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The investigation of hepatitis A virus and hepatitis E virus co-infection in humans and animals in China

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Summary. – The aim of this study was to investigate the prevalence of co-infection of hepatitis A and hepatitis E virus (HAV/HEV) in patients with acute hepatitis as well as in different animal species. A total of 46 serum samples from patients diagnosed as hepatitis A or hepatitis E and 675 fecal samples of 11 animal species were collected. The IgM class antibodies to HEV and HAV, respectively, were detected by enzyme-linked immunosorbent assay. HEV and HAV RNAs were extracted from serum and fecal samples for the nested reverse transcription polymerase chain reaction. At least 10.9% (5/46) of the patients were co-infected with both HAV and HEV. Fifteen percent (18/120) of rabbit fecal samples and 17.5% (7/40) of swine fecal samples were positive for HEV RNA, but only 1% (2/200) of ferret fecal samples were positive for HAV RNA. Our study showed that co-infection with both HAV and HEV in patients and animals is infrequent. At least in our study, we showed that ferrets may represent the potential HAV hosts.

Keywords: hepatitis A virus; hepatitis E virus; co-infection; zoonosis; prevalence

Introduction

Both hepatitis A virus (HAV) and hepatitis E virus (HEV) are the leading causes of acute viral hepatitis in developing countries due to poor sanitary conditions and still a significant public health concern (Ankcorn and Tedder, 2017; Melhem *et al*., 2014). Although the main transmission of these viruses takes place by the fecal-oral route, the HEV infection can be transmitted from animals to humans as a zoonotic disease (Jacobsen and Wiersma, 2010; Chang *et al*., 2009).

Hepatitis A virus has a global distribution and tens of millions of individuals worldwide are estimated to become infected each year (Stanaway *et al*., 2016). HAV is a 27-nm non-enveloped RNA virus belonging to the *Picornaviridae* family (Cohen *et al*., 1987). HAV has six genotypes (I–VI) but only one single serotype. After a mean incubation period of 28 days, HAV can produce asymptomatic or symptomatic infection in humans. Hepatitis A normally remains self-limited and does not progress to chronic liver diseases. In China, a national vaccination program against hepatitis A was launched in the late 1990s and hepatitis A vaccines are highly effective in preventing the spread of the disease (Cui *et al*., 2009).

Hepatitis E virus infection globally leads to acute viral hepatitis and China is considered as endemic area for hepa-

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Abbreviations: HAV = hepatitis A virus; HEV = hepatitis E virus; RT-nPCR = reverse transcription nested PCR

titis E, where 120,000 patients of Xinjiang province were infected with HEV in 1986 and 1988 (Chandra *et al.*, 2008). It is estimated that more than 20 million HEV infections occur each year in the world, causing about 55,000 deaths (Ankcorn and Tedder, 2017). HEV is a non-enveloped, single-stranded, positive-sense RNA virus belonging to the *Hepevirus* genus, the family *Caliciviridae*. Currently, HEV are grouped to 8 genotypes (HEV1–8) (Smith *et al.*, 2016). HEV1 and HEV2 infect humans only; HEV3-8 can infect both humans and animals (Ankcorn and Tedder, 2017). Although its clinical symptoms are usually mild, HEV infection in pregnant women can have more severe consequences with average of 25% mortality (Chandra *et al.*, 2008). Moreover, the chronic hepatitis E and relevant extrahepatic diseases have attracted great attention recently (Grewal *et al.*, 2014; Kamar *et al.*, 2008; Pischke *et al.*, 2014). Hepatitis E vaccine, HEV239, is now commercially available in China but it is not yet routinely available outside of China (Ankcorn and Tedder, 2017), neither has it been introduced as a part of any vaccination program in China yet.

