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Hepatitis E Virus in South Africa: Seroprevalence of Anti-HEV IgG in Swine and Detection of the Virus in Swine Faecal Specimens and Domestic Sewage Samples

by

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"Ek verklaar dat die verhandeling wat ek hiermee aan die Universiteit van Pretoria vir die MSc (Geneeskundige Virologie)-graad voorlê, my eie werk is en nie vantevore deur my aan enige ander tersiêre inrigting vir enige graad voorgelê is nie."

"I certify that the thesis hereby submitted to the University of Pretoria for the degree of MSc (Medical Virology) is my own work and has not previously been submitted by me in respect of a degree at any other tertiary institution."

Signature:

Date: 2004 · 11 · 10

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This thesis is dedicated to my parents

"The success of current scientific theories is no miracle. It is not even surprising to the scientific (Darwinist) mind. For any scientific theory is borne into a life of fierce competition, a jungle red in tooth and claw. Only the successful theories survive - the ones which in fact latched on to the actual regularities in nature."

Bas Van Fraassen (b. 1941)

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LIST OF ABBREVIATIONS

aa	Amino acid
ALT	Alanine aminotransferase
Anti-HEV	Antibodies to hepatitis E virus
Anti-HAV	Antibodies to hepatitis A virus
AST	Aspartate aminotransferase
BEVS	Baculovirus expression vector system
bp	Base pair
CDC	Centers for Disease Control and Prevention
cDNA	Complimentary DNA
CI	Confidence interval
CO	Cut-off
CPE	Cytopathic effect
E. coli	*Escherichia coli*
EIA	Enzyme immuno assay
ELISA	Enzyme-linked immunosorbent assay
IFM	Immuno fluorescence microscopy
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCl	Hydrochloric acid
HEV	Hepatitis E virus
HEVAg	Hepatitis E virus target antigen
H_2SO_4	Sulphuric acid
IEM	Immuno electron microscopy
IV	Intravenous
kb	Kilobase

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KCl	Potassium chloride
kDa	Kilo-Dalton
$MgCl_2$	Magnesium chloride
mM	Millimolar
mRNA	Messenger RNA
NS	Non-structural
nm	Nanometre
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
pmol	Picomole
RdRp	RNA-dependant RNA polymerase
rHEV	Recombinant hepatitis E virus
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
SD	Specimen diluent
SNC	Standard negative control
SPC	Standard positive control
UK	United Kingdom
US	United States
UTR	Untranslated regions
UV	Ultraviolet
VLP	Virus-like particles

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HEPATITIS E VIRUS IN SOUTH AFRICA: SEROPREVALENCE OF ANTI-HEV IgG IN SWINE AND DETECTION OF THE VIRUS IN SWINE FAECAL SPECIMENS AND DOMESTIC SEWAGE SAMPLES

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SUMMARY

Hepatitis E virus (HEV) is a major cause of enterically transmitted hepatitis in areas with a tropical or subtropical climate and poor sanitary conditions. Hepatitis E virus is responsible for both waterborne epidemics and sporadic cases of acute hepatitis in many developing countries world-wide, but rarely causes epidemics in more industrialised countries. Hepatitis E occurs predominantly in the adult population, causing mild symptoms that usually resolve within 2 weeks. However, the disease poses a great threat to pregnant women, specifically during the third trimester, with a mortality rate of up to 25% due to liver failure. Many different animal species world wide, especially swine have been shown to have antibodies against HEV. Hepatitis E virus strains isolated from animals showed a remarkable resemblance to human HEV strains, raising the suspicion that these viruses may be zoonotic. Recently direct evidence supporting this suspicion

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was provided by reports of foodborne transmission of HEV from animals to humans in Asia.

Seroprevalence studies conducted in South Africa have suggested that HEV may be endemic in some areas of the country, especially in low socio-economic communities. Only a few clinical cases of hepatitis E, mainly associated with travelling to HEV endemic countries, have been reported. Similar results have been reported in other predominantly non-endemic countries such as Spain, the UK and the US. Since at least some animal strains of HEV are considered to be zoonotic, it is suggested that these strains are being transmitted to humans via water sources contaminated with animal wastes, which results in the eliciting of an anti-HEV response.

The objective of this study was, therefore, to assess the seroprevalence of HEV in swine, as well as to determine what strains of HEV are circulating in South Africa in order to establish the role which swine may play in the transmission of the virus in the country. The seroprevalence of anti-HEV in selected swine populations in this study indicated that HEV is widespread throughout the swine population in South Africa. This is in agreement with results obtained in similar studies conducted in other non-endemic countries. In addition, this study reported the detection and characterisation of four novel isolates of HEV in domestic sewage samples and swine faecal specimens. The two HEV strains isolated from sewage were closely related to strains of genotype I, which includes human-related strains from Asia, Europe, Africa and the Middle East. This indicated that HEV is most likely circulating in the human population in South Africa, despite the absence of clinical disease. The two swine HEV strains detected in the swine faecal specimens showed a relationship with genotypes III and IV respectively, which raises concern, as the strains of these HEV genotypes have shown to cross the species barrier to cause disease in the human population.

Future research on HEV in South Africa includes determining the prevalence and characterisation of the virus in the human, swine and other animal populations throughout

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the country in order to cast more light on the potential health impact they may have on the human population.

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HEPATITIS E VIRUS IN SUID-AFRIKA: DIE VOORKOMS VAN ANTI-HEV IgG IN VARKE EN DIE DETEKSIE VAN DIE VIRUS IN VARK STOELGANGE EN RIOOL MONSTERS

deur

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OPSOMMING

Hepatitis E virus (HEV) is die hoof oorsaak van enteries-oordraagbare hepatitis in tropiese en subtropiese gebiede en waar daar swak sanitêre toestande heers. Hepatitis E virus is verantwoordelik vir beide water-gedraagde epidemies en sporadiese gevalle van akute hepatitis in verskeie ontwikkelende lande wêreldwyd en veroorsaak selde epidemies in ontwikkelde lande. Hepatitis E kom hoofsaaklik in die volwasse populasie voor waar dit matige simptome veroorsaak wat gewoonlik binne 2 weke opklaar. Daarteenoor is die siekte lewensgevaarlik vir swanger vroue, spesifiek gedurende die derde trimester, met 'n mortaliteitsyfer van tot 25% as gevolg van lewerversaking. Daar is wêreldwyd bewys dat verskeie dierspesies, veral varke, HEV teenliggame bevat. Daar word bespiegel dat HEV soönoties oorgedra kan word as gevolg van die ooreenkoms

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tussen HEV stamme wat onderskeidelik in mense en diere voorkom. Hierdie bespiegeling is onlangs bewys deurdat voedsel-oordrag van HEV vanaf diere na mense in Asië gerapporteer is.

Vorige studies wat die voorkoms van HEV teenliggame in Suid Afrika nagevors het, het voorgestel dat HEV endemies is in sommige dele van die land, veral in lae sosio-ekonomiese gemeenskappe. Die paar kliniese gevalle van hepatitis E wat wel aangemeld word, was gewoonlik geassosieer met 'n reis-geskiedenis na 'n endemiese land. Soortgelyke gevalle is ook aangemeld in ander lande, soos Spanje, die Verenigde State van Amerika en die Verenigde Koninkryk, waar HEV nie endemies is nie. Aangesien dit wil voorkom asof ten minste sommige HEV stamme soönoties oorgedra kan word, word daar vermoed dat hierdie HEV stamme oorgedra word na mense via waterhulpbronne wat gekontamineer is met dierlike afval, wat aanleiding kan gee tot 'n HEV teenliggaam reaksie in mense.

Die doel van hierdie studie was om die voorkoms van HEV teenliggame in varke te bepaal, asook om vas te stel watter HEV stamme in Suid Afrika sirkuleer, om sodoende die rol wat varke mag speel in die oordrag van die virus vas te stel. Die voorkoms van HEV teenliggame in geselekteerde vark populasies in hierdie studie het aangedui dat HEV wydverspreid is in die vark populasie in Suid Afrika. Hierdie resultate is in ooreenstemming met soortgelyke studies wat in ander nie-endemiese lande gedoen is. Hierdie studie beskryf ook die opsporing en karakterisering van vier nuwe HEV isolate vanuit riool en varkmis. Die twee HEV isolate wat uit riool geïsoleer was, stem ooreen met mens HEV stamme van genotipe I, wat stamme van Asië, Europa, Afrika en die Midde Ooste insluit. Dit dui aan dat HEV moontlik in die mens-populasie van Suid-Afrika sirkuleer, ongeag die feit dat geen kliniese gevalle voorkom nie. Die twee HEV isolate vanuit varkmis stem ooreen met stamme van genotipe III en IV onderskeidelik, wat kommerwekkend is, aangesien hierdie HEV genotipes die vermoë het om verskillende spesies te infekteer, wat aanleiding kan gee tot die oordrag van die virus na die menslike populasie.

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Toekomstige navorsing op HEV in Suid Afrika sluit in die opsporing en karakterisering van die virus in die mens, vark en ander dier-populasies regoor die land om sodoende meer uitsluitsel te kry oor die potensiële gesondheidsimpak wat HEV mag hê.

