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Short Communications

Current status of hepatitis E virus infection at a rhesus monkey farm in China

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ABSTRACT

Rhesus and several other species of monkeys are susceptible to genotypes of hepatitis E virus (HEV), and these species are thus commonly used as animal models for experimental HEV infection. However, information regarding HEV infection in monkeys in nature or at monkey farms is limited. To investigate the status of HEV infection in rhesus monkeys at farms, we collected 548 serum and 48 fecal samples from a rhesus monkey farm in China, and analyzed their levels of anti-HEV IgG antibodies and HEV RNAs. An enzyme-linked immunosorbent assay using genotype 3 HEV-like particles as antigen revealed anti-HEV IgG-positivity in 388 (70.8%) monkeys. The antibody-positive rates in the 1-year-old and 2-year-old monkeys were significantly lower than those in monkeys > 3 years old. The antibody-positive rate was greatly increased from 7.4% in the 2-year-old monkeys to 100% in the 3-year-olds, suggesting that the latter received HEV infection at a high frequency. HEV RNA was detected in one of 88 sera from 1- and 2-year-old monkeys and 10 of 48 fecal specimens from 3-year-old monkeys by reverse transcription-polymerase chain reaction. Phylogenetic analyses revealed that the HEV strain RmKM15 was present in a serum sample that belonged to subtype 4b in genotype 4, whereas 10 strains detected in the fecal specimens belonged to subtype 4 h, suggesting that two genetically different strains were circulating at the farm. However, no significant clinical signs were observed in these monkeys. Further studies are required to identify the source of infection and to evaluate the pathogenicity of HEV in rhesus monkeys.

1. Introduction

Hepatitis E is a public health concern in many Asian and African countries where sanitation conditions are insufficient (Emerson and Purcell, 2003). In industrialized countries, clinical cases of hepatitis E usually occur sporadically (Meng, 2010). Hepatitis E is caused by hepatitis E virus (HEV) infection, which is transmitted primarily by the fecal-oral route (Balayan et al., 1983). HEV is a positive-sense single-stranded RNA virus that belongs to the genus *Hepevirus* in the family *Hepeviridae* (Smith et al., 2014).

In humans, hepatitis E is caused mainly by four genotypes of HEV, genotype 1 (G1) to genotype 4 (G4) (Meng, 2010). In addition to human HEV, many HEV-like viruses have been identified in animals, including wild boars, rabbits, rats, minks, moose, ferrets, red foxes, camels, kestrel, little egrets, chickens, bats, and cutthroat trout (Rasche et al., 2016; Reuter et al., 2016a, b; Smith et al., 2014). HEV in the family *Hepeviridae* has been divided into two genera, *Orthohepevirus* and *Piscihepevirus* (Smith et al., 2014). Because many species of animals, such as pig, wild boar and wild deer, are known to serve as a reservoir of G3 and G4 HEV, zoonotic infection is another route of HEV infection (Li et al., 2005; Meng et al., 1997; Tei et al., 2003). To date, sporadic zoonotic infections have been attributed to each of G3, G4, and G7 HEV (Lee et al., 2016).

Several species of monkeys, including Japanese, rhesus, and cynomolgus monkeys, are susceptible to HEV infection and are frequently used as an animal models for experimental HEV infection (Li et al., 2004; Liu et al., 2013; Tsarev et al., 1995; Yamamoto et al., 2012). Since HEV causes a zoonotic infection, the possibility of HEV infection during the breeding and rearing of monkeys should be considered. In fact, HEV antibodies have been detected in wild Japanese monkeys (36.2%) and imported cynomolgus (10.5%) and rhesus monkeys (3.6%) in Japan (Hirano et al., 2003), and in wild rhesus (36.7%), bonnet (19.1%) and langur monkeys (2%) in India (Arankalle et al., 1994). Although HEV RNA has not been detected in these monkeys, these

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Table 1
Primers used to amplify the entire genome of HEV subtype 4b.

