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Hepatitis E Vaccines
Progress and Prospects

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Abstract

Hepatitis E accounts for the major part of enterally transmitted non-A, non-B hepatitis worldwide. Its agent, the hepatitis E virus (HEV), is a small, single-stranded RNA virus. Only one serotype of HEV is recognised. Infection results in protective immunity with long-lived neutralising antibodies. In developing countries with poor sanitary conditions and high population density, hepatitis E causes water-borne epidemics with substantial mortality rates in pregnant women. In addition, more than 50% of cases of acute hepatic failure and sporadic acute hepatitis are due to hepatitis E. The overall prevalence rates of antibodies to the HEV in populations native to these areas rarely exceed 25%. Hence, many individuals remain susceptible to hepatitis E infection, making hepatitis E an important public health concern.

In this context, the development of an HEV vaccine is warranted. Because HEV does not grow adequately in cell cultures the development of a vaccine based on inactivated or attenuated whole-virus particles is not feasible. HEV vaccines currently under study are based on recombinant proteins derived from immunogenic parts of the HEV capsid gene. Other approaches such as DNA-based vaccines or transgenic tomatoes have also been developed. Several recombinant protein-based vaccines elicited neutralising antibodies and protective immunity in vaccinated non-human primates. One such vaccine has passed phase I trial and is currently under further evaluation in field trials. Even so, several questions remain to be answered before vaccination programmes could be implemented.

Hepatitis E virus (HEV), a small, single-stranded, hepatotropic RNA virus with an enteral route of transmission is endemic to most tropical and subtropical areas of the world. Infection causes acute, self-limited and generally icteric hepatitis, clinically indistinguishable from hepatitis A. In endemic areas, hepatitis E occurs sporadically in the form of water-borne epidemics (the largest epidemic involved several thousand individuals).[1,2] With the introduction of broadly available serological tests for antibodies to HEV in the early 1990s, it became obvious that hepatitis E accounts for the major part of enterically transmitted non-A, non-B hepatitis. In recent years, rare sporadic cases emerged in industrialised countries in patients without travel history.[3-7] Therefore, it was simultaneously recognised that HEV circulates in several animal species native to developing and developed countries. In particular, pigs[8-13] and rats[14,15] were shown to be highly susceptible. It was therefore speculated that these and other species may serve as a zoonotic reservoir.[16] This hypothesis was further supported

by experimental cross-species transmission studies, for example swine isolates to non-human primates[17,18] and the close genetic relatedness of human and animal strains native to the same region.[10,12,13] Hence, sporadic cases, some of which were probably food-borne,[19,20] may be encountered even in countries where HEV is not endemic (see Worm et al.[21]).

However, the burden of HEV-associated morbidity and mortality falls predominantly in areas with poor hygienic conditions and high population density. In many developing countries, such as India or Nepal, more than 50% of cases of acute hepatic failure,[22,23] as well as sporadic acute hepatitis,[24,25] are caused by hepatitis E. Both public health and socioeconomic reasons are the factors behind the need for the development of an HEV vaccine, even though the cost of vaccine programmes for populations at risk will have to be balanced with related costs for the improvement of sanitary conditions and water supplies.[26]

1. Populations and Groups at Risk for Hepatitis E Virus (HEV)

The occurrence of outbreaks (despite being of variable magnitude) has been surveyed in Southeast and Central Asia, the Middle East, northern and southern parts of Africa, and Mexico (figure 1). Local outbreaks or even larger epidemics are reported nearly annually in parts of the Indian subcontinent, such as the Kashmir Valley in India or the Kathmandu Valley in Nepal (see Labrique et al.[27]). Considering that the prevalence of anti-HEV antibodies in areas of high endemicity with an annual occurrence of outbreaks and constant presence of sporadic disease is about 25%, the rate of hepatitis E cases can be roughly estimated for such populations. The case-to-infection ratio during outbreaks and under sporadic conditions was reported to be 28–30 per 100 infections.[28] Converted into population figures this would mean that 6 of 100 individuals living in a highly endemic area would experience acute hepatitis E once in their life (in the case of waning antibodies the number would be even greater). In fact, this would mean >30 000 annual cases for a country such as Nepal, or several hundred thousand annual cases for the population in India, with a mean anti-HEV antibody prevalence between 4% and 15% (others have even estimated 2.2 million annual cases[29]).

1.1 Epidemic Occurrence of HEV Infection

Several studies of large epidemics identified faecal contaminated water supplies (such as riverine water, shallow wells, bore holes or inadequately serviced pipes) as the primary source of HEV transmission.[1,2,30-36] Hence, populations that depend on water supplies or riverine water for both sanitary purposes and source of (untreated) drinking water are at constant risk of HEV infections. Sewage of human, and potentially also of animal origin (naturally or in the form of manure), disposed near riverbanks may contaminate water supplies during rainy seasons as a result of flooding. In addition, undiluted sewage drained into inadequately chlorinated water supplies may lead to a high load of HEV particles. Such contamination may accidentally occur in large water pumping stations as was the case in the first reported epidemic in New Delhi, India, in 1955–6,[1] or later in 1991 in Kanpur, India,[2] involving thousands of individuals.

Epidemiological studies on these outbreaks reported attack rates in a range between 2% and 15%, with a peak incidence in male adults aged 15–40 years and a low rate among children. The observed fatality rate varies from 0.07% to 0.6%. However, the mortality rate in pregnant women in the third trimester was much higher (10–20%).[1,30,34,35] In addition, higher rates of premature delivery[34,37] and the potential of vertical transmission[38] make women of reproductive age residing in endemic areas a main target group for HEV vaccines.

The majority of outbreaks, which are generally smaller in size, occur in rural areas. In rural populations living in poor hygienic conditions, prevalence rates of anti-HEV antibodies are much higher[39,40] and protective immunity may develop as a result of frequent low-dose exposure to HEV or following early subclinical infection during childhood. Differences in living conditions, duration of breast-feed-

Fig. 1. Global distribution of hepatitis E virus (HEV) infection. The burden of HEV-associated morbidity is depicted for different countries and graduated into four different zones. Within one country substantial differences may exist for various regions. The countries are classified according to the region with the highest score. Zone A (black): nearly annual occurrence of HEV epidemics; high burden of HEV-associated morbidity and mortality; implementation of vaccination programmes would be recommendable; vaccination would probably be recommendable to travellers to these regions. Zone B (vertical stripes): outbreaks have been reported; overall prevalence rates of anti-HEV antibodies are >25%; implementation of vaccination programmes would be recommendable for specific groups at risk of HEV infection. Zone C (horizontal stripes): outbreaks have not been reported; sporadic cases do occur and the anti-HEV antibody prevalence rates are >5%; vaccination would probably not be recommendable as specific groups at risk have not been defined. Zone D (white): low or undefined probability of sporadic HEV infection; low or undefined anti-HEV antibody prevalence rates.

ing or attendance of local institutions may explain the wide range of anti-HEV antibody prevalence rates reported for endemic areas. Anti-HEV antibody prevalence rates in children <5 years and <10 years old may vary (even if the same test was used) between 0% and 5% (reported for Pune, India)[41] and up to approximately 35% and 61% (reported for rural Egyptian communities),[40] respectively. By late childhood, the anti-HEV antibody prevalence in endemic areas never reaches more than 90%, as is the case for anti-hepatitis A virus (HAV) antibody prevalence,[41] thus exposing a substantial number of adults to overt disease.

Molecular studies of sewage samples from a sewage treatment plant in Pune, India, detected a significantly higher overall prevalence of HAV RNA compared with HEV RNA ($p = 0.023$) and negative results for HAV RNA after sewage treatment.[42] However, sewage treatment did not result in reduction of HEV RNA-positive samples. Therefore, despite treatment, sewage may remain infectious for HEV particles.

Outbreaks may occur after natural disasters and military operations may force refugees to use uncertain water sources.[43] Long periods of dryness in regions with constant water shortage increase the

pressure on remaining water supplies or may concentrate HEV particles, thereby increasing the infectious load.[35]

The risk of person-to-person transmission of HEV seems to be rather low (1–2%)[1,30,44] even though some studies have suggested a higher rate.[34,45] However, if a vaccine is available in the future, vaccination of household members of an index case will not be recommendable as most secondary diseases occur within 2 weeks of the index case.

