Hepatitis E Virus: Molecular Mechanisms and Opportunities for Diagnostics

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Summary

Hepatitis E Virus infection is an emerging cause of acute viral hepatitis and gastro-enteritis around the world. It is believed that every year about 10,000 people die because of the consequences of an infection with this virus. As early as 1983, a researcher voluntarily infected himself with HEV to study the pathogenesis (Balayan *et al.,* 1983). Despite this effort, nowadays still only limited knowledge of HEV is present. Recently, a cell culture infection system has become available which can lead to a breakthrough in our knowledge on this virus. Here we present an overview of the present understanding of HEV in which we focus on the molecular mechanisms of this threat, the technical possibilities of and the challenges in diagnosis of present or recent HEV infection.

Introduction

A large outbreak of acute viral hepatitis in New Delhi 1955-56 was retrospectively found to be caused by the, until then, unknown hepatitis E Virus (HEV). Clinically this virus resembles hepatitis A, however, even though many clinical features of these viruses are similar, these viruses are genetically unrelated and belong to different families. During the past few decades much knowledge has been generated on epidemiological and clinical features of infection with the hepatitis E virus, however, due to the absence of an efficient cell culture system other characteristics, such as molecular mechanisms, are still largely to be elucidated. This lack of knowledge resulted in a more difficult and less efficient development of diagnostic tools and vaccines. Nowadays, several serology- and nucleic acid-based diagnostic tests have been developed, although their accuracy is regarded as not yet optimal. This review deals with general features of the HEV, in particular the clarified molecular mechanisms and the struggle to develop efficient diagnostic tools.

Classification

The mammalian hepatitis E virus is the only member of the genus *hepevirus*. This species, together with the avian hepatitis E virus in an unassigned genus, are the only species present in the family *hepeviridae* (International Committee on Taxonomy of Viruses *et al.,* 2009).

Only one serotype has been reported, however, due to the high genetic variability, mammalian isolates have been categorized into 4 major genotypes (1-4), based on full-length genomic sequences. Although more precise classifications in genotypes and subgenotypes have been proposed, no standardization has been accomplished and therefore, we will describe the present four genotypes only.

Using the neighbor joining method (as first described by Saitou *et al.,* 1987), a phylogenetic tree has been created, based on the full sequences of 75 HEV isolates (figure 1). Within each of the genotypes a variability exists of up to 11.8%, 19.3% and 17.0% for genotypes 1, 3 and 4, respectively (Okamoto, 2007). For genotype 2 only one full genome sequence was available at the time of this study.

To elucidate the evolutionary history of HEV, a Bayesian analysis has been performed on the non-overlapping region of open reading frame 2 (ORF2). The Time to Most Recent Common Ancestor (TMRCA) for all genotypes ranged from 536 to 1344 years ago (Purdy *et al.,* 2010). This MRCA gave rise to a human-based and a non-human based species. Subsequently, these species split up and created genotypes 1-4, which eventually gave rise to all modern detected HEV isolates (figure 2). The predecessor of genotypes 1 and 2 developed differently from the predecessor of genotypes 3 and 4. The geographic distribution and several viral features of these genotype pairs are similar.

Epidemiology

Although the virus is relatively unknown, HEV seropositivity is globally present and endemic in large parts of Africa, Latin-America and Asia (Figure 2, Teo, 2011). However, the use of ELISA's based on genotype 1 and 2 derived antigens may result in a lower accuracy of these tests for detecting antibodies against genotype 3 and 4 antigens (Bendall *et al.,* 2010). The data shown in figure 3, and other similar data for HEV, are therefore believed to be an underestimation for

Figure 1. Phylogenetic tree of HEV-isolates. Full-length or nearly full-length sequences of 75 HEV isolates, of which 54 human-, 8 swine-, 4 boar-, 1 mongoose- and 1 deer-isolates, were used to generate this tree by the neighbor-joining method. After the isolate name and GenBank accession number the country of isolation is depicted. Isolates from pigs, wild boars, deer and mongoose are incidated by one, two three or four asterisks, respectively. The bar indicates 0.1 substitutions per site. Numbers 1, 2, 3 and 4 specify each of the genotypes. (Okamoto, 2007)

genotypes 3 and 4.

According to estimations about 2 billion people live in regions in which HEV is endemic (Chandra *et al.,* 2008b). In general, HEV epidemics occur in regions with suboptimal sanitary conditions, in which drinking water is mixed with sewage. Hence, a feco-oral infectious route is considered the main route of transmission, although other routes are also possible (see section 3.3 transmission).

The four different genotypes, which are able to infect humans, are differently distributed across the globe and seem to have different characteristics. Genotypes 1 and 2 are not able to infect swine (Cooper *et al.,* 2005), whereas genotypes 3 and 4 are (Ijaz *et al.,* 2009; Meng *et al.,* 1998b). Moreover, genotypes 1 and 2 are known to cause epidemics, whereas type 3 and 4 are sporadically transmitted, zoonotic infections (As described by Purdy *et al.,* 2011). The four genotypes are found in different regions as illustrated by figure 3 and 4. Genotype 1 is mostly found in Asia and Africa, genotype 2 in Africa and Mexico. Presence of genotype 3 has been shown all over the world except for Africa, whereas genotype 4 is exclusively found Asia. Genotypes 3 and 4 are mainly found in industrialized countries.

An overview of large outbreaks of the hepatitis E virus

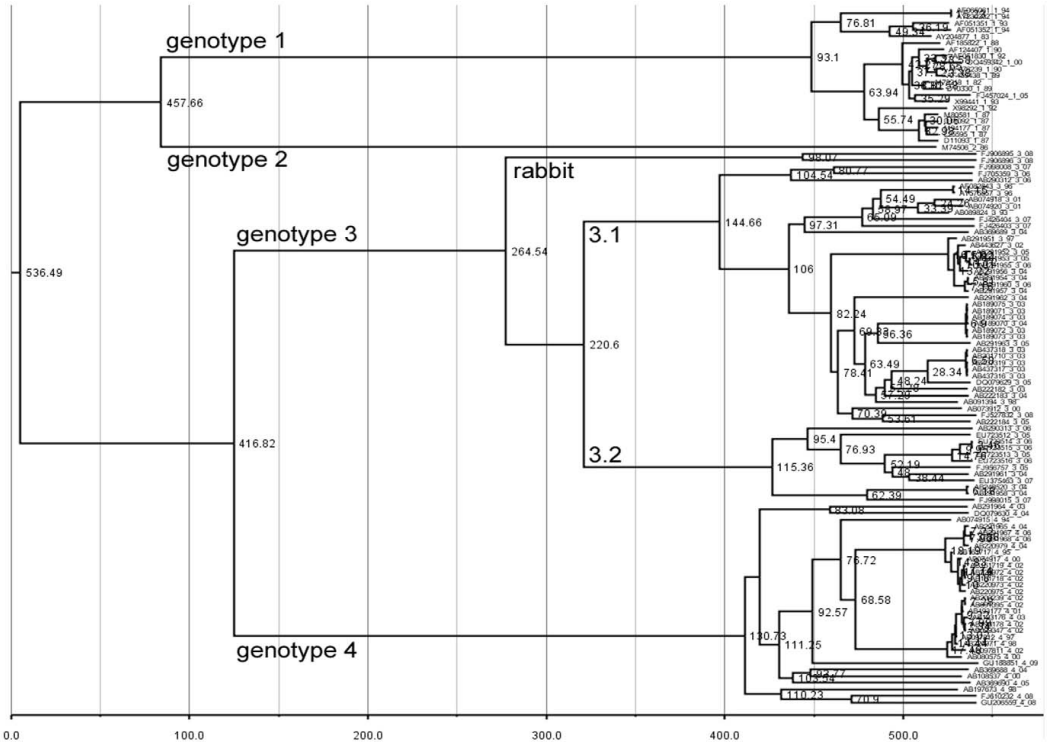

Figure 2. Evolutionary history of different HEV isolates. This tree was generated using non-overlapping sequences of ORF2. The numbers at each node and the x-axis indicate the Time to Most Recent Common Ancestor (TMRCA). The clade of genotype 3 has been divided in 3 clades: rabbit, 3.1 and 3.2. Each of the isolates has been labeled with its GenBank accession number, its genotype and (two digits). (Purdy *et al.*, 2010)

