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Full-length sequence analysis of hepatitis E virus isolates: showing potential determinants of virus genotype and identity

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Abstract The complete genome sequence of a genotype 4 strain of hepatitis E virus (CH-YT-HEV02) from a patient (in Yantai, China) has been determined. Phylogenetic analysis showed that CH-YT-HEV02 belongs to genotype 4, subtype 4a. However, the phylogenetic analysis indicated that it was most closely related to JKO-CHiSai98C (AB197673) strain, sharing only 91.6 % sequence identity with it. Judging from the phylogenetic tree based on the full-length nucleotide sequences of all 70 genotype 4 HEV isolates retrieved from GenBank up to May, 2013, the CH-YT-HEV02 isolates could serve as a Yantai-indigenous strain. A broader comparison with other genotype isolates revealed that there are a few conserved amino acids in the HVR region of different HEV genotypes, and two amino acid motifs in ORF2 and ORF3 might serve as signatures of genotype diversity of HEV.

Keywords Hepatitis E virus · Genotype diversity · Conserved amino acid · HVR · Signatures of genotype diversity

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Introduction

Hepatitis E is an important public health disease in many developing countries of Asia and Africa [1]. Sporadic cases of hepatitis E have also been reported in many industrialized countries [2, 3]. Hepatitis E virus (HEV), the causative agent of hepatitis E, is a non-enveloped virus with a single-stranded, positive-sense RNA virus genome of ~7.2 kb and is currently classified as the sole member of the genus *Hepevirus,* family Hepeviridae [4, 5]. The genome of HEV contains a short 5′ noncoding region (5′ NCR) followed by three open reading frames (ORFs) and a 3′ NCR: ORF2 overlaps ORF3, but neither ORF2 nor ORF3 overlaps ORF1 [6, 7]. The putative functional domains in the ORF1 protein include methyltransferase, protease, helicase, and RNA-dependent RNA polymerase (RdRp) domains [5, 8]. ORF2 encodes the virial capsid protein, whereas ORF3 codes for a small, multifunctional, cytoskeleton-associated phosphoprotein that is involved in many aspects of viral replication and pathogenesis [9–11].

Although all mammalian HEV isolates are believed to belong to a single serotype phylogenetic, analysis of HEV sequence has led to the recognition of at least four major genotypes and several subtypes within each genotype: genotypes 1 and 2 exclusively infect human, and they are often associated with outbreaks or large epidemics in developing countries. HEV genotypes 3 and 4 are zoonotic, and they are often associated with sporadic hepatitis E [7, 12]. This poses it the interesting question as why genotypes 3 and 4 were able to cross species barriers, whereas genotypes 1 and 2 strains were not. In addition, increasing reports have shown that different HEV genotypes are associated with differing epidemics range [1].

HEV 1, 3, and 4 are all prevalent in China, but genotype 4 HEV has been found to be the dominant cause of

hepatitis E in China since 2000 [3, 13–17]. Although molecular and seroepidemiological investigations of HEV have been performed in many provinces in China, there is shortage of epidemiological data from Shandong province, and the genotypes have not been characterized previously. Therefore, we have now determined the full genome sequence of Yantai endemic strain, CH-YT-HEV02. During the past 20 years, a lot of researches on swine and human HEV molecular epidemiology and phylogeny have been performed in the world. Whereas, no summarized determinates of virus genotype has been reported. Therefore, in order to identify specific nucleotide/amino acids influencing the verity of HEV genotype, full-length genomic HEV sequence were retrieved from GenBank up to May, 2013 for an extensive comparison, including the CH-YT-HEV02 strain.

