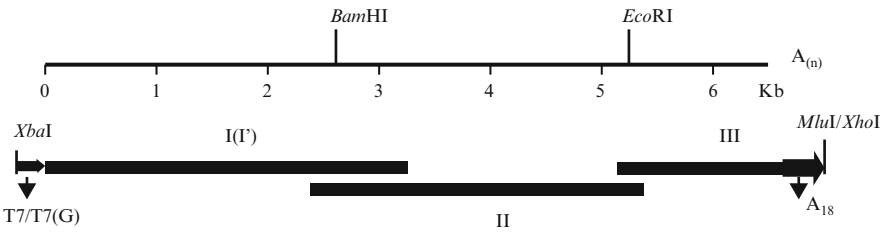

Fig. 8.1 Construction of full-length cDNA clones of HEV. BamHI and EcoRI are unique restriction sites naturally present in the HEV genome and were utilized to construct the full-length cDNA clones

8.5 Applications of Cell Culture

8.5.1 Viral Thermal Stability Studies

In viral thermal stability studies, there were differences between different genotypes and different strains of HEV. Mex14 strain (genotype 2) had a higher tolerance than Akluj strain (genotype 1) and Sar55 strain (genotype 1). And in genotype 1, Sar55 strain has a higher tolerance than Akluj strain. Mex14 strain could not be inactivated by 56 °C heat treatment for 1 hour, and about 80 % could be inactivated by 60 °C treatment. For Sar55 strain, about 50 % could be inactivated by 56 °C treatment, and about 96 % could be inactivated by 60 °C treatment, while for Akluj strain, about 50 % could be inactivated by 45–50 °C treatment, and 100 % could be inactivated by 60 °C treatment [8].

8.5.2 HEV Genome Structure and Function Analysis

5′ end and 3′ end of HEV genomic RNA contain 26 nt and 65 nt NCR, respectively, and these two regions play a key role in viral replication and infection (Fig. 8.2). m7G cap at 5′ end that confirmed by immuno-capture methods was indispensable for HEV infection [22, 52]. It is important for the stability and translation initiation of viral RNA. In infectious clone studies, lack of cap caused significant expression reduction of viral protein and infection deficiency of progeny virus. And in animal studies, HEV genome RNA transcripts without m7G cap could not cause infection by intrahepatic injection [6, 7]. It was indicated that lack of cap leaded to the decrease in HEV RNA stability and binding capacity to translation initiation complexes and resulted in the decrease of viral protein expression efficiency.

In negative strand synthesis, the RdRp binds HEV positive strand RNA through 3′ end NCR [1]. In HEV 1, 2, and 3, the 3′ end NCR forms a same secondary structure with different nucleotides; at the same time, there were differences in the pORF1 amino acid sequence of three genotypes. By above clues, it was inferred that the amino acid sequence differences were corresponded with the nucleotide sequence differences in 3′ end NCRs among different genotypes. In HEV 1 studies

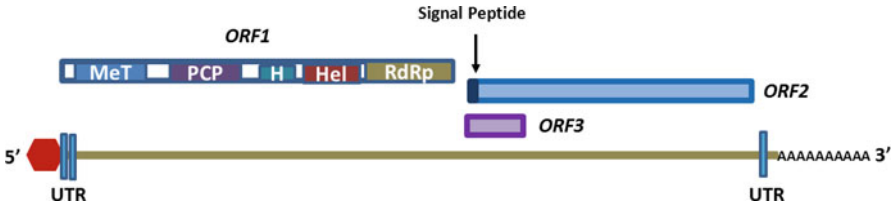

Fig. 8.2 Genome structure of HEV

by infectious clones, a G to T mutation at 7106 nt of Sar55 strain (in Sar55 strain, ORF2 ended at 7125 nt) caused a significant infectivity decrease in animals. RNA secondary structure prediction showed that the mutation destroyed the neck-ring structure of 7082–7161 nt. When the former C that paired with G was mutated to A to form a new pair with the mutated A, the virulence of the virus recovered [6]. Further substitute 3′ NCR of genotype 1 with genotypes 2 and 3 to generate chimerical infectious clones that have same neck-ring structure but different nucleotide sequence at 3′ end, there were no significant changes observed. These results indicated that the binding of RdRp and 3′ end NCR was structure but not sequence dependent [12].

Experimental animal studies showed that HEV genomic/sub-genomic RNA had three fragments, including 7.2 kb, 3.7 kb, and 2.1 kb [45]. But in cell culture studies, only 7.2 kb and 2.2 kb fragments were found. After the ORF1 was frameshift mutated, 2.2 kb fragment could not be detected in infectious clone-transfected cells, which indicated the 2.2 kb fragment was synthesized by ORF1 coding RdRp [13]. RNA sequence analysis showed that the 2.2 kb fragment of Sar55 strain started at 5122 nt. It located at the downstream of traditionally considered initiation codon of ORF3 (5104 nt) and also located downstream of the second ATG codon of traditionally considered ORF3. To analyze the start codon of ORF3, a T was inserted between the second and third ATG codon of ORF3 to generate a frameshift mutation. Transfection result showed that pORF3 expression was not affected. When the third ATG of ORF3 was mutated to GCA, pORF3 expression was disrupted. Thus, it was inferred that the start codon of ORF3 was located at 5131 nt but not traditionally considered 5104 nt in Sar55 strain. Using G418 resistance gene (*neo*) as screen marker, when *neo* was fused at downstream of ORF2 or ORF3, Huh-7 cells transfected with chimeric clones could grow in medium containing fatal dose of G418. By this result, it could be inferred that pORF2 and pORF3 were all translated with the 2.2 kb transcript [13, 20]. Thus, it can be inferred that 7.2 kb RNA is responsible for the synthesis pORF1, and 2.2 kb RNA is responsible for the synthesis pORF3 and pORF2.

8.5.3 pORF1 Posttranslational Processing and HVR Function Analysis

HepG2 cells were transfected with infectious clone of HEV 1, and ^{35}S-methionine-labeled viral proteins in cell lysates were affinity purified with anti-pORF2, pORF3, methyl-transferase (Met), helicase (Hel) and RdRp antibody. Besides pORF2 (~72 kDa) and pORF3 (~13.5 kDa), individual Met (~35 kDa), Hel (~38 kDa) and RdRp (~36 kDa) could be detected too. Met, Hel, and RdRp appeared at different time points after transfection. In the early stage after transfection, Met and Hel appeared, while RdRp appeared at the late stage. In the anti-Met and anti-Hel antibody purified proteins, some high molecular weight proteins were detected too. These high

molecular weight proteins were likely to be pORF1 (~186 kDa) shearing intermediates. It indicated that pORF1 was sheared after translation to generate viral enzymes performing different function [33].

High amino acid variation in HVR region (indicated with "H" in Fig. 8.2) of pORF1 is the main cause of the length differences in HEV genome RNA, but the role of HVR in HEV infection and replication is not clear [18, 36]. Shukla et al. found an HEV strain, whose HVR was inserted 57 amino acid-coding fragments (171 nt) of swine ribosomal protein S17, had replication advantages in cell culture. With the passages raising, the population of insertion mutants got an absolute dominance [38]. It had been found that HVR could be inserted with a variety of gene fragments, such as 117 nt human ribosomal protein S19 gene fragment or 114 nt human GTPase gene fragment [24, 31]. These phenomena indicated that amino acids coded by HVR were not required for HEV replication. Partly deletion of HVR in HEV1, HEV3, and avian HEV did not affect the expression efficiency in cells and virulence in experimental animals. But complete deletion of HVR leads to a significant decrease of virulence in progeny virus [34]. Using infectious clones inserted with swine S17 fragment, the function of HVR in HEV infection and replication was analyzed. When the insertion fragment was removed partly or completely, the expression efficiency of pORF2 decreased. When the insertion fragment was synonymously mutated, the expression efficiency of pORF2 did not change significantly. When the 171 nt insertion fragment was substituted with 174 nt fragment of green fluorescent protein gene, the expression efficiency of pORF2 decreased, which was consistent with the efficiency of completely removed clones. Above results indicated that the pORF2 expression efficiency related to the amino acid sequence coded by insertion fragment, but not related to the length or nucleotide sequence of insertion fragment [39]. Although HVR was not necessary for the HEV, but it did not lose, it still had some biological function and affected the infection efficiency and host range of HEV.

8.5.4 pORF2 Posttranslational Processing and Its Function in Virus Assembly and Determination of Host Range

As the capsid protein, pORF2 posttranslation processing has great significance to the study of HEV structure and vaccine development. Tanaka et al. detected cell culture supernatant using anti-pORF2 monoclonal antibody. WB results showed two bands, a 65 KDa main band and a 74 kDa band of lower content [47]. Molecular weight of full-length HEV3 pORF2 is about 72 kDa; it contains 111 amino acids of signal peptide at the N-terminal, and molecular weight after signal peptide cut is about 62 kDa. In addition of this, there are three potential glycosylation sites and one oligosaccharide chain having a molecular weight of about 3 kDa [21]. It was therefore presumed that pORF2 in virions would cut off the N-terminal 111 amino acids of the signal peptide and contained at least one oligosaccharide chain. Study

results indicated that the splicing manner of pORF2 in cell culture was same as in insect cells, but the glycosylation ratio is lower.

A C-terminus sequence of pORF2(585-606aa) could bind 5′ end of the genomic RNA and package a virus particle [25, 40]. Three potential glycosylation sites were not located in the core area of the assembly function [50]. To study the function of glycosylation and amino acid sequence in virus assemble, Graff et al. mutated the three glycosylation sites, respectively (Asn137, named G1 clone; Asn310, named G2 clone; Asn562, named G3 clone) and all of three sites (named G123 clone) in infectious clone (HEV1). Transfecting cells with above clones, genomic RNA could replicate and pORF2 and pORF3 could express, but infectious progeny virus could not be detected in the supernatant of mutant clone-transfected cells. In supernatant of G1, G2, and G123 transfected cells, intact virus particles could not be detected. This case indicated that any one mutation of two glycosylation sites at Asn137 and Asn310 resulted in a deficiency in pORF2 package function. pORF2 in supernatant of wild-type and G123 clone-transfected cells was purified and analyzed electrophoretically. There was no significant difference in mobility. It indicated that the majority of the natural pORF2 was not glycosylated, and therefore glycosylation deletion was not the main reason for virus can not package. To further clarify whether the glycosylated amino acid mutations or oligosaccharide chain deletions resulted failure of virus assemble, researchers designed three mutant clones. In which, X and Ser/Thr in glycosylation motif (Asn-X-Ser/Thr) were mutated to other amino acids that does not affect glycosylation (X mutated to any other amino acid other than Pro, Ser mutated to Thr, Thr mutated to Ser). Complete virus particles could not be detected in all mutant clone-transfected cells. Further, single point mutation in a nonglycosylated Asn (Asn445) also caused a deficiency in virus assemble. These results indicated that the factor affected pORF2's function in viral packaging was the amino acid sequence of pORF2, rather than glycosylation [14].

The traditional view is that the host range of the virus is determined by the viral capsid proteins. In vitro results showed that Sar55 and Akluj strain of genotype 1 could infect swine kidney cells [38], indicated that genotypes 1, 3, and 4 used the same receptor at least. In infectious clone studies, "JR + ORF3 + ORF2 + 3′NCR" section of genotype 4 was connected with "5′NCR + ORF1" section of genotype 3 to generate chimeric infectious clone. The resulting chimeric virus could infect HepG2/C3A cells and pigs [11]. When the "JR + ORF3 + ORF2+ 3′NCR" section of genotype 3 or 4 was connected with the "5′NCR + ORF1" section of genotype 1 or when the "JR + ORF3 + ORF2 + 3′NCR" section of genotype 1 was connected with the "5′NCR + ORF1" section of genotype 3 or 4, only progeny virus of 1 + 4 chimeric clones could infect HepG2/C3A cells. After chimeric infectious clone-injected pigs, no infection symptoms produced. HEV RNA could be detected in biles of genotype 1 "JR + ORF3 + ORF2 + 3′NCR" plus genotype 3 "5′NCR + ORF1" chimeric clone-injected pigs, but no stool virus shedding and viremia, indicated that this chimeric virus could establish very low levels of infection [4]. These results indicated that the pORF2 difference among genotypes was not a major factor in determining host range.

8.5.5 *pORF3 Role in the Envelope Formation and Virus Release*

Cell culture studies showed that HEV virions in culture supernatant might contain envelope. The density of fecal HEV virions was 1.21 g/cm^3 in gradient ultracentrifugation, while it was 1.15 g/cm^3 for the cell culture supernatant virions, and both HEV with different density could infect and propagate in cells [35]. Treating HEV in supernatant with detergent digitonin, virions density increased from 1.16 g/ml to 1.21 g/ml. It indicated that the HEV virions in cell culture supernatant had envelope structure.

pORF3 is related to the formation and release of enveloped virions in cell culture. After ORF3 was silently mutated in HEV 1 or 3 infectious clones, transfected cells could produce progeny virus and release it into supernatant, but the concentration of progeny virus in supernatant was 1/10 of wild type, approximately [37, 51], whereas the concentration of progeny virus within mutant transfected cells was equal with wild type. Mutant progeny virus in cell lysates could infect hepatoma cells [9], but could not infect rhesus monkeys [6]. Therefore, it could be deduced that pORF3 mediates the formation of envelope and virus release from infected cells but has little relationship with the viral infection.

pORF3 has 1 or 2 PSAP motif; the second PSAP motif played an important role in the formation of envelope and release of HEV [10, 28, 29]. Five PSAP mutant clones were derived from JE03-1760F strains. Of which, four mutant clones (LSAP, PSAL, LSAL, and PLAP/PSAP) produced progeny virus as efficient as wild-type clones, and their progeny virus had envelope (density 1.16 g/ml). PLAP/LSAL mutant clones produced progeny virus with less efficient than wild-type clones and equal with ORF3 deletion mutant clones. Gradient centrifugation analysis showed that the density of PLAP/LSAL mutant progeny virus was 1.26–1.27 g/ml. And progeny virus could only be captured by anti-pORF2 mAb but not by anti-pORF3 mAb, whether it was or not treated with sodium deoxycholate. It was indicated that PLAP/LSAL mutant of pORF3 caused the absence of virus envelope. Analyzing the expression of pORF3, intracellular expression was not affected, but pORF3 could not be detected in supernatant. These result showed the PSAP motif was the key motif of pORF3 to mediate the formation of envelope and release of virus, and these functions might be further confirmed by the interaction of pORF3 with two key proteins of vacuolar transport system: tumor susceptibility gene 101 (tumor susceptibility gene 101, Tsg101) and CD63 [28–30].

These results are summarized: HEV has an envelope at the time of its releasing from hepatocytes, and virion-embedded pORF3 plays a decisive role in the envelope formation. For the feces HEV, envelope is destroyed by bile, and embedded pORF3 was digested by trypsin, but for the HEV in blood, these two components were reserved (Fig. 8.3).

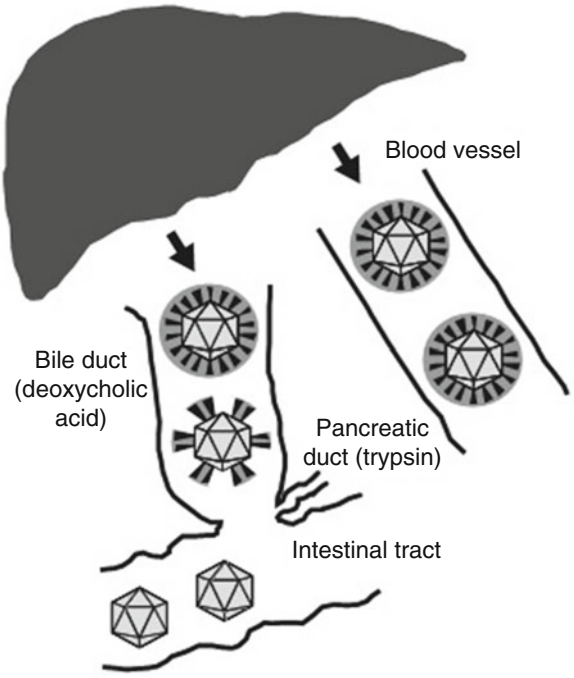

Fig. 8.3 Schematic diagram of the "enveloped" and "non-enveloped" HEV particles in infected hosts [32]

8.5.6 Neutralization Analysis

As mentioned in the previous discussion, envelope existed in culture supernatant virus but not in fecal virus. Therefore, to clarify the structure of HEV virions and distribution of viral proteins in envelope and capsid is the preconditions of neutralization analysis. Studies were carried out by analyzing the ability of anti-pORF2 or pORF3 mAb to capture detergent treated/untreated viruses. Using anti-pORF2 and anti-pORF3 mAbs, the supernatant virus capture rate was 5.3 % and 7.1 %, respectively. After treatment with sodium deoxycholate, capture rate rose to 84.2 % and 55.9 %. After treated with trypsin, capture rate of anti-pORF2 mAb rose slightly to 7.1 %, but capture rate of anti-pORF3 mAb decreased dramatically to 0.2 %. Treating with trypsin and sodium deoxycholate simultaneously (simulate the structure of fecal virus), capture rate of anti-pORF2 mAb rose to 94.1 %, and capture rate of anti-pORF3 mAb decreased to 0.2 % [30]. These results indicated that, in the culture supernatant HEV virus particles, pORF3 simultaneously presented in the viral envelope and capsid surface and might be released from the envelope or capsid by trypsinization. Similar analysis was performed with serum and feces virus, results showed that HEV in feces had no envelop and pORF3, and enveloped viruses in serum contained a large amount of pORF3. HEV virus in cell lysates existed in both cases [30, 42].

Previous studies indicated that anti-pORF2 antibodies could neutralize the infection of virus from feces [37, 55]. But in studies of Takahashi et al., the HEV from livers of pigs or deers could infect and replicate efficiently despite the existence of anti-pORF2 antibodies [43]. While the fecal virus was used as the inoculation virus, infection could be neutralized by anti-pORF2 antibodies in cell culture [47]. Also, because pORF3 is abundant in envelope, so the anti-pORF3 antibodies exhibited some neutralizing activities, while the culture supernatant was used as inoculation virus [42]. Structure differences can explain the neutralization results using the virus of different sources.

8.6 Summary and Outlook

In recent years, the incidence of HEV increased significantly, and HEV had become the most important pathogens of acute viral hepatitis. The combination of cell culture and infectious clone makes HEV researchers get a great progress in recent years, but many questions about HEV need to be further investigated, especially the constraint factors of host range, the cellular receptor of HEV, the reason of high fatal rate in pregnant women, etc.

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Chapter 9
Animal Models for Hepatitis E Virus

Lin Wang and Ling Wang

Abstract Animal models are one of the most important tools in the study of human hepatitis E virus (HEV) infection. They are particularly important in light of the major limitations of the cell culture system for HEV. Besides nonhuman primates, which are extremely valuable because of their susceptibility to HEV genotypes 1–4, animals like swine, rabbit, and chicken are also potential models for studies of pathogenesis, cross-species infection, and the molecular biology of HEV. Identification of the most useful animal model for human HEV infection studies is crucial to further investigations into this ubiquitous yet poorly understood virus.

