Research

Cloning of full genome sequence of hepatitis E virus of Shanghai swine isolate using RACE method
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Abstract

Genotype 4 hepatitis E virus (HEV) was reportedly transmitted freely between humans and swine in eastern China. The full-length genomic sequence of Shanghai swine isolate (SH-SW-zs1) recovered from feces sample of a pig which was infected with HEV RNA positive swine serum was determined using RT-PCR and RACE (Rapid Amplification of cDNA Ends) methods. The full genome of the SH-SW-zs1 isolate was 7265 nucleotides in length and phylogenetic analysis indicated that this isolate belonged to genotype 4. Comparison of the 3' UTR sequence with the corresponding regions of other 38 HEV strains from different region revealed that the Shanghai swine isolate is 21–49 bp longer than the other stains.

Introduction

Hepatitis E is an important public health disease in many developing countries of Asia and Africa and also occurs sporadically in some industrialized countries [1-4]. The disease mainly affects young adults and has a relatively high mortality of up to 25% in affected pregnant women [1]. The main mode of transmission of hepatitis E virus (HEV) is fecal-oral route, primarily through contaminated water supplies [1]. HEV is single-stranded, positive-sense RNA virus without an envelope [5]. The genome of HEV is approximately 7.2 Kb and consists three open reading frames (ORF1–3) [6]. ORF1 locates at the 5' end of genome and encodes non-structural proteins, including the methyltransferase, protease, helicase and RNA-dependent RNA polymerase (RdRp) [7]. ORF2 maps to the 3' terminus and encodes for a major structural protein, and ORF3 overlaps both and encodes a thus far unknown function [6]. Based on sequence analysis, HEV sequences have been classified into four major genotypes (1–4). Genotype 1 is the main cause of hepatitis E in developing countries in Asia and Africa, and genotype 2 has been documented in Mexico and Nigeria. Genotype 3 or 4 have been described in the United States, European countries, China, Taiwan, and Japan [8,9]. The virus is also prevalent in swine, and isolates from swine are genetically closely related to that from humans [10-12]. Lots of researches showed that genotype 4 and genotype 1 were the major genotype in China, recently genotype 3 HEV was reported in swine of Shanghai suburb [13]. For the further research, such as genomic characteristics and phylogenetic analysis, the full genome of the isolate which was proved prevalent in Shanghai swine was determined in the current study.
Materials and methods

Samples

132 serum samples of swine were collected from Shanghai suburb in China. These samples were tested for HEV RNA, using reverse transcriptase-polymerase chain reaction (RT-PCR). One HEV RNA positive swine serum sample was used for experimental infection of pigs [14]. HEV RNA positive swine fecal samples were stored as 10% suspension in aliquots at 70°C. About 10 g of HEV RNA positive fecal sample was converted to 10% (w/v) suspensions in PBS (0.01 M, pH 7.2–7.4, added 0.1% DEPC) for determining the full genomic sequence of HEV.

Viral RNA extraction

One hundred microlitre of fecal suspensions was mixed with 1 ml of trizol (invitrogen, USA). The mixture was homogenized and incubated for 5 min at room temperature. Two hundred microlitre of chloroform was added and the mixture was vigorously shaken for 15 s and incubated at room temperature for 3 min. The aqueous phase was transferred to a fresh microfuge tube after centrifugation at 12 000 g for 15 min at 4°C. Five hundred microlitre of isopropl alcohol was added and the mixture was incubated for 15 min at room temperature. Then centrifuging at 12 000 g at 4°C for 15 min. After discarding the supernatant, RNA pellet was washed with 1 ml 75% ethanol. The RNA pellet was dried at room temperature for 5 min after centrifuging at 12 000 g for 5 min at 4°C. And Discarding the supernatant. RNA sample was dissolved with 20 µl DEPC treated water and used to reverse transcription immediately.