Materials and methods

Fecal specimen, patient information, and extraction of RNA

A fecal specimen containing a human HEV isolate (CH-YT-HEV02) was collected from a 34-year-old male patient (a taxi driver) who was sent to hospital following 2 weeks of asthenia, anorexia, and progressive jaundice (15 Mar 2012). He had no recent history of traveling abroad. The diagnostic criteria of hepatitis E was as described elsewhere [13]. Routine biochemical test revealed evidence of acute hepatitis, with total bilirubin (TBiL) of 149 μmol/L, direct bilirubin (DBiL) 100 μmol/L, alanine transaminase (ALT) 114 U/L, and aspartate transaminase (AST) 436 U/L. The serum sample was detected for anti-HEV IgM, anti-HEV IgG, anti-HAV IgM, HBsAg, anti-HCV IgG using commercial enzyme immunoassay (EIA) kits (Beijing Wantai Biological Pharmacy Enterprise Co., Beijing, China). All assay procedures were carried out according to the manufacturer's instructions. Hepatitis A, hepatitis B, and hepatitis C viruses were all negative, whereas anti-HEV IgM and IgG were both positive.

Fecal sample collected from the patient was diluted to make a 10 % fecal suspension (1 g faces suspended in 10 ml phosphate-buffered saline, pH7.4) and the suspension was mixed thoroughly and clarified by centrifuging ant 8,000 rpm at 4 °C for 20 min. The supernatant was stored at −80 °C for RNA extraction. Total RNA was extracted from 100 μl of fecal supernatant using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Amplify the entire genome of CH-YT-HEV02 strain

All the non-terminal reverse transcripts (RTs) were synthesized with the SuperScriptTM III First-strand synthesis system (Thermo, USA) using the specific external antisense primer and the first-strand cDNA were used immediately for PCR.

Nested polymerase chain reactions (n-PCR) using ten sets of specific external and internal primer pairs (Table 1) was performed to amplify the entire viral genome. The 5′ and 3′ ends of the genome were determined using a rapid amplification of cDNA ends (RACE) kit (TaKaRa, Dalian, China) according to manufacturer's instructions.

Sequencing and sequence analysis

PCR products were purified and ligated into pGEM-T Easy vector (Promega) and amplified in *Escherichia coli* DH5a. At least three positive clones were selected at random and sequenced (Invitrogen Beijing Co., Ltd) in both directions using an ABI model 3,730 automatic DNA sequencer (ABI, CA, USA). Nucleotide sequences were assembled and analyzed using the MEGA 4.0 software package (version 4.0, http://www.megasoftware.net, Tempe, AZ, USA) and ALIGNX software (vector NTI package version 11.0, MD, USA). Phylogenetic trees were constructed by the neighbor-joining method with the aid of the MEGA 4.0 software package. Genetic distances were calculated using the Kimura two-parameter method. One thousand re-samplings of the data were used to calculate the percentages of bootstrap support for the branches obtained.

Results

Genome organization of HEV isolate CH-YT-HEV02

The full-length genome of HEV isolate CH-YT-HEV02 was 7,238 nucleotides (nt) long, excluding the 3′poly (A) tail. The genome contained a 5′UTR of 25 nt covering nucleotides 1–25, and it shared both length and complete nucleotide sequence identity with other genotype 4 HEV isolates. ORF1 extended from nucleotides 26 to 5,143 (5,118 nt), ORF2 started from nucleotides 5,143 to 7,164 (2,022 nt), and ORF3 partially overlapped ORF2 in a second reading frame and extended from nucleotides 5,171to 5,512 (342 nt). The 3′UTR comprised 74 nt from nucleotides 7,165 to 7,238 and was followed by a poly (A) tail of 12 A residues. An additional G residue preceding the poly (A) tail was found, which was again consistent with what has been observed in other genotype 4

