CHAPTER 2

Literature Review

2.1 Introduction

Hepatitis E virus (HEV) has emerged world-wide as a significant infectious cause of clinical and sub-clinical liver inflammation in humans, particularly in some developing countries, where it is endemic and frequently epidemic (Favorov *et al.*, 2000; Schlauder and Mushahwar, 2001; Van der Poel *et al.*, 2001). Discovered in the late 1970's, HEV was initially referred to as enterically transmitted non-A, non-B hepatitis virus, but since has been classified as the type species of the genus *Hepatitis E-like viruses* (Wong *et al.*, 1980; Pringle, 1999; Schlauder *et al.*, 1999; Wang *et al.*, 1999; Berke and Matson, 2000).

Outbreaks of hepatitis E, primarily associated with faecally contaminated drinking water, have been reported in Asia, the Middle East and Africa (Hunter, 1997, Van der Poel *et al.*, 2001; Grabow, 2002). Hepatitis E virus is widespread in Africa, with notable epidemics reported in Algeria, the Ivory Coast, Sudan, Somalia and Djibouti (Bradley, 1992). The first occurrence of HEV in southern Africa was a waterborne outbreak that occurred in Namibia in 1983 (Isaäcson *et al.*, 2000), followed by a second waterborne outbreak of HEV in northern Botswana in 1985 (Byskov *et al.*, 1989). The occurrence of HEV in South Africa has been confirmed by reports in seroprevalence studies (Grabow *et al.*, 1996; Tucker *et al.*, 1996), as well as sporadic cases of hepatitis E associated with a travel history to Asian countries where the disease is endemic (Grabow, 1997; South African Virus Laboratories Surveillance Bulletin, 2003).

Hepatitis E is generally a short, self-limited infection with a mortality rate of 0.07% to 0.6% (Purcell, 1996; Krawczynski *et al.*, 2000; Worm *et al.*, 2002). A unique feature of hepatitis E is the unusually high mortality rate (25%) that is observed in pregnant women, in whom the disease is exacerbated by the development of fulminant hepatic disease (Purcell and Ticehurst, 1997; Jameel, 1999; Van der Poel *et al.*, 2001).

Antibodies to HEV (anti-HEV) and molecular evidence for natural HEV infection have been found in several wild and domestic animal species native to developing and industrialised countries world-wide (Meng, 2000; Pina *et al.*, 2000; Garkavenko *et al.*, 2001; Van der Poel *et al.*, 2001). Hepatitis E virus may be more widespread than previously thought, as novel HEV strains unrelated to prototype strains from endemic areas were detected in patients from various non-endemic countries (Pina *et al.*, 2000; Schlauder *et al.*, 2000; Worm *et al.*, 2000). These novel HEV strains may have originated from animal HEV strains that have undergone genetic reversion to strains causing clinical disease in humans (Meng *et al.*, 1998; Hsieh *et al.*, 1999). This assumption might explain the similarity between these novel HEV strains and the swine HEV strains detected in the same geographical area (Wu *et al.*, 2000).

The objective of this study was to assess the seroprevalence of anti-HEV immunoglobulin G (IgG) in selected swine populations, as well as to detect HEV in domestic sewage and swine stool specimens, which may cast light on the endemic presence and genetic characteristics of HEV in South Africa.

2.2 Classification of Hepatitis E Virus

The existence of an enterically transmitted non-A, non-B hepatitis virus was suggested in the early 1980's when sensitive serological assays for hepatitis A excluded hepatitis A virus (HAV) as the etiological agent responsible for waterborne epidemics of hepatitis in India (Wong *et al.*, 1980; Khuroo *et al.*, 1983). The existence of a hepatitis virus other than HAV and hepatitis B virus (HBV) was confirmed when a human volunteer developed clinical hepatitis 36 days after ingesting diluted faecal material pooled from nine patients with non-A hepatitis (Balayan *et al.*, 1983).

Two major epidemiological differences distinguish HEV infection from HAV infection:

1) In countries where both diseases are endemic, seroconversion to HAV usually occurs in young children, whereas seroconversion to HEV occurs mainly in young adults between the ages of 15 and 40, and

2) Hepatitis E presents a greater risk of fatality in pregnant woman (Tsega *et al.*, 1992; Khuroo *et al.*, 1995; Husaini *et al.*, 1997).

Hepatitis E virus was assumed to be a RNA virus when first detected by immuno electron microscopy (IEM) and it was suggested to group this virus into the *Picornaviridae* family (Balayan *et al.*, 1983). It was later established that HEV is antigenically and biophysically unrelated to the picornaviruses (Worm *et al.*, 2002). Although sub genomic messenger RNA (mRNA) was detected in infected liver tissue, which was suggestive of a relationship with the caliciviruses, the sequence of HEV does not resemble the sequence of any other recognised virus (Miller, 1995; Purcell, 1996).

Hepatitis E virus has a smaller particle size than non-enveloped caliciviruses and surface spikes and indentations are more subtle (Worm *et al.*, 2002). The sedimentation coefficient in sucrose and the buoyant density of HEV are similar to members of the *Caliciviridae* family, and in addition, the 5' non-structural (NS)/3' structural genomic organisation is also found in caliciviruses (Worm *et al.*, 2002). Analysis of the RNA helicase and RNA-dependant RNA polymerase (RdRp) regions of HEV, showed that HEV forms a phylogenetically distinct group closer to the rubella virus (an enveloped RNA virus currently classified in the family Togaviridae, genus *Alphavirus*) and the beet necrotic yellow vein virus (a plant virus, family Togaviridae, genus *Furoviridae*) than the members of *Caliciviridae* (Purcell, 1996; Berke and Matson, 2000; Worm *et al.*, 2002). New recommendations of the International Committee on the Taxonomy of Viruses (http://www.ncbi.nlm.nih.gov/ICTV/) now place HEV in a separate genus called *Hepatitis E-like viruses*, with HEV being the type species of this genus (Pringle, 1999; Berke and Matson, 2000).

Phylogenetic and sequence analysis of various HEV strains indicated that at least nine different groups of HEV could be defined (Table 2.1): group 1 includes the prototype Burmese isolate and related strains from Asia and Africa, group 2 includes the prototype Mexican strain and isolates from Nigeria, group 3 includes the human US isolates, which are related to the swine isolate from the US, group 4 includes the Italian isolate, which is

similar to the swine isolate from New Zealand, group 5 includes an isolate from Greece and two isolates from Spain, which are similar to an HEV sequence from swine sewage, group 6 includes a second isolate from Greece, while group 7 includes the Argentine and Austrian isolates, group 8 and group 9 are represented by the two China isolates, Ct1 and Cs15 (Schlauder and Mushahwar, 2001). One or more additional groups could be represented by related but distinct isolates from China and Taiwan off the same branch as the Cs15 and Ct1 isolates (Schlauder and Mushahwar, 2001). An alternate scheme for the distribution of HEV isolates could be defined by four major genotypes (Table 2.1) (Schlauder and Mushahwar, 2001).

Table 2.1 Potential genotypic designations for isolates of HEV (Schlauder and Mushahwar, 2001)

Isolates	Scheme 1[a]	Scheme 2[b]	Schlauder[c]	Wang[d]	Arankalle[e]	Tsarev[f]
B1, B2, I2	I	1	1	1a	IA	I1b
P1, C1-4	I	1	1	1b	IB	I1a
I1	I	1	1	1c		I1c
Cb6, Cb7, Cs13	I	1	1	1c		
Mo12, Mo23	I	1	1	1e	IC	
Uz, Ki, Chad	I	1		1d	IC	I2
M	II	2	2	2	II	II
Ni[g]	II	(12)[h]				
US1, US2, swUS1	III	3	3	3	III	III
It1	III	4	5			
SwNZ1	III	4				
G1, Sp1 (VH1), Sp2	III	5	6			
(VH2)	III	6	7			
Gr2	III	7	8			
Ar1, Ar2	III	7				
Au1	IV	8				
Ct1	IV	9	4	4a		
Cs15, Ch3	IV	(10)		4b		
Cs5, Cb3, Cb4	IV	(11)		4c		
Cs9	IV	(10-11)[h]				
Ct705, Ct825, Ct845						

[a]Scheme 1: Genotypes of HEV; [b]Scheme 2: Groups of HEV; [c]Schlauder *et al.*, 2000; [d]Wang *et al.*, 1999; [e]Arankalle *et al.*, 1999; [f]Tsarev *et al.*, 1999; [g]Nigerian 1, 4-7 and 9; [h]Overlapping sequence with other isolates not available for comparison

Hepatitis E viruses of genotype I exhibit a low level of diversity but do not show genotypic distribution based on the geographical origin of the isolates (Schlauder and Mushahwar, 2001). Genotypes II, III and IV consist of HEV isolates with a wide degree of diversity (Schlauder and Mushahwar, 2001). Nomenclature for types and subtypes of

HEV isolates have been proposed in several recent publications (Chatterjee *et al.*, 1997; Van Cuyck-Grandré *et al.*, 1997; Schlauder *et al.*, 1998; Arankalle *et al.*, 1999; Erker *et al.*, 1999b; Schlauder *et al.*, 1999; Tsarev *et al.*, 1999; Wang *et al.*, 1999; Schlauder *et al.*, 2000).