In summary, populations living in areas with poor sanitary conditions and unsafe water supplies constitute the main target group for HEV vaccination programmes. Figure 1 refines the global risk for HEV infection for different countries. The risk for HEV-associated morbidity is graduated into four different zones, each depicting the possible use of HEV vaccination in the various countries.

1.2 Groups at Risk for Sporadic HEV Infection

Even though multiple serostudies have been conducted, special risk factors for sporadic infections with HEV have been poorly defined until now. This section describes the current evidence for the use of a potential vaccine in specific risk groups. The evidence is derived from papers published since 1990, searched for in Medline and EMBASE using the keywords 'HEV or hepatitis E virus or viral hepatitis E' alone or in combination with keywords such as 'risk factors or risk groups'. Studies were included if they could demonstrate an increased risk for HEV-associated morbidity.

1.2.1 Blood Transfusion Recipients

Parenteral transmission of HEV is unusual, as the viraemic phase of infection is usually brief. A possible risk of HEV transmission via blood transfusions has only been suggested for highly endemic areas.[46] At present there is insufficient evidence to recommend vaccination for patients undergoing multiple blood transfusions.

1.2.2 Haemodialysis

Several studies showed an increased prevalence of anti-HEV antibodies in patients on maintenance haemodialysis (see Fabrizi and Martin[47]). A case-control study conducted in Saudi Arabia reported a prevalence rate of IgM anti-HEV antibodies significantly higher in patients on maintenance haemodialysis than that found in controls (odds ratio [OR] 8.9; $p = 0.037$).[48] Serum ALT levels were only moderately elevated and mostly in those with concomitant chronic viral hepatitis. Despite elevated IgM anti-HEV antibodies patients remained clinically asymptomatic. The investigators speculated that patients with chronic renal disease may fail to recognise clinical episodes of hepatitis as a result of their pre-existing condition and less pronounced jaundice under haemodialysis treatment. A single case of elevated serum ALT levels and subsequent anti-HEV seroconversion was reported in Germany.[49] Without doubt, rising serum ALT levels in patients undergoing long-term haemodialysis, which is often encountered, will usually not lead the physician to test for HEV infection. However, the low risk of HEV-associated severe morbidity and a possible lower response rate to vaccination would have to be balanced with potential advantages. There is no evidence that vaccination should be recommended for patients undergoing long-term haemodialysis.

1.2.3 Pre-existing Hepatic Diseases

Patients with chronic hepatic diseases may be at higher risk of HEV-associated morbidity and liver failure. In Chad, a country with extremely high hepatitis B virus infection rates, acute and fulminant hepatitis is more often seen if acute HEV infection is conjuncted with either chronic or acute hepatitis B.[50] A study from Pakistan reported several cases of severe liver decompensation in patients with chronic liver disease and superimposed acute HEV infection.[51] A mono-centre study from India conducted within a period of 18 months reported nine cases of HEV-induced acute liver decompensation in patients with chronic liver diseases (such as Wilson's disease, chronic hepatitis B infection or autoimmune hepatitis).[52] Two recently published case reports from Japan[53] and France[54] described fulminant hepatic failure due to HEV superinfection in patients with pre-existing hepatic diseases. The Japanese patient was a hepatitis B surface antigen

(HbsAg) carrier with thyrotoxicosis and acute HEV infection. The patient from France had underlying cirrhosis and fulminant hepatic failure necessitated liver transplantation. Vaccination of patients resident in endemic areas with pre-existing liver disease as well as those travelling to such regions will be recommendable.

1.2.4 Travellers to Endemic Areas

Only a few cases of travel-associated acute HEV infections have been reported. In fact, a review of the risk of hepatitis E infection to travellers identified only 161 cases of travellers who developed acute hepatitis between 1986 and 1999.[55] Interestingly, such cases are mainly reported from a few laboratories that are well acquainted with HEV tests, suggesting a gross under-reporting. In a study from Taiwan conducted in patients with acute non-A, non-B, non-C hepatitis, hepatitis E infection was common (18/47; 38%) and mainly in those with a travel history to endemic areas such as China (OR 22.2; 95% CI 4.7, 105.8).[56] Because of the lack of larger studies, future recommendation of vaccination for tourists will have to be judged on an individual basis. Pregnant women should be cautious.[57] Travellers with pre-existing liver disease could be regarded as candidates for vaccination, especially if their journey goes to endemic areas during seasons with known recurrent epidemics and unsafe water supply.

1.2.5 Occupational Risk

An elevated risk for sporadic acute HEV infection or even outbreaks may exist for personnel working under poor hygienic conditions, such as military staff crowded together in camps or soldiers undergoing operations. Even if strict hygiene regulations are followed, HEV infection cannot be prevented in any case.[43] Such HEV-associated outbreaks occurred in Nepal in over 600 soldiers at an isolated military training camp[28] and over 100 patients were hospitalised in a military unit in Pakistan.[58] Smaller clusters or solitary cases have been reported in French soldiers sent to endemic areas such as Somalia, where seven acute HEV infection cases occurred during the Restore Hope international mission in 1993.[43] Soldiers or peacekeepers sent to endemic areas or living in endemic areas constitute a specific risk group and vaccination would be recommendable.

Nosocomial spread from a pregnant woman to staff members of an operating theatre team was reported in South Africa.[59] In Germany, seroprevalence rates for antibodies to HEV were significantly higher in persons working in emergency admissions or in surgery.[60] Therefore, medical personnel, especially operating theatre staff and surgeons, residing in HEV endemic areas would be recommended to have vaccination.

Since it has been recognised that HEV isolates circulate in several animal species, several serostudies have been conducted to assess a potential higher risk for occupations that involve close contact to animals. Significantly higher prevalence rates were reported for veterinarians working with swine in the US,[60] pig handlers in Taiwan,[60] field workers in Iowa, USA[61] and swine farmers in Moldova.[62] In the latter study elevated anti-HEV antibodies prevalence was associated with cleaning barns, assisting sows at birth or drinking unpasteurised cow's milk. However, elevated antibody prevalence rates were not associated with a history of jaundice or hepatitis. Zoonotic infection (at least with genotype III animal strains) may result in less severe disease. Overt disease seems to be dose dependent and zoonotic transmission routes may mostly not transmit virus loads large enough to cause overt disease.[63,64]

1.2.6 Food-Borne Risks

Studies on sporadic cases of acute hepatitis (from Sicily, Italy[65] and Hong Kong[66]) reported shellfish ingestion to be an independent risk factor for HEV infection; shellfish are filter feeders that can extract and concentrate viruses if grown in sewage polluted water and are, therefore, prone to cause viral infection.[67] When consumed raw, animal products may cause severe disease, as reported for deer meat[19] and pig liver[20] in Japan. Therefore, where there is a threat of infected animal products, the option would undoubtedly be cooking rather than vaccinating. Occupations involved in manufacturing processes of raw animal products need further evaluation of the risk regarding HEV-associated morbidity.

