Hepatitis E virus (HEV), previously referred to as enterically transmitted non-A, non-B hepatitis, is a non-enveloped virus approximately 32–34 nm in diameter, with a positivesense, single-stranded RNA genome of approximately 7.5 kb (Purcell, 1996; Krawczynski et al., 2000). HEV is the leading cause of enterically transmitted, non-A hepatitis worldwide and is responsible for major outbreaks of acute hepatitis in developing countries, especially in tropical and subtropical regions of the world where outbreaks are usually associated with faecally contaminated drinking water (Irshad, 1999). Sporadic cases of HEV are common in endemic regions and appear to be rare in industrialized countries, where they tend to be imported. However, not all isolated reports of HEV-associated hepatitis in developed nations include a history of travel to regions endemic for HEV (Zaaijer et al., 1993; Zanetti et al., 1994; Tassopoulos et al., 1994; Hsieh et al., 1998; Schlauder et al., 1998, 2000; Worm et al., 2000; Takahashi et al., 2002).

HEV is prevalent in Africa and epidemics have been identified in Algeria, the Ivory Coast, Sudan, Somalia (Bradley, 1992) and Djibouti (Coursaget et al., 1996), with smaller outbreaks reported in Morocco (Benjelloun et al., 1997), Ethiopia (Tsega et al., 1991), Chad (van Cuyck-Gandre et al., 1996) and Kenya (Mast et al., 1994), while sporadic cases of HEV have been identified in Egypt (Tsarev et al., 1999), Nigeria (Buisson et al., 2000) and Tunisia (Coursaget et al., 1996).

The first report of hepatitis E in southern Africa was the published account of a typical waterborne outbreak with 273 cases and at least four deaths in Maun, northern Botswana, in 1985 (Byskov et al., 1989). The disease appeared to have affected 1–2% of the population and pregnant women were most severely affected. An earlier outbreak in southern Africa, which occurred in Namibia’s Kavango region in 1983 and affected people living in settlements lacking potable water and waste disposal facilities, was only recently characterized and identified by molecular methods as epidemic HEV (Isaacson et al., 2000). The disease was usually mild, except in pregnant women who made up six of the seven fatal infections reported in this 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 open reading frame 2 (ORF2) (He et al., 2000). Other indications of hepatitis E infection in southern Africa include reports from the small kingdom of Swaziland, where van Rensburg et al. (1995) examined Mozambican refugees, and South Africa (Grabow et al., 1996; Tucker et al., 1996).

In this paper, we have confirmed for the first time, by amplification and sequencing of isolates, that an outbreak of hepatitis E also occurred in the Kavango region of Namibia in 1995. In addition, we have shown by phylogenetic analysis that this outbreak was not caused by the same strain as the 1983 outbreak in the same area.

A non-A, non-B hepatitis outbreak involving more than 600 people occurred in 1995/6 in the Kavango region of northern Namibia, centred on the town of Rundu (Surveillance Bulletin, 1996). The source of the outbreak was suspected to be the water supply, which was compromised by drought and disturbances in reticulation following work on the pipes approximately 6 months earlier.
Patients included both males and females within the age range between 5 days and 80 years (median 25 years), a total bilirubin range of 17–724 µmol l⁻¹, an alanine aminotransferase range of 26–6780 IU l⁻¹ and an aspartate transaminase range of 25–10 540 IU l⁻¹. Patients presented with symptoms of viral hepatitis (jaundice and hypochondrial pain). There were at least three fulminant cases, including one pregnant woman.

Patients' sera were tested for HEV IgG using the Abbott HEV EIA kit after onset of illness. Mean optical density per cut-off ratio for the four sequenced specimens 8039, 7948, 7801 and 7798 were, respectively, 2·56, 6·36, 1·79 and 1·79 (positive cut-off 0·15). HEV antibodies were detected in sera specimens of approximately 75 % of icteric patients. The incidence was 13 % in patients aged < 10 years and 81 % in those aged ≥ 50 years. No antibodies were detected in any of the children under the age of 5 years (Surveillance Bulletin, 1996).