2.3 Characteristics of Hepatitis E Virus

Hepatitis E virus was first described in 1983 as a spherical, non-enveloped particle 27-34 nm in diameter, containing a polyadenylated, positive strand RNA genome of approximately 7.5 kilo-bases (kb) (Figure 2.1) (Jothikumar *et al.*, 1993; Purcell, 1996; Aggarwal *et al.*, 1999). The HEV particles were first visualised by IEM in the faeces of a volunteer orally infected with stool extracts from presumed enterically transmitted non-A, non-B hepatitis (Balayan *et al.*, 1983). Intravenous (IV) inoculation of cynomolgus monkeys with the virus-containing stool extract of the infected volunteer resulted in hepatitis, excretion of the virus-like particles (VLP's) and an antibody response (Khuroo *et al.*, 1993). Reyes and colleagues (1990) used virus-enriched gall-bladder bile from cynomolgus macaques infected with the second-passage Burma isolate to construct a complimentary DNA (cDNA) library in plasmid Ygt10. Hybridisation analyses of human faecal material collected from outbreaks of enterically transmitted hepatitis in geographically separate locations indicated a common pathogen responsible for the majority of enterically transmitted hepatitis world-wide (Reyes *et al.*, 1990).

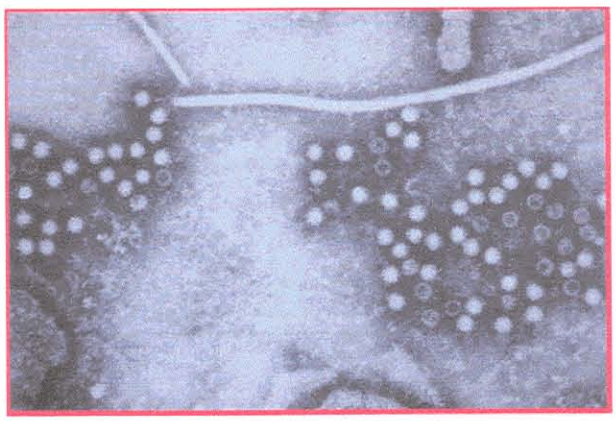

Figure 2.1 Electron micrograph of HEV (Hepatitis A-E slide set, www.cdc.gov)

Sequence analysis revealed that the HEV genome contains two large potential open reading frames (ORF's) (Figure 2.2) within the full-length genomic transcript, and a third small positive-polarity ORF was identified by the immunoreactive epitope that it encodes (Zafrullah *et* al., 1997; Aggarwal *et al.*, 1999; Aggarwal and Krawczynski, 2000; Tyagi *et al.*, 2001). Antibodies directed against epitopes that are present on the proteins, encoded by the translated ORF's, are found in infected humans and experimental animals, which suggests that all three ORF's are expressed during viral infection (Khudyakov *et al.*, 1994; Panda *et al.*, 1995). Apart from the ORF's, the HEV genome contains short 5' and 3'-untranslated regions (UTR's) of 26 and 68 nucleotides respectively, which have the potential to form secondary structures, such as stem-loops and hairpins (Tam *et al.*, 1996). Secondary structures are found within a conserved 58-nucleotide region within ORF1 (Tam *et al.*, 1996).

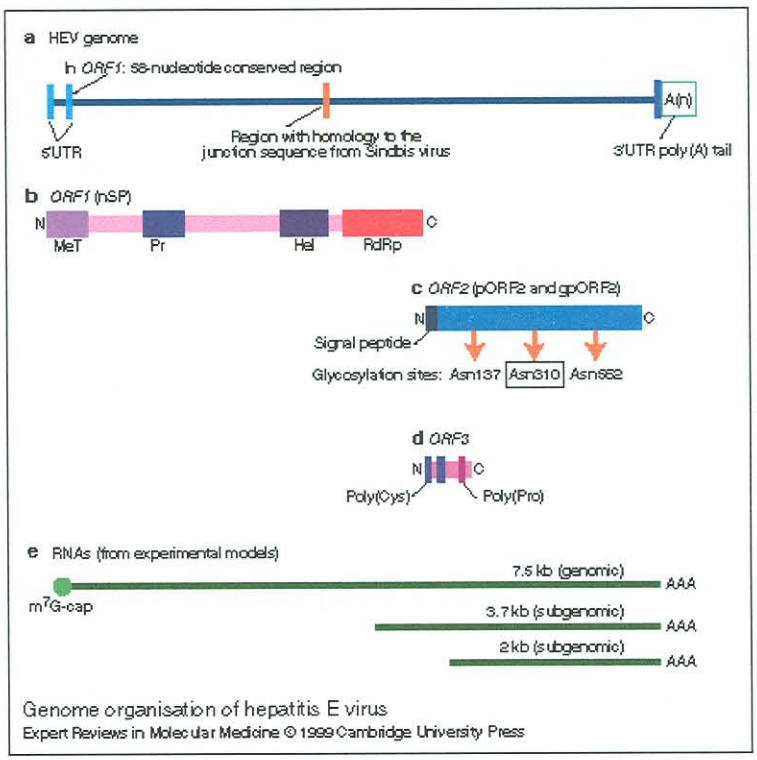

Figure 2.2 Schematic representation of the HEV genome organisation (Jameel, 1999)

2.3.1 Open Reading Frame 1 of the Hepatitis E Virus Genome

Open reading frame 1 is situated at the 5' end of the viral genome starting after a 27 base pair (bp) non-coding sequence and extends 5 079 bp to the 3' end (Burmese prototype strain) (Purcell, 1996; Aggarwal *et al.*, 1999; Worm *et al.*, 2002). Open reading frame 1 encodes 1 693 amino acids (aa), which encompass NS, enzymatically active proteins responsible for viral genome replication and viral protein processing (Purcell, 1996; Aggarwal *et al.*, 1999; Worm *et al.*, 2002). Open reading frame 1 contains domains encoding a RdRp, an RNA helicase, a papain-like cysteine proteinase (also found in alphaviruses and rubella virus) and X and Y domains with unknown function (also found in rubella virus) (Worm *et al.*, 2002).

2.3.2 Open Reading Frame 2 of the Hepatitis E Virus Genome

Open reading frame 2 extends 1 980 bp, starting 41 bp downstream from ORF1 and terminating 65 bp upstream of the poly-A tail at the 3' of the viral genome (Worm *et al.*, 2002). Open reading frame 2 encodes 660 aa, which encompass one or more structural or capsid proteins (Purcell, 1996; Aggarwal *et al.*, 1999; Worm *et al.*, 2002). The ORF2 protein is a ~88 kilo-Dalton (kDa) glycoprotein synthesised as a precursor, then processed through signal sequence cleavage into the mature protein and glycosylated in three potential glycosylation sites (Worm *et al.*, 2002). *In vitro* assays suggest that the ORF2 protein is cotranslationally translocated across the endoplasmic reticulum and is expressed intracellularly as well as on the cell surface (Zafrullah *et al.*, 1999).

2.3.3 Open Reading Frame 3 of the Hepatitis E Virus Genome

Open reading frame 3, the smallest one, overlaps with both ORF1 (1 nucleotide) and ORF2 (328 nucleotides) (Aggarwal *et al.*, 1999; Worm *et al.*, 2002). Open reading frame 3 encodes for a 123 aa, ~13.5 kDa non-glycosylated protein (Aggarwal *et al.*, 1999; Worm *et al.*, 2002). The ORF3 protein is phosphorylated at a serine residue (Ser-80) by a mitogen-activated protein kinase (Zafrullah *et al.*, 1997). The phosphoprotein was

found to associate with the hepatocellular cytoskeleton and to form a complex with the capsid protein of ORF2, suggesting a possible role as a cytoskeletal anchor site for viral particle assembly (Zafrullah *et al.*, 1997).

2.3.4 Replication and Expression of the Hepatitis E Virus Genome

Based on similarities and sequence homology to other characterised +RNA viruses, Reyes and his colleagues (1993) proposed a general model for the replication and gene expression of HEV (Figure 2.3). Studies in rats suggested that extrahepatic tissues such as peripheral blood monocytes, spleen, lymph nodes and the small intestine are involved in the replication of HEV (Maneerat *et al.*, 1996). Viral genomic RNA is translated in the cytosol of infected cells to synthesise the ORF1-encoded NS polyprotein, as seen in Figure 2.3 (Reyes *et al.*, 1993). The NS-polyprotein includes a RdRp, which plays a role in the generation of both negative- and positive-strand viral RNA (Reyes *et al.*, 1993). The RdRp is only detectable in the early phase of replication (Panda *et al.*, 2000). It is unknown whether or not the replication is initiated by a cap or by internal sites such as stem-loop structures (Worm *et al.*, 2002). Studies indicated that structural proteins might be expressed from two sub genomic RNA's (3.7 kb and 2.0 kb in length), which are encoded by ORF2 and ORF3 (Tam *et al.*, 1991). Encapsulation of the genomic RNA, involving the ORF3 phosphoprotein, results by the association with the basic capsid proteins (Zafrullah *et al.*, 1997). It remains unknown how the viral particles are excreted from the infected hepatocytes into the blood and bile (Worm *et al.*, 2002).

