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CHARACTERIZATION OF HEPATITS E VIRUS IN SOUTHERN AFRICA

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Dissertation submitted in compliance with the requirements for the degree of Master of Science

in the department of Health Science at the University of the Witwatersrand

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ABSTRACT

Endemic circulation of hepatitis E virus (HEV) in Namibia was suspected from serological data during an outbreak of non-A non-B hepatitis in Rundu, in 1995. The source of the outbreak was surmised to be the water supply, which had been compromised approximately six months earlier. This study examines nucleotide and amino acid sequence data obtained from the open reading frame 2 (ORF2) region towards the carboxy terminal end (3'-end) of the HEV genome extracted from the stool of Namibian patients infected during this outbreak. The overall aim is to establish a polymerase chain reaction (PCR) for molecular diagnosis of HEV, to isolate HEV from specimens collected from acute viral hepatitis outbreaks such as this one in Namibia, to characterize, at the genomic level, the strain of the virus involved, to compare the strain to those involved in other outbreaks of HEV in southern Africa and to relate this information to available data from other parts of the world. A nested reverse transcriptase-polymerase chain reaction (RT-PCR) was developed for molecular diagnosis of HEV and four representative HEV isolates from this outbreak have been successfully amplified, sequenced and analysed over a 451 base pair (bp) region of a subgenomic fragment from the 3'-end of the genome in ORF2. Phylogenetic analysis showed the four Namibian HEV isolates clustered with a Mexican isolate in genotype II and shared a 85.8-86.3 percent (%) nucleotide identity with the 1987 Mexican isolate but were only 77.6-79.6 % similar to other African isolates. HEV isolated from the same region of Namibia in 1983 was reported to cluster into genotype I and this analysis clearly indicates the difference between the strains involved in the two outbreaks. Virus from sporadic cases of HEV isolated in 1997/8 in Nigeria were also found to be from

genotype II. This is the first study performed in South Africa to isolate, amplify by PCR and sequence the HEV. It is also the first to characterize HEV as the causative agent of the hepatitis outbreak that occurred in Namibia in 1995. As it reports the presence of a second unique HEV strain in southern Africa, we conclude that HEV genotypes may be more widely distributed than previously thought.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in the Faculty of Health Sciences at the University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other university.

_____

(Hazel T. Maila)

_____ day of _____ ,2004

To my Mom and my Fiancé

Thank you for encouraging and believing in me always especially throughout this study; and to my Dad, you left just when you were about to see the end of this project. R.I.P Daddy

ABBREVIATIONS

A/a	Alanine/adenosine
aa	amino acid
Abb	Abbottabad
Ahm	Ahmedabad
AIDS	acquired immune deficiency syndrome
ALT	alanine aminotransferase
AMV-RT	avian myeloblastosis virus-reverse transcriptase
Anti	antibody
AST	aspartate aminotransaminase
bp	base pair
C	cytidine
CDC	Center for Disease Control and Prevention
cm^3	centimetre cube
CPE	cytopathic effect
CsCl	cesium chloride
cynos	cynomolgus macaques
dATP	deoxy-adenosine triphosphate
dCTP	deoxy-cytidine triphosphate
ddATP	dideoxy-adenosine triphosphate
ddCTP	dideoxy-cytidine triphosphate
ddGTP	dideoxy-guanosine triphosphate
ddTTP	dideoxy-thymidine triphosphate

ABBREVIATIONS

dGTP	deoxy-guanosine triphosphate
DNA	deoxyribonucleic acid
DNR	department of national resources
dNTPs	deoxy ribonucleoside triphosphates
ds	double stranded
dTTP	deoxy-thymidine triphosphate
E	glutamic acid (glutamate)
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetate
EIA	enzyme immunoassay
ELISA	enzyme linked immunosorbent assay
EthBr	ethidium bromide
ET-NANB	enterically transmitted non-A, non-B
FITC	fluorescein isothiocyanate
FTAI	French Territories of Afars and Issas
g	gram/force of gravity
G	Glycine/guanosine
GBS	gelatine buffered saline
GC	guanine-cytosine
HAV	hepatitis A virus
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus

ABBREVIATIONS

HCl	hydrochloric acid
HCV	hepatitis C virus
HDV	hepatitis delta virus
HEV	hepatitis E virus
HEV-S	swine hepatitis E virus
HIV	human immunodeficiency virus
I/i	isoleucine
IDU	injecting drug users
IEM	immune electron microscopy
IgG	immunoglobulin G
IgM	immunoglobulin M
IPTG	isopropylthio-β-d-galactoside
IU/L	international units per litre
kb	kilobase
KCl	potassium chloride
kD	kilodalton
KH_2PO_4	potassium phosphate
Kol	Kolhapur
L/l	Leucine/liter
LB	Luria broth
M	molar
$MgCl_2$	magnesium chloride

ABBREVIATIONS

$MgSO_4$	magnesium sulphate
ml	millilitre
mM	millimolar
MPG	magnetic glass particles
N/n	asparagine
NaCl	sodium chloride
Na_2HPO_4	disodium hydrogen phosphate
NANB	non-A, non-B
NANBNC	non-A, non-B, non-C
NaOH	sodium hydroxide
NAT	nucleic acid testing
NC	non coding
ng	nanogram
NICD	National Institute for Communicable Diseases
NIV	National Institute for Virology
nm	nanometre
nM	nanomolar
nt	nucleotide
OD/CO	ratio of absorbency to cut-off
ORF	open reading frame
P/p	proline
PBS	phosphate buffered saline

ABBREVIATIONS

PCR	polymerase chain reaction
pmol	picomolar
Q	glutamine
RdRp	RNA-dependent RNA polymerase
REs	restriction endonucleases
RNA	ribonucleic acid
RNAsin	RNase inhibitor
rpm	revolutions per minute
RT	reverse transcriptase/transcription
RT-PCR	reverse transcriptase-polymerase chain reaction
S/s	Serine/sedimentation
Sar	Sarghoda
SCA	sickle cell anaemia
SPIEM	solid phase immune electron microscopy
TBE	tris borate/EDTA
T/t	Threonine/thymidine
U	units
UN	United Nations
UNMIH	United Nations mission in Haiti
USA/US	United States of America
USSR	Union of Soviet Socialist Republics
V/v	Valine/volts

ABBREVIATIONS

VLPs	virus-like particles
W/w	tryptophan
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-idolyl-β-d-galactoside
XUAR	Xinjiang Uighur Autonomous Region
5’-end	amino terminal end
3’-end	carboxy terminal end
%	percent
°C	degrees celsius
μg	microgram
μl	microlitre
μmol/μM	micromolar

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CHAPTER 1: LITERATURE REVIEW

1.1 Discovery and history of the hepatitis E virus

Hepatitis E was not recognized until the early 1980s when sensitive and specific tests for antibody to hepatitis A virus (HAV) were first applied to the study of epidemic waterborne hepatitis in India, all of which had, in the past, been thought to be caused by the hepatitis A virus (Wong *et al*., 1980). Direct experimental evidence for an additional waterborne hepatitis agent was first reported in 1983 when Balayan *et al.* (1983) described the successful faecal-oral transmission of hepatitis to a volunteer from patients with hepatitis A-like disease in Tashkent, Uzbekistan. The volunteer, who had been infected with HAV in the past, developed severe clinical hepatitis 36 days after ingestion of a faecal suspension from the patients but did not have a serological response to HAV or hepatitis B virus (HBV). He did, however, develop antibodies to virus-like particles (VLPs) which were recovered from his faeces on days 28 and 45 post-exposure and determined by immune electron microscopy (IEM) to be 27 to 30 nanometres (nm) in diameter (Purcell, 1996).

This form of non-A, non-B hepatitis was referred to as epidemic non-A, non-B hepatitis, or enterically-transmitted non-A, non-B (ET-NANB) hepatitis, and the agent of the disease was subsequently found to be the major cause of sporadic hepatitis cases in regions where the epidemic form was known to exist (Khuroo *et al*., 1983; Arankalle *et al*., 1993). IEM and primate transmission studies remained the only means of studying ET-NANB hepatitis until 1990 when Reyes *et al.* succeeded in cloning and sequencing a

part of the genome of the virus. The agent was renamed hepatitis E virus (HEV; Reyes *et al*., 1990) and its first full genome sequence was reported by Tam *et al.*, (1991).

A significant proportion of acute viral hepatitis occurring in young to middle-aged adults in Asia, Africa and the Indian subcontinent is now recognized to be caused by HEV. Hepatitis E has been shown to occur in both epidemic and sporadic endemic forms and is primarily associated with the ingestion of faecally contaminated drinking water. The first documented case of hepatitis E involved an outbreak of 29,000 cases of icteric hepatitis in New Delhi, India, in 1955 which followed widespread faecal contamination of the city's drinking water (Bradley, 1994). Both this and a similar epidemic of viral hepatitis which occurred between December 1975 and January 1976 in Ahmedabad city, India, also due to contaminated water supplies, were concluded to be caused by hepatitis E after retrospective serological analysis of paired serum specimens showed the aetiological agent to be neither HAV nor HBV.

Careful review of records and other related documents and manuscripts by Bradley, during a visit to the former Union of Soviet Socialist Republics (USSR), showed that the epidemiological and clinical features of the large hepatitis epidemics observed in the Kirgiz Republic, USSR, between 1955 to 1956, were remarkably similar to those associated with the 1955-56 New Delhi outbreak. During these outbreaks 10,800 cases of acute viral hepatitis were documented in young to middle-aged adults and approximately 18 % of infected pregnant women died (Bradley, 1994). Although these outbreaks were never described in the western scientific literature, Bradley concluded that HEV was the most likely aetiological agent.

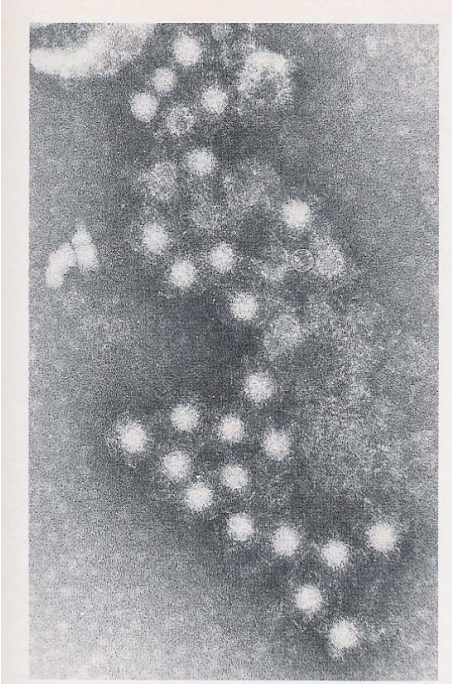

Figure 1.1 Electron micrograph of typical 32nm virus particles recovered from stool of a case of ET-NANB occurring in Telixtac, Mexico, during a 1980 outbreak. Particles are morphologically indistinguishable from those recovered from cases in Borneo, Burma, India, Somalia and Tashkent (adapted from Bradley, 1992).

1.2 The hepatitis E virus

1.2.1 Classification and morphology

The virus has a non-enveloped icosahedral particle that was originally described as a spherical 27 to 30 nm particle (Balayan *et al*., 1983), although in subsequent studies this diameter reportedly varies from 32 to 34 nm (Purcell, 1996; Krawczynski *et al*, 2000). HEV has been grouped with the Caliciviridae, a family of small, round-structured viruses (Figure 1.1), which includes agents associated with nonbacterial gastroenteritis in humans and animals (Reyes, 1997). However, the phylogenetic position of HEV, by analysis of non-structural genes, is outside the caliciviruses (Berke and Matson, 2000) and HEV remains taxonomically unclassified (Fauquet and Pringle, 1999). The morphological features of HEV are similar to those of Norwalk virus and both have a 7.5 kilobase (kb) single-stranded, positive sense RNA genome which contains three open reading frames (ORFs; Bradley, 1994). However, although the sequence of HEV is related to Norwalk virus, it most closely resembles the sequences of Rubella virus,

currently classified as a Togavirus and Beet necrotic yellow vein virus, a plant Furovirus (Fry *et al*., 1992). In addition, codon usage most closely resembles that of Rubella virus (Tam *et al*., 1991). It has thus been proposed that, on the basis of genetic relatedness and genomic organization, the taxonomy of positive sense RNA viruses should be reorganized (Koonin and Dolja, 1993). Under such a scheme, Rubella virus, Beet necrotic yellow vein virus and HEV would be placed in separate but related families (Purcell, 1996).

On the basis of morphology, HEV cannot be reliably distinguished from other "small round viruses" found in faeces (Purcell, 1996). HEV appears to be substantially different from Picornaviruses including HAV (Irshad, 1999). However, based on hybridization experiments, IEM and immunoflourescence blocking studies, HEV has been found to be the single major agent responsible for the majority of ET-NANB hepatitis cases seen worldwide.

1.2.2 Genomic organization

The cloning of HEV (Krawczynski and Bradley, 1989a; Reyes *et al*., 1990; Yarbough *et al*, 1991) was followed by the sequencing of its genome (Tam *et al*., 1991; Tsarev *et al*., 1992). Molecular analysis of the HEV genome has shown that it is a positive sense, single stranded RNA of approximately 7.5 kb in length with short 5' and 3'-noncoding regions. The RNA codes at least three separate ORFs and there is little variation among different isolates.

The putative nonstructural genes are located at the 5' end of the HEV genome and the structural genes are located at the 3' end (Figure 1.2; Irshad, 1999). The first open reading frame (ORF1) begins 28 nucleotides (nt) from the 5' end and extends for 5,079 base pairs

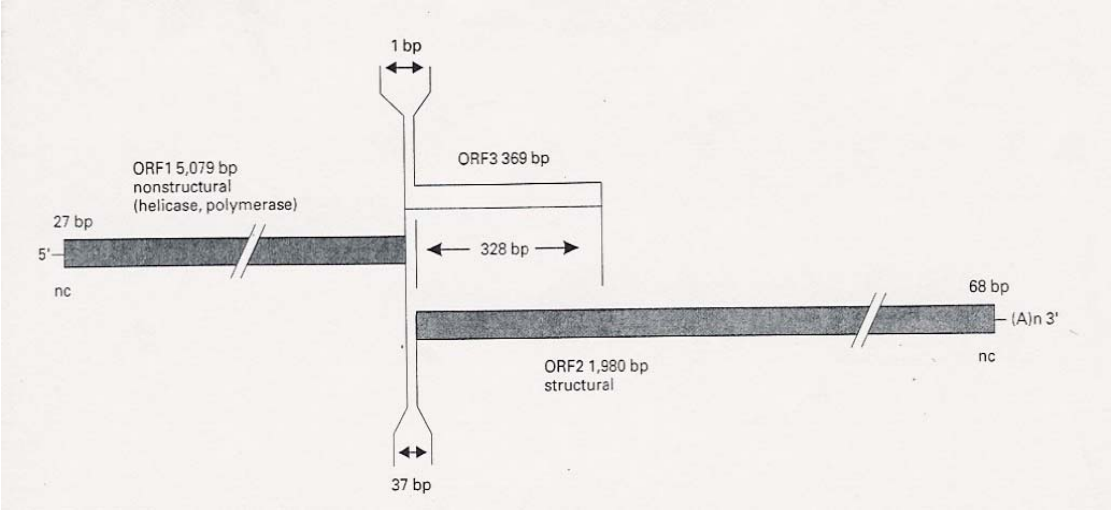

Figure 1.2 Genomic organization of HEV (adapted from Irshad, 1999).

(bp) ending at nucleotide 5,107. The 5' untranslated region (27 bp) is thought to play a role in transcription and expression of the virus. Analysis of the nucleotide sequence of the Burmese isolate revealed the presence of consensus sequences in ORF1 which encode both an RNA-dependent RNA polymerase (RdRp) and an RNA helicase. These are located at the extreme carboxy-terminal portion of the ORF1 poly-protein. A methyl transferase has been found located at the amino-terminal of the polyprotein which is a strong indication that HEV is capped (Zhang *et al*., 2001). Other domains in ORF1 include a 'Y' domain of unknown function, a putative papain-like cysteine protease domain and an 'X' domain of unknown function, acting as a flexible hinge (Figure 1.3). ORF1 thus appears to encode the nonstructural proteins with enzymatic activities.

Open reading frame 2 (ORF2) which is located at the 3' end of the RNA molecule begins at nucleotide 5,147 and extends for 1,980 bps terminating 65 bps upstream of the poly (A) tail (Irshad, 1999). This ORF encodes the structural proteins. These include a 660 amino acid glycosylated protein used in transport and a polypeptide with a high percentage of basic amino acids. Major immunological epitopes have been identified in this reading frame (Irshad, 1999).

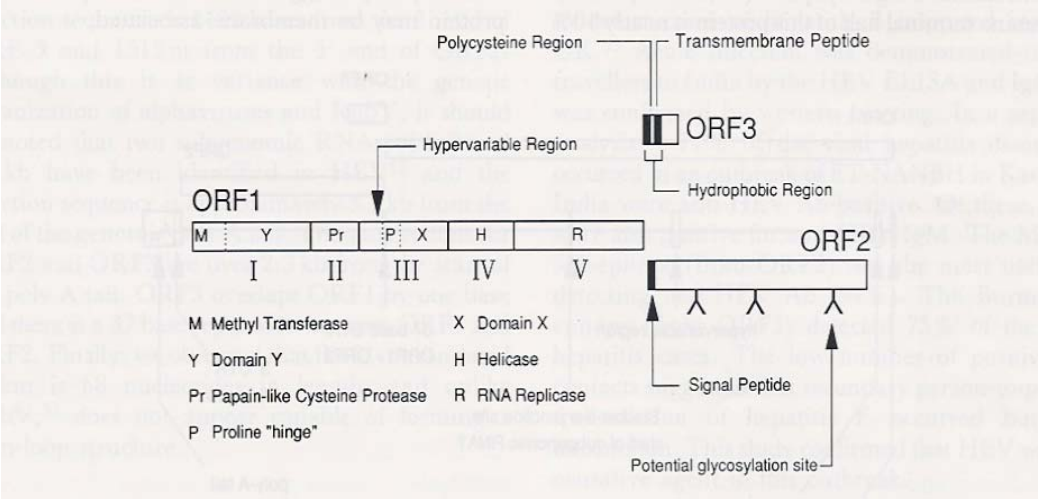

Figure 1.3 Genome map of the hepatitis E virus open reading frames (ORFs). The putative function of the genes contained in ORF1 is shown in the shaded boxes and the legend at the bottom of the figure. ORF2 is assumed to contain the structural gene(s) of HEV. A putative signal sequence at the amino-terminus of the ORF2 peptide is denoted by a shaded box at the amino end of ORF2. The inverted Ys denote potential glycosylation sites. The function of ORF3 is unknown but its carboxyl half is hydrophilic and contains epitopes recognized by antibodies from patient sera. Its amino half contains two hydrophobic regions separated by a small hydrophilic segment. The first hydrophobic region at the amino-terminal end of ORF3 terminates with a poly-cysteine region and the second hydrophobic region may be a transmembrane sequence. M, methyl transferase; Y, domain Y; Pr, papain-like cysteine protease; P, proline 'hinge'; X, domain X; H, helicase; R, RNA replicase (adapted from Purdy *et al.*, 1993).

The smallest ORF, ORF3, is located at the end of ORF1 between, and partially overlapping, the other two reading frames (Irshad, 1999). It encompasses 369 bps and overlaps the first ORF1 at its 5' end by one nucleotide only whereas the 328 bps at its 3' end overlap the 5' end of ORF2. The function of the proteins encoded by ORF3 are still unknown. One of these encodes a protein 123 amino acids long which has an immunoreactive epitope at its carboxy-terminus and a transmembrane sequence at its amino-terminus.

1.2.3 Physiochemical properties

The buoyant density of hepatitis E viral antigen and/or virus particles have been reported to be 1.35g/cm^3 (Balayan *et al.*, 1983) and 1.39 to 1.40g/cm^3 in CsCl (Favorov *et al*., 1989) while others have found the virus to be labile in CsCl (Bradley *et al.,* 1987). The buoyant density of HEV is 1.29g/cm^3 in potassium tartrate and glycerol (Bradley, 1990). The sedimentation coefficient of HEV is 183s (Bradley, 1992). HEV is unstable when stored at temperatures between -70 °C and +8 °C and readily degrades when freeze-thawed but it is stable in liquid nitrogen (Bradley *et al*., 1987). Hepatitis E virions appear to be unaltered by exposure to triflourotrichloroethane (Ticehurst, 1991). Survival of HEV in the intestinal tract suggests that the virus is relatively stable in acid and mild alkaline conditions (Purcell, 1996).

1.3 The natural history of HEV

1.3.1 Course and clinical features of infection

On August 31, 1990, Chauhan deliberately infected himself with HEV prepared from the stool of a patient involved in an epidemic of ET-NANB hepatitis and recorded the course and clinical features of the disease (Chauhan *et al*., 1993). Anicteric hepatitis accompanied by epigastric pain and discoloured urine began 30 days post-inoculation (average 40 days, ranges to 60 days; www.cdc.gov). The icteric phase started on day 38 and lasted until day 120. This was accompanied by a rise in serum alanine aminotransferase (ALT) and bilirubin. Stools collected on day 34 and 37 showed characteristic VLPs and transmitted disease to three monkeys. The stools first became positive for HEV reverse transcription-polymerase chain reaction (RT-PCR) with onset of the icteric phase on day 38 and remained positive until the ALT activity peaked on day

46. Illness resulted in severe nausea and vomiting. HEV was first observed in serum on day 22 before the symptoms of hepatitis developed and persisted during the pre-icteric phase, declined during the icteric phase and had disappeared by the time ALT peaked on day 46. The HEV antibody first appeared on day 41 and persisted for the duration of this study. Two years after the transmission IgG anti-HEV was still present in this individual. A distinctive feature of disease in this volunteer was the unusually long phase of abnormal serum ALT, lasting about three months, in contrast to two to three weeks in monkeys. The volunteer recovered completely and follow-up over two years has shown no further clinical or biochemical evidence of liver disease. The course of HEV infection in laboratory infected primates (Figure 1.4) follows a very similar pattern to that described by Chauhan although the immune response appears late in the incubation period or during the acute phase of the illness (Tsarev *et al*., 1992). Not all HEV infections are clinically apparent although the majority of clinical cases in epidemics experience jaundice, anorexia and hepatomegaly (Purcell, 1996). Approximately half of the cases experience abdominal pain and tenderness, nausea, vomiting and fever. Like hepatitis A, hepatitis E never progresses to chronicity.

To better characterize HEV infections, viremia, faecal shedding and antibody responses to HEV infections were examined in 67 patients with acute markers for hepatitis E who were admitted to the Infectious Disease Hospital in Kathmandu, Nepal in 1993 (Clayson *et al*., 1995). In this study day zero refers to the onset of symptoms and therefore correlates with day 30 of the initial study outlined above. It was found that the proportion of RNA-positive sera increased from day zero to day three and day 8 to 11 in the presence of decreased ALT activity (Figure 1.4) suggesting an uncoupling of viral replication and liver injury.

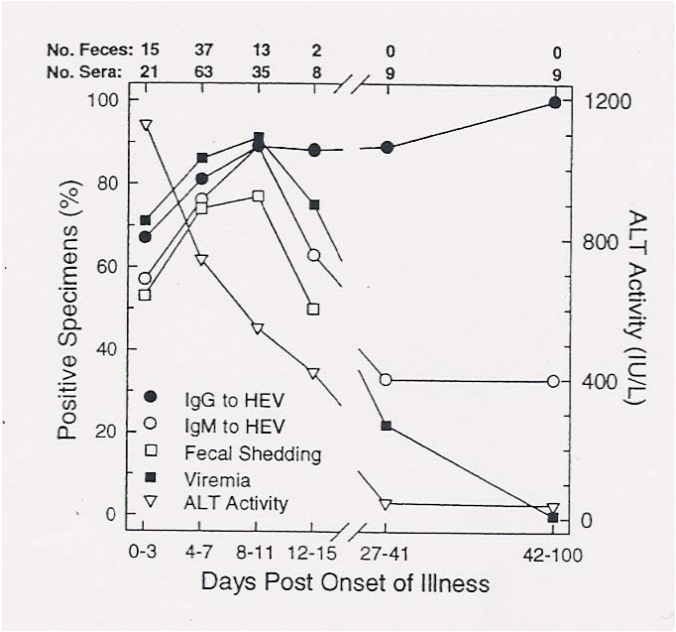

Figure 1.4 Changes in viremia, faecal shedding, IgM and IgG to HEV, and serum alanine aminotransferase (ALT) activity at various days after onset of illness. Numbers of faecal and serum samples examined for each time period are listed at the top of figure (adapted from Clayson *et al.*, 1995).

One possible explanation is that HEV virions may be released from hepatocytes by a mechanism other than cell lysis as in Poliovirus and Simian virus 40 (Kallman *et al.*, 1958; Clayson *et al.*, 1989). Another possibility is that virus detected in serum after the decrease in ALT activity was released after replication in tissue(s) other than the liver. HEV antigens were previously detected in the cytoplasm of cells in the small intestine, spleen and lymph nodes of rats and swine experimentally infected with HEV, suggesting that HEV replicates in these cells (Corcoran *et al.*, 1993; Maneerat *et al.*, 1996). Perhaps HEV replicates in tissues other than the liver in humans as well. At least initially the period of viremia was found to coincide with the period of IgM and IgG responses to HEV infection (Clayson *et al.*, 1995). The detection of prolonged viremia in the presence of antibodies raises the possibility that acute-phase immunoglobulin may lack neutralizing activity.

In one report, protracted viremia of 45 to 112 days duration was detected by PCR in approximately one-fourth of patients presenting with acute sporadic hepatitis E (Reyes, 1997). A prolonged period of virus shedding in faeces (to day 52) was also seen in one patient from whom samples were available. These findings indicate that protracted viremia my be more common in hepatitis E than previously thought and may contribute to prolonged infectivity and endemicity in the community. In another report, serum samples were obtained from four boys aged 5 to 10 years diagnosed with acute hepatitis in Egypt (Schlauder *et al*., 1993). For all four patients, the first sample drawn two to three days after onset of symptoms was PCR positive for HEV RNA while the samples obtained 10 to 20 days after onset of illness were negative. These results show that HEV does not produce a persistent viremia in all the patients.

1.3.2 Pathogenesis

Because serologic and molecular tests for HEV were developed only recently, the pathogenesis of HEV is poorly understood as outlined in paragraph 1.3.1 above. However, Purcell (1996) summarizes the data and reaches some speculative conclusions as shown in Figure 1.5. Entry of the virus into the host is believed to be primarily by the oral route via contaminated water. The site of primary replication is not known but is presumed to be in the intestinal tract. It is not clear how the virus reaches the liver but it is presumably via the portal vein. It replicates in the cytoplasm of hepatocytes and is released into the bile and blood.

Reyes (1997) reports that the key pathologic features of HEV include “cholestatic hepatitis, proliferation of bile ductules and pseudoglandular arrangement of hepatocytes surrounding distended bile canaliculi with frank parenchymal necrosis. The complete

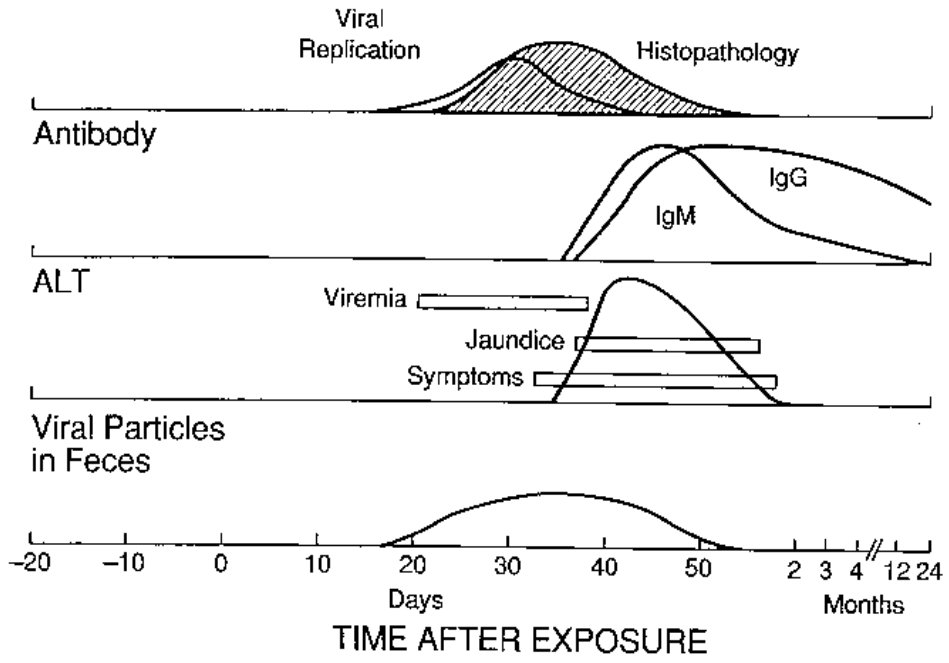

Figure 1.5 Diagrammatic illustration of the clinical and serologic events in a typical case of acute hepatitis E. Antibody pattern is depicted as measured by ELISA. Viremia and faecal shedding patterns are based on PCR data (adapted from Purcell , 1996).

pathologic picture includes mild portal and lobular inflammation with a predominantly polymorphonuclear leukocyte and macrophage infiltration."

Lau *et al.* (1995) detected the HEV genome in 50 to 60 percent of hepatocytes of two patients with fulminant hepatitis E. Viral transcript and antigens were found to be in the cytoplasm of infected cells exclusively, consistent with viral cytoplasmic replication (Reyes, 1997). The presence of a minimal inflammatory infiltrate was consistent with the hypothesis that the majority of the hepatic injury associated with HEV fulminant hepatitis is due to a direct cytopathic mechanism rather than to a host-mediated immune injury. Cytopathic effects *in vitro* have also been observed (Huang *et al*., 1992).

HEV is implicated in fulminant hepatitis on only a sporadic basis (Lau *et al*., 1995). Pre-existing conditions may contribute to fulminant hepatic failure (Reyes, 1997). In one case HEV-associated fulminant hepatic failure developed in a 6-year-old patient with

Wilson's disease (Sallie *et al*., 1994). The patient developed progressive deterioration in liver function with serum, liver and bile all positive for HEV by RT-PCR.

