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Seroepidemiology and genetic characterization of hepatitis E virus in western Yunnan Province

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ABSTRACT

Objective: To investigate the seroepidemiology and genetic characterization of hepatitis E virus (HEV) in western Yunnan Province. **Methods:** Questionnaire survey was conducted among 1638 residents in western Yunnan Province using stratified sampling method. Enzyme−linked immunosorbent assay was used to detect serum anti−HEV IgG and IgM. HEV RNA was extracted from patients with serum anti−HEV IgM positive. The open reading flame 2 (ORF2) of HEV that was amplified by nested RT−PCR was sequenced and compared with standard HEV genotypes 1−4. **Results:** Serum anti−HEV positive was found in 13.92% (228/1638) residents. The HEV infection rate in males was significantly higher than that in females with a ratio of 1.47 ($P<0.01$). 20−30 and 30−40 years old young men showed the highest incidence, 20.57% and 20.78%, respectively. While 10−20 and 20−30 years old young women exhibited the highest infection rate, 11.85% and 15.60%, respectively. According to occupation, the highest HEV infection rate was observed in farmers (20.35%) and migrants (16.50%). We isolated 10 individual HEV isolates from 31 patients with serum anti−HEV IgM positive. Homology analysis and phylogenetic analysis indicated that these 10 HEV isolates belonged to HEV genotype 4 with the homology of 78.65%−94.71%. **Conclusions:** The HEV infection rate is high in western Yunnan Province. HEV genotype 4 is the leading cause of HEV infection and young farmers and migrants are the main infected population.

1. Introduction

Hepatitis E virus (HEV) is a nonenveloped virus with a single−stranded and positive−sense RNA genome of an approximately 7.2 kb length[1]. HEV is divided into the mammalian HEV and avian HEV according to infection of host[2]. The mammalian HEV mainly infects human and other mammals. Researchers have suggested that zoonotic infection may play a key role in HEV transmission[3]. 4 distinct genotypes have been indentified in HEV isolates, and further dividing 4 genotypes into 24 subtypes has been proposed[4]. Genotypes 1 and 2 are exclusively found in humans, while genotypes 3 and 4 have been identified in humans and several animal species[5]. In China, genotypes 1 and 4 are the leading cause of HEV infection[6]. HEV, an

intestinal virus, transmits via water, food and close contact and can result in widespread outbreaks[7]. Furthermore, HEV infected pregnant women are characteristic of high mortality[8]. HEV infection also leads to premature birth and dead fetus[8].

HEV, an acute infectious disease, is mainly transmitted via the faecal−oral route. HEV has been considered as an amphixenosis. It once was a pandemic disease in Xinjiang Province, China[9]. HEV is one of the common types in sporadic viral hepatitis. The western Yunnan Province, including Chuxiong, Dali, Nujiang, Baoshan and Dehong city, is inaccessible and lack of transport. The living standard of resident here is relatively low. Accordingly, they eat raw or undercooked food and contact with animals frequently. All these above causes lead to a higher infection rate of HEV in Yunnan than other areas[10]. We randomly selected 1638 residents from different age and occupation for sampling survey. We detected the infection rate of HEV in this area and analyzed the correlation between HEV infection and several features including age, gender and occupation. Furthermore, the HEV RNA that was isolated

from HEV positive patients was cloned and sequenced for genotyping.

2. Materials and methods

2.1. Patients and specimens

A total of 1 638 residents were selected including 936 males [(37.41±18.53) years] and 702 females [(32.85±18.53) years], who live in western Yunnan Province including Chuxiong, Dali, Nujiang, Baoshan and Dehong city. The blood was collected and used after obtaining informed consent. The serum was separated and frozen in −80 ℃.

2.2. Serum anti–HEV antibody detection

Samples were subjected to anti–HEV IgG and IgM detection using a commercial kit (Beijing Wantai Biological Pharmacy Enterprise Co. Ltd., Beijing, China) according to the manufacturer's guidelines. P/N≥2 was considered as anti–HEV antibody positive.

2.3. Virus RNA extraction

Samples with anti–HEV IgM positive were subjected to virus RNA extraction using a RNA extraction kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. Total RNA that was dissolved in 20 μ L RNase–free water was subjected to reverse transcription or cryopreservation in −80 ℃.

2.4. Virus ORF2 amplification and sequencing

Nested RT–PCR was used to amplified a 348bp length conserved fragment in open reading frame 2 (ORF2) of HEV as previously described[11]. The following primers were used for amplification: outer primers 5–AAYTATGCWCAGTACCGGGTTG–3, 5–CCCTTATCCTGCTGAGCATTCTC–3; inner primers 5–GTYATGYTYTGCATACATGGCT–3, 5–AGCCGACGAAATYAATTCTGTC–3. The amplification products were separated by agarose gel electrophoresis and the 348bp length products were collected. The PCR products were inserted into a pMD18T vector (Takara Bio, Shiga, Japan). Recombinant plasmids were transfected into DH5 α bacteria and positive clones were subjected to sequencing by Sangon Biotech (Shanghai) Co. Ltd. (Shanghai, China).

