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MOLECULAR FEATURES OF HEPATITIS E VIRUS FROM FARMED RABBITS IN SHANDONG PROVINCE, CHINA

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Abstract: This study was undertaken to investigate the genetic variability of hepatitis E virus (HEV) from farmed rabbits in Shandong province, China. A total of 50 fresh faecal samples from 5 rabbit farms were collected and subjected to reverse transcription and nested polymerase chain reaction (RT-nPCR) for a fragment sequence of HEV capsid gene. The results demonstrated that HEV RNA was observed in 6 faecal samples (6/50, 12.0%). In addition, the result of phylogenetic analysis showed that the 6 HEV isolates were classified into HEV-3 genotype with other rabbit HEV isolates from other countries, and shared 85.2-87.2%, 81.5-83.1%, and 77.0-78.6% nucleotide similarities with rabbit HEV isolates from Korea, the United States and France, respectively. To sum up, the HEV isolated in this study from farmed rabbits belongs to the HEV-3 genotype, and the zoonotic ability and pathogenesis of the rabbit HEV merit further study due to the fact that HEV-3 genotype has the potential to trigger zoonotic infections.

Key Words: HEV, farmed rabbit, RT-nPCR, phylogenetic analysis, nucleotide identity.

INTRODUCTION

Hepatitis E virus falls into the genus *Orthohepevirus* of the family *Hepeviridae.* The genus *Orthohepevirus* is composed of 4 species (*Orthohepevirus* A to *Orthohepevirus* D), and *Orthohepevirus* A comprises 7 genotypes (HEV-1 to HEV-7) (Johne *et al.,* 2014; Smith *et al.,* 2014; Krzowska-Firych *et al.,* 2018). Among the 7 genotypes, HEV-1 and HEV-2 can only infect people and the infection route is mainly associated with drinking water contaminated by HEV (Purcell and Emerson, 2008; Pérez-Gracia *et al.,* 2014; King *et al.,* 2018). In comparison, HEV-3 and HEV-4, recognised as zoonotic pathogens (Meng, 2010a, 2011), not only infect people but can also infect many animal species, such as pigs, rabbits and deer, and the main infection route may be related with HEV-contaminated animal meats or internal organs (Tei *et al.,* 2003; Yazaki *et al.,* 2003; Zhao *et al.,* 2009; Meng, 2010b; Pavio *et al.,* 2015; Li *et al.,* 2017). Wild boars have become an important reservoir for HEV-5 and HEV-6; HEV-7 has been isolated from camels, and has the potential to infect people (Woo *et al.,* 2014; Lee *et al.,* 2016; Sridhar *et al.,* 2017). Although hepatitis caused by HEV is usually self-limiting, the HEV infection can result in chronic hepatitis among patients with immunosuppression (Emerson and Purcell, 2003; Kamar *et al.,* 2008; Fujiwara *et al.,* 2014; Frias *et al.,* 2018).

So far, HEV infections among rabbits have been detected in several countries, such as China (Zhao *et al.,* 2009), the United States (Cossaboom *et al.,* 2011, 2012), France (Izopet *et al.,* 2012), Germany (Eiden *et al.,* 2016), Italy (Di Bartolo *et al.,* 2016) and Korea (Ahn *et al.,* 2017). For example, the detection rates of HEV RNA in farmed rabbit serum samples in China, the United States and Mongolia were 2-7.5%, 16%, and 71.6%, respectively (Cossaboom *et al.,* 2011; Geng *et al.,* 2011; Jirintai *et al.,* 2012; Zhao *et al.,* 2009). The prevalence of HEV RNA in wild rabbit

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liver samples was 5-23% and 60% in France and the Netherlands, respectively (Izopet *et al.,* 2012; Lhomme *et al.,* 2015; Burt *et al.,* 2016).

HEV isolates from rabbits have a genome of approximately 7.2 kb, containing 3 open reading frames, genetically close to HEV-3 (Cossaboom *et al.,* 2012; Smith *et al.,* 2014). Rabbit HEV can not only cause mild hepatitis in rabbits (Liu *et al.,* 2013), but can also replicate in A549 and PLC/PRF/5 cells of human origin (Jirintai *et al.,* 2012). In addition, scientists have found a rabbit HEV isolate (HEV-3 genotype) genetically close to human HEV in France, indicating that zoonotic transmission of the rabbit HEV-3 may be possible (Izopet *et al.,* 2012).