HEV is now confirmed as a zoonotic virus. Several animal species have been identified as sources of HEV and swine are considered as the major reservoir of HEV (Meng, 2010). Since the first rabbit HEV (rHEV) was isolated in China in 2009, rabbits have been considered as HEV animal hosts that are as important as swine (Zhao *et al.*, 2009). Besides, more and more animal species have been implicated as potential host animals, such as cynomolgus monkeys, wild rats, ferrets, camels, dogs, sheep, tree shrews, minks, foxes and so on (Li *et al.*, 2004; Hirano *et al.*, 2003; Raj *et al.*, 2012; Woo *et al.*, 2014; Liang *et al.*, 2014; Sanford *et al.*, 2013; Yu *et al.*, 2016; Krog *et al.*, 2013). However, currently there is no definite evidence of HAV in animal hosts, although there are some studies showing that HAV can experimentally infect nonhuman primates and pigs (Le Duc, 1983; Chitambar *et al.*, 2001; Song *et al.*, 2016).

Several studies have indicated that the superinfection of HEV or HAV in patients with hepatitis B or hepatitis C is common and can result in severe disease and poorer outcome, which means that emphasis should be given in order to take appropriate measures for the diagnostics and treatment of such patients (Zhang *et al.*, 2010; Ke *et al.*, 2006; Vento, 2000). However, there are few studies about the co-infection of HEV and HAV in patients (Joon *et al.*, 2015; Rendon *et al.*, 2016). HAV and HEV have similar virological features, including transmission mainly by fecal-oral route. Therefore, it is likely that patients can be infected by these two viruses at the same time. The aim of the current study was to determine the prevalence of co-infection of HEV and HAV in patients, and to investigate HEV and HAV infection among various animals which can serve as potential hosts.

Materials and Methods

Ethics statement. The animal experiments were approved by the Committee of Laboratory Animal Welfare and Ethics, Peking University Health Science Center, which rules are in strict accordance with the protocol for the review on Laboratory Animal Welfare and Ethics, Peking University Health Science Center (LA2017047). The study in question was also approved by the Ethics Committee of Peking University People's Hospital (Beijing, China, RDY2016-13).

Sample collection. In this study, 675 fecal samples were collected from rabbits (120), pigs (40), dogs (50), camels (70), mice (20), tree shrews (30), minks (107), ferrets (200), foxes (27), monkeys (5) and sheep (6) from several stock farms in Beijing and Hebei. A total of 46 human blood samples were collected from Peking University People's Hospital, including 22 diagnosed as HEV positive and 24 diagnosed as HAV positive. All samples were stored at -80°C.

Antibody detection. All the serum samples were tested for IgM anti-HEV and IgM anti-HAV antibodies using commercially available enzyme-linked immunosorbent assay (ELISA) kits developed by Wantai Biopharmaceutical (Beijing, China). The assay procedures of ELISA were conducted according to the manufacturer's instructions.

RNA extraction and detection. According to the manufacturer's instructions, TRIzol reagent (Invitrogen, Canada) was used for the total RNA extraction from 100 µl of serum or a 10% fecal suspension solution (i.e., 1 g of feces dissolved into 10 ml of phosphate-buffered saline, pH 7.4). The extracted RNA was finally dissolved in 20 µl of ribonuclease (RNase)-free water. All samples were tested for positive-stranded HEV or HAV RNA by reverse transcription PCR (RT) nested polymerase chain reaction (nPCR) using specific primers (Table 1) (Fu *et al.*, 2010; Wang *et al.*, 2013). The RT-nPCR was carried out as previously described (Song *et al.*, 2016; Liu *et al.*, 2013). Negative and positive controls were included in all assays.

Sequencing and phylogenetic analysis. The targeted second-round PCR products of positive-stranded HEV or HAV RNA were

Table 1. Sequences of primers for HEV and HAV RT-nPCR

Primer*	Sequence**	Site (nt)
HEV1 (EF)	ATGTYCGYATYYTWGTCCA	5887-5905
HEV2 (IF)	TGGCGYTCKGTTGAGACCTCY	5967-5987
HEV3 (IR)	CDGCCGACGAAATCAATTCTG	6369-6349
HEV4 (ER)	GWCGGTCYTGCTCATGYTG	6549-6531
HAV1 (EF)	ATGTTACTAACAAAGTTGGAGAT	2195-2218
HAV2 (IF)	GCTCCTCTTTATCATGCTATGGAT	2172-2196
HAV3 (IR)	GATCCTCAATTGTTGTGATAGCT	2380-2357
HAV4 (ER)	CAGGAAATGTCTCAGGTACTTTCT	2415-2391