PCR amplification

Full-length primers: 18 sets of degenerate primers were designed based on a multiple sequence alignment of entire genome from isolates AY594199, DQ279091, DQ450072 and AB108537 (Table 1). Reverse transcription was carried out at 42°C for 1 h with 1 ul (200 units) of AMV Reverse Transcriptase (TakaRa, Japan) and 1 ul (25 mM) of external antisense primer. The first round PCR was carried using 10 ul of the synthesized cDNA and an external set of forward and reverse primers with Ex Taq DNA polymerase (TakaRa, Japan). A nested PCR was carried out with internal primer set and 5 ul of the first PCR product. The PCR parameters of all amplification reactions included an initial incubation at 95°C for 9 min, followed by 39 cycles of denaturation at 94°C for 1 min, annealing for 1 min at a temperature varied according to the Tm of different primers, and extension at 72°C for 1.5 min, with a final incubation at 72°C for 7 min. The resulting PCR products were excised from agarose gel and purified using the Axyprep DNA Gel Extraction Kit (AXYGEN, USA). The purified PCR products were ligated into pMD18-T vector (TakaRa, Japan) using T4 DNA ligase (TakaRa, Japan) at 16°C overnight. The recombinant plasmid was transformed into DH5α competent Escherichia coli cells (TakaRa, Japan). Plasmids containing the insert fragment were identified by PCR. Three of the positive clones were sequenced.

5’RACE

The 5’RACE was carried out with the 5-Full RACE Core Set (TaKaRa, Japan) following the manufacturer’s instructions. Briefly, 1st strand cDNA was synthesized by reverse transcription using 5’end-phosphorylated RT Primer which was specific to the swine HEV (5’-p-GTCATRCCRT-GGCG-3’). The PCR reaction mixture was incubated for 2 min at 94°C followed by 35 amplification cycles, comprising denaturation at 94°C for 30 s, annealing at 65°C for 30 s and extension at 72°C for 30 s. The reaction was extended for another 7 min at 72°C to insure the full extension. Fifteen ul of 1st Strand cDNA was treated with RNase H in a total 75 µl reaction mixture containing 15 ul of Hybrid RNA Degeneration Buffer for 1 h at 30°C. The mixture was then precipitated at -20°C for 30 min, being added 100 ul of H2O and 500 ul 100% ethanol. The supernatant was discarded and the pellet was washed with 75% ethanol after centrifuging at 12 000 g for 5 min. The pellet was dissolved with 8 ul of RNA (ssDNA) Ligation Buffer and 12 ul of H2O after dried at room temperature for 5 min. 20 ul of 40% PEG-6000 and 1 ul of ligase were added and incubated at 16°C overnight. Fifteen microliters of cycled cDNA was then used as template for nested PCR using ExTaq DNA polymerase (TaKaRa, Japan) with two sets of primers: 5’-CGGAGTGGCCGCTGCTAGAG-3’ (external forward primer, nucleotide position numbers 104 to 84), 5’-TGTACTGJTITGTGCTGAGAC-3’ (external reverse primer, nucleotide position numbers 482 to 501), 5’-ATGGGTGATTCCACAG(A)AACCTC-3’ (internal forward primer, nucleotide position numbers 225 to 203), and 5’-ATCCACACAG(T)GAGCT(p)GAGCAC-3’ (internal reverse primer, nucleotide position numbers 236 to 256). The PCR reaction mixture was incubated for 2 min at 94°C followed by 35 amplification cycles, comprising denaturation at 94°C for 30 s, annealing at 65°C for 30 s and extension at 72°C for 30 s. The reaction was extended for another 7 min at 72°C to insure the full extension. The final PCR product was analyzed on 20 g/L agarose gel.

3’RACE

The 3’RACE was carried out with the TaKaRa RNA PCR Kit (TaKaRa, Japan) following the manufacturer’s instructions. Briefly, ten microliters of the HEV RNA was used as template to synthesize cDNA with AMV Reverse transcriptase for 1 h at 42°C. The external reverse primer (HE17A) which has a poly (T) tract was used to prime the cDNA synthesis. The cDNA was then amplified by nested PCR with the external forward primer (5’-CGCTACTACATATC- CAGCAG-3’, nucleotide position numbers 6763–6782)
Table 1:

| Primer name | Nucleotide position | Nucleotide sequence (5'-3') |
|-------------|---------------------|-----------------------------|
| HE0ES       | 104-84              | CGGAGTTGGCGCCTGCTAGAG        |
| HE0EA       | 482–501             | TGACT(5'T)GGCTGCTAGAC        |
| HE0IS       | 225-203             | ATGGGCTATTCCACAG(A)AACTCT    |
| HE0IA       | 236–256             | ATCCAAAC(T)GGCTT(C)GAGCA     |
| HE1ES       | 11–32               | TATGTTGCTGAGCGCCGATGAGG      |
| HE1EA       | 528-509             | GCCCTTTATCTGACGCA            |
| HE1IA       | 573-554             | ATACCGTGCCGAGCCATTGC         |
| HE2ES       | 482–501             | TGACTTTTTGCTGCTAGAC          |
| HE2EA       | 956–975             | ACAGGGAGCGCCATGAAATGT        |
| HE2IA       | 437–454             | CTTCCACCTGT(C)GAT(C)CGG      |
| HE2IA       | 1000–983            | AAGCATG(A)GACCCTGTCCC       |
| HE3ES       | 671–692             | CGTGGCA(T)GTG(A)ATTACATAT(C)GAGG |
| HE3EA       | 1336–1317           | CCACCCGTCGGAA(G)CAGCTG(A)GCAT |
| HE3IS       | 742–762             | GATCCGTGACC(G)ACT(C)AAGGTCAC |
| HE3IA       | 1314-1293           | AA(C)CTG(C)CAA(G)CTG(A)CGA(G)CCAGGGA |
| HE4ES       | 984–1005            | GGGACAGGGCTTTATGCTTTTG       |
| HE4EA       | 1528–1508           | TGCCCTTATATCATAAATGGC        |
| HE4IS       | 956–975             | ACCTTATCCTCCGTCCTGT          |
| HE4IA       | 1703–1684           | GGGCGTCG(A)GAGAGCGG(G)TCCAG |
| HE5ES       | 1331–1348           | CCGTGTTGCTAG(T)GTTCCCTG(G)TC |
| HE5EA       | 1792–1746           | GTTGAG(A)AAGGTT(C)TATATG(T)G |
| HE5IS       | 1310–1329           | C(G)AGTTTTGATGACCCTGTCCC     |
| HE6ES       | 1803–1785           | GGAC(A)GACATAC(T)TGCTCT(C)G |
| HE6EA       | 1508–1528           | CAGGGTCG(T)GATAG(C)GAGGCG    |
| HE6IA       | 2529–2510           | GGGAAACG(T)CTG(A)GTGAAT(A)GC |
| HE7IS       | 1679–1700           | GGTGAGC(A)GTC(T)CTGCTAT(G)CCG |
| HE7EA       | 2477–2457           | GTGTTA(G)GAGCT(G)CGATTA(G)AC |
| HE7IA       | 2028–2048           | TGTGGTAC(T)CAG(T)CTCGAGGGGC |
| HE7IA       | 2144–2123           | CTCTACACT(C)CGG(T)ACTGCTGCG |
| HE7IS*      | 2830–2850           | GTAAGGCGGTGAAAGGGTGGGC       |
| HE7IA*      | 2913–2893           | ACTTCACTGTCGCGGAGAGTCTAAC    |
| HE8ES       | 2751–2772           | GCCCTGGGAAAGTACCCCG          |
| HE8EA       | 3366–3347           | GTCTGGGATGTCAT(T)TTTT(C)GGGTAGCC |
| HE8IS       | 2714–2733           | GCCCGC(T)ATATATAAGGT(C)AC   |
| HE8IA       | 3438–3416           | GCCCTGGGTG(A)ATAT(C)ACCAAG(T)C(T)GCTTCT(C)G |
| HE9ES       | 3209–3228           | GGTGAC(T)CCC(T)TATAT(C)AAATCCC |
| HE9EA       | 3948–3929           | GGGCGTCACTGCGAGGTG         |
| HE9IS       | 3312–3334           | GATGC(T)CGCGGTGAG(C)TGCTGTA(G)GAG |
| HE9IA       | 3810–3791           | GGTGCA(G)TGCCCAAGGC(T)TCCTC |
| HE10ES      | 3764–3781           | CAGTTTATGCTG(T)GATCTAC(T)CAG |
| HE10EA      | 4432–4413           | ATCATTCTCAAAAACCTTAC        |
| HE10IS      | 3587–3605           | ACG(T)GAGAGA(A)TGTTGGTGCTG(C)G |
| HE10IA      | 4518–4496           | CACTCC(T)CTCGATATGCTACTCT  |
| HE11ES      | 4290–4311           | TGTTCTGCCCC(G)TGGTTCTTGG    |
| HE11EA      | 4752–4733           | CGATGCTACATACGAGCAC         |
| HE11IS      | 4355–4375           | TATGTTGATGCA(G)TGATGAG(A)GAC |
| HE11IA      | 4736–4717           | GCACAACAGAAATCATCCTCCC      |
| HE12ES      | 4607–4625           | TGGAAAGGAA(G)CAT(C)TCTGGTG |
| HE12EA      | 5253–5233           | CGGTTGGCGCGGCGACATAG        |
| HE12IS      | 4496–4518           | GAGTGTAAACATGAGG(A)GAGTG    |
| HE12IA      | 5347–5366           | GGTGGATGGAATAGGAGGGA        |
| HE13ES      | 4977–4997           | CGAGTGTGCTGCGTTTGGG         |
| HE13EA      | 5451–5431           | GCAACGGGCAAACCGAGGGC        |
| HE13IS      | 5020–5039           | CGGTTGATTGTCCCCGCTTTGG      |
| HE13IA      | 5392–5371           | GTTGGAAGTTCGATGCGAAGG       |
| HE14ES      | 5347–5366           | TCCCCCTATATTCATCCAACC       |
| HE14EA      | 5956–5934           | TGATTG(T)CGATAG(A)TGCAAGGGCTC |
| HE14IS      | 5233–5252           | CATATGCTGCCGGCGCAAGC        |
| HE14IA      | 5980–5957           | GAGTGTCTCAACT(C)GAG(A)CGCCAA(G)CCC |
and internal forward primer (5'-CTAAGACCTTCTTTGTTCTGCC-3', nucleotide position numbers 6787–6808) with ExTaq DNA polymerase (TaKaRa, Japan). The PCR reaction mixture was incubated for 2 min at 94°C, followed by 35 amplification cycles comprising denaturation at 94°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 30 s. The reaction was extended for another 7 min at 72°C to ensure the full extension.