Table 1 Sequence of primers for RT-nPCR and RACE

Set	Primer	Primer sequence (5′–3′)	Product size (bp)
1	15–1316F	TATGTGGTCGACGCCATGGAGG	1,255
	15–1316R	CGGCGGCACTGRGCRTAAAAC	
	23–1278F	GCCATGGAGGCCCAYCAGTT	
	23–1278R	GATRTARTCACGCCCRGACTT	
2	956–2566F	TCAYGCCGTCCCTGTGCATA	1,488
	956–2566R	CCCGTCGGACATGATAAACT	
	984–2472F	GGGAYAGGCTTATGCTYTTTGG	
	984–2472R	TTRGAGGCATTRACCAGCCA	
3	2009–3982F	GCGGCATTCYCTTATTGGAG	1,806
	2009–3982R	GCCRACAAGAGTYGACAGGA	
	2127–3933F	CTTTAYACTCGCACYTGGTC	
	2127–3933R	CGRCAGTGCACWATGTCCGT	
4	3724–4691F	CTCGTGGTACTATAAGCAGCAAC	900
	3724–4691R	ATGCCGCAGCTTTAAGGTCA	
	3751–4651F	ATACGCTTGATGCATTCCC	
	3751–4651R	GCTATAACCGCCATGTTCCAGA	
5	4494–5339F	AGTGTATAATCATGGAGGAGTGT	625
	4494–5339R	GGTTGGATGAATATAGGGGA	
	4605–5230F	GGAAGAARCACTCTGGTGAGC	
	4605–5230R	CGGTGGCGCGGGCARCATAGG	
6	4975–5434F	GAATGTGGCYCAGGTTTGTGT	317
	4975–5434R	GGACTGGTCRCGCCAAGCGGA	
	5039–5363F	CATAACCTGATYGGRATGCT	
	5039–5363R	GCTGGAATGTCAGATGCGAAGG	
7	5229–5963F	GCCTATGYTGCCCGCGCCACC	585
	5229–5963R	CAGCGACRCCAGAGGTCTCAA	
	5339–5924F	TCCCCTATATTCATCCAACC	
	5339–5924R	ATTKCGATARTGCAGGCGCTCAC	
8	5689–7046F	ACYAATACYCACATYATGGCT	1,272
	5689–7046R	CAGGGCAGAARTCATCAAAAG	
	5714–6978F	AAGCWTCYAATTATGCCCAGT	
	5714–6978R	GCAGARTGAGGGGCRAGGACA	
5′RACE	480–R	CGGCAAAAGTGCAGCCAGA	454
	454–R	CCGTCAAAGCAGTATGTACGAT	
3′RACE	6773–F	AGTATTCTAAGACYTTCTATG	367
	6874–F	GCTAGYGAYCAGATCCTGATT	

HEV isolates. The full-length sequence of the CH-YT-HEV02 isolate obtained in this study has been deposited in the GenBank database under accession number KC492825. The HEV full-length sequences used during the phylogenetic analysis and nucleotides/amino acids comparison.

Phylogenetic analysis

A phylogenetic tree was constructed based on the entire genomic sequence of the CH-YT-HEV02 isolate and 28 prototype HEV isolates, using an avian HEV as an outgroup (Fig. 1). Sequence analysis showed that the CH-YT-HEV02 isolate shared 74.4–74.9, 73.2, 74.4–74.7, and 82.3–91.6 % identity with prototype isolates from genotypes 1, 2, 3, and 4, respectively, and from this appeared to belong to genotype 4. A bigger phylogenetic tree depicting sub-genotypic status of all 70 genotype 4 HEV isolates based on full-length sequence was built (Fig. 2). According to the phylogenetic tree, the bootstrap values between KC492825 and the neighboring isolates is only 58, so the CH-YT-HEV02 could serve as a Yantai-indigenous strain.

