Identification of a novel hepatitis E virus in Nigeria

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Sporadic cases of acute hepatitis E among ten native Nigerian adults were reported in Port-
Harcourt (Nigeria). Hepatitis E virus (HEV) was detected in serum and/or faecal samples of seven
patients by RT–PCR of the open reading frame (ORF)-1 polymerase region and the 3’-end of ORF2.
Restriction analysis widely used to distinguish genotypes I and III showed that all Nigerian strains
have a pattern similar to the Mexican strain (NotI, nt 286; Smal, nt 397; no KpnI restriction site)
but displayed a BsmI restriction site at nt 213 as do most African HEV strains sequenced so far.
Sequence analysis performed from internal ORF1 and ORF2 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
genotype III (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 II (75%'
homology in ORF1, 77% homology in ORF2). Genetic divergence up to 15% in ORF2 with the
Mexican genotype clearly defined a new subgenotype within genotype III. At the amino acid level,
Nigerian strains showed more homology with genotype III (96%) than with genotype I (92%). This
study clearly determined the co-existence of genotypes I and III in Africa. These Nigerian HEV
strains belonging to genotype III, but sharing some properties with genotype I, could be one of the
missing links between African and Latin American HEV and could help us to determine the
phylogenetic evolution of HEV from the ancestral virus.

Introduction

Hepatitis E virus (HEV) is a widespread enterically
transmitted agent, responsible for at least 50% of acute non-A,
non-B hepatitis in developing countries. HEV is considered to
be endemic in many areas of Asia, Africa and Latin America.
HEV strains from different geographical origins share antigenic
properties. Nevertheless, genetic heterogeneity exists, so that
genotyping represents a reliable epidemiological tool. The
sequence of open reading frame (ORF)-2, encoding the viral
capsid, and ORF3, encoding a protein of unknown function, are
well conserved, whereas one small region of ORF1 displays
significant genetic diversity among HEV isolates (Huang et al.,
1992; Tsarev et al., 1992). Four main human genotypes are
currently defined: (I) the Asian cluster, comprised of Burmese,
Pakistani and Chinese-Xinjiang subgenotypes, (II) the USA
genotype, (III) the Mexican genotype (Tsarev et al., 1999), and
(IV) Chinese-Beijing genotype (Wang et al., 1999). Only
limited data on the geographical distribution of USA and
Mexican genotypes, classified as genotypes II and III re-
respectively, are available. Moreover, the Mexican genotype
described in 1992 is still limited to the prototype strain. Recently, European isolates from Italy and Greece were proposed as new genotypes (Schluder et al., 1999).

African HEV isolates have been characterized from Tunisia, Morocco (Chatterjee et al., 1997), Algeria, Chad (van Cuyck-Gandré et al., 1997) and Egypt (Tsarev et al., 1999). All these strains are close to the Asian genetic cluster, but they constitute a separate subtype within genotype I (Meng et al., 1999; Tsarev et al., 1999). Hepatitis E is presumed endemic in west African countries (Krawczynski et al., 1991), but previously no HEV strain had been available for genetic analysis until now. Here we report clinical and biological features of sporadic cases in African countries (Krawczynski et al., 1991). The genetic analysis of ORF1 and ORF2 regions from Nigerian HEV isolates provides evidence for a co-existence of two HEV genotypes in Africa.

These results were presented in part at the 38th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Diego, CA, September 24–27, 1998 (abstract H 162).

Methods

Patients. From November 1997 to June 1998, ten sporadic cases of acute hepatitis were recorded among Nigerian workers of industrial companies attending the Occupational Health Service. Patients were male adults, 25 to 33 years old, living in Port-Harcourt, employed as engineers, chauffeurs, gardeners, watchmen or helpers. They had no history of blood exposure, drug consumption or household contact with patients with jaundice. Initial symptoms were gastrointestinal complaints and fever, leading to clinical diagnosis of malaria and ineffective treatment. All patients displayed jaundice, fatigue, anorexia and hepatomegaly but no splenomegaly; one patient complained of pruritus. No urticaria, arthralgia, vasculitis or haemorraghic signs were observed. All patients recovered within 2 months of onset, jaundice disappearing by 15 days, AST and ALT serum levels normalizing by 2 or 3 weeks, but fatigue and dyspepsia persisting several weeks. Relapse, protracted cholestasis and fulminant hepatitis were not observed.

