

## Seroprevalence and evolutionary dynamics of genotype 4 hepatitis E virus in Shandong Province, China

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### Abstract

**AIM:** To investigate the seroprevalence and evolutionary dynamics of hepatitis E virus (HEV) and assess the ancestor of HEVs in China's Shandong Province.

**METHODS:** A total of 2028 serum, 60 fecal and 82 bile samples were collected from the general human population, patients and swine, respectively. This seroepidemiological study was conducted using an immunosorbent assay and HEV RNA was detected by the reverse transcription-nested polymerase chain reaction (RT-nPCR) method. Complete genome sequences of the

prevalent strains (CH-YT-HEV01, CH-YT-HEV02 and CH-YT-sHEV01) were determined, and the sequences were analyzed phylogenetically. In addition, the evolutionary dynamics of three HEV isolates were determined using the framework of coalescent analysis in the program package BEAST, and the time of the most recent common ancestors (TMRCA) of China-indigenous genotype 4 HEV isolates was calculated.

**RESULTS:** The overall viral burden in the general human population was 0.1%, and the positive rates of anti-HEV IgG and IgM in the serum specimens were 25.1% (509/2028) and 2.3% (51/2028), respectively. In addition, IgG positivity increased with age. The phylogenetic analysis based on the full-length nucleotide sequences showed that the strain CH-YT-HEV02 was directly related to CH-YT-sHEV01 with a 94% identity, suggesting that they were involved in cross-species transmission. The isolate CH-YT-HEV01 was close to HB-3 and CHN-SD-sHEV with a bootstrap value of 100%, sharing a 96.1%-96.4% identity with each other. Surprisingly, the HB-3 strain was a representative strain prevalent in swine in Hubei, and the isolate CHN-SD-sHEV was obtained from swine in Shandong in a previous report. TMRCA for the clade of CH-YT-HEV01 and HB-3 was 2003, which was consistent with the TMRCA for the clade of CHN-SD-sHEV and HB-3, and they were both earlier than the TMRCA for the clade of CH-YT-HEV01 and CHN-SD-sHEV (2004).

**CONCLUSION:** The strains CH-YT-HEV01, CHN-SD-sHEV and HB-3 are involved in trans-regional transmission, and the ancestors of HEVs in Shandong come from Hubei Province.

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**Key words:** Hepatitis E virus; Zoonotic; Cross-species transmission; Trans-regional transmission; Evolutionary dynamics

**Core tip:** This is the first study to investigate the genetic history of hepatitis E virus (HEV) based on the complete genome sequences in China. The results suggested that the strains CH-YT-HEV02 and CH-YT-sHEV01 were involved in cross-species transmission between swine and humans in Shandong Province. The strains CH-YT-HEV01, CHN-SD-sHEV and HB-3 were involved in trans-regional transmission between Hebei and Shandong, and the coalescent analysis suggested that the ancestors of HEVs in Shandong come from Hebei Province.

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## INTRODUCTION

Hepatitis E virus (HEV), the causative agent of hepatitis E, is an important public health concern in many parts of the world. Outbreaks resulting from fecal contamination of drinking water have been reported in developing countries, and industrialized countries were previously thought to be free of HEV. However, hepatitis E has also become an emerging problem in developed areas, with a number of sporadic cases<sup>[1,2]</sup>. HEV is non-enveloped with a single-stranded positive RNA genome of 7.2 kb, which is capped at the 5' termini and polyadenylated at the 3' termini, and it is the sole member of the *Hepenviridae* family and the *Hepivirus* genus<sup>[3]</sup>. Phylogenetic analysis of various mammalian HEV isolates showed that HEV has at least four genotypes, representing a single serotype<sup>[4]</sup>. Genotypes 1 and 2 have been identified exclusively in humans, while genotypes 3 and 4 have been found in humans and animals<sup>[5]</sup>. HEV genotypes 3 and 4 are recognized as an emerging pathogen in industrialized countries, and can cause chronic hepatitis in immunocompromised individuals, leading to rapid fibrosis of the liver<sup>[6]</sup>. The HEV genome contains three open reading frames (ORF). ORF1 encodes a nonstructural polyprotein with six conserved domains and one hypervariable region<sup>[7]</sup>. ORF2 encodes the capsid protein, and ORF3 encodes a phosphoprotein necessary for infection *in vivo*<sup>[8]</sup>.

The host range of HEV is ever-expanding. In addition to humans, animal species from which HEV isolates have been discovered include domestic and wild pigs, chickens, deer, ruminants, rats, rabbits and many other animal species which were seropositive for HEV antibodies<sup>[1,2,9]</sup>. Experimental infections have confirmed the cross-species transmission of swine strains to humans and of human strains to non-human primates<sup>[10,11]</sup>. Accumulating evidence indicated that genotypes 3 and 4 were

involved in cross-species transmission in some cases<sup>[12]</sup>. Hepatitis E is now recognized as a zoonotic disease, and pigs are more likely to be reservoirs than other animal species<sup>[13,14]</sup>. However, a few reports have shown close phylogenetic relationships between sequences identified in swine and in humans<sup>[15]</sup>.