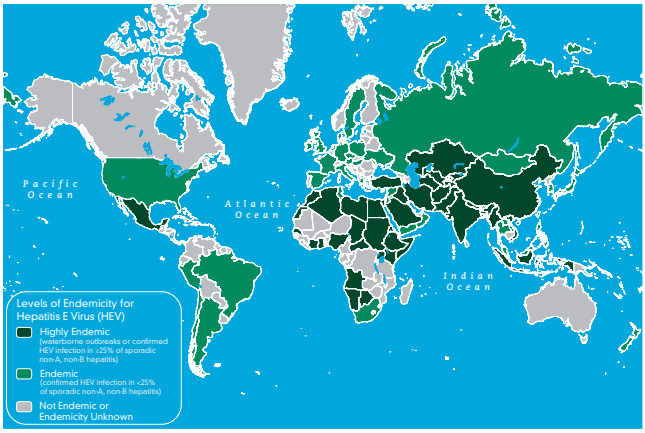

Fig 3. Global prevalence of HEV. Countries in which HEV is endemic or highly endemic are colored. (Teo, 2011)

Genotype	Geographic region				
	Asia	Africa	America	Europe	Oceania
1	Bangladesh Cambodia China India Japan[a] Kyrgyzstan Myanmar Nepal Pakistan Uzbekistan Vietnam	Algeria CAR Chad Djibouti Egypt Morocco Namibia South Africa Sudan Tunisia	–	Russia UK[a]	–
2	–	CAR Chad DRC Egypt Namibia Nigeria	Mexico	–	–
3	Cambodia Japan Korea Kyrgyzstan Taiwan Thailand	–	Argentina Canada Mexico USA	Austria France Germany Greece Hungary Italy Netherlands Russia Spain UK	Australia New Zealand
4	China India Indonesia Japan Taiwan Vietnam	–	–	–	–

[a] Most likely imported from countries endemic for genotype 1 HEV.

Figure 4. Geographic distribution of the different HEV genotypes across the different continents. (Okamoto, 2007)

is shown in figure 5 (Teshale *et al.,* 2010b). Outbreaks, for which the genotype has been determined, were caused by genotypes 1 and 2.

Hepatitis E virus infection of genotypes 1 and 2 is usually found in people of 15-40 years old. Fatality rates during epidemics of genotypes 1 and 2 range from 0.2-4% (Teshale *et al.,* 2010b).

Transmission

Several routes of transmission of HEV have been identified. Most of the epidemics described in literature of genotypes 1 and 2 have one thing in common: mixing of drinking and sewage water. Without a proper drainage system and water pipelines, the mixing of these two streams results in a high-potential source of infection in a feco-oral transmission route. This transmission route was confirmed by Balayan *et al.*, of which the primary author infected himself with 9 HEV-positive stool samples (Balayan *et al.,* 1983). However, other transmission modes have been shown to exist for these genotypes, although their relative contribution to the total amount of infections is probably low. Vertical transmission is highly potent (Khuroo *et al.,* 1995), and blood transfusions in endemic regions are likely to cause transmissions (Khuroo *et al.,* 2004). Spread of HEV by person-to-person transmission is thought not to be efficient since intrafamilial transmission is rare in both epidemic and sporadic hepatitis E cases (Somani *et al.,* 2003), although a more recent study supports the existence of person-to-person transmission during a large outbreak in Uganda (Teshale *et al.,* 2010a).

Prevalence of circulating antibodies against HEV in individuals in non-endemic regions (see figure 3, epidemiology) might be due to zoonotic transmission, although the occurrence of zoonotic events of all genotypes is limited (Dong *et al.,* 2011). HEV-related viruses have been found in swine, pigs, deer and wild boar (For references see Chandra *et al.,* 2008b). Eating raw contaminated meat might lead to direct transmission (Chandra *et al.,* 2008b; Emerson *et al.,* 2005; Tei *et al.,* 2003; Tei *et al.,* 2004). Besides, occupational exposure to pigs is likely to increase the risk of transmission (Krumbholz *et al.,* 2011; Lewis *et al.,* 2010), however, no direct significant evidence supporting this route of transmission has been published so far. Even though currently most HEV-infections in humans are asymptomatic, continued viral infection might eventually lead to evolution of HEV (Ijaz *et al.,* 2005; Zheng *et al.,* 2006).

Clinical presentation and pathogenesis

HEV targets hepatocytes, however, no hepatocyte-specific receptor molecules have been identified that could mediate infection (Protzer *et al.,* 2012). Instead, liver infection is thought to be mediated by the functional clearance of blood of pathogens. HEV particles are thought to be endocytosed by liver cells and somehow escape subsequently denaturation.

HEV is a self-limiting disease in immunocompetent individuals and it therefore does normally not lead to a chronic infection. However, chronic HEV

Year(s); location	No. of persons affected	Putative mode of transmission
1955–1956; India	29,300	Waterborne
1978–1979; Kashmir	>270	Waterborne
1986; Mexico[a]	>200	Contaminated well water
1988–1989; Ethiopia	>750	After monsoon rains
1991; India	79,000	Contaminated river water (Ganges)
1991; China[b]	119,000	Waterborne
1994; Vietnam	>300	After heavy rains
2004; Sudan[b]	>2600	Safe water insufficient
2008; Uganda[b]	>10,000	Substantial person-to-person

[a] Caused by HEV genotype 2.
[b] Caused by HEV genotype 1.

Figure 5. Major outbreaks of HEV. (Teshale *et al.*, 2010)

infection has been observed in immunocompromised transplant recipients (Haagsma *et al.,* 2008; Kamar *et al.,* 2008). Symptoms resemble those of hepatitis A virus infection, which include fever, fatigue, loss of appetite, nausea, vomiting, abdominal pain, jaundice, dark urine, clay-colored stool and joint pain. However, most HEV infections remain without symptoms. The symptomatic to asymptomatic infection ratio has been reported between 1:2 and 1:13 (National Center for HIV, Viral hepatitis, STD and TB prevention, 2009). Other possible outcomes of HEV infection include a more severe form in which substantial liver injury can cause (sub) acute liver failure and might eventually lead to retransplant or death (Schlosser *et al.,* 2012).

Reliable data on clinical manifestation of acute HEV infection have been obtained in two volunteer studies. In both studies researchers deliberately infected themselves by ingestion of preparations derived from stool samples of patients with acute hepatitis E from endemic areas (Balayan *et al.,* 1983; Chauhan *et al.,* 1993). On day 36 and 30 after inoculation, respectively in the first (Balayan *et al.,* 1983) and second (Chauhan *et al.,* 1993) study, the first symptoms of hepatitis E virus infection were observed: frailty, abdominal pain, nausea, vomiting and anorexia, followed by dark urine and eventually jaundice. In the first study alanine transaminase (ALT) levels (a diagnostic marker of hepatocellular injury) peaked at 3,010 IU/L (international units per liter, a value of 5 – 60 IU/L is considered normal, depending on test sensitivity), whereas in the second study a value of more than 100,000 IU/L was reached. In the second study HEV could be detected in serum and stool samples by means of RT-qPCR (see the "diagnosis and serological assessment" section) at day 34. Present PCR-methods are more sensitive and could probably detect HEV RNA presence in an earlier stage. Presence of virions in the stool samples confirms the potential fecal-oral transfection route. HEV particles could be detected in stool samples starting about 1 week before the start of symptoms, and ending about 2 weeks after the illness (Balayan *et al.,* 1983; Chauhan *et al.,* 1993).