An analogy to the alphavirus replication is presumed, as a sequence stretch in the HEV negative strand RNA is present, which is similar to the junction sequence found in the RNA replicative intermediate of the Sindbis alphavirus (Reyes *et al.*, 1993; Jameel, 1999). This junction sequence serves as a sub-genomic promoter for transcription of the structural region mRNA, and therefore, the proposed sub-genomic positive strand HEV RNA can be translated into the structural proteins at late stages in the viral replication (Reyes *et al.*, 1993).

Evidence for the above-mentioned model of replication was provided by Tam and associates (1996), as they showed the presence of one HEV genomic (~7.5 kb) and two sub-genomic (~3.7 kb and ~2 kb) RNA molecules in experimentally infected cynomolgus macaques. Nanda and colleagues (1994a) demonstrated the presence of HEV positive- and negative-strand RNA in the liver of the rhesus macaque model, which is the primary site of viral replication in this animal model. Positive-strand viral RNA was found in the serum and bile (Nanda *et al.*, 1994a). This supported the above-mentioned model, as it indicates that the HEV genomic RNA replicates through a negative-strand RNA intermediate (Jameel, 1999). It was recently shown that the genomic RNA of HEV is capped and that such an addition, together with a short 5' UTR is compatible with cap-mediated translation of the HEV genomic RNA (Kabrane-Lazizi *et al.*, 1999b). The conserved secondary structures at the 5' and 3' ends of HEV RNA are presumed to act as binding sites for the replicase and host factors (Jameel, 1999).

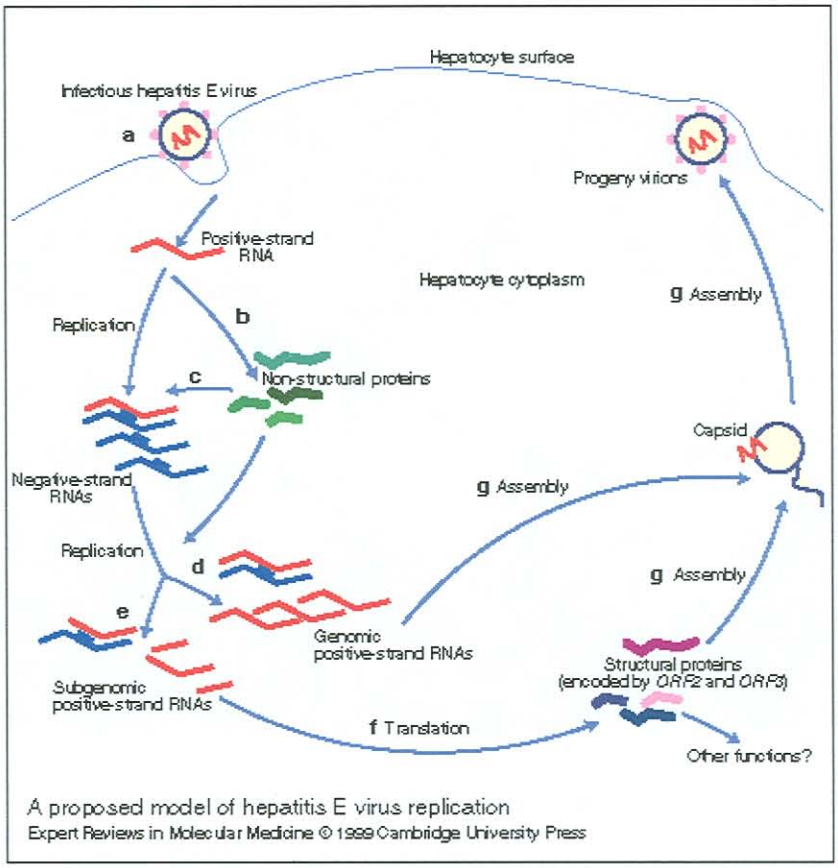

Figure 2.3 A proposed model of HEV replication (Jameel, 1999)

2.4 Epidemiology of Hepatitis E Virus

Hepatitis E virus is restricted to tropical and subtropical countries, where it causes epidemics of viral hepatitis, often involving large numbers of patients (Hunter, 1997; Jameel, 1999; Schlauder and Mushahwar, 2001). Notable epidemics which have occurred include New Delhi, India, 1955-56 (29 000 cases), Kirgiz Republic, Soviet Union, 1955-56 (10 000 cases), Kathmandu Valley, Nepal, 1973-74 (10 000 cases), Mandalay, Myanmar, 1976-77 (20 000 cases), Kashmir, India, 1978-82 (52 000 cases), Xinjiang, China, 1986-88 (120 000 cases) and Kanpur, India, 1991 (79 000 cases) (Table 2.2) (Hunter, 1997). Hepatitis E epidemics have been identified in a number of African countries such as Algeria, Ivory Coast, Somalia, Sudan (Bradley, 1992), while smaller outbreaks have been reported in Morocco (Benjelloun *et al.*, 1997), Ethiopia (Tsega *et al.*, 1991), Chad (Van Cuyck-Gandré *et al.*, 1997), as well as South Africa's neighbouring countries Botswana and Namibia (Byskov *et al.*, 1989; Isaäcson *et al.*, 2000; Maila *et al.*, 2004).

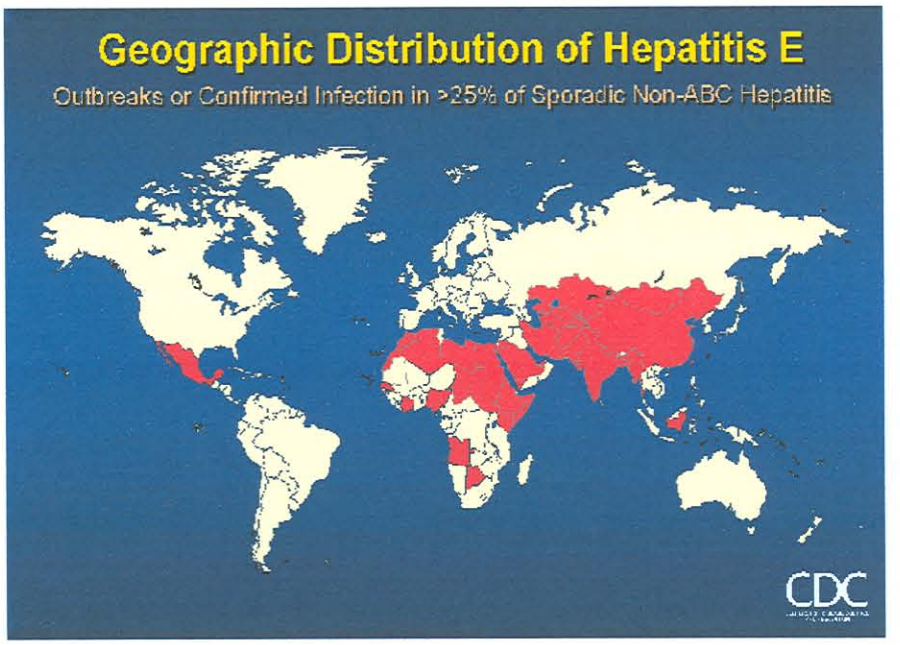

Figure 2.4 **Geographic areas with confirmed HEV infection or large outbreaks of hepatitis E (Mast and Alter, 1993)**

Table 2.2 Outbreaks of HEV (Hunter, 1997; ProMed, 2004a, b, c)

Site	Dates	Number of cases	Source of infection
New Delhi, India	1955-56	29 000	Contaminated water
Kirkiz Republic, USSR	1955-56	10 812	Not determined
Kathmandu, Nepal	1973-74	10 000	Not determined
Ahmedabad, India	1975-76	2 572	Contaminated water
Mandalay, Burma	1976-77	20 000	Contaminated water
Kashmir Valley, India	1978-79	275	Contaminated water
Calcutta, India	1980	226	Contaminated water
Madhya Pradesh, India	1980	38	Contaminated water
Medea, Algeria	1980-81	788	Contaminated water
Kolhapur City, India	1981	1 200	Contaminated water
Kathmandu, Nepal	1981-82	6 000	Not determined
Rangoon, Burma	1982-83	399	Contaminated water
Central Asia, USSR	1982-83	?	Not known
Constantine, Algeria	1983	6	Contaminated water
Namibia	1983	?	Contaminated water
Tortiya, Ivory Coast	1983-84	623	Not known
Chad	1983-84	38	Contaminated water
Eastern Sudan	1985	2 012	Contaminated water
Karachi, Pakistan	1985	Several cases	Not determined
Maun, Botswana	1985	273	Contaminated water
Refugee camps, Somalia	1985-86	2 000	Contaminated water
Huitzililla, Mexico	1986	94	Contaminated water
Telixtac, Mexico	1986	129	Contaminated water
South Delhi, India	1986-87	61	Contaminated water
Xinjang, China	1987	125	Contaminated water
Kathmandu, Nepal	1987	370	Contaminated water
Sargodha, Pakistan	1987	133	Contaminated water
South Delhi, India	1987	43	Contaminated water
Borneo, Indonesia	1987-88	2 000	Contaminated water
Somalia	1988	11 413	Contaminated water
Northern Ethiopia	1988-89	423	Contaminated water
Rairangpur Town, India	1989-90	127	Contaminated water
Madhya Pradesh, India	1989-90	801	Contaminated water
Kanpur, India	1990-91	>79 000	Contaminated water
Delhi, India	1992	N/A	Contaminated water
Delhi, India	1994	27	Not determined
Rundu, Namibia	1995	?	Not known
Otjiwarango, Namibia	2001	17	Contaminated water
Sadr City, Iraq	2004	100	Contaminated water

