Transmission of Human Enterovirus 85 Recombinants Containing New Unknown Serotype HEV-B Donor Sequences in Xinjiang Uighur Autonomous Region, China

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Abstract

Background: Human enterovirus 85 (HEV85), whose prototype strain (Strain BAN00-10353/BAN/2000) was isolated in Bangladesh in 2000, is a recently identified serotype within the human enterovirus B (HEV-B) species. At present, only one nucleotide sequence of HEV85 (the complete genome sequence of the prototype strain) is available in the GenBank database.

Principal Findings: In this study, we report the genetic characteristics of 33 HEV85 isolates that circulated in the Xinjiang Uighur autonomous region of China in 2011. Sequence analysis revealed that all these Chinese HEV85 isolates belong to 2 transmission chains, and intertypic recombination was found with the new unknown serotype HEV-B donor sequences. Two HEV85 isolates recovered from a patient presenting acute flaccid paralysis and one of his contacts were temperature-insensitive strains, and some nucleotide substitutions in the non-coding regions and in the 2C or 3D coding regions may have affected the temperature sensitivity of HEV85 strains.

Conclusions: The Chinese HEV85 recombinant described in this study trapped a new unknown serotype HEV-B donor sequence, indicating that new unknown HEV-B serotypes exist or circulate in Xinjiang of China. Our study also indicated that HEV85 is a prevalent and common enterovirus serotype in Xinjiang.

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Introduction

Human enteroviruses (HEV) are members of the genus Enterovirus within the family Picornaviridae, order Picornavirales, and contains 4 species: HEV-A, HEV-B, HEV-C, and HEV-D [1]. Human enterovirus 85 (HEV85) belongs to the species HEV-B, which currently consists of 60 serotypes, including echovirus (ECHO, serotypes 1–7, 9, 11–21, 24–27, 29–33), coxsackievirus group A (CVA, serotypes 9), coxsackievirus group B (CVB, serotypes 1–6), recently identified HEV serotypes to be designated HEV69, HEV73–75 [2–5], HEV77–80 [6–9], HEV99 [10], HEV97–98 [9,11], HEV100–101 [9], HEV106–107 [12], HEV110 [13], and the simian enterovirus SA5. HEV-B viruses cause a variety of diseases such as acute aseptic meningitis, acute flaccid paralysis (AFP), hand, foot, and mouth disease, and acute myocarditis, and others [14–17].

The genome of HEV (approximately 7,450 nucleotides) is positive-sense, single-stranded, and contains a long open reading frame (ORF) flanked by a 5'-untranslated region (UTR) and a 3'-UTR. The 5'-UTR is about 740 nucleotides in length and comprises a secondary structure named the internal ribosome entry site [18] which is involved in the replication and internal initiation of translation of the genomic RNA [19]. A single polyprotein translated from the RNA strand is first cleaved into 3 polyprotein precursors: P1, P2, and P3. The P1 protein encodes the 4 structural polypeptides, VP1–VP4, while P2 and P3 are precursors of the nonstructural proteins 2A–2C and 3A–3D, respectively. A short 3'-UTR, approximately 100 nucleotides in
length, is located between the ORF and the poly (A) stretch, and comprises structural domains involved in RNA replication [20].

HEV85 is a newly identified serotype within the species HEV-B. Its prototype strain (BAN00-10353/BAN/2000) was identified in a stool sample from a patient presenting with AFP in Bangladesh in 2000, and at present, only one nucleotide sequence of HEV85 (the complete genome sequence of the prototype strain) is available in the GenBank database [9].

In the present study, we analyzed 33 complete VP1 nucleotide sequences (5 of which also have the complete genome sequences) of the HEV85 isolates recovered from one AFP patient and 32 of his health contacts in the Hotan and Kashgar prefectures, in the southern part of the Xinjiang Uighur autonomous region of China, in 2011. The results indicate that Chinese HEV85 strains are all recombinants with a new unknown serotype HEV-B and circulate in the southern part of Xinjiang.