Clinical samples. Blood samples drawn during the acute phase of hepatitis were centrifuged and locally analysed for serum enzyme levels, coagulation tests, malaria, anti-HIV antibodies, hepatitis A, B and C markers. These analyses confirmed acute hepatitis but excluded types A, B and C virus. Then, HEV serology and molecular biology tests were both performed in France. Moreover, the molecular biology testing was extended to stool samples from three patients.

Detection of IgG and IgM anti-HEV antibodies by ELISA. Anti-HEV antibodies were searched for by two tests: a commercial kit (Abbott) and a new recombinant immunoassay. In the Abbott HEV EIA test, two recombinant proteins from a Burmese strain are used as antigens: SG-3 encoded by the 3’ sequence of ORF2 and 8-5 encoded by the complete ORF3 (Dawson et al., 1992). Samples were considered as anti-HEV reactive when the ratio absorbance to cut-off (OD/CO) was over 1. The new test is based on the fusion of the immunodominant region located at the C terminus of the ORF2 protein to a particular carrier, the hepatitis B virus core antigen (HBcAg) (Touze et al., 1996). This 42 amino acid long HEV sequence is inserted within the major antigenic loop of the HBcAg in order to delete the HBc reactivity. This chimeric gene is inserted into a recombinant baculovirus used to infect insect cells. The HEV–HBc chimeric protein produced self assembles in structures resembling viral capsomer particles. Such particles exhibited HEV reactivity but no HBcAg reactivity. Half of the wells of a microtitre plate (Maxisorp, Nunc, Life Technologies) were coated at 100 ng each with purified HEV–HBc particles, and then postcoated with newborn calf serum (1% NBS). Duplicate wells (one test and one control) were incubated with sera diluted 1/100 in 1 × PBS containing 1% NBS and 2% Tween 20. Following incubation at 45 °C for 90 min and four washes, bound antibodies were detected with mouse anti-human IgG or IgM antibodies covalently linked to horseradish peroxidase (Southern Biotechnology Associates). After incubation at 45 °C for 90 min and four washes, 100 µl of substrate solution containing o-phenylenediamine and H2O2 was added. The reaction was stopped after 30 min by adding 100 µl of 2 M H2SO4 and the results were obtained by measuring absorbance at 492 nm with a photometer and comparison of the absorbance between test and control wells (Microplate Autoreader EL311, Biotek Instruments). For each sample, a positive result was scored when the ratio OD/CO was over 1.

Previous studies have shown the ability of these assays to detect anti-HEV antibodies in acute and convalescent sera collected from various geographical regions (Dawson et al., 1992).

RNA extractions and RT–PCR amplification. Purified RNA was obtained from 50 to 140 µl of serum or from 100 µg of 10% stool suspension using, respectively, a QiAamp viral RNA kit (Qiagen) and an InViSorb enterovirus RNA isolation kit (InViTek Berlin-Buch) according to the manufacturer’s instructions. In both cases, RNA was recovered in a final volume of 50 µl of molecular grade water.

Two independent regions of the HEV genome, ORF1 and ORF2, were targeted for RT-nested-PCR amplification. The nucleotide positions of the primers refers to the Burmese strain (Tam et al., 1991). Primers matching nucleotides 6581 to 6660 and 7130 to 7111 were used as outer primers for reverse transcription of the ORF2 3’–end. The inner primers were positioned at nucleotides 6553 to 6671 and 7100 to 7081 (van Cuyck-Gandré et al., 1996; Yarbrough et al., 1994). The polymerase gene located in the ORF1 region was amplified using primers positioned at nucleotides 4406 to 4483 and 4954 to 4931 for the RT–PCR, 4412 to 4432 and 4910 to 4891 for the nested PCR (Gouvea et al., 1998).

All thermal procedures were performed in a GeneAmp 2400 thermal cycler (Perkin Elmer). Briefly, a 10 µl aliquot of RNA was submitted to RT–PCR using the Titan One Tube RT (Boehringer Mannheim). The reverse transcription step consisting of a 45 min incubation at 50 °C was followed by PCR amplification with the following cycling program: 1 × (94 °C, 7 min; 45 °C, 45 s; 68 °C, 45 s), 39 × (92 °C, 30 s; 45 °C, 30 s; 68 °C, 45 s). For nested PCR, 5 µl from the first ampiclons was amplified under the following conditions: 1 × (94 °C, 7 min; 50 °C, 45 s; 72 °C, 45 s), 39 × (92 °C, 30 s; 50 °C, 30 s; 72 °C, 45 s). PCR products were analysed on a pre-stained ethidium bromide 1:5% agarose gel in 1 × TAE buffer, and visualized under UV irradiation. Then they were transferred to a positively charged nylon membrane under denaturing conditions (0.3 M NaCl and 0.4 M NaOH). Sheets were hybridized with a 3’–end digoxigenin-labelled oligonucleotide.

