SEROLOGIC EVIDENCE FOR HEPATITIS E VIRUS INFECTION IN MONGOOSE

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Abstract. Although pig and wild boar are considered to be the reservoirs of hepatitis E virus (HEV) in Japan, the spread of HEV to other animals is unknown. Serum samples from 84 mongooses (Small Asian mongoose; Herpestes javanicus) collected in Okinawa, Japan were examined for antibodies to HEV by enzyme-linked immunosorbent assay and RNA was analyzed by reverse transcription–polymerase chain reaction. Seven (8.3%) of 84 mongooses were positive for IgG antibodies to HEV, and the antibody-positive rate increased with body weight and size, whereas HEV RNA was not detected in these samples. These results are consistent with the possibility that mongooses in Okinawa are occasionally infected with HEV; however, they are not considered the major zoonotic reservoir of HEV.

INTRODUCTION

Hepatitis E virus (HEV) is the most important cause of acute hepatitis in many developing countries in Asia, the Middle East and north Africa,1 and was recently classified as the sole member of the genus Hepevirus in the family Hepaeviridae.2 Hepatitis E was first recognized when a large waterborne hepatitis outbreak occurred in India in 1955, where the antibody-positive rate of hepatitis A virus is extremely high in all age groups.3 Because HEV is transmitted via an oral-fecal route, contaminated drinking water and food are the primary source of the infection. Although hepatitis E is self-limiting and neither chronic nor persistent infection is observed in the adult population in general, a high mortality rate of 15–20% is reported in pregnant women.2

Hepatitis E virus is a nonenveloped, single-stranded positive-sense RNA virus.5 Phylogenetic analysis has identified at least four major genotypes of HEV.6 Genotype 1 (G1) HEV was isolated from Asia and Africa,1,4 genotype 2 (G2) from Mexico,7 and genotypes 3 (G3) and 4 (G4) from the United states, European countries, China, Japan, and Vietnam.8–15 These viruses are believed to compose a single serotype.14

Although most cases were imported into developed countries, recent studies have shown that hepatitis E occurred in patients who had never been abroad.9,10 Genetically similar G3 and G4 HEVs isolated from pigs, deer, and wild boars,8,15,16 and serum antibodies to HEV in a variety of animals including pigs, deer, wild boars, wild rats, dogs, cats, and cows17,18 suggest that hepatitis E is a zoonosis. Recently, direct evidence of G3 HEV transmission from deer and wild boar meats to humans was clearly provided in Japan, suggesting that wild animals are the zoonotic reservoir of HEV in this country.15,16 Transmission from visceral organs of pigs to humans has also been suspected.19

Okinawa is located southwest of Japan, where mongoose (Small Asian mongoose; Herpestes javanicus), an exotic animal, was introduced from India in 1910 for the control of a poisonous snake (habu) and rats. The number of mongooses and their living area increased quickly because there was no natural predator of this animal in Okinawa. This caused a disruption of the ecology. Since wild boars also live on this island, mongooses have an opportunity to be exposed to HEV from infected boars.

MATERIALS AND METHODS

Mongoose sera. Wild H. javanicus (54 males and 30 females) were captured between July 2004 and May 2005 in Okinawa. The mongooses were transferred to the laboratory and anesthetized. Their sex was identified, and their body weights and head and body length (body size) were measured. Blood was collected by cardiac puncture under anesthesia, and allowed to clot at room temperature for one hour. The serum fraction was collected by centrifugation and stored at −20°C until use.