Results

Transmission of HEV85 in Xinjiang in 2011

The VP1 region nucleotide sequence alignment included all 33 Chinese HEV85 strains and the prototype strain. The pairwise distance among the 33 Chinese HEV85 strains ranged from 0.000 to 0.026 divergence, and from 0.115 to 0.128 divergence compared with the prototype strain. On the basis of the high nucleotide and amino acid identities of the VP1 region of all 33 Chinese HEV85 isolates, strain HTYT-ARL-AFP02F, which was isolated from an AFP patient, was selected as the representative strain and depicted the scatter diagram, the plot of the amino acid sequence identity versus nucleotide sequence identity (Figure 1). HEV-D was omitted in this figure because few HEV-D sequences are available in the GenBank database. The plotting curve showed obvious grouping among HEV-A, HEV-B, and HEV-C, and the nucleotide and amino acid sequences of the strain HTYT-ARL-AFP02F had the lowest similarity values with HEV-A and HEV-C species. Intermediate values were observed with HEV-B species, ranging from 55.9% to 69.7% and from 57.3% to 75.2% for nucleotide and amino acid sequences, respectively (Table 1), which confirmed it was a member of HEV-B species. Finally, there were high nucleotide and amino acid sequence similarities values (89.4% and 98.6% for the nucleotide and amino acid sequences, respectively) between strain HTYT-ARL-AFP02F and the HEV85 prototype strain.

Phylogenetic analysis was performed on the basis of the alignment of VP1 region sequences described above (Figure 2). Clearly all 33 Chinese HEV85 strains are derived from the same origin, and a 2.0% nucleotide divergence was found among these strains. They were all circulated in the Hotan and Kashgar prefectures of southern Xinjiang from September to November 2011.

Full-length genomic characterizations of Chinese HEV85 strains

The full-length genome sequences of 5 selected Chinese HEV85 strains were determined (randomly selected on the basis of their genetic relationships; Figure 2). The results showed that they were similar to the only reported genome of HEV85 (the prototype strain), with 7,422–7,424 nucleotides, including a 5'-UTR of 742–744 nucleotides (0–2 nucleotide insertion compared with the prototype strain), a single ORF of 6,579 nucleotides encoding a single polyprotein of 2,191 amino acids, and a 3'-UTR of 101 nucleotides preceding the poly (A) tract. All these sequences shared 98.34–99.08% nucleotide sequence identities with each other, validating the circulation of HEV85 in Xinjiang of China.