Keywords HEV • Animal models • Pathogenesis • Cross-species infection • Vaccine study

Abbreviations

ALT	alanine aminotransferase
AST	aspartate aminotransferase
ET-NANB	enterically transmitted non-A, non-B
GE	genome equivalents
HEV	hepatitis E virus
IEM	immune electron microscopy
IHC	immunohistochemistry
i.v.	intravenous
PR	pregnant rabbit
rHEV	rabbit HEV
sHEV	swine HEV

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SPF	specific pathogen free
uPA-SCID	immune-deficient urokinase-type plasminogen activator-severe combined immune deficiency
USB	uPA-SCID-beige
VLP	virus-like particle
wpi	week post inoculation

Animal models are of prime importance in studies regarding viral pathogenesis and development and evaluation of antiviral agents and vaccines. They are also valuable tools for the study of HEV infection. Nonhuman primates, particularly, are suitable animal models for HEV infection studies, because of their susceptibility to HEV genotypes 1–4. Swine are the primary reservoir of HEV-3 and HEV-4 and are, therefore, a suitable model for studying HEV-3 and HEV-4 infections. Recently, rabbit HEV has been isolated in China and thus provides an alternative small animal model of HEV infection. Chickens infected with avian HEV can display symptoms such as hepatitis-splenomegaly syndrome, egg drop, regressive ovaries, and acute death. Such presentations in liver are, in some aspects, similar to human HEV infection, yet chickens can only be infected by avian HEV. In this section, we will discuss both the advantages and limitations of widely used animal models in the study of HEV.

9.1 Nonhuman Primate Models

Nonhuman primates are important animal models for HEV research. Although they are not the natural hosts of HEV, their susceptibility to HEV genotypes 1–4 has led to the prominent use of nonhuman primates in studies of HEV.

There are some deficiencies in this model: limited presentations of clinical diseases, ethical concerns, high cost, and difficulty in operation. However, many studies have been carried out by using these animals.

9.1.1 Pathogenesis

The first investigations into enterically transmitted non-A, non-B (ET-NANB) hepatitis virus, which is now known as HEV, were in 1983 [5]. The human volunteer, Balayan himself, ingested stool samples collected from Afghan patients and subsequently developed clinical symptoms consistent with acute viral hepatitis. Virus-like particles (VLPs) were visualized by immune electron microscopy (IEM) in stool samples during preclinical and early post-clinical phases. Intravenous inoculation of cynomolgus monkeys with the virus-containing stool extract resulted in histopathologically and enzymatically confirmed hepatitis, VLP shedding, and a VLP-specific antibody response.

Following the first breakthrough in the investigation of HEV, further characterization in macaques and other nonhuman primates was undertaken. Experimental infection of cynomolgus and rhesus macaques and tamarins induced clinical signs, coinciding with acute viral hepatitis with occasional excretion of VLPs in the stool, and detection of antiviral antibodies [3, 7, 49, 50]. However, results in chimpanzees and tamarins were more complex [2, 7, 35]. Not all tamarins developed infection, while chimpanzees [2], pig-tailed macaques [49, 50], vervets [50], owl monkeys [63], squirrel monkeys [50], and patas monkeys were all susceptible to experimental infection. According to these studies, it was accepted that chimpanzees, rhesus monkeys, and cynomolgus monkeys were susceptible to HEV genotypes 1–4, although chimpanzees and tamarins were used less frequently, and levels of virus shedding, liver enzyme elevation, and histopathologic changes in liver showed different results between different species [15, 19, 49, 50]. Infection in pregnant rhesus monkeys failed to induce any severe outcomes or fulminant hepatitis E, outcomes that have been observed previously in pregnant women [19, 51].

9.1.2 Cross-Species Infection

In studies of HEV cross-species infection, rhesus monkeys are broadly utilized animal models [29]. All four genotypes of HEV can induce infection in rhesus monkeys, with development of viremia, fecal shedding, and specific antibody responses. Genotype 3 swine HEV infection has been established in rhesus monkeys and chimpanzees with manifestations of acute viral hepatitis, viremia, fecal shedding, mild elevations of alanine aminotransferase (ALT), and seroconversion to anti-HEV antibodies [38]. Rhesus macaques were also susceptible to an Indian strain of genotype 4 swine HEV as manifested by viremia and seroconversion to anti-HEV antibodies [4]. Most recently, rabbit HEV has been identified in Gansu, China [60], and subsequent experiments have shown that a rabbit strain of HEV, CHN-BJ-R14 (GenBank Accession number: JX109834), could infect cynomolgus monkeys [31]. During the infection, clinical symptoms like jaundice and malaise were observed, along with viremia, fecal shedding, elevations of liver enzymes, and detection of anti-HEV antibodies, suggesting the possibility that the rabbit HEV may infect humans. It has been demonstrated that avian HEV cannot infect rhesus macaques [24, 31]. In addition, rat and ferret strains of HEV could not infect nonhuman primates in experimental settings [30, 42] as evidenced by no detection of viremia, fecal shedding, or seroconversion to anti-HEV antibodies.

9.1.3 Vaccine Studies

Nonhuman primates often serve as surrogates of humans in HEV studies, including vaccine studies. Rhesus and cynomolgus macaques were proven to be desirable models in preclinical immunization and challenge HEV vaccine trials. Vaccinated rhesus monkeys were inoculated with HEV genotypes 1, 2, and 3, and no sign of

infection was observed [41, 57]. One recombinant vaccine was subsequently proved to be efficacious in phase II clinical trials in young men, with 95% protection against HEV genotype 1 in Nepal [46]. A second recombinant vaccine, HEV 239 (Hecolin; Xiamen Innovax Biotech, Xiamen, China) was also administered to rhesus monkeys in order to evaluate its immunogenicity and protective efficacy. The vaccinated macaques developed anti-HEV antibodies and were protected against challenge with homologous and heterologous HEVs. This vaccine also showed promising protection and long-term efficacy against HEV genotypes 1 and 4 in phase III clinical trials in China [58, 59, 61].

9.2 Rabbit Models

The first strain of rabbit HEV (rHEV) was isolated in farmed rabbits in Gansu Province, China, in 2009 [60]. Since then, researchers in the United States [12], France [25], Italy [8], and Germany [14] have also discovered several different strains of rHEV in a wide range of rabbit breeds. Subsequent studies have shown that rHEV can transmit across the species barrier to infect specific pathogen-free (SPF) pigs [13] and nonhuman primates [31], suggesting the potential risk of zoonotic transmission to humans. As the rabbit is becoming recognized as a noteworthy reservoir of HEV alongside swine, researchers are beginning to evaluate the usefulness of the rabbit model as an alternative for HEV study.

The deficiencies exist in this model including limited presentations of clinical diseases and not being susceptible to HEV-1 and HEV-2.

9.2.1 Pathogenesis

Rabbits were found to be naturally infected with rHEV in many countries and regions. The ability for cross-species transmission to pigs [13] and nonhuman primates [31] in an experimental setting provoked interest in the pathogenesis of this virus, especially in its natural host, the rabbit.

In a pathogenesis study, a total of 42 SPF rabbits were used and randomly divided into 11 groups, with 1 group serving as negative control. Several strains of rHEV (GenBank No: FJ906895, FJ906888, FJ906890, FJ906896, FJ906893) isolated from Gansu [60] were inoculated intravenously (IV) into these SPF rabbits [33]. The inocula were diluted from 10^1 to 10^7 genome equivalents (GE). Rabbits became infected with HEV after inoculation; fecal shedding could be detected at 1–2 weeks post inoculation (wpi), and viremia occurred at 4 wpi. The study ended at 14 wpi, and fecal and serum HEV RNA remained detectable in some of the rabbits. Elevated ALT levels were also observed during the late period of fecal shedding. The value peaked at 9–11 wpi with a fourfold elevation over baseline level. Liver histology was investigated for pathological signs of HEV infection. Multifocal lymphohistio-

cytic infiltrates and local hepatocellular necrosis was observed. All rabbits inoculated with non-passaged rHEV strains seroconverted by 3 months post-inoculation, and the severity of the disease in rabbits was dose-dependent. A second study reproduced these findings [10]. In this second study, rabbits intravenously inoculated with rHEV strains (GenBank No: JQ065065, JQ065068) showed similar manifestations of acute hepatitis E. In addition to IV inoculation, the authors also administered the rHEV strains orally in 15 rabbits. However, the infectivity was low, with only two rabbits showing virus shedding and seroconversion [10]. These studies combined indicated that the pathogenesis of rHEV in rabbits is very similar to acute HEV infection observed in humans, with fecal shedding of HEV RNA, viremia, seroconversion, and elevated ALT levels, although IV administration of a high dose of virus was required to establish infection.

Increasing numbers of chronic hepatitis E cases caused by HEV-3 have been reported in developed countries [26] and recently a confirmed HEV-4-associated chronic hepatitis E patient has been found in China [18]. The definition for chronic hepatitis E is elevated aminotransferase levels, positive serum HEV RNA, and suggestive histologic findings for at least 6 months [26]. Interestingly, in a recent study [22], rabbits inoculated IV with rHEV isolate CHN-BJ-rb14 (GenBank No. JQ768461) showed fecal shedding of virus RNA over a period of 9 months. Liver histopathology results showed chronic inflammatory cell infiltrations and obvious portal fibrosis, which indicated the chronicity of HEV infection in rabbits. The prolonged viremia and fecal shedding in rHEV-infected rabbits corresponded to that observed in human chronic HEV infection. All rabbits, except one, seroconverted at about 5 wpi, and the high antibody levels were maintained until the end of the study; one rabbit seroconverted at 22–25 wpi, but virus became undetectable thereafter. In a more recent study, this group [53, 54] discovered that two SPF rabbits inoculated IV with 10^6 copies/mL rHEV, but not with 10^4 or 10^5 copies/mL, developed, respectively, a 40-week (R1) and a 20-week (R2) persistent infection. The liver and kidney tissues from R1 showed signs of chronic hepatitis and kidney injury. HEV ORF3 proteins were detected in the kidney tissues. Additionally, HEV RNA was detected in the urine of R1 and was infectious to healthy SPF rabbits. These findings demonstrated that rHEV could induce chronic infection and extrahepatic injury in rabbits, although many specific details that are crucial to the chronicity remain to be investigated thoroughly in the future.

An important consideration in human HEV infection is its pathogenesis during pregnancy. A mortality rate of up to 20 % has been reported in HEV-infected pregnant women, and the underlying mechanism of this increased mortality rate is still unclear [39, 48]. A recent study used the rHEV isolate CHN-BJ-R14 to investigate its pathogenesis and effects in pregnant rabbits [55]. HEV infection was successfully established in six pregnant rabbits (PR). Two of six infected PR miscarried and three of the remaining PR died. All rabbits had fecal shedding of virus from 3 days post-inoculation, with persistent or transient viremia. Elevations of ALT and AST were observed. The seroconversion to anti-HEV antibodies occurred at 3–7 wpi. Both positive and negative strands of HEV RNA were detected in the placental tissues of the infected PR, and positive staining for HEV antigen was observed in

placental tissue by immunohistochemistry (IHC). Furthermore, vertical transmission was observed by analysis of the first defecation of the offspring of the one PR survived, and the newborns seroconverted at 3 months of age, which suggested that the anti-HEV antibodies were more likely induced by infection rather than passively acquired from their infected mothers. This study reproduced, for the first time, the severe outcome in pregnancy-associated human HEV infection in an animal model, including the high mortality rate, miscarriage, and vertical transmission.

In summary, rabbits infected with rHEV present signs of acute HEV infection, including viremia, fecal shedding, elevated ALT level, and seroconversion. Chronic infection of rHEV in rabbits was also discovered with persistent fecal shedding and typical histopathological changes. High mortality in HEV-infected pregnant rabbits was also reproduced. Further investigation into factors that may affect virus pathogenicity in rabbits is required.

9.2.2 Cross-Species Infection

Rabbits are susceptible to HEV-3 isolated from rabbit and HEV-4 from both human and swine origins. Efficient infections could be established when inoculating high doses of these strains of viruses, with viremia, fecal shedding, elevation of ALT, and histopathologic changes in liver or other organs [10, 22, 33, 58, 59]. Although rHEV has been assigned to HEV-3, human HEV-3 could not infect SPF rabbits [10]. HEV-1 was also administered to SPF rabbits, but no sign of infection was observed [33].

9.2.3 Vaccine Studies

The world's first commercial vaccine against HEV infection, HEV 239 (Hecolin; Xiamen Innovax Biotech, Xiamen, China), was approved by China's State Food and Drug Administration. The results of a phase III trial [61] and long-term efficacy analysis [58] showed good performance in protecting humans against HEV-1 and HEV-4 infections. Yet, it is also important to evaluate the protective ability of this vaccine in preventing HEV infection in animals because of the zoonotic nature of HEV. As previously mentioned, the HEV-239 vaccine could confer protection on nonhuman primates against HEV-1 and HEV-4 infections [17]. In a later study, rabbits vaccinated with 30 μg HEV 239 at 0, 2, and 4 weeks, showed complete immunization against rHEV and swine HEV-4 (sHEV-4) [32]. However, the vaccine dosage used in the study was designed for humans, which is costly for large-scale immunization of animals. Thus, Zhang et al. conducted a study to explore a more cost-effective immunization strategy to protect rabbits against HEV infection [59]. Three strategies were designed: two doses of 10 μg, two doses of 20 μg, or one dose of 30 μg. The results indicated that two doses of 10 μg could not only protect rabbits

against homologous and heterologous HEV infections but also was more economical and thus provided an optimal strategy for large-scale vaccination of rabbits. This may even be applicable to other animal reservoirs of HEV worldwide. Cheng et al. vaccinated the rabbits with three doses of 20 μg of another candidate vaccine, HEV p179 (Changchun Institute of Biological Products Co. Ltd, Chinese National Biotech Corporation, Changchun, China). The vaccinated rabbits produced anti-HEV, which completely protected the rabbits against infection with 10^5 GE HEV-4 [10]. These studies suggest that rabbits may serve as an animal model for vaccine evaluation, not only for evaluating the vaccine efficacy for humans but also for investigating the management of zoonotic transmission.

9.3 Pig Models

It was 1997 when the first strain of swine HEV was discovered in pigs in the United States [37]. To date, after numerous follow-up studies, swine is recognized as the primary reservoir of HEV-3 and HEV-4 in the world, responsible for almost all sporadic hepatitis E cases worldwide [27]. Therefore it is not unreasonable that pigs come to mind as possibly the most suitable model for HEV-3 and HEV-4 studies.

However there are some limitations in this model including limited presentations of clinical diseases, not being susceptible to HEV-1 and HEV-2, high cost, and difficult in operation.

9.3.1 Pathogenesis

As the natural host of HEV-3 and HEV-4, pigs have been utilized as an animal model for HEV pathogenesis studies. Yet results showed pigs infected by swine or human HEV develop only a subclinical infection with mild-to-moderate histopathologic lesions of hepatitis [36]. Experimental infections of pigs usually used parenteral administration because the oral route of inoculation often yielded an inefficient infection, with, in some case, no detection of viremia, fecal shedding, or seroconversion to anti-HEV IgG [9, 28]. Intravenous inoculation is more efficient in producing infection in pig models, yet overt hepatitis-like clinical signs were still lacking [21].

9.3.2 Cross-Species Infection

The pig model has been utilized to investigate cross-species infection. SPF pigs are susceptible to human HEV-3 and HEV-4, but not HEV-1 or 2 [11, 16, 36]. Swine can also be infected by rabbit HEV, but not rat HEV [13].

9.3.3 Vaccine Studies

Pigs with prior infection with swine HEV-3 could induce immunity against HEV-3 and HEV-4 infection [43]. Additionally, truncated recombinant capsid antigens derived from three different animal strains of HEV could induce strong anti-HEV IgG responses in pigs and could confer immunity to cross protect against a genotype 3 mammalian HEV [44].

9.4 Chicken Models

Avian HEV was first identified in the United States in 2001 in chickens with what was referred to as hepatitis-splenomegaly syndrome (HSS) [23]. Another syndrome, referred to as big liver syndrome, was discovered in chickens from Australia, with sequences acquired from isolates being closely related to avian HEV [34, 40]. Although the avian HEV strains share only around 60 % nucleotide sequence identity with human HEV strains [34], the overt clinical signs have raised attention to its potential as an animal model for the study of HEV.

However, the presentations of clinical signs were not typical to those found in infected patients and, most importantly, chicken is not susceptible to human or swine HEV.

9.4.1 Pathogenesis

Birds experimentally infected with avian HEV presented with inflammatory cellular infiltrations within the liver parenchyma, lymphocytic periphlebitis, and phlebitis in the liver; enlarged and hemorrhagic livers were observed in approximately 25 % of the infected birds [6, 62]. In addition, oronasal delivery of virus inocula in the chicken model induced similar patterns of infection to humans, with viremia, seroconversion, and clinical and pathologic lesions. Oronasal inoculation resulted in delayed appearance of seroconversion to anti-HEV than intravenous administration group [6]. Although avian HEV is genetically distanced from mammalian HEV, the particular hepatic disease symptoms occurring in infected chickens may offer potential for the study of at least some aspects of HEV in this model.

9.4.2 Vaccine Study

Chickens immunized with avian HEV capsid protein induced protection against avian HEV infection [20], thus providing an animal model for vaccine study.

9.5 Mice with Humanized Liver

HEV has been isolated from rats, and the isolates are now recognized as HEV-C1 [47]. In experimental settings, the rat is not susceptible to HEV genotype 1, 2, or 3 [42]. Successful infection of a HEV-4 strain in gerbils has been reported, which suggested a potential small animal model [56], but the value of this animal model requires further investigation.

Recently, several studies reported a mouse model with a humanized liver for studying HEV infection [1, 45, 52]. Immune deficient urokinase-type plasminogen activator-severe combined immune deficiency (uPA-SCID) mice or uPA-SCID beige (USB) mice were used, and human hepatocytes were transplanted into the mice. Subsequent experiments have shown that these mice were susceptible to HEV-1 and/or HEV-3, and chronic infections were observed. In the work done by Allweiss et al. [1], USB mice were inoculated either intravenously or orally, with serum- or feces-derived HEV-1 and HEV-3 strains (10^5 IU/mouse) isolated from clinical patients. The successful establishment of infection was only seen in mice inoculated intravenously with feces-derived virus inocula. Interestingly, cohousing of an HEV-1-infected mouse (serum titers of 10^5 IU/mL) with three naïve humanized USB mice led to successful HEV infections in all three mice, indicating HEV infection can be transmitted via the fecal-oral route in humanized mice. HEV ORF2 proteins and HEV RNA were seen in both HEV-1 and HEV-3 infected USB mice by immunofluorescence and RNA in situ hybridization. However, HEV ORF3 proteins were only detected in HEV-1-infected mice. Sayed et al. [45] performed a similar study in humanized uPA-SCID mice. Chronic-type infection was also observed in both HEV-1 (10^6 IU/mouse) and HEV-3 (10^5 IU/mouse) infected mice. The two studies tested the efficacy of ribavirin in HEV-infected humanized mice. A significant reduction of serum and fecal HEV RNA load was observed, which highlighted the potential usefulness of this mouse model in novel antiviral preclinical testing.

The mouse with humanized liver model represents an attractive small animal model for the study of HEV infection, including virus replication and antiviral testing. However attractive it is, there are still some questions requiring further investigation. Future studies should focus on how to rebuild a functional adaptive immune system in these mice in order to investigate the pathogenesis of the disease and on the investigation of the effect of HEV strains representing different genotypes or subtypes in infection studies.

9.6 Conclusion

The inefficiency of the cell culture system for the study of HEV hinders research into this important human pathogen. Thus, finding suitable animal models to study human HEV infection is critical. Currently, the abovementioned animal models for HEV study are widely used. However, major deficiencies in these models still exist.

Recently, evidence of naturally infection of HEV in SPF animals has been found, indicating that careful screening of HEV before relevant experiments are conducted [53, 54]. Some aspects of clinical disease could not be faithfully reproduced in these animal models, including the chronic infection seen in immunocompromised patients and extrahepatic manifestations observed in HEV-infected patients, such as neurological disorders and kidney diseases. We suggest that SPF rabbits can be the most versatile animal model because of their susceptibility to HEV-3 and HEV-4, the development of acute and chronic infections in experimental settings, and the reproduction of the severe outcome in HEV-associated pregnancy, although HEV-1 could not infect SPF rabbits and no overt clinical signs could be observed during infection. Future endeavors will be focused on finding new natural hosts of HEV with the potential to be animal models and developing a more efficient cell culture system.