Although molecular and seroepidemiological investigations of HEV have been performed in many provinces in China<sup>[16-20]</sup>, few studies have shown the relationship between a strain in swine and an isolate from the human population in the same area based on complete genome sequences analysis<sup>[21]</sup>. The aim of the present study was to investigate the molecular epidemiology and genetic history of HEV infection in swine and humans in Shandong Province, China.

## MATERIALS AND METHODS

### Sample collection

We conducted a cross-sectional seroepidemiologic study of HEV infection in 2011. A total of 2028 serum specimens were collected from the general population (947 males and 1054 females, age range 2-75 years) in rural communities in Shandong Province, China. Anti-HEV IgG and IgM levels were determined using commercial ELISA kits (Beijing Wantai Biological Pharmacy Enterprise Co., Beijing, China). In addition, 60 fecal samples were collected from hospitalized patients with hepatitis E from August 2011 to July 2012 in Shandong. The patients had autochthonous HEV infection and had no travel history outside Shandong. The diagnostic criteria for hepatitis E were as follows: an elevation of alanine aminotransferase (ALT) level (> 2.5 ULN); positive result for anti-HEV IgM or at least a 4-fold increase in IgG levels during hospitalization<sup>[22]</sup>. The experimental protocols were approved by the Animal Care and Protection Committee of the Institute of Health and Environmental Medicine. All participants gave written consent after receiving a full explanation of the study. Eighty-two swine bile samples were also obtained from pigs aged 26 to 28 wk in 2011. The swine bile samples were collected from five farms in Shandong. The patient fecal and swine bile samples were obtained from Yantai, Penglai, Haiyang and Laiyang in Shandong. Penglai lies approximately 65 km from Yantai, Haiyang lies approximately 99 km from Yantai and Laiyang lies approximately 106 km from Yantai.

### Detection of HEV RNA and phylogenetic analysis

Fecal samples collected from patients were diluted to obtain a 10% fecal suspension (1 g feces suspended in 10 mL phosphate-buffered saline, pH 7.4), and the suspension was mixed thoroughly and clarified by centrifuging at 10000 × g for 20 min at 4 °C. The supernatant was stored at -80 °C for RNA extraction. Total RNA was extracted from 100 µL of patient fecal supernatant or swine bile using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruc-

tions. cDNA was synthesized from 4 µL RNA with a SuperScript™ III First-strand Synthesis System for the RT-PCR kit (Thermo, United States) according to the manufacturer's instructions, and the first-strand cDNA was used immediately for PCR. HEV RNA was amplified using nested reverse transcription PCR for the ORF2 gene as described<sup>[23,24]</sup>, and the PCR products were sequenced on amplified strands in both directions using an ABI model 3730 automatic DNA sequencer (ABI, CA, United States). Nucleotide sequences were assembled and analyzed using the MEGA 5.0 software package (version 5.0, <http://www.megasoftware.net>, Tempe, AZ, United States). A phylogenetic tree was constructed by the neighbor-joining method with the aid of the MEGA 5.0 software package. The phylogenetic tree was produced using the neighbor-joining method with a bootstrap of 1000 replicates.

### Amplification of entire genome and full-length genome sequences analysis

Three prevalent strains were designated to determine the complete genome sequences. The first-strand cDNA was synthesized with the same kit used for HEV RNA detection. Nested polymerase chain reactions (n-PCR) using specific external and internal primer pairs were 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. PCR products were sequenced as mentioned above. The complete genome sequences and the fragments were assembled using the MEGA 5.0 software. Furthermore, nucleotide sequences and amino acid sequences of ORF1-3 were analyzed and compared with the full-length genome HEV sequences which were retrieved from GenBank up to September 2013 using the MEGA 5.0 software package and ALIGNX software (vector NTI package version 11.0, MD, United States). The phylogenetic tree was produced using the same techniques as described above.

### Coalescent analyses of the three HEV isolates in this study

The genetic history of the three HEV isolates in this study was determined using the framework of coalescent analysis in the program package BEAST with some modifications<sup>[25]</sup>. In brief, the complete genome sequences of China-indigenous genotype 4 isolates with known sampling dates were analyzed. Seven subtype 4a isolates and nine subtype 4d isolates, including three isolates in the present study, were added to reconfirm the time of the most recent common ancestors (TMRCA) of China-indigenous subtype 4a and 4d, respectively. The combination of a strict clock model and the exponential growth coalescent model was analyzed<sup>[26]</sup>. Markov chain Monte Carlo (MCMC) sampling was performed for at least 10<sup>8</sup> generations, and a tree was sampled every 10000 generations. The program, Tracer, (<http://tree.bio.ed.ac.uk>) was used to determine whether appropriate mixing of the

**Table 1** Prevalence of hepatitis E virus antibodies and RNA in the general human population in Shandong Province, China in 2011

Type of antibody	Positive samples/total analyzed of HEV antibodies, 95%CI	Positive samples/total analyzed of RNA, 95%CI
IgG <sup>+</sup> /IgM <sup>-</sup>	473/2028 (23.3%), (21-25)	0/473, -
IgM <sup>+</sup>	51/2028 (2.5%), (2-3)	2/51, (3.9%), (89-100)

HEV: Hepatitis E virus.

posterior target distribution had been achieved (effective sample size > 200), and the program, TreeAnnotator, was used to construct a phylogeny that best summarized the set of credible trees, called the maximum clade support phylogeny<sup>[25]</sup>.