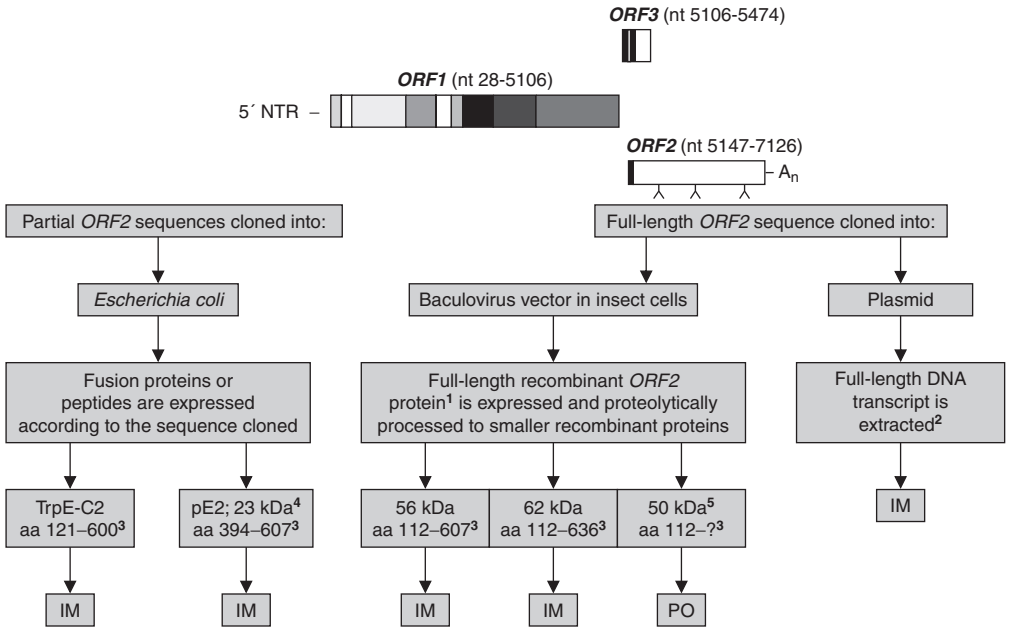

Fig. 2. Structure of the hepatitis E virus (HEV) genome and HEV vaccines derived from the HEV capsid gene. HEV vaccines are generated from the HEV capsid gene (*ORF2*). The *ORF2* sequence is cloned into various expression systems. The shading in the schematic presentation used for ORF gene mapping defines: for the *ORF1* gene putative functional regions such as (beginning at the 5′end): Methyltransferase, Domain Y, region of unknown function, papain-like cysteine protease, hypervariable region, proline 'hinge', domain X, helicase, RNA replicase; for the *ORF3* gene: the black bars correspond to a hydrophobic region at the 5′end with a polycysteine region and a transmembrane sequence; for the *ORF2* gene: the black bar corresponds to a signal peptide at the 5′end, at the 3′end is a 3′-nontranslated region (nt 7127-94) followed by A_n, the *ORF2* has three potential glycosylation sites (reversed Y). **1** full-length recombinant *ORF2* protein (pORF2); 72 kDa, amino acids 1–660; **2** full-length *ORF2* is expressed in cells of the vaccinee; **3** depicts amino acids corresponding to the full-length pORF2; **4** forms 42 kDa homodimers; **5** assemble into virus-like particles; **5′ NTR** = 5′-nontranslated region (nucleotides nt 1–27); **aa** = amino acids; **A_n** = polyadenylated 3′end; **IM** = intramuscular; **ORF** = open reading frame; **PO** = oral application.

2. HEV

2.1 Molecular Biology of HEV

The HEV was first described in 1983 by means of immune electron microscopy (IEM) as spherical, 27–30nm virus-like particles (VLPs).[68] After oral ingestion the virus mainly infects and replicates in hepatocytes, and the virus particles are excreted via bile in the faeces. Even though the virus has been shown to be labile when exposed to high concentrations of salt, freeze-thawing and pelleting,[69] infectious particles can persist in sewage for a significant period.[70] The HEV genome is a single-stranded, positive-sense, polyadenylated RNA molecule of approximately 7.2Kb with short 5′- and 3′-noncoding regions (figure 2).[71,72] It contains three partially overlapping open reading frames (ORFs): *ORF1*, at the 5′end of the genome, encodes about 1700 amino acids encompassing nonstructural, enzymatically active proteins probably involved in the virus replication and viral protein processing.[72,73] *ORF3* encodes for a 123-amino acid phosphorylated protein of unknown function.[74] *ORF3* significantly overlaps *ORF2*, which is located at the 3′end and encodes a 660-amino acid structural or capsid protein. Proteins derived from all three ORFs elicit an antibody response during the acute state of infection.[75,76] However, recombinant proteins and peptides used in enzyme-linked immunoassays (EIAs) or for vaccination studies are predominately expressed from *ORF2* epitopes.

2.2 Genetic Diversity of HEV

With the exception of a hypervariable region within *ORF1*, the genome of HEV seems to be relatively stable.[77] However, prototype strains from locations around the world or even strains from separate sporadic cases within one region may show considerable diversity. The overall nucleic acid identity of the most diverse strains is around 70%, whereas the deduced amino acid identity is over 80%.[78] Variations in nucleic acid sequences are often conservative substitutions that do not result in differences at the amino acid level. In particular, the epitope regions of *ORF2* and *ORF3* are highly conserved.[79]

Defining an HEV genotype as viruses having nucleotide divergence of not more than 20% of the nucleotides in the *ORF2* region,[80,81] at least four major genotypes can be separated of which the largest group is genotype I (including strains from Asia and Africa). Genotype II is a small group represented by single isolates from Mexico and Nigeria, whereas genotype III comprises several strains isolated from sporadic cases of acute HEV infection in the US, parts of Europe (Austria, Italy, Greece, Spain), Argentina and Japan, and also swine isolates from various countries such as the US, The Netherlands, New Zealand, Canada and Korea[82] (see Worm et al.[21,80]). Genotype IV consists of Chinese, Japanese and probably Taiwanese isolates as well as swine isolates from India and Bali.[83] In India, human strains are grouped within genotype I and strains isolated from pigs into genotype IV.[84] In Japan, genotype III and IV isolates were reported in both animals and humans.[12]

Experimental transmission from genotype III swine isolates to non-human primates caused less severe hepatitis than did human genotype I and II isolates.[16,63] Sewage samples of human origin collected from sewage drains in various industrialised countries such as Spain, the US and France showed a high rate of contamination with HEV strains (e.g. in Barcelona the rate was 43%; 20/46).[85] The HEV isolates were clustered predominantly into genotype III. Therefore, it was speculated that these less virulent genotype III HEV isolates with probably zoonotic transmission routes (e.g. from swine to humans) could cause little or no disease in humans, but account for the 0.4–3% anti-HEV antibody prevalence in non-diseased blood-donor populations reported for various industrialised countries. A newly identified avian HEV strain was also shown to share antigenic epitopes with the swine and human HEV. Convalescent sera of experimentally infected chickens cross-reacted in EIAs for antibodies to the human and swine HEV.[86] Therefore, the agent causing the anti-HEV reactivity in industrialised countries remains to be determined.

Despite this genetic diversity all genotypes seem to represent a single serotype. Diverse HEV strains seem to share at least one epitope region within *ORF2* which elicits neutralising antibodies. This is an important prerequisite for the development of a broadly protective vaccine.

3. Hepatitis E Vaccine Development

A further prerequisite for successful implementation of HEV vaccine programmes would be the ability of an HEV vaccine to induce antibodies with broadly HEV neutralising activity, and long-term immunity and protection against both clinical disease (i.e. significant elevation of serum ALT levels and liver damage) and infection (i.e. replication in host liver cells, vertical transmission and excretion into the faeces). Vaccination with inactivated or attenuated whole-virus particles would best mimic natural or experimental infection and result in similar broad immunity. Because HEV does not adequately grow in cell cultures, the development of a vaccine based on inactivated or attenuated whole-virus particles is not feasible. Most HEV vaccines currently under study are based on recombinant proteins derived from immunogenic parts of the HEV capsid gene. However, monovalent recombinant proteins only derived from parts of the viral genome could fail to elicit neutralising antibodies or result in a narrow strain-specific immunity. Therefore, vigourous studies are crucial to assess the neutralising ability of such vaccines.

3.1 Naturally Acquired Antibodies and their Detection

Natural infection with HEV elicits an antibody response and results in protective immunity. After an incubation time (ranging between 15 and 60 days) anti-HEV IgM antibodies appear in the serum of infected patients with the onset of symptoms and remain detectable for about 2–3 (up to 6) months.[87] Anti-HEV IgG antibodies can be detected promptly after the IgM response. The IgG titre increases throughout the acute phase of infection and falls during the convalescent phase. Longitudinal studies demonstrated persistence of anti-HEV antibodies up to 12[88] and 14[89] years. Repeated infections in the same individual have not been reported. However, several studies have suggested that in a significant proportion of patients, mostly children, anti-HEV antibodies may be short lived,[90-93] and projection analyses of anti-HEV antibody prevalence rates suggested partial waning of antibodies.[93,94] This may explain the relatively low anti-HEV antibody prevalence rates in adults native to endemic areas.