Apart from epidemic hepatitis, HEV causes rampant sporadic hepatitis in endemic areas during intra-epidemic periods (Nanda *et al.*, 1994b; Coursaget *et al.*, 1998; Jameel, 1999; Favorov *et al.*, 2000; Schlauder and Mushahwar, 2001). Sporadic hepatitis E in Africa has been reported in Egypt (Tsarev *et al.*, 1999), Nigeria (Buisson *et al.*, 2000), Central African Republic (ProMed, 2004c) and Chad (Coursaget *et al.*, 1998). Figure 2.4 shows the regions of the world where more than 25% of sporadic non-ABC hepatitis cases are due to HEV infection.

Hepatitis E virus is most prevalent in tropical and subtropical regions of the world where it is the leading cause of enterically transmitted, non-A hepatitis (Irshad, 1999; Aggarwal *et al.*, 1999; Wang *et al.*, 1999; Favorov *et al.*, 2000; Schlauder and Mushahwar, 2001; Van der Poel *et al.*, 2001). Hepatitis E is found most frequently in certain geographical regions, as well as refugee camps, where faecal contamination of the drinking water is frequent (Jameel, 1999).

A hypothesis has arisen that HEV may be zoonotic, since HEV or HEV-like viruses and anti-HEV have been reported in a wide variety of animal species (Hsieh *et al.*, 1999; Kabrane-Lazizi *et al.*, 1999a; Meng, 2000; Choi *et al.*, 2003; Meng, 2003; Tei *et al.*, 2003; Kuno *et al.*, 2003). This hypothesis is based on indirect proof, such as the high frequency of anti-HEV in animal handlers and that the local HEV swine strains are similar to human HEV strains in the same district (Tei *et al.*, 2003).

Direct evidence for the zoonotic transmission of HEV has recently been reported in Asia (Matsuda *et al.*, 2003; Tei *et al.*, 2003; Yazaki *et al.*, 2003; ProMed, 2004a). Four individuals were admitted to the same hospital within two weeks of each other, complaining of fever, nausea and general malaise (Tei *et al.*, 2003). All patients were negative for serological markers of hepatitis A, B and C viruses, but were positive for HEV RNA and both anti-HEV immunoglobulin M (IgM) and IgG, leading to the diagnosis of hepatitis E (Tei *et al.*, 2003). The patient histories revealed that all four patients had eaten uncooked meat of two wild-caught Sika deer three times in the week preceding the onset of disease (Tei *et al.*, 2003). The same HEV strain was identified in

all the patients, and when compared to a strain found in the meat of one of the deer, it was found to be identical, which confirmed the suspicion that the uncooked deer meat was responsible for the transmittance of HEV (Tei *et al.*, 2003). Similar reports involving the ingestion of uncooked swine liver have been published (Matsuda *et al.*, 2003; Yazaki *et al.*, 2003; ProMed, 2004a).

Although of minor importance, other routes of transmission such as vertical transmission *in utero* from infected mothers to their new-born have been documented (Khuroo *et al.*, 1995; Singh *et al.*, 2003). The possibility of parenteral transmission in endemic areas has been suggested (Chauhan *et al.*, 1993). Person-to-person transmission is not, however, a major factor in the evolution of the outbreaks as secondary waves of hepatitis E usually do not occur following epidemics (Arankalle *et al.*, 2000). Inapparent secondary household transmission may however occur, as indicated by elevated serum transaminase levels, which have been detected in up to 20% of household contacts of patients with hepatitis E (Khuroo, 1980). Nosocomial HEV transmission, presumably by person-to-person contact, has been suggested to occur in hospitals (Robson *et al.*, 1992). No evidence of chronic infection has been detected in long-term follow-up of patients involved in several hepatitis E outbreaks (Khuroo *et al.*, 1983).

2.4.1 Detection of Hepatitis E Virus in Water Environments

Sewage contaminated water is considered to be the most important vehicle for the transmission of HEV and has been the common feature preceding large outbreaks of hepatitis E (Hunter, 1997; Favorov *et al.*, 2000; Schlauder and Mushahwar, 2001; Vaidya *et al.*, 2002; Worm *et al.*, 2002). Molecular methods for detecting HEV in environmental water, especially those strains associated with outbreaks, have been developed (Jothikumar *et al.*, 1993; Erker *et al.*, 1999a; Grimm and Fout, 2002). Hepatitis E virus has been detected in sewage in both endemic and non-endemic countries, such as Spain (Pina *et al.*, 1998; Pina *et al.*, 2000; Clemente-Casares *et al.*, 2003) and India (Jothikumar *et al.*, 1993; Vaidya *et al.*, 2002). Sewage acts as a reservoir for HEV and the presence of

the virus in the sewage of a population reflects both clinical and sub-clinical HEV infections prevalent in that population (Vaidya *et al.*, 2002).

2.4.2 Hepatitis E in South Africa

As far as we are aware, the epidemiology of HEV in South Africa has not thoroughly been investigated. In a sero-epidemiological study by Tucker and colleagues (1996), the prevalence of anti-HEV IgG was found to be 6.6% in selected urban areas, compared to 15.3% in selected rural areas. Individuals using unchlorinated river water for domestic purposes appeared to be at great risk, which was shown by the 17.4% seroprevalence of anti-HEV IgG in these communities (Tucker *et al.*, 1996). The seroprevalence of anti-HEV IgG in canoeists with regular exposure to sewage-contaminated recreational water was found to be 1.8% (Grabow *et al.*, 1994).

The rare diagnosis of clinical cases of hepatitis E in South Africa remains to be explained, but similar results have been reported from other parts of the world (Grabow, 1997). It was thought that clinical cases of hepatitis E in South Africa were limited to a small number of imported cases, but it would appear that there might be more local cases of clinical disease (Grabow *et al.*, 1994; Grabow, 1997; South African Virus laboratories Surveillance Bulletin, 2003). The absence of clinical cases of hepatitis E in South Africa is surprising, as outbreaks have been recorded in the neighbouring countries Namibia and Botswana (Byskov *et al.*, 1989; Grabow *et al.*, 1996; South African Virus Laboratories Surveillance Bulletin, 2001).

2.4.3 Hepatitis E During Pregnancy

Compared to other forms of viral hepatitis, hepatitis E has an increased incidence and severity in pregnant women (Khuroo *et al.*, 1981). In areas where the disease is endemic, HEV is an important cause of death due to liver failure in pregnant women, with mortality rates of up to 25% (Grabow *et al.*, 1996; Hamid *et al.*, 1996; Hussaini *et al.*,

1997; Jameel, 1999; Van der Poel *et al.*, 2001). None of the other hepatitis viruses causes such severe hepatitis during pregnancy (Jameel, 1999).

The hypothesis put forward by Purcell and Ticehurst (1997) to explain the pathogenesis of fulminant hepatitis E in pregnancy, suggested that HEV damages the liver sinusoidal cells, particular the Kupffer cells. This results in the diminishing of the ability of these cells to protect hepatocytes against endotoxins that originate from Gram-negative bacteria found in the intestinal tract (Purcell and Ticehurst, 1997). Hepatocytes can be damaged directly by endotoxins or indirectly by eicosanoids (20-carbon chain polyunsaturated fatty acids) that cause platelet aggregation, inflammation and other effects (Purcell and Ticehurst, 1997). Chemotactic attraction of inflammatory neutrophils can be triggered by the release of prostaglandins, a type of eicosanoid (Purcell and Ticehurst, 1997). This can lead to the swelling of the tissue by water accumulation (oedema) and the arrest of bile flow (cholestasis) (Purcell and Ticehurst, 1997). Pregnant women have an enhanced sensitivity to such an endotoxin-mediated effect and, thus, might explain the high mortality of hepatitis E in pregnancy (Purcell and Ticehurst, 1997).

2.4.4 Prevalence of Antibodies to Hepatitis E Virus

The prevalence of anti-HEV IgG in humans has been investigated in several developing countries where waterborne outbreaks of hepatitis E have been reported (Pujol *et al.*, 1994; Perez *et al.*, 1996; Clayson *et al.*, 1997). The seroprevalence in these hepatitis E endemic countries was found to be between 4.6% and 31% (Pujol *et al.*, 1994; Perez *et al.*, 1996; Clayson *et al.*, 1997). Sero-epidemiological studies in industrialised countries indicated a 1-5% prevalence of anti-HEV IgG in the healthy human population, even in the absence of clinical cases (Paul *et al.*, 1994), which indicates that HEV infection appears to be more prevalent in industrialised nations than previously thought (Lau *et al.*, 1995).