A comprehensive comparison of nucleotide sequence and deduced amino acid sequence identities between the 5 Chinese
| Region | Nucleotide identity (%) | [Amino acid identity (%)] |
|--------|-------------------------|--------------------------|
|        | HT-LYKH202F             | HTYT-ARL-AFP02F          | HTYT-ARLH403F | HTTPS-MJH21F | HTTPS-MKLH04F |
| S'-UTR | 91.5                    | 79.6–88.4                | 91.3          | 79.4–88.3    | 91.2          | 79.3–88.3    | 91.2          | 79.2–88.2    |
| VP4    | 89.3 (100)              | 69.0–81.1 (75.3–95.0)    | 89.8 (100)    | 68.5–81.1 (75.3–95.0) | 89.8 (98.5) | 68.5–81.1 (75.3–94.2) | 89.8 (100) | 69.0–81.6 (75.3–95.6) | 90.3 (100) | 69.0–80.6 (75.3–95.6) | 89.6 (98.4) | 64.9–72.4 (75.5–83.2) |
| VP2    | 89.5 (98.8)             | 64.9–72.7 (75.5–83.2)    | 89.2 (98.4)   | 64.9–72.2 (75.5–83.2) | 89.6 (98.8) | 64.5–72.9 (75.5–83.5) | 89.1 (98.8) | 65.0–72.9 (75.5–83.5) | 89.6 (98.4) | 64.9–72.4 (75.5–83.2) |
| VP3    | 90.0 (98.7)             | 63.1–71.0 (66.9–81.0)    | 89.9 (98.7)   | 63.1–71.1 (66.9–81.1) | 88.9 (99.1) | 63.2–70.4 (66.9–81.1) | 89.3 (98.7) | 62.8–70.7 (66.9–81.1) | 89.9 (99.1) | 63.1–70.7 (66.9–81.9) |
| VP1    | 89.4 (98.9)             | 55.5–69.8 (57.3–75.0)    | 89.4 (98.6)   | 55.9–69.7 (57.3–75.2) | 89.1 (98.6) | 55.6–69.0 (57.0–75.6) | 89.0 (97.9) | 55.5–69.7 (57.0–75.2) | 89.6 (98.4) | 55.5–69.1 (57.3–75.6) |
| 2A     | 79.3 (94.6)             | 74.8–81.5 (84.6–96.0)    | 79.5 (94.6)   | 74.8–81.5 (84.6–96.0) | 79.7 (94.6) | 75.3–81.1 (85.3–96.6) | 80.2 (95.3) | 74.8–81.5 (85.3–96.6) | 80.0 (94.6) | 75.5–81.7 (84.6–96.0) |
| 2B     | 80.1 (94.9)             | 75.0–83.5 (93.9–98.9)    | 80.4 (94.9)   | 75.0–83.5 (93.9–98.9) | 80.1 (94.9) | 75.0–83.5 (93.9–98.9) | 80.8 (94.9) | 76.0–83.1 (93.9–98.9) | 79.4 (94.9) | 75.0–83.1 (93.9–98.9) |
| 2C     | 83.8 (98.4)             | 74.3–84.9 (96.3–99.0)    | 84.3 (98.7)   | 74.9–84.9 (96.6–99.3) | 84.1 (99.0) | 79.5–85.2 (96.9–99.6) | 84.3 (98.7) | 79.6–85.1 (96.6–99.9) | 84.0 (97.8) | 79.1–84.7 (85.7–99.0) |
| 3A     | 80.1 (97.7)             | 76.0–85.3 (91.0–98.8)    | 80.5 (97.7)   | 76.0–85.7 (91.0–98.8) | 80.5 (97.7) | 76.0–85.7 (91.0–98.8) | 79.4 (97.7) | 74.9–84.6 (91.0–98.8) | 80.1 (97.7) | 75.6–85.3 (91.0–98.8) |
| 3B     | 84.8 (100)              | 69.6–87.8 (90.9–100)     | 72.7–87.8 (90.9–100) | 72.7–87.8 (90.9–100) | 72.7–87.8 (90.9–100) | 71.2–89.3 (90.9–100) | 86.3 (100) | 71.2–89.3 (90.9–100) | 86.3 (100) | 71.2–89.3 (90.9–100) |
| 3C     | 83.9 (98.9)             | 75.9–85.7 (92.8–99.4)    | 83.7 (98.3)   | 75.9–85.6 (92.3–99.4) | 83.9 (98.9) | 76.1–85.7 (92.8–99.4) | 84.3 (98.9) | 76.1–86.1 (92.8–99.4) | 84.1 (98.9) | 75.7–85.4 (92.8–99.4) |
| 3D     | 84.9 (97.6)             | 77.5–85.6 (95.0–98.2)    | 85.5 (98.0)   | 78.1–86.1 (95.4–98.1) | 85.4 (97.8) | 77.8–86.2 (95.2–98.4) | 85.1 (97.8) | 77.7–85.7 (95.2–98.4) | 85.6 (98.0) | 77.9–86.0 (95.4–98.7) |
| 3'-UTR | 87.5                    | 74.0–91.3                | 87.5          | 75.0–91.3    | 88.4          | 74.0–92.3    | 87.5          | 73.0–91.3    | 88.4          | 74.0–92.3    |

Table 1. Pairwise nucleotide and amino acid sequence identities between human enterovirus 85 strains and prototype strains of the HEV-B species.
HEV85 strains and the prototype strain of HEV-B viruses including HEV85 is shown in Table 1. The nucleotide sequence identities between the 5 Chinese HEV85 strains and the prototype HEV85 strain were 86.46–86.73% in the full-length genome sequence and 89.28–89.67%, 82.06–82.70%, and 84.15–84.68% in the P1, P2, and P3 coding regions, respectively. The deduced amino acid sequence identities between the 5 Chinese HEV85 strains and the prototype HEV85 strain were 98.60–98.95%, 96.54%–97.23%, and 98.02–98.28% in the P1, P2, and P3 coding regions, respectively. Interestingly, in the capsid region, the nucleotide sequences of the 5 Chinese HEV85 isolates were closer to the HEV85 prototype strain (89.28–89.67%) than to other HEV-B prototype strains (58.8–68.3%, Table 1); while in the noncapsid region, the nucleotide sequences of the 5 Chinese HEV85 isolates and all HEV-B viruses including HEV85 were almost equidistant from each other (74.3–81.8%) and did not cluster with regard to all known serotypes HEV-B viruses, which indicated the occurrence of recombination events in the noncapsid region.