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CHAPTER 1

Introduction

Hepatitis E virus (HEV), the etiological agent of hepatitis E, was first discovered in the late 1970's after it had become evident that there was a hepatitis virus other than hepatitis A virus and hepatitis B virus (Wong *et al.*, 1980). Hepatitis E virus was initially classified in the family *Caliciviridae*, but is currently recognised as the type species of the genus *Hepatitis E-like viruses* (Pringle, 1999; Berke and Matson, 2000).

Hepatitis E virus is transmitted via the faecal-oral route and is an important cause of acute epidemic viral hepatitis in some developing countries (Favorov *et al.*, 2000; Schlauder and Mushahwar, 2001; Van der Poel *et al.*, 2001). Outbreaks of hepatitis E are primarily associated with faecally contaminated drinking water (Favorov *et al.*, 2000; Schlauder and Mushahwar, 2001; Van der Poel *et al.*, 2001), and have been reported from countries in Asia, Africa and the Middle East (Byskov *et al.*, 1989; Mushahwar *et al.*, 1993; Swanepoel *et al.*, 1995; Hunter, 1997; Van der Poel *et al.*, 2001; Grabow, 2002).

Hepatitis E occurs predominantly in the young adult population, while it tends to be asymptomatic in the younger age groups (Van der Poel *et al.*, 2001). The onset of disease follows an incubation period of one to eight weeks (mean of 40 days) (Purcell, 1996; Van der Poel *et al.*, 2001). In areas where the disease is endemic, HEV is an important cause of death due to liver failure, especially in pregnant woman during the third trimester, with mortality rates of up to 25% (Grabow *et al.*, 1996; Van der Poel *et al.*, 2001). Chronic HEV infections have not been observed and the disease is usually mild and resolves within 2 weeks (Van der Poel *et al.*, 2001).

Hepatitis E virus may be endemic in certain areas of South Africa (Tucker and Kirsch, 1994; Tucker *et al.*, 1996; Grabow *et al.*, 1996). 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.*,

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1994; Grabow, 1997; South African Virus Laboratories Surveillance Bulletin, 2003). The rare diagnosis of clinical cases of hepatitis E in South Africa is therefore surprising, since outbreaks have been recorded in the neighbouring countries Namibia and Botswana (Byskov *et al.*, 1989; Swanepoel *et al.*, 1995; South African Virus Laboratories Surveillance Bulletin, 2001).

Although a reservoir of HEV has not yet been established, indirect evidence has suggested that at least some strains of HEV may be zoonotic (Meng *et al.*, 1997; Hsieh *et al.*, 1999; Kabrane-Lazizi *et al.*, 1999; Tei *et al.*, 2003). An increase in HEV infection among persons with occupational exposure to swine has been reported (Meng *et al.*, 1999; Drobeniuc *et al.*, 2001). Recently a few cases of foodborne HEV have been reported after the consumption of raw pig liver and deer meat, which provides direct proof of zoonotic transmission of HEV (Matsuda *et* al., 2003; Tei *et al.*, 2003; Yazaki *et al.*, 2003; ProMed, 2004).

Rare cases of hepatitis E without a travel history have been reported in non-endemic countries, in which case the transmission of the virus remains uncertain (Hsieh *et al.*, 1998; Erker *et al.*, 1999; Schlauder *et al.*, 1999; Zanetti *et al.*, 1999; Favorov *et al.*, 2000). It has been established, however, that the genomic sequences of the HEV strains detected in these patients were more related to the swine HEV strains prevalent in the swine population of the same area than to human HEV strains (Wu *et al.*, 2000). These swine HEV strains may undergo genetic reversion to HEV strains that cause clinical disease in humans similar to that in many parts of the world where hepatitis E has major public health implications (Meng *et al.*, 1998; Hsieh *et al.*, 1999).

The detection of anti-HEV IgG in swine populations from both developing and industrialised countries has been documented (Clayson *et al.*, 1995; Meng *et al.*, 1997; Chandler *et al.*, 1999; Hsieh *et al.*, 1999; Meng *et al.*, 1999; Pina *et al.*, 2000). It was found that swine herds from developing and industrialised countries contained many pigs that were seropositive for HEV, which suggests that HEV may be enzootic in swine regardless of whether HEV is endemic in the human population (Meng *et al.*, 1999).

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Since the waterborne transmission of HEV is well established and animal strains of HEV are closely related to the human strains, it would appear to be possible that the virus may be transmitted from animals to humans via water sources polluted with animal wastes. It is, therefore, important to determine what the prevalence of HEV is in the swine population of South Africa as well as the role that swine may play in the transmission of the disease. The purpose of this study was to determine the seroprevalence of anti-HEV IgG in swine sera and to detect HEV strains in swine faecal specimens and domestic sewage samples. This could cast light on the prevalence of the virus in selected areas of South Africa, as well as the possible relationship of these viruses with known strains of HEV from other parts of the world.

The objectives of this study were to:

1. Validate and optimise an enzyme-linked immunosorbent assay (ELISA) for the detection of anti-HEV IgG in swine serum samples.
2. Assess the seroprevalence of anti-HEV IgG in swine from selected areas of the Gauteng and Limpopo Provinces of South Africa by means of the ELISA.
3. Validate and optimise a reverse transcriptase-polymerase chain reaction (RT-PCR) procedure for the detection of HEV RNA.
4. Attempt to detect HEV RNA in swine faecal specimens by means of the RT-PCR.
5. Analyse selected domestic sewage samples for the presence of HEV by means of the RT-PCR.
6. Determine the relationship of HEV viruses detected in swine excreta and sewage to those described in other parts of the world using nucleotide sequence analysis.

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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).

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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

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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

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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 (VH2)	III	5	6			
Gr2	III	6	7			
Ar1, Ar2	III	7	8			
Au1	III	7				
Ct1	IV	8				
Cs15, Ch3	IV	9	4	4a		
Cs5, Cb3, Cb4	IV	(10)		4b		
Cs9	IV	(11)		4c		
Ct705, Ct825, Ct845	IV	(10-11)[h]				

[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

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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).

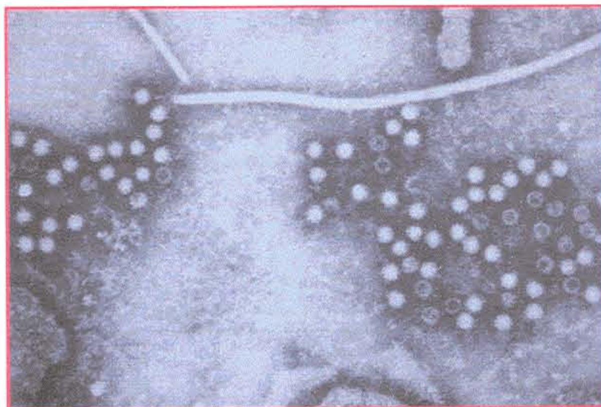

Figure 2.1 Electron micrograph of HEV (Hepatitis A-E slide set, www.cdc.gov)

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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).

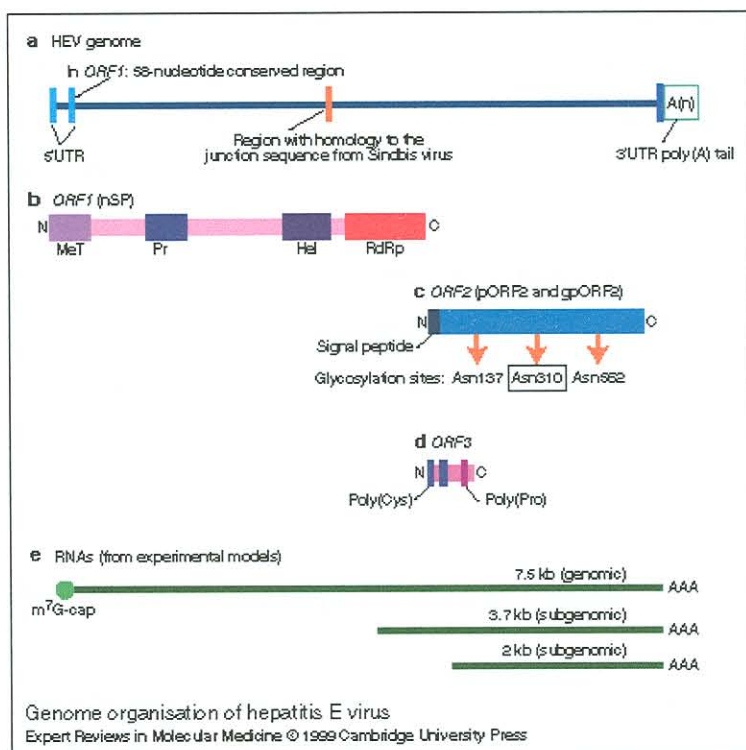

Figure 2.2 Schematic representation of the HEV genome organisation (Jameel, 1999)

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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

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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).

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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).

