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Molecular Characterization of a Hepatitis E Virus Isolate from Namibia

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Abstract
Hepatitis E virus (HEV) causes sporadic and epidemic acute viral hepatitis in many developing countries. In Africa, hepatitis E has been documented by virus detection (reverse transcriptase polymerase chain reaction, RT-PCR) in Egypt, Chad, Algeria, Morocco and Tunisia. Cases of presumptive hepatitis E also have been documented by detection of antibody to HEV in the Sudan, Kenya, Ethiopia, Somalia, Djibouti and South Africa. Recently, we reported the recovery of 9 isolates of HEV from feces collected during an outbreak of jaundice in Namibia. These specimens were stored frozen for many years at the South African Institute for Medical Research awaiting new methods to determine the etiology of jaundice. HEV genomic sequences were detected by antigen-capture RT-PCR with primers that amplified 2 independent regions of the HEV genome (ORF-2 and ORF-3). To further characterize the HEV 83-Namibia isolates, we determined the nucleotide (nt) sequence of the 3′ end of the capsid gene (296 of 1,980 nt in ORF-2) and ORF-3 for 1 isolate. The capsid gene sequence shared 86% identity with the prototype Burma strain and up to 96% identity with other African strains at the (nt) level, and 99% identity with Burma or other Africa strains at the amino acid level. A 188 (nt) fragment amplified from ORF-3 was also highly homologous to other HEV but was too short for meaningful comparison. Phylogenetic analysis indicated that HEV 83-Namibia is closely related to other African isolates, and differs from Burmese, Mexican and Chinese HEV. These data link the HEV causing the 1983 Namibia outbreak to more recent HEV transmission in northern and sub-Saharan Africa, suggesting this subgenotype of HEV is firmly established throughout the continent.

Introduction

Hepatitis E virus (HEV) is the major cause of acute hepatitis in many developing countries. HEV is transmitted mainly be the fecal-oral route and large epidemics due to this virus have been associated with contaminated water. Hepatitis E has its highest attack rate in young to middle-aged adults. HEV transmission, both sporadic and epidemic, is common in Africa having been identified in Egypt [8], Djibouti [7], Sudan [16], Kenya [18], Ethiopia [27], Somalia [4], South Africa [28], Algeria and Chad [29] and Morocco and Tunisia [5]. HEV isolates from 6 Africa countries have been partially sequenced [5, 19, 26, 29] and found to be related, whereas the nucleo-

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tide sequences of HEV isolates collected from distant geographic locations were more divergent. Analysis of recently reported human HEV sequences from China [15] and also from swine herds in the United States [20] suggests HEV variants may exist which are phylogenetically distinct from the classical Mexican and Burmese strains. Recently, we reported the isolate of HEV from archived samples collected during a 1983 jaundice outbreak in Namibia [17]. These isolates represented the first available HEV from southern Africa. To establish their phylogeny, 1 of 9 Namibia HEV isolates was selected for partial sequence analysis of fragments amplified from ORF-2 and ORF-3. Phylogenetic analysis of this first HEV isolate from southern Africa revealed a close relationship to HEV from northern and sub-Saharan Africa.

Materials and Methods

Stool Specimens and Mouse Anti-HEV ORF-2 Sera

Stool specimens were collected during a 1983 hepatitis outbreak in Kavango, Namibia [17]. We designated the HEV selected for analysis as 83-Namibia. Hyperimmune serum to HEV ORF-2 (Burmese strain) was generated by immunizing mice with an HEV DNA vaccine [10].