The case fatality rate of hepatitis E infection varies in different reports but an overall rate of one to three percent exists which is high when compared to 0.2 percent for hepatitis A (www cdc.gov). The mortality of hepatitis E in pregnancy increases with each succeeding trimester and may reach 20 percent (Purcell, 1996). In experimentally infected rhesus monkeys, the HEV incubation period in pregnancy may be considerably shortened but the disease is not necessarily more severe (Arankalle *et al*., 1993). In this study, none of the pregnant animals developed fulminant hepatitis, but there was apparent transmission to one of three infants as well as a macerated foetal still-birth.

In a vertical transmission study, six of ten pregnant women presenting with hepatitis E developed fulminant hepatic failure (Khuroo *et al*., 1995). PCR on cord blood or early birth samples from the infants showed five with detectable HEV sequences and elevated ALT levels. This study clearly establishes intrauterine infection with HEV as a significant cause of perinatal morbidity and mortality (Reyes, 1997). Premature deliveries with a high infant mortality (33 %) have also been reported (Purcell, 1996).

1.3.3 Diagnosis

Viral antibody or the presence of VLPs in clinical specimens was in the past detected by IEM using specimens from infected animals or humans. Solid phase immune electron microscopy (SPIEM) analysis was also used as another IEM method to detect VLPs in the bile of infected monkeys and in human stools (Chauhan *et al*., 1994). Fluorescent antibody blocking assay is another microscopy method used to screen sera for total immunoglobulin to HEV. This assay measures the ability of serum to block binding of a

fluorescein isothiocyanate (FITC)-labeled antibody probe to native HEV antigens in liver sections from experimentally inoculated monkeys (Clayson *et al.*, 1995). Another blocking assay uses HEV recombinant antigens as blocking reagents to inhibit anti-HEV from binding to the solid phase antigen made from polystyrene beads coated with synthetic peptides (Dawson *et al*., 1992). It was later realized that IEM was not practical due to low levels of virus excretion in faeces (Reyes, 1997). The estimated titre of HEV in stool is 100-fold less (estimated by PCR) than that seen with HAV. Purification of a presumed viral-specific antigen containing fractions from faecal specimens was the basis for one of the earliest diagnostic tests (Sarthou *et al*., 1986). These tests were problematic because of the variability and difficulty in standardizing reagents. Assays of this sort have been largely supplanted by the ability to detect viral-specific nucleic acid by PCR (Reyes, 1997) and agarose gel electrophoresis.

Enzyme-linked immunosorbent assay (ELISA) is now the preferred screening method for large-scale seroprevalence studies. ELISAs based on recombinant antigens and synthetic peptides are commercially available for HEV. Both open reading frames 2 and 3 of the HEV genome encode major immunogenic epitopes at their carboxyl-termini that are immunoreactive with sera from infected animals or humans (Yarbough *et al*., 1991). The prototype HEV ELISA used the '3-2' derived ORF2 protein and the '4-2' derived ORF3 protein from the HEV Mexico and HEV Burma strains, respectively, expressed in *Escherichia coli (E. coli)*. Another commonly used ELISA is the Abbott HEV ELISA (Abbott GmbH Diagnostica, Wisbaden, Germany) assay which uses two recombinant antigens 'SG-3' from ORF2 and '8-5' the full length of ORF3 from the Burmese strain expressed in *E. coli* and coated on polystyrene beads (Christensen *et al*., 2002). Current tests are capable of detecting IgM anti-HEV in up to 90 percent of acute infections if a

serum sample is obtained one to four weeks after onset of disease (Bryan *et al*., 1994). By three months, IgM anti-HEV is no longer detectable in 50 percent or more of hepatitis E cases (Arankalle *et al*., 1994). IgM anti-HEV reaches peak titers of 1:1,000 to 1:10,000 during the first four weeks after onset of hepatitis. IgG anti-HEV peaks in titer (1:1,000 to 1:100,000) between two and four weeks after onset of hepatitis and diminishes relatively rapidly thereafter (Purcell, 1996). A more recent assessment of the diagnostic value of commercial IgG and IgM anti-HEV ELISA tests (manufactured by Genelabs Diagnostics) compares the sensitivity (86.7 and 53.3 %, respectively) and specificity (92.1 and 98.6 %, respectively) of these tests using serum samples taken from patients with HEV RNA detected by RT-PCR tests (Lin *et al.*, 2000) and concludes that they provide a good diagnostic screening test for acute hepatitis E even in nonendemic areas.

The cDNA, RNA and protein, hybridization diagnostic methods appeared to be more sensitive for detecting HEV than standard IEM (Balayan, 1993). These methods include Southern blot hybridization (Ray *et al*., 1991; Turkoglu *et al*., 1996), Northern blot hybridization (Tam *et al*., 1991) and a sensitive and specific western blot assay for both IgM and IgG anti-HEV. The latter has been shown to be capable of detecting anti-HEV in acute and convalescent phase sera (Krawczynski *et al*., 1989b; Favorov *et al*., 1992; Hyams *et al*., 1992; El-Zimaity *et al*., 1993).

1.4 Host range

Labrique *et al*. (1999) debated the possibility of a zoonotic reservoir of HEV. Wild-caught primates have occasionally been found to have anti-HEV IgG before laboratory inoculation. Antibodies to HEV have also been found in domestic swine, cows, sheep, goats, chickens and water buffaloes. The serum of 44 to 94 % of wild rats captured from

different regions of the United States (US) contained antibodies to a human strain of HEV (Kabrane-Lazizi *et al*., 1999). These animals are not limited to antibody reactivity; in Nepal, HEV RNA and anti-HEV were found to be highly prevalent in domestic swine (Clayson *et al*., 1995). Wild rodents captured in the Kathmandu Valley of Nepal were also found to harbour HEV RNA with nucleotide sequences similar to those of human strains from that area. Of particular interest as a reservoir in the US is domestic swine. A novel virus was identified in 1997 in the US domestic swine population and was designated "swine HEV" (HEV-S; Meng *et al*., 1997). This virus, which is commonly present in domestic swine older than three months, was found to have nucleotide sequences (79-85 %) and amino acid sequences (77-92 %) that were closely related to those of some human HEV strains. In fact, the US-1 (human) strain is genetically more similar to the swine HEV (> 97 % nucleotide identity in ORF1 and ORF2) than to other human strains of HEV (Meng *et al*., 1998; Schlauder *et al*., 1998). Swine HEV is also serologically cross-reactive with human HEV. Inoculation of nonhuman primates with this swine variant caused infection. While Labrique *et al.* (1999) speculate whether swine HEV circulates or causes illness in humans and whether human HEV has an animal reservoir, Karetnyi *et al.* (1999) presented evidence of possible interspecies transmission (see paragraph 1.5.4).

While chimpanzees have been shown to be susceptible to infection with HEV, only two isolates, from Ahmedabad, India and Telixtac, Mexico, have been shown to cause liver disease in this host (Bradley, 1994). Intravenous inoculation of chimpanzees with other isolates proven by IEM to contain HEV, including those from Burma, USSR (Tashkent) and Nepal, have not caused disease in these animals. These results suggest that adaptation can occur in at least one host species and that some isolates of HEV may be

more virulent than others.

1.5 Transmission of HEV

HEV has relatively low infectivity, with a secondary attack rate of about 2 % among household contacts (Bradley *et al*, 1991). HEV is believed to have a lower environmental stability, which may result in decreased secondary transmission. Furthermore, a larger infective dose may be necessary to cause overt disease among contacts of HEV patients (Labrique *et al*., 1999) and nosocomial spread has been reported in South Africa (see paragraph 1.5.3 below). Also accidental transmission of HEV in the laboratory has been reported; one Calcutta outbreak involved 21 people in a research facility where HEV was being studied. (Neogi *et al*., 1995).

No secondary cases of hepatitis have been reported among sexual partners of cases under conditions of epidemic or sporadic disease (Longer *et al*., 1993; Myint *et al*, 1985). However a high incidence of HEV antibodies was found in a small study of homosexual men in Italy (33/162 or 20.4 %. Montella *et al*., 1994). The higher prevalence of HEV found among homosexual men indirectly confirms the role of transmission through the faecal-oral route facilitated by sexual practices.

Vertical transmission of HEV from mothers to their infants has been reported, with associated morbidity and mortality (as discussed in paragraph 1.3.2 above).

Outbreak investigations reveal faecally contaminated drinking water as the major source of HEV and although transmission in developed countries is discussed below, the causes outlined ultimately equate to faecally contaminated water.

1.5.1 Contaminated drinking water

HEV infection is common in many tropical and subtropical hot climate countries including India, Nepal, Myanmar, Pakistan, Afghanistan, Borneo, China, Mexico, North Africa (Egypt and Algeria in particular), East Africa (Djibouti, Somalia, Ethiopia and Sudan), West Africa (Ivory Coast) and, more recently, southern Africa (Botswana and Namibia). Many of the epidemics both large and small are associated with sewage contaminated drinking water such as the 1955 outbreak in New Delhi in which 30,000 cases were reported (Viswanathan, 1957) and the Chinese outbreak with greater than 100,000 cases in the Xinjiang Uighar region (Zhuang *et al*., 1991).

Adult anti-HEV seroprevalence rates of 4 to 11 % have been reported in various developing countries such as China (Lee *et al.*, 1994), Turkey (Thomas *et al.*, 1993), Venezuela (Pujol *et* al., 1994) and South Africa (Grabow *et al*., 1994). However, since none of the anti-HEV positive individuals had a record of clinical viral hepatitis which could be related to HEV infection they would appear to be sporadic cases of sub-clinical infection (Grabow *et al.,* 1996). A study by Tucker *et al.* (1996) suggests that contaminated water may play a major role in the spread of HEV in South Africa. In this study the prevalence of anti-HEV was determined among 407 urban and 360 rural black South African adults living in formal housing, squatter camps or mud huts in the Western Province. Rural subsistence farmers living in isolated clusters of traditional mud huts who use unchlorinated river water were found to be at greatest risk (17.4 %) of HEV infection. Rural people living in formal housing within villages had a significantly reduced risk in comparison (5.3 %). However, there was no difference in prevalence between the urban formal and squatter communities despite poor living conditions within the majority of squatter areas where non-water-borne sewerage disposal methods are

used. Urban squatter communities do, however, unlike their rural equivalents, rely entirely on chlorinated tap water for domestic use.

1.5.2 Transmission in developed or non-endemic areas

Non-endemic areas, corresponding to industrialized nations, are considered to be free of natural foci of HEV infection and HEV is thus regarded as unimportant since only rare sporadic cases are reported. However seroprevalence of 0.4 to 3.2 % has been described in the general population of developed countries (Moaven *et al*., 1995; Lavanchy *et al.*, 1994).

1.5.2.1 Sewage polluted shellfish

It is suspected that in Mediterranean countries, such as Italy and Spain, cases of HEV infection could be causatively related to the consumption of shellfish cultivated in sewage-polluted waters (Balayan, 1993).

1.5.2.2 Travelling

Most sporadic cases of HEV disease which have been reported in Westerners who develop jaundice occur shortly after their return from endemic areas. A seroprevalence study of 104 selected sera from sporadic cases of non-A, non-B, non-C (NANBNC) hepatitis encountered in metropolitan areas of the former southern Transvaal province of South Africa during 1993 found only five HEV positive reactors (Swanepoel *et al*, 1996). Three of these sera reacted weakly (OD < 1.000), whereas two sera, from patients who had visited the Indian subcontinent, reacted strongly (OD > 1.000).

1.5.3 Nosocomial transmission

Robson *et al.* (1992) relate a case study in which a 34-year-old woman from Cape Town presented at 32 weeks gestation at the maternity unit of Groote Schuur Hospital with diarrhoea, jaundice and episodic confusion. She had recently returned from holiday in Bombay, India. She also had a history of contact with hepatitis in India. Samples taken at the onset of illness were tested for antibodies to HEV (anti-HEV) and shown to contain both IgG and IgM anti-HEV by western blotting. Six weeks after attending this patient, the referring physician and two theatre sisters from the maternity unit, attended the liver clinic with features compatible with acute self-limiting viral hepatitis. Serum from one theatre sister, taken six weeks after exposure, was positive for anti-HEV-IgG and -IgM. There was no serological evidence of HEV infection in the physician or the other theatre sister though they may have had very low grade HEV infection which could not be confirmed serologically by antibody tests. The mode of transmission of HEV in these cases was presumptive. The physician had helped the patient from a bedpan, while the nurses had transferred the confused, unco-operative patient to the operating theatre for repair of vaginal lacerations. Thus all had been exposed to blood and stool. Hepatitis E must be suspected in travellers returning from the Indian subcontinent and appropriate care taken especially since pregnant women with acute hepatitis E infection may progress to severe fulminant hepatitis.

1.5.4 Interspecies transmission

Recently, HEV-like virus was isolated from swine in Iowa (Karetnyi *et al*., 1999). Swine production is a major industry in Iowa with the potential for human exposure to swine in and around industrial and family farm operations. One study with the objective to

determine whether individuals in Iowa are exposed to HEV was undertaken (Karetnyi *et al*., 1999). Anti-HEV prevalence in four selected Iowa populations was determined. Sera were collected from 204 patients with NANBNC hepatitis; 87 staff members of the Department of Natural Resources (DNR); 332 volunteer blood donors in 1989 and 111 volunteer blood donors in 1998. All sera were tested for anti-human HEV IgM and IgG by ELISA. Both the patients with NANBNC hepatitis (4.9 %) and the healthy field workers from the Iowa DNR (5.7 %) showed significantly higher prevalence of anti-HEV IgG compared to normal blood donor sera collected in 1998. It was concluded that human HEV circulates in Iowa. At risk human populations with occupational exposure to wild animals and environmental sources of domestic animal wastes have increased seroprevalence of HEV antibodies. These findings support the presence of endogenous HEV in the United States and raises the possibility of interspecies transmission.

1.5.5 Parenteral transmission

Although HEV infection frequently results in subclinical infection and does not lead to chronicity, transfusion of HEV-positive blood to hepatitis B surface antigen (HBsAg) carriers, pregnant women or persons with ongoing replication of other hepatitis viruses, can result in fulminant hepatic failure leading to death (Arora *et al*., 1996). It is because 95 % of HEV infections remain subclinical (Arankalle *et al*., 1995) and viremia persists for at least two weeks prior to serum alanine amino-transferase (ALT) changes (Chauhan *et al*., 1993) and HEV has a predilection for young adults, a population which corresponds to eligible blood donors, that the possibility of viremic blood being transfused is not remote, particularly in a region of high endemicity.

Arankalle and Chobe (1999) found 1.5 % of a group (N = 200) of voluntary blood donors in India to be HEV RNA positive. This study also showed that a significantly higher proportion of blood donors older than 20 years of age had prior exposure to HEV (22.7 %) as opposed to those under 20 years of age (9.8 %). Interestingly, 76 % of the paid plasma donors tested (N = 71) were found to be anti-HEV IgG positive. The higher exposure of the plasma donors to HEV was not related to poor living conditions as had been found in commercial blood donors (N = 191) and infection may have resulted from contamination of the plasmapheresis machine. Although Tsarev *et al.* (1997) found the HEV titre to be at least 1000-fold lower in serum than in faeces and HEV has been shown to be unstable at +4 ^{o}C, these results indicate a possible risk of transfusion-associated hepatitis E in hyper-endemic areas, particularly if transfusion is immediate (Chobe *et al*., 1997).

Three percent (2/66) of injecting drug users (IDU) were found to contain HEV antibodies in a small study in Italy to determine other risk factors (Montella *et al*., 1994). With regard to IDUs, HEV could be transmitted in this population group through injection or ingestion of contaminated drugs, even though direct person-to-person contact because of poor hygiene cannot be ruled out.

1.6 Prevention and control

1.6.1 Prevention

Environmental factors are a major contributor to the endemic and epidemic spread of HEV. Management of the community outbreak involves identification and correction of the environmental factors promoting faecal contamination of drinking water. Seasonal monsoons can lead to floods that can overwhelm water treatment facilities leading to

contaminated drinking water (Reyes, 1997). The correction of inadequate water treatment facilities is essential. Consideration should be given to protecting pregnant women from potential sources of infection in an epidemic situation. Simple hand-washing procedures should suffice to interrupt any person-to-person transmission.

In outbreak settings, the handling and disposal of human waste must follow strict sanitary guidelines (Drabick *et al*., 1997). In sporadic cases, the patients' excreta must be disposed of hygienically, with care being taken to avoid contact with these waste products. Improvements in drinking water storage, treatment and distribution should be encouraged as a means of reducing HEV transmission. Better community sanitation and sewage management would also reduce rates of HEV infection worldwide, especially in flood-prone areas.

Health education about personal and environmental hygiene in high-risk communities might reduce the likelihood of HEV outbreaks (Tsega *et al*., 1991). It is probable that outbreaks may be prevented by adequate chlorination of water supplies that are not severely contaminated. However, when an HEV epidemic is suspected, all drinking water should be boiled or imported from other non-endemic areas, since chlorination alone may be unsuccessful in controlling epidemics (www.cdc.gov). To reduce the risk of HEV infection, travellers to HEV-endemic areas should be advised to practice prudent hygienic practices, including avoidance of untreated drinking water and iced beverages of unknown quality. Uncooked shellfish, fruits or vegetables should also be avoided. In laboratory settings, the use of iodinated disinfectants or autoclaving is believed to destroy HEV (Ticehurst, 1999; Labrique *et al*., 1999).

1.6.2 Control

Controversy remains as to whether immune globulin prophylaxis is efficacious, even if it is prepared from HEV-endemic populations (Labrique *et al*., 1999). Passive immunization was not successful in preventing infection in two nonhuman primate studies (one used immune globulin from a human volunteer with historical HEV infection, and the other used late convalescent plasma from a previously infected cynomolgus monkey (cynos; Tsarev *et al*., 1994a; Chauhan *et al*., 1998). The use of immune globulin in an HEV outbreak in India did not reduce disease rates in a controlled study (Khuroo and Dar, 1992). Furthermore, treatment of pregnant women with immune globulin did not demonstrate a significant reduction in adverse events (Arankalle *et al*., 1998). Since there is no specific treatment for hepatitis E infection, patient management is primarily supportive.

Further studies reported the use of recombinant HEV proteins as a candidate HEV vaccine in cynos (Krawczynski and Bradley, 1989a). A recombinant protein encoded by two-thirds of HEV ORF2 (Burma strain), C51, was used to immunize cynos. When the animals were subsequently challenged with intravenous inoculation of the HEV Burma strain, a significant improvement of symptoms was observed. In another study, cynos immunized with trpE-C2 protein did not develop hepatitis after challenge with wild-type HEV from either a Burmese or Mexican stool isolate (Purdy *et al*., 1993). Studies at the National Institute of Health, Bethesda (USA) using an ORF2-derived 55-kD protein from HEV (strain SAR-55 from Pakistan) as a vaccine in cynos showed similar results demonstrating full protection against challenge with wild-type HEV. Protection against genetically distinct strains was provided by this vaccine. Multiple doses of vaccine increased the protective effect of the vaccination against an HEV challenge. A 62-kD

recombinant protein derived from the putative capsid protein encoded by ORF2 was used in studies conducted at the Center for Disease Control, Atlanta (USA). Full protection against HEV infection was reported in animals receiving a 62-kD HEV vaccine, as opposed to the control animals showing symptoms of HEV disease. Post-exposure vaccination has been shown not to be protective against disease (Irshad, 1999).

1.6.3 Treatment

Presently there is no treatment for HEV infection. The most serious and life-threatening complication is the development of fulminant hepatic failure. The therapy of acute liver failure has been reviewed (Caraceni and van Thiel, 1995). Medical management in an intensive care unit is indicated in the case of progressive deterioration and development of fulminant hepatic failure as a result of acute viral hepatitis. A variety of nonspecific measures can result in effective symptomatic relief. Bed-rest is indicated for the symptomatic patient. Dietary restrictions are unnecessary with the exception that alcohol and other potential hepatotoxic drugs should be avoided. Hepatic encephalopathy is a hallmark of acute liver failure and therapies are generally directed to a reduction in any factor that might exacerbate or predispose to its development. Cerebral oedema is a severe complication of stage III/IV hepatic encephalopathy in 50 to 85 % of patients. Intracranial pressure monitoring is useful in prompting early intervention for and prevention of serious sequelae (e.g brain stem herniation) by treatment with mannitol (Caraceni and van Thiel, 1995). Other complications in acute liver failure include bacterial and fungal infections and coagulopathy. Prophylactic antibiotic therapy and fresh frozen plasma are useful in decreasing the infection rate and complications due to bleeding, respectively (Caraceni and van Thiel, 1995). There are no hepato-protective or anti-inflammatory agents proven efficacious in acute liver failure. Survival rates may

increase to 60 to 80 % with orthotopic liver transplantation (Reyes, 1997).

CHAPTER 2: EPIDEMIOLOGY

Historically HEV epidemiological study designs involved retrospective investigations of sera from reported ET-NANB epidemics. More recently, however, many cross-sectional prevalence studies of healthy high and low risk populations, as well as hospital based case-control studies of patients admitted with acute hepatitis have been described. Laboratory methods used in these studies include exclusion of hepatitis A, B and C, IEM, ELISA, western Blot, RT-PCR and sequence analysis (see paragraph 1.3.3).

Outbreaks and high seroprevalence of HEV have been reported from many, mainly tropical regions, including India, Pakistan, Bangladesh, Nepal, Myanmar, Borneo, Algeria, Somalia, Sudan, Ivory Coast, Mexico, Saudi Arabia, China, Chad, Egypt, Ethiopia, Botswana, some former Soviet republics, including Kazakstan, Tajikistan, Turkmenistan and Uzbekistan and more recently Kenya, French Territories of Afars and Issas (FTAI), Morocco, Nigeria and Namibia (Figure 2.1 and Table 2.1). Although hepatitis E is most commonly recognized to occur in large outbreaks, HEV infection also accounts for > 50 % of acute sporadic hepatitis in both children and adults in some highly endemic areas (www.cdc.gov). In the United States and other non-endemic areas, where outbreaks of hepatitis E have not been documented to occur, a low prevalence of anti-HEV (2 % or less) has been found in healthy populations, indicating that HEV is responsible for both sporadic illness and in-apparent infection. Between these two extremes are countries, normally neighboring endemic areas, with little or no sporadic HEV where studies report considerably more than a 2 % prevalence of HEV antibodies in cohorts of healthy individuals. Since none of the individuals had a history of contact with

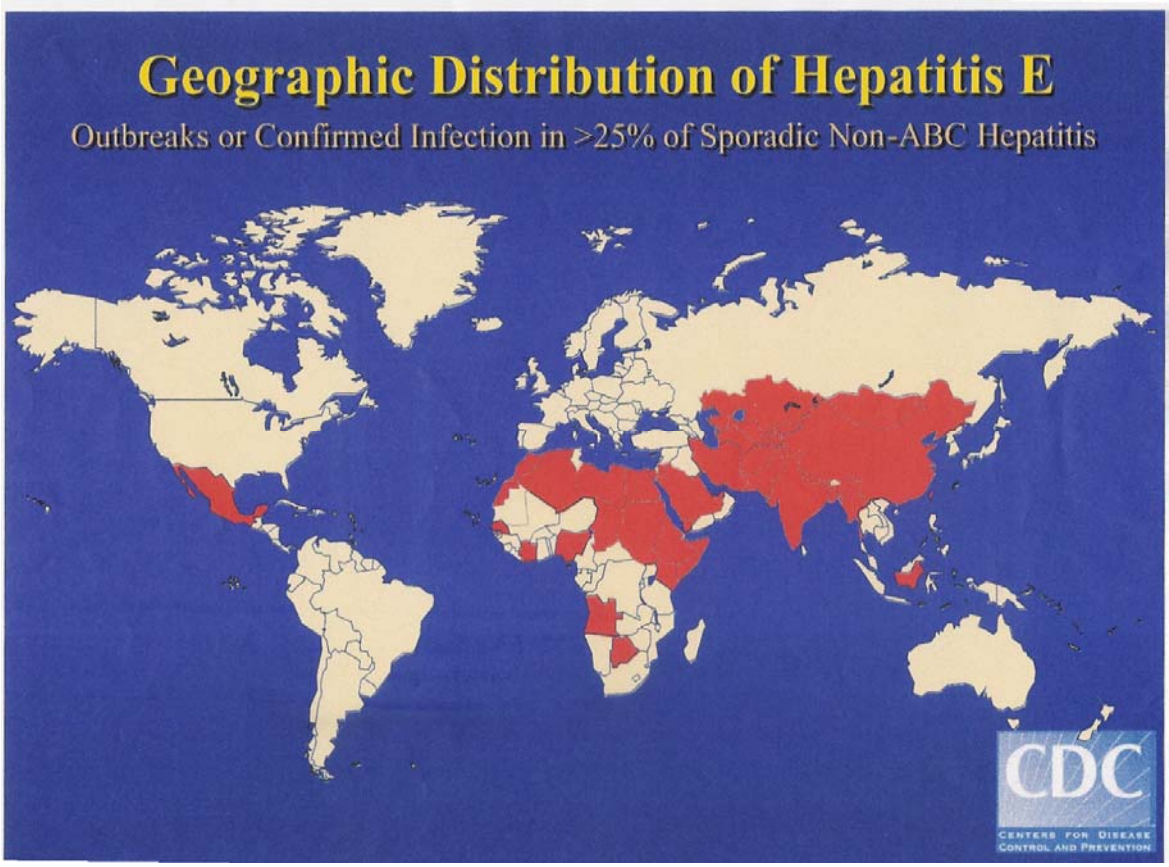

Figure 2.1 Global distribution of hepatitis E virus. Red regions represent areas where documented outbreaks of HEV infection have occurred or where >25% of sporadic non-A, non-B, non-C hepatitis is attributable to HEV. Adapted from the Center for Disease control website (http://www.cdc.gov/ncidod/diseases/hepatitis/slideset/hep_e/slide_5.htm).

others who were involved in outbreaks of viral hepatitis or related diseases, it appears that they represent cases of sporadic subclinical HEV infection.

2.1 Epidemic outbreaks and prevalence studies in regions in which HEV is endemic

Large epidemics have been reported in Africa, Asia and Eurasia and outbreaks are reported on a recurring basis on the Indian subcontinent (Table 2.1). Epidemiological and clinical characteristics, which are characteristic of hepatitis E outbreaks in these regions, include:

- Faecal-oral transmission
- Occurrence of water-borne outbreaks
- Typical age distribution pattern with predominant involvement of teenagers and

Table 2.1 Documented epidemics or sporadic outbreaks of enterically transmitted NANB hepatitis

Site	Dates	Number of cases	Method(s) used	Reference
Asia				
India				
New Delhi	1955-56	29,300	RA, X, EI	Labrique *et al*., 1999
Ahmedabad	1975-76	2,572	RA, X, EI	Labrique *et al*., 1999
Kashmir	1978	275	EI	Labrique *et al*., 1999
Gulmarg (Kashmir)	1978	20,000		Labrique *et al*., 1999
Sopore (Kashmir)	1979	6,000		Labrique *et al*., 1999
Ambala/Karnal	1980-1981	865	X	Labrique *et al*., 1999
Azamgarh	1980	152	X	Labrique *et al*., 1999
Kolhapur (Pune)	1981	1,169	RA, EI	Labrique *et al*., 1999
Jammu	1981	206	X	Labrique *et al*., 1999
Harwara (Kashmir)	1981-1982	11,000	X	Labrique *et al*., 1999
Kupwara (Kashmir)	1981-82	15,000	X	Labrique *et al*., 1999
Ahmedabad	1982	1,072	RA, EI	Labrique *et al*., 1999
Ahmedabad	1984	118	RA, EI, IEM	Labrique *et al*., 1999
Baroda	1984	3,005	RA, EI	Labrique *et al*., 1999
Surat	1985	1,395	RA, EI	Labrique *et al*., 1999
Ahmedabad	1986	1,015	RA, EI	Labrique *et al*., 1999
South Delhi	1987	43	X	Labrique *et al*., 1999
Karnal, Haryana	1987	1,273	EI	Labrique *et al*., 1999
Ahmedabad	1987	2,215	RA, EI	Labrique *et al*., 1999
Jammu	1988	518	RA, X, PCR	Labrique *et al*., 1999
Khadakwasla	1989	276	RA, EI	Labrique *et al*., 1999
Akluj	1990	139	RA, EI	Labrique *et al*., 1999
Beed	1990	>3,000	RA, EI	Labrique *et al*., 1999
Rewa	1990	517	RA, EI	Labrique *et al*., 1999
Bijapur	1990	>158	X, IEM	Labrique *et al*., 1999
Hyderabad	1990	>158	X, IEM	Labrique *et al*., 1999
Kolhapur	1991	1,442	RA, EI	Labrique *et al*., 1999
Kanpur	1991	79,091	X, PCR	Labrique *et al*., 1999
Aligarh	1991	>77	PCR	Labrique *et al*., 1999
Karad	1993	2,427	RA, EI	Labrique *et al*., 1999

Table 2.1 continued on next page

Table 2.1 (continued)

Site	Dates	No. of cases	Method(s) used	Reference
Asia				
Nepal				
Kathmandu	1973-74	10,000	RA	Labrique *et al*., 1999
Kathmandu	1981-82	4,337	X	Labrique *et al*., 1999
Kathmandu	1987	370		Bradley, 1992
Kathmandu	1995	32	EI	
Pakistan				
Mardan	1984	>8	RA, X	Labrique *et al*., 1999
Rawalpindi	1985	>11	RA, X	Labrique *et al*., 1999
Quetta	1985	>53	RA, X	Labrique *et al*., 1999
Karachi	1986			Irshad, 1999
Sargodha	1987	133	IEM, RA, X, EI	Labrique *et al*., 1999
Abbottabad	1988	190	RA, EI	Labrique *et al*., 1999
Islamabad	1993-94	3,827	EI	Labrique *et al*., 1999
Bangladesh				
UN Haiti unit	1995	4	EI	Labrique *et al*., 1999
USSR				
Kirgizstan, Republic	1955-1956	10,812		Irshad, 1999
	1982-1983			Irshad, 1999
Myanmar				
Mandalay	1976-1977	20,000	RA, X	Labrique *et al*., 1999
Rangoon	1982-83	399	X	Irshad, 1999
Yangon	1989	111	EI	Labrique *et al*., 1999
China				
Xinjiang	1986-88	120,000		Cao *et al*., 1991

Table 2.1 continued on next page

Table 2.1 (continued)

Site	Dates	No. of cases	Method(s) used	Reference
Borneo				
Indonesia	1987-1988	2,000		Bradley, 1992; Irshad, 1999
Central America				
Costa Rica				
San José	1976	5		Irshad, 1999
Mexico				
Telixtac	1986	129		Bradley, 1992
Huizililla	1986	94		Irshad, 1999
Middle East				
Saudi Arabia				
Qatar	1981	91		Irshad, 1999
Africa				
Algeria				
Mostaganem	1979-1980	26	PCR, EIA	Coursaget *et al*.,1996; van Cuyck-Gandre *et al*.,1997
Medea	1980-1981	788		Bellabes *et al*., 1985; Bradley, 1992; Irshad, 1999
Botswana				
Maun	1985	273	X	Byskov *et al*., 1989
Chad				
N'Djamena	1983-1984	38	EIA, PCR	Bradley, 1992; van Cuyck-Gandre *et al*.,1997
Ethiopia				
Asmera	1988-1989	>750	EI	Tsega *et al.,* 1992
Kenya				
refugees	1991	151	EI	Mast *et al*., 1994

Table 2.1 continued on next page

Table 2.1 (continued)

Site	Dates	Number of cases	Method(s) used	Reference
Kenya				
	1991	*151*	EIA	Favorov *et al*., 1994
FTAI				
Djibouti	1992-1993	*45*		Coursaget *et al*.,1996
Somalia				
Medea	1980-1981	57	X	Belabbes *et al*., 1985
Refugee camps	1985-1986	129		Bradley, 1992; Irshad, 1999
Tug Wajale	1986	>2,000	IEM	CDC, 1987
Shebli	1988-1989	>145	EI	Mushahwar *et al*., 1993
Sudan				
E. Sudan	1985	2,012	X	Bradley, 1992; Irshad, 1999
Namibia				
Kavango	1983	16	EIA, PCR	Isaacson *et al* 2000; He *et al*., 2000
*Ivory **Coast***				
Tortiya	1983-1984	623	X	Sarthou *et al*., 1986
Morocco				
Casablanca	1994		EI, PCR	Benjelloun *et al.,* 1997; Meng *et al.,* 1999
Nigeria				
Harcourt	1997-1998	10	PCR	Buisson *et al*. 2000
Egypt				
Cairo	261	58	EI, PCR	Tsarev *et al*., 1999

RA, retrospective analysis of sera; EI, enzyme immunoassay; IEM, immune electron microscopy; PCR, polymerase chain reaction; X, exclusion of hepatitis A, B, and C viruses. A blank cell indicates that no data were available. For some large outbreaks, a smaller sample of cases was serologically examined.

young adults (15 to 40 years old)

- Low secondary attack rate among household contacts
- Severe fulminant hepatitis with a high case-fatality rate among sick pregnant women and
- The absence of chronic sequelae.