Primers	Sequences	Products
Forward primer F1 (1-21)*	5'- GCAGACCACGTATGTGGTCG-3'	
Reverse primer R497 (497-518)	5'-GAGACAGGTATTGCTCTTTACT-3'	518 bp
Forward primer F445 (445-464)	5'-TGCTGATCGGACGTATTGTT-3'	
Reverse primer R1442 (1423-1442)	5'-AGCAGCAGAACTTGTGAGCT-3'	998 bp
Forward primer F1364 (1364-1383)	5'-CGTGTGCTTGTCTTTGATGA-3'	
Reverse primer R2337 (2317-2337)	5'-ACATTGGTCGAAGTGGGACTA-3'	947 bp
Forward primer F2145 (2145-2165)	5'-TATCAGGCTTTTCGAGTTGCT-3'	
Reverse primer R3264 (3244-3264)	5'-AGACCGGCATGCTCAAAATCA-3'	1120 bp
Forward primer F3172 (3172-3192)	5'-CCTACTCCTGTTGCATATGCA-3'	
Reverse primer R4289 (4270-4289)	5'-AGGTTTTGCTCCACGCAGAT-3'	1118 bp
Forward primer CS (4187-4209)	5'-TCGCGCATCACMTTYTTCCARAA-3'	
Reverse primer CASN (4598-4623)	5'-CCAGGCTCACCRGARTGYTTCTTCCA-3'	436 bp
Forward primer F4496 (4496-4515)	5'-ATCATGGAGGAATGTGGGAT-3'	
Reverse primer R5347 (5347-5366)	5'-AAGGGGTTGGTTGGATGAAT-3'	871 bp
Forward primer F5196 (5196-5216)	5'-TCTTCTGTTTCTGCTCCTCGT-3'	
Reverse primer R6060 (6060-6026)	5'-GTAAGGCGTATTAGTATAAGA-3'	831 bp
Reverse primer 044 (5943-5969)	5'-CAAGGHTGGCGYTCKGTTGAGAC-3'	
Reverse primer TX30SXN	5'-GACTAGTTCTAGATCGCGAGCGGCCGCCCTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3'	~1300 bp

* Numbers in parentheses show the positions of the primers corresponding to the entire genome of a swine HEV (EU676172).

observations suggest that HEV could infect monkeys in nature. Indeed, an outbreak tentatively attributed to G3 HEV was reported at a Japanese monkey facility in Japan (Yamamoto et al., 2012). However, the source of HEV infection in monkeys remains elusive.

Since the monkeys used in animal experiments are mainly obtained from monkey farms, it is necessary to determine whether the monkeys at these facilities are infected with HEV. To investigate the status of HEV infection in rhesus monkeys at monkey farms, we collected 548 serum and 48 stool samples from a rhesus monkey farm in China, and we analyzed the presence of anti-IgG antibodies and RNA genomes in the samples.

2. Materials and methods

2.1. Serum and fecal specimens of rhesus monkeys

Serum samples were collected at a rhesus monkey farm in Kunming City, Yunnan Province, China from December 2017 to May 2018; nearly 3000 monkeys are maintained at this facility. At this farm, the monkeys are weaned at 6 months after birth, then raised together in an indoor feeding facility. For the first 6 months in the facility, they are kept in individual cages containing two to three monkeys, then for the next 2 years they are kept in large cages containing 12 to 15 monkeys. When they reach 3 years of age, the monkeys are moved to an outdoor feeding facility. The ages of the monkeys ranged from 1 to 22 years, and 11–61 serum samples were collected from each group; because there were few monkeys older than 18 years, the 18- to 22-year-old monkeys were treated as one group.

A total of 548 serum samples were collected from 1- to 22-year-old monkeys, and 48 fecal specimens were collected from 3-year-old monkeys. The fecal specimens were diluted with 10 mM phosphate-buffered saline (PBS) to prepare a 10% (w/v) suspension. The suspension was shaken at 4 °C for 1 h, clarified by centrifugation at 10,000 *g* for 30 min, passed through a 0.45-μm membrane filter (Millipore, Bedford, MA), and stored at −80 °C until use. All of the experiments were reviewed by the Ethics Committee of Institute of Medical Biology, Chinese Academy of Medical Science and carried out according to the guidelines for humane treatment of animals.