Thomas and colleagues (1997) detected anti-HEV IgG in 21.3% of blood donors, 15.9% of homosexual men and 23% of injection drug users from Baltimore, Maryland. In

contrast, 1.2% to 1.4% of blood donors were found to be seropositive for anti-HEV IgG in Northern California (Mast *et al.*, 1997). Similar results have been reported in other non-endemic countries including the Netherlands, Italy, Sweden, Germany, Greece, England, Spain and Taiwan (Jardi *et al.*, 1993; Johansson *et al.*, 1995; Peng *et al.*, 1995; Zaaijer *et al.*, 1995; Bernal *et al.*, 1996; Gessoni and Manoni, 1996; Langer and Frösner, 1996; Psichogiou *et al.*, 1996; Hsieh *et al.*, 1998).

In a previous study conducted by Arankalle and colleagues (1995), the age-specific prevalence of the antibodies to HAV (anti-HAV) and anti-HEV in an endemic area in India was investigated. The data indicated that by the age of 3 years, 73% of the children had seroconverted to HAV and virtually the entire population was anti-HAV positive by the age of 15 years (Arankalle *et al.*, 1995). In the case of HEV, however, fewer persons had seroconverted to HEV, with a peak anti-HEV prevalence of 33% in the age group 20 to 35 years, remaining at that level in older age groups (Arankalle *et al.*, 1995). The prevalence of anti-HEV among infants and children (ages 7 months to 10 years) was ~5%, compared to the prevalence of anti-HAV of ~95% in the same age group (Arankalle *et al.*, 1995). This age-specific pattern of anti-HEV was similar to that reported in other population-based studies in Somalia, Hong Kong and Turkey (Lok *et al.*, 1992; Mushahwar *et al.*, 1993; Thomas *et al.*, 1993). This pattern suggested a sporadic transmission of HEV that accumulates over age, which was consistent with the predominantly sub-clinical nature of the disease, short periods of viremia and the limited pool of HEV infection in the community (Jameel, 1999).

There are several possible explanations for the age-specific prevalence of anti-HEV that has been observed. The lower anti-HEV prevalence could be artefactual, resulting from the rapid decay of anti-HEV after infection (Arankalle *et al.*, 1995). It is not clear how long anti-HEV can be detected after infection with HEV (Arankalle *et al.*, 1995). In a study conducted in Egypt of HEV disease in children, the seropositivity rate fell from 38% at the time of hospitalisation to 17% one year later (Goldsmith *et al.*, 1992). However, in other studies there was no decline in the anti-HEV seropositivity rate in young adults with serologically documented HEV infection after several years (Khuroo *et*

al., 1993; Bryan *et al.*, 1994; Arankalle *et al.*, 1995). Failure of young children to mount an effective anti-HEV response, when compared to adults, could lead to a lower prevalence of anti-HEV in younger age groups (Arankalle *et al.*, 1995). It is, however, documented that during an epidemic of HEV among schoolchildren in Talegaon, India, some children developed high titres of anti-HEV (comparable with adults) after infection with HEV (Arankalle *et al.*, 1988; Arankalle *et al.*, 1995). It has been reported that there is a high prevalence of anti-HEV immunoglobulin M (IgM) in adults with a HEV infection, which is evident of a primary infection (Bryan *et al.*, 1994). Thus, it is possible that adults may be more exposed to primary HEV infection as a result from sexual contact, increased exposure to high risk environments through work or travel, or by increased exposure secondary to increased volumes of ingested food and water (compared to infants and children) (Arankalle *et al.*, 1995).

2.5 Animal Models for Hepatitis E Virus

The reservoir of HEV has not yet been established, although indirect evidence has suggested the possibility of zoonotic transmission of the virus from pigs to humans, especially in non-endemic areas (Balayan *et al.*, 1990; Meng *et al.*, 1997; Hsieh *et al.*, 1999; Kabrane-Lazizi *et al.*, 1999a). This possibility was supported by the identification of anti-HEV in domestic farm animals in Thailand and the identification of HEV RNA and HEV-like sequences in swine from China, New Zealand, Thailand and the US (Clayson *et al.*, 1996; Meng *et al.*, 1997; Hsieh *et al.*, 1999; Garkavenko *et al.*, 2000; Wu *et al.*, 2000). Several non-human primates have been used for HEV transmission studies, with cynomolgus macaques and rhesus macaques being the most useful (Bradley, 1995; Panda and Jameel, 1997). Pigs and rats have been found to be susceptible to infection with HEV (Maneerat *et al.*, 1996; Meng *et al.*, 1997).

Antibodies to HEV have been detected in swine, wild rats, mice and monkeys from areas where HEV is endemic as well as in domestic swine, rats and colony-raised macaques in the US (non-endemic) (Kabrane-Lazizi *et al.*, 1999a). Experimental models of infection have been developed using swine, sheep, laboratory rats and non-human primates such as

cynomolgus macaques, owl monkeys, rhesus monkeys, African green monkeys and colony-borne chimpanzees (Balayan *et al.*, 1990; Bradley, 1995; Maneerat *et al.*, 1996; Pina *et al.*, 1998; Kabrane-Lazizi *et al.*, 1999a; Favorov *et al.*, 2000).

In a study by Wu *et al* (2002) 1.1% to 1.6% of pigs in different areas of Taiwan tested positive for HEV by use of the reverse-transcriptase polymerase chain reaction (RT-PCR). Similar results were found in the swine population in the US (Hsieh *et al.*, 1999). This leads to the speculation that HEV may circulate in the swine population world-wide and it was suggested that HEV might be spread unnoticed among farms and countries through trading (Wu *et al.*, 2002). It is possible that these animal HEV strains may undergo genetic reversion to HEV strains which cause clinical disease in humans, which can have major public health implications (Meng *et al.*, 1998; Hsieh *et al.*, 1999). Changes in the restrictive conditions would expose the South African population to the same health implications typical of the disease in other parts of the world (Grabow *et al.*, 1996; Grabow, 1997).

Swine HEV isolates with high sequence identity (90%-91%) to human HEV strains (US-1 and US-2) have been detected in pigs in the US (Meng *et al.*, 1997; Erker *et al.*, 1999b; Hsieh *et al.*, 1999; Schlauder *et al.*, 1999). Similar results were obtained in a study in which a swine isolate of HEV was closely related to the HEV strain isolated from humans with sporadic acute hepatitis in Taiwan (Hsieh *et al.*, 1999). This swine HEV strain was distinct from the HEV isolate found in the US (Hsieh *et al.*, 1999). An avian HEV strain has been identified in the US, which is genetically and antigenically related to human and swine HEV (Huang *et al.*, 2004). In a study by Drobeniuc *et al.* (2001) it was established that there is an increase in HEV infection among persons with occupational exposure to swine, which suggests animal-to-human transmission of this infection.

2.6 Pathogenesis and Clinical Spectrum of Hepatitis E

Hepatitis viruses replicate within the human liver, where they may cause mass destruction of hepatocytes (Grabow, 1997). Although these hepatitis viruses cause liver

damage, they are not directly cytopathic to hepatocytes (Purcell and Ticehurst, 1997; Jameel, 1999). Liver biopsy specimens showed either non-specific inflammatory changes or prominent canalicular bile stasis with pseudoglandular arrangement of hepatocytes around distended bile canaliculi (cholestatic form) (Worm *et al.*, 2002). These patterns are considered characteristic for hepatitis E (Worm *et al.*, 2002). The liver damage results in the failure of the liver to perform basic functions such as the removal of bilirubin from the circulatory blood system (Grabow, 1997). This causes the symptomatic condition known as jaundice (Grabow, 1997). Jaundice causes the excretion of accumulated bilirubin through the kidneys, the digestive tract and also causes the yellow coloration of the conjunctivae and skin due to the deposition of bilirubin in the peripheral blood network (Grabow, 1997). Liver enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are released into the bloodstream as a consequence of the massive liver damage (Zuckerman and Thomas, 1993). The serum levels of ALT and AST are used to diagnose hepatitis (Zuckerman and Thomas, 1993). Following acute liver injury, the clinical manifestations and outcome of viral hepatitis are determined by the host immune response (Jameel, 1999). The pathogenesis of hepatitis E is not well understood, as serological assays for HEV have recently become available (Jameel, 1999). After oral ingestion of HEV by the host, the virus replicates in the intestinal tract (Jameel, 1999). The virus presumably reaches the liver via the portal vein serving the liver (Jameel, 1999). After replication of HEV in the cytoplasm of hepatocytes, the virus particles are released into the bile and bloodstream (Krawczynski and Bradley, 1989; Jameel, 1999).