*EF = external forward; IF = internal forward; IR = internal reverse; ER = external reverse. **R = A / G; Y = C / T; S = C / G; W = A / T; K = G / T; D = G / A / T; M = A / C.

sequenced commercially (Beijing Ruibiotech Institute, China). The sizes of DNA fragments of HAV and HEV were 139 bp and 382 bp, respectively. Nucleotide sequences were assembled and analyzed using the MEGA 5.0 software. The phylogenetic trees were built through the neighbor-joining method and the interior branch test method was used to evaluate the results. When a bootstrap value is less than 70%, it is considered as not providing evidence of a phylogenetic grouping. All reference sequences representing different HAV and HEV genotypes were obtained from GenBank.

Statistical analysis. The quantitative variables were used in the unpaired *t*-test or Chi-square test by SPSS computer program (SPSS, USA). The P-value of <0.05 was taken as significant.

Results

Characteristics and seroprevalence of anti-HEV or anti-HAV IgM in patients

A total of 46 patients, including 29 men and 17 women, with a mean age of 55.7±16.4 years were included in the analysis. Among these 46 patients, 6/46 (13%) had other diseases including acute leukemia, rheumatoid arthritis, hyperthyroidism or severe pneumonia. Furthermore, 3/22 (13.6%) samples coming from patients with HEV infection (HEV group) were positive for anti-HAV IgM; 2/24 (8.3%) samples from the patients with HAV infection (HAV group) were positive for anti-HEV IgM; 5/46 (10.9%) samples were positive for both anti-HEV IgM and anti-HAV IgM (HEV/HAV group) (Table 2). Anti-HEV or anti-HAV IgM indicated the current infection with either HEV or HAV. Table 2 shows the differences in baseline characteristics between the HAV, HEV and HAV/HEV groups.

Table 2 also showed the results of laboratory tests on liver function, biochemistry and clinical symptoms of patients. The group with HAV/HEV had a higher prevalence of jaundice (P < 0.05) as compared with the groups of HAV and HEV alone. There was no significant difference in the levels of other laboratory tests between both groups.

Prevalence of HEV and HAV RNA in patients

For patients diagnosed with hepatitis E, the serum HEV RNA positive rate was 9.1% (2/22); for patients diagnosed with hepatitis A 12.5% (3/24) of the serum HAV RNA were positive; and none of them were positive for both HAV and HEV RNA (Table 2).

A phylogenetic tree based on a portion of the VP1 sequence of HAV and one based on the ORF2 sequence of HEV were constructed (Fig. 1 and 2). Phylogenetic analysis showed that all HAV detected in patients belong to genotype IA and all HEV detected in patients belong to genotype 4.

HEV and HAV RNA detection in animal fecal samples

Eighteen of 120 (15%) rabbit fecal samples and 7 of 40 (17.5%) swine fecal samples were positive for HEV RNA. All the remaining fecal samples from other animal species were negative for HEV RNA (Table 3). Only 2 of 200 (1%) ferret fecal samples were positive for HAV RNA, while the remaining ones from all other animal species were negative for HAV RNA (Table 3).

The phylogenetic trees based on the ORF2 sequence of HEV and on the VP1 sequence of HAV were constructed (Fig. 3 and 4). Their analysis showed that all HEV detected in swine belonged to genotype 4, namely subtype 4d and all HEV detected in rabbits belong to rabbit HEV strains. The phylogenetic analysis also showed that the HAV strains detected in ferrets belong to genotype IA.

