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Genotypic characterization of symptomatic hepatitis E virus (HEV) infections in Egypt

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ABSTRACT

Background: Hepatitis E virus (HEV) is a common cause of acute viral hepatitis (AVH) in many developing countries. In Egypt, HEV seroprevalence is among the highest in the world; however, only a very limited number of Egyptian HEV sequences are currently available.
Objectives: The objectives were to determine the HEV genotype(s) currently circulating in Egypt.
Study design: AVH patients without serologic evidence of hepatitis A, B, and C viruses were evaluated for possible HEV infection using serologic assays for anti-HEV IgM and anti-HEV IgG and real-time PCR for HEV RNA. Stool suspensions from suspected cases were inoculated into rhesus macaques to confirm the presence of HEV. Sequence analysis was utilized to determine HEV genotype.
Results: Of 287 subjects with AVH enrolled, 58 had serologic evidence of acute HEV infection. Stool samples for two of these patients were repeatedly positive for HEV RNA by real-time PCR. Macaques experimentally inoculated with these human stools also developed viremia. Sequence analysis of open reading frame (ORF) 1 demonstrated that these isolates belonged to HEV genotype 1 and were 3.9–9.5% divergent from other genotype 1 isolates. ORF2 was 5.3–8.7% divergent from previously reported Egyptian isolates.
Conclusions: This study strongly suggests that genotype 1 HEV related to other North African isolates is circulating in acute symptomatic patients in Egypt. Further evaluation of genotypic variability is underway in this highly endemic cohort and is considered an important component of our increased understanding of HEV pathogenesis.

1. Background

Hepatitis E virus (HEV) is an enterically transmitted virus that represents a common cause of acute viral hepatitis (AVH) in many developing countries.[1] Although most patients with HEV recover fully, mortality rates of ~1% in the general population and up to 20% in pregnant women have been reported.[2] Based on genomic variability observed among HEV isolates, four genotypes have been identified.[3] As with other hepatitis viruses, genotype may play a role in disease severity.[1,4]

In Egypt, the prevalence of anti-HEV is among the highest of any country in the world, approaching 80%[5–10]; yet, outbreaks have not been reported. A prospective study also estimated the HEV incidence at 41.6/1000 person-years[11]; however, none of the seroconverting individuals gave a history compatible with AVH during the observation period. Similarly, HEV infection among pregnant women was not associated with history of jaundice or liver disease as reported elsewhere.[11] Reasons for the infrequent clinical hepatitis remain unclear but could be the result of early childhood exposure leading to inapparent infection. Alternatively, the predominant HEV genotype(s) in Egypt could be less virulent than those circulating in Asia.

2. Objectives

Only a very limited number of HEV sequences are currently available from North Africa; therefore, we investigated HEV infection and genotypes in two Egyptian communities.

Abbreviations: HEV, hepatitis E virus; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; AVH, acute viral hepatitis; ORF, open reading frame; RT, reverse transcriptase; ULN, upper limit of normal; SD, standard deviation; ALT, alanine transaminase; AST, aspartate transaminase; S/C, sample/cut off ratio; IU, international units; nts, nucleotides; bp, base pair.

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3. Methods

3.1. Study participants and sample collection

In Egypt, patients with acute viral hepatitis (AVH) are routinely referred to specialty fever hospitals. Suspected acute hepatitis patients were enrolled after providing informed consent. Clinical data were obtained by the treating physician and recorded in a pre-designed clinical research datasheet. From 2006 to 2008, we enrolled 287 acute hepatitis subjects. Patients were serologically screened for hepatitis A virus (HAV), hepatitis B virus (HBV), and hepatitis C virus (HCV) using rapid tests for anti-HAV IgM (CTK Bioteck Inc., San Diego, CA), anti-HBV core IgM (IND Diagnostic, Canada), and anti-HCV IgG (Clinpro International, Union City, CA).

3.2. Serologic testing for HEV infection

Sera from patients with no evidence of HAV, HBV, or HCV infection were tested for possible HEV infection using the commercial anti-HEV IgM kit (HEV-IgM ELISA 3.0, MP Diagnostics, formerly Genelabs Diagnostic, Singapore). Anti-HEV IgG was measured as described previously using an in-house ELISA.[12] Using the manufacturer's criteria for positive anti-HEV IgM, 58 acute HEV infections were identified; 44 were subjects with acute symptomatic hepatitis, while 14 were contacts (mostly asymptomatic) of symptomatic cases.

3.3. Experimental HEV infection

Rhesus macaques derived from a hepatitis virus-free colony were housed at Bioqual Inc. (Rockville, MD, USA) and maintained according to the American Association of Accreditation of Laboratory Animal Care guidelines. For the current study, one animal each was inoculated with serum or a 20% suspension of stool derived from HEV-infected Egyptian patient TS00223 or TS00226 to confirm the presence of HEV. This study was approved by the Animal Care and Use Committees of Bioqual Inc., and the National Institute of Allergy and Infectious Diseases (Bethesda, MD, USA).