As an endemic country for hepatitis E (Syed *et al.,* 2018), many epidemiological investigations into anti-HEV antibody in various animal species have been carried out in China, including pigs, deer, chickens, goats, ferrets, minks and cows (Zheng *et al.,* 2006; Geng *et al.,* 2011; Zhang *et al.,* 2015; Huang *et al.,* 2016; Li *et al.,* 2017; Shuai *et al.,* 2017; Wang *et al.,* 2018). However, to our best knowledge, relatively little information about genetic variability of HEV isolated in farmed rabbits has been reported in China to date. Therefore, the current study aimed to investigate whether HEV RNA was present or diffuse in farmed rabbits in Shandong province, China, and to further investigate the molecular features.

MATERIALS AND METHODS

Collection and treatment of rabbit faeces

From March to July, 2017, fresh faecal samples were randomly obtained from 50 New Zealand white breed rabbits from 5 rabbit farms in Shandong province, China (10 rabbits from each farm). The rabbits were raised for meat consumption. The rabbits were between 8 and 10 wk (mean 9 wk) of age, and the animals reared in the 5 farms appeared to be healthy.

A sample of 2 grams of fresh faeces taken from an individual rabbit was mixed with 20 mL of phosphate-buffered saline (PBS) and then centrifuged for 30 min at 1200×*g*. The supernatant was centrifuged for 10 min at 16000×*g*. The supernatant was stored at −70°C for RNA extraction.

RT-nPCR and sequencing

Based on the manufacturer's instructions, 200 µL of supernatant was used to extract HEV RNA with a RNA kit (Invitrogen, USA). Subsequently, a specific primer targeting the HEV capsid gene was used to amplify cDNA using a commercial RT-PCR kit (Invitrogen, USA). The generated cDNA was stored at −20°C for further study.

A reverse transcription and nested polymerase chain reaction (RT-nPCR) method was used to detect the partial fragment of capsid gene of HEV (Ahn *et al.,* 2017). The first PCR reaction condition with specific external primers (Table 1) was as follows: 95°C (5 min), 40 cycles of 95°C (30 s), 50°C (30 s), and 72°C (30 s), followed by an extension for 10 min at 72°C. Subsequently, the first PCR product was subjected to the second PCR with specific internal primers (Table 1), and the amplification condition was as follows: 95°C (5 min), 35 cycles of 95°C (30 s), 56°C (30 s), and 72°C (30 s), followed by an extension for 10 min at 72°C. The second PCR products were first cloned into a Cloning Vector, and then transformed into Competent Cells DH-5α. The positive plasmid was selected and sequenced.

Table 1: Primers used in this study for detecting a fragment sequence of Rabbit HEV.

Primers	Sequence (5'→3')	Location*
External forward primer	CCG ACA GAA TTG ATT TCG GC	6,376-6,398
External reverse primer	CAR AGT GAC YTT AGA CCA ATC AAG	6,766-6,789
Internal forward primer	GTC TCA GCC AAT GGC GAG CC	6, 427-6,429
Internal reverse primer	GCR CCT GTT GCS ACA TTR ACA AAT	6,723-6,746

*Primer location was expressed according to the genome of reference strain HEV FJ906895.

Phylogenetic tree construction and identity analysis

A phylogenetic tree was constructed in this study using the neighbour-joining method, following the instructions of the MEGA 7.0 software package. Reference nucleotide sequences of different HEV genotypes were obtained from GenBank. The nucleotide sequence identity of HEV was also analysed using Bioedit 7.0 software.

RESULTS

A total of 50 fresh manure samples were obtained from 5 rabbit farms in Shandong province, China. The RT-nPCR results showed that 6 out of 50 samples (6/50, 12.0%) were positive for the fragment of HEV capsid gene (243 bp). The 6 HEV positive samples came from 3 rabbit farms (3/5, 60.0%) (Table 2).