Table 2. Characteristics and biochemical markers of patients with acute viral hepatitis

	HAV (n = 22)	HEV (n = 19)	HAV/HEV (n = 5)	P-value
Age (years)	32-84(55.43)	22-92(55.23)	22-66(49.60)	0.863
Sex				0.218
Male	13	10	5	
Female	9	9	0	
Jaundice (%)	9.09 (2/22)	5.26 (1/19)	60 (3/5)	0.028*
ALT (U/L)	333.57±698.72	381.33±597.48	1470.6±1180.2	0.916
AST (U/L)	178.13±324.09	273.67±343.48	745.6±487.8	0.896
Total bilirubin	67.20±102.81	94.52±129.19	267.1±157.8	0.944
anti-HAV IgM (+)	22	0	5	
anti-HEV IgM (+)	0	19	5	
HAV RNA (+)	2 (Genotype IA)	0	1 (Genotype IA)	0.158
HEV RNA (+)	0	2 (Genotype 4)	0	0.266

*Significantly different, P <0.05. ALT = alanine aminotransferase, AST = aspartate aminotransferase.

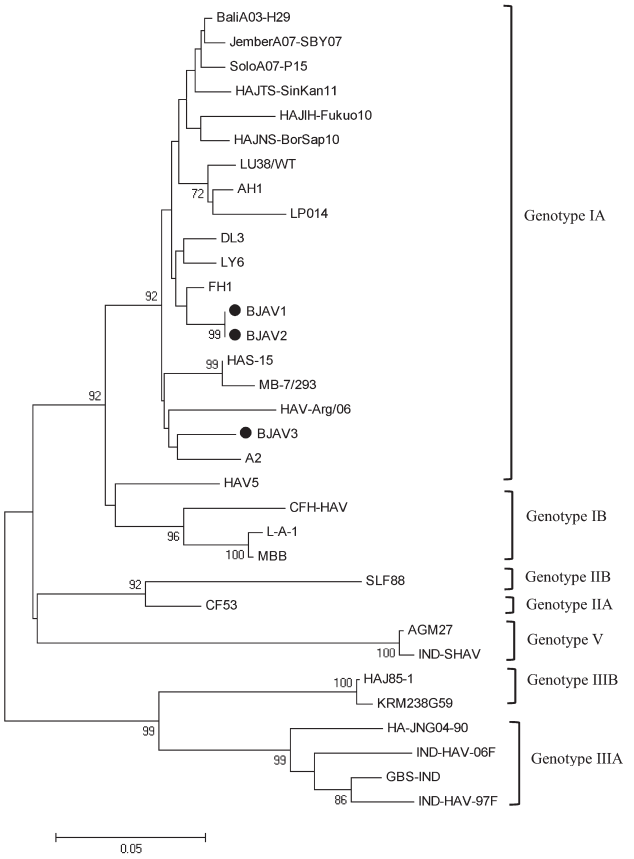

Fig. 1

Phylogenetic analysis of HAV isolates from patients

The phylogenetic tree was constructed by using the neighbor-joining method, a partial nucleotide sequence of the VP1 region and reported HAV sequences in GenBank as references. The internal node numbers indicate the bootstrap values as a percentage of trees obtained from 1000 replicates. The • indicates patients isolates obtained in this study. Scale bar indicates nucleotide substitutions per site.

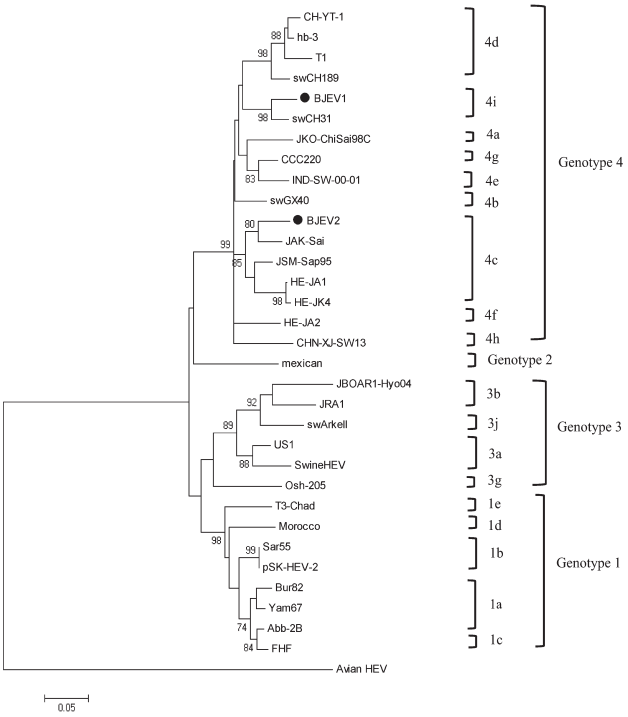

Fig. 2

Phylogenetic analysis of HEV isolates from patients

The phylogenetic tree was constructed by using the neighbor-joining method, a partial nucleotide sequence of the open reading frame 2 region, and reported HEV sequences in GenBank as references. The internal node numbers indicate the bootstrap values as a percentage of trees obtained from 1000 replicates. The • indicates patients isolates obtained in this study. Scale bar indicates nucleotide substitutions per site.