These volunteer studies represent the most common clinical manifestations in highly endemic areas. The acute icteric hepatitis is clinically identical to illness caused by infection of other hepatotropic viruses such as hepatitis A or B viruses.

In rare cases HEV can be persistent, however, so far this has only been reported in transplant recipients, receiving immunosuppressive drugs, or cancer patients receiving chemotherapy, patients with certain hematological conditions and human immunodeficiency virus (HIV)-infected patients (Dalton *et al.,* 2011; Gerolami *et al.,* 2008; Halac *et al.,* 2011; Kaba *et al.,* 2011; Motte *et al.,* 2011; Myint *et al.,* 2006; Pfefferle *et al.,* 2011). These chronic infections might eventually lead to liver cirrhosis (Gerolami *et al.,* 2008). All chronic HEV-infection cases reported so far were classified as genotype 3. No reactivation of HEV has been reported, even in immunocompromised individuals (Legrand-Abravanel *et al.,* 2011).

In case of an outbreak of genotypes 1 or 2, symptoms are slightly different. During the 1955-1956 outbreak in India most patients endured a two-phase clinical development: a 'mild' pre-icteric phase (characterized by fever, loss of appetite, anorexia, constipation, diarrhea and loin pain), and an icteric phase of 10-24 days during which the fever stopped, and in which hepatomegaly was a common feature. In some patients the fulminant disease appeared in this phase, which frequently developed into a fatal condition (Aggarwal, 2011). In total 6.8% of the diagnosed cases were fatal. Certain groups of people appear to have an increased risk of developing the more severe fulminant illness in India (most likely genotype 1 or 2), in particular pregnant woman, especially in their third trimester (Jilani *et al.,* 2007). Mortality rates of this particular group are increased to 15-20% (Khuroo *et al.,* 1981). The precise mechanisms for this observation are unclear, however, immunological (i.e. an increased T-helper 1 cells over T-helper 2 cells ratio) (Navaneethan *et al.,* 2008; Pal *et al.,* 2005) and hormone-induced changes (Arankalle *et al.,* 1998; Boll *et al.,* 1996; Hussaini *et al.,* 1997) during pregnancy have been proposed to play key roles. Notably, this clinical observation has only been made for hepatitis E virus. Pregnant women with jaundice and acute viral hepatitis caused by the hepatitis E virus had higher mortality rates, worse obstetric and fetal outcomes compared to women in the same condition with other types of viral hepatitis (Patra *et al.,* 2007). Even though general virus replication enhancing features or hormonal changes during pregnancy seem to be unlikely to cause this worse outcome, currently only the hepatitis E-associated nature is certain.

In low-endemic areas (mainly industrialized countries) diagnosis of HEV infection used to be due to recent travel to endemic regions. However, in the early 1990s more and more cases of HEV-infected patients were diagnosed in these regions despite the nonattendance of recent travel (Aggarwal, 2011). Cases were diagnosed in Europe, North-America, Oceania and developed countries in Asia (Aggarwal, 2011). Usually a different genotype is found in these patients (see section epidemiology) with a slightly different

clinical manifestation. In low-endemic areas patients suffering acute hepatitis E tended to be older, were predominantly male, and had a higher frequency of liver disease or alcohol abuse compared to patients in highly endemic areas (Dalton *et al.,* 2008; Pavio *et al.,* 2010). For more elaborate data on HEV manifestation in non-endemic areas Aggarwal provides an excellent review (Aggarwal, 2011).

Immunity

In healthy individuals, HEV infection leads to innate and adaptive immune responses. The number of natural killer (NK) and T-cells and their activation state increases during acute hepatitis infection (Srivastava *et al.,* 2007; Srivastava *et al.,* 2008). Besides, the peripheral blood mononuclear cells (PBMCs) of HEV patients reacted on HEV proteins (Srivastava *et al.,* 2007). Moreover, the numbers of both CD8+ and $CD4+^{high}/CD8+^{low}$ T-cells were increased in acute hepatitis E virus infected patients (Husain *et al.,* 2011; Prabhu *et al.,* 2011; Srivastava *et al.,* 2007). A recent paper suggests the association of a specific T-cell response with clearance of infection (Suneetha *et al.,* 2011). It remains to be elucidated whether HEV infections in chronically infected patients were present before immuno suppression or are newly acquired after the immunosuppressive event.

Weakened T-cell responses in immunocompromised individuals may result in the inability to clear the viral infection, which is supported by the finding that HEV infected transplant patients who developed chronic disease had significantly lower CD2, CD3 and CD4 positive lymphocytes than HEV infected transplant patients who cleared the infection (Kamar *et al.,* 2008).

In parallel, an adaptive immune response leads, among others, to the production of anti-HEV IgM and IgG. Although detectable IgG anti-HEV antibody levels rise quickly and peak at around 4 weeks after infection, subsequently they drop until they are undetectable (Corwin *et al.,* 1995). It is believed that IgG antibody presence persists for many years and several reports indicate a gradual decrease of anti-HEV IgG presence in an infected population. IgG presence within infected populations has been reported to decline to 72% after 2 years (Corwin *et al.,* 1995), 46.7% after 14 years (Khuroo *et al.,* 1993) and 4.5% after 30 years (Khuroo, 2011). Khuroo *et al.* hypothesized that a following outbreak can occur when antibody levels are declined to a certain extent and there is fecal contamination of water sources (Khuroo *et al.,* 2010).

Specific anti-HEV IgM antibodies can be detected quickly upon infection with the hepatitis E virus, and decrease after HEV infection has been cleared (3-5 months after clearance). Therefore, these antibodies provide an accurate marker for present or recent HEV infection.

No direct research has been performed on whether infection prevents from reinfection, for instance with a different genotype. However, since vaccination strategies based on the ORF2 capsid protein look very promising (see vaccination chapter), it can be speculated that prior infection prevents disease development for at least several years.

Molecular virology

Study models

Many non-primate animal models have been used to investigate HEV features (for an overview see Krawczynski *et al.,* 2011). Although research with these models generated valuable knowledge on pathogenesis and clinical presentation, and will remain necessary for vaccine and drug testing, current knowledge on the molecular virology of HEV has been mostly obtained using *in vitro* systems.

Finding a suitable cell culture system to propagate HEV has been, and remains a major challenge. Several cell lines have been used to culture HEV (reviewed by Okamoto, 2011a), including human diploid lung fibroblast 2BS (Aggarwal *et al.,* 2011), human lung epithelium A549 (Huang *et al.,* 1995; Wei *et al.,* 2000), and fetal rhesus monkey kidney FRhK (Kazachkov *et al.,* 1992) cells. Human epithelial hepatoma PLC/PFR/5 (Pillot *et al.,* 1987) and primary cynomolgus monkey hepatocytes (Tam *et al.,* 1996) were shown to be infectable with HEV and produced new virions, albeit with low efficiency. A more productive replication was shown with the same human epithelial hepatoma PLC/PFR/5 cells by Tanaka *et al.* (2007). As described by Okamoto (2011b; 2011a) cell culture systems have now been set-up for both genotypes 3 and 4.