Analysis of ORFs

ORF1 of CH-YT-HEV02 consisted of 5,118 nucleotides capable of encoding a protein of 1,706 amino acids. The

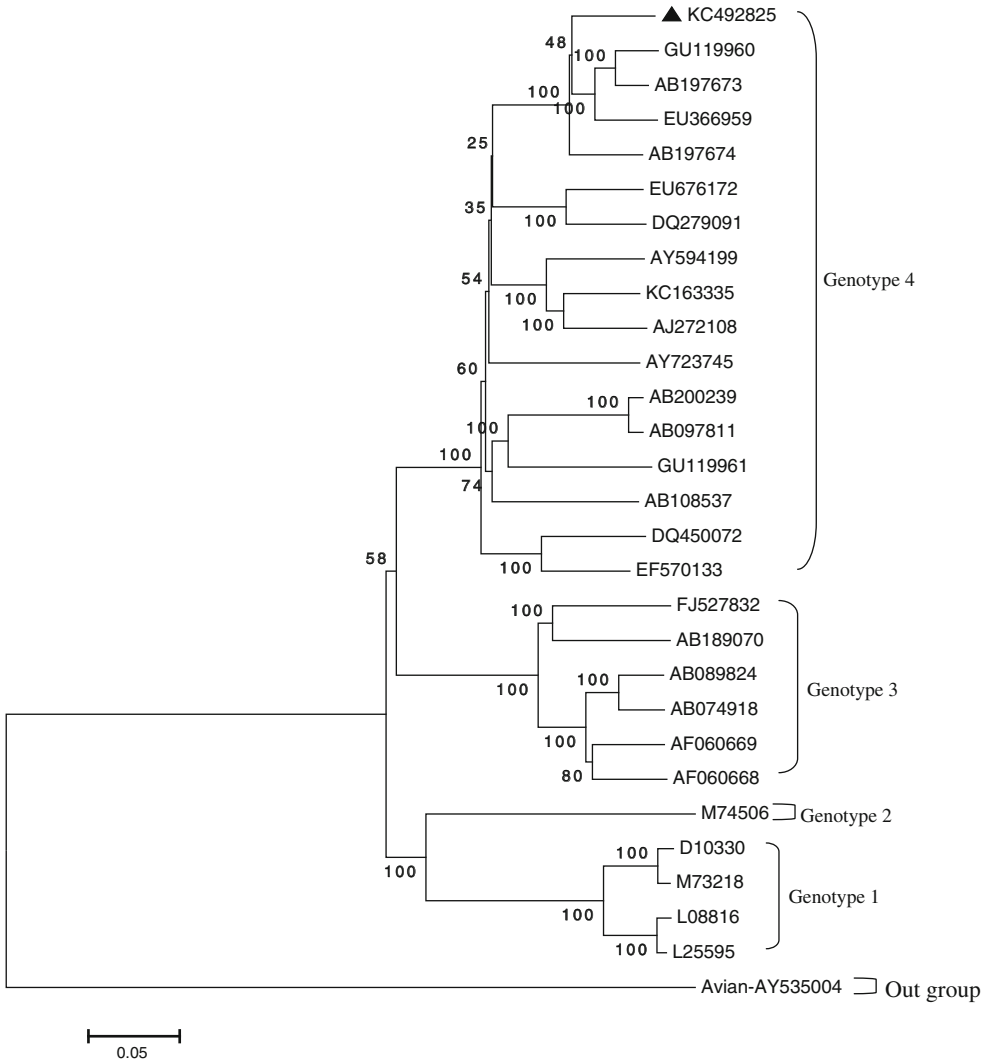

Fig. 1 Phylogenetic tree based on the full-length nucleotide sequences, using the neighbor-joining method. An avian HEV strain (AY535004) is included as an out group. Genetic distances were calculated using the Kimura two-parameter method. The *internal node numbers* indicate the bootstrap values as a percentage of trees obtained from 1,000 replicates. The *Arabic numbers* and the *Roman letters* outside of the *square bars* indicate potential genotypic and sub-genotypic designations

nucleotide and amino acid sequence identities in the CH-YT-HEV02 isolate compared with other genotype 4 isolates were 82.2–91.1 and 93.7–96.7 %, respectively. In agreement with the full-length sequence analysis, CH-YT-HEV02 showed the highest sequence identity to the AB197673 isolate at both the nucleotide (91.1 %) and amino acid (96.7 %) level. When compared with other HEV genotype 4 isolates, eleven unique amino acid substitutions (D728, V729, L744, S753, H754, A1004 V, C1125R, A1220S, A1268 V, A1565 V, M1641A/V) were found in ORF1 of the CH-YT-HEV02 isolate.