3.4. Real-time RT-PCR for HEV infection

Viral RNA was extracted from 20% stool suspensions from humans or macaques using TriZol and resuspended in DEPC-treated water. Viral RNA was extracted from 140 μL of patient serum using the QIAamp Viral RNA kit (QIAGEN, Valencia, CA). To determine the presence of HEV RNA in patient stool samples, two real-time RT-PCR methods were employed. The method of Jothikumar et al. amplifies ORF3 and detects as few as 4 genome equivalent copies of HEV,[13] while the method of Gyarmati et al. amplifies ORF2 and detects 1–20 genome equivalents of HEV.[14] Positive controls included HEV-infected rhesus stool samples corresponding to genotypes 1–4. A negative control with no input RNA was included in each run. 1:100 and 1:1000 dilutions of stool-derived RNA were also tested to reduce the effects of PCR inhibitory substances.

3.5. Reverse transcriptase (RT)-PCR amplification of HEV

To analyze genotype diversity, qualitative RT-PCR assays for ORF1, ORF2, and the ORF2/3 overlap region were employed. Primer sequences for ORF1 were CTG GCA TYA CTA CTG CYA TTG AGC (outer sense; nts 64–87), CCA TCR ARR CAG TAA GTG CGG TC (outer antisense; nucleotides [nts] 459–581), CTG CCY TKG CGA ATG CTG TGG (inner sense; nts 112–132), and GGC AGW RTA CCA RCG CTG AAC ATC (inner antisense; nts 375–398).[15,16] Primer sequences for ORF2 were CTG TTT AAY CTT GCT GAC AC (outer sense; nts 6342–6361), TGA AAG CCA WAG CAC ATC (6633–6650), GAC AGA ATT RAT TTC GTC GGC TGG (inner sense; nts 6380–6403), and CTT GTT CRT GYT GGT TRT CAT AAT C (inner antisense; nts 6552–6576).[15] Primer sequences for ORF2/3 were GCR GTG GTT TCT GGG GTG AC (outer sense; nts 5279–5298), CTG GGM TYG GTC DCG CCA AG (outer antisense; nts 5485–5321), GYT GAT TCT CAG CCC TTC GC (inner sense; nts 5302–5321), and GMY TGG TCD CGC CAA GHG GA (inner antisense; nts 5419–5428).[17] One-step RT-PCR conditions were 50 °C for 60 min, 94 °C for 10 min, and 40 cycles of 94 °C for 1 min, 53 °C for 45 s, and 72 °C for 1 min, followed by an additional 10 min at 72 °C. Nested PCR conditions were 94 °C for 2 min, 40 cycles of 94 °C for 1 min, 53 °C for 45 s, and 72 °C for 1 min, followed by an additional 10 min at 72 °C. Negative controls included one reaction with no reverse transcriptase and a separate reaction with no RNA. PCR products were gel purified and directly sequenced in both directions.

3.6. Phylogenetic analyses

The HEV genotype was determined by direct sequencing of the PCR fragment. All alignments were performed using Clustal X version 1.64b.[18] GenBank references used to identify the HEV genotype included genotypes 1 (AF076239, M80581, AF051830, D11093, AY230202, AY204877, and X98292), 2 (M74506), 3 (AB246676, AF082843, AF060668, and AB189075) and 4 (AJ272108, AB220975, AB220978, and AB220979). Sequences from Egypt and Algeria – AF051350, AF051351, and AF051352[19] – were also included.

4. Results

4.1. Demographic and clinical characterization

58 HEV-infected subjects were identified. 52% of the population was female. The average age was 8 years. Median ALT and AST values were 94 (interquartile range [IR]: 60, 112) and 89 (IR: 51, 920), respectively. These levels were 5.4 (0.3, 52.4) and 3.6 (0.3, 89.7) fold above the upper limit of normal (ULN), respectively. 35 (60%) individuals reported jaundice, while 36 (62%) reported fever. 18 (30%) individuals reported contact with animals. The source of water was public/outside the residence for 24 (41%), residential tap water for 18 (31%), or wells for 16 (28%).