Genome

Hepatitis E virions consist of non-enveloped, round particles of approximately 27-34 nm in size (Krawczynski *et al.,* 2000). They contain a positive sense 7.2 kilobases (kb) RNA genome, which is 5'-capped and 3'-polyadenylated. At both termini a short untranslated region (UTR) is present of 25 and 65 nucleotides (nt) on the 5'- and 3'-side of the coding regions, respectively, which surround three partially overlapping open reading frames (ORFs), named ORF1, ORF2 and ORF3 (figure 6), as established by Tam *et al.* (Tam *et al.,* 1991). ORF1 extends about 5 kb from the

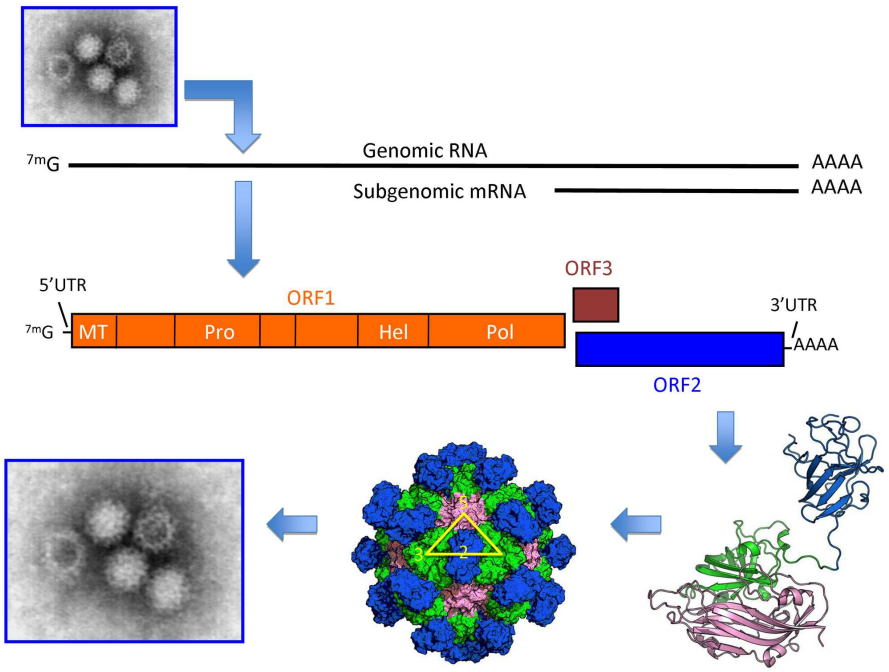

Figure 6. Replication model of HEV. Virions carry a (+)-stranded capped and polyadenylated RNA genome which contains 3 open reading frames. ORF1 codes for a polyprotein which contains a methyltransferase (MT), a papain-like cysteine protease (Pro), a Helicase (Hel) and an RNA-dependent RNA Polymerase (Pol) domain. ORF2 codes the capsid protein whereas ORF3 has several, host-interacting roles. The capsid protein contains 3 domains which allow for virion formation. Upon infection the HEV genome is replicated and expressed, after which new virus particles are assembled. (Aggarwal *et al.*, 2011)

5'-end, ORF2 starts 39 bp downstream, whereas ORF3 partially overlaps both ORF1 and ORF2 and is only 369 bp in length (Tam *et al.,* 1991). Besides, subgenomic RNA was observed of 3.7 and 2 kb in length. The latter has been shown to be a coding for both ORF2 and ORF3 (Graff *et al.,* 2006), however, the presence of the 3.7 kb RNA species was not found in this study, which was confirmed by more recent studies (Ichiyama *et al.,* 2009; Varma *et al.,* 2011), and its presence in the former study may be an artifact.

RNA structure appears to have an important function in viral replication. Mutational analysis of a conserved stem-loop region, situated in between the ORF1 and ORF2/ORF3 regions, showed a significantly inhibited HEV replication (Cao *et al.,* 2010), thereby indicating the necessity of this secondary structure.

Proteins

The three open reading frames present in the HEV genome encode for three polyproteins called ORF1, ORF2 and ORF3, of which the latter two have been shown to be present during human infection by serology-based studies (Khudyakov *et al.,* 2011; Panda *et al.,* 1995).

ORF1

The first polyprotein (ORF1) is generally assumed to contain many non-structural functions. It contains several functional elements such as RNA-dependent RNA polymerase (RdRp), RNA helicase (Hel), papain-like cysteine protease (PCP) and methyltranferase (MeT), as has been predicted by computer-assisted alignments (Koonin *et al.,* 1992). Other elements, homologous to other viruses, have been found, however, their functions remain unclear. These include the Y domain (about 200 amino acids (aa) long), the macro-domain (previously known as the X-domain), and a proline-rich region (V) (Ahmad *et al.,* 2011).

It has to be elucidated whether these biochemically diverse functions appear on separate proteins, or that the polyprotein remains (partially) intact, since several studies gave rise to contradictory results. When transfecting HepG2 hepatoma cells with separate cDNA transcripts of ORF1, ORF2 and ORF3, an infection was established and separate processed products of ORF1 domains could be detected (Panda *et al.,* 2000). However, expression of ORF1 in a vaccinia-based expression system initially only resulted in the full length 185 kDa polyprotein. After extended incubation products of 107 kDa and 87 kDa were observed (Ropp *et al.,* 2000). More recently the very same group could not confirm their findings using a different (plasmid-based) expression method (Suppiah *et al.,* 2011). Probably, a more accurate answer will be available when this issue will be investigated after viral infection in cell culture (Okamoto, 2011b; Okamoto, 2011a).

Viral RNA, transcribed *in vitro* from cDNA, has been shown to be infectious in primates only when capped (Emerson *et al.,* 2001). The methyltransferase domain, predicted by computational alignment (Koonin *et al.,* 1992), is likely to be involved in this process. An HEV cDNA fragment, consisting of nucleotides 1-979, was expressed in insect cells and exhibited guanine-

7-methyltransferase and guanyltransferase activity (Emerson *et al.,* 2001), both crucial steps for capping. However, the initial capping step, the removal of the terminal 5'-gamma-phosphate group is not clarified by these activities.

A shared feature among positive stranded RNA viruses is proteolytic processing of polyproteins to create mature and active proteins. The polyproteins containing the replicase precursor of all animal positive stranded RNA viruses have been shown to be proteolytically processed (Suppiah *et al.,* 2011), which led to the believe that this was true for HEV as well, performed by the putative papain-like protease (PCP) domain on the ORF1 polyprotein. This was initially confirmed by the finding that expression of ORF1 itself by a vaccinia virus-based expression resulted in proteolytic processing (Ropp *et al.,* 2000). However, a later report by the same group contradicted this finding since expression of ORF1 by a plasmid-based expression vector did not result in a processed polyprotein (Suppiah *et al.,* 2011). Besides, analysis of multiple ORF1 sequences revealed that the previously identified putative catalytic site within the papain-like protease domain was not completely conserved (Suppiah *et al.,* 2011). This led the authors to conclude that HEV might be unique among positive stranded RNA viruses to not possess a protease-mediated ability to process its own replication precursor. Instead, the PCP domain was recently shown to exhibit a deubitiquilating activity, indicating a role in antiviral escape (Karpe *et al.,* 2011).

RdRp is a crucial enzyme for RNA-based viruses, since it is able to create negative sense copies of positive sense RNA, an essential replication step in both positive and negative sense-RNA viruses. A computer-assisted alignment of the putative HEV RdRp with poliovirus polymerase predicted several functional motifs important for replication activity, and showed its ability to produce negative stranded viral RNA (Rehman *et al.,* 2008). This study also confirms the ability of the RdRp to specifically bind the 3'-terminus of the HEV genome shown previously (Agrawal *et al.,* 2001) by fluorescence resonance energy transfer (FRET) analysis. For this ability, the presence of two stem-loop regions in the RNA is paramount (Agrawal *et al.,* 2001). Besides, co-localization with the endoplasmatic reticulum was established (Rehman *et al.,* 2008), which indicates an ER-based replication localization of HEV.

