CHAPTER 4

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

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

4.1 Abstract

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

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

4.2 Introduction

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

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

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

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

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

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

4.3 Material and Methods

4.3.1 Domestic Sewage Samples

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

4.3.2 Swine Fecal Specimens

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

4.3.3 Concentration of Viral Particles from Domestic Sewage Samples

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

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

4.3.4 Nucleic Acid Extraction from Domestic Sewage and Swine Fecal Specimens

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

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

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

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

4.3.6 Sequencing and Phylogenetic Analysis of RT-PCR Amplicons

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

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

4.3.7 Nucleotide Sequence Accession Numbers

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

4.4 Results and Discussion

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

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

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

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

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

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

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

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

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

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

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4.5 References

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

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

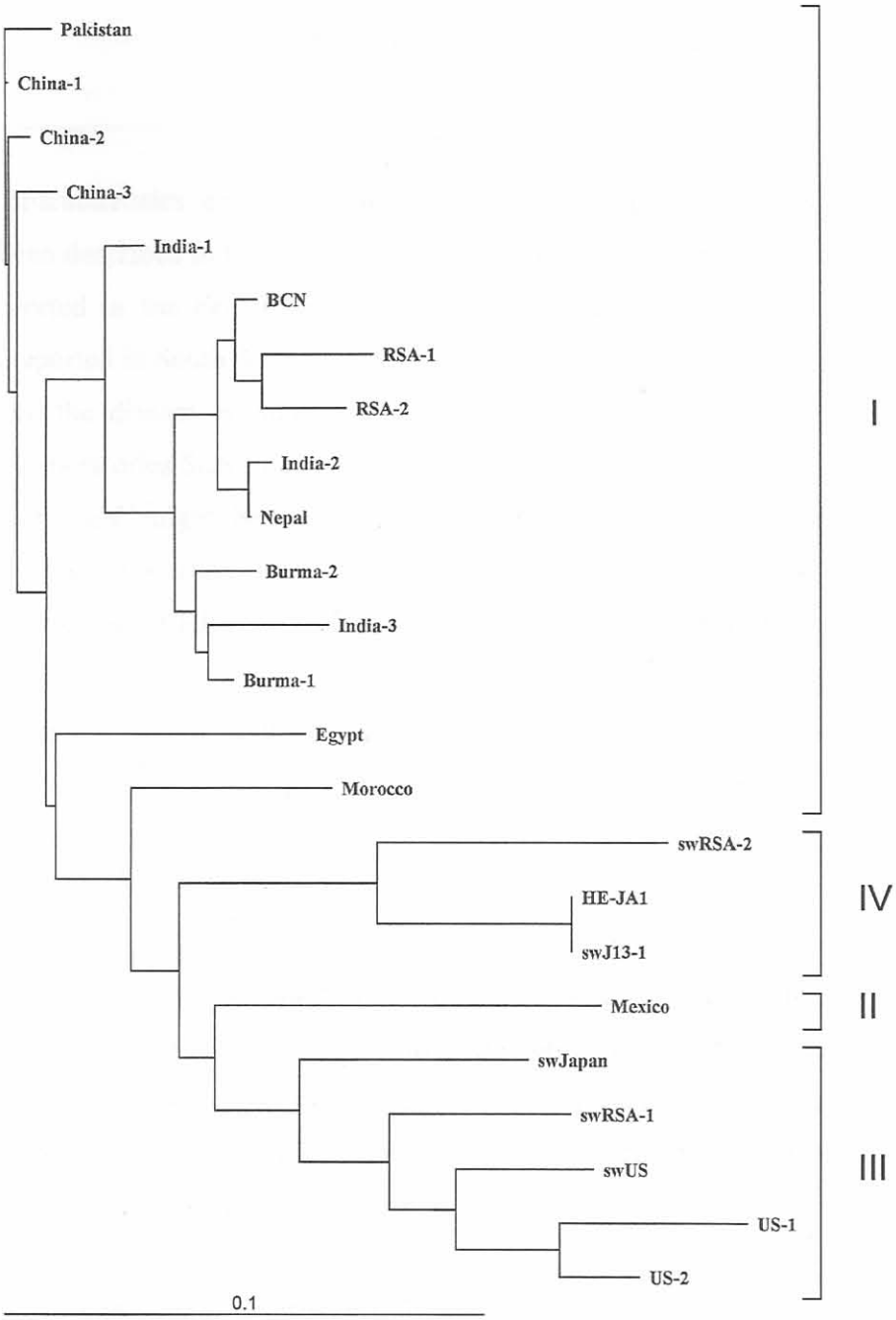

Figure 4.1 **Phylogenetic tree of the ORF2 region for the HEV reference and South African strains**