2.5. Multiple comparison analysis of sequence

DNAMAN 6.0 software (Lynnon Biosoft, Quebec, Canada) was used for multiple comparison analysis of base sequence and phylogenetic tree analysis. The standard sequences of HEV strain were obtained from GeneBank: genotype 1 (AF051830, AF076239, AF459438, and AY230202); genotype

2 (M74506); genotype 3 (AB073912, AB089824, AF082843 and AP003430); genotype 4 (AB074917, AB108537, AB168096, AJ272108, AJ428856, FJ763142 and GU119961).

2.6. Statistical analysis

Data was analyzed by SPSS statistical package for Windows Version 15.0 (SPSS, Chicago, IL, USA), using a Pearson *Chi*–squared test when appropriate. Difference were considered significant when $P<0.05$.

3. Results

3.1. Detection of anti–HEV antibody in western Yunnan Province

We detected anti–HEV IgG and IgM in 1 638 residents from western Yunnan Province. Serum anti–HEV positive was found in 13.92% (228/1 638) residents. In these cases, 12.03% (197/1 638) residents showed anti–HEV IgG positive, while 1.89% (31/1 638) residents showed anti–HEV IgM positive (Table 1).

Table 1
Detection of anti–HEV IgG/IgM in western Yunnan Province.

Anti–HEV antibody	Positive rate (%)	95% *CI*
IgG$^+$/IgM$^-$	12.03 (197/1 638)	10.45–13.60
IgM$^+$	1.89 (31/1 638)	1.23–2.55
IgG$^-$/IgM$^+$	0.73 (12/1 638)	0.32–1.15
IgG$^+$/IgM$^+$	1.16 (19/1 638)	0.64–1.69
Total	13.92 (228/1 638)	12.24–15.60

CI: confidence interval.

3.2. Effect of age and gender on HEV infection rate

As shown in Table 2, HEV infection rate was 16.13 (151/936) in males, while the incidence of HEV was 10.97% (77/702) in females. It affected more men than women with a ratio of 1.47 (χ^2=8.927, P=0.003). 20–30 and 30–40 years old young men showed the highest incidence, 20.57% and 20.78%, respectively. However, 10–20 and 20–30 years old young women exhibited the highest infection rate, 11.85% and 15.60%, respectively.

3.3. Effect of occupation on HEV infection rate

The distribution of HEV infection rate in different occupation was shown in Table 3. The highest HEV infection rate was observed in farmers (20.35%). Then, the migrants and workers showed the second and third highest of HEV infection, 16.5% and 13.06%, respectively. However, the private businessmen, government workers and retirees exhibited the lowest HEV infection rate, 6.67%, 8.53% and 7.02%, respectively.

Table 2

Comparison of HEV infection rate in different gender and age.

Age (years)	Male			Female			χ^2	P
	n	Positive cases	Positive rate(%)	n	Positive cases	Positive rate (%)		
<10	84	4	4.76	69	3	4.35	0.015	0.903
10-	159	24	15.09	135	16	11.85	0.653	0.419
20-	141	29	20.57	141	22	15.60	1.173	0.279
30-	255	53	20.78	156	17	10.90	6.695	0.010
40-	186	30	16.13	93	9	9.68	2.146	0.143
50-	84	9	10.71	66	6	9.09	0.108	0.742
60-	27	2	7.41	42	4	9.52	0.093	0.761
Total	936	151	16.13	702	77	10.97	8.927	0.003*

* $P<0.05$.

Table 3

HEV infection rate in different occupation.

Occupation	n	Positive cases	Positive rate (%)
Preschoolers	66	8	12.12
Students	201	23	11.44
Farmers	285	58	20.35
Migrants	303	50	16.50
Workers	360	47	13.06
Commercial service people	222	26	11.71
Private businessmen	15	1	6.67
Government workers	129	11	8.53
Retirees	57	4	7.02
Total	1 638	228	13.92

3.4. Homology analysis of ORF2 sequence in HEV

We isolated 10 individual HEV fragments, which were amplified from 31 serum specimens with anti-HEV IgM positive. After sequencing, comparison between fragments and 4 subtypes of HEV standard strain were performed. As shown in Table 4, the highest homology was observed between these 10 HEV fragments and HEV genotype 4 standard strain (78.65%-94.71%).

Table 4

Homology alignment between HEV isolates and HEV standard strains.