2.2. Detection of anti-HEV IgG antibody

Anti-HEV IgG antibodies were detected by an enzyme-linked immunosorbent assay (ELISA) using G3 HEV-like particles (HEV-LPs) as the antigen (Xing et al., 2010). Briefly, flat-bottom 96-well polystyrene microplates (Immulon 2; Dynex Technologies, Chantilly, VA) were coated with the purified G3 HEV-LPs (1 μg/ml, 100 μl/well) and incubated at 4 °C overnight. The wells were washed twice with 10 mM PBS containing 0.05% Tween 20 (PBS-T) and then blocked at 37 °C for 1 h with 200 μl of PBS-T containing 5% skim milk (Difco Laboratories, Detroit, MI).

After the plates were washed three times with PBS-T, the monkey serum (100 μl/well) was added in duplicate at a dilution of 1:200 in PBS-T containing 1% skim milk. The plates were incubated at 37 °C for 1 h, and then washed three times as described above. The wells were incubated with 100 μl of peroxidase-conjugated goat anti-monkey IgG-heavy and light chain antibody (Bethyl Laboratories, Montgomery, TX). The peroxidase-conjugated antibody was diluted 1:10,000 with PBS-T containing 1% skim milk.

The plates were incubated at 37 °C for 1 h, and washed three times with PBS-T. Then 100 μl of the substrate orthophenylenediamine (Sigma Chemicals, St. Louis, MO) and H_2O_2 were added to each well, and the plates were incubated in a dark room at room temperature for 30 min. The reaction was stopped with 50 μl of 4 N H_2SO_4, and the absorbance at 492 nm was measured with a microplate reader (Molecular Devices, Tokyo). The cut-off value for anti-HEV IgG antibody was calculated as the mean value of the optical density (OD) plus three times the standard deviation (SD).

2.3. Nested reverse transcription-polymerase chain reaction (RT-PCR) for the detection of HEV RNA

The RNA was extracted from 200 μl of the serum or 10% stool suspensions using a MagNA Pure LC system with a MagNA Pure LC Total Nucleic Acid isolation kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's recommendations. Reverse transcription was performed with a high-capacity cDNA reverse transcription kit (ABI Applied Biosystems, Carlsbad, CA) under a protocol of 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min in a 20-μl reaction mixture containing 1 μl reverse transcriptase, 2 μl of the random primer, 1 μl RNase inhibitor, 2 μl RT buffer, 0.8 μl 10 mM deoxynucleoside triphosphates, 8 μl RNA and 5.2 μl distilled water. Then 5 μl of cDNA was subjected to a nested RT-PCR that targeted a portion of the open reading frame 2 (ORF2) as described previously (Mizuo et al., 2002).

To amplify the entire genome of HEV, reverse transcription (RT) was performed by using Superscript™ II RNase H⁻ reverse transcriptase (Invitrogen, Carlsbad, CA) and a primer TX30SXN (Table 1). The full-length HEV genome was amplified by RT-PCR with the primers based

on the nucleotide sequences of the G4 HEV strain (GenBank no. EU676172) (Table 1). All of the PCR products were purified using a QIAquick Gel extraction kit (Qiagen, Valencia, CA). The nucleotide sequencing was carried out using an ABI 3130 Genetic Analyzer automated sequencer (Applied Biosystems, Foster City, CA). The sequence analysis was performed using the Genetyx ver.11.0.4 software program (Genetyx, Tokyo), and multiple alignments were generated using the CLUSTAL W software program ver. 1.8.1. Phylogenetic trees were constructed by the neighbor-joining method.