Restriction endonuclease analysis (REA). Fast genotyping of the HEV strains was performed for ORF1 and ORF2 using REA (Gouvea et al., 1998). The internal PCR products, 499 nt long for ORF1 amplifiers and 448 nt long for ORF2 amplifiers, were used for analysis. Digestion of 10 µl aliquots of nested PCR products was performed using Smal, NotI, KpnI, BsmI (New England Biolabs, distributed by Ozyme) in a 15 µl reaction volume containing the appropriate buffer. The mixture was incubated for 1 h at 37 °C. Digested fragments were separated on a 1.5% agarose gel pre-stained with 0.5 µg/ml ethidium bromide.
Novel HEV in Nigeria

Results

The ten patients were negative for malaria tests as well as human immunodeficiency virus, HCV and HAV IgM antibodies. Two patients (nos 1 and 9) were anti-HBc-positive, but HBsAg- and anti-HBc- IgM negative. All early sera were strongly reactive for anti-HEV IgG antibodies in the two tests used with mean OD/CO ratios equal to 3.8 in the Abbott HEV EIA kit (range from 3.2 to 6.1) and 3.8 in the test involving a chimeric HEV–HBc antigen (range from 3.5 to 15.5). Likewise, a strong anti-HEV IgM reactivity was underlined in the ten patients with mean OD/CO ratios equal to 3–6.1 (range from 0.4 to 18), confirming the diagnosis of acute hepatitis E (Table 1).

Table 1. Laboratory features of ten sporadic cases of acute non-A, non-C hepatitis in Port-Harcourt, Nigeria.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Onset of disease</th>
<th>AST (UI/l)</th>
<th>ALT (UI/l)</th>
<th>TBIL (mg/dl)</th>
<th>GGT (UI/l)</th>
<th>ALP (UI/l)</th>
<th>Anti-HEV IgG (OD/CO)</th>
<th>Anti-HEV IgM (OD/CO)</th>
<th>Rec-ORF1 RT–PCR</th>
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<td>95</td>
<td>+</td>
<td>+</td>
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Cloning and sequencing. Freshly prepared amplicons were purified on Microspin HR200 columns (Amersham–Pharmacia Biotech) and cloned in a PCR-Script SK(+) plasmid (Stratagene). The recombinant plasmids were sequenced, then purified using the Rapid Pure Miniprep kit (Bio 101). At least three clones of nested PCR products including internal primers of each HEV strains were sequenced using the M13 primers. The sequences were determined for both strands on an ABI 377 automated DNA sequencer (ABI Perkin Elmer, Genome Express).

Sequence analysis. Sequences were assembled using the Sequencher 3.0 software (Gene Codes Corporation). The consensus sequence without the sequence of primers was analysed with the Wisconsin Package version 10.0 (Genetics Computer Group, MA, USA) on an Alpha 8400 computer (Unix). Multiple sequence alignments were performed with a GCG-PileUp program. A consensus evolutionary tree was produced with a GCG-Paupsearch program using Neighbor Joining (NJ) or Maximum Parsimony (MP) methods. Confidence for the grouping in the trees was assessed by the bootstrap method (1000 replicates). Bootstrap values of 75% were regarded as providing evidence for the phylogenetic grouping (Zharkikh & Li, 1992). The graphical outputs of the phylogenetic trees were produced by GCG-GROWTREE.