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Chapter 10
Clinical Manifestations of Hepatitis E

Shaojie Xin and Long Xiao

Abstract The clinical manifestations of hepatitis E are similar to those of other types of viral hepatitis. While acute hepatitis E is usually self-limited, pregnant women and chronic liver disease patients suffering from acute hepatitis E usually present with severe clinical manifestations that may develop into fulminant hepatic failure. Chronic HEV infection is typically only seen in organ transplant patients; most HEV cases are asymptomatic and rarely display jaundice, fatigue, abdominal pain, fever, fatigue, or ascites. The clinical manifestations of HEV infection in neonates are diverse and have varied clinical signs, biochemistry, and virus biomarkers. Lastly, the extrahepatic manifestations and complications of hepatitis E are in need of further study.

Keywords Alanine aminotransferase • Clinical features • Extrahepatic manifestations • Pathology

Abbreviations

ALT	Alanine aminotransferase
AP	Acute pancreatitis
EBV	Epstein Barr virus
ELISA	Enzyme-linked immunosorbent assay
GBS	Guillain-Barré syndrome
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
RT-PCR	Reverse transcriptase polymerase chain reaction

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10.1 Introduction

In 1978, the first case of hepatitis E was identified as non-A, non-B hepatitis. Hepatitis E virus (HEV) infection is believed to be one of the common causes of enterically transmitted acute hepatitis in developing countries, and this disease is rare in developed countries, except for patients with a travel history to HEV-endemic regions. However, an increasing number of chronic HEV infection cases have recently been reported in developed countries [31]. In these countries, immunosuppressed patients with HEV infection, such as organ transplant recipients, human immunodeficiency virus (HIV)-infected patients, or patients with hematological malignancies, can develop chronic hepatitis E. In this chapter, we will describe the clinical manifestations and complications of hepatitis E.

10.2 Acute Hepatitis E

10.2.1 Clinical Manifestations

Acute hepatitis E is usually self-limited, and the course of HEV infection generally lasts 4–6 weeks, although the duration of viremia is sometimes prolonged in patients with normal immune function [52]. Initial manifestations of acute hepatitis E include muscle and joint aches, fatigue, and vomiting. Some patients present with jaundice, itching, light-colored stool, and dark urine, accompanied by increased levels of liver transaminases, bilirubin, alkaline phosphatase, and γ-glutamyl transferase. The alanine aminotransferase (ALT) level in a typical patient with acute hepatitis E increases to approximately 1500 IU/L. In contrast, some patients, even during the viremia period, show only mildly elevated or even completely normal ALT levels. Forty percent of patients with acute hepatitis E develop jaundice.

Pregnant women or patients with chronic liver disease who are suffering from an acute HEV infection usually have severe clinical manifestations, such as coagulation disorders, jaundice, hepatic encephalopathy, and ascites, which sometimes develop into fulminant hepatic failure. These severe clinical symptoms in pregnant women may be associated with the normal hormonal changes that occur during pregnancy and their associated immune system characteristics [58]. Additionally, the high mortality of acute HEV in pregnant women may be related to a decreased expression of the progesterone receptor [12]. The high mortality of HEV infection is also related to a high viral load, and the specific T cell response is weak in fulminant hepatitis E [74].

10.2.2 Pathology

The pathology of acute hepatitis E includes an unclear lobular structure, a slightly enlarged portal area, an infiltration of lymphocytes and polymorphonuclear leukocytes, and a proliferation of Kupffer cells. It also typically includes a certain degree of bile capillary expansion, with cholestasis, ballooning degeneration of hepatocytes, red-stained apoptotic bodies scattered mainly in perilobular areas, relatively concentrated lymphocytes, and focal necrosis of hepatocytes [82].

The pathology of fulminant hepatitis E includes an unclear lobular structure, patchy confluent necrosis of hepatocytes, and degeneration and proliferation of residual hepatocytes with large or double nuclei arranged in piles. Additionally, there is generally an expansion and proliferation of bile capillaries that are filled with concentrated bile and a massive interstitial lymphocyte infiltration.

10.2.3 Diagnosis

The diagnosis of acute hepatitis E is mainly based on epidemiological history, clinical manifestations, and laboratory test results. Acute hepatitis E is typically transmitted through a fecal–oral route, and many patients have histories of drinking unboiled water, eating raw food, dining out, contacting a hepatitis patient, or visiting HEV-endemic areas. The clinical manifestations of acute hepatitis E are similar to those of the acute viral hepatitis caused by other viruses, and it is difficult to distinguish hepatitis E from other viral hepatitis based solely on clinical manifestations.

Laboratory diagnoses of HEV infection include the detection of HEV RNA or virus particles in patient stool using molecular techniques or immune electron microscopy, as well as the serological detection of anti-HEV immunoglobulin (Ig) M or IgG in patient serum. A reverse transcription polymerase chain reaction (RT-PCR) assay can detect HEV RNA in feces 1–2 weeks before the appearance of clinical symptoms, and HEV RNA can sometimes be detected up to 52 days. Additionally, HEV antigen has been detected in hepatic tissue from experimentally infected primates. Serologic diagnoses of HEV infection are usually made based on the results of enzyme-linked immunosorbent assays (ELISAs). Anti-HEV IgM antibodies can be detected during the acute phase of the illness and can last approximately 4–5 months. HEV-specific IgG antibody appears after anti-HEV IgM levels rise and they increase from the acute phase until convalescence. Anti-HEV IgG antibody has been detected up to 4.5 years after the acute phase of hepatitis E. Therefore, an increased level of anti-HEV IgM is indicative of acute infection, and a detectable anti-HEV IgG titer is related to previous contact with HEV.

10.3 Chronic Hepatitis E

Chronic HEV infections were first reported in liver and kidney transplant patients in Europe in 2008 [42]. Since then, chronic hepatitis E cases have been continuously reported in organ transplant recipients [35, 61]. It has been shown that genotype 3 HEV infection can lead to chronic hepatitis in immunosuppressed patients, such as solid-organ-transplant patients, stem-cell-transplant patients, HIV-infected patients, hematology patients receiving chemotherapy, and rheumatology patients receiving immunotherapy [19, 42, 59]. Genotype 4 HEV was also reported to induce chronic hepatitis [32, 33].

No chronic HEV infections have been reported in patients infected by HEV genotypes 1 and 2 [57]. Estimates based on a series of published cohort studies suggest that the prevalence of anti-HEV IgG in solid-organ-transplant patients is approximately 11.6 % and that of genomic viral RNA is 2 %. Furthermore, in the included studies, 65 % of the patients who were HEV RNA-positive developed chronic infection [84]. Compared with patients with self-limited HEV infection, chronically HEV-infected patients have a shorter interval time between organ transplantation and liver injury diagnosis, resulting in a rapid progression to liver fibrosis and cirrhosis. This has been reported in kidney [34], liver [36], and heart [50] transplantation recipients, although the exact risk rate is still unclear. In a large retrospective multicenter study, out of 56 HEV-infected organ recipients, eight developed cirrhosis, two died of decompensated cirrhosis, and two of the liver transplant patients required a second liver transplant.

The combination of HIV infection with HEV infection can result in a persistent HEV infection that may develop into chronic active hepatitis [19, 47]. Recent studies have indicated that, similar to hepatitis B or C, chronic HEV infection is defined by the persistence of HEV RNA in the serum for at least 6 months after infection.

Chronic HEV infection in immunosuppressed patients has been shown to lead to chronic hepatitis and progressive liver fibrosis, culminating in liver cirrhosis [34, 36]. Chronic HEV infection often has no specific symptoms; most patients are asymptomatic and rarely display jaundice, fatigue, abdominal pain, fever, fatigue, or ascites [34, 37, 42, 76]. Chronic HEV infection may result in the development of bilateral pyramidal syndrome, peripheral neuropathy, inflammatory polyradiculopathy, encephalitis, or proximal myopathy [44, 70]. Kamar et al. reported that 6 % of solid-organ-transplant recipients with chronic HEV infection showed neurologic signs and symptoms [44].

Currently, there is no specific treatment program for chronic HEV, but studies have shown that interferon combined with ribavirin treatment is effective at suppressing HEV.

10.4 Extrahepatic Complications of HEV Infection

10.4.1 Neurological Complications

Hepatitis E-related neurological complications are relatively rare in clinical practice. A few specific case reports have described the following neurological complications of HEV infection: Guillain–Barré syndrome (GBS) [15, 18, 53], Bell's palsy [26], neuralgic amyotrophy [30, 68], acute transverse myelitis [55], and acute meningoencephalitis [25]. A recent report showed that GBS appeared in a case of a liver transplant recipient and was associated with necrotizing myositis [23]. Kamar et al. reported a case in which a renal transplantation patient with chronic hepatitis E developed neurological symptoms, specifically peripheral nerve involvement with proximal muscular weakness, leading to limb joint–central nervous system involvement and bilateral pyramidal syndrome. HEV RNA was detected in the serum and cerebrospinal fluid of the renal transplantation patient. The findings in this case report suggest that HEV-associated neurological injury might be linked to the emergence of a neurotropic variant of HEV [43].

GBS is a subacute disorder of the peripheral nerves and nerve roots that results in rapidly progressive weakness and sensory deficits of the limbs, and it can progress to respiratory failure [29]. In a recent case–control study, 201 Dutch patients with GBS were investigated [11], and 5 % of the GBS patients had anti-HEV IgM-positive serology that was indicative of a recent or current locally acquired HEV infection. This proportion was ten times higher than that in the control population. The existence of an association between GBS and HEV is further supported by the detection of HEV genotype 3 RNA in the blood samples of three patients. All the cases of HEV-associated GBS were anicteric with normal results from liver function tests, and most had only mildly elevated liver enzyme levels. The affected patients had typical GBS with respect to disease severity and outcome, and none had detectable anti-ganglioside antibodies.

Neuralgic amyotrophy is characterized by attacks of severe neuropathic pain of the arm and shoulder, followed by patchy weakness, atrophy, and sensory disturbances [80]. The symptoms are largely localized to the brachial plexus, although other peripheral nerves can be involved. Neuralgic amyotrophy is considered to be a post-infection, immune-mediated neuropathy; 50 % of patients reported an antecedent trigger with nonspecific "infectious" symptomatology, and liver function tests were mildly raised in 25 % of cases [81].

Diagnosis of hepatitis E in patients with neurological symptoms is not straightforward. In these cases, the hepatitis is generally mild, the patients are usually not jaundiced, and the results of a liver function test may be normal, particularly in patients who present at a late stage of hepatitis E. The timing of neurological symptoms associated with the onset of HEV infection is uncertain. On the basis of the combination of liver function test abnormalities and the presence or absence of viremia and serological responses, immune-mediated neurological symptoms are likely to follow HEV infection by approximately 2–4 weeks [20].

10.4.2 Thrombocytopenia

Hepatitis E concurrent with thrombocytopenia has only been described in a few case reports [2, 16, 78, 83]. These cases mostly lacked obvious clinical symptoms. With the improvement of liver function, the bone marrow suppression gradually reduced until it disappeared, and the number of platelets gradually returned to a normal level. The diagnosis of HEV as the cause of thrombocytopenia relies on the exclusion of other causes of thrombocytopenia. The platelet counts of hepatitis E patients with thrombocytopenia typically range from 1×10^9/L to 21×10^9/L. These patients generally do not need special treatment; only a few cases have to be treated with immunoglobulins or cortisol hormones. The mechanism of thrombocytopenia in these cases remained unclear. Anti-platelet antibodies were detected in the serum of some patients [73, 78], but this test was not performed in the other cases.

10.4.3 Hemolysis

Hepatitis E concurrent with hemolysis may be related to a deficiency of red blood cell glucose-6-phosphate dehydrogenase (G6PD). A deficiency of G6PD leads to hemoglobin sulfhydryl oxidation and precipitation, with flow and deformation dysfunctions of red blood cells, leading to hemolysis [7]. Hepatitis E concurrent with hemolysis usually presents with an anemic appearance and jaundice, patients may have soy sauce-colored urine, fever, and hepatosplenomegaly, and critical patients may have chills. Patients with hepatitis E concurrent with hemolysis can be given liver protection drugs and symptomatic treatment; hormone therapy may also be considered.

10.5 Other Complications Associated with HEV

10.5.1 Acute Pancreatitis Associated with Hepatitis E

The association of acute pancreatitis (AP) with viral hepatitis is well known. Usually, these cases are attributed to hepatitis A virus [21], hepatitis B virus [22], or hepatitis C virus [4]. Recently, several cases of AP associated with HEV have been reported [10, 39, 40, 54, 56]. In 1999, Mishra et al. reported a group of cases of nonexplosive viral HEV infection leading to AP. Patients with HEV infection that leads to AP are mostly young people, and symptoms usually develop after the appearance of jaundice in the second or third weeks of the course of hepatitis. The infection is characterized by upper abdominal pain and an elevation of bilirubin and serum amylase levels. Approximately one third of patients with AP associated with hepatitis E experienced a complication of AP, such as intra-abdominal collections.

The disease is generally not serious and is usually self-healing. Currently, most of these cases are from India or in people who have recently visited India. The pathogenesis of AP associated with hepatitis E is not clear, but it may be associated with direct damage to pancreatic cells or cellular immunity-mediated injury. To date, all of the reported cases have been genotype 1 HEV [24]. The early diagnosis of AP complicating acute hepatitis E may help in reducing morbidity and mortality. Despite the rarity of the association between AP and non-fulminant acute hepatitis E, HEV infection should be added to the potential etiologies of AP in areas where the disease is endemic.

10.5.2 Autoimmune Disorders Associated with HEV Infection

An autoimmune response can be initiated by HEV infection and often occurs in organ transplantation patients who are being treated with immunosuppressive agents. Several clinical reports support this view. It has been reported that HEV infection could cause rheumatic diseases, skin rash, and joint pain [3], and the immune response to HEV infection which could induce thrombocytopenia [73], hemolytic anemia, and other autoimmune disease manifestations, such as membrane proliferative and membranous glomerulonephritis [2] and allergic purpura [79].

The pathological mechanism of skin rash and joint pain after HEV infection is still not clear. However, some researchers believe that the application of cold globulin can explain this phenomenon. Cold globulin often appears after HEV clearance, and the symptoms can be improved by steroid treatment [63]. Overall, the extrahepatic symptoms following HEV infection respond favorably to steroid hormone treatment. When HEV-infected patients exhibit autoimmune disorders, the cold globulin test should be conducted. Notably, most of the autoimmune disorders are believed to be caused by HEV infection.

10.5.2.1 Allergic Purpura

In 2010, Thapa et al. reported a single case of allergic purpura caused by HEV infection [79]. Allergic purpura is a common allergic disease of blood capillaries; the main pathological change is a wide range of capillary inflammation. The main clinical manifestations of allergic purpura include skin purpura, gastrointestinal mucosal bleeding, joint swelling and pain, and nephritis. A few patients, especially children and young people, exhibit vascular nerve edema, which is typically benign and self-limiting. The deposition of IgA and immune complexes in different tissues and fine blood vessels, which can be caused by bacterial or viral infection, are believed to be the main pathogenesis of allergic purpura. Many viruses can induce allergic purpura, including adenovirus, rubella virus, measles virus, Coxsackie virus, cytomegalovirus, Epstein–Barr virus (EBV), herpes simplex virus (HSV),

hepatotropic viruses (HAV and HBV), HIV, and varicella zoster virus. Several allergic purpura cases caused by HEV infection have been reported. Given the fact that HEV is similar to the viruses mentioned above, HEV infection may also be one of the viral mechanisms causing allergic purpura.

10.5.2.2 Nephritis

Both acute and chronic hepatitis E can cause renal insufficiency, but it is more common with chronic infection. The manifestations of renal lesions include glomerular nephritis, cryoglobulinemia, and a decrease in the creatinine clearance rate. All subtypes of HEV can cause glomerular disease, with pathological changes including value-added membranous nephritis and membranous glomerulonephritis [45]. This phenomenon is more common in patients who have received an organ transplantation. The pathological mechanism of renal injury caused by HEV infection is not clear, but cryoglobulinemia is significant in the pathogenesis of renal injury. When the HEV has been removed, the creatinine clearance rate will increase, the proteinuria will decrease, and the glomerular nephritis will be generally improved.

10.5.2.3 Aplastic Anemia

Shah et al. reported the first case of aplastic anemia caused by HEV infection in 2012 [71]. The aplastic anemia induced by hepatitis E has a poor prognosis with a high mortality (approximately 85 %) [13, 28, 65]. HEV-induced aplastic anemia has different manifestations compared with those of primary aplastic anemia; pancytopenia occurs at 2–3 months after HEV infection, and patients have fever and anemia, bleeding tendencies, and other symptoms. Signs of lymph node enlargement or hepatosplenomegaly are rare. Although immune inhibitors can be used for treatment, the best therapeutic methods for this condition are bone marrow and stem cell transplantation.

10.6 Hepatitis E in Pregnant Women

The prevalence and genotypes of HEV infection vary among different countries and regions. In developing countries, the infection rate of hepatitis E is high, and HEV genotypes 1 and 2 are more common than HEV genotypes 3 and 4. Indeed, several studies already define a clear separation between the two different types of HEV infection. HEV genotypes 1 and 2 are prevalent in developing countries, whereas HEV genotypes 3 and 4 are prevalent in industrialized countries. HEV infection is endemic in Central and Southeast Asia. The HEV infection rate is 15–30 % in Central Africa, approximately 30 % in South Asia, and reaches 84.3 % in the Nile Delta [9, 75]. In China, the positive rates of anti-HEV IgG and anti-HEV IgM were

16.2 and 2.6 %, respectively, in pregnant women, and were 25.3 and 3.2 %, respectively, in nonpregnant women [17]. Seroprevalences of HEV among pregnant women were 14.1 % in Gabon [14] and 28.7 % in Ghana [1]. Iran is an endemic country for hepatitis E, with few suspected outbreaks of HEV [6]. The seroprevalence of anti-HEV IgG is low in pregnant women in Iran, similar to the rates reported from developed countries [69]. Effective health services and the provision of safe water supplies may significantly reduce the incidence of HEV infection.

Although most HEV infections occur in developing countries, HEV appears to be an emerging problem in several industrialized countries and is often associated with traveling to a HEV-endemic area. Further studies are required to evaluate the role of swine in HEV epidemiology in developed areas [62, 66]. In the Western world, the most common genotype causing HEV infection is genotype 3 [5, 67]. Renou et al. reported an overall HEV prevalence of 7.74 % among 315 pregnant women in France. Anti-HEV IgG seroprevalence was higher in the south than in the north of France (29.3 % vs. 3.6 %, $p < 0.0001$), old women with anti-HEV IgG were easier to be detected, and anti-HEV IgG seroconversion or anti-HEV IgM detection was not observed during pregnancy. Lindemann et al. found a low anti-HEV IgM prevalence rate in Spain; 0.67 % of 1040 pregnant women's anti-HEV IgM antibodies were positive [51].

10.6.1 Clinical Features

In most men and nonpregnant women, HEV infections are asymptomatic and self-limited. However, the severity of the disease can range from mild to fulminant in pregnant women. One study reported that nausea, vomiting, and jaundice were the predominant symptoms of HEV infection in pregnant women [72]. Fever was significantly more common as the predominant presentation in nonpregnant patients than it was in pregnant patients. Other symptoms of HEV infection include dark urine, myalgia/arthralgia, right upper quadrant pain, fever, altered sensorium, pruritus, light-colored stools, diarrhea, and hematemesis. Though the occurrence of pruritus, myalgia/arthralgia, and jaundice appeared to be more common in the nonpregnant group, this trend was not statistically significant.