Phylogenetic analysis
Using Clustal × 1.8, multiple alignments of nucleotide sequences was carried out. The phylogenetic status SH-SW-zs1 isolate was assessed employing the software MEGA Version 2.1 [15]. For analysis in MEGA, Jukes Cantor (JC) distance was utilized employing the Neighbor joining (NJ) algorithm. The reliability of different phylogenetic groupings was evaluated by using the bootstrap test (1000 bootstrap replication) available in MEGA. Accession numbers, designations and countries of origin of the full genome sequences employed for analysis in the present study were as follows:

Genotype 1: AF051830, Nepal; X99441, India; AF076239, India; AF459438, India; AF185822, Pakistan; X98292, India; L25595, China; M80581, Pakistan; AY230202, Morocco.

Genotype 2: M74506, Mexico.

Genotype 3: AP003430, Japan, human; AB091394, Japan, human; AB073912, Japan, swine; AY115488, Canada, swine; AF060668, US, human; AF082843, US, swine; AB089824, Japan, human; AB074918, Japan, human; AB074920, Japan, human.

Genotype 4: AB091395, Japan, human; AB097812, Japan, human; AB097811, Japan, swine; AB074915, Japan, human; AB074917, Japan, human; AJ272108, China, human; AB108537, China, human; AB161717, Japan, human; AB161718, Japan, human; AB161719, human; DQ450072, China, swine; AY954199, China, swine; DQ279091, China, swine; AB197673, China, human; EF077630, China, swine; AB197674, human.

Avian Hepatitis E virus (AY535004) was chosen as an outgroup. The sequence reported here has been deposited with GenBank accession no.: EF570133.