2.1.1 The Indian Subcontinent

Apart from the New Delhi outbreaks of 1955 and 1987 and the Ahmedabad outbreaks of 1976, 1982, 1984, 1986 and 1987 (Labrique *et al.*, 1999), epidemic and sporadic non-A, non-B (NANB) hepatitis outbreaks have been documented in Kashmir, northern India (1978, 1979 and 1981-1982; Khuroo, 1991), Central India (1990: Akluj, Beed, Rewa, Bijapur; Arankalle *et al.*, 1994); including Hyderabad (Jameel *et al.*, 1992), Kolhapur in the South West in both 1981 and 1991 (Arankalle *et al.*, 1994; Khuroo, 1991) as well as the largest South Asian epidemic which occurred in Kanpur, India, South East of New Delhi (Ray *et al*., 1991).

Outbreaks of epidemiologically similar cases of hepatitis E have also been reported in other countries of the Indian subcontinent including Myanmar (previously Burma), Bangladesh, Nepal and Pakistan (Labrique *et al*., 1999; Table 2.1).

Between June 1976 and August 1977, more than 20,000 icteric cases occurred in Mandalay, Burma, with a case fatality rate of 18 % in pregnant women. An epidemic of viral hepatitis involving 10,000 cases in the Kathmandu valley, Nepal, in 1973 was also reported to be associated with a high mortality rate in infected pregnant women (Bradley, 1992). Epidemics have been documented in Pakistan in 1984 (Mardan), 1985 (Rawalpindi and Quetta; Malik *et al.*, 1988), 1987 (Sargodha; Ticehurst *et al.*, 1992; Bryan *et al.*, 1994), 1988 (Abbottabad; Yarbough *et al.*, 1994) and 1993/94 (Islamabad;

Rab *et al.*, 1997) and the occurrence of hepatitis E in Pakistan was also recently inferred from the observation of cases of disease imported into the United States (de Cock *et al.*, 1987).

2.1.2 Documented epidemics in the rest of Asia and Eurasia

Stable foci of HEV disease exist in Central Asia in the former USSR as well as both Taiwan and the Chinese mainland (Balayan, 1997) including the Xinjiang Uighur Autonomous Region (XUAR; Yin *et al*., 1993 and 1994), Beijing, Liaoning province, Henian province (Wang *et al*., 1999) and Guangzhou, south of China.

In South-east Asia there are epidemic foci centered in jungle, riverine environments from Indonesia (Corwin *et al*., 1999), Viet Nam (Hau *et al.*, 1999) and Malaysia (Seow *et al*., 1999).

2.1.3 Epidemics in the New World

NANB hepatitis associated with faecally contaminated drinking water has been documented in two rural villages located south of Mexico City, Mexico (Bradley, 1992). More than 90 cases of presumed hepatitis E were recorded in Huitzililla, Mexico between June and October of 1986. Additional cases of NANB hepatitis were seen in Telixtac, Mexico shortly thereafter (Table 2.1). Virological and serological studies of stool and serum specimens obtained from affected persons, suggested that the aetiological agent responsible for disease in North America might be similar to that associated with hepatitis E in other regions of the world. This was later confirmed by Huang *et al*., in 1992 after cloning and sequencing the Mexican isolate.

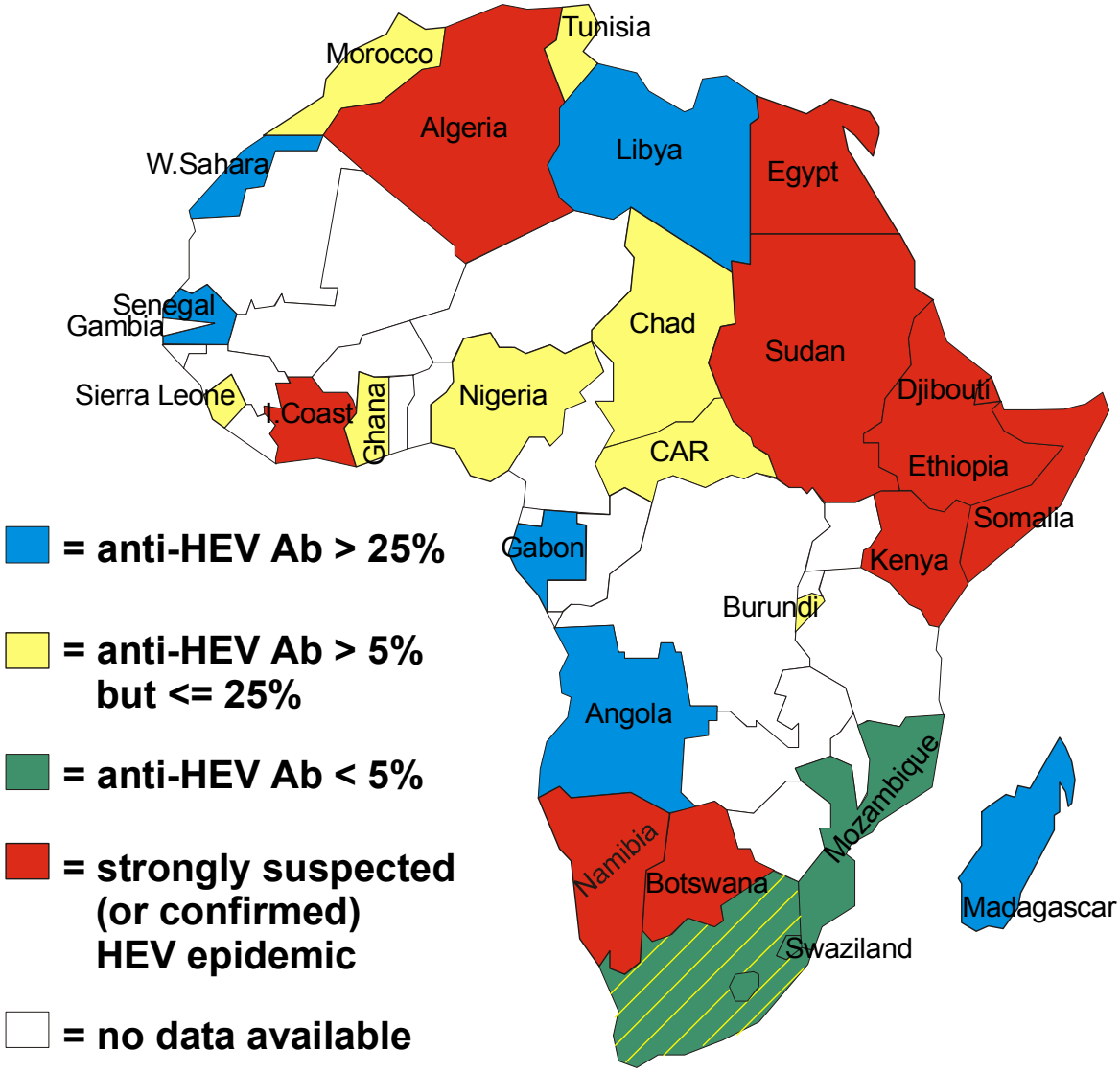

Figure 2.2. Distribution of HEV epidemics (red) in Africa together with regions of high (blue, defined in the text) or intermediate (yellow) endemicity or alternatively non-endemic regions (green). References for this map can been found in the relevant tables: Table 2.1, Table 2.2, Table 2.3 and Table 2.4.

2.1.4 Documented epidemics in North, East, West and Central Africa

Outbreaks of hepatitis E have also been reported in Africa, including Morocco, Algeria, Ivory Coast, Senegal, Nigeria, Egypt, Chad, eastern Sudan, Ethiopia, Somalia, Kenya and Angola (Table 2.1 and Figures 2.1 and 2.2).

Acute hepatitis E was diagnosed in 77.3 % of patients and 10.4 % healthy contacts after an outbreak in the south of Morocco in 1994 (Benjelloun *et al*., 1997). Controls collected from the west of Morocco were also found to be positive for anti-HEV IgG (6.1 %). Between October 1980 and January 1981, more than 780 cases of NANB hepatitis were documented in Algeria and linked to the use of faecally contaminated drinking water (Belabbes *et al*., 1985). Mostly young adults were affected and 9/9 infected pregnant women died as a direct result of NANB hepatitis. A similar outbreak with NANB hepatitis cases (N = 623) was observed in Tortiya, Ivory coast, between 1983 and 1984, again a high mortality rate among infected pregnant women was noted (Sarthou *et al*., 1986).

Large outbreaks of hepatitis E have also been documented in Eritrean and Tigrean refugees from Ethiopia residing in refugee camps in eastern Sudan (Bradley, 1992). More than 2,000 cases occurred in these refugees between June and October 1985, after the onset of heavy rains. Eleven of 63 individuals who died were pregnant women. Between 1985 and 1986, more than 2,000 cases and 87 deaths due to hepatitis E were noted in Ethiopian refugees residing in Tug Wajale and other camps in northwest Somalia (Bradley, 1992). The mortality rate among pregnant refugee women in their second and third trimester was greater than 17 % and standing faecally contaminated rain water was implicated as the vehicle for virus transmission.

Subsequently, an outbreak of acute HEV infection was reported among military personnel in northern Ethiopia between October 1988 and March 1989 in some military barracks in the Keren district (Tsega *et al*., 1991). Twenty-eight of the 30 (93 %) randomly collected sera from the patients were positive for anti-HEV, indicating that HEV infection was the most likely cause of this and other waterborne epidemic outbreaks

in a population already immune to HAV infection. There are indications that ET-NANB epidemics occur frequently in Ethiopia where crowding and poor hygiene prevail.

Somalia has frequently been the centre of massive humanitarian relief efforts. A major part of the relief effort has come in the form of medical aid because of the breakdown of medical care and the high rates of mortality due to preventable infectious diseases. One such humanitarian relief effort involved military troops from more than 20 countries deployed to Somalia on 9 December 1992 by the United Nations in an operation called Restore Hope (Burans *et al*., 1994). In support of this, the United States military established a diagnostic laboratory for infectious diseases, the Joint Forward Laboratory, in Mogadishu, Somalia. Staff members evaluated 31 Somalis, five displaced Ethiopians and three western relief workers who had acute clinical hepatitis. IgM antibody to HEV was found in 20 (65 %) of 31 Somalis, two (40 %) of five Ethiopians and two (67 %) of three western relief workers. These data indicate that HEV was a common cause of acute sporadic hepatitis in Somalia during the initial stages of Operation Restore Hope. The outbreak occurred in multiple, separate locations. This finding suggests that HEV is not just a cause of epidemic disease but that it is endemic in Somalia and a common cause of acute sporadic hepatitis.

HEV infection was confirmed to be the aetiological agent of two further NANB hepatitis epidemics, one observed in Algeria (1979-1980) and the other in Djibouti, the capital of a small East African country, FTAI in 1992 to 1993 (Coursaget *et al*., 1996). Among the cases involved in the NANB epidemics observed in Algeria and FTAI, HEV infections were diagnosed in 81 and 64 % of them respectively.

Apart from the outbreaks in Algeria, Sudan, Somalia, Morocco, Ethiopia and FTAI, other outbreaks have also been observed in Chad (van Cuyck-Gandre *et al*., 1996), Kenya (Mast *et al*., 1994), Nigeria (Buisson *et al*., 2000), the Gambia and Angola, all of which have the characteristics of HEV infections. Sporadic cases of HEV have been identified as well, in Egypt (Tsarev *et al*., 1999), Sudan (Hyams *et al*., 1992), Chad (Coursaget *et al*., 1998), Nigeria (Buisson *et al*., 2000), Tunisia and Burundi (Coursaget *et al*., 1996).

It is of note that imported infections observed in French patients or immigrants to France have been contracted in Algeria, Benin, Ivory Coast, Morocco and Senegal, confirming the results of the detection of HEV infection in African countries (Coursaget *et al*, 1996).

2.1.5 Documented outbreaks in southern Africa

The first indication of hepatitis E in southern Africa was a typical waterborne outbreak with 273 cases and at least four deaths in 1985 in Maun, northern Botswana (Byskov *et al*., 1989). It was known before the outbreak that most adults were immune to HAV, most had markers indicating past infection with HBV and a proportion of people had antibodies to hepatitis delta virus (HDV). The epidemic curve suggested that there was a major common source of infection, the disease appeared to have affected 1-2 % of the population; 90.3 % (214/237) of patients for whom information is available were aged 20 years or older; the disease was generally mild and affected pregnant women most severely. Forty-nine patients were admitted to hospital and at least 4/273 died. The main features of the outbreak conformed to public descriptions of waterborne epidemic NANB hepatitis and it is postulated that the disease was most severe in patients with acute HBV infection and in those with HDV superinfection. Although this is the first description of a possible outbreak of waterborne NANB hepatitis in southern Africa, the first confirmed

outbreak to be molecularly characterized occurred at Rundu in the Kavango region of north-east Namibia in 1983 (Isaacson *et al*., 2000; He *et al*., 2000). The disease was usually mild, except in pregnant women who made up six of the seven fatal infections reported in the outbreak. HEV was detected by RT-PCR in faeces from nine of 16 patients tested and their phylogeny was later established from a consensus sequence of 296 bp derived from the extreme 3' region of ORF2.

2.2 Prevalence of HEV antibody in populations in regions of intermediate endemicity

Countries bordering endemic regions in which sporadic acute hepatitis is rare or absent have often been found to have a fairly high prevalence of HEV antibodies within the healthy population. It is not clear whether this is the result of sub-clinical HEV infection as the individuals were neither sick nor in contact with apparent viral hepatitis. South Africa is an example of one such region (Table 2.2).

Table 2.2 Prevalence of HEV among populations in regions of high and intermediate endemicity

Site	Number tested	% Seroprevalence	Population tested	Reference
Thailand	269	6.5	Hmong people	Louisirirotchanakul *et al*., 2002
Hong Kong	355	16.1		Lok *et al*., 1992
Turkey	1,350	5.9	NANB	Thomas *et al*., 1993
Chile	594	7.4	Healthy children, prisoners & blood donors	Ibarra *et al*., 1994
Brazil	93	11.3	Blood donors	Trinta *et al*., 2001
Tunisia	72	4	NANB	Coursaget *et al*., 1995
Venezuela	223	5.4	Rural Amerindians	Pujol *et al*., 1994
South Africa	555	1.8	canoeists	Grabow *et al*., 1996
	227	2.6	Medical students	Grabow *et al*., 1996
	360	19.1	Rural adults	Tucker *et al*., 1996
	407	5.8	Urban adults	Tucker *et al*., 1996

The first known imported case of hepatitis E into South Africa was a 34-year-old pregnant woman who was admitted to the Groote Schuur Hospital in Cape Town in 1992 shortly after returning from holiday in Bombay, India and this nosocomial outbreak is described in paragraph 1.5.3 (Grabow *et al*., 1996). Hepatitis E was also diagnosed in a 45-year-old female admitted to the H F Verwoerd Hospital, now called Pretoria Academic Hospital, with clinical hepatitis shortly after returning from vacation in India (Grabow *et al*., 1996).

A typical anti-HEV IgG study in South Africa involved healthy, low-risk, medical students and high risk Dusi canoeists (Grabow *et al*., 1994 and 1996). HEV antibodies were found in ten of 555 canoeists (1.8 %) with regular exposure to sewage-polluted water and six of 227 (2.6 %) medical students with minimal exposure in this seroprevalence study. The overall prevalence of 16/782 individuals (2.05 %) suggests that HEV may be endemic in South Africa, particularly since four of the persons had never been out of the country. At least seven of the remaining positive cases had only visited other parts of Africa or Mediterranean countries on rare occasions, suggesting that exposure to the virus occurred in South Africa. None of the above individuals had a history of contact with others who were involved in outbreaks of viral hepatitis or related diseases. It would therefore appear that all of them represent sporadic cases of subclinical infection. The higher seroprevalence of anti-HEV among students (2.6 %) as compared with canoeists (1.8 %) indicates that regular exposure to sewage-polluted water was not a risk factor for HEV infection in the communities concerned. Grabow's studies classify South Africa as a typical non-endemic region. The source of HEV exposure in South Africa remains unknown and no symptoms of clinical disease were recalled. The 782 selected individuals can be considered as fairly representative of middle to high

socioeconomic communities in this country. The communities are not considered at high risk for a disease which is primarily associated with circumstances of inadequate public sanitation and malnourishment.

A study to evaluate the general population of South Africa, and particularly high risk communities, was published by Tucker *et al* in 1996. This study recorded the seroprevalence of anti-HEV in 360 rural and 407 urban adult black South Africans living in the Western and Eastern Cape provinces and showed a prevalence of 19.1 and 5.8 %, respectively, placing South Africa in an intermediate position between the typical endemic and non-endemic HEV regions of the world.

The seroprevalence for antibodies to HEV was also determined in a large group of Mozambican refugees living in Swaziland, a small landlocked kingdom within South Africa (van Rensburg *et al*., 1995). Serum samples collected from a total of 398 refugees located in two camps (Ndzevane and Malindza) showed a total anti-HEV prevalence of 1.5 %.

Other seroepidemiological surveys of hepatitis E in healthy individuals are shown in Table 2.2. In one survey in Hong Kong conducted to validate the recombinant-based enzyme immunoassay for the diagnosis of hepatitis E, serum from 394 patients with acute viral hepatitis and 355 healthy subjects was tested and 65 (16.5 %) patients with hepatitis were found to be positive for IgM anti-HEV and 57 (16.1 %) of the healthy subjects were found to be positive for IgG anti-HEV (Lok *et al*., 1992). In Turkey, the seroprevalence and risk factors for infection with HEV were analyzed in five different areas where antibodies to HEV were found in 80 (5.9 %) of 1 350 subjects (Thomas *et al*., 1993). The presence of anti-HEV was investigated in blood samples from 166

healthy children, 66 male prisoners and 237 blood donors from Chile. Sixty of 166 (36 %) healthy children were found to be positive for both anti-HEV and anti-HAV. Since there were no reports of clinical hepatitis in any of the children the infection must have occurred subclinically (Ibarra *et al*., 1994).

2.3 Incidence of HEV and reports of rare sporadic outbreaks in non-endemic regions

Acute sporadic hepatitis E has been reported from every continent: North America (Erker *et al.,* 1999; Schlauder *et al.,* 1998), Europe (Schlauder *et al.,* 1999; Balayan, 1993) including the United Kingdom (Wang *et al.,* 2001), industrialized regions of Asia including Taiwan (Hsieh *et al.*, 1999) and Japan (Takahashi *et al.*, 2002a and 2002b), Malaysia (Seow *et al.,* 1999), Australia (Moaven *et al.,* 1993) and Africa (Grabow *et al*., 1996). The high rate of prevalence of anti-HEV in healthy subjects is thought to be an indication of the existence of considerable subclinical infection (Lin *et al.*, 2000) although the constant presence of anti-HEV among normal populations particularly in non-endemic regions has yet to be adequately explained (Table 2.3). In one study the seroprevalence of antibodies to HEV in the normal blood donor population and two aboriginal communities in Malaysia were examined (Seow *et al*., 1999). IgG anti-HEV were detected in 45 (44 %) of 102 samples and 15 (50 %) of 30 samples from the two aboriginal communities respectively, compared to only 2 (2 %) of 100 normal blood donors and this indicates that the aboriginal communities are at high risk of exposure to HEV as compared to the normal (urban) population.

Table 2.3 Prevalence of HEV among normal/healthy populations in non-endemic countries

Site	Number tested	% Seropositive	References
Italy	2,233	1.5	Rapicetta *et al*., 1999
Haiti	105	3	Gambel *et al*., 1998
Belgium	556	2.2	Vandenvelde *et al*., 1994
Switzerland	94	3.2	Lavanchy *et al*., 1994
Venezuela	204	3.9	Pujol *et al*., 1994
Israel	1,139	2.6	Karetnyi *et al*., 1995
Netherlands	1,275	1.5	Zaaijer *et al*., 1993
UK	1,500	1	Balayan, 1997
Germany	972	2.1	Balayan, 1997
France	1,007	0.9	Balayan, 1997
Russia	168	1.2	Balayan, 1997
Ukraine	1,721	0.5	Balayan, 1997
Australia	279	0.4	Balayan, 1997
USA	386	2.1	Balayan, 1997
Malaysia	100	2	Seow *et al*., 1999
Vietnam	646	9	Hau *et al*., 1999
Spain	54	5.5	Jardi *et al*., 1993
Taiwan	984	4.4	Lee *et al*., 1994

No outbreak of clinically overt hepatitis E has ever been documented in the United States of America (USA), Canada, Europe, Japan, Australia and New Zealand. In these countries, only sporadic cases are occasionally observed among immigrants and travellers returning from disease-endemic areas. However, seroprevalence studies of different population groups suggest that 0.4-3.2 % of the population have been exposed to the virus (Lavanchy *et al*., 1994; Paul *et al*., 1994; Zaaijer *et al*., 1993; Zanetti *et al*., 1994). Recent findings in the Netherlands (Zaaijer *et al*., 1993), Italy (Zanetti *et a*l.,

1994), Greece (Tassopoulos *et al*., 1994), Taiwan (Hsieh *et al*., 1998), Argentina (Schlauder *et al*., 2000), Austria (Worm *et a*l., 2000), USA (Tsang *et al*., 2000; Schlauder *et al*., 1998) and Japan (Takahashi *et al*., 2001 and 2002a) suggest that the lack of a foreign travel history does not necessarily exclude the diagnosis of hepatitis E infection (Table 2.3). Sporadic cases in Italy and Spain have been attributed to the consumption of shellfish from sewage-polluted waters (Langer and Frosner, 1996; Table 2.4).

Table 2.4 Sporadic cases in non-endemic countries

Site	Source of study group	Number tested	% Seroprevalence
Netherlands	NANBNC hepatitis patients	6	2.1
Australia (Victoria)	Acute with travel history	one case	100
USA	Acute with travel history	6	100
Taiwan	NANBNC hepatitis patients	?	10
Italy	NANBNC hepatitis patients	10	6.5
Greece	NANBNC hepatitis patients	2	100
Argentina	Acutely infected patients	2	100
Japan	NANBNC hepatitis patients	87	13
Spain	NANBNC hepatitis patients	90	5.6
Austria	NANBNC hepatitis patients	one case	100

References: Jardi *et al*., 1993; CDC, 1993; Hau *et al*., 1999; Hsieh *et al*., 1998; Mizuo *et al*., 2002; Moaven *et al*., 1993; Schlauder *et al*., 2000; Tassopoulos *et al*., 1994; Zaaijer *et al*., 1993; Zanetti *et al*., 1994.

2.4 Molecular Epidemiology of HEV infection

Thus, HEV is not only an important cause of sporadic and epidemic disease in the developing world, but the widespread global mobility we enjoy today makes this infectious agent a concern for developed nations as well. Travel has increased the propensity for the rapid spread of infectious diseases; pathogens can be imported into susceptible populations, where they may easily become established and spread (Drabick

et al., 1997). Incidents of hepatitis E in persons from non-endemic countries is usually associated with a history of recent travel to an endemic area. As an export disease, HEV has been carried by migrant workers to host nations. As a result of the many recent export cases, physicians in the US and Europe, as well as in developing countries, are now being advised to consider HEV in the differential diagnosis of acute hepatitis (Labrique *et al*., 1999).

The advent of RT-PCR and sequence analysis has enabled characterization of the virus and global comparison of the various strains of HEV as well as providing a means of nucleic acid testing (NAT) for HEV. However, molecular assays are dependent on the presence of nucleic acid, viremia persists for only a few weeks and part of this time is prior to the onset of illness or symptoms (section 1.3.1). Thus of necessity, anti-HEV IgG assays must still be used to detect past HEV infection in normal healthy individuals particularly in residents of non-endemic areas (Table 2.2 and 2.3).

The genomes of several HEV strains from Asia: Myanmar (previously Burma; Tam *et al*., 1991; Aye *et al*., 1993), Pakistan (Tsarev *et al*., 1992; van Cuyck-Gandre *et al*., 2000), India (Panda *et al*., 2000; Donati *et al*., 1997), Nepal (Gouvea *et al*., 1998), China (Aye *et al*., 1992; Bi *et al*., 1993; Yin *et al*., 1994; Wang *et al*., 2000); US (Schlauder *et al*., 1998; Erker *et al*., 1999); and Mexico (Huang *et al*., 1992) have been entirely sequenced, and many partial sequences are available.

Phylogenetic clustering of HEV was previously observed to be geographically related. HEV sequences from Asia were closely related to, but could be distinguished from, those first found in Africa (Chatterjee *et al*., 1997; van Cuyck-Gandre *et al*., 1997; Tsarev *et al*., 1999; Meng *et al*., 1999; He *et al*., 2000). A single isolate from Mexico remains the

most genetically distant isolate (Huang *et al*., 1992) sequenced to date. Two human HEV isolates and a swine HEV isolate from the United States were described recently and they differed from the Asia-African group to the same extent as from the Mexican HEV strain (Schlauder *et al*., 1998; Erker *et al*., 1999). A novel strain of HEV closely related to US strains was recently identified from a patient returning from vacation in Thailand (Kabrane-Lazizi *et al*., 2001).

HEV sequences, orginally classified into three major genetic groups, have, with the characterization of the unique Chinese isolate, T1, been tentatively classified into four genotypes, I to IV (Widdowson *et al*., 2003; Figure 2.3). Genomic characterization of multiple isolates from Burma and from neighbouring locations in Northern Pakistan and Southwest China have demonstrated that isolates from close geographic regions tend to be more similar than isolates from distant regions (Aye *et al*., 1993; Yin *et al*., 1994). Genotype I consists of Asian and African isolates (Meng *et al*., 1999), genotype II includes the prototype Mexican isolate (Meng *et al*., 1999; previously designated as genotype III, Tsarev et al., 1999; Buisson *et al*., 2000; van Cuyck-Gandre *et al*., 2000), the United States isolates comprise genotype III (Schlauder *et al*., 1998; Erker *et al*., 1999; previously designated as genotype II, Tsarev *et al*., 1999; Buisson *et al*., 2000; van Cuyck-Gandre *et al*., 2000) and the unique HEV isolate, designated T1, is the prototype of genotype IV (Wang *et al*., 2000). Genotype IV also includes the Taiwanese isolate characterized by Meng *et al*., 2000.

From these data and others, it is evident that HEV infections are frequent in East and North African countries, and present as rare outbreaks in western and southern Africa.