Preparation of recombinant virus-like particles. A recombinant baculovirus, Ac5480/7126, that harbors the G1 HEV capsid protein gene with a 111-amino-acid deletion at the N-terminal was constructed as previously described.20 Briefly, Tn5 cells (High Five™; Invitrogen, Carlsbad, CA) were infected with Ac5480/7126 at a multiplicity of infection of 10 and incubated at 26.5°C for 7 days. The intact cells and cell debris were removed from the culture medium, and the recombinant virus-like particles (VLPs) with a molecular mass of 53 kD were concentrated by centrifugation at 100,000 × g for 2 hours in an SW28 rotor (Beckman Instruments, Inc., Fullerton, CA). The VLPs were further purified by isopycnic binding in a CsCl gradient.21 Recombinant baculoviruses that express N-terminal truncated capsid proteins of G3 and G4 HEV were similarly prepared, and the 53-kD VLPs were also prepared (Li T-C and others, unpublished data).

Detection of antibodies to HEV in mongoose. Flat-bottom 96-well polystyrene microplates (Immulon 2; Dynex Technologies, Inc., Chantilly, VA) were coated with the purified VLPs (1 µg/mL, 100 µL/well). The plates were incubated overnight at 4°C. Unbound VLPs were removed, and the wells were washed twice with 10 mM phosphate-buffered saline containing 0.05% Tween 20 (PBS-T) and blocked at 37°C for 1 hour with 200 µL of 5% skim milk (Difco Laboratories, Detroit, MI) in PBS-T. After the plates were washed four times with PBS-T, mongoose 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 hour and washed four times as described above. The wells were incubated with 100 μL of peroxidase-conjugated goat anti-cat IgG (heavy plus light chain) (Kirkegaard and Perry Laboratories, KPL, Gaithersburg, MD) or anti-cat IgM (Kirkegaard and Perry Laboratories) containing 1% skim milk. The plates were incubated at 37°C for 1 hour and washed four times with PBS-T. One hundred microliters of substrate (o-phenylenediamine; Sigma Chemical Co., St. Louis, MO) and H2O2 was added to each well. The plates were incubated in the dark at room temperature for 30 minutes and 50 μL of 4 N H2SO4 was added to each well. After the plates were incubated at room temperature for 10 minutes, the absorbance at 492 nm was measured.

**Western blot assay.** Approximately 1 μg of VLPs was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skim milk in 50 mM Tris–HCl, pH 7.4, and 150 mM NaCl and incubated with mongoose serum (1:500 dilution), followed by horseradish peroxidase (HRP)-conjugated goat anti-cat IgG (heavy plus light chain) (1:1,000 dilution). The membrane was treated with electrogenerated chemiluminescence detection reagent (Amersham Biosciences, Piscataway, NJ) according to the manufacturer’s instruction and exposed to FP-3000B45 film (Fuji, Tokyo, Japan).

**Detection of HEV RNA by reverse transcription–polymerase chain reaction (RT-PCR).** Total RNA was extracted with RNAzol-LS reagent (Tel-Test Inc., Friendswood, TX) using 200 μL of the mongoose serum and resuspended in 20 μL of DNase-, RNase-, and proteinase-free water. Reverse transcription was performed at 42°C for 50 minutes and 70°C for 15 minutes with 1 μL of oligo (dT) primer, 1 μL of superscript II reverse transcriptase (Gibco-Bethesda Research Laboratories, Gaithersburg, MD), 0.5 μL of 0.1 M dithiothreitol, 4 μL of 5x reverse transcription buffer, and 1 μL of 10 mM deoxyribonucleoside triphosphates. Two microliters of the resulting cDNA were amplified in a 50-μL nested PCR with ExTaq DNA polymerase (Takara Shuzo Co. Ltd., Kyoto, Japan) and an external sense primer HEV-F1 (5’-GGBGTBGCGAGGACGAGGC-3’, nucleotide residues 5903-5922 of the G1 Myanmar strain D10330) and an antisense primer HEV-R1 (5’-TGYTGGTTRTCRTARTCCTG-3’, nucleotide residues 6316-6467 of the G1 Myanmar strain D10330) using the GeneAmp PCR System 9700 (Perkin Elmer Biosystems, Foster City, CA). Each cycle consisted of the denaturation at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds, and extension at 72°C for 60 seconds, followed by a final extension at 72°C for 7 minutes. The nested PCR was conducted with the internal sense primer HEV-F2 (5’-TAYCGHAAYCAAGGHTGGC-3’, nucleotide residues 5939-5958) and the internal antisense primer HEV-R1 (5’-CGACGAAAATYATCTCTGTCG-3’, nucleotide residues 6316-6297) using the same conditions.16