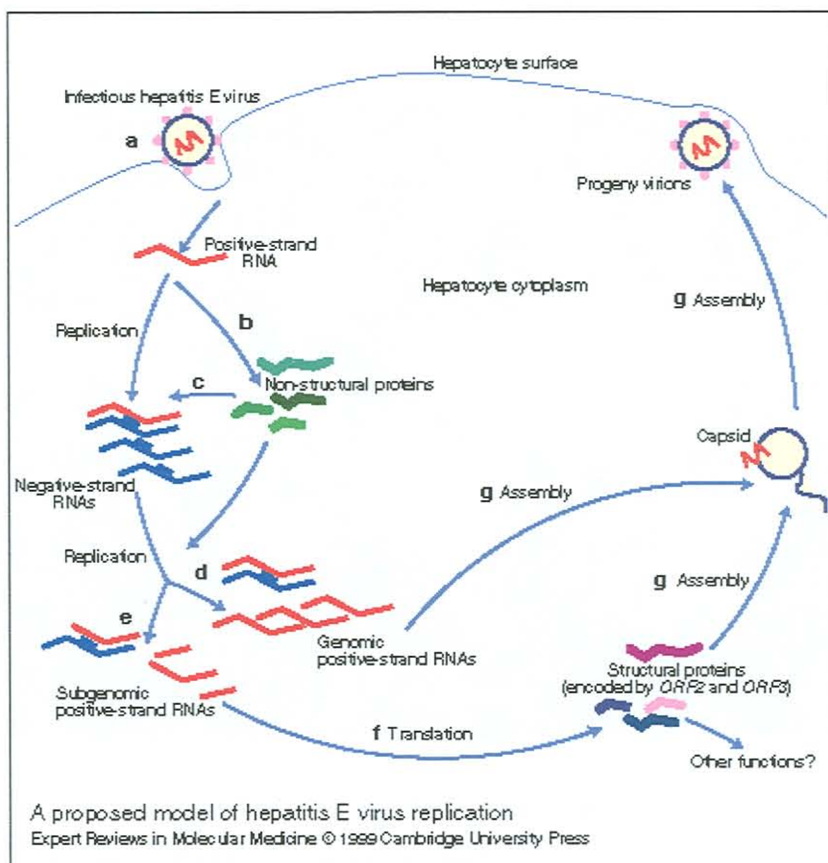

Figure 2.3 A proposed model of HEV replication (Jameel, 1999)

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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).

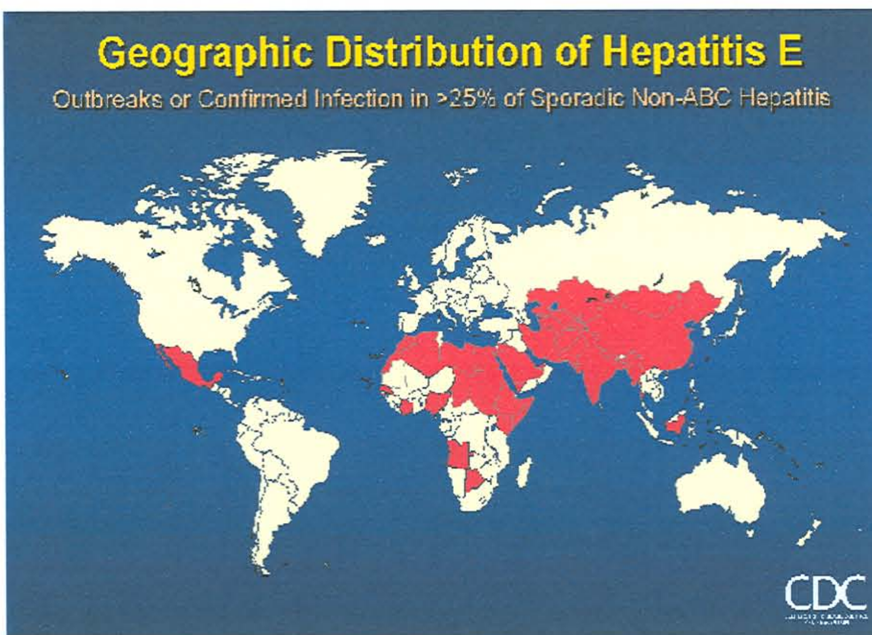

Figure 2.4 **Geographic areas with confirmed HEV infection or large outbreaks of hepatitis E (Mast and Alter, 1993)**

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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

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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

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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

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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.*,

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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

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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*

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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

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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

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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.

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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).

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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.

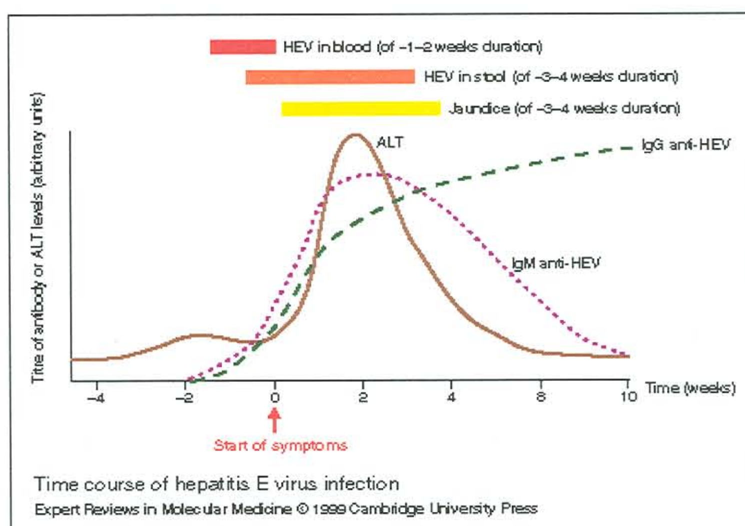

Figure 2.5 Correlation between clinical disease, viremia and antibody response to HEV (Jameel, 1999)

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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).

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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;

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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).

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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).

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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).

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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

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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

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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

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(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).

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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

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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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CHAPTER 3

Seroprevalence of Anti-HEV IgG in Swine from the Gauteng and Limpopo Provinces of South Africa

The editorial style of the *International Journal of Epidemiology* was followed in this chapter

3.1 Summary

3.1.1 Background

Hepatitis E virus (HEV) causes faecal-oral transmitted epidemic acute viral hepatitis in some developing countries. Outbreaks are predominantly associated with faecally contaminated drinking water. Hepatitis E virus may cause sporadic cases of acute hepatitis in some developed countries. The majority of these cases are, however, associated with a travel history to areas where HEV is endemic. It has been shown that some strains of HEV may be zoonotic. Swineherds in HEV endemic and non-endemic countries were found to contain pigs that are seropositive for HEV. The objective of this study was to assess the seroprevalence of anti-HEV immunoglobulin G (IgG) in selected swine populations in South Africa.

3.1.2 Methods

The study population comprised 192 swine from various regions of the Gauteng and Limpopo Provinces, South Africa. Swine serum was analysed for the presence of anti-HEV IgG by use of an enzyme-linked immunosorbent assay (ELISA).

3.1.3 Results

The prevalence of anti-HEV IgG in the Gauteng and Limpopo Provinces was 14.29% and 15.84%, respectively, with an overall prevalence of 15.10%.

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3.1.4 Conclusions

The prevalence of anti-HEV IgG in swine determined in this study was in the same order as reported in HEV non-endemic countries. According to the results, HEV may be prevalent in the swine population throughout South Africa and swine may play a meaningful role as reservoirs for the virus.

Keywords

Anti-HEV IgG, hepatitis E virus, South Africa, swine, zoonotic

3.2 Introduction

Hepatitis E virus (HEV) was initially mistaken for hepatitis A virus (HAV) as these viruses share basic clinical and epidemiological properties (1). Hepatitis E virus was first discovered in the late 1970s after it had become evident that there was a hepatitis virus other than HAV and hepatitis B virus (2). Hepatitis E virus was initially classified in the family Caliciviridae, but is currently recognised as the type species of the genus Hepatitis E-like viruses (3, 4).

Hepatitis E virus is an important public health concern in some developing countries, where it causes faecal-oral transmitted epidemic acute viral hepatitis (5-7). Hepatitis E outbreaks, primarily associated with faecally contaminated drinking water, have been reported in countries such as India, Nepal, Burma, Pakistan, Afghanistan, Borneo, China, Mexico, Egypt, Algeria, Ethiopia, Somalia, Sudan, the Ivory Coast, Botswana and Namibia (5, 7-12).

Sporadic cases of HEV have been reported in some developed countries (5, 6, 13). The majority of these cases were probably imported because they had a travel history to countries where the disease is endemic (5, 6, 13). Rare cases of sporadic hepatitis E have been reported without a history of travel, in which case the transmission of the virus remains uncertain (5, 14-17). It has, however, been established that the genomic

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sequences of the HEV strains detected in these patients were more related to the swine HEV strains prevalent in the swine population of the same area than to the human HEV strains (18). These swine HEV strains may undergo genetic reversion to HEV strains which cause clinical disease in humans similar to that in many parts of the world where hepatitis E is endemic (19, 20).

Although a reservoir of HEV has not yet been established, recent findings have shown that at least some strains of HEV may be zoonotic (21-27). In support of this view, Drobeniuc et al. (2001) established that there is an increase in HEV infection among persons with occupational exposure to swine, which suggests animal-to-human transmission of HEV.

