Short Communication

High Prevalence of Hepatitis E Virus Infection in Imported Cynomolgus Monkeys in Japan

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SUMMARY: Cynomolgus monkeys are important experimental animals for hepatitis E virus (HEV) infection. In Japan, cynomolgus monkeys are mainly imported from Asian countries for use at animal facilities and institutions. However, the status of HEV infection in cynomolgus monkeys remains unclear. Overall, 187 pairs of serum and fecal samples were collected from cynomolgus monkeys (*Macaca fascicularis*) imported from China and Cambodia to detect anti-HEV immunoglobulin (Ig) G and IgM antibodies, as well as HEV RNA. Based on an enzyme-linked immunosorbent assay using HEV-like particles derived from genotype 3 HEV as the antigen, 183 of 187 (97.9%) and 102 of 187 (54.5%) samples tested positive for anti-HEV IgG and IgM antibodies, respectively. In contrast, all 45 serum samples collected from cynomolgus monkeys bred and grown at the Tsukuba Primate Research Center, Japan tested negative for both antibodies. However, real-time quantitative reverse transcription polymerase chain reaction detected no HEV RNA in any of the 187 serum and fecal samples. These results strongly indicated that HEV infection is common in imported cynomolgus monkeys. A source of HEV-free monkeys for HEV studies is urgently needed.

Hepatitis E virus (HEV) is a non-enveloped, positive-sense, single-stranded RNA virus belonging to the genus *Hepevirus* in the family *Hepeviridae* (1). Based on nucleotide sequence divergence, hepeviruses are divided into 2 genera: *Orthohepevirus* and *Piscihepevirus*. The orthohepeviruses are further subdivided into 4 distinct species, *Orthohepevirus* A–D (1). *Orthohepevirus* A contains 8 genotypes, genotype 1 (G1) to G8, including HEV strains isolated from humans, monkeys, swine, wild boar, deer, camels, mongooses, and rabbits (2,3). In humans, hepatitis E is mainly caused by 4 HEV genotypes (G1 to G4), and many species, such as pig, wild boar, wild deer, monkeys, and rabbits, serve as reservoirs of G3 or G4 HEV (4–6). Zoonotic infections are another important route of HEV infection. Recently, the increasing incidence of hepatitis E associated with zoonotic infection has drawn public attention in industrialized countries.

Several species of monkeys, including Japanese, rhesus, and cynomolgus monkeys, are susceptible to HEV infection and are frequently used as animal models for experimental HEV infection and vaccine development (7–9). These monkeys are particularly useful as nonhuman primate models to evaluate the possibility of zoonotic HEV infection. In fact, the

potential for zoonotic infection with G5, G7, G8, and rabbit HEV has been suggested based on infection experiments using cynomolgus monkeys (*Macaca fascicularis*) (10–13).

In Japan, cynomolgus monkeys are imported from Asian countries for animal experiments; however, the status of HEV infection in these monkeys is unclear. To understand the current status of HEV infection in imported cynomolgus monkeys, we collected a pair of serum and fecal samples from each of 187 monkeys to detect anti-HEV immunoglobulin (Ig)G and IgM antibodies, as well as HEV RNA.

The 3- to 4-year-old cynomolgus monkeys used in this study included 123 from farm A and 19 from farm B, both in China, as well as 30 monkeys from Cambodia. All these monkeys were imported to Japan in 2017, except for 4 from farm A and 11 from farm B, which were imported in 2018. Serum and fecal samples were collected from individual monkeys upon their arrival in Japan. The fecal specimens were diluted with 10 mM of phosphate-buffered saline (PBS) to prepare a 10% (w/v) stool suspension. The suspension was shaken at 4°C for 1 h, clarified by centrifugation at 10,000 × g for 30 min, and passed through a 0.45µm membrane filter (Millipore, Bedford, MA, USA). The serum samples and stool suspensions were stored at –80°C until use. The serum samples were diluted to 1:200, and the antibodies were detected by an enzyme-linked immunosorbent assay (ELISA) using G3 HEV-like particles (HEV-LPs) as the antigen, as described previously (14). The anti-HEV IgG was detected using horseradish peroxidase (HRP)-conjugated goat anti-monkey IgG heavy- and light-chain antibody (Bethyl Laboratories, Montgomery, TX, USA), and anti-HEV IgM antibody was detected using

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HRP-conjugated goat anti-monkey IgM (μ) antibody (KPL, Gaithersburg, MD, USA). Both goat antibodies were diluted (1:10,000) with 10 mM of PBS containing 0.05% Tween 20 (PBS-T) and 1% skim milk (Difco Laboratories, Detroit, MI, USA).