In the case of foodborne transmission of HEV described by Tei and colleagues (2003), one person ingested a very small amount of the contaminated deer meat without contracting clinical hepatitis E. The titre of HEV RNA in the infected meat was in the region of 10^5 copies.g^{-1} (Tei *et al.*, 2003), and therefore, it is suggestive that HEV has a high infectious dose. This high infectious dose may also explain why person-to-person transmission of HEV does not play a major role in the epidemiology of the virus.

The clinical illness of HEV resembles other forms of acute viral hepatitis, especially acute viral hepatitis caused by HAV (Grabow, 1997; Jameel, 1999; Van der Poel *et al.*, 2001). The onset of the disease follows an incubation period of 1 to 8 weeks (mean 40 days) (Grabow, 1997; Van der Poel *et al.*, 2001). An incubation period of 32 days has been measured accurately in a single case of transmission of HEV to a human volunteer (Chauhan *et al.*, 1993). In the case of foodborne transmission of HEV, an incubation period of 66 days has been reported (Tei *et al.*, 2003). Clinical hepatitis E occurs predominantly in the young adult population (15 to 40 years), while it tends to be asymptomatic in the younger age groups (<15 years) (Arankalle *et al.*, 1995; Grabow, 1997; Van der Poel, 2001).

Clinical symptoms of acute hepatitis E include abdominal pain, anorexia, dark urine, fever, hepatomegaly, jaundice, malaise, nausea and vomiting, with less common symptoms such as arthralgia, diarrhoea, pruritus and urticarial rash (Table 2.3) (Tucker and Kirsch, 1994; Clayson *et al.*, 1995b). Liver histology of patients presenting with hepatitis E reveals portal triaditis, cholestatis, lobular inflammation and degeneration of the liver, which are all suggestive of acute viral hepatitis (Jameel, 1999). The disease is usually mild and resolves within 2 weeks. Chronic HEV infections have not been observed (Van der Poel *et al.*, 2001). Hepatitis E is more severe than hepatitis A, with a mortality rate of 1-2% (Jameel, 1999). In areas where the disease is endemic, HEV is an important cause of death due to liver failure caused by acute fulminating hepatitis, especially in pregnant woman, with mortality rates up to 25% (Grabow *et al.*, 1996; Grabow, 1997; Van der Poel *et al.*, 2001).

Infectious viral particles can be detected in the bile and faeces during the late incubation phase of hepatitis E and persist for a week or two following the onset of the illness (Jameel, 1999; Van der Poel *et al.*, 2001). Patients may excrete viral particles for more than seven weeks (Clayson *et al.*, 1995b; Scharschmidt, 1995). Viremia can be detected during the late phase of the incubation period and in the acute phase of the illness (Chauhan *et al.*, 1993; Aggarwal *et al.*, 2000; Van der Poel *et al.*, 2001). The period of infectivity following acute infection has not been determined (Van der Poel *et al.*, 2001).

Table 2.3 Clinical findings in patients with hepatitis E (Worm *et al.*, 2002)

Symptom	Percentage
Jaundice	~100
Malaise	~100
Anorexia	66-100
Abdominal pain	37-82
Hepatomegaly	10-85
Nausea, vomiting	29-100
Fever	23-97
Pruritis	14-59

Immunoglobulin A (IgA), IgG and IgM anti-HEV appear during the course of the disease (Jameel, 1999). IgM antibodies are detectable during the acute phase of the disease and disappear after 3-6 months, whereas IgG antibodies persist for 2-13 years, and thus, lifelong immunity does not occur after infection with HEV (Chauhan *et* al., 1993; Clayson *et al.*, 1995b; Panda and Jameel, 1997; Jameel, 1999; Aggarwal and Krawczynski, 2000). The correlation between clinical disease, viremia and antibody response to HEV is shown in figure 2.5.

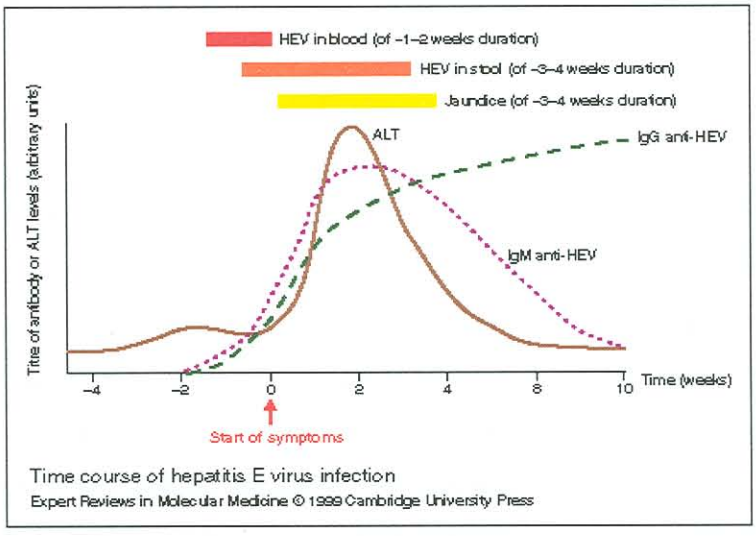

Figure 2.5 Correlation between clinical disease, viremia and antibody response to HEV (Jameel, 1999)

During a study by Nanda *et al* (1995) viremia was observed in the absence of anti-HEV antibodies, in approximately 10% of patients with a HEV infection. Seroconversion might be a critical marker for early clearance of HEV from the bloodstream (Jameel, 1999). Short persistence of anti-HEV IgG/IgM or a negative anti-HEV IgM status may hamper the diagnosis of acute HEV infection (Clayson *et al.*, 1995b; Worm *et al.*, 2002).

2.7 Therapeutic Approaches to Hepatitis E Infection

No therapeutic compounds against hepatitis E are currently available, with the only treatments being supportive in nature (Aggarwal and Krawczynski, 2000). Tandon and colleagues (2002) investigated the possible benefit of glycyrrhizin therapy for acute sporadic hepatitis E. Glycyrrhizin is a drug developed from the *Glycyrrhiza glabora* plant (Tandon *et al.*, 2002). The pharmacological actions of glycyrrhizin includes: 1) natural interferon stimulation, 2) immunomodulation, 3) antiviral properties, 4) anti-inflammatory properties, and 5) hepatoprotective properties (Finney and Somers, 1958; Pompei *et al.*, 1979; Abe *et al.*, 1982; Kimura *et al.*, 1992). Currently, glycyrrhizin is extensively used in chronic hepatitis B and C, and has been successfully used in sub-acute liver failure in India (Acharya *et al.*, 1993; Jardi *et al.*, 1993; Takahara *et al.*, 1994; Tan *et al.*, 1995; Arase *et al.*, 1997; Krawczynski, 1998; Zhang *et al.*, 2000).

2.8 Diagnostic Detection of Hepatitis E Virus

In areas where HEV is endemic, the first-line work-up for individuals with icterus and signs of hepatitis is laboratory testing for HEV (Worm *et al.*, 2002). In areas with a low incidence of hepatitis E, patients that present with jaundice, a recent travel history to an HEV endemic area or elevated ALT levels (>2.5-fold of the upper reference-range; DeRitis-Quotient (ALT/AST) <1) can be considered for laboratory testing of HEV infection (Worm *et al.*, 2002). In cases where there is no travel to HEV endemic areas, laboratory testing can be carried out after more common causes of hepatitis and cholestasis are ruled out (Worm *et al.*, 2002).

2.8.1 Immuno Electron Microscopy and Immuno Fluorescence Microscopy

Immuno electron microscopy is used for the detection of HEV VLP's in clinical specimens (Balayan *et al.*, 1983), but is only offered by a few specialised laboratories (Worm *et al.*, 2002). Native antibody to HEV, derived from acute- or convalescent-phase sera, is used to precipitate HEV particles (Worm *et al.*, 2002). This precipitation technique has been used in the detection of Noroviruses (formally Norwalk-like viruses), rubella virus, HBV and viruses associated with diarrhoea (Chrystie, 1996). Immuno electron microscopy has been used routinely to serotype viruses, such as rotaviruses, enteroviruses and adenoviruses (Chrystie, 1996).

Immuno fluorescence microscopy (IFM) is a semi-quantitative technique, which detects antibodies that react against HEV antigen (Worm *et al.*, 2002). Binding of fluorescein-conjugated anti-HEV IgG to HEV antigen is blocked by anti-HEV in frozen liver tissue (Worm *et al.*, 2002). The anti-HEV concentration is estimated semi-quantitatively (Krawczynski and Bradley, 1989). Both IEM and IFM are laborious and expensive and, therefore, not routinely used for the diagnosis of HEV infection (Worm *et al.*, 2002).

2.8.2 Enzyme Immuno Assays for the Detection of Antibodies to Hepatitis E Virus

Enzyme immuno assays (EIA's) are used to detect anti-HEV IgG and IgM (Worm *et al.*, 2002). To avoid false positive results, test procedures should be carried out during the acute phase of hepatitis E (Anderson *et al.*, 1999). False positive results for anti-HEV IgM occur infrequently, but may occur in cases of autoimmune hepatitis and rheumatologic diseases (Worm *et al.*, 2002). Acute infection is indicated by a positive result for anti-HEV IgM and the diagnosis of acute hepatitis E may be supported by the presence of a high or increasing titre of anti-HEV IgG (Mushahwar *et al.*, 1996; Gouvea *et al.*, 1997; Worm *et al.*, 2002).