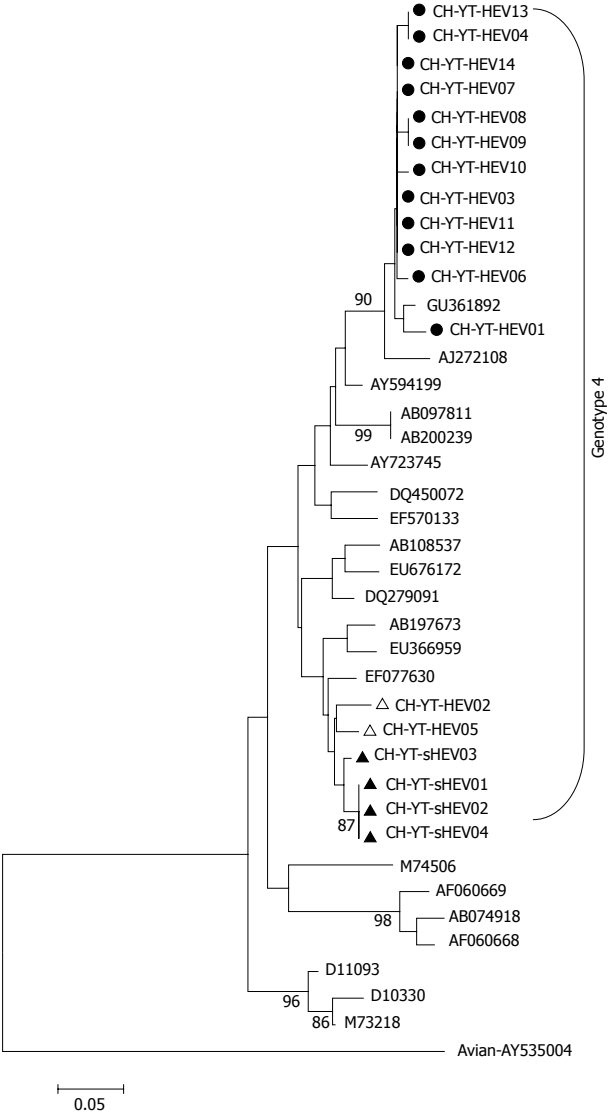
## RESULTS

### Occurrence of HEV

Table 1 shows the prevalence of HEV antibodies and RNA in 2028 serum samples collected from the general human population in Shandong Province, China. The 524 serum samples which were positive for HEV antibodies (IgG or IgM) were used for HEV RNA detection by RT-nPCR. The results showed that 51 (2.5%) individuals were currently infected (*i.e.*, they were reactive for anti-HEV IgM)<sup>[3]</sup>, and 3.9% (2/51) of these infections were accompanied by viremia (*i.e.*, they were positive for viral RNA). Therefore, the overall viral burden of subjects in the present study was estimated to be 0.1% (2/2028). This probably approximates to the viral burden of the general human population in Shandong. Phylogenetic analysis based on the partial ORF2 sequences showed that these two isolates belonged to genotype 4 (Figure 1, CH-YT-HEV13, CH-YT-HEV14). In addition, none of the 473 serum samples which were only positive for IgG tested positive for HEV RNA. Table 2 shows the age-specific positivity of anti-HEV among the general population in Shandong Province, China in 2011. The 2-19 age group showed the lowest positive rate of anti-HEV IgG (11.3%), while the 40-59 age group showed the highest prevalence rate of anti-HEV IgG (35.2%), and the positivity of IgG was found to increase with age.

### Detection of HEV RNA in human cases and swine herds and sequences analyses

HEV was detected by RT-nPCR in 20% (12/60) of patient fecal samples and in 4.9% (4/82) of swine bile samples. The 150 bp PCR products of the 16 isolates were sequenced and designated as CH-YT-HEV01 to CH-YT-HEV12, and CH-YT-sHEV01 to CH-YT-sHEV04 (Figure 1). Phylogenetic analysis based on the partial ORF2 sequences showed that these isolates belonged to genotype 4, and the 16 sequences could be clearly grouped into two main clades (Figure 1), one of which consisted of 10 HEV isolates sharing a 97%-100% identity with each other. The other clade included six isolates (two in patient feces and four in swine bile) sharing a 96%-100% iden-



**Figure 1** Phylogenetic tree based on partial ORF2 sequences. An avian hepatitis E virus (HEV) strain (AY535004) is included as an outgroup. The strains CH-YT-HEV01 to CH-YT-HEV12 were isolated from human patients, and the strains CH-YT-HEV13 and CH-YT-HEV14 were isolated from the general human population. The strains CH-YT-sHEV01 to CH-YT-sHEV04 were isolated from swine bile.

tity with each other. The endemic strains revealed a close relationship within the clade. Therefore, the strains CH-YT-HEV01, CH-YT-HEV02 and CH-YT-sHEV01 were designated to determine the complete genome sequences.