Amino acid deletions or insertions occurred mainly within the hypervariable region (HVR, 711–798 aa) which has been found previously to show variations in size [15]. The CH-YT-HEV02 isolate showed 47.1–74.1 % amino acid sequence identity within the hypervariable region (HVR) compared with other genotype 4 isolates, and 1 amino acid (residue 795) deletions was found in this region. Furthermore, the special amino acid substitutions in the HVR region of different HEV genotypes were shown in Table 2. The intergenotypic amino acid sequence identity in the HVR among HEV isolates in different genotypes differed by as much as 71 % [18], whereas the intragenotypic spcecific amino acids among isolates within the same genotype differed by 26 amino acids among genotype 1 isolates, 10 amino acids among genotype 3 isolates, 10 amino acids among genotype 4 isolates. The conserved amino acids of the HVR in genotype 2 is unknown, since only one strain of genotype 2 HEV has been sequenced to date. These conseved amino acids may be proved to be useful diagnostic indicator of genotype and species of origin. In addition, the results showed that seven amino acids 708S, 709G, 711S, 712S, 714F, 716P, and 792P were common to all strains of zoonotic origin (genotypes 3 and 4), while two amino acids 720G, 759P were corporate in all strains only isolated from humans (genotypes 1 and 2). In addition, the comparison results suggested that the HVR involved in variety of HEV isolates (data not shown).