It has been reported that anti-HEV IgG was detected in swine populations from developing countries such as Nepal, China, and Thailand, as well as from developed countries such as the US, Canada, Australia, and Spain (20, 29-33). Swine herds in both HEV endemic and non-endemic countries were found to contain many pigs that are seropositive for HEV, which suggests that the virus may be enzootic in swine regardless of whether HEV is endemic in the human population (32).

In South Africa clinical cases of hepatitis E are rarely seen. Most of those on record are imported cases. However, HEV seems to be endemic in certain areas in the country because seroprevalence studies revealed that up to 15% of individuals in certain developing communities are anti-HEV positive (34). The seroprevalence of anti-HEV IgG in swine has not yet been investigated in South Africa. The objective of this study was to assess the seroprevalence of anti-HEV IgG in selected swine populations in the Gauteng and Limpopo Provinces, which may cast light on the endemic presence of the virus in the country.

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3.3 Materials and Methods

3.3.1 Swine Serum Samples

Swine serum samples were collected from swine of various ages at the Agricultural Research Council in Irene, the experimental farm and Faculty of Veterinary Sciences of the University of Pretoria, and selected abattoirs in the Gauteng and Limpopo Provinces, South Africa. The blood samples (5 ml) were centrifuged (Sorvall® Super T 21) at 1 000 × *g* for 10 min at room temperature (23°C) to obtain clear non-haemolysed specimens and stored at -70°C until the samples were processed.

3.3.2 Detection of Anti-HEV IgG in Swine Serum

Anti-HEV IgG was detected by ELISA, using a mosaic protein, composed of recombinant proteins from immunoreactive epitopes of the HEV open reading frames (ORF) 2 and 3 as the target antigen (35, 36). The ELISA protocol was based on the procedure previously described (36). Immulon 2HB Styrene ELISA wells (Dynatech Laboratories Inc., Chantilly, VA) were adsorbed with 100 µl of phosphate buffered saline (PBS) (0.01 M, pH 7.2-7.4) containing 15 ng of target antigen (HEVAg) (CDC, Atlanta, GA) and incubated overnight at 4°C. Each specimen was diluted 1:10 in first specimen diluent (FSD) (CDC) in non-coated wells (Evergreen Scientific Inc., Los Angeles, CA) and incubated overnight at 4°C. Each well of the HEVAg-coated Immulon 2HB plate was washed 5 times with 200 µl 1× wash solution (CDC). Ninety microlitres of second specimen diluent was dispensed to each well of the HEVAg-coated plate, followed by the addition of 10 µl of negative control (NC), positive control (PC) and specimens, pre-diluted 1:10 in FSD, to the appropriate wells according to the ELISA protocol to attain a final dilution factor of 1:100. The plate was sealed and incubated for 45 min at 37°C. During the incubation period the rabbit anti-swine horseradish peroxidase IgG conjugate was diluted 1:3000 in conjugate diluent. After the incubation each well was washed 5 times with 1× wash solution to remove any unbound antibody, followed by the addition of 100 µl of conjugate working solution to each well. The plate was sealed and incubated for 45 min at 37°C. Each well was washed 6 times with 1× wash solution, followed by

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the addition of 100 µl of substrate solution (*o*-phenylenediamine dihydrochloride) (CDC). The plate was incubated avoiding strong light for 10 min at room temperature (23°C) for colour development. The enzyme reaction was stopped by the addition of 50 µl of 1 N sulphuric acid. The optical density (OD) of the substrate solution in each well was read using an enzyme immuno assay reader (Titertek® Multiskan Plus MkII) at an absorbency of 492 nm with a reference filter of 630 nm.

The standard NC was tested four times and the standard PC was tested once for each assay run. The OD value of the PC had to be ≥0.600, the OD value of individual NCs had to be ≤0.120, and the OD value of the substrate blank had to be ≤0.040 for the assay results to be considered valid. The cut-off (CO) value was calculated as the mean NC OD + 0.300. Serum specimens with an OD value below the CO value were considered non-reactive, while serum specimens with an OD value equal to or greater than the CO value were considered reactive. Reactive specimens were re-tested in duplicate to be considered positive for anti-HEV IgG.

3.3.3 Statistical Analysis

Each pig was classified as seropositive or seronegative after the ELISA based on the criteria described above. The exact 95% confidence intervals (CI) were calculated as appropriate, using the Smith's Statistical Package version 2.5 (2001).

3.4 Results

Swine serum specimens were randomly obtained from 192 pigs from different regions in the Gauteng and Limpopo Provinces, South Africa. The overall prevalence of anti-HEV IgG was 15.10% (95% CI, 14.39-15.81%) in the serum specimens tested (Table 3.1). The prevalence of anti-HEV IgG in swine from the Gauteng and Limpopo Provinces was 14.29% (95% CI, 13.25-15.33%) and 15.84% (95% CI, 14.85-16.83%), respectively (Table 3.1). In addition, swine serum specimens were obtained from 132 pigs from one

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farm in the Warmbaths District, Limpopo Province. The prevalence of anti-HEV IgG in these serum specimens was 28.03% (95% CI, 27.17-28.89%) (Table 3.1).

3.5 Discussion

In this investigation the prevalence of anti-HEV IgG in swine from the Gauteng and Limpopo Provinces was in the same order (15%) as that reported in HEV non-endemic countries such as Spain (25%), the US (18.3%), Canada (18.1%), and Korea (15%), and significantly lower than that reported in HEV endemic countries such as India (54.6-74.4%), Nepal (32.7%), Thailand (30.7%), and China (26.8%) (22, 29, 32, 33, 37, 38). Anti-HEV IgG was detected in swine from other provinces in South Africa (results not shown), such as Mpumalanga, North West, Kwa-Zulu Natal and Western Cape, which suggests that HEV may be prevalent in the swine population throughout the country. A significant difference in anti-HEV IgG prevalence could be demonstrated between swine from the Gauteng and Limpopo Provinces (14.29% vs 15.84%, P = 0.0332). This difference may be attributed to different densities and geographical conditions of the pig farms in these two provinces. The prevalence of anti-HEV IgG in swine that were in close contact with each other was found to be significantly higher than in swine from different geographical regions (28.03% vs 15.10%, P<0.0001), which suggests that the transmission of swine HEV may be associated with close contact among swine within a particular herd.

The data on anti-HEV in swine reported here indicated that swine may serve as a reservoir for certain strains of HEV in South Africa, as has been suggested for other parts of the world, such as Canada and Korea (32). In terms of observations elsewhere, it would appear that these HEV strains, or mutants of these strains, might cause sub-clinical infections in humans, eliciting an anti-HEV response (19, 28, 32, 39). This could explain the detection of HEV antibodies in seroprevalence studies. Although HEV seems to be endemic in certain areas of South Africa, detailed genetic characterisation and epidemiological analysis of HEV strains, circulating among humans and swine in the

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country, may cast light on the absence of clinical cases of hepatitis E. The relatively high prevalence (17.4%) of anti-HEV in communities living under conditions of poor hygiene and lack of sanitation (34) suggested that water may play a role in the transmission of HEV strains among humans, or from swine to humans (12).

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Table 3.1 Prevalence of anti-HEV IgG in swine from the Gauteng and Limpopo Provinces of South Africa

Sample Origin	No*	% Pos†	95% CI‡
Limpopo Province	101	15.84	(14.85-16.83%)
Gauteng Province	91	14.29	(13.25-15.33%)
TOTAL	**192**	**15.10**	**(14.39-15.81%)**
Warmbaths, Limpopo Province§	132	28.03	(27.17-28.89%)

*Number of serum specimens tested

†Percentage seropositive

‡95% Confidence interval

§Serum specimens of swine from one farm in the Warmbaths District, Limpopo Province

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CHAPTER 4

The Detection of Hepatitis E Virus in Domestic Sewage and Swine Fecal Specimens in the Gauteng Province of South Africa

The editorial style of *Emerging Infectious Diseases* was followed in this chapter

4.1 Abstract

Hepatitis E virus (HEV) has emerged worldwide as a cause of epidemic and acute sporadic hepatitis. The majority of HEV epidemics have occurred in developing countries where HEV is endemic, however, the disease has been reported in non-endemic countries, in which case the majority of cases was associated with a travel history. No information is available on the prevalence of HEV in the swine and human populations of South Africa. The purpose of this study was to investigate if HEV is present in sewage and to determine whether HEV occurs in swine in South Africa. Reverse transcription-polymerase chain reaction (RT-PCR) was used to detect HEV RNA in sewage samples and swine fecal specimens. The HEV strains identified in the sewage samples clustered within genotype I, while the swine-related HEV strains were more divergent, showing similarities to genotypes III and IV, respectively. The findings of this study suggested that swine might be involved in the transmission of HEV in South Africa.

Key words: Hepatitis E virus, RT-PCR, sewage, South Africa, swine

4.2 Introduction

HEV is an important cause of epidemic and acute sporadic hepatitis, which is usually associated with fecally contaminated drinking water (1-3). Foodborne transmission of HEV has been reported (4-6). Many waterborne outbreaks of hepatitis E have been reported in areas of Asia, Africa and the Middle East (7). Several cases of acute sporadic

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hepatitis E, mainly associated with traveling to endemic areas, have been reported in non-endemic countries such as the USA (1).