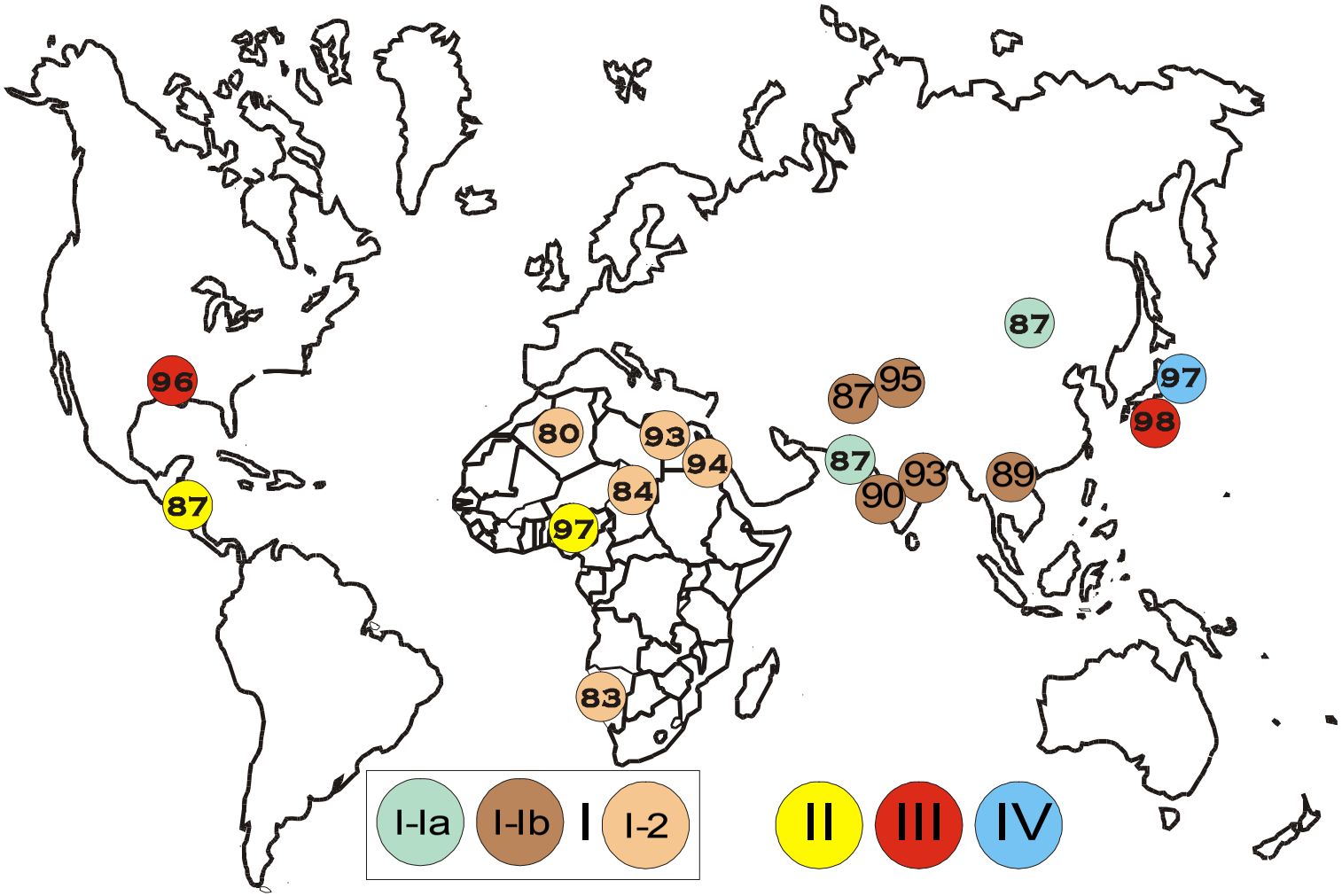

Figure 2.3. Distribution of HEV genotypic groups I—IV globally. Genotype I forms two subgroups, the Asian subtype 1-1 and the African subtype 1-2. Asian subtype 1-1 itself forms a Central Asian (1-1a) and a Burmese-India (1-1b) subcluster. Genotypes are represented by colour coded circles and the numbers within these represent the year of the outbreak from which the sequenced strain originated.

2.4.1 HEV genotype I

Many Asian and African isolates comprise genotype I. Two major subtypes of genotype I are apparent from phylogenetic analysis, namely Asian subtype 1-1 and African subtype 1-2. Within the Asian subtype, there are several clades referred to as the Central Asia (1-1a) and Burma-India (1-1b) clusters. Hepatitis E has been the predominant type of acute hepatitis in Nepal both in adults and children, in sporadic and epidemic forms. Six HEV isolates obtained during an 8-year period, from 1987 to 1995, in the Kathmandu valley of Nepal (Gouvea *et al*., 1997) showed close genetic relatedness among themselves (> 96.4 % identity) and with the Burmese (> 95.5 % identity) and Indian (> 95.3 % identity) isolates, and less so with the African (> 94.4 % identity) and the Chinese (> 91 % identity) isolates over the helicase, polymerase and capsid genes. Nepali isolates form part of the Burma-India evolutionary branch of subgroup 1-1 and the oldest isolate, TK78/87, is more similar to the Burmese isolates whereas the most recent isolates are closer to the Indian ones.

Partial genomic sequences of epidemic-associated Chinese strains of HEV were obtained by direct sequencing of PCR-amplified DNA. Comparison with previously published HEV sequences showed a clear relatedness of all Chinese strains to each other and to a Pakistani strain (Sar-55; Iqbal *et al*., 1989; Ticehurst *et al*., 1992; Tsarev *et al*., 1992). All eight Chinese strains examined had very similar sequences (98.5-99.8 % homology) in the regions examined and were much closer to the Pakistani strain, Sar-55, (97.9-98.4 % homology) than to the Burmese strains (92.5-93.3 % homology; Yin *et al*., 1993 and 1994) comprising the Asian sub-genotype (van Cuyck-Gandre *et al*., 2000). A second Pakistan HEV from a 1988 outbreak in Abbottabad (van Cuyck-Gandre *et al*., 2000) also belongs to the Central-Asian cluster of the Asian sub-genotype.

The African isolates, Morocco (Meng *et al*., 1999); Tunisia (Chatterjee *et al.,* 1997); Egypt (Tsarev *et al*., 1999); Namibia (He *et al*., 2000); Algeria and Chad (van Cuyck-Gandre *et al*., 1996 and 1997), most of which have been partially sequenced, are found to be closely related to, but distinct from, the Asian isolates in genotype I and they cluster into their own sub-genotype away from the Asian sub-genotype (Tsarev *et al*., 1999; He *et al*., 2000). In one study, HEV genome was detected by RT-PCR in faecal samples of two sporadic cases of hepatitis E in Cairo, Egypt. Sequences of the complete ORF2 and complete ORF3 were determined for the two HEV isolates (Tsarev *et al*., 1999). Four regions of the HEV genome were used for phylogenetic analysis. In all four regions, African strains were always clustered together, thus further confirming the geographic origin of HEV strain divergence.

2.4.2 HEV genotype II

In all parts of the genome, the Mexican strain was the most different of the sequences studied (Purcell, 1996). The 5' non-coding (NC) region of most strains was 26 to 27 nucleotides in length, but the 5' NC region of the Mexican strain (originally reported to be three nucleotides in length; Huang *et al*., 1992) is now reported to be 24 nucleotides long (Zhang *et al*., 2001). In the ORF1 region, the Mexican strain was over 25 % different from the Asian strains at the nucleotide level, whereas the Asian strains differed from each other by less than 10 %. The degree of genetic heterogeneity of the Asian strains in ORF2 was similar to that in ORF1 but they differed from the Mexican strain by approximately 20 %. ORF3 was the most highly conserved of the ORFs (with the exception of the 3' end), varying by less than 2 % among the Asian strains and by approximately 10 % in the Mexican strain. Therefore, due to its diversity from the Asian isolates, the Mexican strain is the prototype strain of genotype II (Meng *et al*., 1999)

previously designated as genotype III (Tsarev *et al*., 1999; van Cuyck-Gandre *et al*., 2000).

Recently discovered Nigerian isolates cluster together with the Mexican isolate in genotype II (Buisson *et al*., 2000). Sporadic cases of acute hepatitis E among 10 native Nigerian adults were reported in Port-Harcourt, Nigeria. HEV was detected in serum and/ or faecal samples of seven patients. Restriction analysis used to distinguish genotypes I and II showed that all Nigerian strains have a pattern similar to the Mexican strain but displayed a *BsmI* restriction site at nucleotide 213 as do most African HEV strains sequenced so far. Sequence analysis performed from PCR products displayed strong homogeneity between the HEV isolates, determining a regional cluster. Phylogenetic analysis of nucleotide sequences revealed that these strains were more related to the Mexican prototype in genotype II (87 % homology in ORF1, 80 % homology in ORF2) than to either the African strain genotype I (74 % homology in ORF1, 77 % homology in ORF2) or the USA strain genotype III (75 % homology in ORF1, 77 % homology in ORF2). Genetic divergence up to 15 % in ORF2 with the Mexican genotype clearly defined a new sub-genotype within genotype II. The Nigerian strains were more homologous to genotype II strains (96 %), at the amino acid level, than with genotype I strains (92 %). This study determined the co-existence of genotypes I and II in Africa.

2.4.3 HEV genotype III

Isolates clustering together and away from the Asian, African and Mexican isolates have been identified in patients with acute hepatitis in the US and Europe. The latter include isolates from Austria (Worm *et al*., 2000), Italy (Zanetti *et al*., 1999), Argentina (Schlauder *et al*., 2000), Greece (Schlauder, *et al*., 1999), Spain (Pina *et al*., 2000),

United Kingdom (Wang *et al*., 2001), and the Netherlands (Widdowson *et al*., 2003). These US and European isolates comprise genotype III (Mizuo *et al*., 2002; previously designated genotype II).

A variant of HEV, designated HEV US-1, was identified in a hepatitis patient in the US; the patient had no history of travel to areas where HEV is endemic (Schlauder *et al*., 1998). The HEV US-1 strain is significantly divergent from other human HEV isolates with nucleotide identities ranging from 76.8 to 77.5 %. Phylogenetic analysis indicate that HEV US-1 and a recently discovered HEV variant from swine (Meng *et al*., 1997), may represent separate isolates of a new strain of HEV, significantly divergent from the Mexican and Burmese strains, (Schlauder *et al*., 1998). Later, the near full-length sequences of HEV-US1 and a second US isolate (HEV-US2) were reported (Erker *et al*., 1999). HEV-US2 was identified in a US patient suffering from acute viral hepatitis. These sequences verify the presence of a new HEV strain in North America and provide information as to the degree of variability between variants. The HEV-US nucleotide sequences are 92 % identical to each other and only 74 % identical to the Burmese and Mexican strains. Amino acid and phylogenetic analysis also demonstrate that the US isolates are genetically distinct, clustering away from the Burmese and the Mexican isolates.

2.4.4 HEV genotype IV

Extensive diversity has been noted among HEV isolates from patients with acute hepatitis in China (Wang *et al*., 1999), Taiwan (Wang *et al*. 2000) and Japan (Takahashi *et al*., 2002b), which are distinct from the original Chinese isolates and comprise genotype IV (Mizuo *et al*., 2002). This suggests that HEV of two distinct genotypes

circulate in China.

2.5 Specific objectives of this study

Serological and molecular tests for HEV were developed only recently and the first molecular characterization of the virus in southern Africa was only published in the year 2000 (He *et al*., 2000). Prevalence studies based on serological assessment from different populations, have suggested activity by this agent in South Africa (Grabow *et al*., 1996; Tucker *et al*., 1996) but the virus has never been detected in material from patients with clinical episodes of hepatitis. At the National Institute for Virology (NIV), now called the National Institute for Communicable Diseases (NICD), where the study was conducted, all but one of the hepatitis E cases that have been diagnosed in the routine diagnostic laboratories were in-patients returning from the Indian subcontinent and this also has been the experience of other academic laboratories (Grabow *et al*., 1994).

Although several HEV strains from different geographical areas have been sequenced (Aye *et al*., 1992, 1993; Huang *et al*., 1992; Tsarev *et al*., 1992; Bi *et al*., 1993; Yin *et al*., 1994; Panda *et al*., 1995; Chatterjee *et al*., 1997; Donatti *et al*., 1997; Gouvea *et al*., 1998; van Cuyck-Gandre *et al*., 1997), data are limited from southern Africa. Recent reports (Buisson *et al*, 2000 and He *et al*., 2000) confirmed that HEV is present in southern Africa and characterized the strain of the viruses that resulted in the outbreaks which occurred in Nigeria (1997/8) and in Namibia (1983) respectively. The authors compare sequence data from portions of ORF1 and ORF2 of the HEV genome from patient specimens obtained during the outbreaks.

The objectives of this study are thus:

- To establish an HEV PCR for molecular diagnosis of HEV and to use this PCR to detect HEV nucleic acid in specimens collected from an acute viral hepatitis outbreak in Rundu, Namibia, in 1995 and from a previous outbreak in Maun, Botswana, in 1983.

- To characterize, using partial sequence analysis, the HEV strain involved in the outbreaks and to compare the sequenced strains with other HEV strains circulating in sub-Saharan Africa.

- To relate the sequence information to available data from other parts of the world.

CHAPTER 3: MATERIALS AND METHODS

3.1 Introduction

A non-A non-B hepatitis outbreak involving more than 600 people occurred in 1995/6 in the Kavango region of northern Namibia, centered on the town of Rundu (Surveillance Bulletin, 1996). Patients presented with symptoms of viral hepatitis (jaundice, fever, vomiting, dark urine, diarrhoea, hepatomegaly, haemorrhagic signs and hypochondrial pain) and there were at least three fulminant cases, one of whom was a pregnant woman. Blood specimens were obtained from 564 patients who were aged from 5 days to 80 years (median, 25 years) and only 536 are on record with full details. Patients' sera were tested for HEV IgG using the Abbott HEV enzyme immunoassay (EIA) Kit (Abbott GmbH Diagnostika, Wiesbaden-Delkenheim, Germany) after onset of illness. Samples were considered anti-HEV reactive when the ratio of absorbance to cut-off (OD/CO) was greater than 1. Of the 536 patients tested for HEV IgG, 351 tested positive, 151 cases tested negative and the rest of the patients were not tested for HEV IgG. The patients presented with a total bilirubin range of 17-724 μmol/l, ALT range of 26-6,780 IU/L and an aspartate transaminase activity (AST) range of 25-10,540 IU/L. Based on the normal reference ranges for the chemistries, which are as follows: ALT in males ranges from 1-42 IU/L, in females it is 1-32 IU/L; AST in males ranges from 1-37 IU/L, in females it is 1-31 IU/L; total bilirubin during 0-14 days ranges from 0-20 umol/L and over 14 days it ranges from 0-7 umol/L; it was observed that only three of 536 patients on record, two females and one male, had chemistries within the normal ranges. One example of a normal was a 33-year-old male who had the following chemistries: total bilirubin-28

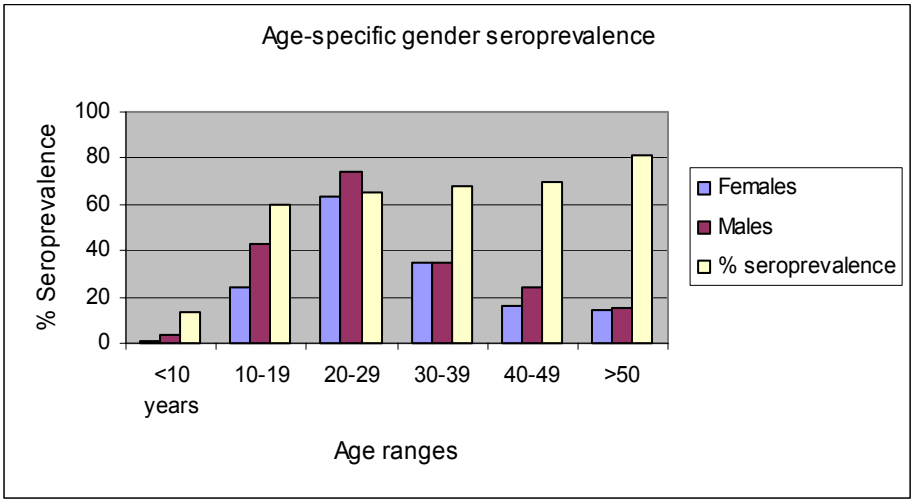

Figure 3.1 HEV seroprevalence obtained from blood samples from 564 patients suspected of HEV infection. The highest antibody prevalence (81 %) was observed in the > 50 years age group and a low prevalence of 13 % was observed in patients aged < 10 years and 60-70 % prevalence in the other age groups, that is, 10-19, 20-29, 30-39 and 40-49 age groups (male to female ratio 1.4:1).

umol/L, AST-25 IU/L and ALT-27IU/L, on his seventh day of illness.

The source of the outbreak is suspected to be the water supply which was compromised by the drought and disturbances in reticulation following work on the pipes approximately six months earlier. HEV antibodies were detected in approximately 75 % of sera specimens from icteric patients with a prevalence of 13 % in patients aged < 10 years and 81 % in those aged > 50 years. The highest antibody prevalence (81 %) was observed in the > 50 years age group with about 60-70 % prevalence seen in the other age groups, that is, 10-19, 20-29, 30-39 and 40-49 age groups (Figure 3.1).

Antibodies were not detected in any of the children under the age of five years and therefore a diagnosis of HEV could not be made conclusively. IgG avidity tests performed on a portion of specimens from icteric patients demonstrated low avidity indices with some indices < 10 %. Approval to undertake the study was obtained from the Ministry of Health and Social Services of the Republic of Namibia and consent was

obtained from the patients in order to use their specimens for HEV studies (Appendix C).

Another outbreak of suspected water-borne epidemic non-A non-B hepatitis with 273 cases occurred from June to December 1985 in northern Botswana (Byskov *et al*., 1989), as mentioned in section 2.1.5. The serum samples were separated into three groups: acute phase taken 0-2 weeks after onset of jaundice, early convalescence taken at 3-6 weeks, and late convalescence taken about nine months after the clinical episode. Residual samples of seven sera from the first group, 42 from the second and 28 from the third, (total 77), which had been stored at –70 ºC were sent to NICD for HEV antibody tests using the Abbott EIA kits (Swanepoel *et al*., 1995). Anti-HEV IgG antibody was detected in 7/7 (100 %) acute, 36/42 (85.7 %) early convalescent, and 19/28 (67.9 %) late convalescent phase sera. Avidity index of the antibody, an index of recent primary exposure, was significantly greater in specimens taken nine months after the onset of illness than in sera taken during the first 30 days of infection.

Clinical specimens, including human stool and serum samples, were obtained from the two outbreaks mentioned above in order to characterize the virus that caused the outbreaks. The hypothesis is that hepatitis E was the causative agent responsible for the two outbreaks.

Two independent regions of the HEV genome, ORF1 and ORF2 were targeted for RT-nested-PCR amplification, in particular the polymerase (RdRp) region in ORF1 and the ORF2 region towards the carboxy terminal end (3'-end) of the genome. The ORF1 region was targeted because a plasmid with an HEV ORF1 insert was available as a positive control for PCR amplification. The ORF2 region was targeted because it is a highly immunogenic epitope (Reyes *et al*., 1993) and most of the published HEV

sequences from Africa are from this region and this would allow for a meaningful comparative sequence analysis.

Some of the clinical specimens that were used in this study had been stored for more than 15 years. The virus is, reportedly, difficult to isolate (Ray *et al*., 1991; Turkoglu *et al*., 1996) and this Chapter provides a detailed account of the procedures used to optimize PCR conditions for effective amplification of both the ORF1 and ORF2 regions of the HEV genome. The aim was to generate a sensitive and reproducible HEV RT-PCR and to use the fragments generated to characterize the HEV strain responsible for the two outbreaks.

3.2 Patients and clinical material

Clinical material, including human stool and serum samples obtained from 536 patients from a documented outbreak of hepatitis E in Rundu, Namibia in 1995 and serum specimens obtained from 77 patients from Maun, Botswana in 1983, were stored at -70 ºC and were available for this study. From the 536 specimens from Namibia, 183 specimens (88 stool and 95 serum specimens) were chosen randomly and only 16 from a total of 77 serum specimens from Botswana were used in this study. The patients from both outbreaks (Namibia and Botswana) suffered from mild disease and presented with jaundice, hypochondrial pain, dark urine, fever, arthralgia, mild haemorrhagic signs and fatigue (Surveillance Bulletin, 1996; Swanepoel *et al*., 1996). The Namibian patients' age ranged from 5 days to 80 years (median, 25 years), (Surveillance Bulletin, 1996). Out of 237 patients from the Botswana outbreak, 214 (90.3 %) of all patients were aged 20 years or older (Swanepoel *et al*., 1996).

A plasmid DNA was used initially as a positive control in this study and was obtained from Dr T Tucker (then at the University of Cape Town) with permission from Dr S A Tsarev (then at the Walter Reed Army Institute of Research). The plasmid consists of an HEV insert of 2,002 bp cloned into the circular 3 kb pCR1000 DNA vector which was constructed from an *E. coli* phage and contains a kanamycin resistant site (www.invitrogen.com). The cloning site disrupts the β-galactosidase marker gene and recombinants (colourless plaques) were differentiated by the plaque assay system. The inserted DNA fragment was from the Pakistani Sar55 strain and included most of the helicase region (699 bp) and the polymerase/replicase region (1303 bp), both in ORF1 (Yin *et al*., 1994).

3.3 Plasmid DNA preparation

3.3.1 Transformation of competent cells

Fifty microliters of JM109 bacterial high efficiency competent cells were added together with 1.5 µl of plasmid in a 1.5 ml microcentrifuge tube, gently mixed and left on ice for 20 minutes. The mixture was heat shocked for 45 seconds in a water bath at 42 ºC, followed by another 2 minutes on ice. SOC medium (Appendix A1.1) was added to the mixture and incubated in a 37 ºC G24 shaking incubator (250 rpm; New Brunswick Scientific Co. Inc. Edison, N.J, USA) for 1.5 hours. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal)/isopropyl-β-D-thiogalactopyranoside (IPTG) Luria broth (LB) agar plates were prepared by spreading 40 µl 50 mg/ml IPTG and 30 µl 100 mM X-Gal onto petri dishes with LB agar (Appendix A1.2) containing 10 mg/ml kanamycin antibiotic. Fifty microliters of the transformed mixture was spread onto X-Gal/IPTG LB agar plates and incubated overnight at 37 ºC. *E. coli* cells infected with the vector will, in the

presence of the *lac* operon inducer IPTG, produce a functional ß-galactosidase enzyme which hydrolyses the substrate X-gal to give a blue dye. Appropriate insertion of foreign DNA interferes with the production of the enzyme thus recombinant vectors give colourless plaques on an *E. coli* lawn and can be picked out and grown.

One millilitre of LB growth medium (Appendix A1.3) containing 5.0 µl kanamycin (50 µ g/ml) was inoculated with two colourless plaques and incubated at 37 ºC in a G24 shaking incubator (250 rpm) for 2 hours, followed by addition of 3 ml LB growth medium and incubated for 16 hours (overnight) in a 37 ºC G24 shaking incubator (250 rpm) and used to make glycerol stocks.

3.3.2 Plasmid DNA stock preparation

Glycerol stocks of the plasmid DNA were made by adding 150 µl of glycerol to 850 µl of the bacterial culture incubated for 16 hours. These were mixed, quick cooled with liquid nitrogen and stored at -70 °C.

3.3.3 Plasmid DNA extraction and purification

Extraction and purification of plasmid DNA was performed by using four different commercial kits in order to obtain optimal yields and quality of plasmid DNA. The methods were used according to the manufacturer's protocols as described for each briefly below.

● ***The Nucleon plasmid minipreps kit*** (Amersham International plc, Buckinghamshire, England) protocol required cells from 3 ml of bacterial culture harvested after 16 hours and centrifuged in an Eppendorf 5415C centrifuge (Eppendorf-Netheler-Hinz GmbH,

Hamburg, Germany) at maximum speed (14 000 rpm). The supernatant was discarded and the cell pellet resuspended in solution 1. Solution 2 was added to the re-suspended pellet for alkaline cell lysis then solution 3 was added for precipitation of cellular debris. The neutralized lysate was centrifuged and the supernatant mixed with DNA binding resin and centrifuged again. The pellet was then washed twice with the 1 X wash buffer. The pellet was left in an open tube for 5 minutes at 60 °C to remove all traces of ethanol from the wash buffer. The pellet was resuspended in the elution buffer and centrifuged to separate the eluate containing the adsorbed plasmid DNA from the supernatant.

● ***The Wizard (TM) Plus Minipreps DNA Purification Systems kit*** (Promega Corporation, Madison, USA) protocol also required a cell pellet from 3.0 ml of bacterial culture obtained as above and resuspended and lysed using the cell resuspension and cell lysis solutions respectively. The cells were neutralized and mixed with the resin suspension. The mixture was applied to a minicolumn followed by wash solution. The minicolumn was attached to a 1.5 ml microcentrifuge tube and centrifuged to dry the resin. The minicolumn was then attached to a new microcentrifuge tube and 50 µl of distilled water was added to the column and centrifuged to elute the DNA.

● ***The QIAGEN plasmid purification kit*** (QIAGEN, GmbH, Germany) used cell stocks which were cultured differently from the two methods above. One hundred millilitres of LB containing 500 µl of 50 µg/ml kanamycin antibiotic was inoculated with a loop which had been dipped in the glycerol stock and incubated in a 37 °C G24 shaking incubator (300 rpm) for 16 hours. The bacterial culture was then centrifuged and the pellet resuspended in buffer P1 containing RNase A. The lysis reaction was initiated by adding buffer P2 followed by the addition of buffer P3 for precipitation. The reaction mixture

was then incubated and centrifuged at 20,000 g for 30 minutes at 4 °C. The supernatant was re-centrifuged for 15 minutes, applied to the QIAGEN-tip containing the resin and allowed to enter the resin by gravity flow. The QIAGEN-tip was then washed with buffer QC and the DNA eluted with buffer QF. The eluted DNA was precipitated by adding isopropanol, and then washed with 70 % ethanol and centrifuged. The pellet was air-dried and the DNA re-dissolved in distilled water.

● ***The High Pure Plasmid Isolation miniprep kit*** (Roche Diagnostics Corporation, Mannheim, Germany) protocol uses a cell pellet from 4 ml of bacterial culture resuspended in suspension buffer containing RNAse followed by addition of lysis buffer. The mixture was incubated at room temperature for 5 minutes when a chilled binding buffer was added and the mixture incubated on ice for another 5 minutes and centrifuged. The pellet was discarded and the supernatant centrifuged in a filter tube and washed twice with buffer I and II respectively. The pellet was resuspended in the elution buffer and centrifuged to elute a purified plasmid DNA.

3.3.4 Agarose gel electrophoresis

The eluted plasmid DNA (5 µl) was mixed with 5 µl bromophenolblue/xylenexyanol loading dye and analysed by electrophoresis at 100 volts (V) for an hour on a 1 % agarose gel (Appendix A2.1 and A2.2) containing 0.5 mg/ml ethidium bromide (EthBr) in tris borate/EDTA (TBE) buffer. Five microlitres (0.25 µg) of 1:5 DNA molecular weight marker IV was used to assess the size of the plasmid DNA which was expected to be approximately 4,980 bases. The DNA was detected by ultraviolet illumination and pictorial records were kept using the GelDoc system (UVP, Upland, California, USA).

3.3.5 Purification of plasmid DNA

Plasmid DNA was ethanol precipitated and the concentration of monovalent cations adjusted by dilution with 0.5 M sodium acetate (pH 5.0, Sambrook *et al*., 1989) to remove impurities. One hundred and thirty microlitres of DNA was mixed with 2 volumes of ice-cold ethanol and placed at -70 °C for 30 minutes. The mixture was then centrifuged at 12,000 g for 10 minutes and the supernatant discarded. Five hundred microlitres of 70 % ethanol was added to wash the cell pellet which was then centrifuged again at 12,000 g for 2 minutes and the supernatant discarded. The tube containing the cell pellet was then left open at room temperature, for evaporation of ethanol, and the cell pellet resuspended in 50 µl distilled water.

3.4 Verification of the Plasmid

3.4.1 Restriction enzyme digestion and verification of the Plasmid

Ten microlitres of vector plus insert was digested separately with 10 units of one, or more, of the following restriction endonucleases (REs; Boehringer Mannheim), *Hind III*, *Sma I* and *Sac I,* using the buffers supplied and the conditions recommended by the manufacturers in a final reaction volume of 15 µl. After digestion, the entire reaction volume was mixed with gel-loading dye and analyzed by electrophoresis as outlined in section 3.3.4. *Hind III* RE has only one cutting site in the plasmid vector (1,163 bp) and none in the HEV insert. *Hind III* digestion was also performed with a control plasmid, the pMosblue vector (2,887 bp) containing a Kaposi sarcoma virus insert (804 bp; Alagiozoglou *et al*., 2000). *Hind III* has two cutting sites for the control plasmid, one in the insert and one in the vector. *Sac I* RE cuts the plasmid vector once (1,143 bp) and the

Table 3.1 Primers used for PCR amplification

ORF	Primer	Location	Sequence
ORF1[a]	External sense	3853-3877	ACA TTT GAA TTA ACA GAC ATT GTG C
	External antisense	4926-4903	ACA CAT CTG AGC TAC ATT CGT GAG
	Internal sense	4120-4140	GAC GTG TCC AGG ATC ACC TTC
	Internal antisense	4678-4654	ACT CAC TGC AAA GCA CTA TCG AAT C
ORF2[b]	External sense	925-947	CGC AAC CTC ACC CCT GGT AAC AC
	External antisense	2012-1991	CAG AAA GAA GGA GGG CAC AAG C
	Internal sense	1256-1277	CTC AGC AGG ATA AGG GTA TTG C
	Internal antisense	1983-1963	CTA TAA CTC CCG AGT TTT ACC

[a] Nucleotide locations of primers are numbered from the start of ORF1 with respect to the sequence isolated from the Xinjiang epidemic (GenBank Accession no.: NC_001434)
[b] Primer sequences are numbered from the start of ORF2 with respect to the sequence isolated from the Xinjiang epidemic (GenBank Accession no.: NC_001434)

insert twice (3,497 and 4,096 bp). *Sma I* RE cut once in the plasmid vector (1,395 bp) and once in the insert (2,987 bp).