Table 3. HEV and HAV RNA detection in animal fecal samples

Animal species (scientific name)	Numbers	HEV RNA positive	HAV RNA positive	HEV/HAV RNA positive
Rabbit (*Oryctolagus cuniculus*)	120	18	0	0
Swine (*Sus scrofa domesticus*)	40	7	0	0
Dog (*Canis lupus familiaris*)	50	0	0	0
Camel (*Camelus ferus*)	70	0	0	0
Mouse (*Mus musculus*)	20	0	0	0
Tree shrew (*Tupaia belangeri*)	30	0	0	0
Mink (*Neovison vison*)	107	0	0	0
Ferret (*Mustela putorius furo*)	200	0	2	0
Fox (*Alopex lagopus*)	27	0	0	0
Monkey (*Macaca fascicularis*)	5	0	0	0
Sheep (*Ovis aries*)	6	0	0	0
Total	675	25	2	0

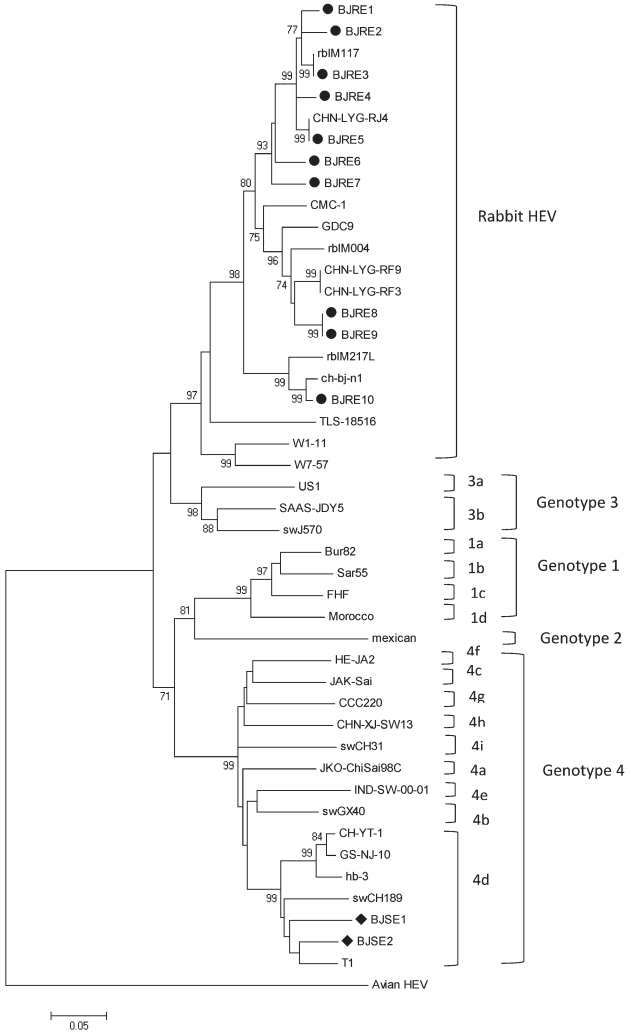

Fig. 3

Phylogenetic analysis of HEV isolates from animals
The phylogenetic tree was constructed by using the neighbor-joining method, a partial nucleotide sequence of the open reading frame 2 region, and reported HEV sequences in GenBank as references. The internal node numbers indicate the bootstrap values as a percentage of trees obtained from 1000 replicates. Rabbit HEV and swine HEV strains in this study were designated with ● and ◆, respectively. Scale bar indicates nucleotide substitutions per site.