HEV is a non-enveloped RNA virus, which is currently recognized as the type species of the genus *Hepatitis E-like viruses* (8). Phylogenetic analysis of the sequences of various HEV strains indicates that these strains fall into several clusters (9). The largest cluster includes the Asian and African strains, the second cluster contains a variant identified in Mexico, while the third cluster includes human and swine strains isolated in the USA (3). Atypical Chinese strains along with partial sequences from two Taiwanese variants have been included in a new group (10, 11). New sub-groups, which are sufficiently diverse, are represented by individual isolates from Argentina, Greece, and Italy (9, 12). Strains of HEV isolated in non-endemic countries (Italy, Greece, Spain, and UK) form a group of isolates, which are genetically divergent compared to strains from endemic countries (13). Large outbreaks among humans are only associated with the African/Asian and Mexico groups (3).

Contaminated water is considered to be the most important vehicle for the transmission of HEV and sewage acts as a reservoir for this virus (14). The presence of HEV in sewage reflects both clinical and sub-clinical infections prevalent in the human and animal populations (14). Contamination of drinking water with sewage can lead to infections among those consuming the contaminated water in sufficient quantity (15). Sewage contaminated drinking water has been the common feature preceding large outbreaks of HEV (7).

Molecular methods have proved to be effective in detecting HEV in environmental samples without the use of cell culture (3, 16). It is likely that non-intact HEVs are rapidly degraded in environmental samples due to the sensitivity of the genomic RNA, and therefore, RT-PCR could indicate the presence of either infectious, or recently inactivated virus (3, 17).

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In this study HEV infection was investigated in the Gauteng Province of South Africa, where HEV is considered to be non-endemic. This was done by detecting the virus in the sewage of various geographical areas within the Gauteng Province. Swine fecal specimens were also analyzed for the presence of viral RNA to determine whether HEV infection occurs in pigs in South Africa.

4.3 Material and Methods

4.3.1 Domestic Sewage Samples

From May to October, 2002, 199 sewage samples (100 mL each) were collected from the East Rand Water Board once a week in sterile containers. The sewage samples were from different geographical regions in the Gauteng Province, South Africa. The samples were stored at 4°C for <8 h until the samples were processed.

4.3.2 Swine Fecal Specimens

From May to August, 2003, 199 swine fecal specimens were collected from pigs of various ages at different abattoirs in the Gauteng Province, South Africa. These swine fecal specimens were from pigs from different geographical regions. A 10% fecal suspension in phosphate buffered saline (0.01 M, pH 7.2-7.4) (Sigma, St. Louis) was prepared for each swine fecal specimen. All fecal suspensions were stored at 4°C for molecular analysis.

4.3.3 Concentration of Viral Particles from Domestic Sewage Samples

The polyethylene glycol (PEG) hydroextraction method was used for the recovery of viral particles from the domestic sewage samples as described previously (18). Briefly, 0.17 g of sodium chloride (Merck, Darmstadt, Germany) and 14 g of polyethylene glycol 6000 (Merck) were dissolved in each 100 mL sewage sample and kept at 4°C for 8 h. The solution was centrifuged (Sorvall® Super T 21) in 50 mL centrifuge tubes (Corning Inc., Corning, NY) at 5,000 × *g* for 30 min at 4°C to pellet all viral particles. The resulting pellet was resuspended in 10 mL phosphate buffered saline followed by sonication (10

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amplitude microns) (Soniprep 150) and centrifuged (Sorvall® Super T 21) at 1,200 × *g* for 10 min at 4°C. The supernatant, containing the viral particles, was stored at -70°C for molecular analysis.

4.3.4 Nucleic Acid Extraction from Domestic Sewage and Swine Fecal Specimens

Aliquots (100 µl) of swine fecal suspensions were pretreated with an equal volume of 1, 1, 2-trichloro-trifluoroethane (Sigma) prior to extraction of total RNA. One milliliter of each concentrated viral suspension obtained from the sewage samples was centrifuged (Sorvall® Super T 21) at 19,000 × *g* for 3 h at 4°C in a 1.5 mL microcentrifuge tube. The resulting pellet was resuspended in 140 µL of nuclease-free water (Promega Co., Madison, WI). Total RNA was extracted from each domestic sewage sample and fecal suspension with a TRIzol reagent (Invitrogen Life Technologies, Paisley, Scotland) according to the manufacturer's instructions. Briefly, 140 µL of each viral suspension was added to 500 µL of TRIzol reagent and incubated for 5 min at room temperature (23°C), followed by the addition of 100 µL of chloroform (Merck). Each sample was gently mixed by inverting the microcentrifuge tube and incubating at room temperature (23°C). The samples were centrifuged (Sorvall® Super T 21) at 11,600 × *g* for 15 min at 4°C, after which 300 µL of the upper phase was transferred to sterile 1.5 mL microcentrifuge tubes containing 600 µL of absolute ethanol (Merck) and 30 µL of 3 M sodium acetate (pH 5.2). The mixture was incubated at -20°C for 8 h and centrifuged (Sorvall® Super T 21) at 11,600 × *g* for 15 min at 4°C. The supernatant was discarded and the pellets washed by adding 300 µL of 70% ethanol (Merck), followed by centrifugation (Sorvall® Super T 21) at 11,600 × *g* for 5 min at 4°C. The pellets were air-dried for 10 min after which the total RNA was dissolved in 30 µL of nuclease-free water and stored at -70°C. Nuclease-free water was included as a negative control for each extraction procedure.

4.3.5 Molecular Detection of HEV in Domestic Sewage Samples and Swine Fecal Specimens

A two-step RT-PCR was used for the rapid amplification of HEV RNA isolated from domestic sewage and swine fecal specimens as previously described (16). Promega Co.

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manufactured all the reagents used in the RT-PCR, unless otherwise stated. The reverse transcription step was performed for 60 min at 42°C (Px2 Thermal Cycler) in a reaction volume of 40 µL containing 62.5 mM KCl, 12.5 mM Tris-HCl (pH 9.0), 0.125% Triton X-100, 3.125 mM $MgCl_2$, 0.5 mM PCR nucleotide mix, 10 pmol primer HEVORF2con-a1 (5'-CTTGTTCRTGYTGGTTRTCATAATC-3') (Sigma-Genosys, NSW, Australia) and 9 units of avian myeloblastosis virus reverse transcriptase. After the reverse transcription step, 10 µL containing 150 mM KCl, 30 mM Tris-HCl (pH 9.0), 0.3% Triton X-100, 5mM $MgCl_2$, 10 pmol primer HEVORF2con-s1 (5'-GACAGAATTRATTTCGTCGGCTGG-3') (Sigma-Genosys) and 2.5 units of Taq polymerase was added to the cDNA. After 35 cycles (20 s at 94°C, 30 s at 55°C, and 30 s at 72°C) and additional incubation for 10 min at 72°C (Px2 Thermal Cycler), 20 µL of the PCR mixture was analysed by agarose gel electrophoresis (2%) (Seakem® LE agarose, Cambrex Bio Science Rockland Inc., Rockland, ME). The ethidium bromide-stained bands were visualized on an UV transilluminator (UVP ImageStore 5000).

4.3.6 Sequencing and Phylogenetic Analysis of RT-PCR Amplicons

The amplicons obtained after RT-PCR were purified with Exonuclease I/Shrimp Alkaline Phosphatase (Fermentas, Lithuania). Both strands of the purified DNA amplicons were sequenced using a Spectrumedix SCE2410 genetic analysis system with the ABI BigDye Terminator cycle sequencing kit version 3.1 (Applied Biosystems, Foster City, CA) as specified by the manufacturer. Polymerase chain reaction amplicons of the appropriate size, which could not directly be sequenced, were cloned with the pGEM-T Easy Vector System (Promega Co.) as described by the manufacturer (Appendix A). Nucleotide sequences were compiled using the program CHROMAS version 1.45 (Griffith University, Queensland, Australia). The sequences reported in this study were compared to HEV sequences present in the GenBank database by using the BLAST program of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Nucleotide sequences were entered into a database in PC/GENE version 6.80 (IntelliGenetics Inc., Geneva, Switzerland). CLUSTALX version 1.8 (ftp://ftp-igbmc.u-stras-bg.fr/pub/ClustalX/) was used for the alignment of the nucleic acid sequences of

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reported isolates and the reference strains (19). Phylogenetic trees were generated using the CLUSTALX version 1.8 program. The robustness of the trees was determined by bootstrap resampling of the multiple-sequence alignments (1,000 sets) with the CLUSTALX version 1.8 program. The graphical output of the phylogenetic trees was created with the TREEVIEW version 1.6.6 program (University of Glasgow, Glasgow, Scotland; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

4.3.7 Nucleotide Sequence Accession Numbers

The sequences reported in this article have been deposited in the GenBank database and assigned the following accession numbers: RSA-1 (AY621662), RSA-2 (AY621663), swRSA-1 (AY621664), and swRSA-2 (AY621665). Accession numbers of the sequences used for analysis in this study are shown in Table 1.