Serodiagnostic assays (Western blots or EIAs) have been developed using recombinant proteins and synthetic peptides derived from antigenic domains within *ORF2* and *ORF3* to detect IgM and IgG class antibodies to the HEV. EIAs are the preferred screening format. The two prototype EIAs for routine diagnosis of HEV infection and serosurveys employ either four short recombinant proteins derived from the 3′termini of *ORF2* (42-amino acid) and *ORF3* (33-amino acid) from Burmese and Mexican prototype sequences,[90,95] or two recombinant proteins derived from complete *ORF3* (123-amino acid) and from a sequence of *ORF2* (327-amino acid) from the Burmese strain.[96]

Recently, attempts have been made to generate recombinant peptides or proteins corresponding to conformational epitopes, thus mimicking the tertiary or quaternary structure of immunogenic parts of the HEV structural *ORF2* protein.

1. A 23 kDa peptide corresponding to amino acid residues 394–604 of the *ORF2* sequence was shown to naturally interact with one another forming homodimers under physiological conditions.[97] These peptides strongly recognised HEV reactive human sera, but only in their dimerised form.

2. Two 111-amino acid N-terminal truncated *ORF2* proteins, a 53 kDa protein derived from a Pakistani strain[98] and a 50 kDa protein derived from a Burmese strain,[99] were shown to spontaneously form VLPs when excreted into the culture medium. These VLPs resembled the whole virus particles when visualised with IEM but were smaller in size (approximately 24nm compared with 27–30nm). Both the dimerised 23 kDa peptides and the 50 kDa Burma recombinant VLPs, have been shown to improve the sensitivity of EIAs to detect convalescent-phase antibodies to HEV and were, therefore, used for vaccination studies.

Comparison of various EIAs and Western blots showed a low overall concordance in the detection of anti-HEV antibodies in a non-diseased population.[100] Recently, a WHO anti-HEV IgG antibodies standard was defined, which could be relevant to appropriately assess immune response in vaccine programmes.[101]

3.2 Passive Immunisation

Studies on passive immunisation are sparse and results are conflicting. In experimental settings passive transfer of convalescent sera from humans and experimentally infected monkeys protected cross-challenged monkeys against disease but not against HEV infection,[102,103] whereas others reported no efficacy of passive immunisation of rhesus monkeys with human convalescent-phase serum.[88] In epidemic settings in Mexico and India, preliminary studies with low-dose human serum immunoglobulin showed no beneficial effects on the severity of the disease,[33,44] whereas a study conducted during an outbreak at Karad, India, reported a decline of disease severity in pregnant women.[104] The effectiveness of a serum immunoglobulin largely depends on its titre of protective antibodies and the dosage used. The data available do not allow definite recommendations regarding passive immunisation.

3.3 Serological Cross-Reactivity and Neutralising Antibodies

Preliminary studies showed that acute- and convalescent-phase sera from cases of HEV infection occurring in geographically distinct regions blocked antibody binding in a fluorescent antibody test.[105] Therefore, hepatitis E isolates with substantial genetic diversity seem to possess at least one major cross-reactive epitope. Experimentally infected nonhuman primates elicited neutralising antibodies, making the animals immune to challenge with homologous and heterologous HEV prototype strains. Cynomolgus macaques (cynos) infected with the Burma strain were immune to re-infection with the Mexico strain[106] and rhesus monkeys infected with HEV from the former USSR developed immunity during infection to divergent Indian HEV isolates.[107] Long-term follow-up of the rhesus monkeys showed persistence of anti-HEV antibodies (though partly declined to a low titre) and protection against disease (but not necessarily against infection) when re-challenged with Indian HEV isolates.[108]

On the other hand, preliminary studies showed only limited neutralising capability for vaccine-induced antibodies. An *in vitro* study suggested a limited cross-neutralisation activity for antibodies elicited against diverse *ORF2* recombinant proteins.[109] The study used a polymerase chain reaction-based cell culture neutralisation assay.[110] Naturally or experimentally acquired convalescent-phase antibodies from humans and cynos showed broad HEV-neutralising activity, whereas post-immunisation antibodies from guinea pigs and rabbits had limited neutralising activity.[109] Other studies suggested that the antigenic structure of surface-expressed HEV proteins were complex and HEV-neutralising epitopes were conformation dependent.[111] Although neutralising monoclonal antibodies (Mabs) to linear epitopes of *ORF2* have been detected,[112,113] Mabs specific for conformational epitopes were more effective in blocking convalescent-phase antibodies in a competitive EIA compared with Mabs specific for linear epitopes.[114] These results indicate the importance of the structure of recombinant proteins used for vaccination. In addition, the results showed that the efficacy of a recombinant protein in inducing protective immunity cannot be predicted even if their linear epitopes had been characterised.

3.4 Experimental Vaccination Studies

Serostudies demonstrated that antibodies to *ORF3* proteins were predominantly found in the acute phase of HEV infection and that these antibodies were more strain specific.[115] Therefore, HEV vaccine research focused on *ORF2*-derived recombinant proteins (pORF2). Several immunodominant epitopes have been described within the *ORF2* sequence.[79,111,112,114,116] These *ORF2* sequences can be cloned into various expression systems, such as *Escherichia coli* and baculovirus (figure 2). In the *E. coli* expression systems, full-length or partial *ORF2* sequences are cloned into the *E. coli* genome using fusion sequences.[117,118] Expressed recombinant proteins correspond exactly to the cloned sequence but include transcribed fusion sequences. Therefore, the linear epitopes are conserved but conformation-dependent immunogenic capability could be impaired. When expressed from a baculovirus vector in insect cells the full-length 72 kDa *ORF2* protein is further processed to smaller proteins ('truncated proteins'), probably by baculovirus proteinase(s).[98,99,119] These truncated proteins seem to include conformation-dependent epitopes.

3.4.1 HEV Vaccines Based on Recombinant Proteins or Peptides

Recombinant Proteins or Peptides Expressed in *Escherichia coli* Expression Systems

One of the earliest studies reported the immunisation of cynos with intramuscular injection of a 436-amino acid pORF2 derived from a Burmese strain (designated TrpE-C2, expressed as a trpE-HEV ORF2 fusion protein in *E. coli*).[117] Immunisation was only seen if the monkeys had received three doses (the third composed of unadjuvanted trpE-C2 protein, which seemed to induce different antibodies compared with the adjuvanted one). Monkeys vaccinated with three doses showed protection against

homologous challenge but signs of infection when challenged with a heterologous isolate.[117] A recent study used a short, 23 kDa peptide (designated pE2) expressed in *E. coli* from a region located near the 3′terminal of ORF2 of a Chinese strain.[118] The 23 kDa peptide naturally forms homodimers. It was speculated that such homodimers mimic more closely the conformation of the tertiary structure of immunogenic parts of the capsid protein. Vaccination of three rhesus monkeys with two doses prevented experimental infection with a homologous strain in two of three rhesus monkeys and reduced viral excretion in one.[118]

Recombinant Proteins or Peptides Expressed in Baculovirus Expression Systems

Advantages of baculovirus expression systems in insect cells (e.g. *Spodoptera frugiperda*, Sf-9) have become apparent. EIAs based on baculovirus-expressed proteins proved a superior performance in detecting convalescent-phase anti-HEV antibodies than fusion proteins made in *E. coli*. It was assumed that baculovirus-expressed proteins may, therefore, be more likely to induce neutralising antibodies. The 72 kDa baculovirus-expressed full-length protein of the HEV ORF2 capsid gene is further processed to smaller proteins with structures similar to that of the native protein (figure 2).[98,99,119]

Three of these proteolytically processed proteins which are excreted into the culture medium were challenged in different vaccination schedules.