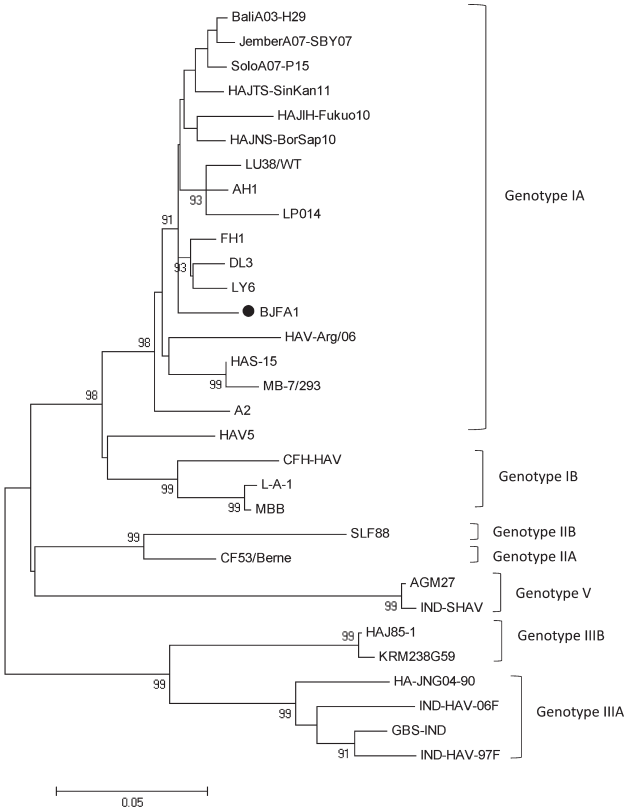

Fig. 4

Phylogenetic analysis of HAV isolates from animals
The phylogenetic tree was constructed by using the neighbor-joining method, a partial nucleotide sequence of the VP1 region, and reported HAV sequences in GenBank as references. The internal node numbers indicate the bootstrap values as a percentage of trees obtained from 1000 replicates. The HAV strain isolated from ferrets in this study were designated with ●. Scale bar indicates nucleotide substitutions per site.

Discussion

Hepatitis A and E virus are the main pathogens of acute viral hepatitis and cause health problems globally (Ankcorn and Tedder, 2017; Melhem *et al*., 2014). Although there are studies concerning the co-infection of HAV and HEV in some developing countries, only a few data are available regarding the prevalence of co-infection of HAV and HEV in Chinese patients (Joon *et al*., 2015; Rendon *et al*., 2016). This study focused on the comparison of clinical symptoms and liver function-related clinical markers with patients infected with HAV or HEV and the patients co-infected with HAV and HEV in China, in order to investigate the difference of clinical symptoms between patients infected with single virus or multiple viruses.

In our study, for HAV and HEV co-infection we regarded those patients, in whom both the anti-HAV and anti-HEV IgM were positive. The anti-HAV or anti-HEV IgM positivity is usually the clinical diagnostic criteria of infection with HAV or HEV. Our results showed that the co-infection of HAV/HEV was 10.9% (5/46) in these patients and this observation was consistent with the other studies, which showed that the co-infection of these enterically transmitted viruses (HAV/HEV) is 33.3% in Cuba 11.5% in India and 4.5% in Egypt (Joon *et al*., 2015; Rendon *et al*., 2016). However, there are fewer reports about HAV and HEV co-infection in developed countries. The spread of HAV

and HEV is mainly transmitted by the fecal-oral route and may have a same source of infection. The reason why co-infection mainly occurs in developing countries may be due to the poor sanitation, contamination of drinking water and food. In our study, samples which were positive for both anti-HAV and anti-HEV IgM were not positive for both HAV and HEV RNA, showing no co-infection of HAV and HEV technically. The reason may be the low specificity of HEV ELISA kit.