3.4.2 Sequencing of cloned plasmid DNA

Plasmid DNA was sequenced using the Thermosequenase cycle sequencing kit (Amersham International plc, Buckinghamshire, England) which relies on amplification of signal intensity by asymmetric PCR. The plasmid was diluted to 0.15 µg in 16 µl distilled water to which 2 µl of 2 pmol/µl primers was added. The primers used were universal fluorescent primers (M13 reverse primer and M13-20 forward primer). Four, 0.5 ml microcentrifuge PCR tubes labeled A, C, T and G, (four for each plasmid run) were filled with 2 µl of the appropriate A, C, T or G dideoxy termination nucleotide. Six microlitres of template/primer mix was added to the four tubes containing the dideoxy termination nucleotides and then immediately placed on the GeneAmp PCR System 2400

thermal cycler (The Perkin Elmer Corporation, Norwalk, USA) with the following cycling conditions: 96 °C for 5 minutes, 30 cycles of denaturation at 96 °C for 30 seconds and priming and amplification at 60 °C for 30 seconds followed by one cycle of denaturation for 30 seconds and 5 minutes of amplification. The reaction was kept at 4 ° C until 4 µl of stop solution was added. Samples were denatured at 90 °C for 3 minutes before 4.0 µl was loaded onto a single well of a 5 % Long Ranger gel (Appendix A6) of the ALFexpress^{TM} DNA Sequencer (Amersham Pharmacia Biotech Ltd., Buckinghamshire, England).

3.5 PCR optimization using plasmid DNA

External sense (nt 3853-3877) and antisense (nt 4926-4903) ORF1 primers (Table 3.1) were used for PCR amplification of a 1,074 bp target of the polymerase region in ORF1 of the HEV genome. The activity of two enzymes, Taq DNA polymerase (Roche Diagnostics Corporation, Mannheim, Germany) and Supertherm DNA polymerase (JMR Holdings, UK) were compared in parallel experiments with annealing temperature optimization. The PCR reaction mixture contained a final concentration of the following reagents: 400 nM of both sense and anti-sense primer, 0.125 µl (0.625 U) of the DNA polymerase (5.0 U/µl) being evaluated, 5 µl of 10X PCR reaction buffer (Roche Diagnostics Corporation, Mannheim, Germany) consisting of 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate [(dNTPs) – 1 mM diluted 1:5 for each stock concentration – deoxy-adenosine triphosphate (dATP), deoxy-guanosine triphosphate (dGTP), deoxy-cytidine triphosphate (dCTP) and deoxy-thymidine triphosphate (dTTP)], and 2.0 µl of pooled plasmid DNA (maximum 0.6 µg/µl) in a final volume of 50 µl made up with distilled water. For annealing temperature optimization the Robocycler gradient

40 machine (Stratagene, California, USA) was used with the following thermal cycling conditions: 35 cycles of a 95 °C denaturing step for 30 seconds, annealing at 42 to 56 °C for 60 seconds (42, 44, 46, 48, 50, 52, 54, 56 ºC - temperature gradient of 2 °C), and amplification at 72 °C for 90 seconds. Five microlitres of the PCR products were analyzed on a 1 % agarose gel by electrophoresis as described in section 3.3.4.

3.6 Nucleic acid extraction from clinical specimens

3.6.1 Clarification of stool specimens

The stool specimens were clarified as follows: using a 1.0 ml syringe, 500 µl of chloroform was added to a McCartney bottle containing 10 ml of 0.5 % gelatin buffered saline (GBS, Appendix A3) with glass beads. A 20 % suspension was made by adding 2.0 g of stool using an applicator stick. The suspension was shaken vigorously for 20 minutes and 1.0 ml of sample was added to a 1.5 ml microtube (Sarstedt AG & Co., Germany) and centrifuged at 23,000 rpm for one hour at 4 °C using the Jouan MR22*i* centrifuge (The Scientific Group, PTY/Ltd, Johannesburg, S.A). The supernatant was discarded and the pellet resuspended in 140 µl phosphate buffered saline (PBS, Appendix A4) for RNA extraction.

3.6.2 Viral RNA extraction

RNA extraction from both stool and sera specimens was performed using either the High Pure Viral RNA Kit (Roche Diagnostics Corporation, Mannheim, Germany) or the QIAamp® Viral RNA isolation kit (QIAGEN, GmbH, Germany) as recommended by the manufacturers. Using the High Pure Viral RNA Kit, 400 µl of binding/lysis buffer was added to 200 µl of sample (either clarified stool or serum) and mixed well. The sample

was then transferred into a high pure filter column which was placed in a collection tube and centrifuged at 10,000 rpm for 15 seconds. The column was then placed into a new collection tube and washed twice with 450 µl of wash buffer. This ensured that the bound nucleic acids are purified from salts, proteins and other impurities. Finally viral RNA was eluted in 50 µl of elution buffer.

Alternatively RNA was extracted using the QIAamp® Viral RNA isolation kit (QIAGEN, GmbH, Germany) according to the manufacturer's instructions: Five hundred and sixty microlitres of lysis buffer was added to 140 µl of the sample and incubated at room temperature for 10 minutes. This ensured isolation of intact viral RNA as well as the inactivation of RNases. After adding an equal volume of 100 % ethanol, the mixture was loaded onto the QIAamp spin column. Salt and pH conditions ensure that RNA binds to the silica-gel column. Proteins and other contaminants were washed through the column using two different wash buffers. Finally, the RNA was eluted in a RNase-free buffer.

3.7 Amplification of clinical specimens

Stool and sera specimens obtained from the 1995 Namibia outbreak were used for a nested RT-PCR using the same external and internal primers (Table 3.1) and conditions (section 3.5) optimized with the plasmid to amplify a first round fragment of 1,074 bp and a second round fragment of 559 bp within the HEV polymerase region of ORF1.

A second region of the HEV genome, located towards the carboxy terminal end (3'-end) of ORF2, was targeted to amplify a first round fragment of 1,088 bp and a second round fragment 728 bp. Primers for both regions were designed using the Primer Detective and DNASIS software (version 7.8).

3.7.1 Titration of magnesium ion concentration

The PCR conditions for plasmid DNA described in section 3.5 were optimized for the ideal $MgCl_2$ concentration to amplify the ORF1 region from clinical specimens using the Supertherm DNA polymerase (JMR Holdings, UK). The following six reaction buffers were evaluated: 10X Supertherm PCR reaction buffer (JMR Holdings, UK) consisting of 15 mM $MgCl_2$, 10X Roche PCR reaction buffer (Roche Diagnostics Corporation, Mannheim, Germany) also consisting of 15 mM $MgCl_2$ and four 10X in-house buffers containing 10, 15, 20 and 25 mM $MgCl_2$ respectively (Appendix A5). The Biometra thermal cycler (Biomedizinische Analytik, GmbH, Göttingen, Germany) was used with the following cycling conditions: First round RT-PCR - 42 °C for one hour (reverse transcription [RT] step), followed by 35 cycles of 95 °C for 30 seconds, 50 °C for 1 minute and 72 °C for 90 seconds followed by a last elongation step at 72 °C for 7 minutes. The reaction mixture was used as described in section 3.5 and the final reaction conditions were as mentioned below (section 3.7.2). Nested PCR (100 µl) was performed with 5 µl of first round PCR product for each of the six reaction buffers evaluated. The nested PCR reaction mixture was the same as the first round PCR reaction mixture (section 3.5) but with the ORF1 inner sense and antisense primers (Table 3.1) and the same cycling conditions used for the first round PCR with the exception of the RT step. Ten microlitres of the PCR product was analyzed on a 1 % agarose gel by electrophoresis as described in section 3.3.4.

The RT-PCR conditions described for ORF1 above were optimized for amplifying the 3'-end of ORF2. The Supertherm DNA polymerase was used together with its 10X corresponding PCR reaction buffer (JMR Holdings, UK). Titration of magnesium ion

concentration was only optimized with the Supertherm PCR reaction buffer and was used at two different initial concentrations of $MgCl_2$, that is, 10 mM and 15 mM $MgCl_2$, respectively. The annealing temperature was also optimized for ORF2 using the Robocycler gradient 40 machine (Stratagene, California, USA) with the following thermal cycling conditions: 42 °C for 60 minutes, 35 cycles of a 94 °C denaturing step for 60 seconds, annealing at 42 to 56 °C for 60 seconds (42, 44, 46, 48, 50, 52, 54, 56 ºC - temperature gradient of 2 °C), and amplification at 72 °C for 80 seconds. The final concentration of the PCR reaction mixture was the same as described for ORF1 (section 3.5) but with the use of ORF2 external sense and antisense primers (Table 3.1). Second round (nested) PCR (100 µl) was performed with 10 µl of first round PCR product and 10 µl of the nested PCR products were analyzed on a 1 % agarose gel by electrophoresis (section 3.3.4).

3.7.2 Final RT-PCR

Final RT-PCR concentrations for amplifying the polymerase region in ORF1 were as follows: First round RT-PCR - 400 nM of both ORF1 external sense and antisense primers (Table 3.1), 200 µM each dNTPs, 0.625 U of Supertherm DNA polymerase (JMR Holdings, UK), 1.0 mM $MgCl_2$ in-house PCR reaction buffer (Appendix A5.1), 6 U of Avian Myeloblastosis Virus Reverse Transcriptase (AMV RT, 24 U/µl), 10 U RNase inhibitor (RNASin, 40 U/µl), 10 µl of extracted RNA and the final volume made up to 50 µl. The Biometra thermal cycler (Biomedizinische Analytik, GmbH, Göttingen, Germany) was used with the cycling conditions described above (section 3.7.1). This was followed by the nested PCR using 5.0 µl of the first round PCR products with the ORF1 inner sense and antisense primers (Table 3.1). The nested PCR reaction mixture

was the same as the first round PCR reaction mixture with the exception of AMV RT and RNAsin in a final volume of 100 µl made up with distilled water. The same cycling conditions used for the first round PCR were used for the nested PCR except for the RT step. Five microlitres of the nested PCR products were analyzed on a 1 % agarose gel by electrophoresis as described in section 3.3.4.

The first and second round PCR reaction mixtures for amplifying the 3'-end of ORF2 was the same as for amplifying the ORF1 except that external and internal ORF2 primers (Table 3.1) were used, respectively with the Supertherm DNA polymerase and its corresponding 10X PCR reaction buffer (JMR Holdings, UK) at 15 mM $MgCl_2$ concentration. The GeneAmp thermal cycler (The Perkin Elmer Corporation, Norwalk, USA) was used with the following final cycling conditions: First round PCR - 43 °C for 45 minutes (RT step), followed by 35 cycles of 94 °C for 45 seconds, 48 °C for 45 seconds (annealing temperature) and 72 °C for 1 minute followed by a last elongation step at 72 °C for 7 minutes. Thereafter, the nested PCR was initiated with 10 µl of the first round PCR products using the same cycling conditions as the first round PCR except for the RT step and the use of the ORF2 inner primers. Ten microlitres of the nested PCR products were analyzed on a 1 % agarose gel by electrophoresis as described in section 3.3.4.

3.7.3 RT-PCR of older specimens

3.7.3.1 The SuperScript™ II RT-PCR system

The PCR conditions described for amplifying ORF2 in section 3.7.2 above, using RNA from the Namibian specimens, were applied to the 1983 Botswana sera specimens and were found to be non-ideal for these older less well preserved specimens. Thus primers were modified as follows: One primer, Mor-Sn (5'-ATA CCC TGG ATT ACC CTG C, nt 1835-1853), based on the sequence of the Moroccan isolate (GeneBank Accession no. AF065061) was used as both external and internal sense primer (designed as described in section 3.7) and used with both the external antisense and internal antisense ORF2 primers (Table 3.1) for first and second round PCR, respectively. The specimens were first clarified and ultracentrifuged as described in section 3.6.1 and reverse transcription was then performed with the SuperScript™ II Rnase H- Reverse Transcriptase (GIBCO BRL®, Life Technologies, Scotland) according to the manufacturer's protocol using the GeneAmp thermal cycler (The Perkin Elmer Corporation, Norwalk, USA), because this enzyme is reputed to give superior amplification with degraded RNA specimens (Gerard *et al*., 1986). Briefly, the RT reaction was primed with 1.0 µl oligo(dT) (500 µg/ml) which was added to 10 µl RNA (final volume made up to 20 µl) and the reaction mixture heated to 70 ºC for 10 minutes and quick chilled on ice. The following were then added to each reaction: 4.0 µl 5X First Strand buffer, 2.0 µl of 0.1 M DTT and 1.0 µl of 10 mM dNTPs. Final volume was thus 27 µl, and tubes were incubated briefly at 42 ºC for 2 minutes. Then, 1.0 µl of SuperScript II (200 U/µl) was added and the RT reaction performed at 42 ºC for 50 minutes. The enzyme was then inactivated by heating at 70 ºC for 15 minutes and the cDNA used as a template for PCR amplification. The thermal

cycling conditions were as follows: First round PCR: 43 ºC for 45 minutes, 35 cycles at 94 ºC for 45 seconds, 48 ºC for 45 seconds and 72 ºC for 1 minute, and a final extension step at 72 ºC for 7 minutes. This was followed by a nested PCR, initiated with 10 µl of the first round PCR product and the same PCR cycling conditions as the first round PCR less the RT step. A 2.5 % Metaphore agarose gel mixed with 0.5 µg/ml EthBr (final concentration) was prepared (Appendix A2.1 and A2.3) and 10 µl of the PCR products were mixed with 1.0 µl bromophenol blue/xylenexyanol loading dye and analyzed by electrophoresis in TBE buffer at 100 V for one hour against the DNA molecular weight markers V and VIII.

3.7.3.2 Platinum Pfx DNA polymerase

PCR using Platinum Pfx DNA polymerase enzyme (GIBCO BRL®, Life Technologies, Scotland) was also tried on the difficult Botswana specimens. The cDNA generated by using SuperScript II as described in section 3.7.3.1 was used. The Mor-Sn primer was used with ORF2 primers as described above. The Platinum Pfx DNA polymerase was used according to the manufacturer's protocol. The reaction mixture consisted of 1.0 µl Platinum Pfx DNA polymerase (2.5 U/µl), 5.0 µl of 10X Pfx amplification buffer, 200 µM (final concentration) of each dNTP, 1.0 µl of 50 mM $MgSO_4$, 10 µM ORF2 primers (Table 3.1), 10 µl template DNA, and the final volume made up to 50 µl with distilled water. The GeneAmp thermal cycler (The Perkin Elmer Corporation, Norwalk, USA) was used with the following cycling conditions: first round PCR: 94 °C for 2 minutes and 35 cycles of 94 °C for 15 seconds, 48 °C for 30 seconds and 68 °C for 1 minute with a final extension step at 68 °C for 1 minute. Nested PCR was performed using 10 µl of the first round PCR products with exactly the same cycling conditions as the first round

PCR. The amplification products were analyzed on a Metaphore agarose gel as described in section 3.7.3.1 with the use of DNA molecular weight marker VIII.

3.8 Sequencing of PCR products

3.8.1 Sequencing reaction

Cycle sequencing of PCR positive products was performed using the ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (The Perkin Elmer Corporation, California, USA) according to the manufacturer's protocol. Briefly, 30 ng/µl PCR product was mixed with 8.0 µl Terminator Ready Reaction Mix and 3.2 pmol of inner sense primer (Table 3.1) for forward reaction and the final volume made up to 20 µl with distilled water. The reaction mixture was subjected to the following cycling conditions with the GeneAmp thermal cycler (The Perkin Elmer Corporation, Norwalk, USA): 25 cycles of 96 ºC for 10 seconds, 50 ºC for 5 seconds and 60 ºC for 4 minutes and maintained at 4 ºC after cycling.

3.8.2 Sequencing reaction purification

The sequencing reaction was purified from excess dye terminators by using the Centri-Sep Spin-Columns purification kit (Princeton Separations Inc., USA) according to the manufacturer's protocol. Briefly, the dry gel columns were hydrated with 800 µl RNAse-free water for at least 2 hours at room temperature. Excess water was allowed to drain by gravity flow followed by a 3,000 rpm spin for 2 minutes in a microcentrifuge. Twenty microliters of the dideoxy sequencing reaction mixture was added onto the hydrated gel column and centrifuged as above. Purified samples were then collected and dried in a vacuum centrifuge for one hour. The dried pellets were resuspended in 6 µl of loading

dye before loading onto a gel for sequencing. The automated sequencing was performed using the ABI PRISM 377 DNA Sequencer (The Perkin Elmer Corporation, California, USA).

3.9 Molecular analysis of PCR products

3.9.1 Construction of Phylogenetic trees

The nucleotide sequences were aligned using DNAMAN software (version 4.0, Lynnon BioSoft). Phylogenetic distances and phylogenetic trees were generated, for both regions, using the same software. The robustness of the trees was determined by 100 bootstrap replicates of multiple-sequence alignments. Bootstrap values of greater than 70 % were regarded as providing evidence for a phylogenetic grouping (Muerhoff *et al*., 1997). The final graphical output of the trees was created using TREEVIEW software (Win32, version 1.6.1).

3.9.2 Construction of consensus sequences

Nucleotide sequences were translated to amino acid sequences using DNASIS software (version 2.5). A consensus amino acid sequence was generated from the Namibian isolates and aligned against the published amino acid sequences using DNAMAN software (version 4.0, Lynnon BioSoft). Percentage amino acid homologies were calculated using the same software.

CHAPTER 4: RESULTS

4.1 Plasmid DNA preparation and sequencing

4.1.1 Comparison of four methods of plasmid DNA extraction

The Nucleon plasmid DNA preparation kit (Amersham International plc, Buckinghamshire, England, section 3.3.3) gave very low yields of cloned plasmid DNA, estimated to be less than 0.05 μg/μl since the DNA could not be visualized by gel electrophoresis (section 3.3.4). Both the Wizard DNA purification kit (Promega Corporation, Madison, USA) and the High Pure plasmid purification and isolation kit (Roche Diagnostics Corporation, Mannheim, Germany) produced adequate amounts (< 0.1 μg/μl) of plasmid DNA. The DNA was, however, found to be impure by gel electrophoresis analysis. The control DNA bands (Kaposi Sarcoma virus; Alagiozoglou *et al*., 2000) were visually clear and sharp compared with the plasmid DNA bands which were smudged in appearance. The plasmid DNA had to be subsequently purified by ethanol precipitation (section 3.3.5). The Qiagen plasmid purification kit (QIAGEN, GmbH, Germany) included isopropanol precipitation and an ethanol wash and produced high yields (> 0.2 μg/μl) of pure plasmid DNA which gave clear sharp bands when visualized by gel electrophoresis (section 3.3.4).

4.1.2 Characterization of the plasmid DNA

The plasmid DNA was used as a positive control in the initial PCR optimization experiments. The plasmid DNA sequence was confirmed by sequencing and restriction enzyme digestion prior to using clinical material. The plasmid DNA was sequenced by using the Thermosequenase cycle sequencing kit (Amersham International plc, Buckinghamshire, England) as outlined in section 3.4.2 and was found to consist of a 2,002 bp HEV insert cloned into the 2,922 bp pCR1000 vector. The HEV insert is from the nonstructural ORF1 region of the Pakistan Sar55 isolate, and constitutes most of the helicase region and all of the polymerase (RdRp) region.

The restriction enzyme digestion performed using *Hind III* RE linearized the plasmid (Figure 4.1) since it has only one cutting site in the plasmid vector (1,163 bp; Figure 4.2), producing a band of about 5 kb after gel electrophoresis (Figure 4.1, lanes 4 and 6). *Hind III* digestion performed with a control plasmid (section 3.4.1) produced three clear bands, the uncut plasmid band of 3,690 bp, and the two cut plasmid bands of 2,867 and 823 bp (Figure 4.1, lane 2).

The restriction enzyme digestions were also performed using *Sac I* and *Sma I* REs. The five bands of sizes 2,670; 1,029; 624; 599 and 510 bp produced after simultaneous *Sac I* and *Sma I* digestion and gel electrophoresis of the plasmid with insert are shown in Figure 4.3, lane 5 and explained in Figure 4.2 and legend.

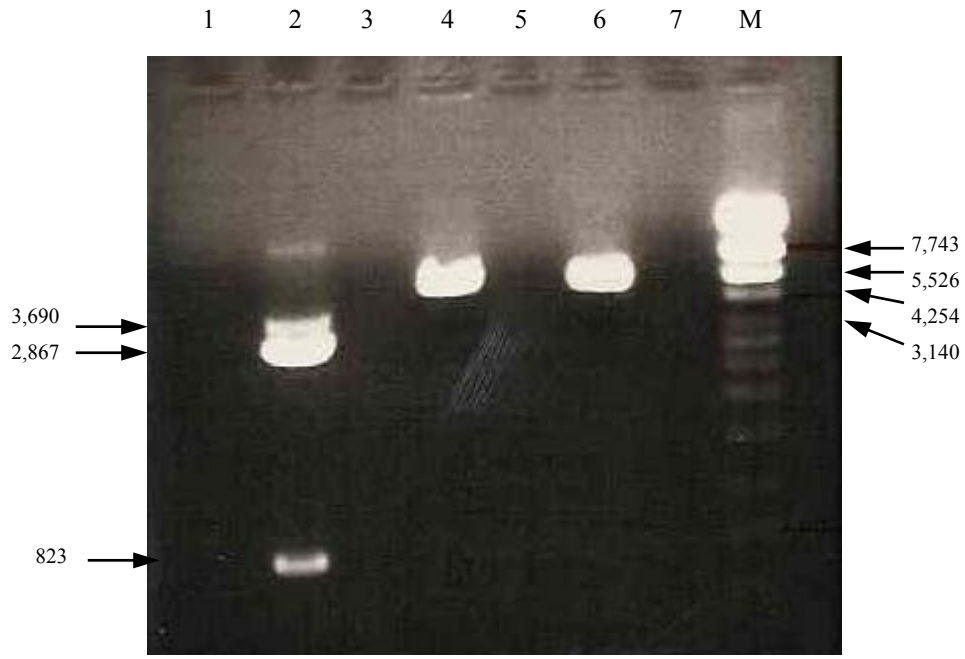

Figure 4.1 Electrophoretogram showing the linearized HEV plasmid vector (lanes 4 and 6) after restriction enzyme digestion with *Hind III* restriction endonuclease. Lane 2 represents the *Hind III* digested control plasmid (consisting of a fragment of the Kaposi sarcoma virus inserted in the pMosblue vector, see text). The molecular weight marker IV (M) is in Lane 8. The HEV plasmid vector has a size of 4,924 bp and runs slightly ahead of the 5,526 bp band of the size marker as expected. Lanes 1, 3, 5 and 7 are empty.

4.2 PCR optimization

4.2.1 PCR optimization using plasmid DNA

PCR optimizations with the plasmid DNA using the Supertherm DNA polymerase (JMR Holdings, UK) with the 10X Roche PCR reaction buffer (Roche Diagnostics Corporation, Manneheim, Germany), as described in section 3.5, produced the best results and the external primers were found to work optimally at an annealing temperature of 50 °C (section 3.5.1) as shown in Figure 4.4.

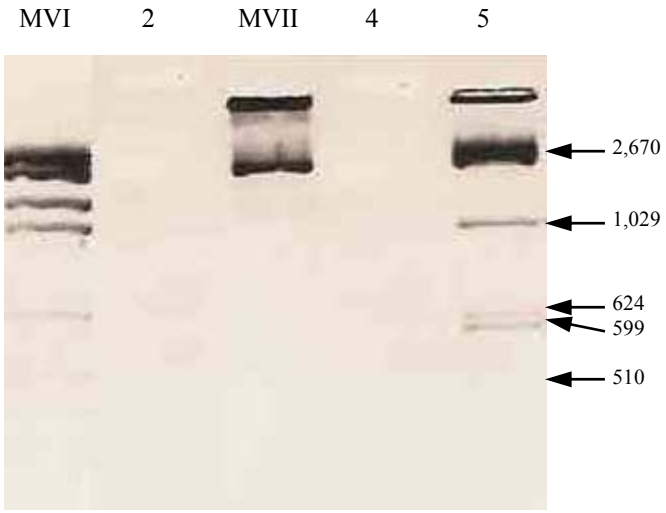

Figure 4.3 Restriction enzyme digestion of the HEV plasmid vector with *Sac I* and *Sma I* restriction endonucleases (lane 5; see Fig. 4.2 for full explanation of sizes observed). Lanes 1 (MVI) and 3 (MVII) contain molecular weight markers VI and VII, respectively, and lanes 2 and 4 are empty.

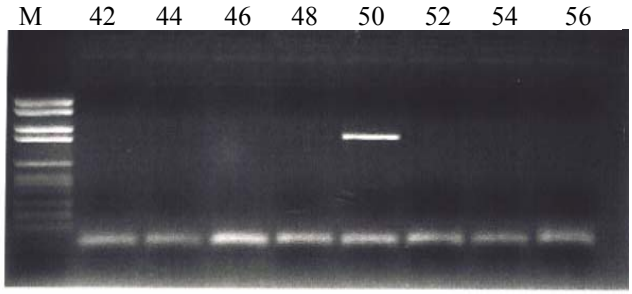

Figure 4.4 Electrophoretogram depicting ORF1 first round RT-PCR reactions using plasmid DNA at 8 different annealing temperatures (from 42 °C to 56 °C, shown above). Lane M is the molecular weight marker VI.

4.2.2 RT-PCR using clinical material

4.2.2.1 PCR evaluation for viral RNA amplification of ORF1

Titration of magnesium ion concentration was performed with six different PCR reaction buffers (section 3.7.1) and it was observed that the in-house buffer containing a final concentration of 1.0 mM $MgCl_2$ (Appendix A) produced the best PCR positive results as compared to the other buffers and gave an amplicon with the expected band size of 559 bp (Figures 4.5(a) and 4.5(b); see paragraph 3.7.2 for ORF1 final reaction conditions for

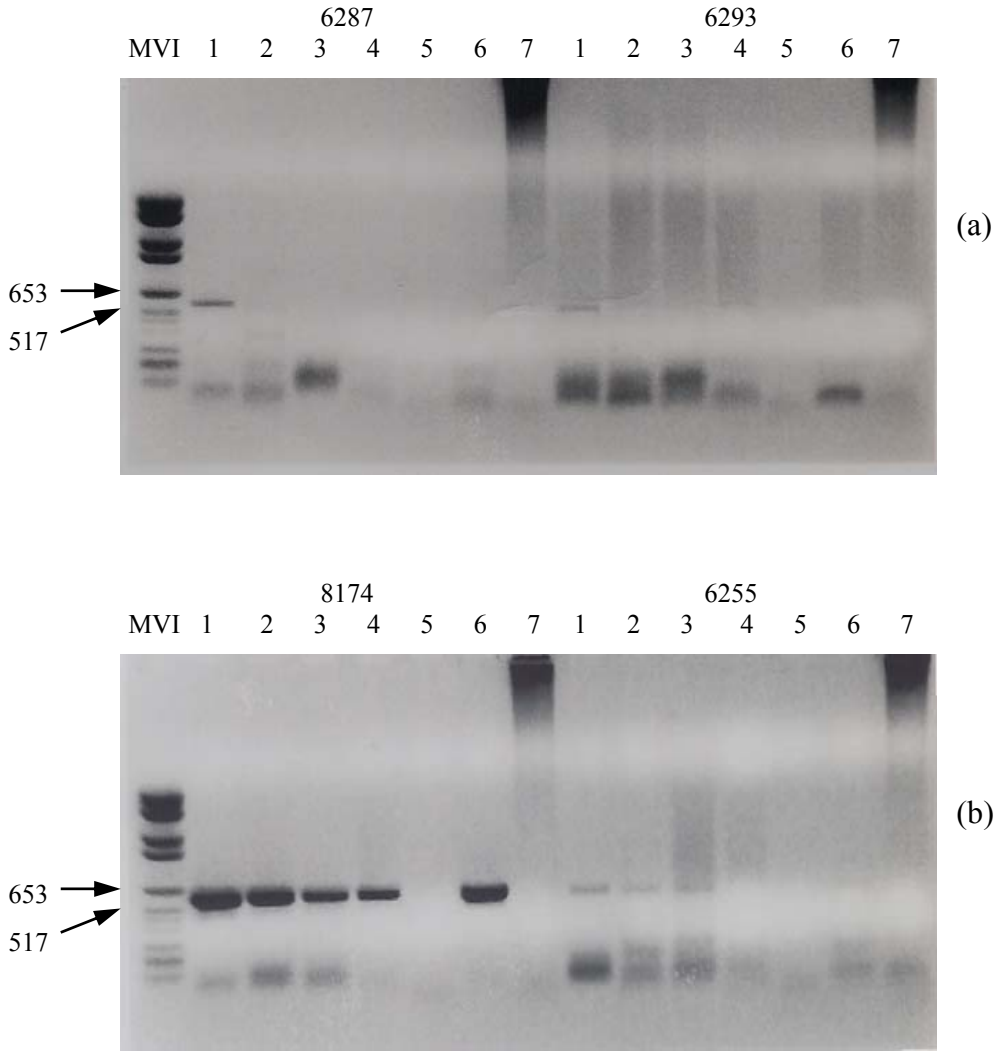

Figure 4.5 (a) and (b). Viral RNA isolated from 4 specimens were reverse transcribed and amplified in the ORF1 region with 6 different $MgCl_2$ titration buffers. Lanes 1, 2, 3 and 4 from both figures represent the in-house buffers containing 1.0 mM, 1.5 mM, 2.0 mM and 2.5 mM $MgCl_2$ final concentrations, respectively. Lanes 5 and 6 (both figures) represent the 10X Supertherm reaction buffer and the 10X PCR reaction buffer (Roche) both with 1.5 mM $MgCl_2$, respectively. Lane MVI (both figures) represent the molecular weight marker VI and lanes 7 (both figures) contain water controls.

clinical specimens).

RNA was amplified from 9.1 % of the specimens using ORF1 PCR. Amplified fragments were obtained from 7/74 stool specimens and 8/90 sera. While this region may be useful for diagnostic PCR, to compare our data with other African data, another region of the HEV genome was also targeted for PCR amplification.