Results
3'RACE
As shown in Figure 1, 3’RACE band of the expected size was obtained. The 3’ terminus of this study had 93 nucleotides upstream of the polyA. The sequence of 3’UTR was: TTT ATT CTT CTT GTA CCT CCC CTT CGG TTC TGT TTC TTT TTA TTT CTC CTT TCT GCG TTC CGC GCT CAC TAC TAT CCA GCA GGA TCC ATG TTG. Comparison of the 3’UTR sequence with the corresponding regions of other 38 HEV strains from different region of the world revealed that the Shanghai swine isolate is 21–49 bp longer than all the other stains (additional file).

Analysis of Full-Length Genome of Shanghai Isolate
The genomic length of the SH-SW-zs1 isolate was determined to be 7265 nucleotides (nt) excluding poly (A) tail at 3’ terminus and contained three open reading frames (ORFs) similar to earlier reported human and swine HEV isolates. The genomic organization consisted of 5’ untranslated region (5’UTR) of 25 nt (1–25), ORF-1 of 5127 nt (26–5152), ORF-2 of 1983 nt (5190–7172), ORF-3 of 372 nt (5249–5520) and 3’UTR of 93 nt (7173–7265), followed by a poly (A) tail of 26 residues. The
length of 5'UTR was same as that of other type 4 isolates and had nucleotide G at the extreme 5' end of the genome as other reported genotype 4 sequences. Whole genome-based phylogenetic analysis confirmed classification of Shanghai swine in genotype 4 (Fig. 2). The phylogenetic tree showed that genotype 4 could be divided into 3 main subgroups. SH-SW-zs1 isolate closely clustered with isolate DQ450072 which was isolated from eastern China, and they shared 89.3% identity (with divergence of 11.3%) with each other and represented a distinct subgroup among the genotype 4 isolates with a bootstrap value of 100%.

**Discussion**

HEV is the major cause of enterically transmitted non-A, non-B, non-C hepatitis and is responsible for significant morbidity and mortality in developing countries [16]. Outbreaks of hepatitis E have been described in Asia, Africa and Mexico [16-18], while sporadic cases have been reported in the United States, Japan and other developed countries [8]. It has been shown that HEV is a zoonotic virus [19,20]. Hitherto, the lack of an efficient cell-culture system for HEV has greatly hampered detailed analysis of the virus replication cycle in infected cells, which makes it difficult to resolve many important questions. Meanwhile, cloning full-length genome of HEV is an efficient way to analysis molecular character, viral replication and other problems. Some reports indicated that genotype 4 and genotype 1 were the major genotype in China, though genotype 3 HEV was recently found in swine of Shanghai suburb [13]. Recent observations suggested that the HEV genotype influences the severity of hepatitis E, and that genotype 4 is associated more strongly with the severe form of hepatitis E than genotype 3 [21]. Therefore, the genomic full-length of the Shanghai isolate was determined in this study for further demonstrating the HEV strain prevalent in eastern China. The full genome of the SH-SW-zs1 isolate was 7265 nucleotides in length and phylogenetic analysis indicated that this isolate belonged to genotype 4. This isolate closely clustered with isolate DQ450072 and they shared 89.3% identity (with divergence of 11.3%) with each other and represented a distinct subgroup among the genotype 4 isolates with a bootstrap value of 100%, thus suggested that they may come from one common strain. Result of comparison showed that the 3'UTR of this Shanghai isolate was 21–49 bp longer than all the other stains so far available on-line. By blast the 21-nt-fragment in GenBank, we found it has many homologous sequences which shared more than 85% identity with it. So we presumed that this fragment may come from the recombination of genome HEV and its host or other microorganism. The true origin of this short fragment and its specific function need to be further studied.
Figure 2
Phylogenetic trees constructed using MEGA software depicting genotypic status of SH-SW-zs1 on the basis of full-length genome sequence of 39 HEV isolates. Genbank accession numbers for the full genome were marked at each branch. Percent bootstrap support is indicated at each node. The abbreviations Ch and Ja stand for China and Japan, respectively.
Additional material

Additional file 1
Comparison of length in the 5'UTR of different HEV strains. The numbers in the brackets show the genotype designation.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1743-422X-4-98-S1.tif]

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