1. A 56 kDa protein derived from a Pakistani strain was studied extensively and is discussed in section 3.5.
2. A 62 kDa protein (designated r62K), derived from the HEV Burma isolate, was purified and used to immunise three cynos.[119] Two sequential doses of 20μg alum-precipitated r62K protein were injected intramuscularly. When challenged with a heterologous Mexican isolate, two of the vaccinated monkeys were immune to both disease and infection, whereas one monkey showed a delayed appearance of the infection (breakthrough infection) without disease 4 weeks after challenge.[120]
3. A 50 kDa protein, derived from a Burma strain, was shown to self-assemble spontaneously as VLPs after secretion into the culture medium.[97,99] The VLPs resemble wild-type HEV particles but are somewhat smaller in size. They can be dispensed via the oral route. In a preliminary study purified VLPs were orally administered to mice four times in a concentration ranging between 50 and 100μg.[121] No adjuvants were used. The oral immunisation induced both systemic and intestinal antibody response. Induced serum IgG and faecal IgA reacted with native HEV antigen, as determined by a Western blot. Subsequently, the VLP vaccine was studied in two cynos. The cynos were immunised orally with four subsequent doses of 10mg rHEV VLPs during a 36-day period and an additional booster dose at day 80. Both cynos showed a serum IgM, IgG and IgA response. When subsequently challenged with a homologous HEV strain both cynos were protected against disease. One cyno showed secretion of the virus into stool for 3 days.[122] A recent study suggested that HEV VLPs could be utilised as an orally inoculated carrier molecule for foreign antigenic epitopes inserted into VLPs either by incorporating an epitope[123] or a plasmid DNA.[124]

3.4.2 HEV DNA Vaccines

A completely different approach to HEV vaccination is the development of DNA vaccines. Purified plasmid DNA containing coding sequences with immunogenic activity is administered intramuscularly (figure 2). Similar to natural infection, the antigen is subsequently expressed in liver cells in their native form. A recent study used the plasmid construct pJHEV containing the full-length HEV *ORF2* sequence.[125,126] The injection of three sequential doses of the HEV DNA vaccine in mice elicited high levels of serum IgG to HEV in less time than the usual incubation period of natural HEV infection. It was speculated that such a DNA vaccine could possibly be used for post-infection vaccination.[126] A recent study evaluated the efficacy of such a DNA vaccine using an expression vector containing the full-length *ORF2* from the Burma strain of HEV to immunise cynos.[127] Four cynos were immunised intramuscularly with four sequential doses of the DNA vaccine. Vaccination induced

an anti-HEV antibody response in all cynos. Interestingly, the humoral immune response was only modest and a lack of boosting effect of repeated vaccination on antibody levels was observed. Only two of the four vaccinated cynos were protected against HEV infection with a heterologous strain of the virus. Recently, this DNA vaccine was successfully administered by gene gun to cynos.[128]

3.4.3 Transgenic Plant Vaccine

Recently, *ORF2* partial gene (representing amino acid residue 394–604, designated HEV-E2 gene) was transferred to tomato plants to develop a plant-derived HEV oral vaccine.[129] A plant expression vector of the partial HEV antigen was constructed and transformed into tomatoes with *Agrobacterium tumefaciens*. EIAs demonstrated the expression of HEV-E2 derived proteins at low levels (average 47.9 ng/g fresh weight leaves and 62 ng/g fruits) with specific antigenicity in fruit tissue of transgenic tomatoes.

3.5 A Vaccine Candidate

Thus far, a single vaccine candidate has passed phase I trial (safety study)[130] and is currently being tested in a field trial in Nepal. The vaccine is based on a recombinant baculovirus expressed 56 kDa *ORF2* protein derived from a Pakistani strain.[98] The vaccine was developed and manufactured by the Hepatitis Viruses section of Novavax (Bethesda, MD, USA), under the sponsorship of Glaxo SmithKline Biologicals (Rixensart, Belgium).

In a preliminary study eight cynos were immunised by either one or two intramuscular injections with 50μg of the adjuvanted 56 kDa protein.[102] All vaccinated cynos were protected against disease when challenged with the Pakistani strain. Only the twice-vaccinated animals showed no excretion of the virus into the faeces. A subsequent dose evaluation study tested the efficacy of four different doses in a range between 0.4 and 50μg of the recombinant 56 kDa protein.[131] Sixteen rhesus monkeys were vaccinated by intramuscular injection of two sequential doses 1 month apart. Vaccine dose and induced anti-HEV antibodies titre correlated directly. All animals were protected against hepatitis, but viraemia and faecal excretion were seen in most animals following both homologous and heterologous challenge. The high rate of infection was most likely due to the very high challenge dose used. A further study evaluated the usefulness of a third dose and the duration of protection following two doses.[132] It was shown that a third dose could improve vaccination efficacy, with protection lasting for at least 6 months. A recent preclinical trial showed that two doses of the vaccine were essential and that the 1μg formulations were equally effective compared with 10μg formulations.[133]

The phase I trial used 1, 5, 20 and 40μg formulations.[130] The 1μg formulation seemed to be less immunogenic, the other formulations resulted in seroconversion rates between 88.9% and 95% in healthy volunteers.

4. Prospects and Conclusion

Substantial progress has been made in developing an HEV vaccine. Further and crucial tests will have to determine the long-term efficacy of HEV vaccines. In case these tests show acceptable efficacy rates, vaccination will constitute an important tool to reduce HEV-associated morbidity. However, the complex epidemiological profile of hepatitis E will need to be counteracted by: (i) strategies to improve the safety of potable water; (ii) defining populations and specific groups exposed to the burden of HEV-associated morbidity and mortality; (iii) defining the cost effectiveness of HEV vaccination programmes for various HEV risk zones; and (iv) implementation of institutionalised vaccination programmes for populations and specific risk groups susceptible to HEV infection. The burden of HEV-associated morbidity and mortality falls predominantly in areas with poor hygienic conditions. Therefore, international health organisations will have to be involved to ensure that HEV vaccination will not be withheld from HEV susceptible populations in developing countries.