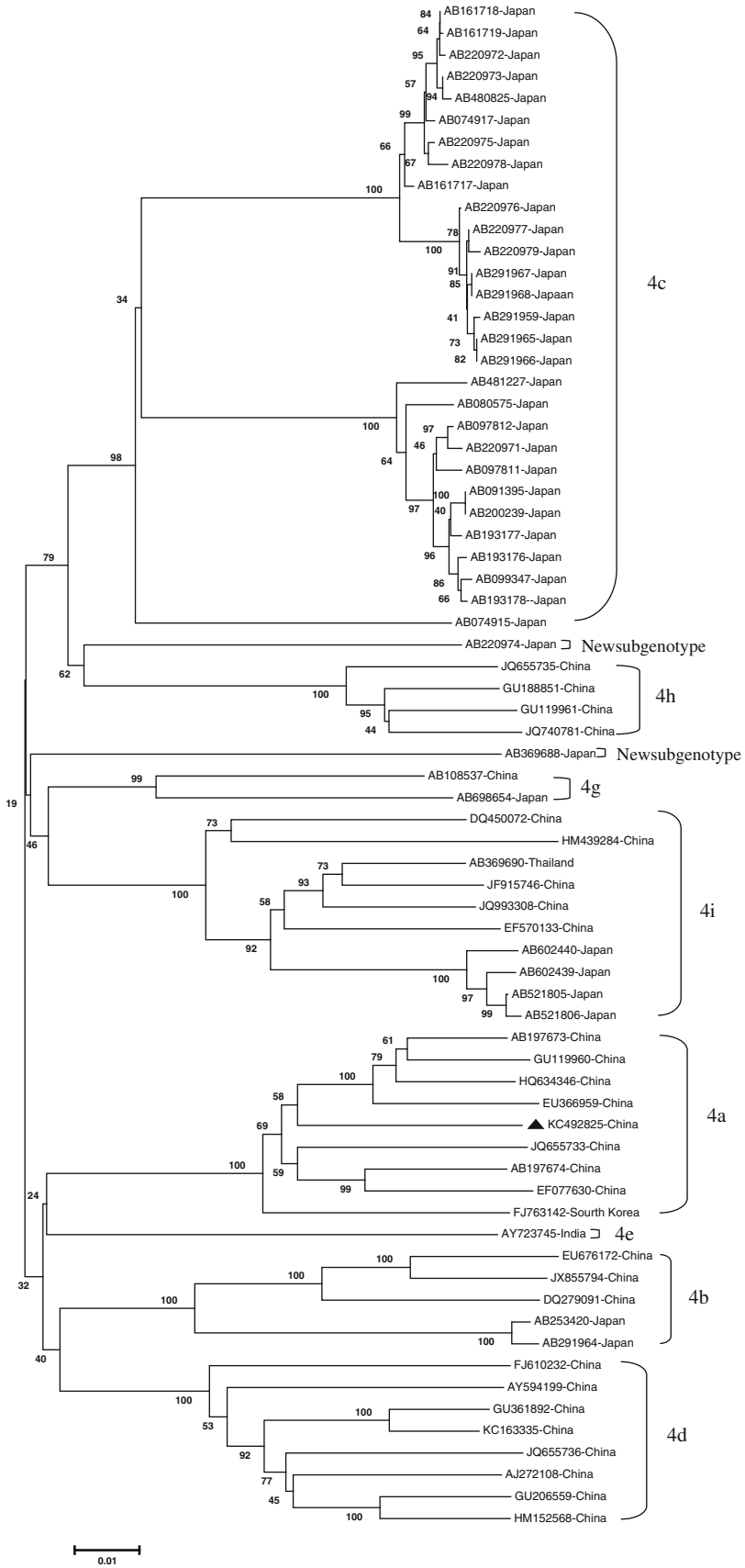

Fig. 2 Phylogenetic tree depicting sub-genotypic status of all 70 genotype 4 HEV isolates based on full-length sequence, using the neighbor-joining method. Genetic distances were calculated using the Kimura two-parameter method. The *internal node numbers* indicate the bootstrap values as a percentage of trees obtained from 1,000 replicates. The *Arabic numbers* and the *Roman letters* outside of the *square bars* indicate potential sub-genotypic designations

Table 2 Conserved amino acids in the HVR region of different HEV genotypes

Genotype	Amino acids and sites
Genotype 1	707E, 709D, 710A, 714P, 717–720(PDLG), 723–725(SEP), 729S, 731A, 737A, 741P, 753A, 757–760(EPA), 769–771(AIT), 773Q, 776R, 777H
Genotype 2	720G, 759P
Genotype 3	708–712(SGFSS), 714F, 716P, 717P, 736P, 792R
Genotype 4	708S, 709G, 711–716(SSCFSP), 778P, 792R

The amino acid positions are in accordance with M73218 (genotype 1), M74506 (genotype 2), AF060669 (genotype 3), KC492825 (genotype 4)

The ORF2 of CH-YT-HEV02 was 2,022 nt long and potentially encoded a structural protein of 674 amino acids. The ORF2 of this strain did not overlap with ORF1, which was defined here according to Graff et al. [19] and Huang et al. [20]. The termination codon of ORF2 was TGA, which was consistent with all genotype 4 isolates. The CH-YT-HEV02 ORF2 had nucleotide and amino acid sequence identity of 86.3–92 and 96.7–98.3 % to other genotype 4 isolates, respectively. It showed the highest degree of identity to the AB197673 isolate, both at the nucleotide (92 %) and amino acid (98.3 %) level. Comparison of amino acid identities of the CH-YT-HEV02 isolate with other HEV isolates showed that the short region at 80–83 aa (amino acid) within ORF2 may prove to be a useful diagnostic indicator of genotype of an isolate (Table 3), and no amino acid substitutions was found within ORF2 of CH-YT-HEV02.