We also found that the patients co-infected with HAV and HEV had higher prevalence of jaundice than patients infected with HAV or HEV though the cases were limited. Fu *et al.* (2016) however observed that patients co-infected with multiple hepatitis viruses were more likely to have fever but the prevalence of jaundice had no difference. They suggested that fever should be an indicator for patients co-infected with multiple hepatitis viruses. In this study, the reason for the increased probability of jaundice in the HAV and HEV co-infection patients remains unclear and it may be related with the onset of jaundice and the different admission time of patients but it still needs further investigation. Our results also showed that the levels of ALT, AST, and TBil of patients co-infected with HAV and HEV had no significant difference compared with patients infected with HAV or HEV. However, there is still controversy about how the biochemical indicators and liver enzymes change in multiple hepatitis viruses infected patients (Joon *et al.*, 2015; Rendon *et al.*, 2016; Hoan *et al.*, 2015; Hamid *et al.*, 2002; Cheng *et al.*, 2013). A probable explanation for the controversial results between these studies could be the sample size used in different studies and the study area. Thus, a larger number of patients should be adopted in the further study.

The HAV strains detected in patients in our study were all classified into genotype IA. This observation was in accordance with the results of previous studies, which indicated that the epidemic HAV genotype of China is genotype IA (Cao *et al.*, 2011; Wang *et al.*, 2013). Similarly, the HEV strains detected in patients in our study all belonged to genotype 4, which is in consistence with previous study's conclusion that the epidemic HEV genotype of China is genotype 4 (Liu *et al.*, 2012). One of the HEV strains detected in patients belonged to subgenotype 4i and another HEV strain belonged to subgenotype 4c.

Hepatitis E is now considered a zoonotic disease and both domestic and wild animals could serve as potential HEV reservoirs (Meng, 2011). It has been reported that the prevalence of anti-HEV antibodies among domestic animals is high in China (Geng *et al.*, 2010; Zhang *et al.*, 2008). However, to define animal as a host for HEV, HEV RNA in animal feces must be detected, proving the replication of HEV in animal and the potential enteric transmission. The previous study demonstrated that swine and rabbits are the major hosts of HEV in China (Xia *et al.*, 2015). There have been reports showing the direct evidence that both immunocompromised and immunocompetent patients in France and Switzerland were infected by rabbit HEV (Abravanel *et al.*, 2017; Sahli *et al.*, 2019), indicating that rabbit HEV can be transmitted to humans and rabbits are zoonotic reservoir for HEV. So far there have been no reports about humans infected by rabbit HEV in China where HEV4 is the major HEV genotype. Besides, some studies also show other animal hosts for HEV like wild boars, camels and mongoose (Doceul *et al.*, 2016).

Up to date, there are fewer studies related to the animal hosts of HAV, thus the host range of HAV is still restricted in non-human primates. Therefore, whether other species of animals can be the animal reservoirs of HAV remains unclear. It has been reported that anti-HAV antibodies but not HAV RNA can be detected in the serum of pigs in natural environment and HAV can infect pigs experimentally (Song *et al.*, 2016). But these results cannot prove that these animal species are the reservoirs of HAV. Thus, in order to find the animal reservoirs of HAV, a large collection of stool samples from different animal species which have close relationships with human beings were collected in the current study and tested for both HAV and HEV RNA, that indicate that the viruses replicate in the animals and can be the evidence of the animal reservoir of HAV or HEV.

In this study, HEV RNA was only detected in rabbits and swine, indicating that they are the major reservoirs of HEV currently in China, which has been broadly confirmed. Besides, we first detected HAV RNA in 1% (2/200) of ferret fecal samples and no HAV RNA was detected in other animal fecal samples. It has been believed that HAV can only infect humans and certain nonhuman primate species under natural conditions (Balayan, 1992). The detection of HAV RNA in ferrets indicated that HAV may be able to infect and replicate in ferrets and ferrets might be the host of HAV.

In conclusion, HAV and HEV infections are still endemic either in China or worldwide. Since HAV and HEV are transmitted by similar routes, the prevention and surveillance of HAV/HEV exposure can have tremendous public health benefits. It is evident from our study, patients and animals infected with both the HAV and HEV are infrequent. In our cohort, swine and rabbits were the main animal hosts of HEV. Further, our findings suggest that ferrets might be the potential animal host of HAV. These data will be essential for the investigation of co-infection of HAV and HEV in their animal hosts in future.

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