Clone	Homology (%)			
	Genotype 1	Genotype 2	Genotype 3	Genotype 4
HEV1	67.23-82.78	66.41	64.56-79.04	79.45-90.58
HEV2	69.01-83.45	69.23	63.12-77.28	81.56-92.76
HEV3	68.57-81.74	67.49	66.38-78.14	83.65-94.71
HEV4	66.47-82.47	65.18	61.78-77.45	80.81-90.53
HEV5	69.76-86.36	70.14	67.34-79.23	78.65-88.69
HEV6	72.43-85.27	71.26	70.38-83.59	81.45-91.47
HEV7	69.65-80.37	73.75	71.23-86.48	78.91-90.58
HEV8	71.35-82.58	68.56	64.48-77.56	83.26-93.07
HEV9	68.83-83.12	69.34	68.36-80.34	79.57-92.88
HEV10	66.98-79.54	72.29	65.74-82.15	82.26-93.24

3.5. Phylogenetic analysis of ORF2 in HEV

The isolated ORF2 of HEV was subjected to phylogenetic analysis. As shown in Figure 1, 4 HEV genotypes showed 4

different branches in the phylogenetic tree. HEV isolates in this study distributed in the branch of HEV genotype 4. 5 HEV isolates (HEV1, 3, 4, 8 and 9) distributed in cluster and were highly homologous. The other 5 HEV isolates showed diffused distribution in the branch of HEV genotype 4.
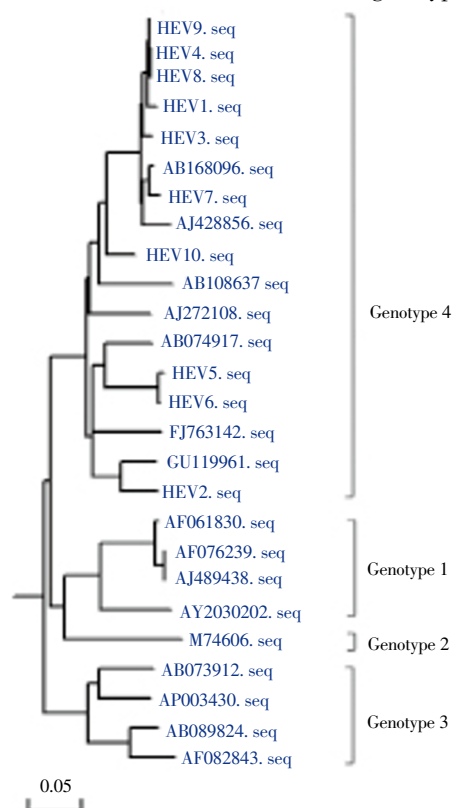
Figure 1. Phylogenetic tree of ORF2 in HEV isolates.

HEV1, HEV3, HEV4, HEV8 and HEV9) distributed in cluster and were highly homologous. HEV2, HEV5, HEV6, HEV7 and HEV10 showed diffused distribution in the branch of HEV genotype 4.

4. Discussion

Detection of anti-HEV IgG and IgM in serum can be used to analyze the HEV infection rate in specific area. In this study, the total HEV infection rate was 13.92% in western Yunnan Province, which is consistent with previous reports[12,13].

Next, we analyzed the correlation between HEV infection rate and age or gender. We found that the incidence of HEV in males was significantly higher than that in females with a ratio of 1.47, which was lower than prior study (3:1)[14]. Among various kinds of occupation, farmers and migrants showed the highest HEV infection rate, which was consistent with former report [15]. Poor health condition, poor living environment and contacting with animal frequently may cause above phenomenon.

Detection of the HEV genotype is important for confirming the initiation, progression, evolution and epidemiology of HEV. Moreover, it also plays a critical role in developing proper HEV vaccines and disease prevention[16]. There are 3 ORF in HEV and ORF2 is the most conserved one, which is usually applied to identify HEV genotype[17]. We isolated 10 individual clones from 31 patients with serum anti-HEV IgM positive through amplification of a conserved sequence in ORF2 using nested RT-PCR. After sequencing and comparison with 4 types of HEV standard strains in GeneBank, we found that all HEV isolates had the high homology with HEV genotype 4. Phylogenetic analysis indicated that 10 HEV isolates distributed in the branches of HEV genotype 4, suggesting that 10 HEV isolates in our study belonged to HEV genotype 4. It was worth noting that 5 HEV isolates (HEV1, 3, 4, 8 and 9) distributed in cluster and were highly homologous, indicating that they may come from a common ancestor of HEV. The other 5 HEV isolates showed diffused distribution in other branches of HEV genotype 4. These results demonstrate that HEV in western Yunnan Province is independent and shows some geographical features[18].

In conclusion, we determine the HEV infection in 1638 residents from western Yunnan Province using sampling survey. The incidence of HEV in western Yunnan Province is slightly higher than that in other area. The HEV infection rate in males is higher as compared with that in females. According to occupation, the farmers and migrants show the highest infection rate. Molecular biological assays indicate that HEV genotype 4 is the leading cause of HEV infection, which settles a good foundation for HEV prevention in western Yunnan Province.

Conflict of interest statement

We declare that we have no conflict of interest.

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