Clinical materials, including human stool and serum samples, obtained from patients were stored at −70 °C and used for this study. 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 to use their specimens for HEV studies.

Stool samples were clarified by vigorous shaking in a 20 % suspension of chloroform containing 0·5 % gelatin-buffered
Fig. 1. Bootstrapped trees (100 sets) showing the conventional segregation of HEV specimens into four genotypes (I–IV). Bootstrap values for the most robust groupings are shown. GenBank accession numbers with corresponding codes used in the text are as follows: Namibia7798 (AY370686), Namibia7801 (AY370687), Namibia7948 (AY370688); Namibia8039 (AY370689); 82-Burma (M73218); 89-Myanmar (D10330); 87-China-Xinjiang (D11092); 87-China-HeBei (M94177); China-K52-87 (L25595); 87-China-Ugh179 (D11093); T1 (AJ272108); 92-Fulminant (X98292); 90-India-Hyderabad (AF076239); 93-India-Madras (X99441); Nepal TK15/92 (AF051380); 97/98-Nigeria5 (AF060669); 93-ChadT3 (U62121); 93-Egypt (AF051351); 94-Egypt (AF051352); 94-Morocco (AF065061); 87-Namibia (AF105021); 97/98-Nigeria1 (AF172999); 97/98-Nigeria4 (AF173000); 97/98-Nigeria5 (AF173001); 97/98-Nigeria6 (AF173230); 97/98-Nigeria7 (AF173231); and 98-Nigeria9 (AF173232). Trees are rooted with the T1 sequence and clades are labelled in accordance with the nomenclature of Wang et al. (2000) and Widdowson et al. (2003). (a) Phylogenetic tree of HEV isolates from nt 1307 to 1757 of the ORF2 region. (b) Phylogenetic tree of HEV isolates, including 83-Namibia and 97/98-Nigeria isolates, from nt 1577 to 1756 of the ORF2 region.
saline and then centrifuged for 30 min at 6000 r.p.m. at 4 °C (model J-21B; Beckman Instruments). The supernatant was filtered (0.45 μm Millex-HA millipore filters; Millipore) and stored at −20 °C.

The virus in a 1 ml sample of clarified stool or sera was concentrated by centrifugation at 23 000 r.p.m. for 1 h at 4 °C (Jouan MR22i centrifuge; The Scientific Group). The pellet was resuspended in 140 μl PBS and viral RNA extracted using the QIAamp Viral RNA isolation kit (Qiagen) according to the manufacturer’s protocol.

First-round PCR and reverse transcription were carried out in a 50 μl reaction mixture with 10 μl purified RNA, 400 nM of each primer, 200 μM of each dNTP, 0.625 U Super-therm DNA polymerase and 1× PCR reaction buffer (JMR Holdings), 1.5 mM MgCl₂, 6 U AMV reverse transcriptase and 10 U RNasin (Roche Diagnostics). Viral RNA was reverse transcribed at 43 °C for 45 min and the cDNA amplified by 35 cycles of PCR (94 °C for 45 s, 48 °C for 45 s and 72 °C for 1 min) and a final extension step at 72 °C for 7 min. Second-round reactions were carried out in a volume of 100 μl with 10 μl first-round product and the same PCR reaction conditions.


Sequencing of PCR products was performed using the ABI Sequencing Ready Reaction kit (Perkin Elmer) as specified by the manufacturer. The sequencing reaction was purified from excess dye terminators using the Centri-Sep Spin-Columns purification kit (Princeton Separations). Automated sequencing was performed using the ABI PRISM 377 DNA Sequencer (Perkin Elmer).