3. Results

3.1. High positive rates of anti-HEV IgG antibody detected in monkeys

We used a total of 548 serum samples from rhesus monkeys for the detection of anti-HEV IgG antibody at a dilution of 1:200. Because there is little difference in antigenicity among the HEV genomes G1, G3, G4, G5, G6, and G7 in *Orthohepevirus* A, we used G3 HEV-LPs as the antigen to detect the antibody (Li et al., 2015, 2017; Zhou et al., 2015). Since younger monkeys have less opportunities to be exposed to HEV, we examined 88 serum samples collected from 1-year-old and 2-year-old monkeys in the indoor breeding facility to determine the cut-off value of the ELISA.

The distributions of the OD values of 88 serum samples are shown in Fig. 1A. The OD values ranged from 0.018 to 3.453, and except for four samples with values > 0.452, all of the values were under 0.235. Because these four serum samples were positive for anti-HEV IgG by western blotting (data not shown), we used the remaining 84 serum samples to determine the cut-off value. The mean OD value of anti-HEV IgG antibody in the serum samples was an 0.053 with an SD of 0.045. The cut-off value was thus calculated as 0.188 on the basis of the mean OD values plus three times the SD ($0.053 + 3 \times 0.045$).

Our examination of the antibody in the serum samples revealed that 70.8% (388/548) of the samples were positive for anti-HEV IgG antibody (Fig.1B). The positive rate of the low-age group (1-year-olds and 2-year-olds) was 9.1% (8/88), and this rate was significantly higher at 82.6% (380/460) in the monkeys > 3 years old ($p < 0.01$) (Fig.1B). The comparison of the IgG-positive rate in each age group demonstrated higher positive rates in the 3-, 4-, 5- and 6-year-old group with 100%, 97.9%, 100% and 92.5% positive rates, respectively, and then the positive rate tended to decrease with age. These results confirmed that HEV infection undoubtedly occurred at the rhesus monkey farm. The positive rate of anti-HEV IgG was greatly increased from 7.4% in 2-year-old monkeys to 100% in 3-year-old monkeys, indicating that 3-year-old monkeys have a higher risk of HEV infection on this farm.