Generation of HEV cDNA Fragments for Sequence Analysis

cDNA for sequence analysis was generated from HEV RNA in 10% fecal suspensions using affinity-capture reverse transcriptase polymerase chain reaction (RT-PCR) [11]. Briefly, microcentrifuge tubes were coated with 100 µl of antimouse IgG (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) diluted 1:200 in sterile phosphate-buffered saline (PBS) and were incubated at room temperature for 1 h. Then, 200 µl of blocking solution (1% bovine serum albumin fraction V in PBS) was added. Tubes were incubated an additional 15 min at 37°C, overnight at 4°C, aspirated, and were washed thrice with 300 µl of wash buffer (0.05% Tween 20, 0.02% sodium azide in PBS). They were loaded with 45 µl of mouse anti-HEV diluted 1:180 and an equal volume of 10% fecal suspension. These mixtures were incubated for 1 h at 25°C, then overnight at 4°C. After 3 washes, a total 85 µl of reaction solution (GeneAMP, Perkin-Elmer, Branchburg, N.J.) containing 10 µl of 10× PCR buffer, 8 µl of 2.5 m*M* dNTP mix, 50 pmol antisense primers, and nuclease-free water was added to each tube. Tubes were heated at 95°C for 5 min, then immediately cooled on dry ice. Primers [29] used for detection of HEV genome derived from the ORF-2 and ORF-3 of the HEV Burma sequence. After thawing, 5 µl (2.5 U) reverse transcriptase (M-MuLV, Life Technologies, Rockville, Md.) was added to each reaction tube. The reaction was carried out at 42°C for 60 min. Then, 10 µl containing 50 pmol of forward primer and 2.5 U of Taq were added for PCR amplification. Cycles were carried out using three programs sequentially: program 1: 95°C for 2 min, program 2: 95°C for 1 min, 55°C for 1 min, 72°C for 3 min, total 29 cycles, and program 3: 95°C for 1 min, 55°C for 1 min, 72°C for 10 min, 1 cycle. Samples were cooled at 4°C. The amplified products were analyzed using 1% agarose gel electrophoresis.

Table 1. HEV sequences used for analysis

Name[1]	Gene Bank accession No.	Reference No.
82-Burma	M73218	24
89-Myanmar (Burma)	D10330	2
90-India (Hyderabad)	U22532	22
93-India (Madras)	X99441	Unpublished
87-Nepal	AF020495	9
87-Nepal	AF020497	9
87-China-A	D11092	1
87-China-B	M94117	3
87-China-C	L25595	31
87-China-D	D11093	Unpublished
87-Pakistan-Sar	M80581	25
92-Fulminant (India?)	X98292	Donati et al., in press
80-Algeria	U40046	29
84-Chad	U62121	29
93-Egypt	AF051351	26
93-Egypt	AF051352	26
83-Namibia	AF105021	This paper
87-Mexico	M74506	14
95-USA (human)	AF035437	23
95-USA (human)	AF060669	23
96-USA (swine)	AF011921	20
99-Morocco	AF065061	19

[1] Figure indicates year of isolation. When an epidemic lasted more than one calendar year and from a publication it was not clear the year of the isolate was assigned the middle or the last year of that epidemic.

DNA Sequencing

PCR products were purified by using the QIAquick PCR purification kit (Qiagen, Santa Carita, Calif.). The two DNA fragments, (ORF-2 and ORF-3 regions) were sequenced on an Applied Biosystem Model 377 Sequencing System (Applied Biosystems, Foster City, Calif.) using the recommended Tag Dye-Deoxy Terminator Cycle Sequencing kit (*PE* Applied Biosystems, Warrington, UK). DNA fragments were sequenced in both directions using reverse and forward-nested PCR primers.

Computer Analysis of HEV Sequences

Sequences were assembled using Sequencher 3.0 (Gene Codes Corporation, Madison, Wisc.) and were analyzed with the Wisconsin Package Version 9.1 (for UNIX), Genetics Computer Group (GCG, Madison, Wisc.) on an Alpha 8400 computer. Multiple sequence alignments were performed with the GCG-PileUp program.

A consensus evolutionary tree was produced with GCG-PAUP search program using Neighbor Joining (NJ). Confidence for the grouping in the trees was assessed by the bootstrap method (1,000 replicates). Bootstrap values of ≥ 70% were taken as evidence for phylogenetic grouping [12, 32]. The graphic output of the phylogenetic trees was created with the Macintosh version of the TREEVIEW program [21]. Table 1 shows the accession numbers of the HEV sequences used for analysis.