The nucleic acid sequences and the deduced amino acid sequences were compared with the following genome sequences: China A (GenBank accession D11092) (Aye et al., 1992); China B (GenBank accession M94177) (Bi et al., 1993); China C (GenBank accession L55959) (Yi et al., 1994); Pakistan (Sa-55) (GenBank accession M80581) (Tsarev et al., 1992); China D (GenBank accession D11093) (T. Uchida, unpublished data); fulminant hepatitis (GenBank accession X98292) (Donati et al., 1997); Burma (GenBank accession M73218) (Tam et al., 1991); Mexico (GenBank accession M74506) (Huang et al., 1992); Egypt-93 (GenBank accession AF051351) (Tsarev et al., 1999); US1 and US2 human (GenBank accession AF060068, AF060069) (Erker et al., 1999); USA swine (GenBank accession AF011921) (Meng et al., 1998); Chad-83 (GenBank accession U62121) (van Cuyck-Gandré et al., 1997); Algeria-80 (GenBank accession U40046) (van Cuyck-Gandré et al., 1997); Haiti (Bangladesh) (GenBank accession AF047864) (Drabick et al., 1997).
Table 2. REA of the ORF1 polymerase and 3′-end of ORF2 amplimers for the Asian (Burmese) and Mexican genotypes and Nigerian strains

ORF1 pol amplimers, 499 nt; 3′-end of ORF2 amplimers, 448 nt. Numbers in parentheses indicate the nucleotide position of the restriction sites.

<table>
<thead>
<tr>
<th>Strains</th>
<th>ORF1</th>
<th>ORF2</th>
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<tbody>
<tr>
<td></td>
<td>SmaI</td>
<td>KpnI</td>
</tr>
<tr>
<td>Burmese (genotype I)</td>
<td>–</td>
<td>+ (164)</td>
</tr>
<tr>
<td>Mexican (genotype III)</td>
<td>+ (397)</td>
<td>–</td>
</tr>
<tr>
<td>Nigerian</td>
<td>+ (397)</td>
<td>–</td>
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</table>

Fig. 1. Bootstrap 75% majority rule consensus phylogenetic tree of HEV isolates based on the 3′-end of ORF2 (410 nt). The internal node numbers represent the bootstrap values (expressed as percentage of all trees) obtained from 1000 replicates with parsimony analysis. A scale in percent of genetic differences is shown.

Restriction endonuclease analysis

Unique enzyme sites within the internal ORF1 and ORF2 PCR products were used to distinguish Mexican and Asian genotypes.

The 499 ORF1 PCR product of all Nigerian strains was efficiently digested by SmaI, yielding the 397 bp and 102 bp expected fragments. ORF2 analysis was performed using NotI, KpnI and BsmI. None of the PCR products obtained were digested using KpnI. For all Nigerian strains, digestion by NotI gave a restriction pattern similar to that expected for the Mexican prototype (site at nt 286). Interestingly, a BsmI site at nt 213 was detected in the Nigerian strains, whereas this site is located at nt 382 in the Mexican strain (Table 2).

Phylogenetic analysis

The comparison of sequences from Nigerian HEV isolates (GenBank accession nos AF172999, AF173000, AF173001, AF173230, AF173231, AF173232) to the sequences available in the GenBank database was performed in the 3′-end of the HEV ORF2 (410 nt) for six isolates and in the ORF1 polymerase region (436 nt) for four isolates. Five isolates (97/98-Nigeria 1, 4, 5, 6, 7), displaying 100% identity in ORF2, grouped together. One isolate (98-Nigeria 9) was 2% different from this group. The same data were obtained by statistical analysis of the genome sequences using either NJ or MP. As a cluster, the HEV Nigerian isolates were more related to the Mexican strain (87% homology in ORF1 and 80% in ORF2) than to the Asian genotype I isolates (74% homology in ORF1 and 76% in ORF2) and the USA genotype (75% homology in ORF1 and 77% in ORF2) (Fig. 1). They were less related to previously sequenced African isolates (74% homology in ORF1 and 77% in ORF2).

On the amino acid level, in ORF2, Nigerian strains displayed 96% homology with the Mexican prototype and 92% homology with genotype I, including the prototypes from Burma, Algeria, Chad and Egypt (Fig. 2).

Discussion

An endemic circulation of HEV in Nigeria was suspected from serological data (Krawczynski et al., 1991). It is now confirmed in one area of the Niger delta. The ten cases of HEV detected in Port-Harcourt, spaced out over an 8 month period, shared no apparent common source of contamination, thus they are considered sporadic cases. Sporadic hepatitis E cases in Asia have occurred most often when there has been extensive
transmission of HEV. Such transmission probably happened in the Niger delta, as Port-Harcourt expanded without city planning in the middle of the Niger delta and several of its quarters have been built in swamps. Rainfall is abundant (210 days, 2250 mm per year) and surface water is briny. These features suggest how residents may be exposed to HEV from contaminated water supplies or uncooked shellfish and could explain the occurrence of sporadic cases observed throughout the year.