The viral helicase belongs to helicase superfamily 1. It has been shown to be able to unwind double stranded RNA in a 5' to 3' manner, combined with a NTPase activity to obtain energy (Karpe *et al.,* 2010a). Besides, helicase has been shown to remove the final gamma-phosphate on the 5'-terminus of the unwinded RNA molecule (Karpe *et al.,* 2010b). This suggests that the initial gamma-phosphatase activity necessary for RNA-capping might be executed by the viral helicase.

ORF2

The second open reading frame encodes the capsid protein of 660 aa. This protein has been shown to be translocated across the endoplasmic reticulum (ER) during translation through an N-terminal hydrophobic sequence, which is necessary for its final cell surface localization (Zafrullah *et al.,* 1999). Normally, when a protein is not properly folded or unassembled, it is retrotranslocated from the ER to the cytoplasm for degradation (for a review see Kopito, 1997). This mechanism has been shown before to be abused by viruses (for instance, see Wiertz *et al.,* 1996) and appears to be abused by HEV as well.

The HEV ORF2 protein is N-glycosylated on amino acid positions 137, 310 and 562 (Zafrullah *et al.,* 1999), without which the virus will not be infectious (Graff *et al.,* 2008). Initially the glycosylated ORF2 protein is translocated to the ER. After unglycosylation of ORF2, the majority of the unglycosylated fraction is subsequently retrotranslocated into the cytoplasm (Surjit *et al.,* 2007). The retrotranslocated ORF2 is not degraded but instead appears to be stable in the cytoplasm (Surjit *et al.,* 2007), which indicates that the viral protein mimics a proteasome substrate to get access to the cytoplasm.

Although many studies on this portion of the genome have been performed, a recent study provided strong evidence for its function. Parvez *et al.* showed that baculovirus-mediated overexpression of ORF2 resulted in the production of large amount of ORF2 protein which self-assembled in so-called virus-like particles (VLPs) (Parvez *et al.,* 2011). Basically, VLPs only consists of the protein shell of the virion, and thus lack any genomic material. Moreover, double transfection studies with this ORF2 vector and a defective mutant (HEVΔORF2) provided with a green fluorescent protein (GFP) in place of the ORF2 gene resulted in the transencapsidation of the HEV genome and generated infective virus particles (Parvez *et al.,* 2011), whereas a transfection with the HEVΔORF2 genome did not. This proves that ORF2 encodes the capsid protein and ORF2 expression by itself is sufficient to produce the functionally active viral capsids, yet not able to reproduce due to the lack of genomic material.

Since the ORF2 protein is the only capsid protein

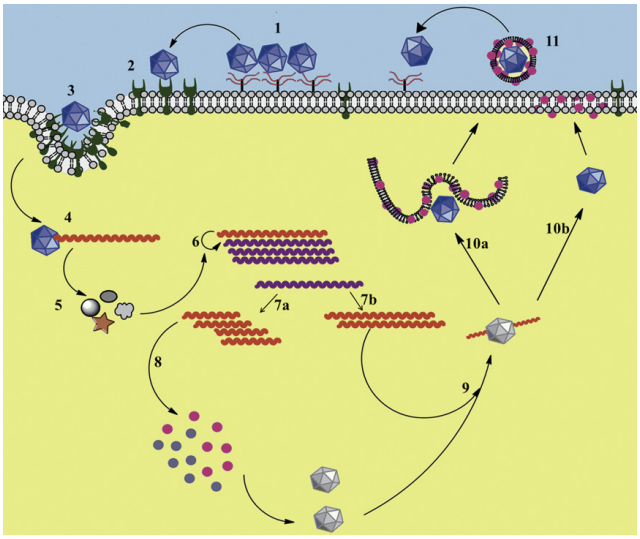

Figure 7. Model of the HEV replication cycle. Initial attachment is mediated by heparan sulphete proteoglycans (1), after which the virion enters the target cell by a specific unknown receptor (2 and 3). Subsequently its genomic material is released in the cytoplasm (4) after which translation of ORF1 and possibly ORF3 result in the expression of non-structural proteins (5) necessary for generation of (-)-stranded RNA intermediates (6), (+) stranded subgenomic mRNAs (sgmRNAs, 7a) and genome replication (7b). Translation of these sgmRNAs leads to expression of ORF2 capsid proteins (blue) and ORF3 (crimson, 8). Capsid proteins for the viral capsid in which a copy of the viral genome in integrated (9). ORF3 proteins are either associated with an intracellular membrane (10a) or the plasma membrane (10b) to mediate virion egress, after which a maturation step (11) results in the formation of new infectious particles. (Ahmad et al., 2011)

present in HEV, it is likely to interact with possible target cells. So far, no definite primary HEV host cell receptor has been identified, although heparin sulfate proteoglycans are required for cellular ORF2 protein binding and viral infection in liver huh-7 and a huh-7 subclone S10-3 cells, respectively (Kalia *et al.,* 2009).

ORF3

The third open reading frame encodes a protein of 123 amino acids at maximum. The exact function of this protein has to be determined, however, contradictory reports describe the localization of ORF3 and its interactions with many host proteins and the viral capsid protein.

These ORF3-host protein interaction include protein tyrosine kinases Src, hemapoietic cell kinase (Hck) and Fyn, phospholipase Cγ, phosphoinosytol 3-kinase, growth factor receptor bound (Grb)-2, an adaptor protein, a mitogen-activated protein kinase (MAPK), and Hepatocyte nuclear factor (HNF)-4 (Chandra *et al.,* 2011; Korkaya *et al.,* 2001).

ORF3 has been shown to modulate host cellular responses in particular. Chandra *et al.* show localization of the ORF3 proteins to early and recycling endosomes, where it causes a delaying effect in trafficking of epidermal growth factor receptor (EGFR) to late endosomes and lysosomes for degradation (Chandra *et al.,* 2008a; Chandra *et al.,* 2010). This EGFR internalization is required by the host phosphorylated signal transducer and activator of transcription 3 (pSTAT3) to translocalize to the nucleus, which may result in a reduced inflammation response, an increased cell survival and thus a higher viral replication (Chandra *et al.,* 2008a).

Also the interaction of ORF3 with α1-microglobulin/bikunin precursor (AMBP) and the ORF3 expression resulting in the export of α1-microglobulin out of the hepatocyte (Tyagi *et al.,* 2004) can be incorporated in this model. Since α1-microglobulin exhibits a granulocyte-migration inhibiting effect (Logdberg *et al.,* 1981), increased α1-microglobulin secretion might decrease an immune response and thus increase viral replication potential.

Besides, ORF3 interacts with microtubules in a microtubule-stabilizing manner (Kannan *et al.,* 2009). Dynein motor proteins, which transport cargo along microtubules, have been shown to play an essential role in the ORF3-microtubule interaction (Kannan *et al.,* 2009). HEV particles are unable to move freely through the cytoplasm of the host cell. This microtubule interaction might allow HEV transport throughout the cell, thereby creating a favorable intracellular environment able to facilitate HEV infection.

In addition, ORF3 has been reported to be present on the surface of cell culture-produced HEV particles (Yamada *et al.,* 2009), indicating a virion egress-facilitating role, which is supported by the observation that a 4 aa sequence in ORF3 protein is necessary for virion release (Nagashima *et al.,* 2011).

Moreover, an ORF2-ORF3-interaction has been shown to take place (Tyagi *et al.,* 2002). Remarkably, ORF3 proteins preferentially interact with the unglycosylated form of ORF2 (Tyagi *et al.,* 2002), indicating a cytoplasmic localization. However, since the exact virion assembly mechanism in respect to ORF2 is still unclear, no direct conclusions can be drawn from these data.

In short, ORF3 is most likely a host cell modulating protein with many interactions. The precise functions it fulfills are far from clear and neither are the involved pathways. ORF3 might play a role in virion assembly. However, more research is needed to reveal the complete extent of the protein interaction network and unravel the precise function of ORF3 in HEV infection.