Antigenic domains have been identified within all three ORF's by epitope mapping studies or with immunoscreening recombinant cDNA libraries (Anderson *et al.*, 1999;

Worm *et al.*, 2002). Various diagnostic and research immunoassays have been developed due to the molecular cloning of the HEV genome and expression of recombinant proteins (Reyes *et al.*, 1990; Ichikawa *et al.*, 1991; Tam *et al.*, 1991; Aye *et al.*, 1992; Huang *et al.*, 1992a; He *et al.*, 1993; Li *et al.*, 1994). Twelve antigenic domains have been identified throughout ORF1, six antigenic domains within the ORF2 protein and three within the ORF3 protein (Khudyakov *et al.*, 1999). Most EIA's use recombinant proteins derived from the C-terminal or large portions of ORF2 and/or the C-terminal or full length of ORF3 (Worm *et al.*, 2002). The antigenic activity of recombinant proteins used in EIA's can be ascribed to three different mechanisms:

i) Recombinant proteins or synthetic peptides can represent the linear epitopes described above. Several peptides derived from different linear epitopes have been aligned to a mosaic fusion protein (Favorov *et al.*, 1996).

ii) Larger recombinant proteins may have conformational antigenic determinants related to their secondary or tertiary structure (Worm *et al.*, 2002).

iii) Capsid-like particles originating from a recombinant 111-aa N-terminal truncated ORF2 protein mimic the three-dimensional structural features of VLP's and have an immunogenic activity represented by their quaternary structure (Li *et al.*, 2000).

Seroprevalence studies have indicated that HEV EIA's based on large antigens expressed from ORF2 or capsid-like particles are superior to those based on short sequences of ORF2 or antigenic epitopes of the ORF3 in detecting convalescent-phase anti-HEV (Ghabrah *et al.*, 1998). Convalescent-phase anti-HEV could not reliably be detected by EIA's based on synthetic peptides and are mainly used for the confirmation of positive results from EIA's based on recombinant proteins and to exclude non-specific reactivity (Mast *et al.*, 1998; Worm *et al.*, 2002). The use of small peptides might increase the specificity to distinguish genotype-specific anti-HEV in the acute-phase serum (Schlauder *et al.*, 1998; Worm *et al.*, 2000).

Presently there are only two commercially available EIA's:

i) the Genelabs®-EIA uses four short recombinant proteins derived from the 3' termini of ORF2 and ORF3 from the Burmese and Mexican prototype sequences, and

ii) the Abbott®-EIA uses two recombinant proteins derived from the complete ORF3 and from a sequence of ORF2 from the Burmese prototype strain.

The reliability of these EIA's from seroepidemiological studies is limited, as the specificity and sensitivity of these tests for detecting convalescence-phase IgG have not been established (Worm *et al.*, 2002).

2.8.3 *In vitro* Cell Culture Propagation of Hepatitis E Virus

The replication strategy of the HEV is poorly understood (Worm *et al.*, 2002). Establishment of a practical cell culture system to allow the propagation of HEV *in vitro* is vital for virological characterisation, as well as for the diagnosis and prevention of HEV infection (Wei *et al.*, 2000). Several strategies for the experimental propagation and production of HEV to study the molecular biology and vaccine development have been published (Huang *et al.*, 1992b; Kazachkov *et al.*, 1992; Huang *et al.*, 1995a; Tam *et al.*, 1996; Huang *et al.*, 1999).

Chinese strains of HEV have been isolated and cultivated in an *in vitro* cell culture using continuous cell lines derived from human lung, kidney or liver (such as 2BS diploid human embryonic lung cells, A549 human lung carcinoma cells or Hep-G2 cells) (Huang *et al.*, 1992b; Huang *et al.*, 1995b; Huang *et al.*, 1999). Cell culture propagation of a Russian strain of HEV using rhesus kidney (FRhK) cells has also been described (Kazachkov *et al.*, 1992). A tissue culture system was developed for the propagation of HEV *in vitro*, however, the replication of HEV in this system appears to be inefficient, as HEV can only be detected by RT-PCR (Tam *et al.*, 1996).

Tam and colleagues (1997) used *in vivo* infected primate liver cells for the *in vitro* replication of HEV in serum-free medium supplemented with growth factors and hormones. Both positive-strand and negative-strand HEV RNA were detected in cellular RNA of the culture cells as well as positive-strand HEV RNA in the culture medium (Tam *et al.*, 1997). The latter is indicative of the shedding of VLP's into the culture medium. No cytopathic effects (CPE's) were observed during these experiments (Tam *et al.*, 1997). A neutralising anti-HEV antibody directed against the ORF2-encoded capsid protein (Tam *et al.*, 1996) blocked the infection of the liver cells. Similar results were obtained when a Chinese strain of HEV was cultivated in A549 (human lung carcinoma) cells under conditions of a relatively high final concentration of $MgCl_2$ (30 mM), a pH of 7.2 and a short (<6 months) preservation time of propagated strains (Huang *et al.*, 1999). Cytopathic effects (including cell rounding and monolayer destruction) were visible at day two post-inoculation and could be neutralised by specific acute-phase anti-HEV (Huang *et al.*, 1999).

Although various authors have reported the experimental propagation of HEV in cell culture, most of these culture systems could not provide authentic HEV particles or high titre VLP's, thereby precluding the study of novel viruses (Wei *et al.*, 2000; Worm *et al.*, 2002).

2.8.4 Molecular Detection of Hepatitis E Virus

Molecular methods and cell cultures are currently used for the screening for waterborne viruses, such as enteroviruses, HAV, astroviruses and adenoviruses (Metcalf *et al.*, 1995). Cell culture is preferred due to the public health implications since it allows the demonstration of virus infectivity (Grimm and Fout, 2002). Although *in vitro* cell culture propagation of HEV has been reported (Huang *et al.*, 1992b; Huang *et al.*, 1995b; Tam *et al.*, 1996; Tam *et al.*, 1997; Huang *et al.*, 1999), a standard cell culture method for the detection of HEV does not presently exist, and therefore, this approach cannot be used for HEV (Grimm and Fout, 2002).

The first strains of HEV were detected, isolated and sequenced during outbreaks in Burma, Pakistan and Mexico involving techniques including IEM, cloning, hybridising specifically to infected source cDNA and immunoscreening of cDNA libraries (Balayan *et al.*, 1983; Worm *et al.*, 2002). Consensus oligonucleotide primers have been developed in various studies to amplify regions in the HEV genome, which encode the helicase, polymerase or parts of the 3' end of ORF2, by RT-PCR (Meng *et al.*, 1997; Schlauder *et al.*, 1999). Hepatitis E virus RNA can be detected by RT-PCR in acute-phase sera, stool, contaminated water and sewage without the use of cell culture (Erker *et al.*, 1999b; Wang *et al.*, 1999; Pina *et al.*, 2000; Grimm and Fout, 2002). Reverse transcriptase-PCR is more rapid and sensitive than cell culture, but only indicates the presence of viral RNA as opposed to infectious viral particles (Grimm and Fout, 2002). It was suggested that non-intact viruses were degraded rapidly in environmental samples (Tsai *et al.*, 1995; Limsawat and Ohgaki, 1997). Several RT-PCR assays have been developed for the detection of HEV in either clinical or environmental samples (McCaustland *et al.*, 1991; Ray *et al.*, 1991; Jameel *et al.*, 1992; Chauhan *et al.*, 1993; Jothikumar *et al.*, 1993; Clayson *et al.*, 1995a; Huang *et al.*, 1995b; Pina *et al.*, 1998; Erker *et al.*, 1999a). Reverse transcriptase-PCR assays amplify either a subset of HEV strains using a small number of primer sets or the majority of the HEV strains using a large number of degenerate primers (Grimm and Fout, 2002). In a study conducted by Grimm and Fout (2002), three RT-PCR assays were developed for the detection of any HEV strain in water sources.

2.9 Recombinant Hepatitis E Viral Proteins

Sufficient quantities of HEV proteins are not available for diagnostic use, since there is only limited growth of HEV reported in cell culture systems (Huang *et al.*, 1992a; Sehgal *et al.*, 2003). An economical method to produce HEV target proteins in large quantities is either to express them using the Baculovirus Expression Vector System (BEVS) in insect cells (Tsarev *et al.*, 1993; Bryan *et al.*, 1994; McAtee *et al.*, 1996; Li *et al.*, 1997b; Sehgal *et al.*, 2003) or to express the ORF2 antigen in *E. coli* (Li *et al.*, 1997a). The

expression of recombinant HEV proteins has allowed the development of numerous diagnostic and research immunoassays (Anderson *et al.*, 1999).

All three ORF's of HEV have been successfully expressed in different systems including *E. coli*, baculovirus and vaccinia virus (Anderson *et* al., 1999; Worm *et al.*, 2002; Sehgal *et al.*, 2003). In an *in vitro* synthetization, a full-length HEV cDNA clone was constructed in a pSGI vector and the *in vitro* transcribed RNA of the clone was found to be infective in a Hep-G2 tissue culture system (Panda *et al.*, 2000).