**Chinese HEV85 strains recombined with new unknown serotype HEV-B donor sequences**

Alignments of the VP1, P1, P2, and P3 region nucleotide sequences were carried out among the 5 selected Chinese HEV85 strains described above and the prototype strains of HEV-B, and phylogenetic trees were constructed (Figure 3). The phylogenetic tree analysis suggested that Chinese HEV85 strains are monophyletic in the VP1 region, and all 5 Chinese HEV85 strains clustered together with the HEV85 prototype strain (Figure 3a), confirming the classification of these isolates as a single enterovirus type. All 5 Chinese HEV85 strains clustered with the HEV85 prototype strain in the P1 capsid region (Figure 3b), but not in the P2 and P3 noncapsid regions (Figure 3c and 3d), indicating that recombination occurred among Chinese HEV85 strains.

Similarity plot and bootscan analyses revealed recombination between the Chinese HEV85 strains and HEV-B strains at the 2A–2B junction. The Chinese HEV85 strains were all identified as an HEV85 capsid sequence containing an unidentified sequence in the P2 and P3 coding regions that was apparently not related to those of the HEV85 strains (Figure 4). Nucleotide and amino acid sequences in the P2 and P3 regions are highly conserved within an
enterovirus species [21], and P2 and P3 sequences do not correlate with HEV serotypes due to frequent recombination; however, these sequences clearly distinguish different HEV species [9]. Comparison of the P2 and P3 coding region sequences of the Chinese HEV85 strains with those of prototype strains of HEV-A, B, C, and D revealed no sequence match above 84.68%, and showed higher similarity to HEV-B than to HEV-A, C, and D. In addition, the deduced amino acid sequence of the recombinant noncapsid sequences of the Chinese HEV85 strains showed high identity with HEV-B, especially those of prototype HEV88 (97.9%), prototype HEV75 (97.8%), and prototype HEV85 (97.6%). These results confirmed that the recombinant noncapsid sequences were classified into the HEV-B phylogeny (Figure 4).

Because the nucleotide sequence identities between the Chinese HEV85 strain and all the HEV-B prototypes in the noncapsid region ranged from 78.63% (HEV80) to 84.30% (HEV107), the noncapsid donor sequences could not be classified as any known serotype; the donor sequences were from a new unknown serotype within HEV-B.

**Temperature sensitivity**

Three selected Chinese HEV85 isolates (HT-LYKH202F, HTYT-ARL-AFP01F, and HTYT-ARLH403F) were compared to each other with regard to replication capacity at an elevated temperature (39.5°C), and showed different temperature sensitivities (Figure 5). Strain HT-LYKH202F was temperature-sensitive with titer reduction of more than 2 logarithms at 36°C/39.5°C, whereas the other 2 strains showed completely lower temperature sensitivities (titer reduced less than 2 logarithms at 36°C/39.5°C). This indicates that the replication efficiencies of these strains remain the same even at elevated temperatures (Figure 5).

In order to further investigate determinants of temperature sensitivity among these strains, differences in nucleotide and amino acid sequences between the temperature-sensitive strain and the 2 temperature-insensitive strains were summarized (Table 2). The results indicate that the numbers of different nucleotides and amino acid were 46 and 6, respectively. On the basis of the above results and on many previous studies of other serotypes of HEVs [22,23], we believe that a total of 8 nucleotides in the 5’-UTR (nt161), 2C (nt4102 and nt4256), 3D (nt6178, nt6340, nt6719, and nt6972), and 3’-UTR (nt7330) regions are candidate determinant sites for temperature sensitivity in HEV85.