The ORF3 of CH-YT-HEV02 was 342 nt in length, with a coding capacity of 114 amino acids, identical in size to that of most genotype 4 HEVs. One unique amino acid (L74R/Q/P) substitutions was observed in this region, with CH-YT-HEV02 sharing 91.9–95.4 % nucleotide and 85.4–94.7 % amino acid sequence identity with other genotype 4 isolates over this ORF. Interestingly, the highest sequence identity (95.4 % nucleotide, 94.7 % amino acid) was again seen with AB197673. Comparison of amino acid identities of the CH-YT-HEV02 isolate with all other HEV isolates showed that the short region at 66–73 aa within ORF3 may also prove to be a useful diagnostic indicator of genotype of an isolate. The motif is SPPMSPLR in genotype 1, LPQTLPLR in genotype 2, (H/R)(N/S) in genotype 3, T(Y/F)Q in genotype 4 (Table 3).

Discussion

The number of published HEV sequences has increased significantly during the last years which also increase the number of potentially new genetic groups [21–23]. Even the most widely accepted subtype classification system has led to ambiguous results, when new isolates were assigned to particular virus subtypes. Oliveira-Filho et al. [23] have performed phylogenetic analyses based on complete HEV genomic and whole capsid gene sequences. The results modified the current taxonomy of genotype 3 and refined the established system for typing HEV, challenging the current system proposed by Lu et al. [24]. Smith et al. [25] have undertaken a reanalysis of HEV phylogenetic relationships using a variety of methods, and the results indicated that HEV variants most closely related to those infecting humans can be consistently divided into six genotypes. Although few differences were observed with regard their results, the approaches may form the basis for a future genetic classification of HEV and are important to understand the epidemiology of hepatitis E [23, 25]. In China, genotype 4 is responsible for the majority of sporadic cases of hepatitis E in humans. There are at least six subtypes of genotype 4 HEV isolates identified so far [3, 13–15, 17, 26, 27]. In this study, phylogenetic trees were built and nucleotide similarity analysis was performed based on full-length sequences. Although CH-YT-HEV02 was classified into genotype 4, the phylogenetic analysis indicated that it was most closely related to the JKO-CHiSai98C (AB197673) strain, sharing only 91.6 % sequence identity with it, which was determined from a Japanese patient who had travelled to Shanghai. According to the bigger phylogenetic tree, the CH-YT-HEV02 isolates could serve as a Yantai-indigenous strain.

Table 3 Specific amino acids in the ORF2 and ORF3 region among different HEV genotypes

Genotype (total amount)	Amino acids in the ORF2 (amount)	Amino acids in the ORF3 (amount)	Host (amount)
HEV-1 (33)	VTAA (33)	SPPMSPLR (33)	Human (33)
HEV-2 (1)	VAAA (1)	LPQTLPLR (1)	Human (1)
HEV-3 (105)	VVSQ (86), VVPQ (5), VLSQ (5), IVSQ (3), VASQ (2); VISQ (2), VFSQ (1), AVSQ (1)	HN (100), HS (3), RN (2)	Human (47), Animal (58)
HEV-4 (70)	IPAA (40), IPTA (24), ISTA (4); ISAA (1), ISTT (1)	TFQ (50), TYQ (16), TPQ (4)	Human (48), Animal (22)

The amino acid positions are in accordance with CH-YT-HEV02 (KC492825). The amino acids sites are 80–83 aa in the ORF2 and the sites in ORF3 are: genotypes 1 and 2 (66–73 aa), genotypes 3 (72–73 aa) and 4 (71–73 aa)

In this study, eleven specific amino acid substitutions were found in ORF1 of CH-YT-HEV02. Five of these substitutions (D728, V729, L744, S753, H754), were in located in HVR, and 1 amino acid (residue 795) deletion was found in this region. Among the substitutions in ORF1 two (A1004 V, C1125R) were in the helicase domain, close to the putative signature U3148 locus reported in earlier studied [28]. It was surprising to find that four (A1220S, A1268 V, A1565 V, M1641A/V) of the substitutions in ORF1 were located within the region identified carrying an RNA-dependent RNA polymerase (RdRp) motif. Bu et al. have analyzed complete genome sequence of the HEV, isolated from a patient with liver failure. The authors suggested a possible association of specific amino acid substitutions with viral pathogenesis [29]. Therefore, further studies will be needed to confirm the relationship between these variants and functional changes. In addition, monitoring of the viral evolution and further study of disease pathogenesis will improve disease treatment and epidemic control.