4.2.2.2 PCR evaluation for viral RNA amplification of ORF2

A region towards the carboxy terminal end of ORF2 was the next target for PCR amplification of viral RNA from clinical specimens. Primers were designed for this region (section 3.7, Table 3.1) and the PCR test was optimized (sections 3.7.1). The 1995 Namibian specimens were used to amplify the 3'-end of ORF2 (section 3.7.1 and 3.7.2). It was observed that the Supertherm PCR reaction buffer consisting of 1.5 mM $MgCl_2$ together with RT-PCR at an annealing temperature of 48 °C produced the best results (optimization experiment not shown). An example of ORF2 PCR positive stool specimens analyzed by gel electrophoresis is shown in Figure 4.6. These PCR conditions were used to amplify 14 stool and 5 sera specimens. Five stool specimens (5/14) produced PCR positive results (35.7 %) and none from the 5 sera specimens.

When the older (1983) Botswana specimens were used for PCR amplification of the 3'-end of ORF2 no positive results were observed despite using specific reagent kits for amplifying problematic templates (section 3.7.3).

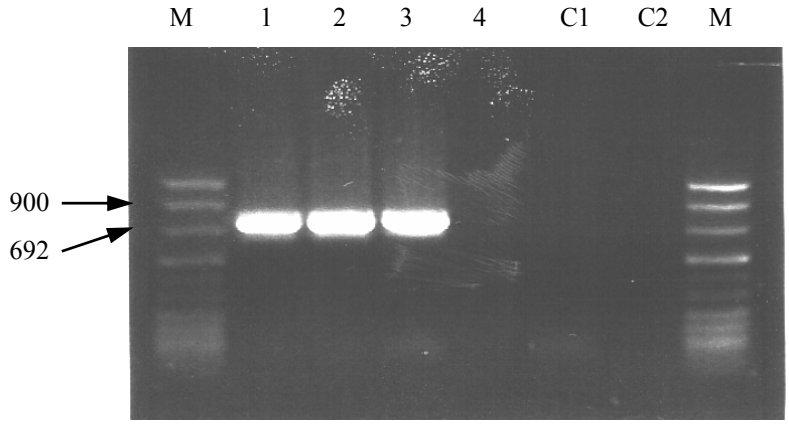

Figure 4.6 Electrophoretogram showing results for ORF2 nested RT-PCR of four specimens in lanes 1 to 4. Lane M represents the molecular weight marker VIII, C1 and C2 are the negative water controls for first round PCR and nested PCR, respectively.

4.3 Sequencing of PCR products

Figure 4.7 shows a representative example of a sequencing electrophoretogram as output from the automated sequencing system which separates sets of fragments specifically terminated with either dideoxy thymidine (ddTTP), dideoxy adenosine (ddATP), dideoxy cytidine (ddCTP) and dideoxy guanosine (ddGTP). The fragments are distinguished by different fluorescent labels, where ddATP is green, ddTTP is red, ddGTP is black and ddCTP is blue (Figure 4.7). Where the software cannot clearly distinguish between different fragments, the ambiguous nucleotide is represented by an "N". Although polymerases have a one in 5,000 error rate, errors at the same spot do not occur sufficiently often to affect signal intervals. Low signals can indicate low amounts of the specific PCR product or contamination by unspecific or primer dimer products. After electrophoresis, the computer identifies the dideoxynucleoside which terminates each oligonucleotide by identifying its characteristic spectrum. Thus, each colour peak in Figure 4.7 represents the specific nucleotide that was recognised at each position in the gel. Below the graph is the nucleotide sequence represented by the peaks as interpreted by the computer.

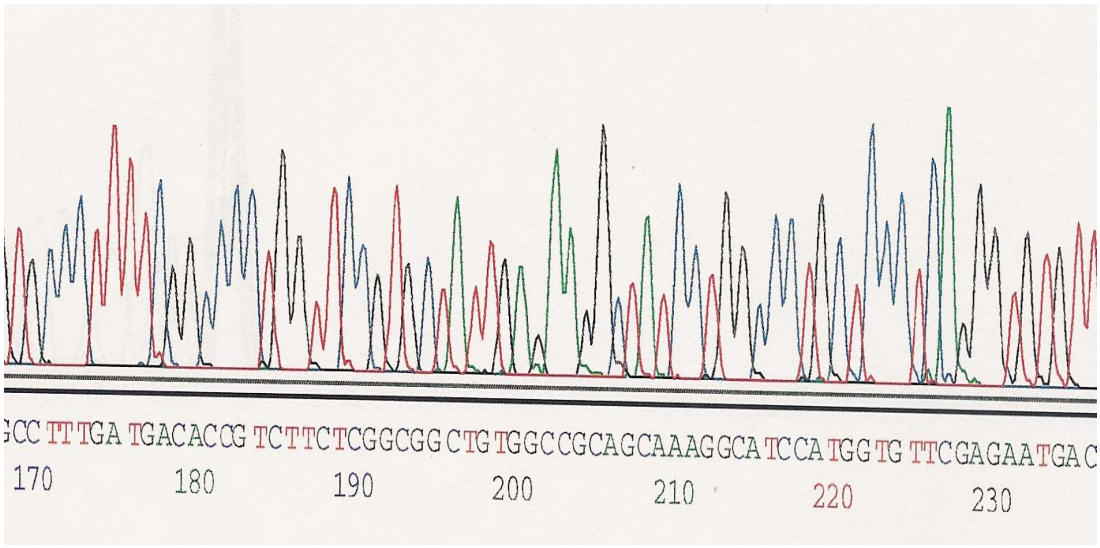

Figure 4.7 A representative graph showing the colour spectrum of each of the four nucleotides obtained from the ABI 377 sequencer.

4.4 Phylogenetic analysis of the carboxy terminal (3') end of ORF2

4.4.1 Nucleotide sequence analysis of ORF2 3' end

To determine the relationship between the Namibian isolates and other known HEV isolates within the ORF2 region, sequence data from four representative Namibian isolates (7798, 7801, 7948 and 8039) were aligned and compared to some of the global isolates (Table 4.1), over a 451 bp region of ORF2 positioned from 1,307 to 1,757 bp towards the carboxy terminal end of HEV genome. The nucleotide alignment is shown in Figure 4.8.

Table 4.1 HEV sequences used for ORF2 analysis.

Name	GenBank Accession No.	Reference
82[a]-Burma	M73218	Tam *et al*. (1991)
83-Chad T3	U62121	van Cuyck-Gandre *et al*. (1997)
87-China-Xinjiang	D11092	Aye *et al*. (1992)
87-China-Hebei	M94177	Bi *et al.* (1993)
87-China-K52	L25595	Yin *et al*. (1994)
87-China-Uigh179	D11093	Unpublished
93-Egypt	AF051351	Tsarev *et al*. (1999)
94-Egypt	AF051352	Tsarev *et al*. (1999)
92-Fulminant (India?)	X98292	Donati *et al*. (1997)
90-India-Hyderabad	AF076239	Panda *et al.* (1995)
93-India-Madras	X99441	Unpublished data
87-Mexico	M74506	Huang *et al*. (1992)
94-Morocco	AF065061	Meng *et al.* (1999)
89-Myanmar	D10330	Aye *et al*. (1993)
83-Namibia	AF105021	He *et al*. (2000)
95-Namibia-7798	AY370686	Maila *et al*. (2004)
95-Namibia-7801	AY370687	Maila *et al*. (2004)
95-Namibia-7948	AY370688	Maila *et al*. (2004)
95-Namibia-8039	AY370689	Maila *et al*. (2004)
92-Nepal-TK15/92	AF051830	Gouvea *et al*. (1998)
97/98-Nigeria-1	AF172999	Buisson *et al*. (2000)
97/98-Nigeria-4	AF173000	Buisson *et al*. (2000)
97/98-Nigeria-5	AF173001	Buisson *et al*. (2000)
97/98-Nigeria-6	AF173230	Buisson *et al*. (2000)
97/98-Nigeria-7	AF173231	Unpublished
98-Nigeria-9	AF173232	Buisson *et al*. (2000)
88-Pakistan-Abb-2B	AF185822	van Cuyck-Gandre *et al*. (1999)
87-Pakistan-SAR-55	M80581	Tsarev *et al*. (1992)
T1-China	AJ272108	Wang *et al*. (2000)
95-USA-1	AF060668	Schlauder *et al.* (1998)
95-USA-2	AF060669	Erker *et al*. (1999)

[a]Year of isolation.

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                      1316      1326      1336      1346      1356      1366
7798                  CACGCGTGGTTATTCAGGATTATGACAATCAGCACGAGCAGGACCGCCCCACCCCCTCAC
7801                  CACGCGTGGTTATTCAGGATTATGACAATCAGCACGAGCAGGACCGCCCCACCCCCTCAC
7948                  CACGCGTGGTTATTCAGGATTATGACAATCAGCACGAGCAGGACCGCCCCACCCCCTCAC
8039                  CACGCGTGGTTATTCAGGATTATGACAATCAGCACGAGCAGGACCGCCCCACCCCCTCAC
Mexico                CGCGTGTGGTCATTCAGGATTATGACAACCAGCATGAGCAGGATCGGCCCACCCCGTCGC
ChadT3                CCCGTGTAGTTATCCAGGACTATGACAACCAACATGAGCAAGACCGACCGACACCTTCCC
94Egypt               CCCGAGTAGTTATCCAGGACTATGACAACCAACATGAGCAAGATCGGCCGACACCTTCCC
Morocco               CTCGTGTAGTCATCCAGGATTATGACAATCAGCATGAGCAGGACCGTCCGACACCTTCCC
China-Xinjiang        CTCGTGTAGTTATTCAGGATTATGACAACCAACATGAGCAGGACCGACCGACACCTTCCC
Fulminant             CTCGTGTGGTCATTCAGGATTATGATAACCAACATGAGCAAGATCGGCCGACACCTTCTC
Pakistan-SAR-55       CCCGTGTAGTTATTCAGGATTATGACAACCAACATGAGCAGGACCGACCGACACCTTCCC
Burma                 CTCGTGTGGTTATTCAGGATTATGATAACCAACATGAACAAGATCGGCCGACGCCTTCTC
Pakistan-Abb-2B       CTCGTGTGGTTATTCAGGACTATGATAACCAACATGAACAGGACCGGCCGACGCCTTCGC
Nepal                 CTCGTGTGGTTATTCAGGATTATGATAACCAACATGAACAAGACCGGCCGACACCTTCTC
India-Madras          CTCGTGTGGTTATTCAGGATTATGACAATCAACATGAACAAGACCGGCCGACGCCTTCTC
T1                    CCCGTGTGGTTATTCAGGATTATGACAACCAACATGAGCAAGATCGTCCCACTCCCTCCC
US-1                  CCCGTGTGGTTATCCAGGATTATGATAACCAGCACGAACAAGATCGACCTACCCCGTCAC
US-2                  CCCGTGTGGTTATCCAGGATTATGATAACCAGCANGAGCAAGACCGACCTACTCCGTCAC
                      * ** ** ** ** ***** ***** ** ** ** ** ** ** ** ** ** ** * *

                      1376      1386      1396      1406      1416      1426
7798                  CTGCACCGTCCCGGTCCTTTTCTGTCCTCCGTGCAAATGATGTGCTTTGGCTGTCTCTCA
7801                  CTGCACCGTCCCGGCCCTTTTCTGTCCTCCGTGCAAATGATGTGCTTTGGCTGTCTCTTA
7948                  CTGCACCGTCCCGGCCCTTTTCTGTCCTCCGTGCAAATGATGTGCTTTGGCTGTCTCTTA
8039                  CTGCACCGTCCCGGCCCTTTTCTGTCCTCCGTGCAAATGATGTGCTTTGGCTGTCTCTTA
Mexico                CTGCGCCATCTCGGCCTTTTTCTGTTCTCCGAGCAAATGATGTACTTTGGCTGTCCCTCA
ChadT3                CAGCCCCGTCGCGCCCTTTCTCTGTCCTTCGGGCTAATGATGTGCTTTGGCTTTCTCTCA
94Egypt               CAGCCCCGTCGCGCCCTTTCTCTGTCCTTCGGGCCAACGATGTGCTTTGGCTCTCTCTCA
Morocco               CAGCCCCGTCTCGCCCTTTTTCTGTTCTCCGGGCCAATGATGTGCTTTGGCTCTCTCTCA
China-Xinjiang        CAGCCCCATCGCGCCCTTTTTCTGTCCTCCGAGCTAATGATGTGCTTTGGCTTTCTCTCA
Fulminant             CAGCCCCATCGCGCCCTTTTTCTGTCCTCCGAGCTAATGATGTGCTTTGGCTTTCTCTCA
Pakistan-SAR-55       CAGCCCCATCGCGTCCTTTTTCTGTCCTCCGAGCTAACGATGTGCTTTGGCTTTCTCTCA
Burma                 CAGCCCCATCGCGCCCTTTCTCTCTGTCCTTCGAGCTAATGATGTGCTTTGGCTCTCTCTCA
Pakistan-Abb-2B       CAGCTCCATCGCGCCCTTTTTCTGTCCTTCGAGCTAATGATGTGCTTTGGCTCTCTCTCA
Nepal                 CAGCCCCGTCGCGCCCTTTCTCTGTTCTTCGAGCTAATGATGTGCTTTGGCTCTCTCTCA
India-Madras          CAGCCCCATCGCGCCCTTTCTCTGCCCTTCGAGCTAACGATGTGCTTTGGCTCTCTCTCA
T1                    CTGCTCCCTCTCGTCCATTTTCTGTTCTTCGTGCTAATGATGTGCTTTGGCTTTCACTTA
US-1                  CTGCCCCCTCCCGCCCTTTCTCAGTTCTTCGTGCCAATGATGTTTTGTGGCTCTCTCTCA
US-2                  CTGCCCCCTCTCGCCCCTTCTCAGTTCTTCGTGCCAATGATGTTTTGTGGCTTTCCCTCA
                      * ** ** ** **  * ** ** *  ** ** ** ** *****  * ***** ** ** *

                      1436      1446      1456      1466      1476      1486
7798                  CTGGGGCCGAGTATGACCAATCTACGTACGGGTCGTCCACCGGCCCGGTTTATGTCTCGG
7801                  CTGGGGCCGAGTATGACCAATCTACGTACGGGTCGTCCACCGGCCCGGTTTATGTCTCGG
7948                  CTGGGGCCGAGTATGACCAGTCTACGTACGGGTCGTCCACCGGCCCGGTTTATGTCTCGG
8039                  CTGGGGCCGAGTATGACCAGTCTACGTACGGGTCGTCCACCGGCCCGGTTTATGTCTCGG
Mexico                CTGCAGCCGAGTATGACCAGTCCACTTACGGGTCGTCAACTGGCCCGGTTTATATCTCGG
ChadT3                CCGCTGCCGAGTATGACCAGTCCACCTACGGCTCCTCGACTGGCCCAGTCTATGTCTCTG
94Egypt               CCGCTGCCGAGTATGACCAGTCCACCTATGGCTCCTCGACTGGCCCAGTCTATGTTTCTG
Morocco               CTGCTGCTGAGTATGATCAGTCCACCTATGGCTCTTCTACTGGCCCAGTCTATGTCTCCG
China-Xinjiang        CCGCTGCCGAGTATGACCAGTCCACTTACGGCTCTTCGACCGGCCCAGTCTATGTCTCTG
Fulminant             CCGCTGCCGAGTATGACCAGTCCACTTACGGTTCTTCGACCGGCCCAGTCTATGTTTCTG
Pakistan-SAR-55       CCGCTGCCGAGTATGACCAGTCCACTTACGGCTCTTCGACCGGCCCAGTCTATGTCTCTG
Burma                 CCGCTGCCGAGTATGACCAGTCCACTTATGGCTCTTCGACTGGCCCAGTTTATGTTTCTG
Pakistan-Abb-2B       CCGCTGCCGAGTATGACCAATCCACTTATGGCTCTTCGACTGGCCCAGTTTATGTCTCTG
Nepal                 CCGCTGCCGAGTATGACCAGTCCACCTATGGCTCTTCGACTGGCCCAGTTTATGTTTCTG
India-Madras          CTGCTGCCGAGTACGACCAGTCCACTTATGGCTCTTCGACTGGCCCAGTTTATGTTTCTG
T1                    CTGCTGCTGAGTATGATCAAACGACTTATGGCTCCTCTACCAACCCTATGTATGTTTCTG
US-1                  CTGCCGCTGAGTACGNCCAGACCACGTATGGGTCGTCCACCAACCCTATGTATGTCTCTG
US-2                  CTGCCGCTGAGTATGACCAGACTACGTATGGGTCGTCCACCAACCCTATGTATGTCTCTG
                      * *  ** ***** *  **  * ** ** ** ** ** **   ***  * *** * ** *
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                    1496      1506      1516      1526      1536      1546
7798            ATAGTGTGACCTTGGTGAATGTAGCGACCGGCGCGCAGGCCGTAGCCCGGTCGCTCGACT
7801            ATAGTGTGACCTTGGTGAATGTAGCGACCGGCGCGCAGGCCGTAGCCCGGTCGCTCGACT
7948            ATAGTGTGACCTTGGTGAATGTAGCGACCGGCGCGCAGGCCGTAGCCCGGTCGCTCGACT
8039            ATAGTGTGACCTTGGTGAATGTAGCGACCGGCGCGCAGGCCGTAGCCCGGTCGCTCGACT
Mexico          ACAGCGTGACTTTGGTGAATGTTGCGACTGGCGCGCAGGCCGTAGCCCGATCGCTTGACT
ChadT3          ACTCTGTGACCTTGGTCAATGTTGGGACCGGCGCGCAGGCCGTTGCTCGGTCGCTCGACT
94Egypt         ACTCCGTGACCTTGGTCAACGTTGCGACCGGCGCGCAGGCCGTTGCTCGGTCGCTCGATT
Morocco         ACTCTGTGACCTTGGTCAATGTTGCGACCGGCGCGCAGGCCGTTGCTCGGTCGCTTGACT
ChinaXin        ACTCTGTGACCTTGGTTAATGTTGCGACCGGCGCGCAGGCCGTTGCCCGGTCACTCGACT
Fulminant       ACTCTGTGACCCTGGTTAATGTTGCGACCGGCGCGCAGGCCGTTGCCCGGTCGCTTGACT
PakiSar55       ACTCTGTGACCTTGGTTAATGTTGCGACCGGCGCGCAGGCCGTTGCCCGGTCACTCGACT
BurmaB1         ACTCTGTGACCTTGGTTAATGTTGCGACCGGCGCGCAGGCCGTTGCCCGGTCGCTCGATT
PakisAbb        ACTCTGTGACCTTGGTTAATGTCGCGACCGGCGCGCAGGCCGTTGCCCGGTCGCTCGATT
Nepal           ACTCTGTGACCTTGGTTAATGTTGCGACCGGCGCGCAGGCCGTTGCCCGGTCGCTCGATT
IndiMadras      ACTCTGTGACCTTGGTTAATGTTGCGACCGGCGCGCAGGCCGTTGCCCGGTCGCTCGATT
T1              ACACTGTTACATTTGTTAACGTAGCGACTGGTGCCCAGGGGGTTTCGCGCTCTCTGGATT
US-1            ATACAGTCACGCTTGTTAATGTAGCCACTGGTGCTCAGGCTGTTGCCCGCTCTCTTGACT
US-2            ACACAGTTACGCTTGTTAATGTGGCTACTGGTGCTCAGGCTGTTGCCCGCTCCCTTGATT
                *    ** **  * ** ** ** *  ** ** ** ****  **  * ** ** ** ** *

                    1556      1566      1576      1586      1596      1606

7798            GGTCCAAGGTTACTCTTGACGGGCGGCCCTTACCTACTATTGAACAATATTCTAAGTCAT
7801            GGTCCAAGGTTACTCTTGACGGGCGGCCCTTACCTACTATTGAACAATATTCTAAGTCAT
7948            GGTCCAAGGTTACTCTTGACGGGCGGCCCTTACCTACTATTGAACAATATTCTAAGTCAT
8039            GGTCCAAGGTTACTCTTGACGGGCGGCCCTTACCTACTATTGAACAATATTCTAAGTCAT
Mexico          GGTCCAAAGTCACCCTCGACGGGCGGCCCCTCCCGACTGTTGAGCAATATTCCAAGACAT
ChadT3          GGACCAAGGTCACACTCGATGGTCGCCCTCTATCTACTATTCAGCAGTATTCGAAGACTT
94Egypt         GGACTAAGGTCACGCTCGATGGTCGCCCCCTTTCTACCATTCAGCAGTACTCGAAGACTT
Morocco         GGACTAAGGTCACACTTGATGGCCGCCCTCTTTCTACTATCCAGCAGTATTCGAAGACTT
ChinaXin        GGACCAAGGTCACACTTGATGGTCGCCCCCTTTCCACCATCCAGCAGTATTCAAAGACCT
Fulminant       GGACCAAGGTCACACTTGATGGTCGCCCCCTCTCCACCATCCAGCAGTATTCAAAGACCT
PakiSar55       GGACCAAGGTCACACTTGATGGTCGCCCCCTTTCCACCATCCAGCAGTATTCAAAGACCT
BurmaB1         GGACCAAGGTCACACTTGACGGTCGCCCCCTCTCCACCATCCAGCAGTACTCGAAGACCT
PakisAbb        GGACCAAGGTCACACTTGACGGCCGCCCCCTCTCCACCATCCAGCAGTACTCGAAGACCT
Nepal           GGACCAAGGTCACACTTGATGGCCGCCCTCTCTCCACCATCCAGCAGTACTCGAAGACCT
IndiMadras      GGACCAAGGTCACACTTGACGGTCGCCCTCTCTCCACCATCCAGCAGTACTCGAAGACCT
T1              GGTCTAAGGTCACTCTCGATGGCCGTCCACTCACCACCATCCAGCAGTATTCTAAGACTT
US-1            GGTCTAAAGTTACTCTGGATGGTCGCCCTCTTACTACCATTCAGCAGTATTCTAAGAAAT
US-2            GGTCTAAAGTTACTCTGGACGGCCGCCCCCTTACTACCATTCAGCAGTATTCTAAGACAT
                ** * ** ** ** ** ** ** ** ** **  *  * **  *  * ** ** ** ***   *

                    1616      1626      1636      1646      1656      1666
7798            TCTTTGTGTTGCCCCTTCGTGGTAAGCTCTCTTTTTGGGAGGCCGGTACAACGAAAGCGG
7801            TCTTTGTGTTGCCCCTTCGTGGTAAGCTCTCTTTTTGGGAGGCCGGTACAACGAAAGCGG
7948            TCTTTGTGTTGCCCCTCCGTGGTAAGCTCTCTTTTTGGGAGGCCGGTACAACGAAAGCGG
8039            TCTTTGTGTTGCCCCTTCGTGGTAAGCTCTCTTTTTGGGAGGCCGGTACAACGAAAGCGG
Mexico          TCTTTGTGCTCCCCCTTCGTGGCAAGCTCTCCTTTTGGGAGGCCGGCACAACAAAAGCAG
ChadT3          TCTTCGTCCTGCCGCTTCGTGGCAAGCTCTCCTTCTGGGAGGCGGGTACTACTAAAGCCG
94Egypt         TCTTTGTCCTGCCCCTCCGTGGCAAGCTCTCCTTTTGGGAGGCAGGTACTACTAAAGCCG
Morocco         TCTTTGTCCTGCCGCTTCGTGGTAAACTTTCCTTCTGGGAGGCAGGTACCACTAAAGCCG
ChinaXin        TCTTTGTCCTGCCGCTCCGCGGTAAGCTCTCCTTTTGGGAGGCAGGTACTACTAAAGCCG
Fulminant       TCTTCGTCCTGCCGCTCCGCGGTAAGCTCTCCTTCTGGGAGGCAGGTACCACTAAGGCCG
PakiSar55       TCTTTGTCCTGCCGCTCCGCGGTAAGCTCTCCTTTTGGGAGGCAGGAACTACTAAAGCCG
BurmaB1         TCTTTGTCCTGCCGCTCCGCGGTAAGCTCTCTTTCTGGGAGGCAGGCACAACTAAAGCCG
PakisAbb        TCTTTGTCCTGCCGCTCCGCGGTAAGCTCTCTTTCTGGGAGGCAGGCACAACTAAAGCCG
Nepal           TCTTTGTCCTGCCGCTCCGCGGAAAGCTCTCTTTCTGGGAGGCAGGCACAACTAAGGCCG
IndiMadras      TCTTTGTCCTGCCGCTCCGCGGCAAGCTCTCTTTCTGGGAGGCAGGTACAACTAAGGCCG
T1              TCTATGTCTTGCCCCTTCGTGGTAAGCTTTCCTTTTGGGAGGCCGGCACCACTAAAGCCG
US-1            TTTATGTTCTCCCGCTTCGNGGGAAGCTGTCCTTTTGGGAGGCTGGTACGACCAAGGCCG
US-2            TTTATGTTCTCCCGCTCCGCGGGAAGCTGTCCTTTTGGGAGGCTGGCACGACTAAGGCCG
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                    1676      1686      1696      1706      1716      1726
7798            GTTACCCCTATAATTACAATACCACTGCTAGTGACCAGATTCTGATCGAGAATGCGGGCG
7801            GTTACCCCTATAATTACAACACCACTGCTAGTGACCAGATTCTGATCGAGAATGCGGCCG
7948            GTTACCCCTATAATTACAACACCACTGCTAGTGACCAGATTCTGATCGAGAATGCGGCCG
8039            GTTACCCCTATAATTACAACACCACTGCTAGTGACCAGATTCTGATCGAGAATGCGGCCG
Mexico          GTTATCCTTATAATTATAATACTACTGCTAGTGACCAGATTCTGATTGAAAATGCTGCCG
ChadT3          GGTACCCTTATAATTATAATACCACTGCTAGCGACCAATTGCTCATCGAGAACGTCGCTG
94Egypt         GGTACCCTTATAATTACAATACCACTGCTAGCGACCAATTGCTTATCGAGAACGCCACCG
Morocco         GGTACCCTTACAATTATAATACTACTGCTAGTGACCAACTGCTCGTTGAGAATGCCGCCG
ChinaXin        GGTACCCTTATAATTATAACACCACTGCTAGTGACCAACTGCTCGTTGAGAATGCCGCTG
Fulminant       GGTACCCCTATAATTATAATACCACTGCTAGCGATCAACTGCTTATTGAGAATGCCGCCG
PakiSar55       GGTACCCTTATAATTATAACACCACTGCTAGTGACCAACTGCTCGTTGAGAATGCCGCTG
BurmaB1         GGTACCCTTATAATTATAACACCACTGCTAGCGACCAACTGCTTGTCGAGAATGCCGCCG
PakisAbb        GGTACCCTTATAATTATAATACCACTGCTAGCGACCAACTGCTCGTCGAGAATGCCGCCG
Nepal           GGTACCCTTATAATTACAACACCACTGCTAGCGGCCAACTGCTTGTCGAGAATGCCGCCG
IndiMadras      GGTACCCTTATAATTATAATACCACTGCTAGCGACCAACTGCTTGTCGAGAATGCCGCCG
T1              GCTACCCTTATAATTATAACACTACTGCTAGTGACCAGATCCTGATTGAGAATGCAGCGG
US-1            GCTACCCGTATAATTATAATACCACTGCTAGTGACCAAATTTTGATTGAGAACGCGGCCG
US-2            GCTACCCTTACAATTATAATACTACCGCTAGTGACCAAATTTTGATTGAGAATGCGGCCG
                * ** ** ** ***** ** ** ** ***** *  **  *  *  * ** ** *     *

                    1736      1746      1756
7798            GGCACCGAGTCTCCATTTCGACCTATACCAC
7801            GCCACCGAGTCTCCATTTCGACCTATACCAC
7948            GCCACCGAGTCTCCATTTCGACCTATACCAC
8039            GCCACCGAGTCTCCATTTCGACCTATACCAC
Mexico          GCCATCGGGTCGCCATTTCAACCTATACCAC
ChadT3          GGCATCGGGTGGCTATTTCCACCTATACCAC
94Egypt         GGCATCGGGTGGCTATTTCTACTTACACTAC
Morocco         GGCACCGGGTGGCTATTTCCACCTACACCAC
ChinaXin        GGCATCGGGTTGCTATTTCCACTTACACCAC
Fulminant       GGCATCGGGTTGCTATTTCCACTTACACCAC
PakiSar55       GGCATCGGGTTGCTATTTCCACCTACACTAC
BurmaB1         GGCACCGGGTCGCTATTTCCACTTACACCAC
PakisAbb        GGCACCGGGTTGCTATTTCCACTTACACCAC
Nepal           GGCACCGGGTTGCTATCTCCACTTACACCAC
IndiMadras      GGCACCGGGTTGCTATCTCCACTTACACCAC
T1              GCCACCGAGTTTGCATCTCTACCTACACTAC
US-1            GTCACCGTGTCGCCATTTCTACTTATACCAC
US-2            GCCACCGTGTCGCTATTTCCACCTATACCAC
                * ** ** **    ** ** ** ** ** **
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Figure 4.8 Nucleotide alignment of global HEV isolates against the Namibian sequences from position 1,307 to 1,757 of the ORF2 region (nucleotide numbering with respect to the HPEGENSA isolate, GenBank Accession no. L08816). The asterisks denote conserved nucleotide positions.

A bootstrapped tree was constructed from 100 replicates of the nucleotide alignment and rooted on the Chinese isolate, T1 (Figure 4.9). The phylogenetic tree grouped the isolates into the four conventional genotypes (nomenclature according to Wang *et al*., 2000 and Widdowson *et al*., 2003). Afro-Asian isolates clustered in genotype I, the Mexican isolate and the newly defined 1995 Namibian isolates in genotype II, the United States isolates in genotype III and the Chinese isolate T1, in genotype IV. These four genetic

clusters are consistent with previous studies. The four 1995 Namibian sequences (bold numbers in Figure 4.9) shared a nucleotide identity of between 98.4 and 99.8 % and were 85.8-86.3 % similar to the Mexican isolate (Table 4.2). The 95-Namibian sequences were only 77.6-79.6 % similar to other African isolates (Table 4.2).