**Discussion**

Molecular serotyping methods have enabled the rapid identification of new HEV serotypes that are untypeable by traditional neutralization of virus isolates in cell culture using standardized antisera, and as a result, sequencing of the complete VP1 capsid region has emerged as the gold standard for HEV typing to distinguish serotypes [24–26]. Molecular identification of recently identified HEV serotypes, including HEV85, provides a tool to assist in the epidemiological investigation of AFP cases that are associated with non-polio enterovirus infection.

In this study, we identified 33 HEV85 isolates in the Hotan and Kashgar prefectures in the Xinjiang region in China from the 6th to 26th Sep 2011, which indicated the prevalence of this serotype of HEV in this region. These 2 prefectures are located in the southernmost part of Xinjiang and have great biological and climatic differences due to the large area (409,800 square kilometers in total), which includes the southern mountain area (tempertate or cold temperate climate), the oasis plain area (warm temperate), and the northern desert area (continental desert climate). The viruses were isolated from the oasis plain area, where it is very suitable for the growth and survival of HEV. HEVs are known to be propagated mainly by fecal-oral transmission, suggesting that the river, the source of water, geographic circumstances, and air humidity are major factors affecting transmission. This is confirmed by the facts that the majority of the prototype strains of novel HEV serotypes were isolated from tropical and subtropical countries and regions such as Bangladesh [9,27], California (USA) [2,9], Egypt [28] and Cote d’Ivoire [9], etc, while the prevalence of these viruses seems to be relatively restricted in the temperate countries.

One of the 33 Chinese HEV85 isolates, strain HTYT-ARL-AFP02F, was derived from an adult patient who presented with AFP in July 2011. Although there is no strong evidence to prove that HEV85 is the causative pathogen of AFP, as the virus was recovered from a stool specimen, it showed high transmissibility based on the fact that it has high nucleotide and amino acid identities with other Chinese HEV85 isolates picked from the contacts of this AFP patient. This strain was also temperature-insensitive and could grow at relatively high temperatures, which is usually an indicator of high virulence or transmissibility [23,29]. These findings suggest that HEV85 is an important human pathogen; however, more data are necessary before HEV85 can be positively associated with any particular human disease.

Strain HT-LYKH202F was a temperature-sensitive strain, whereas strains HTYT-ARL-AFP02F (isolated from an AFP patient) and HTYT-ARLH403F were not. All these 3 strains had high nucleotide and amino acid similarities with each other (a total of 8 nucleotide substitutions of which 6 caused amino acid substitutions), which implies that possibly one or several sites may play significant roles in this biological feature. Our research team is currently using reverse genetics methods to elucidate the mechanism of temperature sensitivity of HEV85, in order to identify several vaccine candidate strains to meet emergency needs in case of epidemic outbreaks triggered by these serotypes in the future.

Recently, increasing numbers of publications have demonstrated that recombination is extremely frequent among HEVs [22,30,31]. Therefore, it can be concluded that HEVs exist as a huge reservoir of genetic material comprising a limited quantity of capsid gene sets defining a finite number of serotypes and a range of non-structural genes that recombine frequently to produce new virus variants. The Chinese HEV85 strains identified in this study are no exception, and have recombined with other HEV-B viruses; this is supported by the genomic features, the phylogenetic tree based on the alignments of the P1, P2, P3 regions, and the similarity and bootscan plots. Together with the isolation of HEV85, a recently identified novel HEV serotype, the trapping of an unknown new serotype HEV-B donor sequence in the Chinese HEV85 recombinant described in this study suggests that new
Figure 4. Similarity plot and bootscan analysis of whole genome of Chinese HEV85 strains. Gene structure organization (a), similarity plot (b), and bootscan analysis (c) of complete HEV-B genomes using a sliding window of 200 nt moving in 20 nt steps. The HTYT-ARL-AFP02F/XJ/CHN/2011 isolate was used as a query sequence and is indicated in the lower right corner, and for each bootscan analysis, the names of viruses of the query sequence are indicated in the upper right corner.

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HEV-B serotypes are in circulation in the Xinjiang region of China. Hence, we have increased HEV surveillance in the Xinjiang region in order to determine the precise donor sequence of the new serotype HEVs.