Structure

Recent studies revealed crystal structures of genotype 1 (Xing *et al.,* 2010), 3 (Yamashita *et al.,* 2009) and 4 (Guu *et al.,* 2009), which confirmed the assumed non-enveloped appearance of the virion. The virion itself adapts an icosahedral configuration, in which the ORF2 protein consists of three domains, S (shell): the continuous capsid, M (middle): tightly associated with the virion shell domains and P (protrusion): a less tightly associated domain which pair-wise forms the typical virion protrusions observed by EM. In various studies expression of the HEV ORF2 protein resulted in the formation of a *T=1* virus-like particle, whereas the HEV virion adapts a T=3 conformation. This incongruence is further elucidated by Xing *et al.* (2010).

Replication

Current knowledge suggests that the HEV life cycle is, to a large extent, similar to other non-enveloped positive stranded RNA viruses. However, only very limited research has been performed on the HEV replication cycle due to the lack of an efficient cell culture system. Figure 7 shows a stepwise model of the life cycle.

The primary host cell attachment factor for HEV is unknown, however, as described before heparin sulfate proteoglycans appear to be crucial for host cell attachment (Kalia *et al.,* 2009). Basically nothing is known about the primary receptor, internalization, uncoating process or location. As described before, tubulin might play a role in intracellular trafficking (Kannan *et al.,* 2009). Once the genomic material is released into the cytoplasm the ORF1 polyprotein is translated, whether or not proteolytically cleaved into distinct functional proteins, which finally results in replication of the genetic material into negative-sense RNA copies of the genomic RNA (Panda *et al.,* 2000). Negative RNA synthesis shows an initial peak production after cell entry, followed by a phase in which the (-)-strand content of the infected cells is stable (Varma *et al.,* 2011). Using these as template, full- and subgenomic positive RNA strands are synthesized. The subgenomic RNA strands would subsequently serve as template for ORF2 and ORF3 translation (Graff *et al.,* 2006; Huang *et al.,* 2007; Ichiyama *et al.,* 2009), resulting in the start of virion capsid protein production. Positive-stranded RNA synthesis shows several peaks during infection (Varma *et al.,* 2011), which indicates cyclical bursts of virion build-up followed by virion egress.

The subsequent mechanisms of virion formation and egress are largely unclear. However, as described before, capsid proteins have to be phosphorylated and translocated to the ER for infectious virion production can be achieved (Graff *et al.,* 2008; Surjit *et al.,* 2007).

Diagnosis and serological assessment

Hepatitis E virus infection is not distinguishable from other types of acute viral hepatitis solely based on clinical features. Therefore, laboratory assessment based on the presence of HEV-specific antibodies or viral RNA is the only way to detect HEV infection.

Basically, two diagnostic methods are used for HEV diagnosis: a serological and a nucleic acid-based test. The first method tests for the presence of antibodies (IgG or IgM) in serum. Since only one serotype of HEV exists and antibodies are shown to be highly cross-reactive (Arankalle *et al.,* 2007; Emerson *et al.,* 2006; Herremans *et al.,* 2007; Zhou *et al.,* 2004), this provides a relatively easy assessment target for all the HEV genotypes. Besides, the serological tests can be used to determine whether an individual has been in contact with the virus, since IgG antibodies against HEV remain present for at least several years. The nucleic acid-based tests can be used to detect viral RNA in serum, bile or in feces. However, nucleic acid-based tests are only effective to test for an active infection, whereas IgM assessment provides information on acute or recent infection.

Although both PCR-based and serological assays can provide information about HEV infection, different kinds of tests can generate contradictory results. To illustrate this an example of a study in which 277 suspected HEV-infected acute hepatitis (of unexplained origin) patients were tested with both a PCR-based and IgM presence-based tests. Hepatitis A, B and C were ruled out in all cases. 11 persons were found positive with both tests, 7 positive for IgM but negative for viral RNA, and 4 positive for RNA but negative for IgM (Echevarria *et al.,* 2011). So, even though 11 patients were tested positive in both tests, another 11 were only tested positive in one of the tests. Various parameters could explain this difference: either of the tests was not sufficiently sensitive, or the infection was in the initial phase in which no antibodies have been produced. Either way, the authors recommend double testing until a reliable testing method has been developed to prevent underdiagnosis.

Based on a study of diagnostic acute markers for HEV, Huang *et al.* concluded the same, although they recommend assessing seroprevalence of IgG, IgM and

the presence of HEV RNA to prevent misdiagnosis (Huang *et al.,* 2010).

Reverse Transcriptase Polymerase chain reaction (RT-PCR)-based assays

PCR-based tests rely on the presence of viral RNA in serum, feces or bile or even possibly highly contaminated sources such as water. By means of RNA purification the viral RNA is isolated and subsequently transcribed into copy DNA (cDNA) using a reverse transcriptase enzyme. Subsequently, this cDNA is multiplied in a quantitative manner using HEV specific primer sets. However, since HEV knows 4 genotypes, and many isolates show minor mutations within the sequence, it is difficult to develop uniformly effective primer sets, capable of binding and replicating all of the different isolates at a similar or preferably identical efficiency. These characteristics make it difficult to develop and standardize a uniformly applicable, reliable and quantitative PCR-based HEV test.

Nowadays several different PCR-based methods have been developed to test for HEV RNA presence, including conventional reverse transcriptase RT-PCR, real-time RT-PCR and reverse transcription loop-mediated isothermal amplification (RT-LAMP) (Lan *et al.,* 2009). A detection threshold as low as 9 copies per reaction was claimed for the latter method (Lan *et al.,* 2009). However, this method is quite laborious compared to regular RT-PCR due to the post-amplification step and relatively sensitive to contamination.

Three different strategies have been developed for (fluorescence-based) real-time RT-PCR: probe-based (such as TaqMan), general DNA dye-based (such as SYBR-green), and molecular beacon-based. The simplest principle is the general DNA dye, which binds all formed DNA, and thus is the least specific and could result in a high background and lacks specific detection. Probes and molecular beacons are sequence-specific fluorophores, which basically only detect specifically amplified DNA, albeit through a different fluorescence activation mechanism.

Some commercial PCR-based HEV-tests are available nowadays, including tests from Altona diagnostics, Germany; PrimerDesign, USA (research only) and Gentaur, Belgium. Many diagnostic laboratories have developed in-house PCR-based HEV tests and several research laboratories are developing more sensitive RT-PCR-based alternatives (for instance see Bendall *et al.,* 2010). Unfortunately, no comparative studies have been performed between these tests. To demonstrate the difference in sensitivity 22 plasma samples of HEV genotype 3 and 4 positive blood donors were sent for testing in 20 laboratories. There was a 100- to 1,000-fold divergence between labs, regardless of the virus strain, although the overall specificity was very good considering none of the negative controls were tested positive (Baylis *et al.,* 2011). This illustrates the need for standardization of these tests to obtain comparable results and therefore Baylis *et al.* proposed a WHO standard for HEV RNA.

Beside these tests, real-time assays have been developed to detect any of the 4 genotypes in a monoplex probe-based reaction (Gyarmati *et al.,* 2007; Jothikumar *et al.,* 2006), or a primer-probe energy transfer (PriProET) probe-based technique (Gyarmati *et al.,* 2007).

Serology-based assays

Nowadays several serology-based assays are commercially available, for instance from Medical Biological Service, Italy; Mikrogen Diagnostik, Germany; Beijing Wantai, China; Beijing Bioneovan, China; BioChain Institute, USA and Diagnostic Automation, USA.

The only viral protein of the intact virion, which is accessible for antibodies, is the capsid protein. Therefore, the antigenic composition of this protein is of considerable importance for vaccine development and diagnostics. Using peptide fragments of this protein to study antigenic potential of each protein domain, 6 antigenic regions have been identified (Khudyakov *et al.,* 1999), of which the termini-located domains generated the most consequent results.

