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Short communication

Rabbit hepatitis E virus is an opportunistic pathogen in specific-pathogen-free rabbits with the capability of cross-species transmission

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ABSTRACT

Hepatitis E virus (HEV) has been detected in rabbits, a recently identified natural reservoir. In this study, anti-HEV antibodies and viral RNA were detected in rabbits sourced from a specific-pathogen-free (SPF) rabbit vendor in Shaanxi Province, China. BLAST results of partial HEV ORF2 genes cloned here indicated that two viral strains circulated in the rabbits. Sequence determination of the complete genome (7302 bp) of one strain and a partial ORF1 gene (1537 bp) of the other strain showed that they shared 90% identity with one another and 78%–94% identity with other known rabbit HEVs. In addition, inoculation with rabbit HEV from SPF rabbits studied here resulted in infection of SPF pigs; this cross-species transmission was evidenced by seroconversion, viremia and faecal virus shedding. These results suggest that to prevent spread of this zoonotic pathogen, rabbits should be tested routinely for HEV RNA in SPF vendor facilities.

1. Introduction

Hepatitis E is an acute, generally self-limiting disease that causes up to 25% mortality in infected pregnant women (Labrique et al., 2012). Hepatitis E virus (HEV) is the causative agent of hepatitis E and belongs to the *Hepeviridae* family including *Orthohepevirus* and *Piscihepevirus* genera (Smith et al., 2014). Within the *Orthohepevirus* genus, four species, designated A-D, infect a wide range of mammalian species (Cossaboom et al., 2011; Meng, 2010; Purcell et al., 2011; Raj et al., 2012; Zhao et al., 2010). Those HEV strains which are able to infect humans have been classified as *Orthohepevirus A* species and are divided into four major genotypes (1–4) (Okamoto, 2007). Currently HEV genotypes 3 and 4 are considered to be zoonotic viruses and are found mainly in pigs and wild boars, their natural reservoir (Purcell and Emerson, 2008).

HEV has been identified in farmed and pet rabbits (Caruso et al., 2015) and these rabbit HEVs are most closely related to genotype 3 strains (Cossaboom et al., 2011). To date, more and more evidences suggested that rabbits may be a new reservoir for HEV (Liu et al., 2013; Ma et al., 2010). Recently, HEV IgG antibodies and partial ORF2 gene were detected in specific-pathogen-free (SPF) rabbits from the China and USA, but the complete genome and the virus showing cross-species infection were not documented (Birke et al., 2014; Wang et al., 2016). In the present study, HEV antibodies and partial ORF2 genes were also detected from a SPF rabbit vendor located in the Shaanxi Province of China. Subsequently, a complete genome of one rabbit HEV strain and a partial ORF1 gene of another strain were amplified and compared with known human and animal HEVs. In addition, an animal experiment was carried out to confirm that the HEV strain from SPF rabbits can successfully cross species barriers and infect pigs.

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2. Materials and methods

2.1. Animal and samples

Eighty sera and 52 faeces were collected from SPF rabbits from the Experimental Animal Center, College of Medicine, Xi'an Jiaotong University (Xi'an, Shaanxi Province, China). This vendor with high quality biosafety provides SPF rabbits for use in animal physiology and anatomy instruction at nearby colleges and universities.

To confirm whether the rabbit HEV from the SPF rabbits can infect across species, six SPF pigs aged 6–8 weeks negative for HEV antibodies and RNA were purchased from HARBIN Veterinary Research Institute, CAAS for the animal experiment.

2.2. ELISA to detect anti-HEV IgG antibodies in sera

Anti-HEV IgG antibodies in the 80 serum samples were detected using two indirect ELISA protocols. One ELISA protocol, employing a commercial kit designed for testing of human sera, used the genotype 1 human HEV ORF2 protein as the coating antigen (Shenyang et al., 2011) (Wantai Biological Pharmacy Co., Beijing, China). The other ELISA, previously developed in-house for testing swine sera, used the genotype 4 swine HEV ORF2 protein as the coating antigen (Wang et al., 2014). Because HEV genotypes 1–4 belong to a single serotype, both indirect ELISAs were used to test anti-HEV IgG antibodies in the rabbit serum samples studied here. The commercial ELISA kit protocol was conducted using the manufacturer's instructions, while the in-house indirect ELISA was conducted according to a protocol described previously (Wang et al., 2014). Briefly, after blocking 96-well flat bottom plates with coating antigen, 100 μl of each rabbit serum sample, diluted 1:100 in 1 mM phosphate-buffered saline (PBS, pH 7.4), was added into each well of the coated plates. The aforementioned protocols were modified by substitution of secondary antibody with horseradish peroxidase-conjugated (HRP) goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) at a 1:5000 dilution. Colour was developed using 3, 3′, 5, 5′-tetramethylbenzidine (TMB; Sigma Chemical Co., St. Louis, MO, USA) for 15 min and stopped with 3 M H_2SO_4. The mean optical density at 450 nm (OD_{450nm}) was read using an automated ELISA plate reader (Bio-Rad, Hercules, CA, USA).