**Phylogenetic analysis of the complete genomes**

The full-length sequences of CH-YT-HEV01, CH-YT-HEV02 and CH-YT-sHEV01 have been deposited in the GenBank database under accession numbers KC163335, KC492825 and KC692453, respectively. A phylogenetic tree based on the full-length nucleotide sequences of the three isolates and all other 69 genotype 4 HEV isolates retrieved from GenBank up till September 2013 (Figure 2) was constructed. Sequence analysis showed that CH-YT-HEV01 was classified into subtype 4d, CH-YT-HEV02 and CH-YT-sHEV01 fell into subtype 4a. According to

Table 2 Relation between age and positivity of hepatitis E virus-specific antibodies <i>n</i> (%)					
Age (yr)	Participants- <i>n</i>	IgG positive	95%CI	IgM positive	95%CI
2-19	453	51 (11.3)	(8.3-14.2)	-	-
20-39	732	165 (22.5)	(19.5-25.6)	17 (2.3)	(1.2-3.4)
40-59	625	220 (35.2)	(31.5-38.9)	24 (3.8)	(2.3-5.3)
≥ 60	218	73 (33.5)	(27.2-39.8)	10 (4.6)	(1.8-7.4)
Total	2028	509 (25.1)	(23.2-27.0)	51 (2.5)	(1.8-3.2)

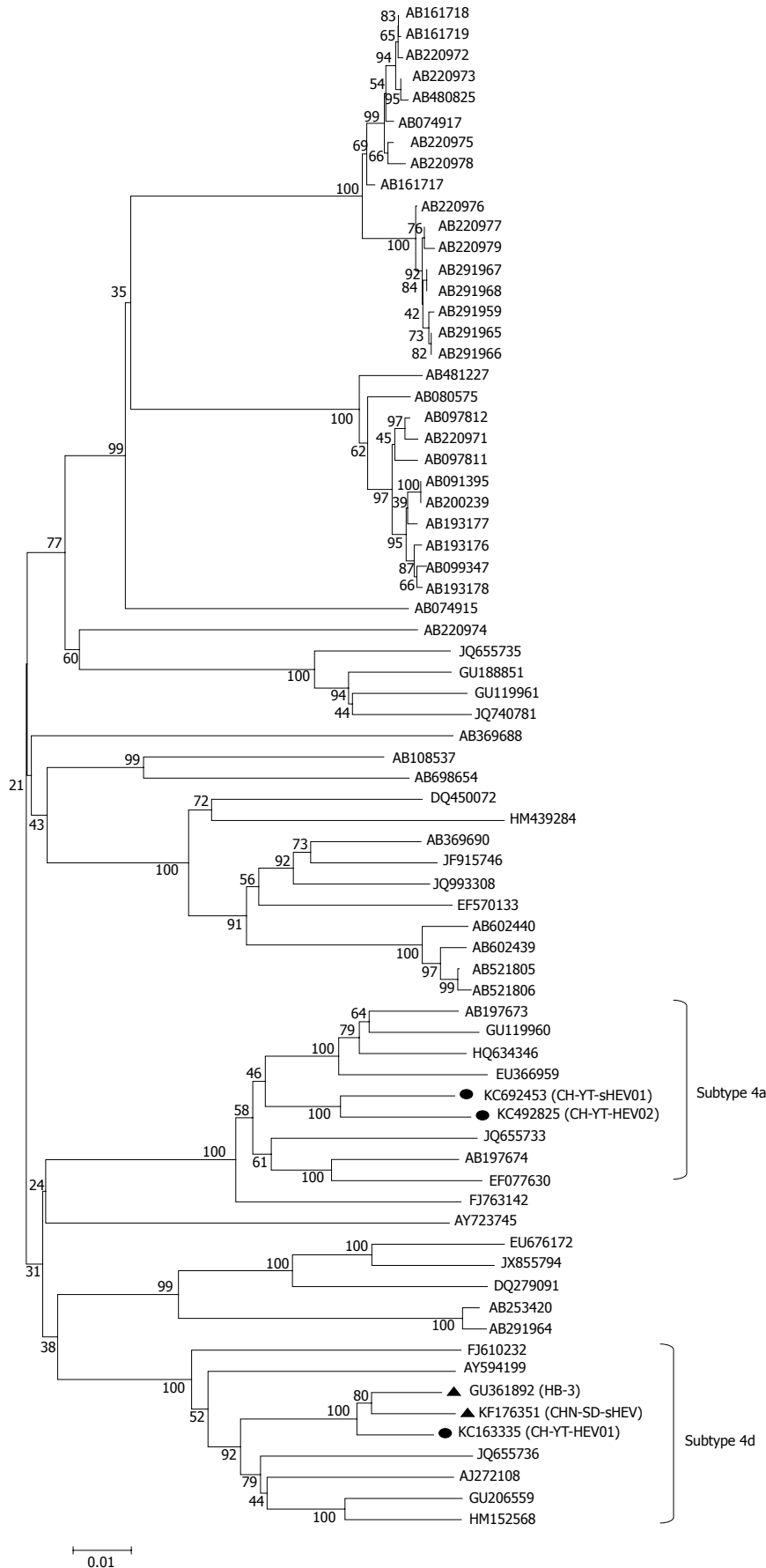
the phylogenetic tree, the human isolate CH-YT-HEV02 was clustered with the swine strain, CH-YT-sHEV01, sharing a 94% sequence identity. In addition, the bootstrap value between this cluster and its neighboring isolate was only 46%, therefore, CH-YT-HEV02 and CH-YT-sHEV01 could serve as Yantai-indigenous strains. The swine isolates HB-3 (GU361892) and CHN-SD-sHEV (KF176351) were most related to the human strain CH-YT-HEV01 in the tree, and they were recovered from swine in Hubei and Shandong, respectively. Furthermore, the three isolates clustered with a bootstrap value of 100%, and shared a 96.1%-96.4% identity with each other.