**RESULTS**

**Detection of mongoose IgG and IgM with anti-cat IgG and IgM.** Because no peroxidase-conjugated antibody to mongoose IgG or IgM is commercially available, we explored the cross-reactivity of mongoose IgG and IgM with those of other animals. Since the mongoose is in the family Herpestidae order Carnivora, the reactivity between mongoose IgG or IgM and anti-cat IgG or IgM was evaluated.

Two-fold dilutions of pooled mongoose sera were used to coat the microplate. After blocking, peroxidase-conjugated goat anti-cat IgG or IgM was added to determine whether anti-cat antibodies are capable of binding to the mongoose antibodies. Peroxidase-conjugated goat anti-rabbit IgG or IgM was added to the control wells. As shown in Figure 1, the mongoose serum reacted with both HRP-goat anti-cat IgG and IgM. The control well did not show any reactivity with these antibodies. These results indicated that the anti-cat antibodies cross-reacted with mongoose IgG and IgM, and that the HRP-goat anti-cat IgG and IgM are useful as the second antibody in detecting mongoose IgG and IgM by enzyme-linked immunosorbsent assay (ELISA).

**Detection of IgG and IgM antibodies to HEV in mongoose sera.** The mongoose serum samples were tested for IgG and IgM antibodies to HEV at a dilution of 1:200 by ELISA. The distribution of optical density (OD) values is shown in Figure 2. The OD values for IgM antibodies to HEV ranged from 0.09 to 0.321, and one serum sample with a titer of 200 had an OD value greater than 0.20. The OD values for IgG antibodies to HEV ranged from 0.011 to 3.751, and 7 sera whose titers ranged from 200 to 12,800 had OD values greater than 0.20 (Table 1).

![Figure 1](image-url)  
**Figure 1.** Detection of mongoose IgG and IgM with anti-cat IgG and IgM by enzyme-linked immunosorbsent assay. Two-fold dilutions of pooled sera from 10 mongooses were used to coat a 96-well microplate. The reactivity of peroxidase-conjugated goat anti-cat IgG (○) and IgM (□) or horseradish peroxidase-conjugated goat anti-rabbit IgG (△) and IgM (▼) was measured. OD492 = optical density at 492 nm.
To determine whether the IgG antibody detected in mongoose sera was specific for HEV, nine serum samples were selected and examined by Western blot assay. The G1, G3, and G4 VLPs were separated by SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane. A serum dilution of 1:500 was used for the assay. As shown in Figure 3, strong bands with a molecular mass of 53 kD corresponding to G1, G3, and G4 VLPs were detected in serum samples 8, 39, 52, and 65. The OD values of these sera ranged from 2.529 to 3.751, as determined by ELISA. Weak bands were detected with in serum samples 52 and 65 whose OD values were 0.383 and 0.387, respectively. No bands were detected in serum samples 60, 41, and 10, which had low OD values, 0.204, 0.175, and 0.387, respectively. When this value was used, the prevalence of IgG antibodies to HEV was 8.3% (7 of 84). The antibody-positive rate was 3.3% in females and 11.1% in males; however, the difference between sexes was not statistically significant ($P > 0.05$). The average body weight and body size were 565.3 grams (range = 182.5–1,037.9) and 553.8 mm (range = 402–654), respectively.

When the antibody-positive rate was analyzed according to body weight, the antibody-positive rate for IgG to HEV was 11.1% in animals with body weights of 500–599 grams, 12.5% in animals with body weights of 600–699 grams, 11.1% in animals with body weights of 700–799 grams, 20% in animals with body weights of 800–899 grams, and 33.3% in animals with body weights greater than 900 grams. IgG antibody to HEV was not detected in animals with body weights less than 500 grams. Thus, the antibody-positive rate increased with body weight.