Reports from China demonstrated that pregnant patients were easier to develop severe hepatitis and hepatic encephalopathy before their jaundice reached its highest level [77]. In contrast, clinical histories of jaundice and liver disease were rare in Egypt, and the prevalence of liver disease was relatively low in subjects with anti-HEV antibodies. The reason for the infrequency of clinical hepatitis in Egypt remains unclear, but some studies speculate that early childhood HEV exposure produces long-lasting immunity, which could account for this difference.

The most common complications of hepatitis E infection during pregnancy include death of the mother and fetus, abortion, premature delivery, or death of a live-born baby soon after birth. The main causes of maternal death are hemorrhage, eclampsia gravidarum, and hepatic failure. The interplay between hormones and the

immune system changes during pregnancy [41, 60]. A recent study demonstrated that the anti-HEV antibody titers were directly proportional to hepatitis E disease severity in pregnant women. HEV during pregnancy was associated with a significant reduction in plasma cytokines despite an increase in the corresponding gene expression in peripheral blood mononuclear cells [64].

10.6.2 Treatment and Prevention

At present, there is no specific treatment for HEV-infected pregnant women. Patients with serious conditions are recommended to terminate the pregnancy, and a routine review of liver function is recommended. It is difficult to distinguish HEV from other forms of acute hepatitis, so a high suspicion of HEV infection is warranted. HEV testing is recommended in any patient with an unexplained elevated level of transaminase. Women who are at risk of HEV infection should get vaccinated before pregnancy. It is necessary to improve blood source control and guarantee transfusion safety because rules requiring the routine screening of blood donations for HEV have not yet been approved. Further studies on the pathogenesis, diagnosis, control, and prevention of HEV are needed.

10.7 HEV Infection in Neonates

HEV seriously threats fetal and neonatal health through the vertical transmission from mothers to children; *neonates* have a similar poor prognosis as HEV-infected pregnant women. Previous studies revealed that the HEV infection rate in newborn babies delivered from pregnant women with gestational HEV infection is high [48], especially when the baby was infected during the intrauterine or perinatal period [8, 38, 49].

The clinical presentations in HEV-infected newborn babies are diverse, and the clinical signs, biochemistry, and virus biomarkers can vary widely in these patients. For example, some HEV-infected babies have an elevated level of ALT, some have an elevated level of bilirubin, and some have elevated levels of both bilirubin and ALT. The increased bilirubin level is caused by the physiological jaundice occurring in the newborn baby. If the level of bilirubin increases after the appearance of physiological jaundice, it usually indicates the infection of HEV. The clinical symptoms, biochemical indices, serum markers, and virus detection in HEV-infected newborn babies are usually self-limiting. Liver function generally returns to a normal level at 8 weeks after birth, and chronic sepsis or ongoing clinical discomfort characteristics do not develop, which may be related to the pathogenesis of direct damage to hepatocytes by HEV [46]. Neonatal death following HEV infection is usually caused by fulminant hepatic failure. Respiratory distress syndrome, sepsis, and liver and spleen enlargement indicate a poor prognosis [27].

10.8 Conclusion

Hepatitis E is an important viral hepatitis that has long been neglected. Over the past decade, with advances in diagnostic techniques and in-depth studies, the understanding and awareness of hepatitis E have significantly progressed. HEV lacks suitable cell and animal models; thus, its pathogenesis has not been completely clarified, and the understanding of HEV is limited. There are no clinical manifestations of HEV that are significantly different from those of other types of viral hepatitis, so more attention must be paid to the role of HEV epidemiology when diagnosing hepatitis E. The detection of HEV RNA is the most direct evidence of HEV infection. Currently, there are no specific drugs or methods for the treatment of hepatitis E. The disease is generally self-limiting, so most HEV patients only need appropriate symptomatic and supportive treatment. For patients with chronic or acute liver failure, it is necessary to conduct a liver transplantation.

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Chapter 11
Laboratory Diagnosis of HEV Infection

Chenyan Zhao and Youchun Wang

Abstract Serological and nucleic acid tests for detecting hepatitis E virus (HEV) have been developed for both epidemiologic and diagnostic purposes. The laboratory diagnosis of HEV infection depends on the detection of HEV antigen, HEV RNA, and serum antibodies against HEV (immunoglobulin [Ig]A, IgM, and IgG). Anti-HEV IgM antibodies can be detected during the acute phase of the illness and can last approximately 4 or 5 months, representing recent exposure, whereas anti-HEV IgG antibodies can last more than 10 years, representing remote exposure. Thus, the diagnosis of acute infection is based on the presence of anti-HEV IgM, HEV antigen, and HEV RNA, while epidemiological investigations are mainly based on anti-HEV IgG. Although significant progress has been made in developing and optimizing different formats of HEV assays, improving their sensitivity and specificity, there are many shortcomings and challenges in inter-assay concordance, validation, and standardization. This article reviews the current knowledge on the diagnosis of HEV infection, including the most common available laboratory diagnostic techniques.

Keywords Anti-HEV IgM • Anti-HEV IgG • Antigen • Assay • Diagnosis • RNA • ELISA

Abbreviations

ALF	acute liver failure
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
EIA	enzyme immunoassay
GGT	γ-glutamyl transferase
HEV	Hepatitis E Virus

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IgA immunoglobulin A
IgG immunoglobulin G
IgM immunoglobulin M
ORF Open reading frame
TBA total iron binding capacity

Hepatitis E is an enterically transmitted or water-borne non-A, non-B hepatitis. Although it is known to cause self-limiting acute infection, it has also been diagnosed as a chronic infection in severely immunocompromised individuals [18, 42]. Hepatitis E virus (HEV) is the causative agent of this disease and was first identified by immune electron microscopy in the feces of a human volunteer [4]. Over the last decades, almost all of the water-borne outbreaks and the majority of the sporadic acute hepatitis cases have been caused by HEV [44, 89].

Over the years, several laboratory tests have been developed to diagnose HEV infection. The early assays used for laboratory diagnosis of hepatitis E included immune electron microscopy [8] and immunofluorescence assay [47]. Although these assays played a critical role in the early identification of the virus and the immune response induced during HEV infection, they were insensitive, technically difficult, and unsuitable as routine tools for clinical diagnosis. Serological assays, including those detecting anti-HEV IgM and IgG, have been developed for the diagnosis of HEV infection and epidemiological study. Detection of viral nucleic acids provides a highly sensitive and specific approach to diagnosing current HEV infection. The nucleic acids assays can also be used to screen blood and blood products before transfusion and to detect HEV RNA in sewage, contaminated water, and other environmental specimens. Recently, antigen-detecting assays have also been developed. This chapter will focus on the significant progress in HEV detection and will describe the most common available laboratory techniques for the diagnosis of HEV infection.

11.1 Dynamic Changes in HEV Markers After HEV Infection

There are usually three phases (latent, acute, and recovery) of the infection process when an individual is infected with the hepatitis E virus (HEV) [19, 46, 48]. The latent phase of hepatitis E infection generally lasts for 15–75 days, and patients with hepatitis E present with clinical manifestations similar to those of other types of acute hepatitis. They display typical patterns of acute viral replication and serological symptoms, and HEV RNA and antigen are first detected in the feces and serum after infection. Geng et al. recently reported that HEV RNA and antigen are also

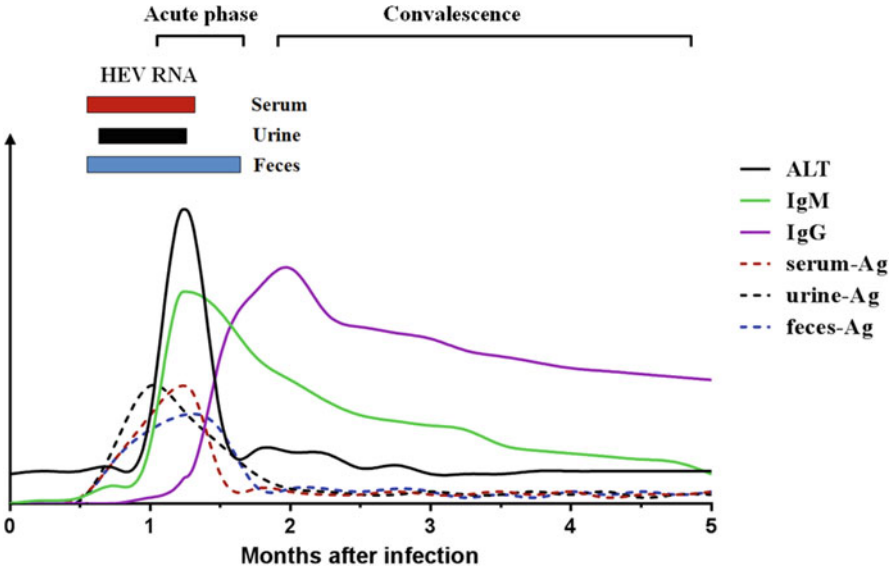

Fig. 11.1 Dynamic changes in HEV markers after HEV infection

detectable in the urine of patients after infection [27]. The detection of HEV RNA and antigen is associated with abnormal liver function indices, including bilirubin, aspartate aminotransferase, alanine aminotransferase (ALT), alkaline phosphatase, and γ-glutamyltransferase, which usually return to normal levels within 3 weeks [61]. Specific immunoglobulin M (IgM) and/or immunoglobulin A (IgA) antibodies are detectable in the serum within 2–4 weeks of infection. These IgM and IgA levels decrease relatively rapidly, within one to several months, or can persist for up to 5 months [21, 72]. Immunoglobulin G (IgG) antibodies appear shortly after the IgM antibodies and commonly persist for 14–20 months and even a long time up to 14 years [9, 19, 45] (Fig. 11.1). IgA antibodies can also be detected in the serum during the acute phase of HEV infection and have been proposed as a diagnostic indicator of acute infection ([11, 35, 72, 74]). However, the role of IgA in acute HEV infections requires further study.

Chronic hepatitis E has been reported in several studies in recent years. These cases are commonly seen in severely immunocompromised individuals, including organ transplant recipients, patients undergoing chemotherapy, and those with acquired immunodeficiency syndrome and concomitant HEV infection [29, 42, 65]. In patients with chronic hepatitis E, the serum, feces, and urine remain positive for HEV RNA for over 6 months, and their transaminase levels remain elevated. However, some of these patients were negative for HEV antibodies and may develop liver cirrhosis [27, 32, 33, 42].

11.2 Clinical Diagnostic Criteria of HEV Infection

The main acute-phase markers of HEV infection include HEV RNA, HEV antigen, IgA antibodies, IgM antibodies, and high-titer or low-affinity IgG antibodies. These markers appear at varying time points, persist for varying periods, and differ in their significance in clinical diagnosis and research. The markers of infectivity are predominantly HEV antigen and RNA, whereas the markers of epidemiological and immune status are IgG antibodies.

In hospitals in China and other countries, HEV RNA is the most commonly used diagnostic indicator of hepatitis E, although the use of IgM antibodies has increased markedly in recent years. Elevated IgG antibodies are only used as a tool for retrospective diagnoses, because they require two serum samples to be collected and quantitative assays of the antibodies to be performed. Low-affinity IgG antibodies are only used as an auxiliary method for diagnosis, because their specificity has not been fully verified. Most previous research has assessed the accuracy of these indicators using HEV RNA as the gold standard. However, the false-negative rate of HEV RNA detection assays cannot be ignored. Patients presenting at hospital who test negative for HEV RNA are often excluded from further assessment, causing selection bias in study samples and the analysis of records. Based on the latest advances in research into the diagnosis of hepatitis E in the international community, Dalton recommended the following criteria for clinical diagnosis of acute hepatitis E: (1) patients have substantially elevated serum transaminases, and (2) patients meet one of the following three conditions, positive for IgM antibodies, elevated IgG levels, or detectable HEV RNA [17]. Similar criteria were proposed in the diagnostic and treatment practices for hepatitis E developed by the Society of Infectious Diseases of the Chinese Medical Doctors Association in 2009, and the critical value of ALT recommended for a diagnosis of hepatitis E is 2.5 times the upper limit of normal [15].

11.3 Assays Used in Laboratory Diagnoses

Laboratory diagnostic methods for HEV infection mainly include the HEV particle assay (immune and immunofluorescent electron microscopy), HEV-specific antibody (e.g., IgG, IgM, or IgA) assays, HEV nucleic acid assay, and HEV antigen assay. Some diagnostic methods based on the HEV markers discussed above have been developed into commercial reagents.

11.3.1 Antigens Used in Antibody-Detecting Assays

Because there is no effective HEV cell culture system, the natural virus cannot be used to test for HEV antibodies. Therefore, assays for HEV antibody detection rely on synthetic polypeptides and recombinant proteins. The majority of antigens

currently used are derived from ORF2 and ORF3 proteins of HEV. Virus-like particles are also used as antigens in enzyme immunoassays (EIAs) [51]. Attempts have been made to express HEV proteins in several expression systems, including *Escherichia coli* [37, 51], insect cells [69, 75, 82], plant cells [54, 88], and *Trichoplusia* [39]. However, when different antigenic fragments of HEV are expressed in various systems, the recombinant antigens and the antigenic epitopes formed show different characteristics. Therefore, the performances of the diagnostic reagents developed based on these antigens also differ. The ORF2 and ORF3 polypeptides have been used to develop HEV antibody detection kits [22, 64, 66]. However, the performance of polypeptide-based diagnostic reagents is generally poor, and their applications are limited [24, 56]. This may be because polypeptides are inferior to recombinant proteins in simulating the conformational epitopes [43, 68, 81].

Although HEV has several genotypes, it has only one serotype [20]. Therefore, antigens expressed from one genotype of the virus, for the use as skeleton antigenic molecules, can theoretically react with antibodies produced to all four viral genotypes. Several studies have shown that the different HEV genotypes have cross-immunoreactive epitopes [2, 35, 87]. However, other studies have suggested that various HEV strains differ in the gene sequences of their epitopes, which may affect the detection assays based on these diagnostic reagents. Ma and colleagues expressed 18 ORF2 and ORF3 protein fragments from genotypes 1 and 4 HEV to prepare HEV IgM and IgG EIA reagents. They found that the ORF3 antigens of genotypes 1 and 4 displayed a higher binding reaction in serum infected with the same HEV genotype than in serum infected with a different HEV genotype. This finding suggests that there are genotypic differences in the antigenicity of the ORF3 proteins of genotypes 1 and 4. The same analysis was performed on the ORF2 antigens of genotypes 1 and 4, but no genotypic differences were detected [53]. The capsid protein of HEV contains several epitopes that may form different structures in different viral strains because their amino acid sequences vary in these strains. Therefore, multiple epitopes can be used to increase the cross-reactivity of recombinant antigens from different HEV strains during the development of diagnostic reagents.

The discovery of neutralizing epitopes has greatly influenced the development of hepatitis E vaccines and has also promoted the development of relevant detection reagents. The expression of recombinant proteins that effectively simulate the neutralizing epitopes of HEV provides an alternative to produce antigens with broad-spectrum reactivity. Several studies have used different expression system, such as *E. coli* and baculovirus, to express the neutralizing epitopes of HEV [52, 59, 75]. These antigens have been used to construct HEV antibody detection kits for large-scale serological and epidemiological surveys of HEV, which can effectively detect different HEV strains and various subtypes of HEV infection [14, 49, 73].

11.3.2 Anti-HEV IgG and IgM Assays

Patient sera that are either positive for specific anti-HEV IgM antibodies or display elevated anti-HEV IgG antibodies can be used as diagnostic criteria for acute HEV infection.

Anti-HEV IgM antibodies appear in the early stage of infection and indicate recent HEV infection, with important implications for clinical diagnosis. An ideal IgM reagent should be sensitive, have a low false-positive rate and short duration, and distinguish between recent and past infections. The use of indirect EIAs in the early development of HEV IgM diagnostic reagents encountered certain problems. For instance, antirheumatoid factor IgM antibodies that may be present in the sera of patients can bind to the recombinant antigens, affecting the detection specificity. Moreover, serum anti-HEV IgG can compete with IgM by binding to the coated recombinant antigens and thus affect the specificity of the assay [10]. To address these issues, the technique of antibody capture can be used in the anti-HEV IgM assay. Anti-IgM μ-chain antibodies are coated onto a plate to capture the IgM in the serum. A specific antigen or antigen–antibody complex is labeled and then used to detect anti-HEV IgM. This method detected the memory IgM responses and recurring IgM antibodies in an HEV-infected chimpanzee model. More importantly, this method reduces the competition between IgG and IgM, thus improving the detection sensitivity of the assay [79]. Commercial anti-HEV IgM kits manufactured by Wantai (Beijing, China) based on the capture method have been used in several studies.

The antigens used in IgM reagents are mainly recombinant antigens or polypeptides expressed from the ORF2 or ORF3 region of HEV. The ORF2 antigen allows the earlier detection of IgM-binding antibodies in the serum, by 1–2 weeks, than the ORF3 antigen. Therefore, the ORF2 antigen is more suited to early diagnosis, and both the N- and C-termini of the protein should be used simultaneously [53]. The discovery of conformational neutralizing epitopes of HEV and the identification of antigens based on conformational epitopes have allowed the development of IgM diagnostic reagents to a new level.

Anti-HEV IgG antibodies appear shortly after IgM antibodies, but persist for longer. IgG antibodies are often used as indicators of past infection and are commonly used in epidemiological surveys. However, a clinically detected increase in IgG antibodies against HEV to more than four times the normal level can be used as a diagnostic criterion for HEV infection. HEV IgG diagnostic reagents are primarily based on indirect EIAs, and the antigen used is usually the ORF2 protein. The N-terminal peptide of the ORF2 protein mainly induces the IgG antibody response in the acute phase of HEV, which persists for only a short period. Therefore, this antigenic fragment is more suited for the preparation of IgG antibody reagents to be used for early diagnoses. IgG antibodies directed against the C-terminal peptide also appear early but persist for longer. Therefore, this antigenic fragment is more suited for the preparation of HEV IgG antibody reagents for epidemiological surveys [53]. In recent years, in-depth studies of the neutralizing epitopes of HEV and

the use of viruslike particles in diagnostics have promoted the development of IgG reagents.

Commercial HEV IgM and IgG diagnostic reagents from several manufacturers have provided data on the epidemiology of HEV, the features of clinical infection, and the pathology of HEV infection [3, 6, 35, 71, 72, 79]. However, these data show large differences, making it difficult to compare and analyze them. These discrepancies may be attributable to the following factors: (1) the gene sequences encoding epitopes vary across the different viral genotypes, which therefore express different epitopes; (2) the recombinant antigens produced in different expression systems have different characteristics and form different epitopes; (3) different fragments of antigens have varying capacities to induce antibodies; and (4) various types of antibodies against different antigen fragments are present in different phases of HEV infection. The prevalence of the different HEV genotypes and the probability and frequency of contact with HEV also differ in various geographic regions. Therefore, the selection of diagnostic reagents must be based on the disease prevalence in the target area. For example, diagnostic reagents with relatively high sensitivity for IgM should be used in regions with a high prevalence of HEV. On the contrary, diagnostic reagents with relatively higher specificity for IgM should be used in regions with a low prevalence of HEV. Whether other factors should also be considered requires further study.

Anti-HEV IgA antibodies can also be used as marker antibodies for recent or acute HEV infections. Their persistence and diagnostic significance in HEV must be confirmed with clinical and epidemiological studies. However, an IgA assay can supplement an IgM assay to confirm the diagnosis of HEV infection in the acute phase.