The nucleotide and amino acid alignments, consensus sequences, distance matrices and phylogenetic analysis were obtained using DNAMAN software (version 4.0, Lynam BioSoft). Using 100 bootstrap replicates, a bootstrap value >70% defined a stable phylogenetic grouping (Muerhoff et al., 1997). Trees were created using TREEVIEW (Win32, version 1.6.1). Nucleotide sequences were translated using DNASIS (Hitachi software, version 2.5).

Four Namibian sequences isolated from four different patients’ stool specimens (HEV IgG positive) were compared with GenBank sequence data from 20 other HEV isolates over a 451 bp region of ORF2 (nt 1307–1757). The bootstrapped tree, rooted on the Chinese isolate T1 (Fig. 1a), grouped the isolates into the four conventional genotypes (nomenclature according to Wang et al., 2000 and Widdowson et al., 2003). Because our sequence data only overlapped previously published sequence data from two other African outbreaks (1997/98 Nigeria and 1983 Namibia) over 180 bp, a second tree was drawn over this shorter region (Fig. 1b) to include these strains. The partitioning of the common species was similar in both trees with the Afro-Asian isolates clustering 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 shared a nucleotide identity of between 98.4 and 99.8% and were 85.8–86.3% similar to the Mexican isolate over the 451 bp region but were only 77.6–79.6% similar to other African isolates.

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**Figure 2.** Histogram representing the distribution of genetic distances calculated for ORF2 region (nt 1307–1757) of HEV.
The 1995 Namibian sequences shared a similar nucleotide identity (98.3–100%) over the 180 bp region and were 88.9–91.1% similar to the 1997/98 Nigerian isolates and 85.6–86.1% similar to the Mexican isolate, all in genotype II, but only 77.8–78.9% similar to the 1983 Namibian isolate in genotype I. Genotype I partitions into two sub-genotypes with different African isolates clustering together and away from the Asian isolates. The 1995 Namibian sequences, however, grouped together as a separate clade of genotype II and were most similar to the 1997/98 Nigerian isolates. The 1997/98 Nigerian isolates were previously shown to cluster together with the Mexican isolate (Buisson et al., 2000) and it is therefore likely that the same HEV strain circulating in Africa at this time caused both the 1995 and the 1997/98 outbreaks.

A histogram was constructed from the 451 bp target region, in which the frequency of evolutionary distances calculated between the 24 HEV isolates, including the four Namibian sequences, was plotted for each 0.02 range between zero and 0.259 (Fig. 2). 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 more 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.

The consensus amino acid sequence of the four 1995 Namibian isolates was compared with the deduced amino acid sequence of the 20 GenBank isolates (Fig. 3) based on the 451 bp target region. Unique, definitive amino acids common to the 1995 Namibian subgroup were observed at residues 477 (A→G), 535 (T→S) and 580 (A→S), and those common to both 1995 Namibia and Mexico were observed.
at residues 517 (T→S), 527 (S→P) and 530 (Q→E). Amino acid changes shared by genotype II isolates but not unique to this group were observed at residues 569 (I→L) and 571 (V→I). The Mexican subgroup of genotype II is defined by unique amino acid changes at residues 494 (V→E) and 529 (I→V). Most change was synonymous and the Namibian consensus sequence shared an amino acid identity of 96–96% with the Mexican isolate and 94–94% with other African isolates.

The first published HEV sequence data from southern Africa were obtained from specimens collected during an outbreak that occurred in 1983 in Rundu, Namibia. These isolates were reported to cluster into genotype I (He et al., 2000), despite the fact that the 1983 and 1995 Namibian outbreaks occurred in the same area. Co-circulating strains within the same country have been reported previously. For example, the 1987 Pakistan-Sar55 isolate clustered with Burmese isolates in subgenotype I-1a, while the 1988 Pakistan-Abb2B isolate clustered with Burmese isolates in subgenotype I-1b (van Cuyck-Gandrè et al., 2000).

By successfully amplifying, sequencing and analysing four 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.

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REFERENCES