The antibody-positive rate for IgG to HEV was 11.1% in animals with a body size of 550–599 mm and 21.2% in animals with a body size of 600–654 mm. IgG antibody to HEV was not detected in animals with body sizes less than 500 mm, which shows that antibody-positive rate also increased with the body size. The mean ± SD OD value of IgM antibody to HEV in these 78 sera was 0.062 ± 0.031. The cutoff value was 0.155 (0.062 + 3 × 0.031). When analyzed with this cutoff value, one mongoose serum sample (no. 8) was positive for IgM antibody; the prevalence rate was 1.2% (1 of 84).

**Detection of HEV RNA by RT-PCR.** All 84 mongoose serum samples tested by RT-PCR were negative for HEV RNA.

**DISCUSSION**

The mongoose is a small, cat-like carnivore that is a member of the family *Herpestidae*. It is between one and four feet in length, and inhabits in Asia, the Caribbean, and southern Europe, comprising more than 30 species. Although the small Asian mongoose was introduced into Okinawa for the control of habu snakes and rats, this animal eats not only habu snakes and rats, but also other small animals.

An ELISA with recombinant HEV VLPs was used to detect IgG and IgM antibodies to HEV in the mongoose. This assay was capable of detecting antibodies to HEV in human sera with high sensitivity and specificity. Although only one serotype was recognized and four genotypes were identified in HEV, we used G1, G3, and G4 VLPs as antigens to compare the reactivity. No significant difference was found between the genotypes. The specificity of the ELISA was confirmed by Western blot assay. Among seven samples positive for IgG antibodies to HEV by ELISA, one sample (no. 60) was be negative by Western blot assay, a result that was probably due to a low antibody titer in the ELISA (OD value of the 1:200 serum dilution = 0.204 and antibody titer = 200) and the lower sensitivity of the Western blot assay.
In this study, only one mongoose was positive for IgM antibodies to HEV and the titer was low (200). We were not able to amplify any HEV sequence in these 84 mongoose serum samples, including the sample positive for IgM antibodies to HEV. Therefore, the genotype of the HEV-infected mongoose in Okinawa is unknown. In experimentally infected monkeys, the period of the viremia was very short, and HEV RNA was detected in serum only 1–2 weeks after seroconversion. This could be one of the reasons why the HEV genome was not detected in serum.

The prevalence of antibodies to HEV was high in domestic pigs and wild boar; they are considered to be possible reservoirs of HEV in Japan. In contrast, the prevalence of IgG antibodies to HEV in mongooses (8.3%) was lower than that in pigs (58%), wild rats (44–94%), or wild boars (44%) (Li T-C and others, unpublished data). This finding suggests that mongooses may not be the major reservoir of HEV in Okinawa, but may occasionally be infected with HEV. The current source and route of infection are not clear. Since many wild boars inhabit Okinawa and were eventually infected with HEV, the virus might spread to the surrounding environment by means of animals’ stool. Mongooses also inhabit the region that wild boars inhabit, and might thus be exposed to HEV. Because mongooses catch insects, crabs, worms, lizards, and other small creatures for food, transmission of HEV may occur if these creatures are infected. The positive rate for IgG antibody to HEV increases with body weight and size, indicating that the exposure to HEV increases with age.

It has been reported that HEV is excreted in low concentrations by humans, and that a small amount of HEV is excreted in experimentally infected monkeys. This may explain the lower efficiency of the transmission of HEV, and may result in the lower prevalence of antibodies to HEV in the mongoose.

In summary, mongooses in Okinawa were infected with HEV; however, the source of infection, the routes of transmission, and their genotype are still unknown. Further serologic and genetic investigations with larger number of specimens are needed.

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