4.2. Virologic detection of HEV infection by real-time RT-PCR

Stool suspensions from 56 individuals were negative for HEV RNA by real-time PCR but were repeatedly positive for two patients—TS00223 and TS00226. TS00223 was a 24-year-old female from the Assuit area who was 4 months pregnant at the time of diagnosis. She presented with acute onset of fatigue, dark urine, yellow sclera, and abdominal pain for 3 days. Serum and stool was collected 3 days after disease onset. She was negative for HAV, HBV, and HCV. ALT and AST values were 94 and 96, respectively; these values are 7.8- and 8.0-fold above the ULN. She recovered completely within 2 weeks and was followed for 9 months with no morbidity. She had a normal delivery of a healthy baby. TS00226 was a 10-year-old female from a distinct village also in the Assuit area. TS00226 was not related to TS00223, although they did share the same well water source. She presented with acute onset of fatigue, loss of appetite, diarrhea, dark urine, and yellow sclera for 4 days. Serum and stool were collected 4 days after disease onset. She was negative for HAV, HBV, and HCV. ALT and AST values were 89 and 85, respectively; these values are 7.4- and 7.1-fold above the ULN. She recovered completely within 2 weeks and was followed for 9 months with no morbidity.

While HEV RNA was detectable in stool samples for TS00223 and TS00226, the corresponding serum samples did not have detectable HEV RNA. Similarly, a subset of individuals with no detectable stool