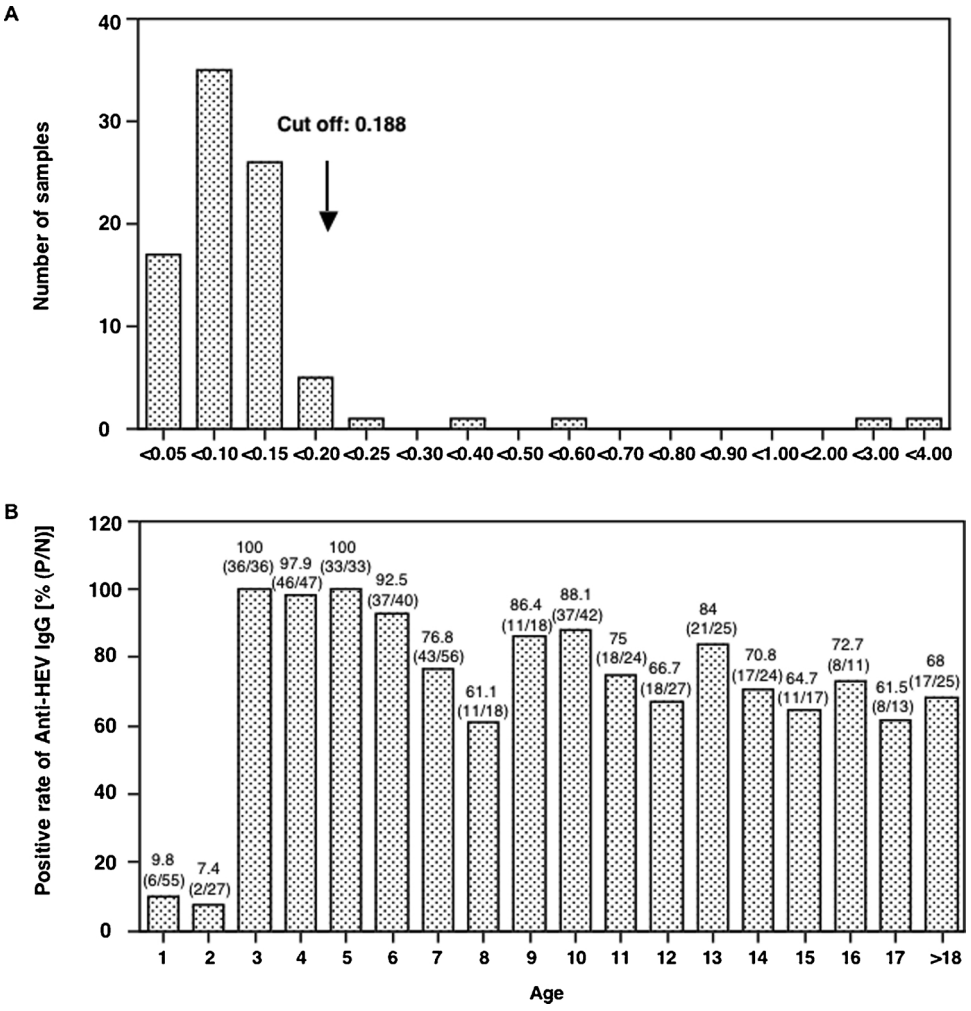

Fig. 1. Detection of anti-HEV IgG antibody in rhesus monkeys. Anti-HEV IgG antibody in monkey sera was detected by an ELISA using G3 HEV-LPs as the antigen. A total of 88 serum samples from 1- and 2-year-old monkeys were tested, and the number of the samples in each OD value was plotted. The arrow indicates the cut-off value (**A**). The anti-HEV IgG antibody was detected by ELISA using 548 serum samples from all age groups of rhesus monkeys, and the antibody-positive rate in each age group is shown (**B**).