11.3.3 Total HEV Antibody Assays

Indirect enzyme immunoassay (EIA) and μ-capture EIA are species-dependent detection methods used to detect the response between hosts and viruses. Because both humans and pigs can be infected by HEV genotype 3 and genotype 4, different reagents must be used in some methods to detect samples from different species sources. Therefore, species-independent detection methods have a great advantage in the HEV detection of these samples. Double-antigen sandwich EIA is one of the methods that uses horseradish peroxidase enzyme-labeled HEV antigens (HEV-Ags) to detect the antibodies captured by the coated HEV recombinant antigens (amino acid residues 394–604 and 452–617 of ORF2 protein are used for labeling and coating, respectively) [78]. Given that double-antigen sandwich EIA is not restricted by the host species (such as human or animal) or antibody type (IgG, IgM, or IgA), it is widely used in epidemiological surveys of HEV from different species and for detecting the total antibody rate of different populations. Thus, this method greatly facilitates investigations of the source of zoonotic HEV. Double-antigen sandwich EIA detected all the positive patient samples (n = 265) that contained

anti-HEV IgM or IgG antibodies, alone or together, while it maintained an excellent specificity of 98.8 % with samples from various patient or healthy control groups (total number of samples, 424) [36]. Compared with the serological status of the specimens, the testing results generated by the double-antigen sandwich EIA had a good level of agreement (99.3 %, with a kappa value of 0.985). Great sensitivity and specificity are shown in the detection of anti-HEV antibodies in pigs, and although anti-HEV antibodies can be detected in many animals (such as bison, cattle, dogs, Norway rats, farmed swine, and feral swine), the positive rate of anti-HEV antibodies in swine (pig) is higher than that in other animals [36].

There are some methods that can be used for rapid screening of HEV antibody, such as immune chromatography. Various serum markers can be detected quickly and easily by an immune chromatograph, and this is especially useful for point-of-care tests. A colloidal gold-labeled HEV antibody–antigen complex can detect the anti-HEV IgM from samples captured by the antihuman IgM antibody. MP Biomedicals developed a colloidal gold diagnostic assay with a recombinant HEV-I ORF2 antigen (amino acid residues 394–660) expressed by *Escherichia coli*. To evaluate the assay, the sera of patients in acute stage or recovery period after HEV infection in Nepal and of patients infected by HAV, HBV, or HCV were used, and the resulting rates of sensitivity and specificity were measured as 93 and 99.7 %, respectively [13, 62].

11.3.4 HEV Antigen Assays

The HEV ORF2 protein is a viral capsid protein that can be detected in the liver and serum during the window period and acute phase of infection [80]. Therefore, researchers have constructed HEV antigen EIA reagents for early diagnosis and treatment monitoring by targeting the ORF2 protein with a double-antibody sandwich method. Using this reagent, the detection of antigens in sera was highly consistent with nucleic acid detection [30, 76]. A test of serial serum samples from monkeys using this reagent showed that the HEV antigen occurs before the seroconversion of anti-HEV IgM antibodies and the elevation of ALT. With the expression of antibodies and the return to normal ALT, serum becomes negative for HEV antigen. HEV antigen and ALT levels are highly consistent. However, HEV antigen persists for a shorter period than anti-HEV IgM antibodies [80]. Testing clinical samples has shown that HEV antigen reagent allows the detection of HEV infection in the window period. The relationships among HEV RNA, HEV antigen, and anti-HEV IgM in acute hepatitis E were investigated. HEV antigen was highly correlated with HEV RNA and elevated ALT, AST, ALP, TBA, and GGT levels. Testing for HEV antigen in combination with anti-HEV IgM is useful for the diagnosis of HEV infection [84]. These results demonstrate that antigen assays play a key role in the early diagnosis of HEV infection. Antigen assays have obvious advantages over IgM antibody assays in the diagnosis of HEV, particularly in HEV-infected patients

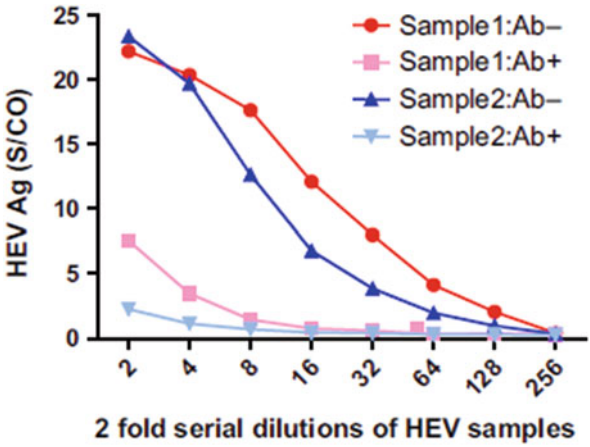

Fig. 11.2 Effect on antigen EIA reactivity of spiking in antibody$^+$antigen$^-$ sera. EIA was conducted after twofold serial dilutions of the two HEV samples with antibody$^+$antigen$^-$ or antibody$^-$antigen$^-$, which were mixed and incubated at 37 °C for 15 min

who cannot produce antibodies, because they are receiving immunosuppressant therapy, or in patients in the recovery phase, who still test positive for IgM antibodies.

To evaluate the sensitivity of antigen assays, and to assess the value of HEV-Ag detection in the diagnosis of HEV infection in comparison with that of HEV RNA detection, serial dilutions of a genotype 4 HEV strain were used to analyze the significant correlation between the EIA (S/CO) and HEV RNA (IU/mL) concentration. The results show that the correlation between them was in the range of $10^{3.5}$–$10^{0.5}$ IU/mL of HEV RNA, and the EIA detection limit was 54.6 IU/mL, compared with 24 IU/mL for HEV RNA using real-time RT-PCR. Therefore, antigen EIA is less sensitive than HEV RNA detection by real-time RT-PCR [83].

In clinical serum samples, the HEV-Ag-positivity rate and the concordance between HEV-Ag and HEV RNA were inversely proportional to the presence of anti-HEV antibody. As shown in Fig. 11.2, the presence of antibodies affected the detection of ORF2 antigen. The HEV-Ag EIA S/CO values of the serum sample and cell culture supernatant mixed with anti-HEV IgG-positive sera were significantly reduced compared with those of the control. This result may help to explain why HEV-Ag was not detected in some blood donors when the corresponding viremias were greater than 10^3 IU/mL [76]. Therefore, in clinical diagnoses of HEV infection, HEV-Ag detection in serum should be combined with antibody detection, followed by less frequent confirmatory testing with molecular assays [83].

The influences of temperature, storage time, and repeated freezing and thawing on HEV-Ag detection were studied. The stability of antigens in serum and stool samples stored at −20 °C was better than those stored at 4 °C or room temperature. However, in urine, the stability of HEV-Ag at 4 °C was better than that of HEV-Ag stored at −20 °C or room temperature. There was little influence of repeated freezing and thawing on the antigen detection.

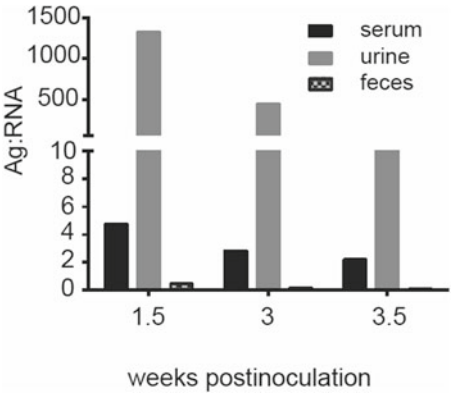

Fig. 11.3 Ratios of HEV-Ag to HEV RNA in monkeys. The ratios of HEV-Ag to HEV RNA in associated urine and serum and fecal samples of infected monkeys at various time points

Using the HEV-Ag reagent, Geng et al. were the first group to detect HEV-Ag in the urine of patients with chronic HEV infection and in the urine of patients with acute hepatitis even though antibodies to HEV were not detectable in the urine samples from these patients [27]. A study of a HEV infection model in cynomolgus monkeys found that HEV-Ag not only persisted for a longer duration in urine than in serum but also had a higher titer in urine. This indicates that HEV-Ag in urine can be used as a meaningful indicator for clinical diagnosis of HEV.

The ratios of HEV-Ag to RNA in the urine, serum, and fecal samples of HEV-infected monkeys were compared. The results show that the ratios of HEV-Ag to RNA in the urine samples were significantly higher than those in the corresponding serum and fecal samples, with the ratios in the fecal samples being the lowest (Fig.11.3). These results may indicate that HEV in urine is not solely in the form of virions. HEV-Ag without HEV RNA, as a free antigen or empty capsid, seems to be quite abundant [27].

The HEV-Ag reagent has been used for blood screening. By testing more than 33,000 serum specimens from blood donors at five blood centers, 18 antigen-positive samples were found, and the risk of transmission after transfusion was therefore reduced [67]. Moreover, the HEV-Ag detection reagent has been used for epidemiological screening in humans, pigs, horses, sheep, rabbits, and minks, among various animals, which has helped to control the risk of HEV transmission in animals.

A study assessing the prevalence of HEV infection in domestic animals in China was performed. A total of 26,561 serum samples, including 16,000 swine, 3880 goat, 1662 rabbit, and 5019 cattle sera, were collected from 31 provinces across the country and tested for HEV-Ag using the HEV-Ag reagent and for anti-HEV antibodies using a total antibody detection reagent. About 4.6 % of the pigs, 3.7 % of the goats, 3.7 % of the rabbits, and 1.5 % of the cattle tested in the study were positive for HEV-Ag. The overall prevalence of anti-HEV antibodies in the animals was 81.6 % in pigs, 12.4 % in goats, 53.4 % in rabbits, and 18.7 % in cattle [25, 26, 28, 86]. Some of the HEV-Ag-positive samples were also positive for HEV RNA. The

study demonstrated that HEV infection is widespread in domestic animals, particularly pigs, in China. To further analyze the relationship among HEV RNA, HEV-Ag, and anti-HEV antibodies during natural HEV infection, 256 serial serum samples were obtained from 32 pigs at ages 0 (cord blood), 15, 30, 60, 75, 90, 120, and 150 days. HEV-Ag, anti-HEV antibodies, and HEV RNA were assayed in these samples, and the results show that the total levels of anti-HEV antibodies and anti-HEV IgG formed two peaks. The first peak occurred at 0–60 days and the second after 75 days. No markers of infection, such as HEV RNA, HEV-Ag, or anti-HEV IgM, were detectable during the first peak. Most newborn piglets (<24 h of age) were negative for total anti-HEV antibodies and anti-HEV IgG. However, colostrum from all of the sows had evidence of these antibodies. Thus, the anti-HEV antibodies in the first peak were assumed to be acquired from maternal milk. HEV-Ag and HEV RNA were both positive at the beginning of the second peak. The antibody present during the second peak may be induced by natural infection with HEV [23]. HEV-Ag showed a close relationship with HEV RNA during natural HEV infection.

11.3.5 HEV Nucleic Acid-Detecting Assays

HEV replicates in the liver after infecting the human body, causing damage to the liver and resulting in viremia. During viremia, HEV nucleic acids can be detected in the serum and feces of HEV-infected patients. HEV nucleic acid detection is a direct standard to determine HEV infection, and HEV nucleic acid-positive cases can be diagnosed as having acute hepatitis (Fig. 11.1). HEV nucleic acid detection methods mainly include conventional reverse transcription-nested polymerase chain reaction (RT-nPCR) and real-time RT-PCR assays.

Conventional RT-nPCR has been extensively used in research on hepatitis E. In early studies, primers were mainly designed in accordance with the sequence of genotype 1 [12, 38, 90]. This detection assay showed high sensitivity and specificity for genotype 1 and played an important role in the clinics at that time. However, along with in-depth research into molecular epidemiology of HEV, it was found that there are at least four genotypes of HEV in the world and at least two genotypes in China. The nucleotide sequences of various genotypes share a homology of less than 80 %. The detection assay using genotype 1-specific primers thus will miss the HEV diagnosis in many cases. Since 2000, a series of degenerate primers have been designed according to the characteristics of prevalent strains, which significantly increased the detection rate of various genotypes of HEV [77]. Conventional RT-nPCR assays were usually based on the ORF1 region, including the methyltransferase, proline-rich hypervariable, and RNA-dependent RNA polymerase regions, and the central portion of the ORF2 genomic region [1]. However, conventional RT-nPCR requires complicated procedures, with strict requirements on the environment and facilities, and improper operation of conventional RT-nPCR can result in nonspecific test results due to contamination.

Real-time RT-PCR instantly quantifies specific products by monitoring the changes in the fluorescence signal intensity during the exponential amplification stage of PCR. This approach has high specificity, high sensitivity, and simple operation compared with conventional RT-nPCR [41, 85]. These assays are based on target amplification where the amplified DNA is detected during the PCR process in real time rather than at the end of the process. Two dominant real-time RT-PCR methods that have been reported to detect HEV are a SYBR Green RT-PCR assay and a TaqMan assay. The SYBR Green RT-PCR [63] is a one-step assay that uses shorter primers (15–16 mer) than those typically used in conventional RT-nPCR assays. The short primers raise the potential for detection problems due to the low Tm of the primers and the possibility of nonspecific amplification of nontarget nucleic acids. The TaqMan assays were designed to target the conserved region of ORF3, allowing the detection of different genotypes of HEV without the use of degenerate primers or probes [40, 55, 85]. HEV subtypes share a nucleotide homology of approximately 75 %, and the distribution of variations is generally uniform. Only an approximately 100-bp fragment in the ORF3 region is highly conserved, with a nucleotide homology of more than 90 %. This conserved region has a high GC content, making it difficult to amplify by conventional PCR techniques. Fluorescent PCR amplifies fragments generally smaller than 100 bp, so it is easy to amplify the high-GC region with this method. Therefore, most real-time RT-PCR assays for detecting HEV RNA have designed primers and probes according to this region. Existing PCR reagents vary greatly in performance. A study in 2011 reported that there exists a 100–1000-fold difference in the sensitivity of different reagents, as revealed by the analysis of nucleic acid amplification technology (NAT) detection results of an HEV serum panel in 20 laboratories from different countries. Real-time RT-PCR assays are usually more sensitive than conventional RT-nPCR assays. To compare the sensitivity of different NAT, the international standards of HEV RNA were constructed and calibrated [5].

The detection of HEV RNA can not only be used to identify HEV infection in typical patients, but it can also be used to diagnose HEV infection in immunosuppressed persons (such as organ recipients) who have no or a low response to the HEV infection and correspondingly negative anti-HEV antibody test results. Furthermore, the use of anti-HEV antibody detection is insufficient for identification of hepatitis E patients with extrahepatic manifestations, so the detection of HEV RNA is very important to evaluate the clinical syndrome caused by the HEV infection.

HEV can be transmitted through transfusions. To date, there have been several instances of posttransfusion HEV reported in Japan, the United Kingdom, France, China, and Saudi Arabia [7, 16, 34, 57, 58]. The apparent high seroprevalence of HEV in blood donors (13.7–31 %) has raised a potential concern for blood safety [60, 67]. However, the HEV seropositivity rate in donors does not provide an estimate for the rate of viremic donations, which are more likely to cause infectious exposures to recipients. Studies in Japan and China have identified acute HEV infections in blood donors, confirmed by the detection of HEV RNA [67, 70]. The detection of HEV RNA in donors would be a useful and feasible measure to estimate

the risk of HEV transmission. In addition to the diagnosis of patients with hepatitis E, the detection of HEV RNA has other important uses. For example, the quantitative evaluation of HEV RNA can be used to monitor the efficacy of treatment for chronic HEV infection. The laboratory detection of HEV RNA can be used for the detection of HEV not only in human and animal samples but also in sewage, water, and other environmental specimens. Unfortunately, the strict requirements for staff, environment, and test facilities and the high cost of this method limit its extensive application in developing countries.

11.4 Immunohistochemical Detection of HEV Proteins in Liver Tissue

HEV proteins can be consistently detected in liver tissues from patients with hepatitis E and from animals that were experimentally infected with HEV by using immunohistochemical assays [27, 31, 47, 50]. An immunohistochemical method using monoclonal antibodies raised against two HEV proteins (pORF2 and pORF3) was used for the detection of HEV-Ags in formalin-fixed, paraffin-embedded liver tissues collected post-mortem from the patients of acute liver failure caused by HEV infection. This test is a valuable tool for the detection of HEV infection in biopsy, autopsy, and explant liver tissues [31]. However, this assay has no clinical utility because, as liver biopsy is an invasive procedure, it is usually not performed in patients with acute hepatitis.

A synthetic peptide-derived, polyclonal antibody-based, immunohistochemical test was developed to detect swine HEV and was compared with in situ hybridization for the detection of HEV in formalin-fixed, paraffin-embedded tissues from experimentally infected pigs. The specificity and sensitivity of the test were both 100 %. The liver was the organ that was the most consistently positive for swine HEV-Ag and RNA by immunohistochemistry and in situ hybridization, respectively [50]. HEV-Ags were also detected immunohistochemically in formalin-fixed, paraffin-embedded needle aspirates of the liver and kidney from the HEV-inoculated monkeys using a mouse anti-HEV ORF2 monoclonal antibody [27]. Therefore, the use of immunohistochemistry as a monitoring and diagnostic tool for confirmation of the presence of HEV can be useful for the management of HEV infection.

11.5 Conclusion and Prospective

In the past two decades of HEV research, significant progress has been achieved in understanding the HEV-specific immune responses, antigenic composition of HEV proteins, and development of serological assays. Nonetheless, the existing anti-HEV antibody assays remain suboptimal, particularly in terms of inter-assay

concordance. The main reason for these issues is the different materials used in the procedures, which is due to the heterogeneity of the HEV genome and the diverse antigenic structure of the HEV proteins. For this reason, seroprevalence data derived using different assays for anti-HEV IgG antibodies might not be comparable. Similarly, the predictive accuracy of positive results from various anti-HEV IgM antibody assays for case diagnosis remains poor. Therefore, the development of more reliable assays for both anti-HEV IgG and IgM antibodies and the strengthening of the standardization of diagnostic reagents should be top priorities in hepatitis E research. In clinical diagnosis, although there is complementarity among the clinical markers, they cannot replace one other, and due to the differing immune statuses among individuals, the dynamic changes in these markers are not the same for all patients. Therefore, comprehensive judgments should be made based on a combination of the HEV infection markers and clinical hepatitis manifestations.

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Chapter 12
Treatment of Hepatitis E

Wei Hui, Linlin Wei, Zhuo Li, and Xinhui Guo

Abstract Hepatitis E virus (HEV) infections are the most common cause of acute hepatitis, but they can also take a chronic course. There is no specific therapy for acute hepatitis, and current treatment is supportive. Choosing ribavirin as the first-line therapy for chronic HEV is advisable, especially in solid organ transplant patients. Pegylated interferon-α has been used successfully for treatment of hepatitis E but is associated with major side effects. Cholestasis is one of the most common, but devastating, manifestations in hepatitis E. Current therapy for HEV aims to treat symptoms. Therapy generally involves several measures, such as vitamins for adequate nutrition, albumin and plasma for supporting treatment, symptomatic treatment for cutaneous pruritus, and ursodeoxycholic acid and S-adenosylmethionine, and Traditional Chinese medicine for removing jaundice. Patients with underlying liver disease may develop liver failure. For these patients, supportive treatment is the foundation. Ribavirin has successfully been used to prevent liver transplantation. Prevention and treatment of complications are important for treatment of liver failure. Liver support devices are intended to support liver function until such time as native liver function recovers or until liver transplantation. Liver transplantation is widely considered as irreplaceable and definitive treatment for acute-on-chronic liver failure, particularly for patients who do not improve with supportive measures to sustain life.