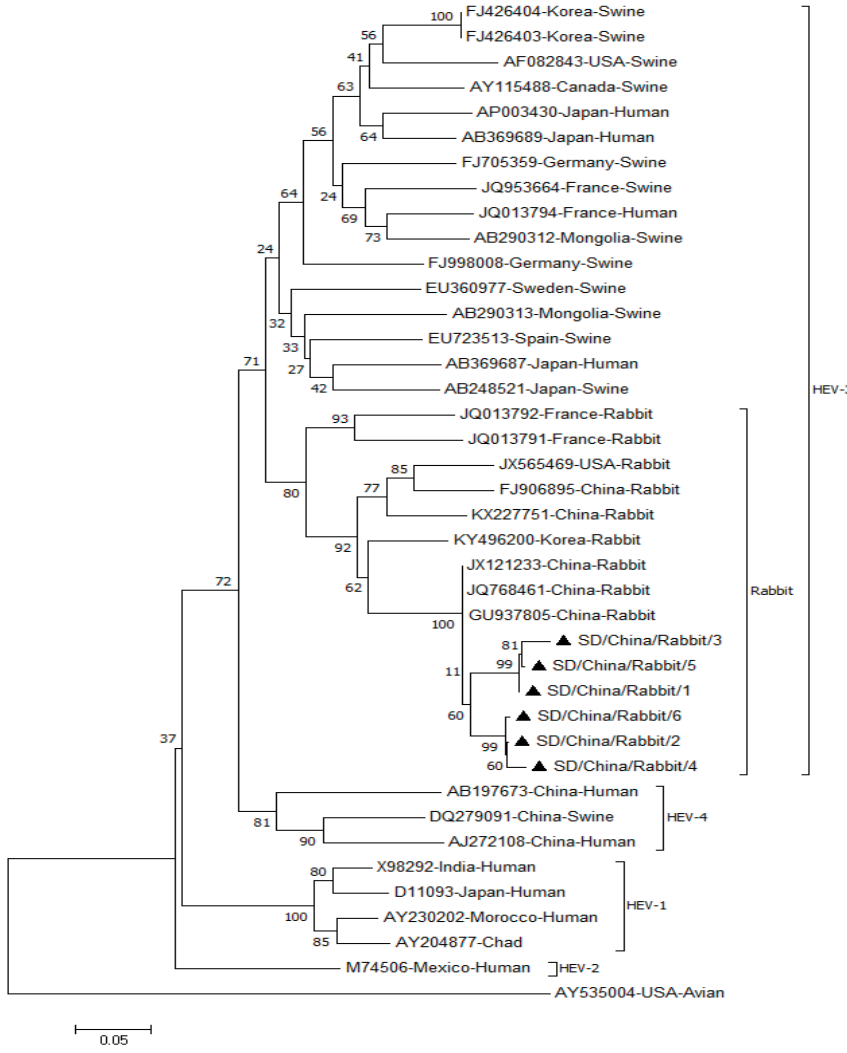

Figure 1: Phylogenetic analysis of a fragment of capsid gene of rabbit HEV. The 6 rabbit HEV isolates in this study are indicated as black triangles.

Table 2: Positive HEV RNA rates in faecal samples of farmed rabbits.

Rabbit farms	Number of faecal sample	Number of HEV RNA-positive samples	Positive rate (%)
A	10	2	20.0
B	10	1	10.0
C	10	0	-
D	10	3	30.0
E	10	0	-
Total	50	6	12.0

Phylogenetic Analysis and nucleotide identity showed that these 6 HEV isolates, designated as SD/China/Rabbit/1-6, were classified into HEV genotype 3 (Figure 1), and shared 85.2–87.2%, 81.5–83.1%, and 77.0–78.6% nucleotide similarities with rabbit HEV isolates from Korea, the United States and France, respectively.

DISCUSSION

Numerous studies have been conducted in several countries to investigate the genetic variability of HEV RNA in rabbits (Zhao *et al.,* 2009; Cossaboom *et al.,* 2011; Izopet *et al.,* 2012; Jirintai *et al.,* 2012), and the HEV RNA shared close similarity with the HEV-3 genotype (Zhao *et al.,* 2009; Ahn *et al.,* 2017; Kaiser *et al.,* 2018; Ryll *et al.,* 2018). Similarly, HEV RNA isolated in this study can be classified into HEV genotype 3.

In addition, a study carried out in Germany showed that HEV RNA was detected in the archived sera in 1989 (Eiden *et al.,* 2016). The research result indicated that HEV infections in rabbits may have occurred long before rabbit HEV was first detected in China (Zhao *et al.,* 2009).

In the current study, the occurrence of HEV in farmed rabbits was detected by RT-nPCR. The results showed that 3 out of 5 sampled rabbit farms (60.0%) were positive for HEV and 12.0% of the collected faecal samples carried HEV RNA. In the United States and Korea, HEV strains have also been found in 15.0 and 17.0% of manure samples of farmed rabbits, respectively (Cossaboom *et al.,* 2011; Ahn *et al.,* 2017). In addition, a previous study conducted in Beijing, China reported that 6 out of 492 faecal samples of farmed rabbits (5.0%) were positive for HEV RNA (Xia *et al.,* 2015). The discrepancy of detection rate of HEV RNA in faecal samples in different studies may be associated with many factors, such as rabbit breed, rabbit age and rearing environments (Cossaboom *et al.,* 2011).

The sampling size in this study was relatively small (only 50 samples from 5 rabbit farms), which was a major limitation of this study, but the findings to some extent reflected the occurrence of HEV in farmed rabbit faeces in Shandong province, China. In addition, it is noteworthy that rabbit HEV isolated in this study belonged to genotype 3 HEV, which has the potential to cause zoonotic infection (Lhomme *et al.,* 2013). Thus, the risk for cross-species and zoonotic infections with rabbit HEV should be evaluated, and the need to carry out extensive molecular epidemiological research into rabbit HEV merits serious consideration.

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