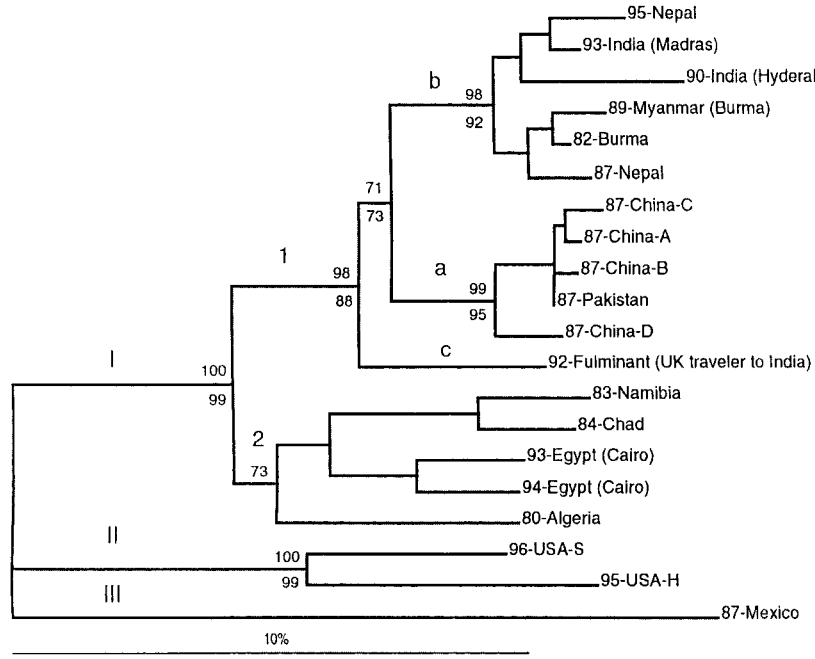

Fig. 1. Phylogenetic analysis of 83-Namibia and 21 other HEV strains.

Results

Analysis of the Consensus Sequences

A consensus sequence of 296 bp was derived from the extreme 3′ region of ORF-2 coding for the capsid protein (Gene Bank accession No. AF 105021). The 83-Namibia sequence had 86% identity with the Burma and 96% identity with other African strains at the nucleotide level (nt). There was 99% identity with Burma and African strains at the amino acid level. A 188 (nt) fragment from 83-Namibia ORF-3 region was also highly homologous to other HEV but was too short for meaningful comparison.

Phylogenetic analysis of 83-Namibia and 21 other HEV strains was performed using NJ (fig. 1). HEV sequences are divided into three genotypes as previously described by Tsarev et al. [26]: Asian-African genotype (I), US genotype (II) and Mexican genotype (III). The Asian-Africa genotype (I) consists of subgenotypes from Asia (I-1) and Africa (I-2). The Africa subgenotype is comprised of a single cluster of isolates including 83-Namibia, whereas the Asian subgenotype has three genetic clusters. All genetic groups were formed with a high level of bootstrap probability except that the NJ bootstrap value for the African subgenotype was 63% (data not shown in fig. 1).

Discussion

Earlier, we and others reported that HEV strains are segregated by geography and that isolates from the same region are usually related [5, 26, 29]. HEV 83-Namibia follows this pattern. Previously in Africa, HEV was isolated only from northern African countries. The strain from Namibia is the first isolate from southern Africa. Interestingly, it is similar to the northern African isolates, particularly 84-Chad, implying that these geographically diverse strains share a close common ancestor. The 63% bootstrap probability value for the Africa subgenotype implies (data not shown in fig. 1) there is some uncertainty that this cluster is a distinct genetic group. We believe this uncertainty will be eliminated as more isolates from Africa are analyzed. The Africa subgenotype displays slightly more diversity than the Asian subgenotype. As in Asia from which the majority of HEV sequences are available, there is not apparent temporal relation among African isolates.

As a rule, HEV isolates from the same geographic area are closely related. On the other hand, there are indications that genetically different HEV strains can coexist in the same country or region. The first indication of such a possibility was reported by Huang et al. [15]. Analyses of a

partial HEV sequence from Guangzhou, China showed high level diversity from other Chinese HEV strains or any known HEV isolates. This observation was confirmed by two independent groups in Taiwan [13, 30]. It is interesting to note that these highly diverse sequences were isolated from sporadic cases of hepatitis E. HEV recovered from patients in the same Asian epidemics (from 1982 to 1992), even spanning many months and hundreds of kilometers, demonstrated no such diversity. More HEV isolates from sporadic African cases must be studied to refute or sustain the hypothesis that all African HEV are similar.

Geographic segregation of HEV is consistent with the hypothesis that HEV is a zoonotic agent. The first evidence for this hypothesis was from Clayson et al. [6] who detected HEV viremia in naturally infected pigs in Nepal. Later HEV was isolated from pigs in the USA [20] and from rats in Nepal [Tsarev et al., unpubl. data]. HEV sequences isolated from animals were very closely related to human HEV sequences from the corresponding area. This strongly suggests that animals could be a reservoir for HEV. Nevertheless, HEV strains can travel most rapidly and widely when infected persons travel. We speculate that movement of people from north to south or vice versa has spread the African subgenotype HEV over the continent.

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