Protein fragments expressed in *Escherichia coli* (*E. coli*) generated more potent virus-neutralizing antibodies than the baculovirus expressed full-length ORF2 protein (Meng *et al.,* 1998a). This might indicate an important role of the protein conformation in respect to antigenic epitopes.

Further characterization of the neutralizing epitope identified a critical neutralizing region at aa positions 452 – 617 of the capsid protein (Meng *et al.,* 2001). This region forms the P-domain of the ORF2 protein as recently elucidated in a reconstructed HEV virion by Xing *et al.* (2011).

Many antigenic epitopes of HEV are available for diagnostic test development. For the development of commercial and in-house serology-based diagnostic tests several of these epitopes have been used, hence resulting in different affinities and efficiencies of such tests. For an overview of reported developed tests, see Khudyakov *et al.* (Khudyakov *et al.,* 2011).

Little research has been done on the quality of these commercially available serology-based HEV tests.

Assay	Sensitivity panel						Specificity panel	
	Genotype (gt) and number of samples reactive					Sensitivity (%)	No. of samples reactive (n = 229)	Specificity (%)
	gt 1 (n = 15)	gt 2 (n = 4)	gt 3 (n = 15)	gt 4 (n = 17)	Total (n = 51)			
I	15	4	14	17	50	98	49	78.5
II	15	3	15	17	50	98	15	93.4
III	15	2	13	12	42	82.4	19	91.7
IV	13	1	10	13	37	72.5	16	93
V	15	4	14	17	50	98	10	95.6
VI	15	3	13	16	47	92.2	9	96.1

Assay I (NIH): ORF2 aa112–606, Pakistan, genotype (gt) 1, expressed in baculovirus; Assay II (CDC): ORF2 aa452–617, gt 1 (Morocco), gt 2 (Mexico), gt3 (US) and gt4 (China) expressed in *E. coli*; Assay III: International Immuno-Diagnostics (Foster City, CA); Assay IV: MP Biomedicals (Singapore); Assay V: Diagnostics Systems (Nizhnii Novgorod, Russia); Assay VI: Mikrogen GmbH (Neuried, Germany).

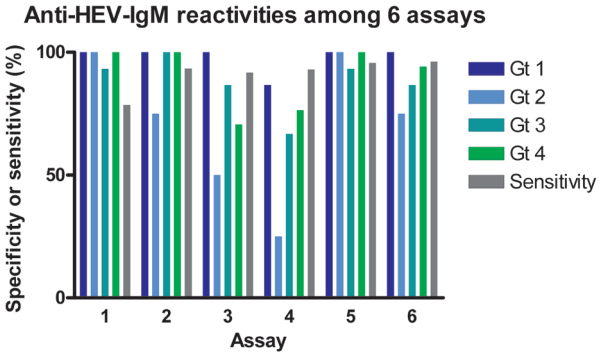

Figure 8. Performance of several serology-based diagnostic assays. Sensitivity and specificity of 4 commercial and 2 in-house IgM anti-HEV assays. (Drobeniuc *et al.*, 2010; Khudyakov *et al.*, 2011)

One of the studies tested 164 randomized human and primate sera in 12 different anti-HEV assays. Of the known-positive samples (human sera obtained 2 months to 13 years after acute hepatitis E) the sensitivity ranged from 17% to 100%. 10 out of 12 tests had negative test results for all of the 22 know-negative samples. However, when testing serum samples collected from patients from a non-HEV-endemic region, there was a high degree of discrepancy between the different assays, which indicates a low specificity of some of the tests in low-endemic areas, indicating that anti-HEV seroprevalence testing was still unreliable at the time (Mast *et al.,* 1998).

An overview of several, more recent studies has been given in figure 9. In the first study, the performance of 5 enzyme immunoassays (EIAs) has been assessed on samples obtained during an HEV outbreak in Indonesia (Myint *et al.,* 2006). Assays were purchased from Abbott, Germany (IgG); Genelabs Diagnostics, Singapore (IgG or IgM); and the Walter Reed Army Institute of Research, USA (IgM or IgG and IgM). The results suggest a relatively low sensitivity for non-symptomatic HEV PCR-positive subjects (Figure 9, 5 upper rows), whereas the sensitivity for symptomatic HEV-cases reaches up to 91%. The specificity in terms of false positives reaches 100% for the two of the assays, whereas other assays score a minimum of 74% (Figure 9). These results emphasize the inaccuracy of both sensitivity and specificity of these assays, and HEV serology tests in general.

Recently, the performance of 6 IgM anti-HEV assays (4 commercially available and 2 in-house assays) was

EIA manufacturer	IgG/IgM	Sensitivity (%)	Specificity (%)	Ref.	Pub. Year
WRAIR	IgM	64 (51$^{\#}$; 91$^{\$}$)	100	Myint *et al.*	2006
GLD	IgM	52 (42$^{\#}$; 72$^{\$}$)	74		
WRAIR	IgG & IgM	54 (39$^{\#}$; 84$^{\$}$)	100		
GLD	IgG	63 (51$^{\#}$; 89$^{\$}$)	86		
Abbott	IgG	60 (45$^{\#}$; 91$^{\$}$)	96		
In-house	IgM	98.0	78.5	Drobeniuc *et al.*	2010
In-house	IgM	98.0	93.4		
IID	IgM	82.4	91.7		
MP Bio	IgM	72.5	93.0		
DS	IgM	98.0	95.6		
Mikrogen	IgM	92.2	96.1		
GLD	IgG	100 / 88*	93	Herremans *et al.*	2007
GLD	IgM	38 / 100*	98		
Mikrogen	IgG	100 / 88*	97		
Mikrogen	IgM	100 / 100*	66		

Figure 9. Overview of sensitivities and specificities of several studies. WRAIR: Walter Reed Army Institute of Research, USA; GLD: Genelabs Diagnostics, Singapore; Abbott: Abbott Diagnostica, Germany, IID: International Immuno-Diagnostics, USA; MP Bio: MP Biomedicals, Singapore; DS: Diagnostics Systems, Russia; Mikrogen: Mikrogen, Germany. In the study by Myint *et al.* sensitivities are specified to asymptomatic ($^{\#}$, n = 174) and symptmatic ($^{\$}$, n = 82) HEV cases. *Sensitivities are given for sera seropositive for genotype 1 and 3, respectively.

tested with different serum samples, representing each of the 4 HEV genotypes. The sensitivity and specificity varied extensively between assays: the assay specificity varied from 78% to 96%, whereas the sensitivity ranged from 72% to 98% (Figure 8 and 9). Besides, the interassay agreement varied broadly (Drobeniuc *et al.,* 2010). (The commercial tests were purchased from International Immuno Diagnostics, MP Biomedicals, Diagnostics Systems and Mikrogen)
Other studies on the sensitivity of commercial assays have been performed, albeit without the testing all 4 existing genotypes, such as performed by Drobeniuc *et al.*, 2010. Most assays have been developed using epitopes from endemic strains only. Basically, this should not be an issue since only one serotype has been shown to exist, however, to test the specificity of such assays in low-endemic areas, enzyme-linked immunosorbent assays (ELISAs, Genelabs Diagnostics) and immunoblotting assays (RecomBlot, Mikrogen) were tested for their ability to specifically detect anti-HEV IgM an IgG in sera obtained from genotype 1 and 3 infected patients. Although both tests scored well on IgM detection (98% and 97%, respectively), detection of IgG positivity was less frequently correctly observed (93% and 66%, respectively) (Herremans *et al.,* 2007). Nonetheless, neither of the assays did perform better on the genotype 1 samples, confirming the hypothesis that these kinds of assays are broadly reactive, despite their development process. Furthermore, Herremans *et al.* conclude that in low-endemic settings, a combination of both assays is necessary to obtain a tolerable specificity and sensitivity for HEV diagnosis (Herremans *et al.,* 2007).
Even though a combination of such assays can generate satisfactory results, the individual assays can be improved, as shown by Arankalle *et al.* (2007). Comparison of baculovirus-mediated, ORF2-expressed protein with a commercially available ELISA (Genelabs) revealed that the commercially available ELISA was less sensitive than the novel ELISAs.