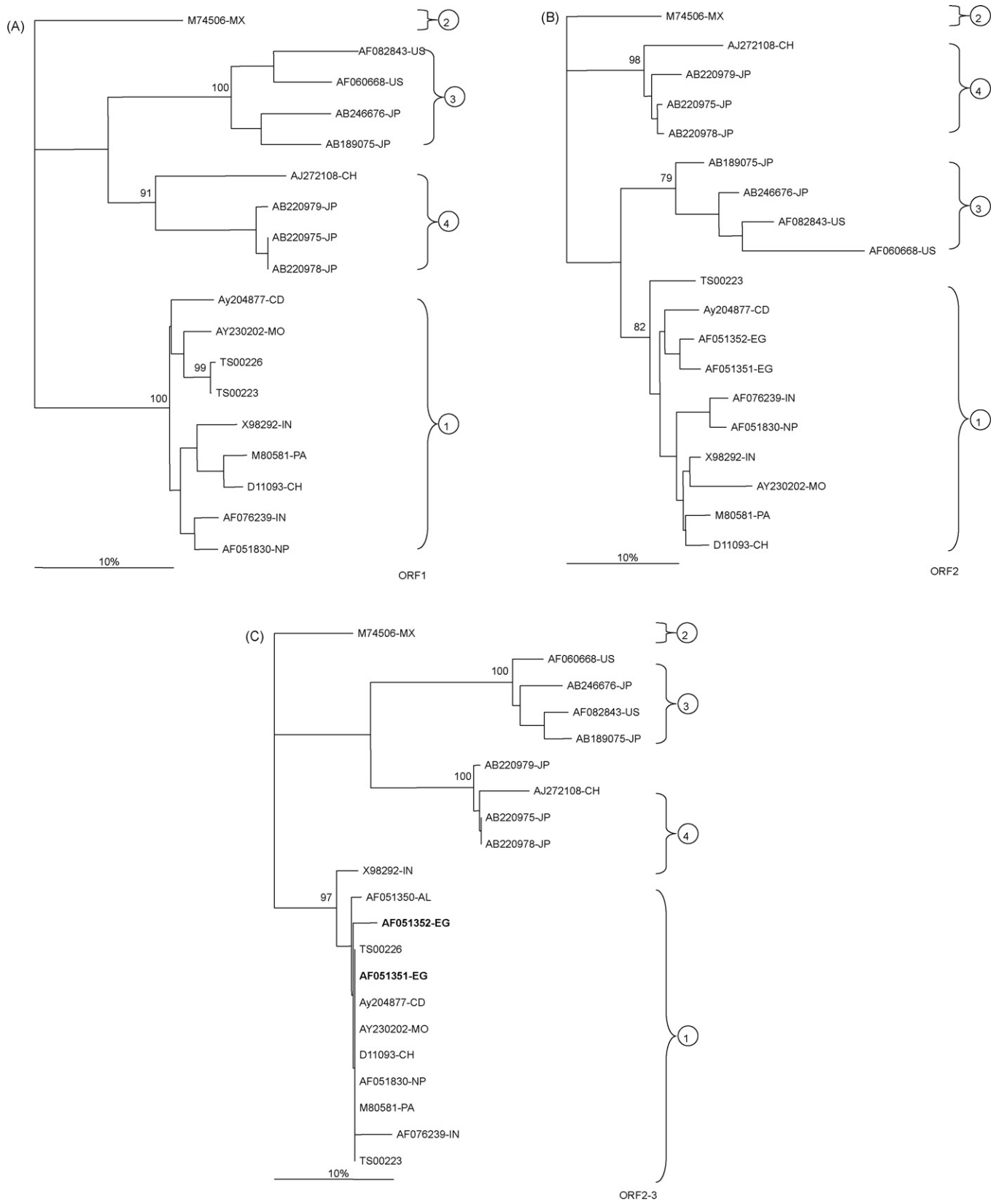

Fig. 1. Phylogenetic analysis of HEV sequences from two symptomatic Egyptian patients. (A) ORF1 (242 base pairs [bp]; (B) ORF2/3 overlap region (97 bp); (C) ORF2 (98 bp). Reference sequences are denoted by their GenBank accession numbers and the following country-specific abbreviations: Mexico (MX), United States (US), Japan (JP), China (CH), Chad (CD), Morocco (MO), India (IN), Pakistan (PA), Nepal (NP), Egypt (EG), and Algeria (AL). The two Egyptian samples characterized in the current study are highlighted in bold. The statistical robustness and reliability of the branching order within each phylogenetic tree were confirmed by bootstrap analysis using 100 replicates.[25] Sequences from the current study – with primer sequences removed – have been submitted to GenBank under accession numbers FJ423076–FJ423080.

HEV RNA was also negative for HEV RNA in their corresponding sera. For TS00223 and TS00226, there was insufficient RNA to determine the HEV genotype by qualitative RT-PCR; therefore, experimental inoculation of macaques with human serum and/or stool samples was attempted. Both animals inoculated with stool suspensions from these patients became positive for HEV RNA by real-time PCR, although neither corresponding serum sample transmitted HEV to the macaques.

4.3. HEV genotype variability

Stool samples from animals that were inoculated with stool suspensions from TS00223 and TS00226 were utilized in qualitative RT-PCR assays to determine the HEV genotype. Analysis of ORF1 demonstrated that TS00223 and TS00226 grouped with other African isolates representing HEV genotype 1 (Fig. 1A). While they were only 0.4% divergent from one another at the nucleotide level, they were 3.9–9.5% divergent from other genotype 1 isolates. No data from other Egyptian isolates were available for this ORF; however, TS00223 and TS00226 were divergent from isolates from Chad and Morocco.

For ORF2, only TS00223 could be amplified. This sample belonged to HEV genotype 1 and was 7.6–11.2% divergent from other previously reported isolates. TS00223 was also 8.7% and 5.3% divergent from isolates AF051351-EG and AF051352-EG, respectively, previously collected from Egypt in 1993–1994 (Fig. 1B).

Analysis of the highly conserved ORF2/3 overlap region also confirmed that TS00223 and TS00226 belonged to HEV genotype 1 (Fig. 1C). Both TS00223 and TS00226 were identical to one another and were 0.0–3.2% divergent from other genotype 1 isolates. TS00223 and TS00226 were identical to Egyptian isolate AF051351-EG but 2.1% divergent from isolate AF051352-EG.

5. Discussion

Universal community exposure is likely responsible for the high seroprevalence of HEV in Egypt.[5,8,11] However, HEV-associated AVH is relatively uncommon, leading some investigators to suggest that HEV strains in Egypt may be less virulent than in other geographic locations.[6,11] Tsarev et al. previously reported on two sporadic cases of HEV infection recovered from male army recruits living in the Cairo area in 1993–1994[19]. These isolates were closely related to other African isolates. Our finding of genotype 1 further supports the circulation of HEV genotype 1 throughout Egypt. Importantly, because our samples were collected in 2006–2008 as part of a large prospective study of HEV infection in the cities of Assuit (Upper Egypt) and Mansoura (in the Delta), they represent the HEV isolates currently circulating in the central region of Egypt. While the intragenotypic variability observed suggests geographic diversity and/or viral evolution with time in this population, continued evaluation of HEV variation in this and other endemic settings is clearly warranted.

The detection of HEV RNA in only two stool samples in the current study is somewhat surprising given that HEV RNA has been detected in the stool in epidemic settings.[20–22] However, the presence of viremia in the serum may be less frequent.[23,24] Furthermore, variable detection rates could reflect differences in the viral extraction methodology, assay sensitivity, and/or a highly divergent HEV strain that is not readily detected by current PCR-based assays. However, in the current analysis, we utilized two highly sensitive, real-time PCR assays that readily detected HEV RNA in human serum and stool in previous studies.[13,14] Additionally, HEV genotype 1 strains related to other North African isolates were detected, suggesting that assay sensitivity and viral divergence were unlikely to explain our findings. Importantly, the low frequency of HEV RNA detection in Egypt could also reflect true low-level viremia in HEV-infected individuals that merits additional investigation. This is further supported by the fact that HEV viremia was detected but only after experimental inoculation of human stool suspensions into macaques. Collectively, these data suggest that an attenuated genotype 1 may be circulating in Egypt and could be responsible for the low rate of HEV mortality observed.

Conflict of interest

None declared.

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Approval for human studies was provided by the University of Cincinnati IRB. Animal studies were approved by the Animal Care and Use Committees of Bioqual Inc., and the National Institute of Allergy and Infectious Diseases (Bethesda, MD, USA).

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