4.4 Results and Discussion

This study documents the prevalence and genetic characteristics of HEV in domestic sewage and swine fecal specimens from the Gauteng Province, South Africa. To determine the prevalence of HEV in the domestic sewage and swine feces, RT-PCR methods were used with primers located in the HEV open reading frame (ORF) 2 region of the viral genome, followed by sequencing analysis of the generated PCR amplicons.

One-hundred-and-ninety-nine domestic sewage samples from 13 different geographical regions and 199 swine fecal specimens from four different geographical regions in the Gauteng Province were collected. The overall prevalence of HEV in these domestic sewage and swine fecal specimens, as indicated by RT-PCR, was estimated to be 4.52% (95% CI 3.82 to 5.22) and 5.02% (95% CI 4.32 to 5.72) respectively. This may be an overestimate of the true prevalence of the virus in these samples, as only 2 (22.2%) of the 9 positive sewage samples and 2 (20%) of the 10 positive swine fecal specimens could be confirmed by sequencing analysis. The PCR amplicons that were sequenced after cloning, contained non-specific nucleotide sequences unrelated to HEV, and therefore, a

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more accurate prevalence of HEV, as confirmed by sequencing analysis, would be 1.01% (95% CI 0.31 to 1.71) for both the domestic sewage and the swine fecal specimens analyzed during this study. Comparison of the 197 bp nucleotide sequences generated by RT-PCR revealed two HEV strains (RSA-1 and RSA-2) in the domestic sewage samples and two swine-related strains (swRSA-1 and swRSA-2) in the swine fecal specimens.

Investigating the viruses present in the sewage of a population may provide meaningful information concerning the viral strains infecting that population (14, 20). The RSA-1 and RSA-2 strains clustered together (Figure 1) with nucleic acid identity of 95.4%. These strains may be related to genotype I, as defined by Schlauder and Mushahwar (13), with nucleic acid identity of 86.3% to 97.5% when compared to other genotype I strains from Burma, China, India, Nepal, Pakistan, and Spain (Figure 1). The RSA-1 and RSA-2 strains are most likely from human origin, as genotype I HEV strains are usually associated with human HEV infection. This suggests that HEV infection does occur in the human population in South Africa, most likely in a sub-clinical form, as only a few imported cases of clinical hepatitis E have been reported to date in South Africa (21).

This is the first report with direct evidence that HEV infection occurs in swine in South Africa. The swRSA-1 and swRSA-2 strains shared a nucleotide identity of 78.7%, and had a nucleotide identity of 78.7% to 85.3% when compared to the RSA-1 and RSA-2 strains. The first of the swine-related strains (swRSA-1) was found to be related to genotype III (Figure 1), showing a nucleotide identity of 86.8% to 92.4% with other genotype III strains from the USA and Japan. The swRSA-2 strain shared a nucleotide identity of 89.8% with other genotype IV strains from Japan.

The majority of the nucleotide substitutions in the strains detected during this study was located in the third codon position, and therefore, did not result in differences at the amino acid level. All strains did, however, show non-conservative substitutions in different positions, which led to differences in the amino acid sequences of these strains. Less distinction could be made between groups based on amino acid than nucleic acid

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identities from the ORF2 fragment, due to the short region (197 bp) used for analysis and the higher level of conservation observed within this region (22). Larger fragments of these HEV strains should be sequenced in order to confirm the results.

Sequence comparisons, genetic distances, and phylogenetic analysis of the 197 bp products revealed that all strains described during this study clustered with previously described Asian, European, American human or swine HEV isolates. Although geographic clustering of swine and human HEV has been suggested in Europe, America, and Asia, this could not be demonstrated during this study. The genotype I strains detected in the sewage did, however, cluster mostly with previously described Asian HEV strains, while the swine strains (genotypes III and IV) described here did not show any geographical clustering. The diversity observed in these novel South African strains showed that a number of diverse strains are simultaneously circulating in the human and swine populations of South Africa.

Swine HEV strains may undergo genetic reversion to strains which are able to cause disease in humans (10). The discovery of swine HEV strains in South Africa similar to human HEV strains, therefore, raises the issue of zoonotic transmission of HEV from swine to humans. These swine strains circulating in South Africa may have caused sub-clinical infections in the human population, eliciting an anti-HEV response, which may explain the prevalence of anti-HEV in South Africa (23, 24), despite the absence of clinical disease.

Additional studies on the prevalence of anti-HEV and HEV-related sequences in the human and swine populations, as well as other animal species, may cast more light on the potential impact they may have on the epidemiology of HEV in South Africa.

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Table 4.1 Nucleotide sequence accession numbers for hepatitis E virus reference strains used for the phylogenetic analysis

Virus Strain	Origin	GenBank Accession No	Reference
BCN	Barcelona, Spain	AF058684	25
US-1	USA	AF060668	22
US-2	USA	AF060669	22
Porcine	USA	AF082843	26
Mexico	Telixtac, Mexico	M74506	27
HE-JA1	Japan	AB097812	28
swJ13-1	Japan	AB097811	28
swJapan	Japan	AB073912	29
India-1	India	X98292	30
India-2	North India	AF459438	31
India-3	Madras, India	X99441	Unpublished
Morocco	Morocco	AY230202	Unpublished
Burma-1	Burma	M73218	32
Burma-2	Burma	D10330	33
Nepal	Nepal	AF051830	34
Pakistan	Sarghoda, Pakistan	M80581	35
China-1	Kashi, China	L25595	36
China-2	Xinjiang, China	L08816	37
China-3	Uigh, China	D11093	Unpublished
93-Egypt	Egypt	AF051351	38
94-Egypt	Egypt	AF051352	38
81-Chad	Chad	U62654	Unpublished
83-Chad	Chad	AY204877	39

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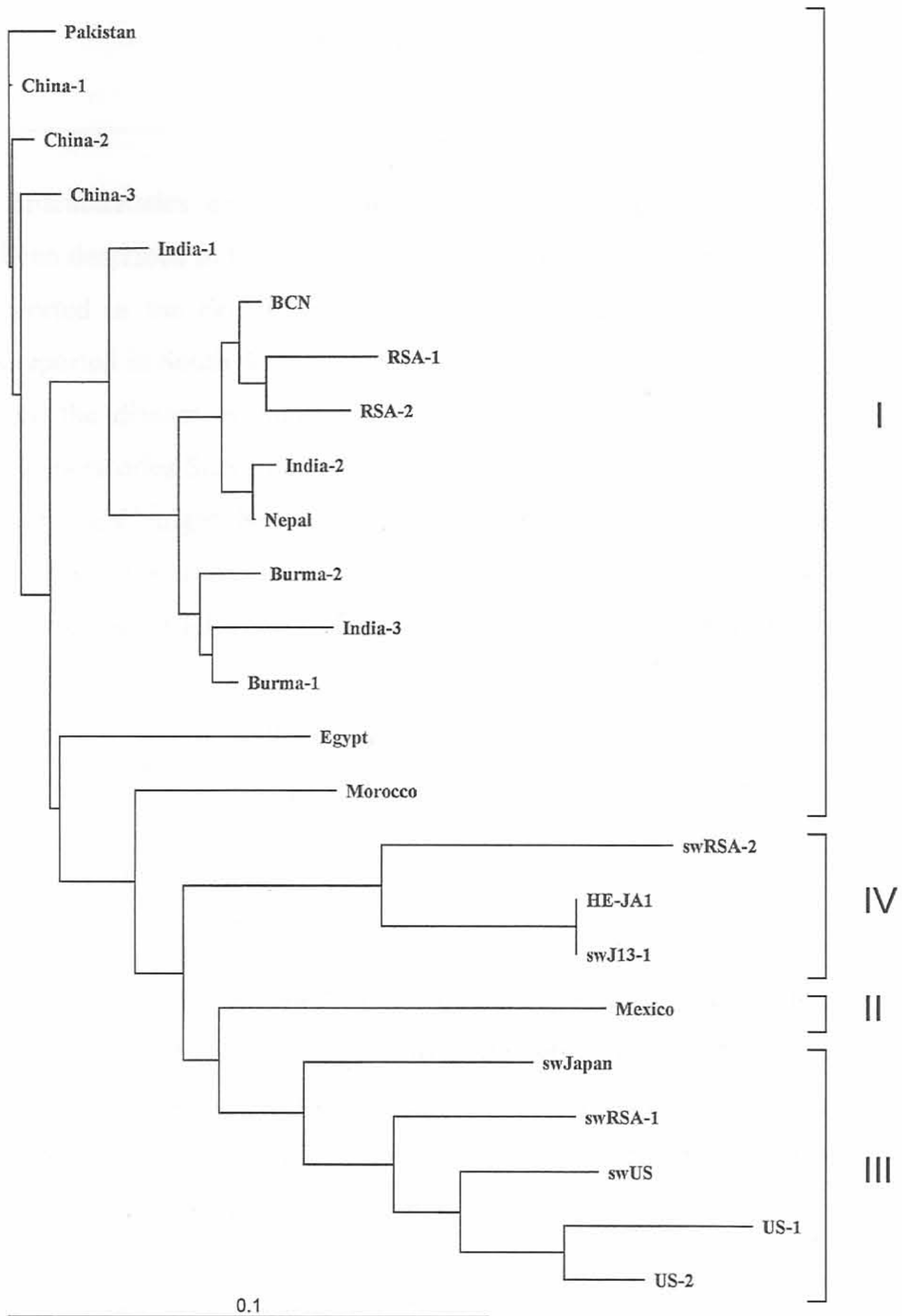

Figure 4.1 **Phylogenetic tree of the ORF2 region for the HEV reference and South African strains**

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CHAPTER 5

Conclusions

The general characteristics and public health risks associated with hepatitis E virus (HEV) have been described in the literature review. Waterborne outbreaks of hepatitis E have been reported in the neighbouring countries Namibia and Botswana, while the clinical cases reported in South Africa were mainly associated with a travelling history to countries where the disease is endemic (Grabow *et al.*, 1994; Grabow, 1997; South African Virus Laboratories Surveillance Bulletin, 2003). Indirect evidence suggested that some strains of HEV might be zoonotic, with swine playing a major role in the transmission of the virus to humans. This suggestion was validated by the reports of foodborne transmission of HEV (Matsuda *et al.*, 2003; Tei *et al.*, 2003; ProMed, 2004). Waterborne and foodborne transmission of HEV have not been recorded in South Africa, and the detection and characterisation of the virus in water resources and animals have not been described in this country. Final conclusions and future research have been summarised in this chapter.