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References

1. Viswanathan R. Epidemiology. Indian J Med Res 1957; 45 Suppl. 1: 1-29
2. Naik SR, Aggarwal R, Salunke PN, et al. A large waterborne viral hepatitis E epidemic in Kanpur, India. Bull World Health Organ 1992; 70: 597-604
3. Schlauder GG, Dawson G, Erker JC, et al. The sequence and phylogenetic analysis of a novel hepatitis E virus isolated from a patient with acute hepatitis reported in United States. J Gen Virol 1998; 79: 447-56
4. Schlauder GG, Desai SM, Zanetti AR, et al. Novel hepatitis E virus (HEV) isolates from Europe: evidence for additional genotypes of HEV. J Med Virol 1999; 57: 243-51
5. Worm HC, Schlauder GG, Wurzer H, et al. Identification of a novel variant of hepatitis E in Austria. J Gen Virol 2000; 81: 2885-90
6. Pina S, Buti M, Cotrina M, et al. HEV identified in serum from humans with acute hepatitis and in sewage of animal origin in Spain. J Hepatol 2000; 33: 826-33
7. Takahashi K, Iwata K, Watanabe N, et al. Full-genome nucleotide sequence of a hepatitis E virus strain that may be indigenous to Japan. Virology 2001; 287: 9-12
8. Meng X-J, Purcell RH, Halbur PG, et al. A novel virus in swine is closely related to the human hepatitis E virus. Proc Natl Acad Sci U S A 1997; 94: 9860-5
9. Hsieh S-Y, Meng X-J, Wu Y-H, et al. Identity of a novel swine hepatitis E virus variant in Taiwan forming a monophyletic group with Taiwan isolates of human hepatitis E virus. J Clin Microbiol 1999; 37: 3828-34
10. Nishizawa T, Takahashi M, Mizuo H. Characterization of Japanese swine and human hepatitis E isolates of genotype IV with 99% identity over the entire genome. J Gen Virol 2003; 84: 1245-51
11. van der Poel WH, Verschoor F, van der Heide R, et al. Hepatitis E virus sequences in swine in the Netherlands are closely related to human hepatitis E virus sequences. Emerg Infect Dis 2001; 6: 970-6
12. Takahashi M, Nishizawa T, Miyaijama H, et al. Swine hepatitis E virus strains from four phylogenetic clusters comparable with those of Japanese isolates of human hepatitis E virus. J Gen Virol 2003; 84: 851-62
13. Arankalle VA, Chobe LP, Walimbe AM, et al. Swine HEV infection in South India and phylogenetic analysis (1985–1999). J Med Virol 2003; 69: 391-6
14. Kabrane-Lazizi Y, Fine JB, Elm J, et al. Evidence for widespread infection of wild rats with hepatitis E virus in the United States. Am J Trop Med Hyg 1999; 6: 331-5
15. Hirano M, Ding X, Li TC. Evidence for widespread infection of hepatitis E virus among wild rats in Japan. Hepatol Res 2003 27: 1-5
16. Meng X-J. Novel strains of hepatitis E virus identified from humans and other animal species: is hepatitis E a zoonosis? J Hepatol 2000; 33: 842-5
17. Meng XJ, Halbur PG, Shapiro MS, et al. Genetic and experimental evidence for cross-species infection by swine hepatitis E virus. J Virol 1998; 71: 9714-21
18. Halbur PG, Kasorndorkbua C, Gilbert C, et al. Comparative pathogenesis of infection of pigs with hepatitis E virus recovered from a pig and a human. J Clin Microbiol 2001; 39: 918-23
19. Tei S, Kitajima N, Takahashi K, et al. Zoonotic transmission of hepatitis E virus from deer to human beings. Lancet 2003; 362: 371-3
20. Yazaki Y, Mizuo H, Takahashi M. Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be food-borne, as suggested by the presence of hepatitis E virus in pig liver as food. J Gen Virol 2003; 84: 2351-7
21. Worm HC, Schlauder GG, Brandstätter G. Hepatitis E and its emergence in non-endemic areas. Wien Clin Wochenschr 2002; 114: 663-70
22. Acharya SK, Panda SK, Saxena A, et al. Acute hepatic failure in India: a perspective from the East. J Gastroenterol Hepatol 2000; 15: 473-9
23. Khuroo MS, Kamili S. Aetiology and prognostic factors in acute liver failure in India. J Viral Hepat 2003; 10: 224-31
24. Das K, Agarwal A, Andrew R, et al. Role of hepatitis E and other hepatotropic virus in the aetiology of sporadic acute hepatitis: a hospital based study from urban Delhi. Eur J Epidemiol 2000; 16: 937-40
25. Shrestha SM, Shrestha S, Tsuda F, et al. Molecular investigation of hepatitis E virus infection in patients with acute hepatitis E in Kathmandu, Nepal. J Med Virol 2003; 69: 207-14
26. Stevenson P. Nepal calls the shots in hepatitis E virus vaccine trials. Lancet 2000; 355: 1623
27. Labrique AB, Thomas DL, Stoszek SK, et al. Hepatitis E: an emerging infectious disease. Epidemiol Rev 1999; 21: 162-79
28. Clayson ET, Vaughn DW, Innis BL, et al. Association of hepatitis E virus with an outbreak of hepatitis at a military training camp in Nepal. J Med Virol 1998; 54: 178-82
29. Nayak NC, Panda SK. Enteric non-A, non-B virus hepatitis in India: current experiences in various settings including transmission studies in the rhesus monkey. In: Shikata T, Purcell RH, Uchida T, editors. Viral hepatitis C, D, and E: proceedings in the international meeting on non-A, non-B hepatitis. Amsterdam: Elsevier Science Publishers BV, 1991: 247-57
30. Khuroo MS. Study of an epidemic of non-A, non-B hepatitis: possibility of another human hepatitis virus distinct from post-transfusion non-A, non-B type. Am J Med 1980; 68: 818-24
31. Corwin AL, Khiem HB, Clayson ET, et al. A waterborne outbreak of hepatitis E virus transmission in southwestern Vietnam. Am J Trop Med Hyg 1996; 54: 559-62
32. Belabbes E-H, Bouguermouth A, Benatallah A, et al. Epidemic non-A, non-B viral hepatitis in Algeria: strong evidence for its spreading by water. J Med Virol 1995; 16: 257-63
33. Benjelloun S, Bahbouhi B, Bouchrit N, et al. Seroepidemiological study of an acute hepatitis E outbreak in Morocco. Res Virol 1997; 148: 279-87
34. Velázquez O, Stetler HC, Avila C, et al. Epidemic transmission of enterically transmitted non-A, non-B hepatitis in Mexico, 1986–1987. JAMA 1990; 263: 3281-5
35. Isaäcson M, Frean J, He J, et al. An outbreak of hepatitis E in northern Namibia, 1983. Am J Trop Med Hyg 2000; 62: 619-25
36. Rab MA, Bile MK, Mubarik MM, et al. Water-borne hepatitis E virus epidemic in Islamabad, Pakistan: a common source outbreak traced to the malfunction of modern treatment plant. Am J Trop Med Hyg 1997; 57: 151-7