Keywords Acute hepatitis E • Chronic hepatitis E • Pegylated interferon • Ribavirin • Treatment • HEV-related liver failure

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Abbreviations

ACLF	acute-on-chronic liver failure
ALSS	artificial liver support system
ELS	extracorporeal liver support
GTP	guanosine 5-triphosphate
HE	hepatic encephalopathy
HEV	hepatitis E virus
ICU	intensive care unit
IFN	interferon
TCM	traditional Chinese medicine

Hepatitis E virus can clinically induce acute and chronic hepatitis. Clinical hepatitis E may sometimes develop into serious consequences and even liver failure. Treatment of clinical hepatitis E has become increasingly important. This chapter reviews the treatment measures used for different types of clinical hepatitis E in the clinic.

12.1 Acute Hepatitis E Treatment

Hepatitis E virus (HEV) is one of the most common causes of acute viral hepatitis worldwide, with an estimated 20 million infections per year. Effective antiviral therapeutics against HEV infections are lacking, despite its worldwide prevalence and association with severe disease. There is no specific therapy for hepatitis E, and treatment for patients with hepatitis E currently is supportive. This disease typically resolves within 4–6 weeks of the onset of symptoms, usually without any long-term consequences [12]. Patients with severe complications of hepatitis E require hospitalization. Hepatitis E may be more severe than hepatitis A [7]. Vulnerable populations, such as pregnant women and persons with preexisting chronic liver disease, should be identified and be provided necessary supportive treatment. To date, immune globulin has not been demonstrated to be effective in preventing hepatitis E in HEV-infected persons, although there is some evidence to suggest that prior infection protects against this disease [6, 35, 52].

Nevertheless, treatment with ribavirin shows significant clinical improvement by reducing the symptomatic period[37].Considering the high mortality caused by acute hepatitis E in acute-on-chronic liver failure (ACLF), ribavirin therapy provides major benefits for those with a poor prognosis or at high risk of fulminant liver failure, such as underlying chronic liver disease. Moreover, a high percentage of immunosuppressed individuals (e.g., human immunodeficiency virus [HIV]-positive individuals or recipients of organ transplants) who eventually develop chronic hepatitis E require early and effective treatment of acute hepatitis E [15, 17, 42, 44, 46, 48].

12.2 Chronic Hepatitis E Treatment

If HEV RNA persists for 3 months, then the patient is unlikely to achieve spontaneous viral clearance without therapeutic intervention [29]. The most important step that should be considered is whether immunosuppression can be reduced. A previous study reported an HEV clearance rate of 25 % by this strategy [4, 24]. However, chances of transplant organ rejection greatly increase when immunosuppression is reduced. Therefore, this situation is referred to as a double-edged sword. Additionally, pegylated interferons have been successfully used at times but are associated with major side effects. These interferons are better options in transplant recipients where reducing immunosuppression is not an option. Another relatively promising option is ribavirin therapy. Ribavirin has been used to successfully treat patients with severe acute hepatitis E with compromised immune systems [48]. There are no convincing data yet on ribavirin to make it a standardized option of HEV treatment. However, two French studies have shown virological responses in two out of two and four out of six patients [26, 39].

Moreover, a retrospective analysis of data from Europe and the United States found that among 85 recipients of solid organ transplants who had HEV infection, 56 (65.9 %) patients developed chronic hepatitis E [27]. The main factor associated with developing chronicity as assessed by multivariate analysis in this study was tacrolimus use for immunosuppression (OR = 1.87; 95 % CI, 1.49–1.97). However, a reduction in immunosuppressive drugs enabled HEV clearance in one-third of the individuals.

When reduction of immunosuppression is impossible or when clearing HEV by immunosuppression cannot be achieved, two alternative therapies for chronic hepatitis E may be pursued: (1) ribavirin monotherapy (dose of 29–1200 mg/d for 1–18 months) [26, 30, 44] and (2) pegylated (Peg) interferon (IFN)-α for 3–12 months [20, 25].

A previous study reported that among 59 patients with chronic HEV after solid organ transplantation, HEV clearance was observed in 95 % of the patients at the end of ribavirin therapy [30]. Additionally, 78 % of the patients achieved a sustained virological response (defined by undetectable serum HEV RNA at least 6 months after cessation of therapy). These patients received ribavirin for a median of 3 months. A longer treatment duration allowed four patients who had recurrence to achieve a sustained virological response. The mechanism of how ribavirin acts against HEV is not clearly understood [31]. However, a recent study suggested that ribavirin exerts an antiviral effect against HEV by depleting intracellular guanosine 5'-triphosphate pools [14]. Further research on this mechanism, as well as other possible mechanisms of action of ribavirin, needs to be performed. Overall data show that ribavirin provides a therapeutic effect in treatment of chronic hepatitis E, with the only major adverse effect of anemia.

Treatment with Peg-IFN-α for hepatitis E has been reported [41, 49]. The duration of therapy ranges from 3 months to 1 year. Most patients are solid organ transplant recipients, and all of them show a favorable outcome in liver enzyme levels,

as well as viral RNA suppression. However, two out of six transplanted patients developed acute allograft rejection after 3 months of Peg-IFN-α therapy [23]. A slight synergistic effect for ribavirin combined with Peg-IFN-α was observed in a recent study in vitro [14]. Successful combination therapy has also been reported for chronic HEV infection in an HIV-positive patient [9]. Decreasing the ribavirin dosage may help reduce anemia and other treatment-associated side effects.

A systematic review indicated that ribavirin monotherapy appears to be an effective and relatively safe treatment for patients with chronic hepatitis E. The choice of ribavirin as the first-line therapy for chronic HEV is reasonable, especially in solid organ transplant patients [18, 22, 43].

Chronic hepatitis E has increasingly become a major clinical problem in immunocompromised individuals [31]. Effective antivirals against HEV are needed. Recent advances with viral replicons and cell culture systems for HEV have led to progress in our understanding of several aspects of the viral life cycle, including HEV's reliance on the ubiquitin proteasome system for replication and particle release. These recent advances in knowledge in these areas combined with an existing knowledge base from other viruses have suggested several potential therapeutic approaches that need to be further studied and used [10, 13, 33, 51].

12.3 Treatment of HEV-Related Cholestatic Hepatitis

Cholestasis is one of the most common, but devastating, manifestations in hepatitis E, especially in older people. Without proper treatment, cholestasis will ultimately result in cirrhosis and hepatic failure.

Current therapy, aiming at treating symptoms, generally involves several measures. These measures include vitamins for adequate nutrition, albumin and plasma for supporting treatment, symptomatic treatment for cutaneous pruritus, and ursodeoxycholic acid or S-adenosylmethionine for removing jaundice.

Wedemeyer et al. showed that short-term steroid medication might be beneficial in HEV-induced acute liver injury. This was especially the case considering that, even in HEV infection, acute liver injury is likely to be immune mediated and not due to viral replication per se [53]. Along this line, recent data have shown non-cytopathic, immune-mediated hepatocyte damage caused by HEV [3, 16].

Traditional Chinese medicine (TCM) is known for its holistic concept, and treatment is based on syndrome differentiation. In TCM, Yin Chen Hao decoction has been widely used in treatment of jaundice. Clinical studies have suggested a significant effect of Chishao at a large dosage for cholestatic hepatitis [21]. In some patients, an artificial liver support system is effective in treating hepatitis E complicated by hyperbilirubinemia on the basis of conventional therapy. An increase in serum levels of total bilirubin can be used to monitor the prognosis of hepatitis E virus infection complicated by hyperbilirubinemia after ALSS treatment.

12.4 Treatment of HEV-Related Liver Failure

Acute viral hepatitis due to HEV is usually an acute, self-limiting illness. However, in two situations HEV may cause serious disease, leading to a high mortality. These situations include pregnant women who may rapidly deteriorate from having HEV to acute liver failure and patients with chronic liver disease who may deteriorate to ACLF. Most deaths from genotype 3 HEV infection are caused by acute or subacute liver failure in patients with pre-existing liver disease. Overall mortality rates of up to 10 % have been reported, most of which were observed in symptomatic cases of HEV infection occurring in patients with comorbidities [28]. When genotype 3 HEV infection occurs in solid organ transplant recipients, it can evolve to cirrhosis and may require liver transplantation [27].

The goals of treatment for hepatitis E are to prevent further deterioration in liver function, reverse precipitating factors, and support failing organs. Liver transplantation is required in selected patients to improve survival and quality of life.

12.4.1 Supportive Treatment

Patients with liver failure require bed rest to reduce physical exertion. Some patients require admission to the intensive care unit to strengthen disease monitoring. They need a proper diet that is high in carbohydrates, low in fat, and has an appropriate amount of protein. For patients with eating disturbances, providing adequate liquid and vitamins intravenously is necessary to ensure that total daily calories are more than 1500 kcal/day. Albumin and/or fresh plasma should be supplied to maintain balance among water, electrolytes, and energy. Drugs should be used for inhibiting inflammatory necrosis and promoting hepatocyte regeneration. The liver microcirculation should be improved and endotoxemia should be reduced. Physicians also need to pay attention to disinfection and isolation and enhance oral care to avoid nosocomial infection.

12.4.2 Etiological Treatment

Although pegylated IFN has been used for treatment of HEV with a good response [23], to patients with liver failure, this drug can cause deterioration of liver function. The current recommendation is to treat these patients with ribavirin because of its satisfactory outcomes [19].

In case series of hepatic failure by genotypes 1 and 3 HEV, ribavirin has successfully been used to prevent liver transplantation [17, 44]. A study from India suggested that ribavirin may be an effective and safe drug for treatment of HEV-ACLF [19].

The investigators of this study treated four patients of HEV-ACLF by ribavirin at a dose of 200–600 mg/d for a median duration of 12 weeks (range, 3–24 weeks). All four patients had undetectable HEV in 3–8 weeks. All of them survived and tolerated ribavirin well without any adverse effects.

The mechanism of action of ribavirin against HEV is unknown. Ribavirin might inhibit HEV replication through depletion of guanosine 5'-triphosphate (GTP) [14]. A study from China [8] showed that acute hepatitis E is associated with more severe disease in patients with chronic hepatitis B virus (HBV) infection and that disease severity correlates with underlying cirrhosis in chronic HBV infection. Anti-HBV treatment cannot improve the prognosis of liver failure caused by HBV-HEV superinfection.

A large retrospective multicenter study showed that ribavirin was efficient for treating patients with solid organ transplantation and chronic HEV infection [24]. Kamar et al. described that the early virological response predicts the virological response to ribavirin in patients with HEV who have organ transplants. These authors found the following: (i) the decrease in viral concentration within the first week of ribavirin therapy was an independent predictive factor for sustained virological response(SVR), and a decreased HEV concentration of 0.5 log copies/ml or greater had a positive predictive value of 88 %; (ii) there were no correlations between ribavirin trough levels on day 7 and at month 2 with the virological response or SVR; (iii) before therapy, HEV RNA levels were significantly higher in patients who received mTOR inhibitor-based immunosuppression compared with those who were provided calcineurin inhibitors. Nevertheless, this immunosuppressive regimen did not affect the response to ribavirin [32].

A study on HEV shedding into the feces of 24 solid organ transplant recipients who were treated with ribavirin for chronic hepatitis E infection highlighted the importance of such HEV excretion as an indicator of treatment failure [1]. This study suggested that testing for HEV RNA in plasma and stool can help determine the duration of antiviral therapy. Patients whose stools were still HEV RNA positive after 3 months of treatment should continue to be treated.

Sebastien Lhomme et al. concluded that the presence of the 1634R variant at ribavirin initiation does not lead to absolute ribavirin resistance. Although its proportion increased in patients for which treatment failed, the presence of the 1634R variant did not compromise the response to a second ribavirin treatment [38].

12.4.3 Prevention and Treatment of Complications

Routine correction of coagulation abnormalities in the absence of active bleeding is rarely indicated [36]. When correction of bleeding abnormalities is required in the presence of active bleeding, thromboelastography, prothrombin time, complete blood count, and activated partial thromboplastin time are used to guide therapy.

Hepatic encephalopathy (HE) is a disturbance in central nervous system function because of hepatic insufficiency. Remove inducement is the key, for instance, gastrointestinal bleeding must be stopped. The intestines need to be emptied of blood. Infections, kidney failure, and electrolyte abnormalities (especially potassium) need to be treated. The mainstay of treatment of HE is the use of lactulose and nonabsorbable antibiotics [34, 40]. The optimal dose of lactulose is not well established; however, titration to two to three semiformed stools per day is recommended.

The current recommendation for treatment of hepatorenal syndrome includes volume expansion with albumin (1 g/kg, maximum 100 g/day for the initial dose, followed by 20–40 g/day) and vasoconstrictors. The goal of treatment is to decrease serum creatinine levels to <1.2 mg/dL.

Because overt signs of infection may be absent, a high index of suspicion is necessary for diagnosis of infection. In patients in whom infection is suspected, early use of broad-spectrum antibiotics is often used. Preferably, antibiotics should be provided within1 h of admission and are highly recommended as is adherence to early goal-directed therapy guidelines [40, 47].

12.4.4 Liver Support Devices

Liver support devices are intended to support liver function until such time as native liver function recovers or until liver transplantation. Liver support devices are categorized into two main types. One type is an artificial liver, using an acellular device, such as albumin dialysis and plasma exchange/diafiltration. The other type of device is a bioartificial device that contains cells from humans, animals, or transformed sources. There have been a total of 74 clinical studies, including 17 randomized controlled trials, on extracorporeal liver support (ELS). These studies included 198 patients with acute liver failure and 157 patients with ACLF. These studies showed that ELS systems appear to improve survival of patients with acute liver failure, but not survival of those with ACLF (risk ratio, 0.87; P = 0.37) [50].

12.4.5 Liver Transplantation

Ramsay et al. reported a case of liver transplantation for acute liver failure because of genotype 3 HEV infection [45]. Liver transplantation is widely considered as an irreplaceable and definitive treatment for ACLF, particularly for patients who do not improve with supportive measures to sustain life. In recent decades, improvement of the survival rate with liver transplants (1- and 5-year survival of 83 and 75%, respectively) has enabled liver transplantation to be a successful therapy for all types of end-stage liver disease [2].

12.5 Evaluation of Antiviral Drugs In Vitro and in Animal Models

Allweiss et al. established an efficient model of HEV infection to test the efficacy of antiviral agents and to exploit mechanisms of HEV replication and interaction with human hepatocytes in vivo. UPA/SCID/beige mice repopulated with primary human hepatocytes were used for infection experiments with genotypes 1 and 3 HEV. Establishment of HEV infection was achieved after intravenous injection of stool-infected animals, but not via inoculation of serum-derived HEV. Finally, 6-week administration of ribavirin led to a strong reduction in viral replication in the serum and liver of GT1-infected mice [5].

Reduction of immunosuppressive therapy, ribavirin, and pegylated interferon has been used with varying success, allowing for viral clearance in up to 78 % of patients. However, failure of ribavirin has been described. Therefore, safer and more effective treatment options are needed. Diet et al. showed that sofosbuvir inhibits the replication of hepatitis E virus GT3 in subgenomic replicon systems, as well as in a full-length infectious clone. Moreover, the combination of sofosbuvir and ribavirin resulted in add-on therapy to ribavirin for the treatment of chronic hepatitis E in immunocompromised patients [11].

12.6 Conclusion

HEV was once considered to induce only acute hepatitis, which is a self-limited disease. Therefore, the treatment of acute hepatitis E was not considered to be important. Since more liver failure and chronic hepatitis induced by HEV have been diagnosed, etiological treatment has become more important. Several antiviral drugs, such as ribavirin and Peg-IFN-α, are effective for hepatitis E. Some TCMs may also be useful for patients. Different treatment measures may be selected for patients with different phases of hepatitis E. Sometimes patients need combined treatment. With the development of culturing HEV in cell lines and the establishment of animal models for clinical hepatitis E, more anti-HEV drugs and treatment measures will be selected and evaluated.

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Chapter 13
Prophylactic Hepatitis E Vaccine

Jun Zhang, Qinjian Zhao, and Ningshao Xia

Abstract Hepatitis E has been increasingly recognized as an underestimated global disease burden in recent years. Subpopulations with more serious infection-associated damage or death include pregnant women, patients with basic liver diseases, and elderly persons. Vaccine would be the most effective means for prevention of HEV infection. The lack of an efficient cell culture system for HEV makes the development of classic inactive or attenuated vaccine infeasible. Hence, the recombinant vaccine approaches are explored deeply. The neutralizing sites are located almost exclusively in the capsid protein, pORF2, of the virion. Based on pORF2, many vaccine candidates showed potential of protecting primate animals; two of them were tested in human and evidenced to be well tolerated in adults and highly efficacious in preventing hepatitis E. The world's first hepatitis E vaccine, Hecolin® (HEV 239 vaccine), was licensed in China and launched in 2012.

Keywords Vaccine • Epitope • Neutralizing antibody • Immunogenicity • Efficacy

Abbreviations

HE	Hepatitis E
HEV	Hepatitis E virus
mAb	Monoclonal antibody
NHP	Nonhuman primate
ORF	Open reading frame
VLP	Virus-like particle

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13.1 Introduction

The awareness of hepatitis E (HE) has increased a lot along with the introduction of better diagnostics and a prophylactic vaccine in recent years [1]. While HEV infection remains as a serious threat to life especially for pregnant women in the developing countries, more and more autochthonous HE cases have been reported in the developed area in recent publications. Good basic sanitation is an effective defense against hepatitis E; however, during the outbreaks in northern Uganda [2] and southern Sudan [3], interventions such as digging latrine, providing clean water, and education on hand washing did not decrease the incidence of new infections. Thus, safe and effective vaccination would be the most efficacious means for the disease control. Since the identification of HEV as a distinct human viral pathogen in 1983 and the first human trial of a hepatitis E vaccine by GlaxoSmithKline (GSK) in 2001, a lot of work has been done to better understand the HE epidemiology and to develop an efficacious vaccine for prophylactic use. As a result, the first commercialized hepatitis E vaccine, Hecolin® (HEV 239 vaccine), was licensed in China and launched in 2012 by Innovax (Xiamen, China) after a large-scale phase III clinical trial [4–6].

13.2 Rationale for Developing a Hepatitis E Vaccine

Anti-HEV antibodies are induced quickly after HEV infection and persist for 14 years or longer in humans. Epidemiologic evidence showed that HEV-infected individuals with naturally induced functional anti-HEV antibodies are protected against severe symptomatic hepatitis E and from viral reinfection [7]. Protective efficacy of candidate HEV vaccines was evaluated in rhesus macaques, which are thought as an excellent challenge animal model. After challenging with clinical HEV isolates of each of the four HEV genotypes, the nonhuman primates develop symptomatic acute hepatitis E with serological, virological, biochemical, and histopathological markers which are similar as those present in human infections [8]. Several lines of evidence from repeated viral challenging experiments, passive immune-prophylaxis, and viral neutralizing activity assessment of the sera from immunized NHPs supported the notion that the presence of functional antibodies is the basis for protection against viral challenge [9–14].

The accumulated results from the past experiments have arrived at the following important conclusions: (1) similar dynamic of antibody responses is elicited by each of the four genotypes of HEV infection [15, 16]; (2) antibodies induced by any of the four genotypes of HEV infection demonstrate HEV-neutralizing activity against different genotypes [17, 18]; (3) immunization with a HE vaccine candidate containing antigen from one genotype or preinfection with one genotype of HEV induces similar protection against subsequent homologous and heterologous genotypes of HEV challenging in animals [19–22]; (4) analogous structural features

harboring functional epitopes are on the virion surface of the four genotypes of HEV [23]; and (5) common cross-neutralization epitopes among different genotypes have been identified [23–25]. Hence, the different HEV genotypes appear to represent a single serotype, and one vaccine seems sufficient to elicit antibodies which are protective against viruses of all four genotypes.