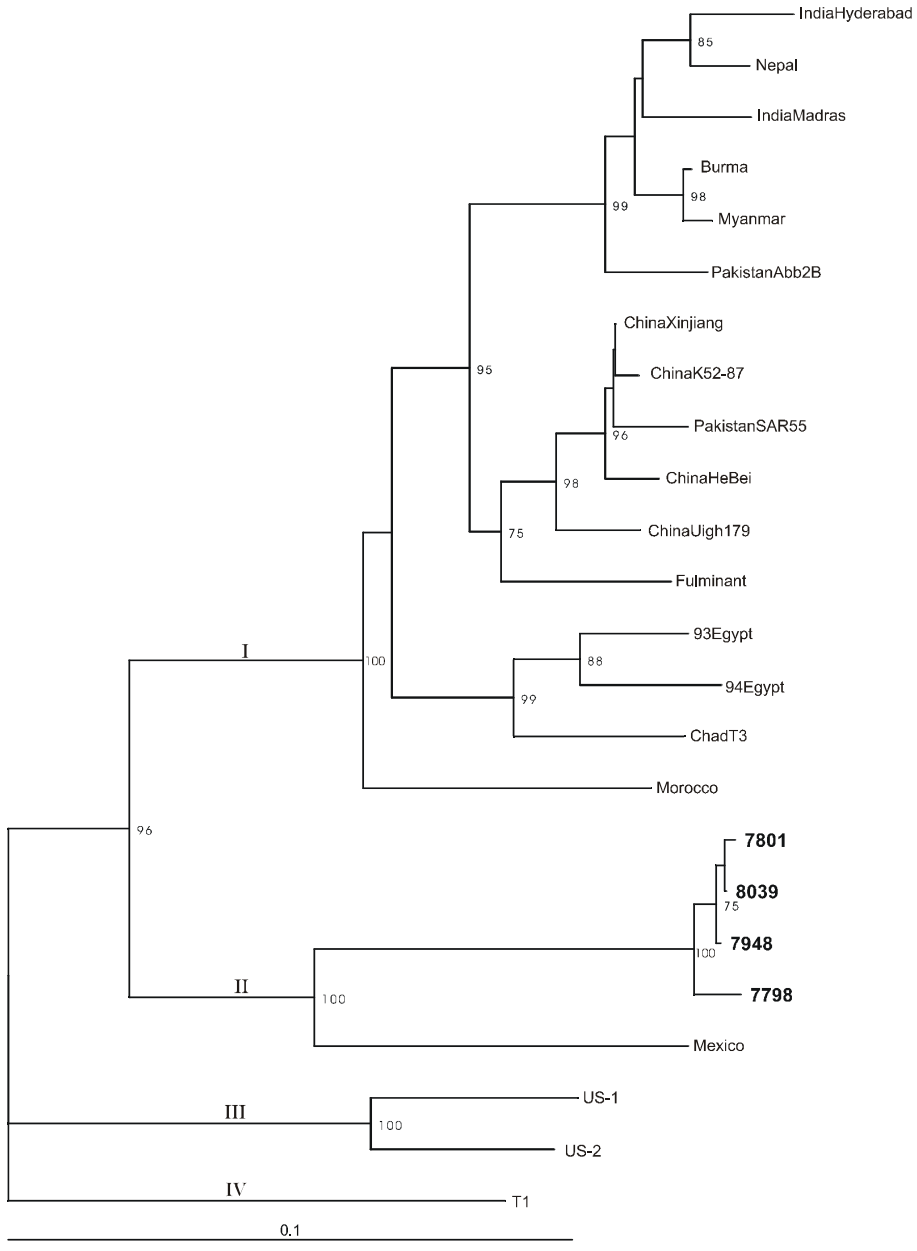

Figure 4.9 Phylogenetic tree of HEV isolates from position 1,307 to 1,757 of the ORF2 region (numbering according to the HPEGENSA sequence, GenBank Accession no. L08816). The tree shows the conventional segregation of HEV isolates into four genotypes (I-IV) and it is rooted with the T1 sequence. Clades are labeled according to the nomenclature of Wang *et al*., 2000. Bootstrap values for the most robust groupings are shown.

Table 4.2 Degree of nucleic acid homology of 1995 Namibian and global isolates of HEV within the 451 bp fragment (1,307 to 1,757 bp) of ORF2 region towards the carboxy terminal end of the genome.

Isolate	7798	7801	7948	8039	Mexico	ChadT3	94Egypt	Morocco	ChinaXin	Fulminant	P-SAR-55	Burma	P-Abb2B	Nepal	I-Madras	T1	US-1	US-2
7798	100																	
7801	98.9	100																
7948	98.4	99.6	100															
8039	98.7	99.8	99.8	100														
Mexico	85.8	86	86	86.3	100													
ChadT3	79.6	79.4	79.4	79.6	80	100												
94Egypt	77.8	77.6	78	77.8	78.9	92.9	100											
Morocco	79.6	79.4	79.4	79.6	80.9	90.2	88.2	100										
ChinaXinjiang	80.3	80.5	80.9	80.7	81.2	91.4	89.1	90.5	100									
Fulminant	78.9	78.7	79.2	78.9	81.2	89.4	88.2	88.5	94.7	100								
PakistanSAR55	79.8	80	80.5	80.3	80.9	90.9	89.1	89.6	98.7	93.3	100							
Burma	79.4	79.6	80	79.8	80.7	88.9	90	87.8	93.1	93.8	92	100						
PakistanAbb2B	80.3	80	80	79.8	80.7	88.7	88.5	88.5	93.1	92.2	92	97.1	100					
Nepal	78.3	78.5	78.9	78.7	78.7	88.9	89.6	88.5	91.8	92.5	90.7	96.9	95.3	100				
IndiaMadras	78.9	78.7	79.2	78.9	79.4	88.7	89.4	88	91.8	92.5	90.9	96.9	95.3	96.2	100			
T1	77.4	78.5	78	78.3	78.3	77.8	78.9	80.3	80	78.9	80.7	79.4	79.4	79.2	78.3	100		
US-1	78.2	78.2	78.2	78.4	78.2	78	78	78.2	78	78.4	77.1	78	76.6	77.5	77.7	80.4	100	
US-2	77.6	77.8	78.2	78	79.3	77.6	77.1	79.3	78.7	78.4	78.4	78.7	78.2	78.2	77.6	82.2	93.1	100

Nigerian HEV isolates, from sporadic cases that were observed during 1997-1998, were previously also shown to cluster together with the 1987 Mexican isolate (Buisson *et al*., 2000). To compare the four 1995 Namibian sequences with the Nigerian isolates and the 1983 Namibian isolate a nucleotide alignment was constructed from a 180 bp region where the sequences overlap (Figure 4.10).

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                      1586      1596      1606      1616      1626      1636
7798            TACCTACTATTGAACAATATTCTAAGTCATTCTTTGTGTTGCCCCTTCGTGGTAAGCTCT
7801            TACCTACTATTGAACAATATTCTAAGTCATTCTTTGTGTTGCCCCTTCGTGGTAAGCTCT
7948            TACCTACTATTGAACAATATTCTAAGTCATTCTTTGTGTTGCCCCTCCGTGGTAAGCTCT
8039            TACCTACTATTGAACAATATTCTAAGTCATTCTTTGTGTTGCCCCTTCGTGGTAAGCTCT
Nigeria-1       TCCCTACTATTGAGCAGTACTCCAAGTCATTCTTTGTGTTGCCCCTTCGCGGTAAGCTCT
Nigeria-9       TCCCTACTATTGAGCAGTATTCCAAGTCATTCTTTGTGTTGCCCCTTCGCGGTAAGCTCT
Mexico          TCCCGACTGTTGAGCAATATTCCAAGACATTCTTTGTGCTCCCCCTTCGTGGCAAGCTCT
ChadT3          TATCTACTATTCAGCAGTATTCGAAGACTTTCTTCGTCCTGCCGCTTCGTGGCAAGCTCT
83Namibia       TATCTACGATTCAGCAGTATTCGAAGACTTTCTTCGTCCTGCCGCTTCGTGGCAAGCTCT
94Egypt         TTTCTACCATTCAGCAGTACTCGAAGACTTTCTTTGTCCTGCCCCTCCGTGGCAAGCTCT
Morocco         TTTCTACTATCCAGCAGTATTCGAAGACTTTCTTTGTCCTGCCGCTTCGTGGTAAACTTT
China-Xinjiang  TTTCCACCATCCAGCAGTATTCAAAGACCTTCTTTGTCCTGCCGCTCCGCGGTAAGCTCT
Fulminant       TCTCCACCATCCAGCAGTATTCAAAGACCTTCTTCGTCCTGCCGCTCCGCGGTAAGCTCT
Pakistan-SAR-55 TTTCCACCATCCAGCAGTATTCAAAGACCTTCTTTGTCCTGCCGCTCCGCGGTAAGCTCT
Burma           TCTCCACCATCCAGCAGTACTCGAAGACCTTCTTTGTCCTGCCGCTCCGCGGTAAGCTCT
Pakistan-Abb-2B TCTCCACCATCCAGCAGTACTCGAAGACCTTCTTTGTCCTGCCGCTCCGCGGTAAGCTCT
Nepal           TCTCCACCATCCAGCAGTACTCGAAGACCTTCTTTGTCCTGCCGCTCCGCGGAAAGCTCT
India-Madras    TCTCCACCATCCAGCAGTACTCGAAGACCTTCTTTGTCCTGCCGCTCCGCGGCAAGCTCT
T1              TCACCACCATCCAGCAGTATTCTAAGACTTTCTATGTCTTGCCCCTTCGTGGTAAGCTTT
US-1            TTACTACCATTCAGCAGTATTCTAAGAAATTTTATGTTCTCCCGCTTCGNGGGAAGCTGT
US-2            TTACTACCATTCAGCAGTATTCTAAGACATTTTATGTTCTCCCGCTCCGCGGGAAGCTGT
                *  * **  *  * ** ** ** ***   ** *  **  * ** ** ** ** ** ** *

                      1646      1656      1666      1676      1686      1696
7798            CTTTTTGGGAGGCCGGTACAACGAAAGCGGGTTACCCCTATAATTACAATACCACTGCTA
7801            CTTTTTGGGAGGCCGGTACAACGAAAGCGGGTTACCCCTATAATTACAACACCACTGCTA
7948            CTTTTTGGGAGGCCGGTACAACGAAAGCGGGTTACCCCTATAATTACAACACCACTGCTA
8039            CTTTTTGGGAGGCCGGTACAACGAAAGCGGGTTACCCCTATAATTACAACACCACTGCTA
Nigeria-1       CTTTTTGGGAGGCTGGTACAACGAAAGCGGGCTACCCTTATAATTACAACACCACAGCTA
Nigeria-9       CTTTTTGGGAGGCTGGTACAACGAAAGCGGGCTACCCTTATAATTACAACACCACAGCTA
Mexico          CCTTTTGGGAGGCCGGCACAACAAAAGCAGGTTATCCTTATAATTATAATACTACTGCTA
ChadT3          CCTTCTGGGAGGCGGGTACTACTAAAGCCGGGTACCCTTATAATTATAATACCACTGCTA
83Namibia       CCTTCTGGGAGGCGGGTACCACTAAAGCCGGGTACCCTTATAATTATAATACTACTGCTA
94Egypt         CCTTTTGGGAGGCAGGTACTACTAAAGCCGGGTACCCTTATAATTACAATACCACTGCTA
Morocco         CCTTCTGGGAGGCAGGTACCACTAAAGCCGGGTACCCTTACAATTATAATACTACTGCTA
China-Xinjiang  CCTTTTGGGAGGCAGGTACTACTAAAGCCGGGTACCCTTATAATTATAACACCACTGCTA
Fulminant       CCTTCTGGGAGGCAGGTACCACTAAGGCCGGGTACCCCTATAATTATAATACCACTGCTA
Pakistan-SAR-55 CCTTTTGGGAGGCAGGAACTACTAAAGCCGGGTACCCTTATAATTATAACACCACTGCTA
Burma           CTTTCTGGGAGGCAGGCACAACTAAAGCCGGGTACCCTTATAATTATAACACCACTGCTA
Pakistan-Abb-2B CTTTCTGGGAGGCAGGCACAACTAAAGCCGGGTACCCTTATAATTATAATACCACTGCTA
Nepal           CTTTCTGGGAGGCAGGCACAACTAAGGCCGGGTACCCTTATAATTACAACACCACTGCTA
India-Madras    CTTTCTGGGAGGCAGGTACAACTAAGGCCGGGTACCCTTATAATTATAATACCACTGCTA
T1              CCTTTTGGGAGGCCGGCACCACTAAAGCCGGCTACCCTTATAATTATAACACTACTGCTA
US-1            CCTTTTGGGAGGCTGGTACGACCAAGGCCGGCTACCCGTATAATTATAATACCACTGCTA
US-2            CCTTTTGGGAGGCTGGCACGACTAAGGCCGGCTACCCTTACAATTATAATACTACCGCTA
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                    1706      1716      1726      1736      1746      1756
7798            GTGACCAGATTCTGATCGAGAATGCGGGCGGGCACCGAGTCTCCATTTCGACCTATACCA
7801            GTGACCAGATTCTGATCGAGAATGCGGCCGGCCACCGAGTCTCCATTTCGACCTATACCA
7948            GTGACCAGATTCTGATCGAGAATGCGGCCGGCCACCGAGTCTCCATTTCGACCTATACCA
8039            GTGACCAGATTCTGATCGAGAATGCGGCCGGCCACCGAGTCTCCATTTCGACCTATACCA
Nigeria-1       GCGACCAGATTTTGATTGAGAATGCAGCCGGCCACCGAGTTGCTATTTCGACCTATACCA
Nigeria-9       GCGATCAGATTTTGATCGAGAATGCAGCCGGCCACCGAGTTGCTATTTCGACCTATACCA
Mexico          GTGACCAGATTCTGATTGAAAATGCTGCCGGCCATCGGGTCGCCATTTCAACCTATACCA
ChadT3          GCGACCAATTGCTCATCGAGAACGTCGCTGGGCATCGGGTGGCTATTTCCACCTATACCA
83Namibia       GCGACCAATTGCTTATCGAGAACGCCGCTGGGCATCGGGTGGCCATTTCTACTTATACCA
94Egypt         GCGACCAATTGCTTATCGAGAACGCCACCGGGCATCGGGTGGCTATTTCTACTTACACTA
Morocco         GTGACCAACTGCTCGTTGAGAATGCCGCCGGGCACCGGGTGGCTATTTCCACCTACACCA
China-Xinjiang  GTGACCAACTGCTCGTTGAGAATGCCGCTGGGCATCGGGTTGCTATTTCCACTTACACCA
Fulminant       GCGATCAACTGCTTATTGAGAATGCCGCCGGGCATCGGGTTGCTATTTCCACTTACACCA
Pakistan-SAR-55 GTGACCAACTGCTCGTTGAGAATGCCGCTGGGCATCGGGTTGCTATTTCCACCTACACTA
Burma           GCGACCAACTGCTTGTCGAGAATGCCGCCGGGCACCGGGTCGCTATTTCCACTTACACCA
Pakistan-Abb-2B GCGACCAACTGCTCGTCGAGAATGCCGCCGGGCACCGGGTTGCTATTTCCACTTACACCA
Nepal           GCGGCCAACTGCTTGTCGAGAATGCCGCCGGGCACCGGGTTGCTATCTCCACTTACACCA
India-Madras    GCGACCAACTGCTTGTCGAGAATGCCGCCGGGCACCGGGTTGCTATCTCCACTTACACCA
T1              GTGACCAGATCCTGATTGAGAATGCAGCGGGCCACCGAGTTTGCATCTCTACCTACACTA
US-1            GTGACCAAATTTTGATTGAGAACGCGGCCGGTCACCGTGTCGCCATTTCTACTTATACCA
US-2            GTGACCAAATTTTGATTGAGAATGCGGCCGGCCACCGTGTCGCTATTTCCACCTATACCA
                * *  **  *  *  * ** ** *     ** ** ** **    ** ** ** ** ** *
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Figure 4.10 Nucleotide alignment of global HEV isolates, including 83-Namibian and 97/98-Nigerian isolates, against the Namibian sequences from position 1,577 to 1,756 of the ORF2 region (numbering according to the HPEGENSA isolate, GenBank Accession no. L08816).

A bootstrapped phylogenetic tree was constructed from 100 replicates of the alignment and rooted on the T1 isolate (Figure 4.11). The 95-Namibian sequences shared a nucleotide identity of between 97.8-100 %, were 88.9-91.1 % similar to the 97/98 Nigerian isolates and 85.6-86.1 % similar to the Mexican isolate (Table 4.3), all in genotype II. The 95-Namibian sequences were only 77.8-78.9 % similar to the 83-Namibian isolate (Table 4.3) in genotype I. Genotype I partitions into two geographical groupings with the African isolates clustering away from the Asian isolates. The 95-Namibian sequences, however, group together, as a separate clade of genotype II and are most similar to the 97/98 Nigerian isolates. This analysis clearly distinguishes between strains of HEV responsible for the 1995 Namibian outbreak and other reported African strains.

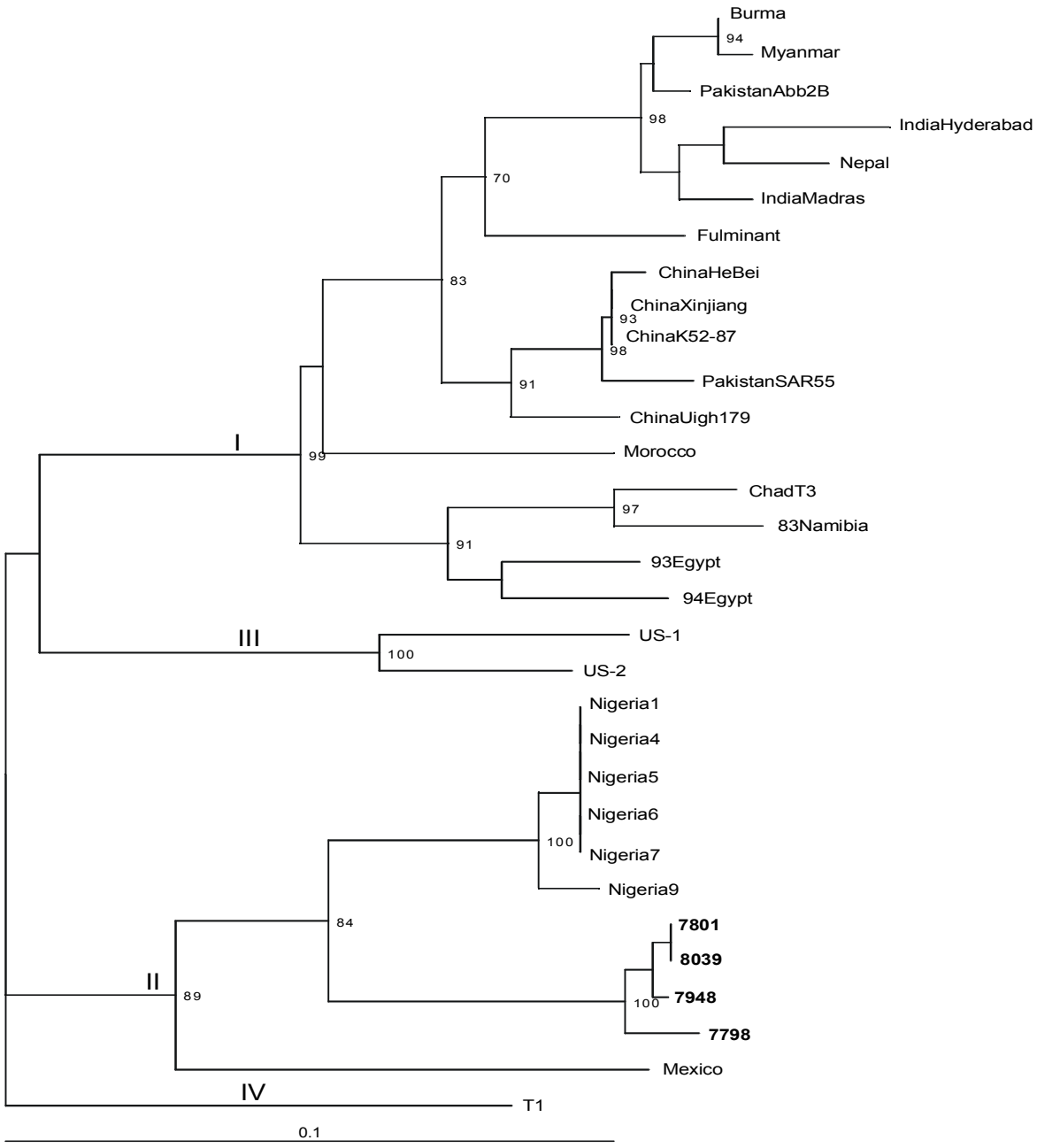

Fig. 4.11 Phylogenetic tree of HEV isolates, including 83-Namibian and 97/98-Nigerian isolates, from position 1,577 to 1,756 (nucleotide numbering with respect to the HPEGENSA isolate, GenBank Accession no. L08816) of the ORF2 region. The tree is rooted with the T1 sequence and clades are labeled according to the nomenclature of Wang *et al.*, 2000. Bootstrap values for the most robust groupings are shown.

Table 4.3 Degree of nucleic acid homology of 1995 Namibian and global isolates of HEV including 83-Namibian and 97/98-Nigerian isolates within the 180 bp fragment (1,577 to 1,756 bp) of ORF2 region towards the carboxy terminal end of the genome.

Isolate	7798	7801	7948	8039	Nig-1	Nig-9	Mexico	Chad	83Nam	94Egy	Moroc	ChinaX	Fulm	Sar-55	Burma	Abb-2B	Nepal	I-Madras	T1	US-1	US-2
7798	100																				
7801	98.3	100																			
7948	97.8	99.4	100																		
8039	98.3	100	99.4	100																	
Nigeria-1	88.9	90.6	90	90.6	100																
Nigeria-9	89.4	91.1	90.6	91.1	98.3	100															
Mexico	85.6	86.1	85.6	86.1	83.9	83.3	100														
ChadT3	79.4	78.9	78.3	78.9	78.9	79.4	80	100													
83Namibia	78.9	78.3	77.8	78.3	77.2	77.8	80.6	95.6	100												
94Egypt	78.3	77.8	78.3	77.8	79.4	78.9	78.3	90.6	91.1	100											
Morocco	78.3	77.8	77.2	77.8	78.3	77.8	79.4	89.4	88.3	86.7	100										
ChinaXinjiang	76.7	77.2	77.8	77.2	79.4	78.9	78.3	87.8	86.1	88.3	90.6	100									
Fulminant	76.1	75.6	76.1	75.6	78.3	78.9	77.2	87.2	87.8	87.2	88.3	93.3	100								
Pakistan-SAR-55	76.1	76.7	77.2	76.7	78.9	78.3	78.3	87.2	84.4	87.8	90	98.3	91.7	100							
Burma	77.8	78.3	78.9	78.3	81.1	80.6	77.8	86.1	86.1	88.3	88.9	92.8	92.2	91.7	100						
Pakistan-Abb-2B	77.8	77.2	77.8	77.2	81.1	80.6	77.8	87.2	86.1	88.3	90	93.3	92.8	92.2	98.3	100					
Nepal	75.6	76.1	76.7	76.1	80	79.4	75	83.9	83.9	87.2	86.1	90.6	91.1	89.4	96.7	96.1	100				
India-Madras	76.7	76.1	76.7	76.1	80	79.4	76.7	86.7	86.7	88.9	88.3	91.7	93.3	90	96.7	97.2	97.2	100			
T1	80.6	82.2	81.7	82.2	81.7	81.1	81.1	77.8	77.8	79.4	82.8	82.2	80	83.3	80.6	80.6	79.4	79.4	100		
US-1	80.4	80.4	79.9	80.4	80.4	79.7	80.4	79.9	79.9	80.4	79.9	80.4	79.9	78.8	78.2	78.2	76.5	78.8	80.4	100	
US-2	78.3	78.9	79.4	78.9	81.7	81.1	81.7	79.4	79.4	78.9	82.8	81.7	80.6	81.7	80.6	80.6	78.9	80	81.7	92.7	100

4.4.2 Analysis of genetic distances within the 3'-end of ORF2

The consensus sequence of the four 95-Namibian isolates was compared to 20 HEV isolates (used in Figure 4.9) over the 451 bp target region of ORF2, and the frequency of evolutionary distances were plotted for each 0.02 range between zero and 0.259 (Figure 4.12). The three peaks of the histogram indicate that isolates within the same subgenotype are less than 4 % different from one another, genotypic groups differ by greater than 18 % and subgenotypes are defined between these two values. Therefore, the suitability of this subgenomic fragment for phylogenetic analysis is demonstrated by both bootstrap analysis and in the clear separation of type and subtype evolutionary distances in the histogram.

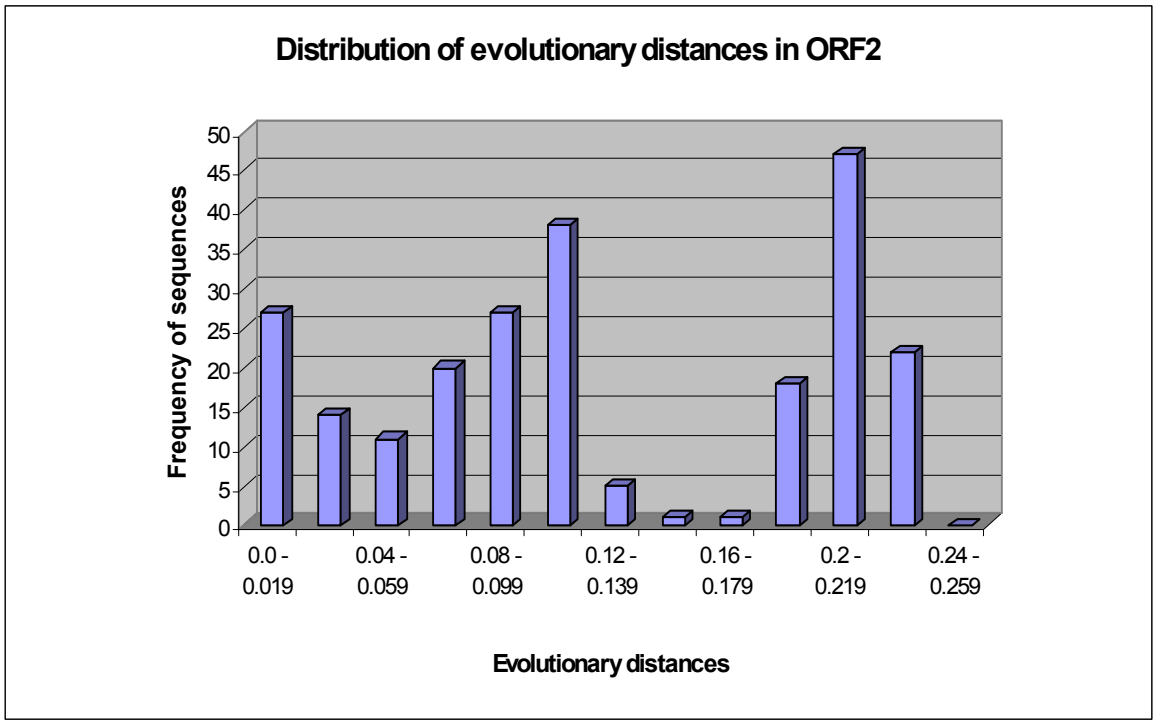

Figure 4.12 Histogram representing the distribution of genetic distances calculated for ORF2 region, from position 1,307 to 1,757 of the HEV genome.

4.4.3 Amino acid analysis of ORF2 3'-end

The consensus sequence of the four Namibian isolates was translated into a consensus amino acid sequence. To observe the relationship between Namibian amino acid sequences and other amino acid sequences from global isolates within the 3'-end of ORF2 region, the Namibian consensus amino acid sequence was compared to the deduced amino acid sequence of 14 GenBank isolates (Figure 4.13) based on the 451 bp target region. Unique, definitive amino acids common to the 95-Namibia consensus sequence only were observed at residues 477 (Alanine 'A' to Glycine 'G'), 535 (Threonine 'T' to Serine 'S') and 580 ('A' to 'S'), and those common to both 95-Namibia and Mexico were observed at residues 517 ('T' to 'S'), 527 ('S' to Proline 'P') and 530 (Glutamine 'Q' to Glutamic acid 'E'). Amino acid changes shared by genotype II isolates but not unique to this group were observed at residues 569 (Leucine 'L' to Isoleucine 'I') and 571 (Valine 'V' to 'I'). The Mexican subgroup of genotype II is defined by unique amino acid changes at residues 494 ('V' to 'I') and 529 ('I' to 'V').

```
                    446       456       466       476       486       496
Consensus       RVVIQDYDNQHEQDRPTPSPAPSRPFSVLRANDVLWLSLTGAEYDQSTYGSSTGPVYVSD
Mexico          ----------------------------------------a----------------i--
ChadT3          ----------------------------------------a-------------------
94Egypt         ----------------------------------------a-------------------
Morocco         ----------------------------------------a-------------------
China-Xinjiang  ----------------------------------------a-------------------
Fulminant       ----------------------------------------a-------------------
Pakistan-SAR-55 ----------------------------------------a-------------------
Burma           ----------------------------------------a-------------------
Pakistan-Abb-2B ----------------------------------------a-------------------
Nepal           ----------------------------------------a-------------------
India-Madras    --------------------------a-----------a-------------------
T1              ----------------------------------------a-----t------n-m----
US-1            ----------------------------------------a---x-t------n-m----
US-2            ----------------------------------------a-----t------n-m----
```

```
                    506       516       526       536       546       556
Consensus       SVTLVNVATGAQAVARSLDWSKVTLDGRPLPTIEQYSKSFFVLPLRGKLSFWEAGTTKAG
Mexico          ------------------------------v-----t--------------------
ChadT3          -------g------------t---------s--q----t--------------------
94Egypt         --------------------t---------s--q----t--------------------
Morocco         --------------------t---------s--q----t--------------------
China-Xinjiang  --------------------t---------s--q----t--------------------
Fulminant       --------------------t---------s--q----t--------------------
Pakistan-SAR-55 --------------------t---------s--q----t--------------------
Burma           --------------------t---------s--q----t--------------------
Pakistan-Abb-2B --------------------t---------s--q----t--------------------
Nepal           --------------------t---------s--q----t--------------------
India-Madras    --------------------t---------s--q----t--------------------
T1              t--f--------g-s---------------t--q----t-y------------------
US-1            t-----------------------------t--q----k-y------------------
US-2            t-----------------------------t--q----t-y------------------


                    566       576       585
Consensus       YPYNYNTTASDQILIENAAGHRVSISTYT
Mexico          ----------------------a-----
ChadT3          ------------l----v-----a-----
94Egypt         ------------l-----t----a-----
Morocco         ------------l-v--------a-----
China-Xinjiang  ------------l-v--------a-----
Fulminant       ------------l----------a-----
Pakistan-SAR-55 ------------l-v--------a-----
Burma           ------------l-v--------a-----
Pakistan-Abb-2B ------------l-v--------a-----
Nepal           ----------g-l-v--------a-----
India-Madras    ------------l-v--------a-----
T1              ----------------------c-----
US-1            ----------------------a-----
US-2            ----------------------a-----
```

Figure 4.13 Amino acid sequence alignment of the 95-Namibian HEV consensus sequence against other global sequences, from position 1,307 to 1,757 of the ORF2 region. The dashes represent regions of homology with the 95-Namibian consensus sequence.