37. Tsega E, Krawczynski K, Hansson BG, et al. Hepatitis E virus infection in pregnancy in Ethiopia. Ethiop Med J 1993; 31: 173-81
38. Khuroo MS, Kamili S, Jameel S. Vertical transmission of hepatitis E virus. Lancet 1995; 345: 1025-6
39. Seow H-F, Mahomed NM, Mak J-W, et al. Seroprevalence of antibodies to hepatitis E virus in the normal blood donor population and two aboriginal communities in Malaysia. J Med Virol 1999; 59: 164-8
40. Fix AD, Abdel-Hamid M, Purcell RH, et al. Prevalence of antibodies to hepatitis E in two rural Egyptian communities. Am J Med Hyg 2000; 62: 519-23
41. Arankalle VA, Tsarev SA, Chadha MS, et al. Age-specific prevalence of antibodies to hepatitis A and E virus in Pune, India 1982 and 1992. J Infect Dis 1995; 171: 447-50
42. Vaidya SR, Chitambar SD, Arankalle VA. Polymerase chain reaction-based prevalence of hepatitis A, hepatitis E and TT viruses in sewage from an endemic area. J Hepatol 2002; 37: 131-6
43. Buisson Y, Coursaget P, Bercion R, et al. Hepatitis E virus infection in soldiers sent to endemic regions [letter]. Lancet 1994; 344: 1165-6
44. Arankalle VA, Chada MS, Mehendale SM, et al. Epidemic hepatitis E: serological evidence for lack of intrafamilial spread. Indian J Gastroenterol 2000; 19: 24-8
45. Khuroo SM, Dar MY. Hepatitis E: evidence for person-to-person transmission and inability of low dose immune serum globulin from an Indian source to prevent it. Indian J Gastroenterol 1992; 11: 113-6
46. Arankalle VA, Chobe LP. Hepatitis E: can it be transmitted parenterally? J Viral Hepat 1999; 6: 161-4
47. Fabrizi F, Martin P. Hepatitis E in end-stage renal disease. Int J Artif Organs 1999; 22: 593-9
48. Ayoola EA, Want MA, Gadour MO, et al. Hepatitis E virus infection in haemodialysis patients: a case-control study in Saudi Arabia. J Med Virol 2002; 66: 329-34
49. Wang C-H, Flehmig B, Jahn G, et al. Hepatitis E virus in haemodialysis [letter]. Vox Sang 1997; 73: 54-5
50. Coursaget B, Buisson Y, N'Gawara MN, et al. Role of hepatitis E in sporadic cases of acute and fulminant hepatitis in an endemic area (Chad). Am J Trop Med Hyg 1998; 58: 330-4
51. Hamid SS, Atiq M, Shehzad F, et al. Hepatitis E superinfection inpatients with chronic liver disease. Hepatology 2002; 36: 474-8
52. Ramachandran J, Eapen C, Kang G, et al. Hepatitis E superinfection produces sever decompensation in patients with chronic liver disease. J Gastroenterol Hepatol 2004; 19: 134-8
53. Hui AY, Chan HL, Chan FK, et al. Fulminant hepatic failure in a patient with inactive HbsAg carrier state, acute hepatitis E and thyrotoxicosis. Hepatol Res 2003; 27 (3): 248-51
54. Nicoluzzi J, Mennecier D, Sogni P, et al. Hepatitis E-associated subacute liver failure: rare indication for liver transplantation [letter]. Am J Gastroenterol 2001; 96: 2278-9
55. Piper-Jenks N, Horowitz HW, Schwartz E. Risk of hepatitis E infection to travellers. J Travel Med 2000; 7: 194-9
56. Wu JC, Sheen IJ, Chiang TY, et al. The impact of travelling to endemic areas on the spread of hepatitis E virus infection: epidemiological and molecular analyses. Hepatology 1998; 27: 1415-20
57. Hussaini SH, Skidmore SJ, Richardson P, et al. Severe hepatitis E infection during pregnancy. J Viral Hepat 1997; 4: 51-4
58. Bryan JP, Iqbal M, Tsarev S, et al. Epidemic of hepatitis E in a military unit in Abbotrabad, Pakistan. Am J Trop Med Hyg 2002; 67: 662-8
59. Robson SC, Adams S, Brink N, et al. Hospital outbreak of hepatitis E [letter]. Lancet 1992; 339: 1424-5
60. Meng XJ, Wiseman B, Elvinger F, et al. Prevalence of antibodies to hepatitis E virus in veterinarians working with swine and in normal blood donors in the United States and other countries. J Clin Microbiol 2002; 40 (1): 117-22
61. Karetnyi YV, Gilchrist MJ, Naides SJ. Hepatitis E virus infection prevalence among selected populations in Iowa. J Clin Virol 1999; 14 (1): 51-5
62. Drobeniuc J, Favorov MO, Shapiro CN, et al. Hepatitis E virus antibody prevalence among persons who work with swine. J Infect Dis 2001; 184 (12): 1594-7
63. Tsarev SA, Tsareva TS, Emerson SU, et al. Infectivity titration of a prototype strain of hepatitis E virus in cynomolgus monkeys. J Med Virol 1994; 43: 35-42
64. Aggarwal R, Kamili S, Spelbring J, et al. Experimental studies on subclinical hepatitis E virus infection in cynomolgus macaques. J Infect Dis 2001; 184: 1380-5
65. Cacopardo B, Russo R, Preiser W, et al. Acute hepatitis E in Catania (eastern Sicily) 1980-1994: the role of hepatitis E virus. Infection 1997; 25 (5): 313-6
66. Chau TN, Lai ST, Tse C, et al. Parenteral and sexual transmission are not risk factors for acute hepatitis E infection in Hong Kong [letter]. Am J Gastroenterol 2001; 96: 3046-7
67. Stolle A, Sperner B. Viral infection transmitted by food of animal origin: the present situation in the European Union. Arch Virol Suppl 1997; 13: 219-28
68. Balayan MS, Andjaparidze AG, Savinskaya NA, et al. Evidence for a virus in non-A, non-B hepatitis transmitted via the fecal-oral route. Intervirology 1983; 20: 23-31
69. Bradley DW. Hepatitis E epidemiology, aetiology and molecular biology. Rev Med Virol 1992; 2: 19-28
70. Jothikumar N, Aparna K, Kamatchiammal S, et al. Detection of hepatitis E virus in raw and treated wastewater with a polymerase chain reaction. Appl Environ Microbiol 1993; 59: 2558-62
71. Reyes GR, Purdy MA, Kim JP, et al. Isolation of a cDNA from the virus responsible for enterically transmitted non-A, non-B hepatitis. Science 1990; 247: 1335-9
72. Tam AW, Smith MM, Guerra ME, et al. Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. Virology 1991; 185: 120-31
73. Koonin EV, Gorbalenya AE, Purdy MA, et al. Computer assisted assignment of functional domains in the nonstructural polyprotein of hepatitis E virus: delineation of an additional group of positive strand RNA plant and animal viruses. Proc Nat Acad Sci U S A 1992; 9: 8259-63
74. Zafrullah M, Ozdener MH, Panda SK, et al. The ORF3 protein of hepatitis E virus is a phosphoprotein that associates with the cytoskeleton. J Virol 1997; 71: 9045-53
75. Kaur M, Hyams KC, Purdy MA, et al. Human linear B-cell epitopes encoded by the hepatitis E virus include determinants in the RNA dependent-RNA polymerase. Proc Natl Acad Sci U S A 1992; 89: 3855-8
76. Khudyakov YE, Khudyakova NE, Fields HA, et al. Epitope mapping in proteins of hepatitis E virus. Virology 1993; 194: 89-96
77. Arankalle VA, Paranjape S, Emerson SU, et al. Phylogenetic analysis of hepatitis E virus isolates from India (1976–1993). J Gen Virol 1999; 80: 1691-700