Diagnosis of ten sporadic cases of non-A, non-B acute hepatitis was suggested by the self-limited course of the disease. Serological evidence of HEV came from the strong anti-HEV reactivity with anti-HEV IgM antibodies in all ten patients. Of these, seven had a viraemia proved by positive RT–PCR at least in the ORF2 region.

In order to relate these isolates to HEV genotypes, we used REA for a preliminary genotype analysis. On account of the African origin of the HEV isolates, digestion with KpnI was initially used to confirm the specificity of ORF2 PCR products (van Cuyck-Gandre et al., 1996). None of the Nigerian strains analysed could be digested by KpnI, as would be expected for Asian–African genotype I. The successful digestion by NsiI at site 286 in ORF2 and by SmaI at site 397 in ORF1, which are widely used to discriminate Mexican genotype III, suggested the relatedness of the Nigerian strains to the Mexican genotype. However, the restriction pattern obtained with BsmI at site 213 shared by most African HEV distinguished the Nigerian strains from genotype III.

In order to determine the relationships between Nigerian and Mexican HEV strains, PCR products from two independent regions of the genome, ORF1 polymerase and the 3′-end of ORF2, were sequenced. The results confirmed and extended the restriction digest analysis by demonstrating that the Nigerian isolates constitute a cluster more related to genotype III (Mexican) than to the genotype I (Asian–African). Nevertheless, genetic divergence up to 15% in ORF2 between the Mexican genotype and the Nigerian strains clearly distinguish the Nigerian cluster as a novel subgenotype. The Nigerian strains are a cluster as they exhibit little variability, a divergence of only 2% being observed at the nucleotide level among the strains. Nigerian HEV are the first new isolates that can be assigned to genotype III, previously represented only by the Mexican strain.

The genetic diversity of HEV genotypes within geographical areas has been suggested by the description of subgenotype I-1 (Burmese cluster) and the novel genotype IV in China (Huang et al., 1995; Wang et al., 1999). Nevertheless, because of the lack of available sequences for Chinese genotype IV in the genome regions used for analysis in previously published studies, the heterogeneity of HEV Chinese isolates is not fully documented. Consequently, comparison of genotype IV to the Nigerian strains could not be well determined. In Africa, the existence of both genotypes I and III clearly suggests that HEV genotypes may be more widely distributed than originally believed. The characterization of these Nigerian strains from endemic cases could provide a major key for the understanding of the phylogenetic evolution of HEV in Africa.

Genotype I is heterogeneous, being divided into two major subgenotypes: I-1 (Asian) and I-2 (African) (Tsarev et al., 1992). Phylogenetic analysis based on the 3′-end of ORF2 has placed all African strains isolated either from endemic cases (Egypt, Morocco, Tunisia) or epidemic cases (Chad, Algeria) in the
same cluster (Tsarev et al., 1999). Our work demonstrates the heterogeneity of genotype III, comprising Mexican and Nigerian subgenotypes. Nevertheless, although clustered within genotype III, Nigerian strains are closer to the previously sequenced African HEV, as shown by the BsmI restriction site at nt 213, than the Mexican prototype is to the African strains. The BsmI restriction site at nt 213 (ORF2 amplicon) shared by Nigerian strains and most African genotype I strains but not by HEV Mexico, differentiates Old World and New World genotype III HEV strains. Therefore, restriction analysis using BsmI could be a reliable and rapid tool to classify new HEV strains as genotype III-1 (Mexican-like) and III-2 (Nigerian). The comparison of ORF2 amino acid sequences revealed Nigerian strains to be closer to genotype III (96% homology) than to genotype I-2 (92% homology), whereas the Mexican and Burmese prototypes exhibited 90% homology to each other. The Nigerian subgenotype thus clearly defines an intermediate step at both the nucleotide and amino acid levels between African and Latin American HEV. These Nigerian HEV could represent one of the missing links between the ancestral virus and the further expansion of HEV variants through the continents.

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The views presented are those of the authors and do not purport to reflect those of the Ministry of Defence, France or U.S. Department of the Army, U.S. Department of the Navy, U.S. Department of Defense.

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