Most change was synonymous and the Namibian consensus sequence shared an amino acid identity of 96.6 % with the Mexican isolate and 94.0-94.6 % with other African isolates (Table 4.4).

Table 4.4 Degree of amino acid homology of the 95-Namibian consensus sequence against other global isolates of HEV within the ORF2 region towards the carboxy terminal end of the genome.

Isolate	Consensus	Mexico	ChadT3	94Egypt	Morocco	ChinaXin	Fulminant	P-SAR-55	Burma	P-Abb-2B	Nepal	I-Madras	T1	US-1	US-2
Consensus	100														
Mexico	96.6	100													
ChadT3	94	94.6	100												
94Egypt	94.6	95.3	98	100											
Morocco	94.6	95.3	98	98.7	100										
ChinaXinjiang	94.6	95.3	98	98.7	100	100									
Fulminant	95.3	96	98.7	99.3	99.3	99.3	100								
PakistanSAR55	94.6	95.3	98	98.7	100	100	99.3	100							
Burma	94.6	95.3	98	98.7	100	100	99.3	100	100						
PakistanAbb2B	94.6	95.3	98	98.7	100	100	99.3	100	100	100					
Nepal	94	94.6	97.3	98	99.3	99.3	98.7	99.3	99.3	99.3	100				
IndiaMadras	94	94.6	97.3	98	99.3	99.3	98.7	99.3	99.3	99.3	98.7	100			
T1	91.3	91.3	90.6	91.3	91.3	91.3	91.9	91.3	91.3	91.3	90.6	90.6	100		
US-1	92.6	92.6	91.9	92.6	92.6	92.6	93.3	92.6	92.6	92.6	91.9	91.9	96	100	
US-2	93.3	94	93.3	94	94	94	94.6	94	94	94	93.3	93.3	97.3	98.7	100

The Namibian amino acid consensus sequence was also compared to the deduced amino acid sequences of 83-Namibia and 97/98-Nigerian isolates over a 180 bp region where the sequences overlap (Figure 4.14). The alignment confirmed that the 83-Namibia sequence belong to genotype I, with its substitutions similar to other genotype I isolates. The Nigerian sequences were identical to the 95-Namibian consensus sequence over this region except for one amino acid change at residue 580 (‘S’ to ‘A’) which differentiated them from the 95-Namibian sequence within genotype II.

```
                    536       546       556       566       576      585
Consensus       PTIEQYSKSFFVLPLRGKLSFWEAGTTKAGYPYNYNTTASDQILIENAAGHRVSISTYT
Nigeria-1       ----------------------------------------------------a-----
Nigeria-9       ----------------------------------------------------a-----
Mexico          --v-----t-----------------------------------------------a-----
ChadT3          s--q----t----------------------------------l----v-----a-----
83Namibia       s--q----t----------------------------------l----------a-----
94Egypt         s--q----t----------------------------------l-----t----a-----
Morocco         s--q----t----------------------------------l-v--------a-----
China-Xinjiang  s--q----t----------------------------------l-v--------a-----
Fulminant       s--q----t----------------------------------l----------a-----
Pakistan-SAR-55 s--q----t----------------------------------l-v--------a-----
Burma           s--q----t----------------------------------l-v--------a-----
Pakistan-Abb-2B s--q----t----------------------------------l-v--------a-----
Nepal           s--q----t--------------------------------g-l-v--------a-----
India-Madras    s--q----t----------------------------------l-v--------a-----
T1              t--q----t-y--------------------------------------------c-----
US-1            t--q----k-y--------------------------------------------a-----
US-2            t--q----t-y--------------------------------------------a-----
```

Figure 4.14 Amino acid sequence alignment of the 95-Namibian HEV consensus sequence against other global sequences, including 83-Namibian and 97/98-Nigerian amino acid sequences, from position 1,577 to 1,756 of the ORF2 region. The dashes represent regions of homology with the 95-Namibian consensus sequence.

The 95-Namibian consensus sequence was observed to be more similar to the Nigerian isolates (98.3 % amino acid identity) than to the Mexican isolate (94.9 % amino acid identity) within genotype II and only 91.5 % identical to the 83-Namibian isolate in genotype I (Table 4.5).

Table 4.5 Degree of amino acid homology of the 95-Namibian consensus sequence against other global isolates of HEV, including 83-Namibian and 97/98-Nigerian isolates, within the 59 common aa in the ORF2 region towards the carboxy terminal end of the

Isolate	Consen-sus	Nig-1	Nig-9	Mexico	ChadT3	83Na-mibia	94Egypt	Morocco	ChinaX	Fulm	P-SAR-55	Burma	P-Abb-2B	Nepal	I-Madras	T1	US-1	US-2
Consensus	100																	
Nigeria-1	98.3	100																
Nigeria-9	98.3	100	100															
Mexico	94.9	96.6	96.6	100														
ChadT3	89.8	91.5	91.5	91.5	100													
83Namibia	91.5	93.2	93.2	93.2	98.3	100												
94Egypt	89.8	91.5	91.5	91.5	96.6	98.3	100											
Morocco	89.8	91.5	91.5	91.5	96.6	98.3	96.6	100										
ChinaXinjiang	89.8	91.5	91.5	91.5	96.6	98.3	96.6	100	100									
Fulminant	91.5	93.2	93.2	93.2	98.3	100	98.3	98.3	98.3	100								
Pakistan-SAR-55	89.8	91.5	91.5	91.5	96.6	98.3	96.6	100	100	98.3	100							
Burma	89.8	91.5	91.5	91.5	96.6	98.3	96.6	100	100	98.3	100	100						
Pakistan-Abb-2B	89.8	91.5	91.5	91.5	96.6	98.3	96.6	100	100	98.3	100	100	100					
Nepal	88.1	89.8	89.8	89.8	94.9	96.6	94.9	98.3	98.3	96.6	98.3	98.3	98.3	100				
India-Madras	89.9	91.5	91.5	91.5	96.6	98.3	96.6	100	100	98.3	100	100	100	98.3	100			
T1	91.5	91.5	91.5	91.5	91.5	93.2	91.5	91.5	91.5	93.2	91.5	1.5	91.5	89.8	91.5	100		
US-1	91.5	93.2	93.2	91.5	91.5	93.2	91.5	91.5	91.5	93.2	91.5	91.5	91.5	89.8	91.5	96.6	100	
US-2	91.5	93.2	93.2	93.2	93.2	94.9	93.2	93.2	93.2	94.9	93.2	93.2	93.2	91.5	93.2	98.3	98.3	100

CHAPTER 5: DISCUSSION AND CONCLUSION

A significant proportion of acute viral hepatitis occurring in young to middle-aged adults in Africa, Asia and the Indian subcontinent is caused by hepatitis E virus (Bradley, 1994). Hepatitis E has major health implications in these countries and outbreaks with many thousands of cases are on record. The disease has been shown to occur in both epidemic and sporadic endemic forms and is primarily associated with the ingestion of faecally contaminated drinking water.

Concern about the disease in South Africa is supported by evidence of typical outbreaks with clinical cases in neighbouring countries such as Namibia and Botswana (He *et al*., 2000; Byskov *et al*., 1989). Whilst the virus has been characterized from many regions in the world, as well as in Africa, only one outbreak has been definitively described from southern Africa (He *et al*., 2000). Prevalence studies based on serological assessment from different populations have suggested activity by this agent in South Africa (Grabow *et al.*, 1996; Tucker *et al*., 1996). Progress in research on the epidemiology of hepatitis E is awaited worldwide and this is particularly important in countries such as South Africa; where the prevalence of the virus has been shown to be intermediately endemic but at the same time conditions exist in many communities which are ideally suited for outbreaks of the disease. Reasons for a relatively low incidence of clinical cases and outbreaks in certain parts of the world, despite the presence of the virus, are not yet fully understood.

The aim of this study was to retrospectively characterize hepatitis E virus from stored specimens collected during two hepatitis outbreaks, one which occurred in 1983, in Maun, Botswana and the other one which occurred in 1995, in Rundu, Namibia. Methods that were developed included extraction of the viral RNA from the clinical specimens, and nested RT-PCR to amplify the viral nucleic acid for sequence analysis and subsequent phylogenetic analysis. Two different regions of the HEV genome were explored. The first region spans 559 bp of the polymerase (RdRp) region within ORF1 and the second region spans 728 bp towards the carboxy terminal (3') end of the genome in ORF2. The ORF1 region was the first region to be targeted for RNA amplification because a plasmid DNA with an HEV ORF1 insert was available as a positive control for optimizing the PCR amplification method. The ORF2 region was then targeted for RNA amplification since it contains a highly immunogenic epitope (Reyes *et al*., 1993) and most of the published HEV sequences from Africa are based on the ORF2 region; this allowed for a meaningful comparative analysis.

Several methods have been used in the past for extraction of HEV RNA from clinical specimens prior to RT-PCR. These include the use of glass powder to bind the nucleic acid nonspecifically after the disruption of the virus (Mc Caustland *et al.*, 1991), and conventional RNA separation by using acid guanidium isothiocyanate-phenol-chloroform extraction (Ray *et al*., 1991). In this study microspin technology was applied (section 3.6.2) in which the virus is first lysed and the lysate in then passed through a silica-based membrane which selectively binds nucleic acid, which is then eluted by passing a small volume of buffer through it (Aggarwal *et al*., 1998).

A nested PCR method was developed for amplification of the ORF1 region of HEV (section 3.7), initially using an HEV insert from amplified plasmid DNA. Another region of the HEV genome, towards the carboxy terminal end of ORF2, was the second region targeted for PCR amplification. The ORF2 region is a well studied region of HEV and especially in Africa, there is a sufficient number of publications which allowed for a more complete comparison of our 1995 Namibian sequences to other African isolates. An optimum nested RT-PCR was developed, in this study, for RNA amplification of the 3'-end of ORF2. Briefly, the specimens were ultracentrifuged at 4 °C with a speed of 23,000 rpm for one hour (section 3.6.1). RNA was extracted from the pellet by use of the High Pure Viral RNA Kit and/or QIAamp Viral Isolation Kit (section 3.6.2). Ten microliters of RNA was then mixed together with the Supertherm DNA polymerase enzyme, 1.5 mM $MgCl_2$ ion concentration in the 10X Supertherm PCR reaction buffer, AMV RT, RNASin and ORF2 primers and the RNA was amplified with the GeneAmp thermal cycler at an annealing temperature of 48 °C (section 3.7.2) The ORF2 RT-PCR method was able to amplify and produce PCR positive results from the 1995 Namibia specimens. The PCR products were then sequenced with the ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (section 3.8).

The ORF2 region was again targeted for RNA amplification of the Botswana specimens and none of the specimens yielded amplicons even after optimizing the PCR using different polymerase enzymes and kits recommended for degraded RNA specimens (section 3.7.3). The Botswana specimens were collected in the year 1983 and were stored at -70 °C for more than 15 years (before they were used in this study) instead of being stored in liquid nitrogen gas, which is ideal for storage of RNA viruses especially over a

long period of time (Chobe *et al*., 1997). Therefore, it was concluded that the nucleic acid within specimens was highly degraded (Ray *et al*., 1991).

Neither PCR was as robust as we would have liked and amplified only a small proportion of the clinical specimens (section 4.2.2.1 and 4.2.2.2). This might have been due to repeated freezing and thawing of the specimens which can lead to the degradation of HEV and the loss of its nucleic acid integrity, and in turn cause difficulties in amplification (Ray *et al*., 1991; Chobe *et al*., 1997).

In addition, difficulties in PCR amplification of both ORF1 and 2 targeted regions, from clinical samples, might have been due to the short duration of HEV RNA in serum (Qi *et al*., 1995). HEV RNA and viral antigens occur in serum from late incubation period to early acute phase of the disease. Viremia in HEV infected persons is usually short-lived, often peaking and waning before the clinical signs of infection are apparent (Yarbough, 1999; Lin *et al*., 2000) and this relates to the timing of virus excretion in faeces (Ray *et al*., 1991; Aubry *et al*., 1997). The difference between the levels and timing of HAV and HEV nucleic acid recovery may be related to a difference in the level of infectious particles of HEV found in faeces and blood during infection (Clayson *et al*., 1995; Purcell, 1996; Divizia *et al*., 1999; Christensen *et al*., 2002). Animal studies suggest that clinical hepatitis E is dose-dependent, that is, for HEV a threshold dose of virus exists below which clinical disease does not occur but infection does (Tsarev *et al*., 1994b, Christensen *et al*, 2002). In other studies no virus was detected in faecal samples by using either IEM (Ticehurst *et al*., 1992) or RT-PCR (Widdowson *et al*., 2003) and this can be due to metabolites and other biological substances interfering with the reactions (Turkoglu *et al*., 1996). One other possible factor contributing to the difficulties in amplifying viral sequences is the fact that variation of nucleotide sequence in the primer

regions among different HEV strains can be as high as 28 % (Huang *et al*., 1995; Lin *et al*., 2000). These and other factors such as 5' and 3'-RNA secondary folding (Huang *et al*., 1992; Agrawal *et al*., 2001) which could prevent primer binding and result in poor cDNA yields, may explain why HEV has never before been isolated from South Africa even though antibody based studies appear to indicate the existence of this virus in the country [Grabow *et al*., 1994 and 1996; Tucker *et al*., 1996; Tucker, personal communication].

Despite the problems encountered, sequence analysis of the PCR fragments obtained did allow adequate molecular characterization of the HEV strain responsible for the 1995 Rundu, Namibia outbreak. Phylogenetic analysis of the carboxy terminal end of ORF2 region revealed the conventional four genetic clusters consistent with previous findings (Tsarev *et al*., 1999; He *et al*., 2000; Worm *et al*., 2000; Schlauder and Mushahwar, 2001; Widdowson *et al*., 2003). The ORF2 analysis clearly distinguishes between the strains of HEV responsible for the 1995 Namibian outbreak and other reported African outbreaks. Genotype I produced a clear division between the Asian isolates and the African isolates from Morocco, Chad, Egypt and Namibia-83. These African isolates clustered together and away from the Asian isolates (Figure 4.9 and Figure 4.11). The 1995 Namibian sequences, however, clustered into genotype II together with the Mexican isolate with a bootstrap value of a 100 (Figure 4.9) and the Nigerian isolates with a bootstrap value of 84 (Figure 4.11), both of which represent statistically significant groupings (Muerhoff *et al*., 1997). This was confirmed by the histogram constructed from the 451 bp target region of ORF2 (Figure 4.12). The three peaks of the histogram indicate that isolates within the same subgenotype are less than 4 % different

from one another, genotypic groups differ by greater than 18 % and subgenotypes are defined between these two values.

The phylogenetic clustering of ORF2 was also confirmed by amino acid analysis based on the 451 bp target region. Unique, definitive amino acids common to the 1995 Namibian subgroup were observed at residues 477 (‘A’ to ‘G’), 535 (‘T’ to ‘S’) and 580 (‘A’ to ‘S’), and those common to both the 1995 Namibian and the Mexican isolates were observed at residues 517 (‘T’ to ‘S’), 527 (‘S’ to ‘P’) and 530 (‘Q’ to ‘E’; Figure 4.13). Amino acid changes shared by genotype II isolates but not unique to this group were observed at residues 569 (‘L’ to ‘I’) and 571 (‘V’ to ‘I’). The Mexican subgroup of genotype II is defined by unique amino acid changes at residues 494 (‘V’ to ‘I’) and 529 (‘I’ to ‘V’). Most change was synonymous and the Namibian consensus sequence shared an amino acid identity of 96.6 % with the Mexican isolate and 94.0-94.6 % with other African isolates (Table 4.4). The same phylogenetic clustering was observed when the 1995 Namibian consensus amino acid sequence was compared with the 1983 Namibian and the Nigerian amino acid sequences (tree not shown but distance matrix shown in Table 4.5). The amino acid alignment in Figure 4.14 also confirmed that the 1983 Namibian sequence cluster into genotype I and is different from the 1995 Namibian and Nigerian isolates.

The Namibia-83 study provided the first published HEV sequence data from southern Africa. These isolates were reported to cluster into genotype I (He *et al*., 2000). This is interesting since the 1983 and 1995 Namibian outbreaks both occurred in the Rundu area of Namibia. Co-circulating strains within the same country have been reported previously. For example, the 1987 Pakistan-Sar55 isolate clustered with other Asian isolates in subgenotype I-1a while the 1988 Pakistan-Abb2B isolate clustered with

Burmese isolates in subgenotype I-1b (van Cyuck-Gandré *et al*., 2000).

Thus, by successfully amplifying, sequencing and analyzing four representative HEV isolates from a non-A non-B hepatitis outbreak in Rundu, Namibia, in 1995 we are able to confirm that the agent was indeed HEV, as suspected from the results of the serological assays at the time. Furthermore we are able to report the presence of a second unique HEV strain in southern Africa, indicating that HEV genotypes may be more widely distributed than previously thought.

ELISAs based on recombinant antigens and synthetic peptides have been commercially available for detection of present and past HEV infection for some years now. Studies using these assays have intimated for almost a decade that healthy populations within certain regions of South Africa have HEV antibody. Since HEV outbreaks have now been confirmed within the borders of at least one of South Africa's neighbours, Namibia 1983 (He *et al*., 2000) and Namibia 1995 (this study) as well as being strongly suspected in Botswana in 1985, travel between other African countries and South Africa could, and probably has, introduced asymptomatic and symptomatic sporadic HEV disease into this country. Not much is known about the survival of the virus in food and water, both implicated in viral transmission, nor the features of the virus or its disease outcome which enable it to cause extensive outbreaks under suitable conditions nor indeed the reasons for the apparent geographic restriction of outbreaks. In the past warnings have been issued to health care personnel to consider hepatitis E as a possible agent in cases of hepatitis in travelers but usually this has been restricted to those returning from Asia, and countries such as India in particular. This is even more critical in the case of pregnant women where disease outcome can be severe. The availability of a nucleic acid test

(NAT) for HEV will enable a more routine inclusion of HEV in hepatitis studies especially in cases of NANBNC hepatitis. The sensitivity and robustness of NAT has recently been significantly improved by "state of the art" methodologies and technologies. Two of the most important of these are based on the MagNa Pure LC instrument which robotically performs ultra-pure nucleic acid extraction and sets up reaction mixtures for real-time PCR using the LightCycler instrument (both from Roche Diagnostics Corporation, Mannheim, Germany). A method employing the real-time quantitative PCR with SYBR-green (ds DNA-binding dye) using the LightCycler instrument which can detect 10 copies of HEV cDNA fragment per reaction tube from human faecal samples after only 3 hours was recently published (Orru *et al*., 2004). The method demonstrated a high linear dynamic range of quantification from 10 to 10^6 molecules of cDNA/reaction with good correlation (r = -1.00). Should our methods and those of others become optimized in this way they will facilitate efficient diagnostic testing for HEV and open the door for meaningful surveillance studies for HEV in this part of the world. Adequate surveillance and rapid, sensitive diagnosis are particularly important since strategies aimed at disease prevention are the only methods for disease control since to date no vaccine or therapy is available.

APPENDIX A: COMPOSITION OF BUFFERS, MEDIA AND GELS

1. Reagents for transformation

1.1 SOC medium:

Bacto-tryptone	10.0 g
Bacto-yeast extract	2.5 g
NaCl	0.25 g
250 mM KCL	10.0 ml

Adjust the pH of the mixture to 7.0 with 5 N NaOH (~0.2 ml), make up to 500 ml with distilled water and autoclave for 15 minutes at 121 °C. Allow the mixture to cool to 60 ° C and add 5.0 ml 2.0 M $MgCl_2$ (19 g $MgCl_2$ in 100 ml distilled water) autoclaved as above. Add 20 ml 1.0 M glucose (18 g in 100 ml distilled water) to the mixture, that is, after the glucose has dissolved make up to 100 ml with distilled water. Filter the complete medium through a 0.2 µm filter unit.

1.2 LB agar plates:

LB agar (Lennox L agar)	6.3 g

Make up to 200 ml with distilled water and autoclave for 15 minutes at 121 °C. Allow it to cool to 60 °C and then add 1.0 ml of 10 mg/ml kanamycin before pouring the plates.

Appendix A

1.3 LB growth medium:

LB broth base (Lennox L broth base)	20.0 g

Make up to 1000 ml with distilled water and autoclave for 15 minutes at 121 ºC. Allow it to cool to room temperature and then add 1 ml of 10 mg/ml kanamycin.

2. Reagents for agarose gel electrophoresis

2.1 10X TBE stock buffer:

Tris-HCL	108.0 g
Boric acid	55.0 g
0.5 M EDTA	40.0 ml

Make up to 1000 ml with distilled water. Dilute 1:10 for 1X TBE and 1:20 for 0.5X TBE running buffer.

2.2 1 % agarose gel:

One gram of molecular grade agarose D-1 LE in 100 ml 1X TBE. Heat in microwave until dissolved. Allow to cool to room temperature and add 5.0 µl of 10 mg/ml ethidium bromide (EthBr) before pouring the gel.

2.3 2.5 % metaphor agarose gel:

Metaphore agarose 2.5 g in 100 ml 1X TBE. Mix and leave for 10 minutes to hydrate the agarose. Heat in microwave until the solution nears boiling. Cool for 30 seconds, swirl and repeat the heating, cooling and swirling until the solution is dissolved. Pour gel and leave it to set at room temperature for 15 minutes followed by 30 minutes at 4 °C.

Appendix A

3. Reagents for clarifying stool samples

3.1 Gelatin buffered saline (GBS) 10X in 5 liters:

NaCl	403.75 g
$Na_2HPO_4.12H_20$	120.0 g
KH_2PO_4	22.5 g
Phenol red 0.4%	250.0 ml
Hot water	5000 ml

Sterilize the complete mixture by filtration.

3.2 0.5 % GBS in 5 liters:

GBS 10X	500 ml
Water	4000 ml
Gelatin (Bacto gelatin)	25.0 g
Water	5000 ml

Add evaporation water 180 ml/5 L for autoclaving.

Appendix A

4. Reagents for resuspending viral RNA

4.1 Dulbecco buffer 10X Mg^{++} free in 1 liter:

NaCl	80.0 g
KCL	2.0 g
Na_2HPO_4 anhydrous	11.5 g
KH_2PO_4	2.0 g

Dissolve the chlorides in 500 ml and use hot water to dissolve the phosphates, add together and make up to 1 liter.

4.2 Phosphate buffered saline (PBS) Ca^{++} Mg^{++} free in 1 liter:

Dulbecco 10X Mg^{++} free	100 ml

Add water up to 1000 ml, adjust pH to 7.4 and sterilize by filtration.

5. In-house PCR reaction buffers

5.1 10X in-house buffer with 10 mM $MgCl_2$:

1 M Tris pH 8.8	100 µl
2.5 M KCL	200 µl
1 M $MgCl_2$	10 µl
5 % Gelatin	20 µl
Triton X-100	10 µl
Water	655 µl

Mix and make 100 µl aliquots in microcentrifuge tubes and store at 4 °C.

Appendix A

5.2 10X in-house buffer with 15 mM $MgCl_2$:

1 M Tris pH 8.8	100 µl
2.5 M KCL	200 µl
1 M $MgCl_2$	15 µl
5 % Gelatin	20 µl
Triton X-100	10 µl
Water	655 µl

Mix and make 100 µl aliquots in microcentrifuge tubes and store at 4 °C.

5.3 10X in-house buffer with 20 mM $MgCl_2$:

1 M Tris pH 8.8	100 µl
2.5 M KCL	200 µl
1 M $MgCl_2$	20 µl
5 % Gelatin	20 µl
Triton X-100	10 µl
Water	655 µl

Mix and make 100 µl aliquots in microcentrifuge tubes and store at 4 °C.

Appendix A

5.4 10X in-house buffer with 25 mM $MgCl_2$:

1 M Tris pH 8.8	100 µl
2.5 M KCL	200 µl
1 M $MgCl_2$	25 µl
Water	675 µl

Mix and make 100 µl aliquots in microcentrifuge tubes and store at 4 °C.

6. Reagents for sequencing

6.1 Sequencing gel

6.1.1 5 % Gelmix 50 ml:

Urea	19.0 g
Baxter water	24.5 ml
10X TBE	7.5 ml
50 % Long ranger	5.0 ml

Mix for 30 minutes to dissolve and filter through Millex HV filter.

6.2 10 % Ammonium persulphate:

Ammonium persulphate	0.5 g
Baxter water	4.5 ml

Mix until it is dissolved, filter through a 0.45 µm filter unit, aliqout into 300 µl amounts and store at -20 °C. Thaw at room temperature before use.

Appendix A

6.3 Gelmix to polymerise 50 ml:

Temed	25.0 µl
10 % Ammonium persulphate	250 µl

Mix gently without making bubbles and leave for 2.5 hours before use.

APPENDIX B: LIST OF SUPPLIERS

ABI PRISM 377 DNA Sequencer	Perkin Elmer Corporation, California, USA
Abbott HEV EIA Kit	Abbott Diagnostika, Wiesbaden-Delkenheim, Germany
Ammonium persulphate	Amersham Pharmacia Biotech Ltd, Buckinghamshire, UK
AMV	Roche Diagnostics Corporation, Mannheim, Germany
ALFexpress^{TM} DNA Sequencer	Amersham Pharmacia Biotech Ltd, Buckinghamshire, UK
Applicator sticks	LASEC, Laboratory and Scientific Equipment, South Africa
Bacto-tryptone	Unipath Ltd, UK
Bacto-yeast extract	DIFCO Laboratories, USA
Baxter water	Adcock Ingram, Sterilab Services, South Africa
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APPENDIX C: APPROVAL FOR UNDERTAKING RESEARCH PROJECT

S-0/0301

Republic of Namibia

Ministry of Health and Social Services

Private Bag 13198 | Ministerial Building | Tel: (061) 2032822
Windhoek | Harvey Street | Fax: (061) 061 227607
Namibia | Windhoek | 264 61 227607
Enquiries: Ms M. Zauana

Date: 19/09/96

OFFICE OF THE PERMANENT SECRETARY

Dear Ms. Bewley

YOUR APPLICATION FOR REGISTRATION OF A RESEARCH PROJECT:

VIROLOGICAL INVESTIGATION OF NON-A NON-B HEPATITIS (NANBH) IN NORTHERN NAMIBIA

The Ministry of Health and Social Services is hereby granting you permission to undertake your research project:

Please note that this permission is subject to the following conditions:

1. There shall be no cost implications on the part of the Ministry.

2. You bear the full responsibility for the project and should it be necessary to deviate from any procedure stipulated in the research proposal, you shall notify the research Management Committee Secretariat, who will obtain the necessary approval from the Permanent Secretary.

3. You shall obtain full consent from patients/subjects or if they are not in a position to do so, to seek their legal guardian's consent.

APPENDIX C

4. You shall submit the results of the project to the Research Management Committee Secretariat, prior to any public statement for publication within a period of 3 months after completion of the project.

5. The following amendments be incorporated into the research project: See Annex.

~~Applicable~~/Not applicable

Yours sincerely,

MINISTRY OF HEALTH AND SOCIAL SERVICES

Mr. O. Akwenye
ACTING PERMANENT SECRETARY

APPENDIX C

SUBJECT INFORMATION SHEET

Your doctor is concerned that you have become sick from a virus called hepatitis E virus, which is causing sickness in a lot of people here at the moment. The doctor will need to take some of your blood (a tiny tube - about 1 teaspoon full), and maybe a stool specimen so that this can be checked. This blood has to be sent to the National Institute for Virology in South Africa so that tests can be done to look for hepatitis E virus as we cannot do these tests here. This will not cost you anything. We would like to ask you though, whether your specimens can be used by the scientists in South Africa to find out more about hepatitis E because we do not know very much about this virus in Africa and it has caused people to get sick in the past in this country. We will also ask some questions which may help us understand how you got this sickness. You do not need to agree to this, it will not change the way we will care for you.

If you do agree we will treat all information as confidential and your doctor will get the results of the tests as soon as they are available. No other testing for viruses like HIV will be done on your blood.

It is important that you understand what is written here before you give your consent. This study will help us to understand more about hepatitis E virus and so help to prevent it. Please ask any questions you may have. You are under no obligation to allow your blood to be studied, your doctor will still get the results he needs to make your diagnosis.

APPENDIX C

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 Maila

CLEARANCE CERTIFICATE **PROTOCOL NUMBER** M990929

PROJECT Characterization of Hepatitis E Virus In Southern Africa

INVESTIGATORS Miss HT Maila

DEPARTMENT Virology Department, National Institute for Virology

DATE CONSIDERED 991001

DECISION OF THE COMMITTEE *

Approved unconditionally

DATE 991004 **CHAIRMAN** .....(Professor P E Cleaton-Jones)

* Guidelines for written "informed consent" attached where applicable.

c c Supervisor: S Bowyer
Dept of Virology Department, National Institite for Virology

Works2\lain0015\HumEth97.wdb\M 990929

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DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and ONE COPY returned to the Secretary at Room 10001, 10th Floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee.

H.T Maila

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

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