13.3 Neutralizing Epitopes

Concentration of viral neutralizing antibodies induced by vaccination usually correlates with the efficacy of the vaccine. The structural basis of an immunogen to elicit neutralizing and protective antibodies is the neutralizing epitopes. The detailed mapping of neutralizing epitopes on the viral subunits or on the viral capsid would facilitate better understanding the working mechanism of vaccine and aid in the rational refinement of future vaccines. Furthermore, improved epitope-specific antigenicity assays which are useful for more precise and more meaningful process control also depend on deeper understanding of the epitopes. It is extremely important to use multi-faceted and orthogonal assays to gauge the critical quality attributes during vaccine process due to the molecular and structural complexity and potential variations in vaccine products.

Both vaccines that undergone clinical evaluation are based on the pORF2, the sole viral capsid protein. Structurally, pORF2 consists of 660 amino acids with three functional domains, the S domain, P1 domain, and P2 domain. HEV is a non-enveloped virus with a 7.2-kb positive-sense RNA genome that contains only three open reading frames (ORFs). The HEV genome is encapsulated in a single-layered icosahedral capsid. The other two proteins encoded by the other two ORFs are nonstructural. pORF1 is a nonstructural polyprotein that may be involved in the inhibition of type I interferons [26]. Protein encoded by ORF3 was thought to be associated with virus release from host cells [27]. The N-terminal segment with 110 amino acid (aa) residues of the capsid protein pORF2 is rich in positively charged arginine residues. Therefore, it is likely responsible for RNA binding during viral packing. The following segment with 118–313 aa, the S domain, is associated with the formation of the basal shell of the capsid. The middle segment of 314–453 aa is recognized as P1 domain. The last functional domain P2 is composed of 153 amino acids from P454 to A606 and is thought to be related to viral antigenicity and immunogenicity [28]. Until now, all the identified neutralizing epitopes of HEV are conformational and mapped to the P2 domain and reside on the dimeric proteins. Based on the results from different epitope mapping methods, the epitopes recognized by neutralizing mAbs comprise of discontinuous amino acid sequences (Fig. 13.1) [29].

The truncated ORF2 protein (aa 112–607) was indicated to contain most of the antigenic epitope regions of pORF2 [30]. Three antigenic epitope regions were identified, localizing at three peptide segments, namely, aa 25–38, aa 341–354, and aa 517–530. By using synthetic peptides, Zhou et al. demonstrated that the N- (aa 12–147) and C- (aa 573–660) termini of pORF2 make up epitopes [31]. A recombinant

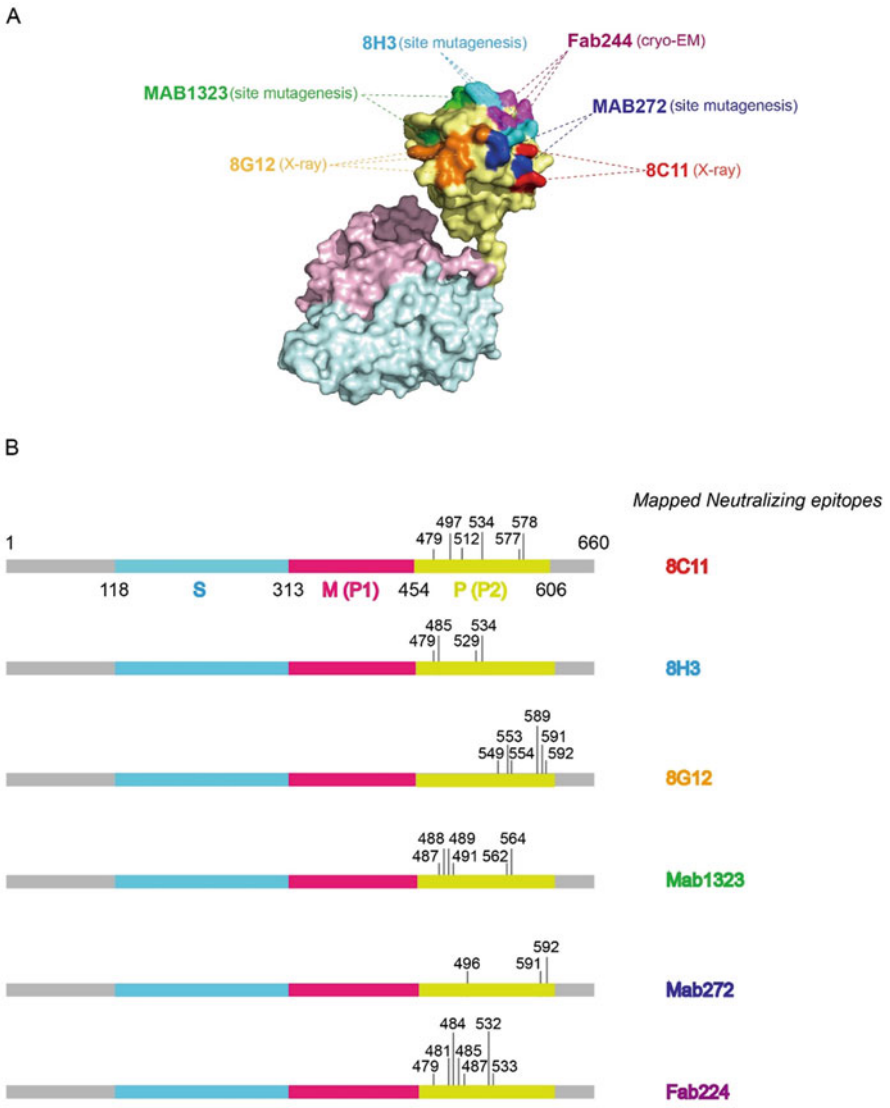

Fig. 13.1 The binding sites of representative neutralizing antibodies on the HEV viral capsid surface. (**A**) The pORF2 monomer is divided into three sections named the S domain (aa 118–313), the P1 domain (aa 314–453) or P domain (aa 320–455), and the P2 domain (aa 454–606) or P domain (aa 456–606), which are shown in color *blue*, *purple*, and *yellow*, respectively. The P2 or P domain is dimeric and harbors all of the identified neutralizing epitopes. The neutralizing epitopes for several neutralizing antibodies are shown in different colors such as E479, D481, T484, Y485, S487, Y532, and S533 for FAB244; S487, S488, T489, P491, N562, and T564 for MAB1323; D496, G591, and P592 for MAB272; E479, Y485, I529, and K534 for 8H3; E479, S497, R512, K534, H577, and R578 for 8C11; and E549, K554, G591, T553, G589, and P592 for 8G12. Ability of the vaccine antigen to these neutralizing mAbs can serve as a surrogate marker for the clinical efficacy of the vaccine. (**B**) Key neutralizing epitopes on P (P2) domain of pORF2. The S domain, M (P1) domain, and P (P2) domain are colored in *blue*, *purple*, and *yellow*, respectively (Reprinted with permission from Taylor and Francis)

capsid protein p166Chn (aa 464–629) was indicated to contain the major antigenic epitopes of pORF2 [32]. Another study identified two immunodominant regions of aa 394–470 and aa 546–580 [33]. Some GST-ORF2 fusion proteins were utilized to identify the antigenic region of aa 394–660 of the pORF2 based on the immunoreactivity with both acute and convalescent sera. On the contrary, a shorter version of the protein with aa 394–473 did not show any substantial binding to these serum samples [34].

The immunodominant epitope with more precise location information was further identified. Using an *E. coli* expression system, a 23 kDa peptide, mapping to aa 394–604 of pORF2, was expressed and formed a dimeric form spontaneously, designated as E2. These homodimers exhibited strong immunoreactivity to sera of clinical hepatitis E patients [35]. Strikingly, the E2 reactivity to serum of HE patients was abrogated when the dimers were dissociated into monomers under harsher experimental conditions. Furthermore, when used as an immunogen, E2 elicited strong protective immune response in animals. Furthermore, the dominant neutralizing epitopes were defined to be localized at aa 458–607 [18]. The initial evidence of neutralizing mAbs recognized as pORF2 was obtained using recombinant mAbs using B cell recloning [11]. Both mAbs recognized the recombinant fragment encompassing aa 112–607 of ORF2 but not the further truncated fragment of aa 112–578. Then, antibodies against short recombinant proteins of ~100 amino acids and a pool of overlapping synthetic peptides derived from pORF2 were developed. These antibodies targeting linear epitope did not exhibit any viral neutralizing activity against HEV in the in vitro assays [36].

Neutralizing epitopes had been endeavored to be characterized with mAbs by cryo-EM (3D-structure reconstruction), X-ray crystal structural determination, and site-directed mutagenesis. The groove region on the surface of the E2s (P or P2) domain was suggested to be the likely surface which directly contact with the neutralizing antibodies. Subsequently, a series of E2 mutants targeting the groove region were expressed, and their binding activity with a neutralizing antibody (8H3) was investigated [25]. The mutants E479A, Y485A, I529A, and K534A independently abrogated the reactivity of mAb 8H3. Based on the first report of the crystal structure of a truncated HEV capsid protein and a dominant type-specific (HEV genotype 1) neutralizing antibody (8C11) [23], the sites of the interaction were determined, comprising of residues E479, S497, R512, K534, H577, and R578 from different peptide segments. Of the residues identified as contact points in the antibody-antigen complex, R512 is the key site for the interaction of the E2s domain with 8C11 and for the neutralization function. This was also confirmed by results from mutational analysis and cell model assays [23]. In addition, the epitope of the other cross-type and highly neutralizing antibody 8G12 was determined via structural analysis [37]. The crystal structures of the E2s (genotype 1) 8G12Fab and the E2s (genotype 4) 8G12Fab immuno-complexes were determined by X-ray crystallography. It revealed identical interacting residues between E2 molecules from two different genotypes of the complexes: E549, K554, G591, T553, G589, and P592. Subsequent site-directed mutagenesis and cell-binding assays further verified the importance of these critical residues at the binding interface. The Fab244 and HEV

VLP-Fab244 immuno-complex were prepared for Cryo-EM determination [38], and the Fab244 was then indicated to recognize a conformational epitope comprising of the residues E479, D481, T484, Y485, S487, Y532, and S533. Another group developed two neutralizing antibodies and identified the corresponding binding sites also at the surface of the P (P2) domain of the capsid protein [39]. The MAB1323 binds to a discontinuous epitope, which consisted of residues, S487, S488, T489, P491, N562, and T564. And MAB272 recognizes an epitope with residues D496, G591, and P592 being the critical contact sites.

All of the key binding sites for neutralizing mAbs were mapped to the E2s domain, further supporting the notion that the E2s domain is a critical region for the virus-host cell interaction. Detailed mapping of the neutralizing epitopes on the E2 protrusion facilitates the characterization of the neutralizing immune response against HEV.

Immunologically, HEV presents as a single serotype, although four major HEV genotypes circulate in human beings. The single serotype may be due to high degree conservation among not only in the amino acid sequences of various HEV genotype capsid proteins but also the three-dimensional structures of the protrusion on the viral capsid. Zhang et al. reported that the neutralizing antibodies in patient serum samples during the HEV convalescent phase were predominantly associated with dimeric, *not the monomeric*, form of the capsid protein [10]. Viral capsid proteins from different HEV genotypes exhibited highly analogous structural features in the protrusion domains (E2s domain) [25]. X-ray crystallographic studies of two immuno-complexes of E2s (from genotypes 1 and 4) and a cross-genotype neutralizing mAb 8G12-Fab showed identical binding scenarios in the crystal structures at the antigen-antibody interface. Furthermore, the mAb 8G12 predominantly outcompetes naturally acquired anti-HEV serum covering genotypes 1, 3, and 4, which supports the single serotype in HEV immunology [37]. The unique structural and immunologic feature of HEV evolution contributes to the single serotype of this virus. This serves as the scientific basis for the vaccine of a single serotype affords protection from infection of all four HEV genotypes.

13.4 Assembly of Virus-Like Particle

Like most of other viral capsid proteins, recombinant pORF2 could self-assemble into a virus-like particle (VLP) [40, 41]. Different truncated versions of the pORF2 can form different sizes of particles. Although certain degree of heterogeneities exists in the assembled particles, most VLPs retain natural epitopes. The polypeptide of aa 112–608 of HEV pORF2 can form T = 1 VLPs, whereas the protein containing aa 14–608 forms predominantly icosahedral T = 3 VLPs. The latter VLPs are highly analogous to the native virions morphologically [39, 42–44]. The p239, containing aa 368–606 of pORF2, can automatically assemble into VLPs with a diameter of 20–30 nm. While polymorphism and irregularity existed in the particle assemblies, the clinically relevant epitopes seem to be properly presented as high

protective efficacy of p239-based vaccine was demonstrated clinically as well as preclinically [20, 41, 45]. The overall structures of the more regularly shaped VLPs (T = 1 and T = 3) have been characterized using cryo-EM and X-ray crystallographic analyses [39, 42–44]. The full length of ORF2 protein can be divided into three parts: the S domain (aa 118–313), the P domain (aa 320–455) or P1 domain (aa 314–453), and the P domain (aa 456–606) or P2 domain (aa 454–606) [39, 42] [15, 78] [39, 42] [39, 42]. The P2 or P domain is localized on the outer surface, named E2s, harboring most of the functional epitopes.

To develop a viable bioprocess for vaccine production, forming the native-like epitopes on the surface of VLPs is the key point. The elicitation of protective neutralizing antibodies depends on the native-like epitopes presenting on the VLP surface and the correct antigen conformation. High local antigen concentration (depot effect of the adjuvant) and the regularly arrayed and high density of epitopes on the bionanoparticles could account for the effectiveness in stimulating protective humoral response. Characterization of the VLP antigen is essential for process control, especially for the morphology and size, to assure lot-to-lot consistency. Various biochemical and biophysical methods are used to characterize the VLPs' properties, including dynamic light scattering (DLS), high performance liquid chromatography (HPLC), transmission electron microscopy (TEM), analytical ultracentrifugation (AUC), Cryo-EM, and atomic force microscopy (AFM). In addition, different immunological tools such as mAbs, pAbs from sera of HEV-infected sera, or vaccines are critically important to define the antigenic properties of the vaccine antigen. The binding activity of the vaccine antigen can serve as a surrogate marker for clinical efficacy.

13.5 Hepatitis E Vaccine Candidates

The development of an effective HEV vaccine was a long journey [6, 46]. The development of a traditional inactivated or live-attenuated vaccine HEV is infeasible because of lacking of an efficient cell culture system for HEV. Therefore, designing a recombinant antigen such as in the case of the highly successful Hepatitis B virus and human papillomavirus vaccines became the most practical approach. Different expression systems such as bacteria, mammalian cells, insect cells, or plant cells have been used; the antigen has always targeted the capsid protein pORF2. Different versions of pORF2 proteins were made, and those that have undergone tests in NHP challenge models are summarized (Table 13.1).

13.5.1 Trp-C2 Protein

The first report of the protective potency of a HEV vaccine candidate was published in 1993 [47]. A recombinant chimeric protein, designated trpE-C2, which contained aa 221–660 of pORF2 was produced in *E. coli*. After three doses, anti-HEV

Table 13.1 Characteristics of hepatitis E vaccine candidates being evaluated by primate challenging models

Designation	Original genotype	Type of vaccine	Expression system	Location in pORF2	Antibody binding	T cell recognizing	Challenge model	Antibody response	Challenge genotype	Challenge outcome
Trp-C2	1	Recombinant protein + alum	*E. coli*	221–660	Patients' serum	Unknown	Cynomolgus macaque	Undetectable after two doses, vigorous after the third dose	1 and 2	Partially protective
E2	1	Recombinant protein + CFA/IFA	*E. coli*	394–606	Patients' serum + neutralizing mAbs	Unknown	Rhesus monkey	Vigorous	1	Completely protective against hepatitis but not infection
HEV 239	1	rVLP + alum	*E. coli*	368–606	Patients' serum + neutralizing mAbs	PBMCs isolated from patients' and immunized mouse	Rhesus monkey	Vigorous	1 and 4	Completely protective against hepatitis; highly protective against infection*
56 kDa	1	Recombinant protein + alum	Baculovirus	112–607	Patients' serum + neutralizing mAbs	Unknown	Rhesus monkey	Vigorous	1, 2 and 3	Completely protective against hepatitis; weakly protective against infection*

T1-ORF2 (56 kDa)	4	rVLP + alum	CHO cell	126–621 (corresponding to 112–607 of genotype 1)	Unknown	Unknown	Rhesus monkey	Vigorous	1 and 4	Completely protective against hepatitis and infection after challenging with 5×10^4 genome copies virus. Not protective after challenging with 5×10^5 genome copies virus.
53 kDa	1	rVLP + alum	Baculovirus	112–578	Do not bind with two neutralizing Fabs	Unknown	Rhesus monkey	Vigorous	1	Not protective against hepatitis; reduce virus replication
62 kDa	1	Recombinant protein + alum	Baculovirus	112–660	Unknown	Unknown	Cynomolgus macaque	Vigorous	2	Protective against hepatitis and infection

(continued)

Table 13.1 (continued)

Designation	Original genotype	Type of vaccine	Expression system	Location in pORF2	Antibody binding	T cell recognizing	Challenge model	Antibody response	Challenge genotype	Challenge outcome
rHEV VLP	1	rVLP (Oral)	Baculovirus	112–608	Patients' serum	Unknown	Cynomolgus macaque	Vigorous	1	Protective against hepatitis and infection
pcHEVORF2	1	DNA (Gene-Gun)	NA	1–660	NA	NA	Cynomolgus macaque	Vigorous	2	Completely protective against hepatitis and infection
Lipo-NE-DP	1	DNA primary, recombinant protein boost	NA, *E. coli*	458–607	NA	NA	Rhesus monkey	Vigorous	1	Completely protective against hepatitis and infection
Lipo-NE-DP	1	DNA primary, recombinant protein boost	NA, *E. coli*	458–607	NA	NA	Rhesus monkey	Vigorous	1	Completely protective against hepatitis and infection

*Highly protective refers to protection rates of 75 % or more in animals vaccinated and challenged afterwards
CFA Complete Freund's adjuvant, *CHO* Chinese hamster ovary, *IFA* Incomplete Freund's adjuvant, *mAb* Monoclonal antibody, *NA* Not applicable, *PBMC* Peripheral blood mononuclear cells, *rVLP* Recombinant virus-like particle

antibodies were induced in two cynomolgus macaques. Animals were protected from developing symptomatic hepatitis after challenging with pathogenic dose of homologous or heterologous HEV [47]. Subclinical infection failed to be prevented in one monkey. There has been no follow-up report on the further development of this candidate vaccine.

13.5.2 56 kDa Proteins

Expression of the full-length HEV ORF2 in insect cells infected by a recombinant baculovirus produced a 72 kDa protein, which is rapidly cleaved into 53 kDa, 56 kDa, or 63 kDa polypeptides [48, 49]. The 53 kDa form, encompassing aa 112–578, was secreted as VLPs, although it existed in monomeric form when purified. The 53 kDa protein induced strong anti-HEV immune response in monkeys; however, protection from developing hepatitis was not observed post challenging with 10,000 monkey infectious doses (MID50) of a genotype 1 virus strain SAR55 [50]. In comparison, monkeys vaccinated with the 56 kDa protein, encompassing aa 112–607, were completely protected from viral infection when challenged with the same dose of the SAR55 strain [9]. One surprising result was that the titers of anti-HEV antibodies induced by both proteins were comparable [50]. Subsequently, rhesus monkeys were vaccinated with two doses of 50 μg, 10 μg, 2 μg, or 0.4 μg of the alum-adjuvanted 56-kDa protein [51]. High levels of anti-HEV titers were induced. All animals were protected from developing hepatitis when challenging with pathogenic dose of SAR55 strain or a genotype 2 strain. Protection was not observed if vaccination occurred after the virus challenging. The protection against hepatitis persisted for at least 12 months after 2 doses [52]. A third dose of vaccine administered 5 months later boosted anti-HEV titers to the same levels achieved after the second dose, suggesting that a vaccination schedule at 0, 1, and 6 months would be reasonable for achieving durable antibody presence.