Seroprevalence studies previously conducted in various communities in South Africa have indicated that HEV may be endemic in the human population of certain low socio-economic communities throughout South Africa (Grabow *et al.*, 1994; Tucker *et al.*, 1996). It is believed that water may play a role in the transmission of HEV strains among humans, or from swine to humans, in these communities, due to conditions of low hygiene and sanitation (Grabow, 2002).

The seroprevalence in swine reported in this study is the first documentation of the presence of anti-HEV IgG in animals in South Africa. It was found that the seroprevalence of HEV in swine in South Africa (15%) compared well with the seroprevalence reported in non-endemic countries (15% to 25%), which was significantly lower than that reported in endemic countries (27% to 74%) (Clayson *et al.*, 1995; Meng

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et al., 1999; Pina *et al.*, 2000; Arankalle *et al.*, 2001; Engle *et al.*, 2002; Choi *et al.*, 2003). Anti-HEV IgG was detected in provinces throughout South Africa, suggesting that swine might be a candidate reservoir for at least some strains of HEV. The differences in the seroprevalence of HEV among the provinces may be due to certain factors, such as different geographical conditions, densities of pig farms in each province and different HEV strains occurring in the provinces.

The prevalence of HEV in the domestic sewage samples and swine faecal specimens, as determined by reverse transcription-polymerase chain reaction (RT-PCR), may have been an over-estimate, as indicated by the confirmation of these results by sequencing analysis. Thus, in order to reflect the true prevalence of HEV in environmental and clinical samples, using this method, sequencing analysis must be done to confirm the results. Sequencing analysis of the RT-PCR amplicons revealed two human-related HEV strains (RSA-1 and RSA-2) detected in the sewage samples and two swine-related HEV strains (swRSA-1 and swRSA-2) detected in the swine faecal specimens. The strains detected in the sewage clustered together within genotype I (human-related Asian strains), while the swine associated strains were more diverse, with swRSA-1 and swRSA-2 related to genotypes III (human- and swine-related US strains) and IV (human- and swine-related Japanese strains) respectively. This is an indication that a number of diverse strains are circulating simultaneously in the human and swine populations in South Africa. Detecting swine-related HEV sequences, similar to that of human HEV sequences, in South Africa raises concern, as these strains may undergo genetic reversion to strains that might possibly cause disease in the human population. The seroprevalence of HEV detected in the human population in South Africa may have been due to swine-related strains being transmitted via water sources causing sub-clinical or low-grade infections in the human population.

It has been suggested that there might be more local cases of clinical hepatitis E in South Africa than previously thought (Grabow *et al.*, 1994; Grabow, 1997). It would, therefore, appear that cases of locally acquired hepatitis E may be occurring undiagnosed in South Africa. Routine testing for HEV infection should be considered in cases of non-A, non-B

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acute hepatitis, even in the absence of a travelling history, in particular in asymptomatic patients with unexplained elevated aminotransferase levels and in pregnant women (Clemente-Casares *et al.*, 2003; Widdowson *et al.*, 2003).

Currently several RT-PCR assays have been developed for the rapid detection of HEV in either clinical or environmental samples. The disadvantage of these assays are, however, that they 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). This is due to the genetic diversity, which exists between the HEV strains, in particular between strains from different geographical regions. Therefore, a single molecular assay (multiplex PCR) must be developed that will simultaneously identify as many HEV strains as possible.

The results reported in this study extends the knowledge of the global distribution of HEV and it is evident that the disease is more widespread than traditionally thought. The seroprevalence of HEV and the prevalence of HEV-related sequences in human populations, as well as swine and other animal species throughout South Africa, will cast more light on the epidemiology of HEV in this country. The identification of additional human- and swine-related HEV isolates and the determination of their full-length genomic sequences will provide fundamental information in order to better understand the genetic divergence of the HEV strains circulating in South Africa.

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APPENDIX A

A1. Cloning of PCR products using the pGEM®-T Easy Vector System (Promega Co., Madison, WI)

A1.1 Preparation of culture media and reagents used during the cloning procedures

A1.1.1 Luria-Bertani (LB) Medium (per Litre)

Bacto-tryptone (Difco, Detroit, MI)	10 g
Bacto-yeast extract (Difco)	5 g
NaCl (Merck, Darmstadt, Germany)	10 g
Deionised water	1 000 ml

Adjust the pH to 7.5 with NaOH (1 M) (Merck).

Sterilise by autoclaving for 20 min at 121°C.

A1.1.2 LB Solid Agar(per Litre)

Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	10 g
Bacto agar (Difco)	15 g
Deionised water	1 000 ml

Adjust the pH to 7.5 with NaOH (1 M).

Sterilise by autoclaving for 20 min at 121°C.

A1.1.3 Preparation of LB/ampicillin/IPTG/X-Gal plates

Prepare LB solid agar as in A1.1.2. After the media has cooled to 50°C ampicillin (Sigma Chemical Co., St Louis, MO) was added to a final concentration of 100 μg/ml. The medium was supplemented with 0.5 mM isopropylthio-β-D-galactoside (IPTG) (Promega) and 80 μg/ml 5-bromo-4-chloro-3-indolyl- β-D-galactoside (X-Gal) (Promega). Thirty millilitres of LB/ampicillin/IPTG/X-Gal

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medium was poured into 90 mm petri dishes (Concorde Plastics, Johannesburg, South Africa) and allowed to solidify. The plates were stored at 4°C.

A1.1.4 Mg^{2+} (2 M) stock

$MgCl_2.6H_2O$ (Sigma)	20.33 g
$MgSO_4.7H_2O$ (Merck)	24.65 g

Add deionised water to 100 ml and filter-sterilise.

A1.1.5 SOC Media (per 100 ml)

Bacto-tryptone	2 g
Bacto-yeast extract	0.5 g
NaCl (1 M)	1 ml
KCl (1 M) (Merck)	0.25 ml
Deionised water	97 ml

Sterilise by autoclaving for 20 min at 121°C and cool to room temperature (23°C). Add Mg^{2+} (2 M) filter-sterilised stock (1.1.4) and filter-sterilised glucose (2 M) (Sigma) solution, both to a final concentration of 20 mM. The final pH should be 7.0.

A1.2 Ligation using 2× Rapid Ligation Buffer

The pGEM®-T Easy Vector and control insert DNA were briefly centrifuged to collect the contents at the bottom of the tubes. The 2× Rapid Ligation Buffer was vortexed before each use and 0.5 ml microcentrifuge tubes (Eppendorf-Netheler-Hinz, Hamburg, Germany), known to have low DNA-binding capacity, were used for the ligation reactions. The preparation of the ligation reactions are summarised in Table A.1. The reactions were mixed by pipetting, followed by incubation for 1 h at room temperature (23°C). Alternatively, the reactions could be incubated overnight at 4°C to obtain the maximum number of transformants.

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Table A.1 Preparation of ligation reactions

Reagents	Standard reaction	Positive Control	Negative Control
2× Rapid Ligation Buffer	5 µl	5 µl	5 µl
pGEM®-T Easy Vector (50 ng)	1 µl	1 µl	1 µl
PCR product	3 µl	-	-
Control insert DNA	-	2 µl	-
T4 DNA Ligase (3 Weiss units/µl)	1 µl	1 µl	1 µl
Deionised water to a final volume of	10 µl	10 µl	10 µl

A1.3 Protocol for transformations using the pGEM®-T Easy Vector ligation reactions

Eschirichia coli JM109 High Efficiency Competent Cells (Promega) were used for the transformations. The tubes containing the ligation reactions were briefly centrifuged to collect the contents at the bottom of the tube. Two microlitres of each ligation reaction was transferred to a sterile 1.5 ml microcentrifuge tube (Eppendorf) on ice. Another tube was set up on ice containing 0.1 ng of uncut plasmid for the determination of the transformation efficiency of the JM109 competent cells. The JM109 competent cells were thawed on ice (approximately 5 min) and gently mixed by flicking the tube. Fifty microlitres of JM109 cells were carefully transferred into each ligation reaction tube (100 µl of cells for the determination of transformation efficiency). The tubes were gently mixed and incubated on ice for 20 min. The cells were heat-shocked for 45-50 s in a heating block at exactly 42°C without shaking and returned to ice for 2 min. SOC medium (950 µl) (A1.1.5) was added to the tubes containing cells transformed with the ligation reactions, while 900 µl of SOC medium (1.1.5) was added to the tube containing cells transformed with uncut plasmid. The tubes were incubated for 1.5 h at 37°C in a shake incubator (150 rpm). Each transformation culture (100 µl) was plated onto duplicate LB/ampicillin/IPTG/X-Gal plates (A1.1.3). A 1:10 dilution was made with SOC medium of the tube containing the cells transformed with uncut plasmid DNA and 100 µl plated onto duplicate LB/ampicillin/IPTG/X-Gal plates (A1.1.3). The plates were incubated for 16-24 h at 37°C. Successful cloning of an insert in the pGEM®-T Easy

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Vector interrupts the coding sequence of β-galactosidase, producing white colonies, which were used for further analysis.