**Analysis of ORFs**

The predicted ORF1 polypeptides of CH-YT-HEV01, CH-YT-HEV02 and CH-YT-sHEV01 were 1707 aa, 1706 aa and 1706 aa, respectively. The closest strain to the CH-YT-HEV01 isolate was CHN-SD-sHEV with a 99.1% identity, and the isolate CH-YT-HEV02 shared the highest identity of 98.2% at the amino acid level in this region with the strain CH-YT-sHEV01. These results were in agreement with the full-length sequences analysis. When compared with other HEV genotype 4 isolates, 11 and 7 unique amino acid substitutions were found in ORF1 of the CH-YT-HEV02 and CH-YT-sHEV01 isolates, respectively, and no unique amino acid substitutions were observed in this region of CH-YT-HEV01. Analysis of the distribution of these substitutions showed that seven [D728, D735, L744, S753, H754 (CH-YT-HEV02), G735, Y754 (CH-YT-sHEV01)] were in the hypervariable region (HVR), two [A1004, C1125 (CH-YT-HEV02)] in the helicase domain, and seven [A1220, A1268, A1565, M1641(CH-YT-HEV02), T1220, T1466, M1641 (CH-YT-sHEV01)] in the RNA-dependent RNA polymerase (RdRp) domain. Amino acid deletions or insertions occurred mainly within the HVR, 711-798aa, which was previously found to show variations in size<sup>[19]</sup>. The CH-YT-HEV01 and CH-YT-HEV02 isolates showed 36%-92% and 35%-86% amino acid sequence identity within the HVR compared with other genotype 4 isolates, respectively. In agreement with the complete genome sequences analysis, the CH-YT-HEV01 and CH-YT-HEV02 isolates showed the highest identity to the CHN-SD-sHEV (KF176351) strain and the CH-YT-sHEV01 isolate within the HVR, respectively.

The ORF2 of CH-YT-HEV01, CH-YT-HEV02 and CH-YT-sHEV01 was 2022 nt in length, with a coding capacity of 674 amino acids. The sequences closest





**Figure 2** Phylogenetic tree depicting sub-genotypic status of all 72 genotype 4 hepatitis E virus isolates based on full-length sequences. The Arabic numbers and the Roman letters outside the square bars indicate potential sub-genotypic designations.

**Table 3** Hepatitis E virus loads in patient fecal samples and swine bile samples

Sample origin	Positive sample numbers and HEV loads	
	RT-nPCR (+)/RT-qPCR (+)	RT-nPCR (-)/RT-qPCR (+)
Patients	12, $6.81 \times 10^4$ - $1.53 \times 10^7$ copies/mL	8, $6.75 \times 10^2$ - $1.35 \times 10^3$ copies/mL
Swine	4, $4.92 \times 10^6$ - $5.46 \times 10^7$ copies/mL	-

The HEV loads were detected by RT-qPCR. Only one patient sample's HEV load was  $1.53 \times 10^7$  copies/mL, while the HEV loads of other samples were less than  $4.92 \times 10^6$  copies/mL. HEV: Hepatitis E virus.

to the isolate CH-YT-HEV01 were the isolates CHN-SD-sHEV and HB-3 with an identity of 99.0%. In this region, the isolate CH-YT-HEV02 was the closest strain to CH-YT-sHEV01, sharing a 98.7% identity. Compared with the other HEV genotype 4 isolates, no amino acid substitution was unique to CH-YT-HEV02 and CH-YT-sHEV01, and two amino acid substitutions unique to CH-YT-HEV01, CHN-SD-sHEV and HB-3 were observed, including Q92P and T619S in this region.