Based on these promising results on NHPs model, vaccine formulation of alum-adjuvanted 56 kDa protein was developed by GlaxoSmithKline (GSK) for additional preclinical studies [19]. NHPs were vaccinated once (10 μg) or twice (1 μg or 10 μg) within 4 weeks between administrations. Robust antibody responses were observed in all animals. Animals that received two vaccine doses were completely protected against hepatitis after challenging with 10,000 MID50 of the virus. This is not the case for animals in the group for which single shot was given. The two-dose vaccination protected most of the animals from viral infection (17/23), while the single-dose schedule was less protective (3/12). No significant differences were observed in the efficacy against the different HEV genotypes. The results indicated that at least two doses of vaccine were essential to achieve optimal protection, and vaccine which originated from one genotype is sufficient to protect against hepatitis caused by different genotypes of HEV.

Using a different expression platform, a similar 56 kDa protein (T1-pORF2) encoded by HEV genotype 4 T1 strain produced in Chinese hamster ovary cells

[53]. Monkeys vaccinated with T1- pORF2 were completely protected from hepatitis and viral infection after challenging with 50,000 genome equivalents of genotype 1 or 4 HEV. Separately, another similar recombinant VLP, designated as rHEV VLP, encompassing aa 112–608 of pORF2 from a Burmese HEV strain (genotype 1), was assembled in insect cells [54, 55]. Two cynomolgus macaques which orally received five 10-mg doses of unadjuvanted rHEV VLPs developed anti-HEV IgM, IgG, and IgA responses. After being attacked with >10,000 MID50 homologous Indian strain (genotype 1), one of them was fully protected from both hepatitis and infection, while the other one was protected only from hepatitis but failed from HEV infection.

13.5.3 HEV E2 Protein and HEV 239 VLP

The HEV E2 protein consists of amino acids 394–607 of pORF2 of a genotype 1 Chinese HEV strain expressed in *E. coli*. The purified HEV E2 protein self-associates into dimeric form. The binding of E2 with sera from patients with hepatitis E and neutralizing mAbs is mainly directed against the dimeric form of the protein [35]. Immunization of rhesus macaques with HEV E2 in Freund's adjuvant resulted in protection from infection in a viral challenge experiment [56]. However, highly purified HEV E2 without Freund's adjuvant was poorly immunogenic in mice and monkey [20]. To improve the immunogenicity, efforts were made to create a particulate antigen. One specific version showing particulate nature with enhanced immunogenicity, designated as HEV 239, contains aa 368–606 of pORF2 with an N-terminal extended version of HEV E2 [57].

The dimeric form of HEV 239 protein assembles into ~23 nm particles. HEV 239 VLP was reactive with sera from hepatitis E patients as well as a panel of neutralizing monoclonal antibodies. Comparative studies in animals demonstrated that HEV 239 VLP is far more immunogenic than the HEV E2 protein and induces a vigorous T-cell response [58]. The vaccine formulation of alum-adjuvanted HEV 239 VLPs was then developed by Innovax (Xiamen, China). In a NHP model, two doses of 5-, 10-, or 20-μg HEV 239 vaccine elicited similar anti-HEV titers. Vaccinated monkeys were completely protected from hepatitis and infection when challenged with 10,000 copies of HEV of either homologous genotype 1 or heterologous HEV genotype 4 [20].

13.6 Clinical Trials of Candidate Vaccines

Two most promising vaccines in animals were tested in human. One is the baculovirus-expressed 56 kDa protein and the other is *E. coli*-expressed HEV 239 protein. The latter was licensed with a trade name Hecolin® in China and launched in 2012 [59].

13.6.1 56 kDa Vaccine

The 56 kDa antigen is adsorbed to 0.5 mg of aluminum hydroxide adjuvant in 0.5 ml of buffered saline. The phase I trial showed that the vaccine is well tolerated and immunogenic, although the 1-μg doses resulted in lower seroconversion rates [60]. Subsequently a double-blind, placebo-controlled phase II trial was conducted in Nepal, an area endemic for HEV genotype 1. The study involved 2000 healthy adults with low or undetectable anti-HEV titers, aged from 18 to 62 years and mostly men (99.6 %). Participants were randomly assigned to receive three 20-μg doses of 56 kDa vaccine or placebo with 0, 1, and 6 months schedule and were followed for a median of 804 days [29]. The vaccine was well tolerated with pains at the injection site being the most common adverse event. No serious adverse events related to vaccination were reported. Among all the participants seroconverted after receiving three doses of the vaccine, half of them lost their antibodies 2 years later. The vaccine showed 95.5 % (95 % CI, 85.6–98.6) efficacy with respect to prevention of hepatitis E in a per-protocol analysis of participants who received all three vaccine doses subjects and of 88.5 % (95 % CI, 77.1–94.2) in an intention-to-treat analysis of those who had received at least one dose. During the interval period between the second and third dose, one vaccine in the vaccine group and seven in the placebo group contracted hepatitis E, suggesting that protection might be achieved after at least two doses of vaccination, although the difference was not statistically significant. The protection against subclinical HEV infection had not been assessed in this study. Additionally, the efficacy of the vaccine against diseases caused by heterogenic HEV was unproved, as only genotype 1 HEV circulated in the study area. The vaccine has not been licensed, and no further development has been undertaken.

13.6.2 HEV 239 Vaccine

The HEV 239 vaccine contains 30-μg HEV 239 particles adsorbed to 0.8 mg (Al (OH3)) of aluminum hydroxide suspended in 0.5 ml of phosphate-buffered saline. The immunization schedule is 0, 1, and 6 months by intramuscular injection.

The phase I trial of the HEV 239 vaccine involved 44 seronegative adults. All the participants received two 20-μg doses of vaccine at 0 and 1 month. The vaccine was well tolerated, with no report of serious adverse events. No clinically meaningful changes in blood biochemistry parameters were found [6].

Subsequently, 155 seronegative volunteers aging from 16 to 18 were randomized to receive three 10-, 20-, 30-, or 40-μg vaccine formulations at 0, 1, and 6 months [4]. The vaccine was safe and all the participants seroconverted. The antibody titers showed a dose-dependent manner from 10.1 to 23.4 WHO U/ml along with the increasing of vaccine doses from 10 μg to 40 μg.

In another component of phase II trial, 457 seronegative adults were randomized assigned into three groups: subjects in the control group were given 5 μg of hepatitis B vaccine; subjects in the 2-dose group were given two doses of 20-μg vaccine formulations at 0 and 6 months; subjects in the 3-dose group was given three doses of 20-μg vaccine formulations at 0, 1, and 6 months. The seroconversion rates were 100 % and 98 % in 3-dose group and 2-dose group, respectively. The peak antibody levels elicited by three doses of vaccine were two times higher than that of two doses. Furthermore, both two- and three-dose schedules showed approximately 85 % efficacy against infection [4].

The vaccine efficacy against hepatitis E was assessed in a large-scale, double-blind randomized phase III clinical trial conducted in Jiangsu Province, China, where endemic circulation of HEV genotype 4 predominates over genotype 1 [5]. The trial involved 112,604 healthy adults between 16 and 65 years of age; about half of them had detectable anti-HEV on day 0. They were randomly assigned to receive three doses of either the HEV 239 vaccine or control vaccine (hepatitis B vaccine).

A vaccine efficacy of 100 % (95%CI, 72–100) in the per-protocol cohort was demonstrated in participants who received three doses of HEV 239, with no cases of hepatitis E developed during the 12 months from 1 month post receiving the final HEV-239 dose, compared with the 15 cases hepatitis E appeared in the control group of volunteers in the same time period. For the participants who received at least one dose (intention-to-treat cohort), the vaccine efficacy was 96 % (95 % CI, 66–99). Similar to the observation made in the Nepal trial of the 56 kDa vaccine, two doses of the HEV 239 vaccine with about 1-month interval provide immediate and complete protection against hepatitis E for at least 5 months until the third dose of vaccine was administered, with efficacy of 100 % (95 % CI 9–100). Hence, vaccination for rapid control of epidemics is justified. Long-term follow-up of all the participants for 55 months since enrollment showed persistent protection against hepatitis E with overall vaccine efficacy of 93 % (95 % CI, 79–98) in the per-protocol cohort and 87 % (95 % CI, 71–94) in those who received at least one dose of vaccine [61]. Besides efficacious against hepatitis E, the HEV 239 vaccine was evidenced to reduce the risk of overall HEV infection, mostly asymptomatic. The overall per-protocol efficacy was 79 % (95 % CI, 68–87). Overall efficacy in subjects who had received at least one dose of vaccine (intention-to-treat analysis) was 77 % (95 % CI, 65–85). Notably, in spite of the genotype 1 originated vaccine antigen used, the majority of HEV isolates from the hepatitis E patients were genotype 4, indicating that the p239 vaccine provides cross-protection against HEV genotype 4.

Three doses of HEV 239 vaccine elicited robust anti-HEV antibody responses in 99.9 % of the subjects without preexisting immunity, with the peak anti-HEV titers of 15 WHO units/ml on month 7; one month after the last vaccination, 87 % of those remained seropositive on month 55, although the titer decreased to 0.27 WHO units/ml. A well-fitted modified power-law model predicted that half of the seronegative vaccines will remain at detectable antibody level for over 30 years after receiving three doses of HEV 239 vaccine, and there would be a long-term plateau of geometric mean concentration (GMC) of anti-HEV IgG, with a 13-year median duration of detectable antibody in this cohort [62].

The seroconversion rates were similar in the pre-negative participants who received less doses (1 or 2 doses) of the vaccine. The antibody levels induced by two vaccine doses were slightly lower than that induced by three doses of the vaccine. The mean anti-HEV level of the seropositive subjects could be boosted to 50 times higher from 0.5 WHO units/ml to 24 WHO units/ml after HEV 239 vaccination.

The HEV 239 vaccine was supposed to be very safe and well tolerated after the large-scale clinical trials with vaccinating more than a hundred of thousands of volunteers. In the phase III trial, the participants of reactogenicity subset comprised 1316 and 1329 subjects in the HEV 239, and control groups were actively followed for adverse events. More local reactions in the HEV 239 group were reported than the placebo group (13.5 % vs. 7.1 %, $p < 0.0001$), of which pain and swelling with itching at the injection site were the main adverse reaction. Reported systemic adverse event (AE) rates were similar between the two groups (20.3 % vs. 19.8 %). Adverse events of grade 3 or higher were reported very rarely. For the whole vaccinated cohort, there was no significant difference in the rates of unsolicited AE or SAEs for the two groups within 30 days of vaccination with each dose. The occurrence of hospitalization and death among all the participants in the two groups was similar during the whole study period; none of these events were estimated to be vaccine related by the Data Safety Monitoring Board.

Pregnancy was one of the exclusion criteria for the phase III trial; while the status of pregnancy was confirmed orally by the female participants instead of the urine pregnancy test, 37 and 31 women who were pregnant inadvertently received the HEV 239 or the control vaccine, respectively. The recorded adverse reactions in these women were similar between the groups and similar to those reported by non-pregnant women. The gestational ages, weights, and lengths of the babies born to the mothers in the two group were comparable. Therefore, these preliminary data suggested that the vaccine is safe for pregnant women, although further studies are needed [63].

Until now Hecolin® was available only in the private market of China, and nearly 300,000 doses of Hecolin® have been distributed after the licensure of the vaccine; the actual doses vaccinated are not clear. Only one case of local adverse event was reported during the post-licensure usage, although the completeness of the report is unclear.

13.7 Critical Quality Attributes of HEV239 Vaccine

A set of quality assessment assays were put in place to support commercial phase production and stability studies. The biophysical, biochemical, immunochemical methods and immunological methods were used to assess the process reproducibility and product consistency of the HEV 239 vaccine. The critical quality attributes of the HEV239 vaccine are summarized in Table 13.2.

Table 13.2 Analytical toolbox for comprehensive characterization of HEV 239 antigen to demonstrate process reproducibility and product consistency during the production of Hecolin®

Method	Characteristics	Parameters of HEV 239
Biochemical methods		
SDS-PAGE	Purity, integrity, molecular weight (kDa)	29.32 ± 0.08
MALDI-TOF MS	Molecular weight (Da)	25,561.64 ± 1.37
LC-MS	Peptide mapping (coverage,%)	100 (three lots)
icIEF	pI, whole column detection capillary isoelectric focusing	6.55 ± 0.03
Biophysical methods		
HPSEC	Size (as reflected by retention time, min)	14.02 ± 0.08
TEM	Size, morphology	20–30 nm particles with certain degree of irregularity
DLS	Hydrodynamic diameter (nm)	28.92 ± 0.20
AUC	Sedimentation coefficient (S)	21.42 ± 0.13
DSC	Thermal stability (°C)	75.58 ± 0.10
Cloud point	Aggregation propensity (°C)	71.83 ± 0.16
CD	Secondary structure	Consistency of secondary structure including α-helices and β-sheets among the lots
UV	Trp/Tyr absorption	Consistent with the UV spectrum of a globular protein with a maximum absorption wavelength at 280 nm
Immunochemical methods		
SPR with mAbs	Affinity to mAbs	Binding activity to five mAbs for different batches of aqueous products with RU(Ag)/RU(Ab)
Sandwich ELISA	Antigenicity	8C11 (capture) and 8H3 (detection) to analyze the consistency of the antigenicity among different batches of p239 aqueous products
Immunological assessment		
Mouse potency	Immunogenicity ED_{50} (μg)	0.025–0.060

13.7.1 Biochemical Methods

Analyses on recombinant protein antigen were carried out to determine the antigen integrity, antigen purity, pI, molecular weight, and protein sequence [45, 64]. The major band on the SDS-PAGE gel indicated an apparent molecular weight (MW) of 29.4 kDa. Data from MALDI-TOF-MS also showed MW values which are close to the theoretical MW of HEV 239. Additionally, the overall amino acid sequence was confirmed using LC-MS-based protein mapping (w/tryptic digestion) with 100 % sequence for all three batches [45].

13.7.2 Biophysical Methods

The HEV 239 VLPs, with certain degree of irregularity and heterogeneity, were found to be 20–30 nm in diameter by TEM [45]. HPSEC analysis showed nearly identical retention times for all the six different lots of HEV 239 products [45]. The AUC profiles demonstrated highly similar sedimentation properties for the six lots [45]. Both UV spectroscopy and circular dichroism methods indicated the consistency of the secondary and tertiary protein structure among the different vaccine lots [45]. The thermal unfolding of the VLP antigen was analyzed by monitoring the T_m values using DSC and confirmed good lot consistency [45, 64]. In addition, the profiles of heat-induced unfolding or aggregation of the VLPs were also highly consistent among all six lots, as determined by both cloud point and DSC.

13.7.3 Immunochemical Methods

Binding activity of different antigen batches to different mAbs was tested. To assess the immunoreactivity of the different antigen batches, the label-free and real-time sensor chip-based SPR method was implemented with five mAbs recognizing different epitopes. With the desired robustness and reproducibility [45], a sandwich ELISA was used to show product consistency in a manufacturing setting [45]. A well-characterized protective neutralizing mAb, 8C11, was used as the capture antibody [41], and 8H3 labeled with HRP was selected as the detection antibody.

13.7.4 Immunological Assessment

In vivo potency of the vaccine was assessed by measuring the ED50 in mice, six batches of amorphous aluminum-based adjuvant absorbed HEV 239 were evaluated, and data suggested comparable ED50 values in the range of 0.025–0.060 μg. Two of the assays including the mouse potency assay (ED50 determination) and the SDS-PAGE are used for routine lot release testing on the final vaccine formulation and filled product. Full antigen recovery was observed with no alterations on antigen characteristics after post-adjuvant dissolution [64].

13.8 Target Populations

The severity of hepatitis E as a public health problem in many developing countries and in certain population groups of developed countries has been recognized. A hepatitis E vaccine could become an effective means in the prevention and control of HEV-related diseases and in reducing mortality particularly in pregnant women.

The first public health target of the vaccine use is to lower the number of hepatitis E-associated maternal deaths. Hepatitis E case fatality can be as high as 20 % among pregnant women [65–68]. The number of hepatitis E-associated maternal deaths was estimated to be nearly 1180 (~10 % of pregnancy-associated death) in Bangladesh each year [69]. This corresponds to ~10,500 HEV-associated maternal deaths annually in Southern Asia if the proportion is representative. However, the data of the safety and effectiveness of the vaccine in pregnant women is very limited, and it is unlikely to be obtained in the near future because of the huge ethnical and practical difficulty on the conduct of a clinical trial in this population group. Hence, the best way to prevent the maternal HEV infection could be to vaccinate the child-bearing women.

Patients with preexisting chronic liver disease can develop acute hepatic failure with very high fatality rate when superinfected with HEV [70, 71]. It had been documented that cirrhotic patients with preexisting chronic hepatic disease were prone to HEV infection which could lead to rapid deterioration of hepatic reserve and high fatality [72]. Also, an increased risk of contracting hepatitis E had been documented in hepatitis B virus (HBV) carriers. The same phenomenon has been recognized with hepatitis A virus (HAV) or HBV superinfection of such individuals and was the basis of the current recommendation that they should be vaccinated against HAV and HBV [73].

In genotype 3 and 4 endemic countries/areas, hepatitis E commonly affects the elderlies. Recent study in China showed that ~65 % sporadic hepatitis E occurred in persons aged 50 or older. HEV infection is the most common cause of acute hepatitis in elderly people, surpassing hepatitis A viral infection in recent years [73]. Outbreaks of hepatitis E, causing fulminant hepatitis and deaths in nursing house for elderly persons, were documented in China. Vaccination in elderly community, especially for those who lived in rural areas popular with pig farming, might be with a highly cost-effective way of reducing the disease burden due to HEV infection.

A limited number of transfusion-transmitted HEV infections have been documented, but recent studies indicate a more frequent occurrence. The prevalence of blood donations containing HEV RNA was in roughly one in twenty to ten thousands [74–79]. Transfusing of contaminated blood and blood components to recipients with immunodeficiency, mostly solid organ transplant recipients, will likely lead to chronic HEV infection [80–82]. Death is a likely outcome for a patient with chronic liver failure requiring transplantation, after infected with HEV [82–89]. Therefore, implementing hepatitis E vaccination in the blood or stem cell donors should be considered.

It is difficult to determine the risk of infection among international travelers. Recent data showed that travel-related hepatitis E contributes to 28 % of reported cases in England and Wales [90]. International travelers in outbreak settings might be at a higher risk of HEV infection [91]. Hepatitis E has occurred among international health workers providing assistance during hepatitis E outbreaks in Africa refugee camps. Recently, the WHO recognized the high risk of HEV infection for travelers, healthcare, and humanitarian relief workers traveling to hepatitis E out-

break areas and recommended that vaccination should be considered in such circumstances [92].

Large epidemics resulting in thousands of cases and death are common in Asia and Africa, particularly among people living under unsanitary conditions. If provision of safe drinking water and improvements of sanitation could not be provided at a level that halts transmission, the epidemics may be prolonged to 1 or 2 years [93]. In such settings, the use of the vaccine to control or mitigate outbreaks, particularly to protect high-risk groups such as child-bearing women, should be considered with priority [92].

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