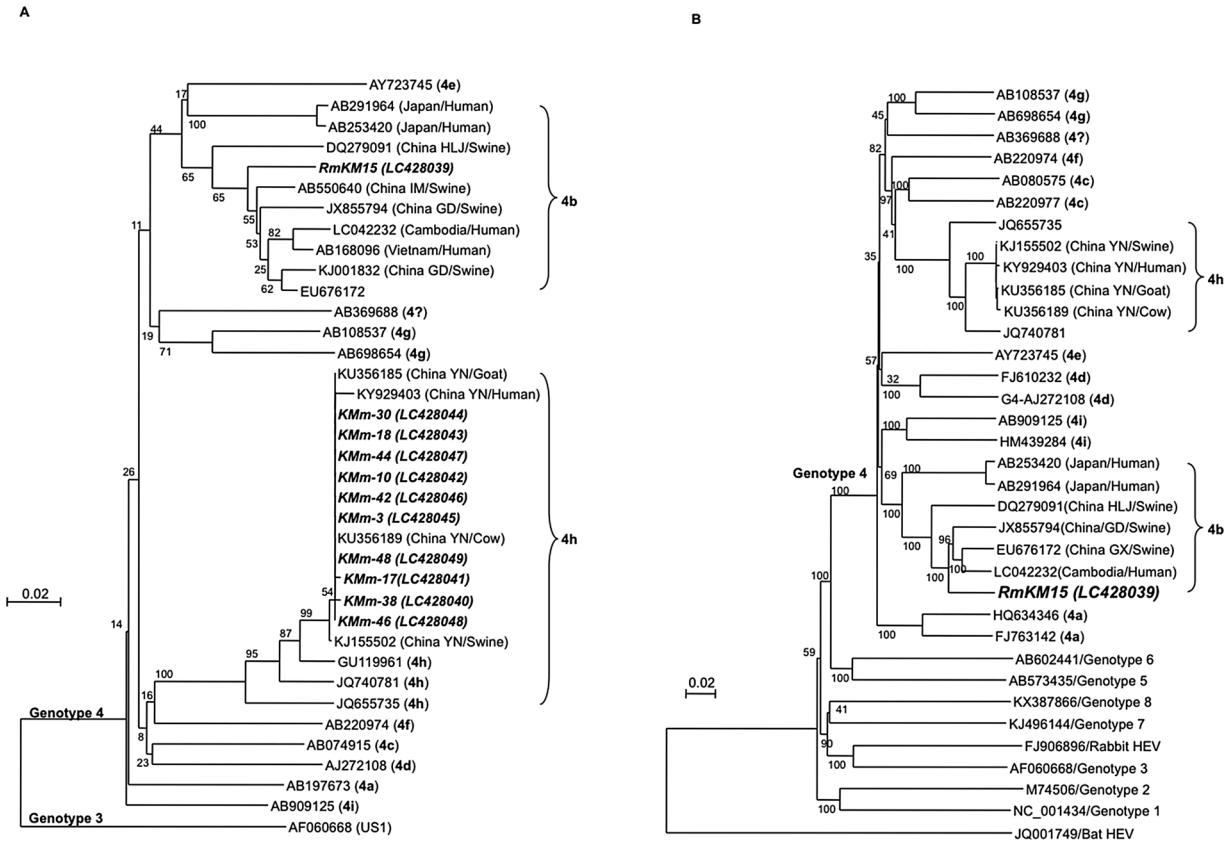

Fig. 2. Phylogenetic analyses of HEV genomes detected in rhesus monkeys. Phylogenetic trees with 1000 bootstrap replicates were generated by the neighbor-joining method based on the partial genome (412 nt) of the HEV ORF2 region (**A**) and the entire HEV genome (**B**). The nucleotide sequence alignment was performed using Clustal X 1.81. The genetic distance was calculated by Kimura's two-parameter method. The scale bar indicates the nucleotide substitutions per site. The HEV strains detected in the present study are shown in bold italic letters. HLJ, Heilongjiang Province; IM, Inner Mongolia; GD, Guangdong Province; GX, Guangxi Province; YN, Yunnan Province.

3.2. Monkeys infected with G4 HEV

A total of 88 serum samples from 1- and 2-year-old monkeys and 48 fecal specimens from 3-year-old monkeys were used for the detection of HEV RNA by RT-PCR. One of the 88 serum samples and 10 of the 48 fecal specimens were positive for HEV RNA. A phylogenetic analysis based on the partial sequences of ORF2 (412 bp) indicated that all of the strains belonged to G 4 HEV (Fig. 3A). The HEV strains KMm-3, -10, -17, -18, -30, -38, -42, -44, -46 and -48 (GenBank accession nos. LC428040–LC428049) were detected in the 3-year-old monkeys grouped into subtype 4 h, and they shared 99.8%–100% nucleotide sequence identities. The HEV strains isolated from humans, swine, cows and goats in the same area, Yunnan Province in China, were classified into the same subtype 4 h, and they shared 99.5%–100% nucleotide sequence identities with the above 10 strains (Fig. 2A), strongly suggesting that these HEV strains were transmitted from the local animals. In contrast, strain RmKM15 detected in the 2-year-old monkeys belonged to subtype 4b, demonstrating that at least two genetically different G4 strains were circulating at the farm (Fig. 2A).

As all of the subtype 4 h HEV genomes were detected after the second round of nested RT-PCR, the copy number of these strains in the fecal specimens was low, and we failed to amplify the entire genome of the subtype 4 h HEV strains.

3.3. The entire genome of subtype 4b HEV detected from rhesus monkeys

A BLAST analysis showed that the partial ORF2 sequence of RmKM15 is most similar to strain EU676172, which was detected from swine in Guangxi Province, China, and the nucleotide sequence identity between them was 94.1%. We thus amplified the full-length genome of RmKM15 by performing an RT-PCR with the primers based on the nucleotide sequences of EU676172 (Table 1).

A total of nine overlapping PCR products ranging from 436 bp to 1300 bp covered the entire genome of the RmKM15 strain. The sequence analyses revealed that the genomes of RmKM15 consisted of 7212 nucleotides (nt) and a poly (A) tail except for the 5′-terminal 21-bp sequence used as the primer (GenBank accession no. LC428039). The RmKM15 strain contains three ORFs: ORF1 (5–5122 nt) encoding 1704 aa, ORF2 (5161–7143 nt) encoding 660 aa as observed in other known G4 HEVs, and ORF3 (5153–5491 nt) encoding 112 aa. Although the sequences of the 5′-terminal untranslated region (5′UTR) could not be identified, the 3′UTR contained 69 nucleotides.