Identifying HEVs recovered from patient specimens has several implications in the medical field, and HEVs should be characterized to allow epidemiological surveillance of the potential epidemic risks associated with new viruses. Indeed, there are more and more novel HEV serotypes that exhibit some features pertaining to epidemic risk; for example, they were isolated from patients, show high prevalence in many regions, and even elicit epidemic outbreaks [32–35]. Therefore, it is considered important to conduct stool surveys in the Xinjiang Autonomous Region, as a few AFP patients have been identified during AFP surveillance activities in support of global polio eradication. However, an effective HEV pathogen surveillance system has yet to be established in mainland China; therefore, current information on HEV85 or other novel HEV serotypes in circulation is not available. Once this is in place, it is expected that monitoring trends in the transmission of HEV85 or other new HEV types will be made possible by molecular epidemiological studies over a wide area. As more laboratories adopt molecular typing methods in order to identify newly described HEV serotypes, it will become possible to address the global distribution of HEV85 and to better understand its epidemiology, disease burden, and spectrum of illness.

Materials and Methods

Viruses
This study did not involve human participants or human experimentation; the only human materials used were stool samples collected from AFP patients or their close contacts at the instigation of the Ministry of Health P. R. of China for public health purposes, and written informed consent for the use of their clinical samples was obtained from all patients involved in this study. This study was approved by the second session of the Ethics Review Committee of the Chinese Center for Disease Control and Prevention. The HEV85 strains used in this study were isolated from stool specimens from one AFP patient and 32 of his healthy close contacts in the Hotan and Kashgar prefectures in the Xinjiang region of China. Viruses were isolated from original stool specimens by propagation in human rhabdomyosarcoma (RD) and human larynx carcinoma (HEp-2) cells by conventional methods and then sequenced [36].

Determination of the complete VP1 nucleotide sequence
Viral RNA was extracted from the viral isolates using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA). The complete VP1/capsid region of each HEV85 strain was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using the primers described previously [9]. RT-PCR was performed with an Access RT-PCR Kit (Promega, Madison, Wisconsin, USA) according to the manufacturer’s instructions. The PCR products obtained were purified using the QIAquick Gel extraction kit (Qiagen), and the amplicons were bi-directionally sequenced using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Hitachi, Japan) [37].

Full-length genome sequencing of Chinese HEV85 strains
First of all, viral RNA was converted to cDNA by a random priming strategy. Then the complete genome sequences of the viruses were acquired according to the published strategies for HEV sequencing [30,38]. Briefly, the overlapping fragments representing the complete genomes were amplified by RT-PCR with the specific, non-degenerate primers listed in Table 3, and the primer-walking strategy was used to close gaps as necessary. The PCR products obtained were purified for sequencing using the QIAquick Gel extraction kit (Qiagen), and the amplicons were then bi-directionally sequenced using fluorescent dideoxy-chain termination and an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA). 5’-segment sequences were determined using a 5’-rapid amplification of cDNA ends core set (Takara Biomedicals, Dalian, China), according to the manufacturer’s instructions [30].