The predicted ORF3 polypeptides of CH-YT-HEV01, CH-YT-HEV02 and CH-YT-sHEV01 were 114 aa in length. No unique amino acid substitutions were observed in this region of the three isolates in this study. The closest HEV strains to CH-YT-HEV01 and CH-YT-HEV02 were HB-3 and CH-YT-sHEV01, with 98.3% and 96.5% identity, respectively.

#### **Estimation of epidemic history and population dynamics of CH-YT-HEV01, CH-YT-HEV02 and CH-YT-sHEV01 using a dated complete genomes dataset**

Coalescent analyses of the dated complete genomes dataset were performed in BEAST under the combination of a strict clock model and exponential growth. The evolutionary rates were  $7.56 \times 10^{-3}$ /substitution site per year (95%CI:  $6.38 \times 10^{-3}$ - $8.84 \times 10^{-3}$ ) and  $3.23 \times 10^{-3}$ /substitution site per year (95%CI:  $2.49 \times 10^{-3}$ - $4.0 \times 10^{-3}$ ) for all Chinese subtype 4a and 4d isolates, respectively. The evolutionary rate for subtype 4a was higher than that for subtype 4d. The TMRCA were calculated based on the clustering of the maximum clade support phylogeny. The TMRCA was 1999 (95%CI: 1997-2000) for all Chinese subtype 4a isolates including sequences in this study, and 2006 (95%CI: 2005-2007) for the clade of CH-YT-HEV02 and CH-YT-sHEV01. The TMRCA was 1979 (95%CI: 1972-1985) for Chinese subtype 4d isolates including CH-YT-HEV01, and the TMRCA for the clade of CH-YT-HEV01 and CHN-SD-sHEV was 2004 (95%CI: 2003-2005). Interestingly, the TMRCA for the clade of CH-YT-HEV01 and HB-3 was 2003 (95%CI: 2002-2004), and the TMRCA for the clade of CHN-SD-sHEV and HB-3 was also 2003 (95%CI: 2002-2004).

## **DISCUSSION**

In this cross-sectional seroepidemiologic study, all serum

samples were collected from the general population. The positive rate of anti-HEV IgG or IgM in the serum specimens was 25.8% (524/2028). This probably approximates to the anti-HEV seroprevalence of the population in this area. Of the 473 subjects who were only positive for anti-HEV IgG, no one had a history of hepatitis E, and none of the 51 individuals with current infection had been admitted to hospital. These results indicated that HEV infection in Shandong (eastern China) was frequently asymptomatic. Although the age group  $\geq 60$  years had the highest positive rate of IgM (Table 2), which was inconsistent with the results of a previous study in central China<sup>[23]</sup>, a seroepidemiologic study of HEV showed that the participants' communities were independent determinants<sup>[27]</sup>. The IgG anti-HEV antibody was detectable in nearly half of the patients who had been affected during a hepatitis E outbreak 14 years previously<sup>[4]</sup>. Therefore, infection earlier in life may result in IgG positivity with increased age. In addition, the HEV burden in swine was much larger than that in humans (Table 3), suggesting that the transmission of HEV infection was likely from swine to humans<sup>[24]</sup>.

In the present study, we used the primers which had been optimized for genotypes 1 and 4 HEV in China, and they also afforded sensitive detection of genotype 3<sup>[27]</sup>. Phylogenetic analysis showed that the isolates all belonged to genotype 4, suggesting that genotype 4 HEV is the main genotype prevalent in this area. Currently, the most widely accepted subtype classification system divides the four major HEV genotypes into 24 subtypes<sup>[28]</sup>. However, the number of published HEV sequences has increased significantly during the last few years, and even the most widely accepted subtype classification system has led to ambiguous results when new isolates are assigned to particular virus subtypes<sup>[29,30]</sup>. Furthermore, the use of short sequences introduced the possibility of discordant subtyping due to the weak phylogenetic signal of the short region. Thus, in order to determine reliable epidemiological results for HEV in Shandong Province, the full-length genome sequences of the endemic strains were determined and phylogenetic analysis based on the complete HEV genome was performed. In the 4a cluster (Figure 2), the human strain CH-YT-HEV02 was found to be directly related to swine strain CH-YT-sHEV01 from the same area with 94% identity. Zoonotic transmission of HEV from swine to humans is well accepted<sup>[15,21,31]</sup>, and HEV has a high mutation rate due to its error-prone RNA-dependent RNA polymerase and is probably present as a quasispecies in an infected host<sup>[32]</sup>. These results suggested that the strains CH-YT-HEV02 and CH-YT-sHEV01 were involved in cross-species transmission between swine and humans in Shandong. In the 4d cluster (Figure 2), the isolates CH-YT-HEV01, CHN-SD-sHEV and HB-3 were closely related to each other, with a bootstrap value of 100% and sharing 96.1%-96.4% identity. The isolates CH-YT-HEV01 and CHN-SD-sHEV were obtained from patients and swine in Shandong, respectively. In addition, the HB-3 strain is one representative genotype 4 strain prevalent in swine in