A phylogenetic analysis based on the entire genome indicated that RmKM15 belonged to subtype 4b along with the strains detected from patients in Cambodia (LC042232) and Japan (AB253420 and AB291964) and from swine in China (Fig. 2B). The detection of the subtype 4b strains from swine has been reported in several provinces in China including Guangdong (JX855794), Guangxi (EU676172), and Heilongjiang (DQ279091) provinces, but these strains had never been reported from Yunnan. In addition, RmKM15 shares 87.1%–94.1% nucleotide sequence identities with other subtype 4b strains, suggesting that this strain is a unique HEV strain circulating at the rhesus monkeys farm in Yunnan Province.

4. Discussion

Monkeys are an important animal model for experimental HEV infection, and many monkeys have been imported from Asian countries

for this purpose. However, cynomolgus monkeys recently imported from China and Cambodia have a high positive rate for anti-HEV IgG (unpublished. data), and it is difficult to obtain monkeys that are HEV-free. To understand the current status of HEV infection during breeding and rearing, we collected serum and fecal specimens at a rhesus monkey farm. We found that the positive rate of anti-HEV IgG was as high as 70.8%, and the monkeys older than 3 years appeared to have an 82.6% positive rate, indicating that a considerable number of rhesus monkeys have been exposed to HEV at this farm. However, none of the monkeys showed significant clinical signs including jaundice, high levels of ALT or AST, vomiting, or fever, indicating that the clinical signs of the monkeys are largely different from those of humans.

After monkeys are born at this farm, they are weaned at 6 months, separated from their parent monkeys, and raised together in an indoor feeding facility. When the monkeys reach 3 years old, they are moved to an outdoor feeding facility and raised with other monkeys > 3 years old. The outdoor feeding facility at this farm is built on a hill surrounded by a high brick fence, and thus natural reservoirs of HEV such as pigs and wild boars cannot enter the facility. The researchers and animal caretakers are required to wear protective equipment including a cap, mask, coat, gloves, and boots to prevent the invasion of pathogens when they enter the farm's facilities.

However, birds and rats have sometimes penetrated the farm's outdoor facility to steal bait, and thus we cannot rule out the possibility that birds and/or rats carried HEV into the facility. The present study's phylogenetic analysis showed that HEVs detected in the monkeys bred in the outdoor facility share as high as 99.5%–100% nucleotide sequences identities with those from swine, cows and goats in this area (Huang et al., 2016; Long et al., 2017; Yu et al., 2014), suggesting that HEV might be transmitted from local animals, and further spread by oral-fecal routes in the farm. These results indicated that the outdoor breeding facility has a high risk for HEV infection.

The RmKM15 strain detected in the 2-year-old monkeys belongs to subtype 4b, and the entire genome sequence of this strain shares 84.1%–84.4% nucleotide sequence identities with strains isolated from humans, swine, cows, and pigs in Yunnan Province, strongly suggesting that this strain is not transmitted from local reservoirs. RmKM15 shows < 94.1% nucleotide sequence identity with other subtype 4b strains, indicating that this strain is a new HEV strain; the transmission route of RmKM15 is not yet known. Since some wild rhesus monkeys were captured and used for breeding at this farm, further studies are needed to determine whether the RmKM15 was transmitted from wild rhesus monkeys.

Because monkeys are an important animal source for experimental infection and vaccine development and are used not only for HEV but also other viruses, the monkey farms are required to control the viral pathogens and to keep the monkeys free from viruses. In the present study, we found that the positive rates of anti-HEV IgG were < 10% among the 1- and 2-year-old monkeys, suggesting that these animals are at a relatively low risk for HEV infection. The separation of the monkeys into small groups post-weaning could be a way to reduce the risk of HEV infection.

Conflict of interest

None to declare.

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