Prevention, vaccines and antiviral measures

Upgrading sanitary conditions, in terms of a separate drinking water supply and sewage disposal, can prevent many infections in epidemics situations, due to the fecal-oral major infection route of the hepatitis E virus. However, as effective as such measures might be, the realization of such conditions in low-income regions remains a challenge. Vaccines might provide an additional measure in the fight against HEV, although the effectiveness of this measure partly depends on the effectiveness of such vaccines.
Nonetheless, the development and availability of these vaccines should not be a reason to delay or dismiss improvement of sanitary conditions in endemic areas.

Vaccines

Several vaccine candidates for hepatitis E virus are in development nowadays, although only two vaccines are in clinical trial stages of development. Since no efficient cell culture systems existed, dead virus and live attenuated virus vaccines have not been developed. In addition to several protein vaccine candidates, expressed in bacterial or insect cells, DNA vaccines might be developed, as reviewed by Kamilli (2011).
Two recombinant vaccines candidates have been developed and assessed in phase 2 or 3 clinical trials. The first developed vaccine consists of a truncated genotype 1 ORF2 protein (aa 112 –aa 607) produced in insect cells (Zhang *et al.,* 2001), which showed a protective immunization in rhesus monkeys. Subsequently, this vaccine (rHEV) was tested in a phase 2 trial among 1,794 young and healthy men (soldiers) (Shrestha *et al.,* 2007), who received 3 doses of the vaccine on 0, 1 and 6 months. One and a half year after the last vaccination, the vaccine efficiency was 95.5% after 3 doses, and 88.5% after one dose. The efficacy of this vaccine was determined by comparison of the number of individuals developing hepatitis E in the vaccine- and placebo-group. Since the vaccinated subjects were mostly young men, the question remains how well this vaccine will perform in a general population. Besides, this study focused on the clinical symptoms rather than HEV infection.
A large-scale phase 3 clinical trial assessed the efficacy and safety of the other recombinant vaccine, HEV 239, which consists of a purified recombinant truncated ORF2 protein (aa 376 – aa 606) (Zhang *et al.,* 2009), in a general population that included healthy men and women of 18 to 65 years of age (Zhu *et al.,* 2010). This study was performed in a region where both HEV genotypes 1 and 4 circulated, and included close to 100,000 participants. After 3 intramuscular injections of the vaccine at 0, 1 and 6 months, none of the participants of the vaccine group (n = 48,693) developed hepatitis E symptoms during a 12 months period starting from the 31st day after the last dose. In comparison, 15 persons of the placebo (hepatitis B vaccine) group (n = 48,663) developed hepatitis E symptoms. A less optimal vaccination schedule, in which only 2 doses were given, still exhibited

maximum protection, whereas only one dose was 95.5% effective. In addition, the incidence of adverse effects was low. The relative safety of the vaccine was also shown in a preliminary study of HEV 239 vaccination during pregnancy (*n*=68), in which no safety issues for mothers or fetuses were reported (Wu *et al.,* 2011). However, the definition of hepatitis E virus infection in the large-scale study is rather strict. A participant was defined to suffer from an acute hepatitis E virus infection if symptoms such as fatigue or loss of appetite remained for at least 3 days, ALT-levels surpassed 2.5-times the normal upper limit, HEV IgM antibodies in combination with HEV RNA or more than a fourfold increase in HEV IgG levels were detected. A more widely accepted HEV case description is defined by increase of ALT levels (≥10 times normal range upper limit) and strong IgM reactivity or rising IgG reactivity or a detectable viraemia by RT-qPCR in case of immunocompetent individuals (Dalton *et al.,* 2008). If one of the three HEV infectious markers (anti-HEV IgG, IgM or HEV RNA presence) would be accepted as definition of a HEV case in the HEV 239 study, 46 additional subjects (25 and 21 in the vaccine- and placebo-group, respectively) would have been identified, which reduces the seemingly perfect HEV protection of the vaccine. In total this would result in 24 and 36 cases of HEV in the vaccine- and placebo-group, respectively, which undermines the efficiency of this vaccine. On the other hand, highly elevated IgG levels are quite common after vaccination and therefore, this marker might be inadequate to distinguish between vaccinated and infected individuals. In addition, the infection itself might not be prevented by vaccination per se, however, vaccination might decrease the severity of the illness. In that case this vaccine can be useful even though infection itself is not prevented.

One of the questions that remain is which groups would qualify to receive such a vaccination. Probably the cost-benefit ratio of vaccinating entire populations is not favorable, however, several distinct risk groups might be eligible. For instance pregnant women and immunocompromised or chronic liver disease-patients, for whom the consequences of HEV infection can be severe, or travelers to endemic regions, might benefit. Epidemics might also be restricted since non-optimal vaccination already exhibits a high protective rate.

How long the vaccinated population remains protected against hepatitis E virus infection remains to be elucidated. So far, only the previously described studies have been published. A follow-up serological assessment may provide additional knowledge of the long-term effectiveness of protection against acute hepatitis E virus infection.

Whether these vaccines only protect against clinical symptoms or also against HEV infection has to be determined. If infection is prevented, these vaccines might actually have a great potential in altering epidemiology, and major epidemics can be largely prevented.

Antiviral measures

If an individual is already infected vaccination might still be effective, however, this has to be elucidated for the two vaccines currently in the pipeline. Several methods of treatment of acute hepatitis E virus infection are being developed or already exist.

In a case-report, an elderly woman exhibited an indolent chronic myelomonocytic leukemia and acute severe thrombocytopenia. Because of high ALT levels she was tested for anti-HEV IgG and IgM, which confirmed an HEV viraemia (Alric *et al.,* 2011). Treatment with ribavirin cleared HEV infection completely, and up to 6 months after treatment no HEV RNA could be detected. These findings are further supported by 8 other case studies (Kamar *et al.,* 2010c; Mallet *et al.,* 2010).

Besides, pegylated interferon alpha has been shown to have an HEV-infection inhibiting and usually clearing effect in chronic hepatitis E (Alric *et al.,* 2010; Kamar *et al.,* 2010a; Kamar *et al.,* 2010b). However, even though this treatment can be successfully applied after liver transplantation (Kamar *et al.,* 2010c), it is contraindicated for for instance kidney transplant patients and thus not generally applicable.

Another potential HEV therapy exploits short hairpin RNA (shRNA) to inhibit the replication of HEV. *In vitro* studies show that several specifically designed shRNAs demonstrate inhibition and might therefore potentially be used as HEV antiviral drugs (Kumar *et al.,* 2010). However, how to deliver these shRNAs to HEV-susceptible cells remains a challenge.

Conclusions

Hepatitis E virus infection is an emerging cause of acute viral hepatitis around the world. Since the first retrospectively identified outbreak of this virus in 1955 much knowledge has been obtained about the virus itself, the epidemiology, transmission route and pathogenesis. Scientists around the world have developed increasingly sensitive and generally applicable diagnostic measures and vaccines.

However, despite this gained knowledge and

increasing scientific effort many of the viral features are unclear or unknown, no WHO standard for diagnostic testing has been developed and no vaccine is currently commercially available. On the other hand, an efficient cell culture system to reproduce HEV has been reported, just as phase II and III clinical trials of the first recombinant vaccines. So even though the final steps in virus knowledge, infection diagnostics and prevention are yet to be taken, current research on these topics appears promising.

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