2.3. Detection of HEV RNA from the samples by RT-nPCR

Rabbit HEV RNA in faecal samples was tested using a reverse transcription nested PCR (RT-nPCR). Fecal samples were prepared as 10% (w/v) suspensions diluted in PBS and clarified by centrifugation at 10,000 × *g* for 40 min. Viral RNA was extracted from a 200 μl faecal suspension volume using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The primers (Table 1) were synthesised for amplification of the partial ORF2 gene (348 bp) of rabbit HEV, as previously described (Geng et al., 2011). Reverse transcription was performed at 50 °C for 30 min using reverse primer and SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Five microlitres of the resulting cDNA was amplified in a 50 μl reaction using Platinum PCR SuperMix High Fidelity (Invitrogen) using two-round nested PCR. The annealing temperatures of both first-round and second-round PCR reactions were both 55 °C and each round was carried out for 39 cycles. Second-round PCR products were electrophoresed on 0.8% agarose gels and positive bands of predicted size were purified with EasyPure Quick Gel Extraction Kit (TransGen Biotech Co., Beijing, China), then sequenced using an ABI3730 Genetic Analyzer (JinSiTe Biotech Co., Nanjing, China). For genetic identification of rabbit HEVs from the faecal samples, the sequences were analysed using BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST).

Table 1
Primers used to test hepatitis E virus RNA and amplify HEV genome in SPF rabbits.

Primer[a]	Sequence, 5′ → 3′[b]	Position[c]
SEBO2	TCYAATTAYGCCCARTAYCGGGTTG	5767–5791
SEEO2	CCCTTRTCYTGCTGYGCRTTCTC	6478–6500
SEBI2	GTYATGCTYTGYATCCATGGCT	6055–6076
SEEI2	AGCCGACGAAATYAATTCTGTC	6381–6402
RHEV-F1	TGAGCCCTGGGCTTGTACA	5068–5086
RHEV-F2	CTGAAACTGTGAAGCCTGTG	5137–5156
RHEV-F3	ACCAGCTGGCTGAGGAGC	3841–3858
RHEV-R3	CGAAGGGGTTGGTTGGATGA	5397–5416
RHEV-F4	CTGATATAGTCCAT(C)TGCCGC(T)	3979–3998
RHEV-R4	ATCAACCCTGTCACCCCAGA	5351–5370
RHEV-F5[d]	GGGTCCCTGTTTGAGTCA(T)GA	2397–2416
RHEV-R5[d]	TC(T)GTG(T)GCCTGCACAGGGCCGAT	4125–4146
RHEV-F6[d]	GCCTCTGTCATGCCTTT(C)TACC	2467–2487
RHEV-R6[d]	GCCATGCGGCAATGGACTATA	3983–4003
RHEV-F7	CCCTGTCCATATTTGGGA	971–988
RHEV-R7	CGAGAA(G)CAAGTCTCACGGT	2632–2650
RHEV-F8	CTGCTGCTCGCGGCTTATGA	1031–1050
RHEV-R8	GGGCAACCGCATGAATGA	2569–2586
RHEV-F9	AGACCACGTATGTGGTCG	4–21
RHEV-R9	AAGCCAGCTATAG(A)AGCCTG	1250–1268
RHEV-F10	CATGGAGGCCCACCAGTTTA	26–45
RHEV-R10	GTAACGCTGGTGGCAGATG	1163–1181
RHEV-R11	AGTACCACCGCTGGACGTCTCTA	362–384
RHEV-R12	GCTCCGACCTCAAGACAACG	279–298

[a] Primers SEBO2, SEEO2, SEBI2, and SEEI2 were synthesised to test for the presence of HEV RNA in rabbit faecal samples as previously described by Geng et al. (Geng et al., 2011). Primers RHEV-F3 to RHEV-R4, RHEV-F5 to RHEV-R6, RHEV-F7 to RHEV-R8, and RHEV-F9 to RHEV-R10 were used to amplify the second, third, fourth, and fifth fragment of the complete genome. Primers RHEV-F1 and RHEV-F2 were used to amplify the extreme 3′ genomic sequence (first fragment) with the 3′ RACE kit. Primers RHEV-R11 and RHEV R12 were used to amplify the extreme 5′ genomic sequence (sixth fragment) with the 5′ RACE kit. Oligo$(dT)_{18}$ primers were used as reverse transcription primers.

[b] Sequences of primers were designed according to the sequences of 6 other known rabbit HEV strains.

[c] Positions of primers located in the complete genome are shown according to the rabbit HEV strain GDC9 from China (GenBank accession no. FJ906895).

[d] The four primers (RHEV-F5 to RHEV-R6) were also used to amplify the 1537-bp sequences of the other rabbit HEV strain using RT-nPCR.

2.4. Amplification of the complete genome and partial ORF1 genes of rabbit HEVs

The complete genomic sequences of one rabbit HEV strain were amplified using RT-nPCR to create six overlapping fragments. For the other isolate, only partial sequences of ORF1 gene were amplified, due to the small number of available clinical samples. Primers were designed based on the six rabbit HEV full-length sequences previously deposited in the GenBank database (Table 1). The RT-nPCR was performed according to the manufacturers' instructions using both SuperScript III Reverse Transcriptase and Platinum PCR SuperMix High Fidelity (Invitrogen). To obtain the complete genome, the 5′- and 3′-terminal noncoding regions of the genome were amplified using SMARTer® rapid amplification of cDNA ends (RACE) 5′/3′ Kit, respectively (TaKaRa, Dalian, Liaoning, China) and the PCR reactions were performed as per the manufacturer's instructions. All PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA) and cloned into the TA cloning vector pMD18-T (TaKaRa). The sequences were collected using an ABI3130 Genetic Analyzer Automated Sequencer (Applied Biosystems, Foster City, CA, USA).

2.5. Sequence analysis

For comparisons of the sequences of partial ORF2 gene from the 24 positive samples, primer binding sites were excluded and a 304-