Phylogenetic and Bioinformatics analyses
The nucleotide and deduced amino acid sequences of the HEV85 isolates were compared to one another and to those of other HEV-B serotypes by pairwise alignment using the MEGA program (version 5.0; Sudhir Kumar, Arizona State University, Tempe, Arizona, USA) [39,40], and the identity matrices were analyzed by plotting VP1 amino acid identities versus VP1 nucleotide identities for each virus pair using the SigmaPlot program (version 10.0; Systat Software Inc., San Jose, CA, USA). Phylogenetic trees were constructed by the neighbor-joining method implemented in the MEGA program using the Kimura-
| Origin or donor Region | Nucleotide Position | Strain (a) | Strain (b) | Strain (c) | Amino acid Position | Strain (a) | Strain (b) | Strain (c) |
|------------------------|--------------------|------------|------------|------------|---------------------|------------|------------|------------|
| HEV85 Sequence 5'-UTR | 161                | C          | T          | T          | /                   | /          | /          | /          |
| VP4                   | 989                | C          | T          | T          |                     |            |            |            |
| VP3                   | 1985               | C          | T          | T          |                     |            |            |            |
| VP2                   | 2174               | T          | C          | C          |                     |            |            |            |
| VP1                   | 2729               | A          | T          | T          |                     |            |            |            |
|                       | 2915               | C          | T          | T          |                     |            |            |            |
| New unknown serotype HEV-B donor sequence 2A | 3449 | C | T | T | | | | |
| 2B                    | 3929               | C          | T          | T          |                     |            |            |            |
| 2C                    | 4102               | G          | A          | A          | 1120                | Ser        | Asn        | Asn        |
|                       | 4256               | T          | A          | A          | 1171                | Asp        | Glu        | Glu        |
|                       | 4565               | T          | C          | C          |                     |            |            |            |
|                       | 4721               | C          | T          | T          |                     |            |            |            |
|                       | 4799               | T          | C          | C          |                     |            |            |            |
|                       | 4898               | C          | T          | T          |                     |            |            |            |
|                       | 4910               | T          | C          | C          |                     |            |            |            |
|                       | 4976               | T          | C          | C          |                     |            |            |            |
|                       | 5006               | T          | C          | C          |                     |            |            |            |
| 3A                    | 5081               | T          | C          | C          |                     |            |            |            |
| 3B                    | 5354               | G          | A          | A          |                     |            |            |            |
| 3C                    | 5549               | T          | C          | C          |                     |            |            |            |
|                       | 5576               | C          | T          | T          |                     |            |            |            |
|                       | 5630               | A          | G          | G          |                     |            |            |            |
|                       | 5645               | G          | A          | A          |                     |            |            |            |
|                       | 5696               | T          | C          | C          |                     |            |            |            |
| 3D                    | 5882               | A          | T          | T          |                     |            |            |            |
|                       | 5999               | G          | A          | A          |                     |            |            |            |
|                       | 6113               | C          | T          | T          |                     |            |            |            |
|                       | 6178               | C          | T          | T          | 1812                | Ala        | Val        | Val        |
|                       | 6212               | A          | G          | G          |                     |            |            |            |
|                       | 6252               | T          | C          | C          |                     |            |            |            |
|                       | 6267               | T          | C          | C          |                     |            |            |            |
|                       | 6284               | T          | G          | G          |                     |            |            |            |
| 3D                    | 6340               | G          | A          | A          | 1866                | Arg        | Lys        | Lys        |
|                       | 6350               | T          | A          | A          |                     |            |            |            |
|                       | 6431               | G          | A          | A          |                     |            |            |            |
|                       | 6719               | C          | A          | A          | 1992                | Asp        | Glu        | Glu        |
|                       | 6725               | T          | C          | C          |                     |            |            |            |
|                       | 6761               | T          | C          | C          |                     |            |            |            |
| 3D                    | 6972               | A          | G          | G          | 2077                | Thr        | Ala        | Ala        |
|                       | 7175               | C          | T          | T          |                     |            |            |            |
2-parameter model. Regions containing alignment gaps were omitted from the analysis. The branch lengths of the dendrogram were determined from the topologies of the trees and were obtained by majority rule consensus among 1000 bootstrap replicates. Bootstrap values greater than 80% were considered statistically significant for grouping.

Recombination analysis

The nucleotide alignment containing the genome sequence of a Chinese HEV85 strain (HTYT-ARL-AFP02F) and HEV-B prototype strains (ECHO-1, 4, 7, 9, 13, 15, 16, 18–21, 24, 27, 29, 30, 33, CVA9, CVB3–5, HEV69, HEV73–75, HEV77, 79–80, HEV97–98, HEV100–101, and HEV107) was generated using the MEGA program (version 5.0; Sudhir Kumar, Arizona State University, Tempe, Arizona, USA) [39,40]. Once aligned, a similarity plot and bootscan analysis were performed using Simplot program (version 3.5.1; Stuart Ray, Johns Hopkins University, Baltimore, Maryland, USA) [41].