A1.4 Calculation of transformation efficiency

The following formula was used to calculate the transformation efficiency of the JM109 cells:

$$\frac{\text{Average number of white colonies obtained}}{0.01 \quad \text{ng of plasmid DNA}}$$

(Note: If the transformation efficiency is lower than 1×10^8 cfu/μg DNA, fresh cells must be prepared.)

A2 Isolation of recombinant plasmid DNA

A2.1 Preparation of solutions used during the isolation of recombinant plasmid DNA

A2.1.1 7 M Guanidine-HCl Solution

Guanidine-HCl (Promega)	66.9 g
Nuclease-free water (Promega)	50 ml

Dissolve the Guanidine-HCl and adjust the solution to a final volume of 100 ml by adding nuclease-free water.

A2.1.2 4/40 Wash Solution

7 M Guanidine-HCl solution (A2.1.1)	30 ml
100% Isopropanol (Merck)	20 ml

Mix and store at 20-25°C.

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A2.1.3 80% ethanol wash solution

100% Ethanol (Merck)	160 ml
Deionised water	40 ml

Store at 20-25°C.

A2.2 Purification of plasmid DNA using the Wizard® PureFection Plasmid DNA Purification System (Promega)

Each white colony obtained after cloning was incubated overnight at 37°C in 10 ml of LB medium (A1.1.1) containing ampicillin to a final concentration of 100 µg/ml. The bacterial cultures were centrifuged at 10 000 × *g* (Sorvall Super T21) for 5 min at room temperature (23°C). The supernatant was discarded and the pellets resuspended in 1.25 ml of Cell Resuspension Solution. The resuspended cells were transferred to sterile 15 ml centrifuge tubes (Corning Inc., Corning, NY). Cell Lysis Solution (1.25 ml) was added to the tubes and mixed by inverting the tubes 6-8 times to ensure cell lysis, followed by incubation for 5 min at room temperature (23°C). Neutralisation Solution (1.75 ml) was added and the tubes mixed immediately by inverting the tubes 6-8 times. The bacterial lysate was centrifuged at 10 000 × *g* (Sorvall Super T21) for 20 min at room temperature (23°C). This centrifugation step was repeated and the supernatant transferred to new 15 ml centrifuge tubes. Endotoxin Removal Resin (0.25 ml) was added to each tube and mixed vigorously. The tubes were incubated for 10 min at room temperature (23°C), shaking the tubes several times during the incubation period. The tubes were placed onto the MagneSil™ Magnetic Separation Unit (Promega) and the solution was allowed to clear for 30 s. The tubes were kept on the magnet while the supernatant was transferred to a new 15 ml centrifuge tube, after which the resin was discarded. Guanidine Thiocyanate (5 M) (1 ml) was added to the supernatant-containing tubes and mixed vigorously. The MagneSil™ Paramagnetic Particles (0.75 ml) were added to each tube and mixed vigorously. The tubes were incubated for 3 min at room temperature (23°C). The tubes were placed onto the magnetic unit and the solution allowed to clear for 30 s. The supernatant was discarded and the particles washed by adding 1 ml of the 4/40 wash solution (A2.1.2). The particles were resuspended by

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vortexing for 10 s. The tubes were replaced onto the magnetic unit and the solution was allowed to clear for 30 s. The supernatant was discarded and the particles washed with 2.5 ml of the 80% ethanol wash solution (A2.1.3). The particles were resuspended by vortexing for 10 s. The tubes were replaced onto the magnetic unit and the solution was allowed to clear for 30 s. The supernatant was discarded. This washing step was repeated 3 times, and after the third wash, the tubes were left open. The particles were allowed to dry while the tubes remained on the magnetic unit. The tubes were removed from the magnetic unit and 1.2 ml of nuclease-free water was added to each tube. The particles were resuspended by vortexing for 10 s, followed by an incubation period of 1 min at room temperature (23°C). The tubes were replaced onto the magnetic unit and the solution allowed to clear for 3 minute to capture all particles. The supernatant was transferred to new 15 ml centrifuge tubes.

A2.3 Precipitation of the eluted DNA

The volume of the above-mentioned supernatant was determined and 0.5 volumes of ammonium acetate (7.5 M) added per volume of supernatant. Absolute ethanol (2.5 volumes) (Merck) was added to each tube and mixed well. The tubes were centrifuged (Sorvall Super T21) at 14 000 × *g* for 15 min at room temperature (23°C). The supernatant was aspirated using a 9-inch Pasteur pipette (Copan Innovation, Italy). The translucent DNA pellets were rinsed in 2 ml of 70% ethanol (Merck) and centrifuged at 14 000 × *g* (Sorvall Super T21) for 5 min. The ethanol was aspirated and the pellets allowed to air-dry for 3-5 min to allow the residual ethanol to evaporate. The DNA pellets were resuspended in 300 µl of nuclease-free water (Promega).

A2.4 Molecular detection of the HEV-like fragments in the purified plasmids

The HEV-like fragments were detected by polymerase chain reaction (PCR) amplification based on the procedure described by Erker *et al.*, 1999. Promega manufactured all the reagents used in the PCR, unless otherwise stated. The PCR was performed in a reaction volume of 49 µl containing 76.5 mM KCl, 15.3 mM Tris-HCl

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(pH 9.0), 0.15% Triton X-100, 3.8 mM $MgCl_2$, 0.6 mM PCR nucleotide mix, 10 pmol of primer HEVORF2con-a1 (5'-CTTGTTCRTGYTGGTTRTCATAATC-3') (Sigma-Aldrich Co., NSW, Australia), 10 pmol of primer HEVORF2con-s1 (5'-GACAGAATTRATTTCGTCGGCTGG-3') (Sigma) and 2.5 units of Taq polymerase. After 35 cycles (20 s at 94°C, 30 s at 55°C and 30 s at 72°C) and an additional incubation for 10 min at 72°C (Px2 Thermal Cycler), 20 µl of the PCR mixture was analysed by agarose gel electrophoresis (2%) (Seakem LE agarose, Bioproducts, USA). The ethidium bromide-stained bands were visualised on an UV Transilluminator (UVP ImageStore 5000). The amplicons obtained were sequenced using a Spectrumedix SCE2410 genetic analysis system with the ABI BigDye Terminator cycle sequencing kit version 3.1 (Applied Biosystems) as specified by the manufacturer.

A2.5 References

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APPENDIX B

The nucleotide sequences within the ORF2 region of the South African HEV strains detected during this study are shown below:

RSA - 1 (GenBank Accession No: AY621662)

```
1     gacagaattg atttcgtcgg ctggcgatca gctgttctac tctcgtcccg
51    tcgtctcagc caatggcgag ctgactgtta agctgtatac atctgtagag
101   aatgctcagc aggataaggg tatcgcaatc ccgcatgata ttgacctcgg
151   agagtctcgt gtggttattc aggattatga caaccaacac gaacaag
```

RSA - 2 (GenBank Accession No: AY621663)

```
1     gacagaaaag atttcgtcgg ctggtggcca gctgttctac tcccgtcccg
51    tcgtctcagc caatggcgag ccgacggtta agctgtatac atctgtagag
101   aatgctcagc aggataaggg tatcgcaatc ccgcatgata ttgacctcgg
151   agagtctcgt gtggttattc aggattatga caaccaacac gaacaag
```

swRSA - 1 (GenBank Accession No: AY621664)

```
1     gacagaattg atttcgtcgg ctgggggcca gttgttctac tcccgcccag
51    tagtctcagc caatggcgag ccgactgtca agttatatac atctgttgag
101   aatgcgcagc aggacaaggg gattgccatc ccacacgata tagatctggg
151   tgattcccgt gtggtcatcc aggattatga caaccagcac gaacaag
```

swRSA - 2 (GenBank Accession No: AY621665)

```
1     gacagaattg atttcgtcgg ctggtggcca gctgttctac tcccgtcccg
51    tcgtctcagc caatggcgag ccgacagtga agcattatac atcagtcgag
101   aatgctcagc aggataaggg tatagctgtt tcacgcaata ccgaccttag
151   tgagtctcga gttgttatct tagattatga taaccagcat gagcagg
```