78. Huang C-C, Nguyen D, Fernandez J, et al. Molecular cloning and sequencing of the Mexico isolate of hepatitis E virus (HEV). Virology 1992; 191: 550-8
79. Yarbough PO, Tam AW, Fry KE, et al. Hepatitis E virus: identification of type-common epitopes. J Virol 1991; 65: 5790-7
80. Worm HC, van der Poel WH, Brandstätter G. Hepatitis E: an overview. Microbes Infect 2002; 4: 657-66
81. Schlauder GG, Mushahwar IK. Genetic heterogeneity of hepatitis E virus. J Med Virol 2001; 65: 282-92
82. Choi I-S, Kwon H-J, Shin N-R, et al. Identification of swine hepatitis E virus (HEV) and prevalence of anti-HEV antibodies in swine and human populations in Korea. J Clin Microbiol 2003; 41: 3602-8
83. Wibawa IDN, Muljono DH, Mulyanto, et al. Prevalence of antibodies to hepatitis E virus among apparently healthy humans and pigs in Bali, Indonesia: identification of a pig infected with a genotype 4 hepatitis E virus. J Med Virol 2004; 73: 38-44
84. Arankalle VA, Chobe LP, Joshi MV, et al. Human and swine hepatitis E viruses from western India belong to different genotypes. J Hepatol 2002; 36: 417-25
85. Clemente-Casares P, Pina S, Buti M, et al. Hepatitis E Virus epidemiology in industrialized countries. Emerg Infect Dis 2003; 9: 448-54
86. Haqshenas G, Huang FF, Fenaux M, et al. The putative capsid protein of the newly identified avian hepatitis E virus shares antigenic epitopes with that of swine and human hepatitis E viruses and chicken big liver and spleen disease virus. J Gen Virol 2002; 83: 2001-9
87. Bryant JB, Tsarev AA, Iqbal M, et al. Epidemic hepatitis in Pakistan: pattern of serologic response and evidence that antibody to hepatitis E virus protects against disease. J Infect Dis 1994; 170: 517-21
88. Chauhan A, Dilawari JB, Sharma R, et al. Role of long-persisting human hepatitis E virus antibodies in protection. Vaccine 1998; 16: 755-6
89. Khuroo MS, Kamili S, Dar MY, et al. Hepatitis E and long-term antibody status [letter]. Lancet 1993; 341: 1355
90. Goldsmith R, Yarbough PO, Reyes GR, et al. Enzyme-linked immunosorbent assay for diagnosis of acute sporadic hepatitis E in Egyptian children. Lancet 1992; 339: 328-31
91. Koshy A, Grover S, Hyams KC, et al. Short-term IgM and IgG antibody responses to hepatitis E virus infection. Scand J Infect Dis 1996; 28: 439-41
92. Worm HC, Wurzer H, Frösner G. Sporadic hepatitis E in Austria [letter]. N Engl J Med 1998; 339: 1554-5
93. Aggarwal R, Shahi H, Naik S, et al. Evidence in favour of high infection rate with hepatitis E virus among young children in India [letter]. J Hepatol 1997; 26: 1425-6
94. Mathur P, Arora NK, Panda SK, et al. Sero-epidemioloy of hepatitis E virus (hev) in urban and rural children of north India. Indian J Pediatr 2001; 38: 461-75
95. Dawson GJ, Chau KH, Cabal CM, et al. Solid-phase enzyme-linked immunosorbent assay for hepatitis E virus IgG and IgM antibodies utilizing recombinant antigens and synthetic peptides. J Virol Methods 1992; 38: 175-86
96. Paul DA, Knigge MF, Ritter A, et al. Determination of hepatitis E virus Seroprevalence by using recombinant fusion proteins and synthetic peptides. J Infect Dis 1994; 169: 801-6
97. Zhang JZ, Ng MH, Xia NS, et al. Conformational antigenic determinants generated by interactions between a bacterial expressed recombinant peptide of the hepatitis E virus structural protein. J Med Virol 2001; 64: 125-32
98. Robinson RA, Burgess WH, Emerson SU, et al. Structural characterization of recombinant hepatitis E virus ORF2 proteins in baculovirus-infected insect cells. Protein Expr Purif 1998; 12: 75-84
99. Li TC, Zhang J, Shinzawa H, et al. Empty virus-like particle-based enzyme-linked immunosorbent assay for antibodies to hepatitis E virus. J Med Virol 2000; 62: 327-33
100. Mast EE, Alter MJ, Holland PV, et al. Evaluation of assays for antibody to hepatitis E virus by a serum panel. Hepatology 1998; 27: 857-61
101. Ferguson M, Walker D, Mast E, et al. Report of a collaborative study to assess the suitability of a reference agent for antibodies to hepatitis E virus. Biologicals 2002; 30: 43-8
102. Tsarev SA, Tsareva TS, Emerson SU, et al. Successful passive and active immunization of cynomolgus monkeys against hepatitis E. Proc Natl Acad Sci U S A 1994; 91: 10198-202
103. Pillot J, Turkoglu S, Dubreuli P, et al. Cross reactive immunity against different strains of the hepatitis E virus transferable by simian and human sera. C R Acad Sci III 1995; 318: 1059-4
104. Arankalle VA, Chada MS, Dama BM, et al. Role of immune serum globulins in pregnant women during an epidemic of hepatitis E. J Viral Hepat 1998; 5: 199-204
105. Krawczynski K, Bradley DW. Enterically transmitted non-A, non-B hepatitis: identification of virus-associated antigen in experimentally infected cynomolgus macaques. J Infect Dis 1989; 159: 1042-9
106. Bradley DW. Enterically-transmitted non-A, non-B hepatitis. Br Med Bull 1990; 46: 442-61
107. Arankalle VA, Chada MS, Chobe LP, et al. Cross-challenge studies in rhesus monkeys employing different Indian isolates of hepatitis E virus. J Med Virol 1995; 46: 358-63
108. Arankalle VA, Chada MS, Chobe LP, et al. Long-term serological follow up and cross-challenge studies in rhesus monkeys experimentally infected with hepatitis E virus. J Hepatol 1999; 30: 199-204
109. Meng J, Pillot J, Dai X, et al. Neutralization of different geographic strains of the hepatitis E virus with anti-hepatitis E virus-positive serum samples obtained from different sources. Virology 1998; 249: 316-24
110. Meng J, Dubreuil P, Pillot J. A new PCR-based seroneutralization assay in cell culture for diagnosis of hepatitis E. J Clin Microbiol 1997; 35: 1373-7
111. Meng J, Dai X, Chang JC, et al. Identification and characterization of the neutralizing epitope(s) of the hepatitis E virus. Virology 2001; 288: 203-11
112. Schofield DJ, Glamann J, Emerson SU, et al. Identification by phage display and characterization of two neutralizing chimpanzee monoclonal antibodies to the hepatitis E virus capsid protein. J Virol 2000; 74: 5548-55
113. Schofield DJ, Purcell RH, Nguyen HT, et al. Monoclonal antibodies that neutralize HEV recognize an antigenic site at the carboxyterminus of an ORF2 protein vaccine. Vaccine 2003; 22: 257-67
114. Riddell MA, Li F, Anderson DA. Identification of immunodominant and conformational epitopes in the capsid protein of hepatitis E virus by using monoclonal antibodies. J Virol 2000; 74: 8011-7
115. Ghabrah TM, Tsarev S, Yarbough PO, et al. Comparison of tests for antibody to hepatitis E virus. J Med Virol 1998; 55: 134-7

116. Khudyakov YE, Lopareva EN, Jue DL, et al. Antigenic domains of the open reading frame 2-encoded protein of hepatitis E virus. J Clin Microbiol 1999; 37: 2863-71

117. Purdy MA, McCaustland KA, Krawczynski K, et al. Preliminary evidence that a trpE-HEV fusion protein protects cynomolgus macaques against challenge with the wild-type hepatitisE virus (HEV). J Med Virol 1993; 41: 90-4

118. Im SW, Zhang JZ, Zhuang H, et al. A bacterially expressed peptide prevents experimental infection of primates by the hepatitis E virus. Vaccine 2001; 19: 3726-32

119. McAtee CP, Zang Y, Yarbough PO, et al. Purification and characterization of a recombinant hepatitis E protein vaccine candidate by liquid chromatography-mass spectrometry. J Chromatogr (B) 1996; 685: 91-104

120. Yarbough PO. Hepatitis E virus: advances in the HEV biology and HEV vaccine approaches. Intervirology 1999; 42: 179-84

121. Li T, Takeda N, Miyamura T, et al. Oral administration of hepatitis E virus-like particles induces a systemic and mucosal immune response in mice. Vaccine 2001; 19: 3476-84

122. Li TC, Suzaki Y. Ami Y, et al. Protection of cynomolgus monkeys against HEV infection by oral administration of recombinant hepatitis E virus-like particles. Vaccine 2004; 22: 370-7

123. Niikura M, Takamura S, Kim G, et al. Chimeric recombinant hepatitis E virus-particles as an oral vaccine vehicle presenting foreign epitopes. Virology 2002; 293: 273-80

124. Takamura S, Nikura M, Li TC, et al. DNA vaccine-encapsulated virus-particles derived from an orally transmissible virus stimulate mucosal and systematic immune responses by oral administration. Gene Therapy 2004; 11: 628-35

125. He J, Binn LN, Caudill JD, et al. Antiserum generated by DNA vaccine binds to hepatitis E virus (HEV) as determined by PCR and immune electron microscopy (IEM): application for HEV detection by affinity-capture RT-PCR. Virus Res 1999; 62: 59-65

126. He J, Hayes CG, Binn LN, et al. Hepatitis E virus DNA vaccine elicits immunologic memory in mice. J Biomed Sci 2001; 8: 224-6

127. Kamili S, Spelbring J, Krawczynski K. DNA vaccination against hepatitis E virus infection in cynomolgus macaques. J Gastroenterol Hepatol 2002; 17 Suppl. 3: 365-9

128. Kamili S, Spelbring J, Carson D, et al. Protective efficacy of hepatitis E virus DNA vaccine administered by gene gun in the cynomolgus macaque model of infection. J Infect Dis 2004; 189: 258-64

129. Ma Y, Lin SQ, Gao Y, et al. Expression of ORF2 partial gene of hepatitis E virus in tomatoes and immunoactivity of expression products. World J Gastroenterol 2003; 9: 2211-5

130. Safary A. Perspectives of vaccination against hepatitis E. Intervirology 2001; 44: 162-6

131. Tsarev SA, Tsareva TS, Emerson SU, et al. Recombinant vaccine against hepatitis E: dose response and protection against heterologous challenge. Vaccine 1997; 15: 1834-8

132. Zhang M, Emerson SU, Nguyen H, et al. Recombinant vaccine against hepatitis E: duration of protective immunity in rhesus macaques. Vaccine 2002; 20: 3285-91

133. Purcell RH, Nguyen H, Shapiro M, et al. Pre-clinical immunogenicity and efficacy trial of a recombinant hepatitis E vaccine. Vaccine 2003; 21: 2607-15

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