In addition to the 187 serum samples, 45 serum samples were collected from cynomolgus monkeys bred and grown at the Tsukuba Primate Research Center, Japan and used for antibody detection at the same dilution. The distributions of the optical density (OD) values of anti-HEV IgG and IgM antibodies are shown in Fig.1A and B, respectively. The OD values of anti-HEV IgG and IgM antibodies of the serum samples

from the Tsukuba Primate Research Center ranged from 0.016 to 0.149 and 0.025 to 0.192, respectively, and no sample provided a notably large OD. Therefore, these 45 samples were used to determine the cutoff for ELISA. The mean OD of anti-HEV IgG antibody in the serum samples was 0.044, with a standard deviation (SD) of 0.046, and the cutoff was calculated as 0.182 based on the mean OD plus 3 times the SD ($0.044 + 3 \times 0.046$). Similarly, the mean OD of anti-HEV IgM antibody in the serum samples was 0.056 with a 0.048 SD, and the cutoff for IgM antibody was calculated as 0.200 ($0.056 + 3 \times 0.048$).

The OD values of anti-HEV IgG and IgM antibodies

Fig. 1. Detection of anti-HEV IgG and IgM antibodies in cynomolgus monkeys. Anti-HEV IgG (A) and IgM (B) antibodies were detected by ELISA. The number of samples in each OD was plotted. White bars, serum samples from the Tsukuba Primate Research Center; Shaded bars, serum samples from Farm A, China; Blue bars, serum samples from Farm B, China; Red bars, serum samples from Cambodia. The arrows indicate the cutoff values.

detected in the serum samples from the imported monkeys ranged from 0.038 to 3.589 and 0.042 to 1.554, respectively. As shown in Fig.1, 97.9% (183/187) and 54.5% (102/187) of the imported cynomolgus monkeys tested positive for anti-HEV IgG and IgM antibodies, respectively. The anti-HEV IgG positivity rates were 100% (127/127) in the monkeys from farm A, 86.7% (26/30) in those from farm B in China, and 100% (30/30) in those from Cambodia. The anti-HEV IgM positivity rates were 53.5% (68/127) in the monkeys from farm A, 56.7% (17/30) in those from farm B, and 56.7% (17/30) in those from Cambodia. Only 2.1% (4/187) of the monkeys tested negative for both anti-HEV IgG and IgM antibodies. These 4 monkeys were imported from farm B in 2017. These findings indicated that HEV infection is common in imported cynomolgus monkeys. It is noteworthy that the serum samples from the 45 monkeys from the Tsukuba Center tested negative for anti-HEV IgG and IgM antibodies.

The RNA was extracted from 200 µL of the serum or 10% stool suspensions, and the HEV RNA was quantified using real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) (15). However, all the fecal and serum samples, including anti-HEV IgM-positive samples, tested negative for HEV RNA. The samples were presumably collected after the acute phase of HEV infection, and HEV RNA was undetectable on RT-qPCR.

Imported cynomolgus monkeys are an important source of experimental animals for HEV studies. Unfortunately, our results showed that 97.9% of the imported monkeys were exposed to HEV. Since no feeding or environmental information about these monkey facilities was available, we could not identify the sources of the infection. Our findings revealed that very few monkeys could be used for experimental HEV infection.

In our previous study, we identified an outbreak of G3 HEV infection at a Japanese monkey facility (9) and found a high prevalence (70.8%) of G4 HEV infection in a rhesus monkey farm in China; the HEV infection was spread via oral-fecal routes in monkey farms (14). Considering the previous and present results together, we conclude that HEV infection is common in monkey farms and that a source of HEV-free monkeys for HEV studies is urgently needed.

Because monkeys are used as an animal model of experimental infection and vaccine development for not only HEV but also other viruses, further studies are required to clarify the status of infection with other

viruses in imported monkeys. Moreover, monkey farms must control viral infection and protect monkeys from particular viral pathogens.

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Conflict of interest None to declare.

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