The ORF2 protein is most suitable as a diagnostic reagent, as it is the major structural protein of HEV (He *et al.*, 1993). An ORF2-encoded 56 kDa protein, truncated at its N- and C-termini, served a highly reactive antigen in detecting anti-HEV antibodies (McAtee *et al.*, 1996). Vaccination using a 56 kDa processed form of the ORF2 protein reduced virus shedding and protected rhesus monkeys when challenged with a high IV dose of HEV (Zhang *et al.*, 2001). The BEVS-expressed ORF2 protein is more sensitive and specific compared to the ORF2 protein expressed in bacteria (Zhang *et al.*, 1997).

2.10 Vaccine Development for Hepatitis E Virus

Many pathogenic viruses and bacteria establish their initial infections through mucosal surfaces (Niikura *et al.*, 2002). Consequently, vaccine strategies that can stimulate mucosal immunity have been widely studied (Bergmann and Waldman, 1988; Ulrich *et al.*, 1998). Mucosal immunity involves a systemic network of mucus throughout the body and plays a crucial role in the defence mechanisms against infection by pathogenic organisms (Boyaka *et al.*, 1999; Czerkinsky *et al.*, 1999). IgA is the predominant species of antibody that plays a role in mucosal immunity (Mestecky and McGhee, 1987; Li *et al.*, 2001). An effective HEV vaccine should have the ability to induce a specific intestinal IgA response (Miller *et al.*, 1992).

Stimulation of mucosal immunity through oral administration of vaccines is beneficial in terms of its convenience (Morrow *et al.*, 1999). Oral immunisation with non-replicating

molecules faces several difficulties, including the low pH in the stomach, proteolytic enzymes in the digestive tract and physical as well as biochemical barriers associated with the mucosal surface itself (Morrow *et al.*, 1999). Once the barriers have been overcome, oral immunisation by non-replicating molecules may stimulate mucosal immunity through the specialised epithelial M cells and the intestinal lymphoid organs (Neutra, 1999).

No practical cell culture system has been developed to allow the growth of HEV (Grimm and Fout, 2002). Following the molecular cloning of the capsid protein gene, ORF2, into a BEVS, insect cells were infected with the recombinant baculovirus, which resulted in the spontaneous assembly of VLP's (Li *et al.*, 1997b). The advantages of VLP's as the mucosal immunogen include the following:

1. The recombinant HEV (rHEV) VLP's are composed of a single protein assembled into particles without nucleic acid, which prevents them from replicating,
2. rHEV VLP's are easy to prepare and purify in large quantities (1 mg of VLP per 10^7 insect cells),
3. rHEV VLP's are antigenically similar to the native virion,
4. rHEV VLP's are highly immunogenic in experimental animals when injected parenterally,
5. rHEV VLP's are stable at low pH, and
6. oral delivery of rHEV VLP's could induce the same immune response as would be elicited by a natural infection (Li *et al.*, 1997b; Xing *et al.*, 1999).

The oral immunisation of recombinant VLP's to prevent disease has been described previously (Breitburd *et al.*, 1995; Suzich *et al.*, 1995; Kirnbauer *et al.*, 1996; Ball *et al.*, 1998; Rose *et al.*, 1999; Estes *et al.*, 2000). Oral immunisation offers many advantages over parenteral immunisation, including easy delivery, more acceptability to recipients, reduction in the purity, and reduction in the number of trained personnel needed to administer injections (Li *et al.*, 2001). It is often necessary to add mucosal adjuvants, such as cholera toxin, to the VLP's in order to induce significant mucosal immunity

(O'Neal *et al.*, 1997; Balmelli *et al.*, 1998; Fooks *et al.*, 1998; Modelska *et al.*, 1998; Yuan *et al.*, 1998; Brennan *et al.*, 1999; Mrsny *et al.*, 1999). A major drawback with cholera toxin as a mucosal adjuvant is its intrinsic toxicity (Scheibner, 2000). The potential of rHEV VLP's as an oral immunogen was analysed by Li and colleagues (2001). The rHEV VLP's were shown to be immunogenic, generating high titres of HEV-specific antibody in serum, which was capable of binding to the native HEV antigen (Li *et al.*, 2001). The rHEV VLP's given orally provided a non-replicate and safe candidate vaccine for HEV (Li *et al.*, 2001).

Alternate strategies for developing anti-HEV vaccines are being conducted in various research laboratories. A naked DNA immunisation approach in which ORF2 was injected as an expression plasmid directly into muscle resulted in moderate anti-pORF2 titres in mice (He *et al.*, 2001).

Although many HEV vaccine candidates have been produced, only one vaccine candidate has progressed to the stage of clinical trials (Wang and Zhuang, 2004). Despite these achievements in vaccine research, many questions remain to be answered by future research before an effective vaccine is produced for HEV.

2.11 Summary

Hepatitis E virus was initially believed to be restricted to the Indian sub-continent, but is now recognised as the major causative agent of enterically transmitted non-A, non-B hepatitis in subtropical and tropical areas world-wide, where it has been shown to be responsible for many outbreaks since the 1950's (Wang *et al.*, 1999; Favorov *et al.*, 2000; Schlauder and Mushahwar, 2001; Worm *et al.*, 2002). Epidemiological field studies have indicated that faecally contaminated drinking water has been the primary source of HEV infection during outbreaks (Wang *et al.*, 1999; Favorov *et al.*, 2000; Schlauder and Mushahwar, 2001; Van der Poel *et al.*, 2001).

Seroprevalence studies have been conducted in both developing (endemic) and industrialised (non-endemic) countries (Paul *et al.*, 1994; Clayson *et al.*, 1997). The seroprevalence of anti-HEV IgG among humans in the endemic countries was relatively high (5-31%) compared to the industrialised nations (1-5%), where clinical disease is rarely seen (Paul *et al.*, 1994; Clayson *et al.*, 1997). Antibodies to HEV, as well as HEV RNA, have been detected in a number of animal species (particularly swine) native to developing and developed countries world-wide (Meng, 2000; Pina *et al.*, 2000; Garkavenko *et al.*, 2001; Van der Poel *et al.*, 2001). The HEV strains detected in industrialised nations were found to be unrelated to the prototype HEV strains from endemic areas (Pina *et al.*, 2000; Schlauder and Mushahwar, 2001; Worm *et al.*, 2002). These novel swine HEV strains, or mutants of these strains, might have caused sub-clinical infections in humans eliciting an anti-HEV response, which may explain the prevalence of anti-HEV IgG in industrialised countries, despite the absence of clinical disease (Balayan, 1997; Meng *et al.*, 1998; Meng *et al.*, 1999; Drobeniuc *et al.*, 2001).

Although the reservoir of HEV has not been established, several reports of foodborne transmission of HEV provided direct evidence for zoonotic transmission of the virus (Matsuda *et al.*, 2003; Tei *et al.*, 2003; Yazaki *et al.*, 2003). Hepatitis E was reported in Japan in a number of patients after the ingestion of uncooked deer meat and pig liver, which was found to be infected with HEV (Matsuda *et al.*, 2003; Tei *et al.*, 2003; Yazaki *et al.*, 2003).

The diagnostic detection of HEV is limited. The use of IEM and IFM is laborious and expensive, which makes these approaches impractical for the routine diagnostic detection of HEV (Worm *et al.*, 2002). Enzyme immuno assays are routinely used to detect anti-HEV IgG and IgM (Worm *et al.*, 2002). The disadvantage of these assays is the limited reliability, due to the unestablished specificity and sensitivity (Worm *et al.*, 2002). *In vitro* cell culture propagation has been reported, however, the reproducibility of these methods have not been confirmed, with the result that no standard cell culture procedure currently exists for the propagation of HEV (Kazachkov *et al.*, 1992; Tam *et al.*, 1996; Huang *et al.*, 1999). To date, molecular detection is the most reliable method for the

detection of HEV in clinical and environmental samples (Grimm and Fout, 2002). Various RT-PCR procedures have been described using specific oligonucleotide primers for the amplification of various regions of the HEV genome (Meng *et al.*, 1997; Schlauder *et al.*, 1999).

The epidemiology of HEV in South Africa has not thoroughly been investigated. A limited number of seroprevalence studies have been conducted, while the veterinary aspects remain untouched (Grabow *et al.*, 1996; Tucker *et al.*, 1996). It has been shown that anti-HEV IgG is more prevalent in the lower socio-economic communities, as well as individuals with a regular exposure to recreational water (Grabow *et al.*, 1996; Tucker *et al.*, 1996). Barring a few imported cases of hepatitis E, clinical cases are rarely diagnosed (Grabow, 1997). This raises the concern as to what extent swine and other animal species play a role in the transmission of HEV in South Africa. This information may be of fundamental importance to strategies aimed at controlling infections and waterborne transmission of HEV, not only in South Africa, but also in other parts of the world, particular in view of the absence of a HEV vaccine.

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