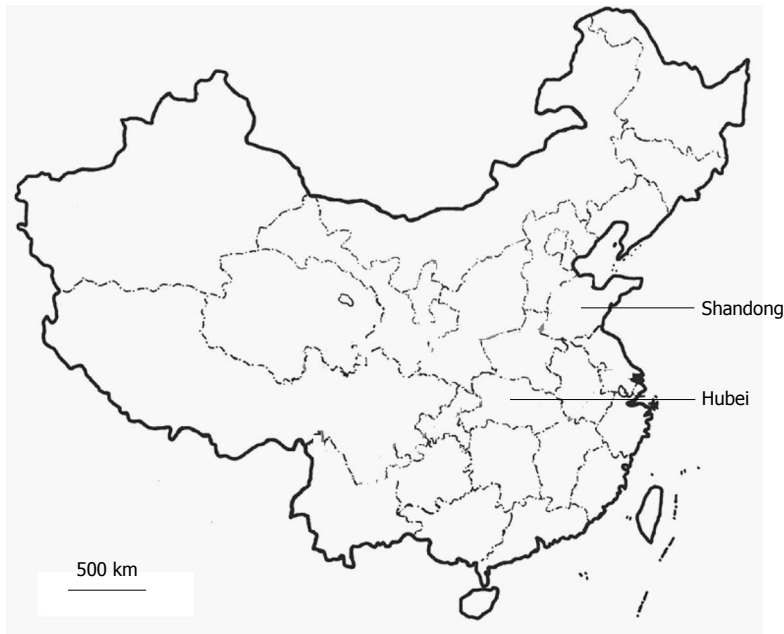


Figure 3 Map showing the geographic distribution of Shandong and Hubei in China. Shandong Province is located 380 km northeast of Hubei Province.

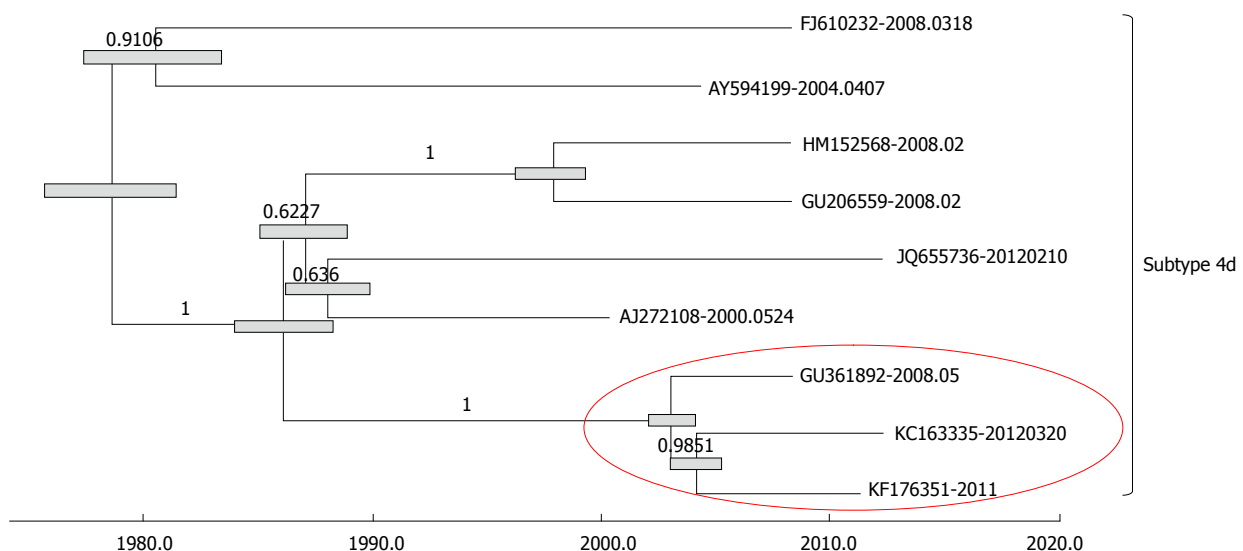


Figure 4 Maximum clade support phylogeny for the dated China-indigenous (subtype 4d) isolates of complete genome sequences. The branch lengths and node heights are in units of years.

Hubei Province<sup>[33]</sup> (Figure 3). Recently, Nakano *et al.*<sup>[34,35]</sup> reported the historical inflow of subtype 3e isolates from Europe to Japan using phylogenetic analysis. Therefore, our results suggest that the strains CH-YT-HEV01, CHN-SD-sHEV and HB-3 were involved in trans-regional transmission between Hubei and Shandong.