Assay for temperature sensitivity

Temperature sensitivities of 3 selected HEV85 isolates (HT-LYKH202F, HTYT-ARLH403F and HTYT-ARL-AFP02F) were assayed on monolayer RD cells in 24-well plates as described before [42]. Briefly, the 24-well plates were inoculated with 50 μl of undiluted virus stocks. Two different incubators were used; the temperature of one incubator was adjusted to 36°C (optimal temperature for virus propagation), while the temperature of the other incubator was adjusted at 39.5°C (supraoptimal temperature for virus propagation). After absorption at 36°C or at 39.5°C for 1 h, the unabsorbed virus inoculum was removed, 100 μl of maintenance medium was added to each well, and the plates were continually incubated at 36°C or at 39.5°C, separately. After 5 time points post-infection (4, 8, 16, 24, and 48 h), the plates were harvested, and the cell culture infectious dose 50% (CCID50) was calculated by the end-point dilution method on monolayer RD cells at 36°C. In this study, virus isolates showing more than a 2-logarithm reduction in titer at different temperatures were considered to be temperature-sensitive [22].

Table 3. PCR and sequencing primers.

| Primer        | Nucleotide position (nt) | Primer sequence (5’–3’) | Orientation | Reference |
|---------------|--------------------------|-------------------------|-------------|-----------|
| 0001548       | GGGGACAAGTTTGTACAAAAAAGCAGGCTTTAAAACAGCTCTGGGGTT | Forward | [43] |
| HEV85-1049A   | 1030–1049                | ACAACAACATTGGCACACTC   | Reverse     | This study |
| HEV85-6365    | 636–655                  | GCCATCGGCTGCTAAATAGA   | Forward     | This study |
| HEV85-1656A   | 1637–1656                | ACCATCCTATCTGGTGCCCA   | Reverse     | This study |
| HEV85-1487S   | 1487–1506                | TGGGCAACTTACACATTC    | Forward     | This study |
| HEV85-2460A   | 2611–2640                | GACCTGAGGGGAAATGGTA   | Reverse     | This study |
| HEV85-2480S   | 2461–2480                | GAAGGGAGAGGAGGGCTAGT  | Forward     | This study |
| HEV85-3406A   | 3406–3425                | TATGCTCCCGACAGCAACT   | Reverse     | This study |
| HEV85-3290S   | 3268–3287                | CGTGGCAACTTACACATTC   | Forward     | This study |
| HEV85-4257A   | 4238–4257                | TTAATTTGTTGCAACTTG    | Reverse     | This study |
| HEV85-4072S   | 4072–4091                | GGCTGCTCAAGAATGTCC    | Forward     | This study |
| HEV85-5062A   | 5043–5062                | CTTGGAGCTTGAACAAAG   | Reverse     | This study |
| HEV85-4930S   | 4930–4949                | GCCATCGAGTCTGGCAG     | Forward     | This study |
| HEV85-5880A   | 5861–5880                | GTGGCAACCAAGGTGATTCC  | Reverse     | This study |
| HEV85-5722S   | 5722–5741                | TACATCCTGCAGGCTGACT   | Forward     | This study |
| HEV85-6619A   | 6600–6619                | GTAGCGCCAGCACTGATACT  | Reverse     | This study |
| HEV85-6436S   | 6436–6455                | GTGGAAGGCAATGCCGCA    | Forward     | This study |
| HEV85-7260A   | 7241–7260                | TACTGAGGGCTTCTTGATC   | Reverse     | This study |
| HEV85-7084S   | 7084–7103                | CAATCAGCCTGCTGTCGCA   | Forward     | This study |
| 7500A         | GGGGACACATTTTGTACAAAGACGGTGGT | Reverse | [43] |
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Nucleotide sequence accession numbers

The 5 full-length genomic sequences of HEV85 strains that were determined in this study have been deposited in the GenBank database under the accession numbers JX989093 to JX989097. The 28 other complete VP1 nucleotide sequences (570 nucleotides) of HEV85 strains that were determined in this study have been deposited in the GenBank database under the accession numbers JX989010 to JX989037.

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Author Contributions

Conceived and designed the experiments: QS YZ WX. Performed the experiments: QS YZ SZ HL GH HL HC XL DY ZZ JL PZ BJ ZT HZ HN. Analyzed the data: QS YZ ZZ. Contributed reagents/materials/analysis tools: ZZ HN. Wrote the paper: QS YZ.

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