Sequence analysis of known HEV strains revealed an HVR with a high degree of variability at both amino acid and nucleotide sequence levels [18]. Fu et al. have compared the CHN-XJ-SW13 isolate with 90 HEV strains covering genotype 1–4 retrieved from GenBank, and the results showed that the one short region at the 5′ end of the HVR region may prove to be a useful indicator of species of origin of an isolate [15]. However, when we analyzed all of the isolates used in our study, amino acid substitutions in the motif led to inconsistencies of their results (data not shown). In our study, comparison of amino acid within the HVR of different HEV genotype isolates showed that the variety of HEV strain involved in different amino acid sequence of HVR, so the HVR region can be served as the identity of a HEV strain. However, there were some special amino acid substitutions in the HVR region of different HEV genotypes (Table 2). These conseved amino acids may proved to be useful diagnostic indicator of genotype and species of origin, and the results showed that seven amino acids were common to all strains of zoonotic origin (genotypes 3 and 4), while two amino acids were corporate in all strains only isolated from humans (genotypes 1 and 2). Although variety of amino acid deletions and protein insertions were found within the HVR of many HEV isolates and did not influence the viability of the virus, the insertions only reported in few isolates of genotype 3 [30–33]. The previous study also showed that the HVR may interact with viral and host factors to modulate the efficiency of HEV replication, and there exists a degree of genotype specificity with respect to the efficiency of virus replication and infectivity [34]. Moreover, the HVR may play a biological role in HEV pathogenesis [18]. In addition, the deletions and the insertion in the HVR region may involve in genotypes and hosts. These results could shed light on that genotypes 1 and 2 strains are restricted to humans, whereas genotypes 3 and 4 are zoonotic.

Besides mutation pressure, natural selection, for instance involving fine-tuning translation kinetics and escape from the host immune system, may also play a role in shaping the HEV genome, particularly in the ORF1 hypervariable region and the ORF2/3 overlapping region [35]. ORF2 of HEV encodes its capsid protein which assembles homodimers to form the capsid shell. Comparison with genotypes 4 (excluding few novel genotype 4 isolates), the ORF2 of genotypes 1, 2, and 3 were 14 amino acids shorter, and the C-terminal amino acid residues are various: l (genotype 1), S (genotypes 2 and 3), Y (genotype 4). Our data comparing all HEV genotypes identified a 4 amino acids (80–83 aa) motif within ORF2, which may prove to be a useful diagnostic indicator of genotype of an isolate. ORF3 of HEV overlapped ORF2 in a second frame, encodes a small protein (ORF3) of 123 or 114 aa, which plays considered role in HEV pathogenesis through promotion of cell survival and downregulation of the innate host responses, and is essential for virion release from HEV infected cells. Interestingly, comparison of amino acid sequences of ORF3 of all HEV genotype isolates showed that the short region 66–73 aa region within ORF3 may also prove to be a useful diagnostic indicator of genotype of an isolate. The two amino acid motifs in ORF2 and ORF3 might serve as signatures of genotype diversity of HEV.

In our study the power of the observations was confirmed by the other 10 positive fecal samples. However, all the 10 isolates divided into subtype 4. Further studies using other genotypes, as well as new isolates, should be carried out in order to confirm the applicability of our results. In addition, we should pay attention to the motifs in genotype 2 in further studies, since only one complete sequence is available for genotype 2 [23].

In conclusion, the HRV region and the two amino acid motifs identified in our study may facilitate to understand the identity, classification, and epidemiology of HEV isolates. In addition, this results may form the basis for new methods for detection the genotype of a HEV isolate.

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Conflict of interest The authors declare no competing financial interests.

Ethical standards The experimental protocols were approved by the Animal Care and Protection Committee of Institute of Health and Environmental Medicine.

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