To further analyze the genetic relationship between human and swine HEV strains, we determined the similarities in amino acid sequences of the ORF1, ORF2 and ORF3 proteins between human and swine sequences. CH-YT-HEV01 shared the highest identity with CHN-SD-sHEV or HB-3 in ORFs1-3, while CH-YT-HEV02 shared the highest similarity with CH-YT-sHEV01. These results were in agreement with the complete genome se-

quence analysis and suggested that these strains were involved in trans-regional or cross-species transmission. In addition, when compared with other genotype 4 isolates, one amino acid (residue 788) deletion was found in the HVR of subtype 4a strains, including CH-YT-HEV02 and CH-YT-sHEV01. The high quasispecies heterogeneity in the regions encoding the PPR (nt 2137-2340, L08816) and the macro domain (nt 2341-2829, L08816), which overlapped with the HVR, was associated with persistence of HEV, and this association may be due to the appearance of mutants in the PPR and macro domain able to modulate the host immune response<sup>[36]</sup>. However, there were some special amino acid substitutions in the HVR of different HEV genotypes and subtypes. There-

fore, further investigation is needed to confirm these associations.

In addition, to obtain more information on the genetic history of the three HEV isolates in the present study, we calculated the TMRCA of subtype 4a and 4d isolates. The date of the latest subtype 4a and 4d isolates in Chinese were close to each other. However, the TMRCA for Chinese subtype 4a and 4d isolates was 1999 and 1979, respectively. Therefore, the earlier TMRCA date for subtype 4a was mainly due to the higher evolutionary rate for subtype 4a than subtype 4d. The TMRCA was 2006 for the clade of CH-YT-HEV02 and CH-YT-sHEV01, and this may shed light on the origin of the Yantai-indigenous strains. The TMRCA for the clade of CH-YT-HEV01 (KC163335) and CHN-SD-sHEV (KF176351) was 2004, suggesting that the ancestor of the strains was introduced into Shandong Province about 10 years ago, and these strains have persisted since then. Moreover, the TMRCA for the clade of CH-YT-HEV01 and HB-3 (GU361892) was 2003, which was consistent with the TMRCA for the clade of CHN-SD-sHEV and HB-3, and they were both earlier than the TMRCA for the clade of CH-YT-HEV01 and CHN-SD-sHEV. These results suggest that the ancestor of the HEVs in Shandong entered Shandong from Hubei province (Figure 4). Nakano *et al.*<sup>[34]</sup> reported that the import of pigs from Europe since the 1960s may be responsible for the introduction of subtype 3e. Therefore, the incident responsible for the introduction of the HEVs in Shandong will be investigated in our future study.

In conclusion, the phylogenetic and coalescent analyses suggested the transmission direction or route of HEV infection, which will be helpful in identifying the genetic relatedness of HEV isolates circulating in China, in the South-East Asian region, and finally worldwide.

## COMMENTS

### Background

Hepatitis E virus (HEV), the causative agent of hepatitis E, is an important public health issue in developing countries, where it causes large waterborne epidemics. Industrialized countries were previously thought to be free of HEV. However, HEV has also become an emerging problem in developed areas, with a number of sporadic cases. China is generally judged to be an HEV-endemic area and both molecular and seroepidemiological investigations of HEV have been performed in many provinces. However, there is a shortage of data on seroprevalence and evolutionary dynamics from Shandong Province.

### Research frontiers

Hepatitis E is now recognized as a zoonotic disease, and pigs are more likely to be reservoirs than other animal species. However, a few reports have shown close phylogenetic relationships between sequences identified in swine and in humans based on full-length genome sequences. The number of published HEV sequences has increased significantly during the last few years, which has also increased the number of potentially new genetic groups. As a result, some studies have reported that analysis of longer or even whole-genome sequences should be more reliable for the accurate identification of genetic relatedness among HEV isolates. Recently, Nakano *et al.* investigated the genetic history of subtype 3e isolates and suggested that the isolates entered Japan from European countries.

### Innovations and breakthroughs

This is the first study to investigate the genetic history of HEV based on the complete genome sequences in China. Three prevalent strains (CH-YT-

HEV01, CH-YT-HEV02 and CH-YT-sHEV01) were designated to determine the complete genome sequences. The phylogenetic analysis based on the full-length nucleotide sequences suggested that the strains CH-YT-HEV02 and CH-YT-sHEV01 were involved in cross-species transmission between swine and humans in Shandong Province, and the strains CH-YT-HEV01, CHN-SD-sHEV and HB-3 were involved in trans-regional transmission between Hubei and Shandong. In addition, the coalescent analysis suggested that the ancestors of HEVs in Shandong entered Shandong from Hubei, and trans-regional transmission may be a risk route for hepatitis E in China.

### Applications

The phylogenetic and coalescent analyses suggested that the ancestors of HEVs in Shandong entered Shandong from Hubei, and trans-regional transmission may be a risk route for hepatitis E in China. Clarification of the transmission direction or route of HEV infection should be helpful in preventing a future endemic or epidemic of HEV infection.

### Peer review

The work determined the HEV seroprevalence and performed phylogenetic analysis on HEV-4 genotypes circulating in Shandong Province, China. Numerous samples from human beings (serum specimens,  $n = 2028$ ; and stools,  $n = 60$ ) and swine (bile samples,  $n = 82$ ) were tested. The study was well designed, full-length genome sequences were obtained for three strains (CH-YT-HEV01, CH-YT-HEV02 and CH-YT-sHEV01), the results are well presented and the paper is well-written.

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