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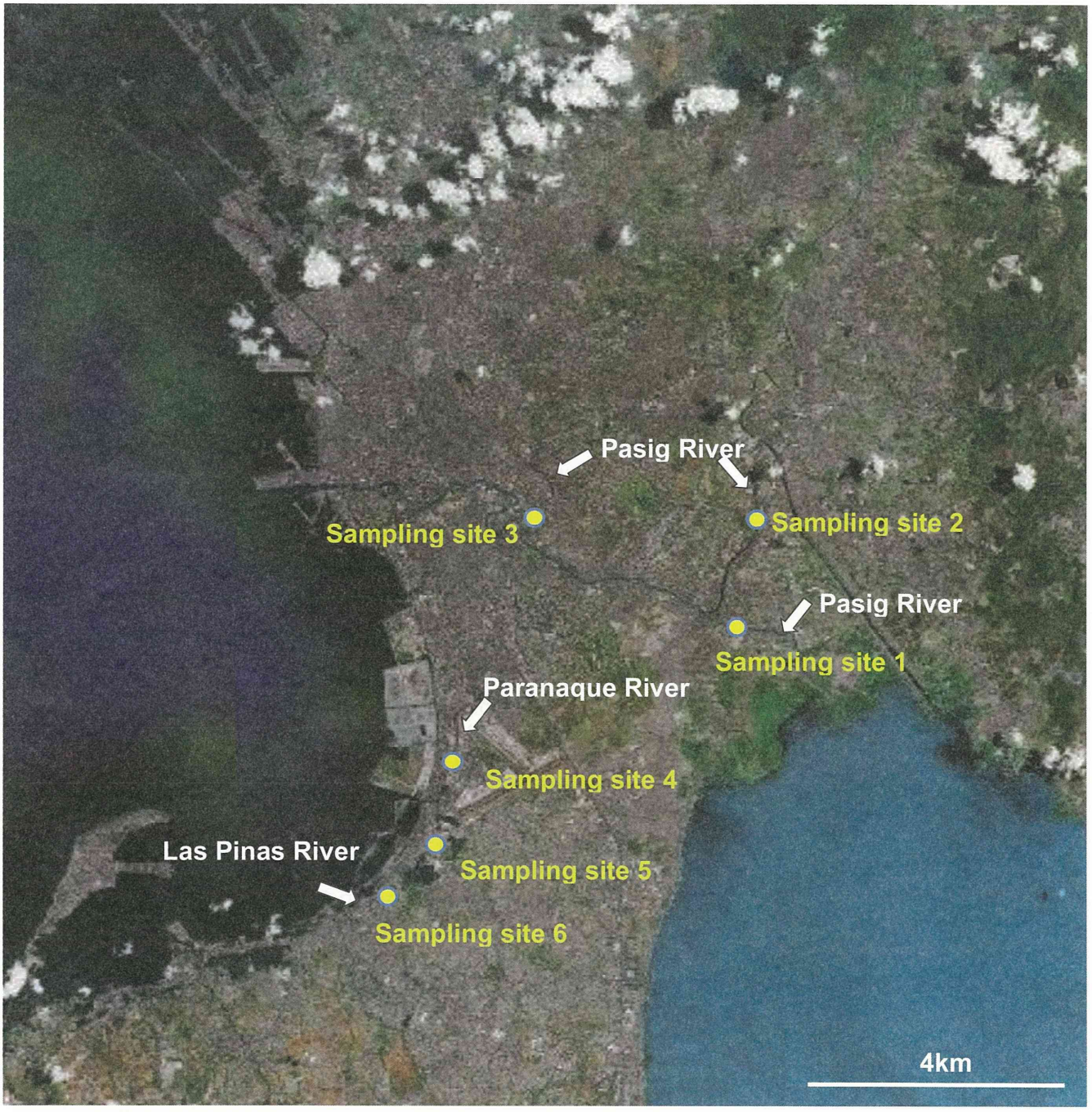
**Figure legends**

**Fig. 1.** Map of Manila City and the sampling sites. Yellow circles indicate the sampling sites. Each river is labeled with an arrow.

**Fig. 2.** Phylogenetic analysis of HEV isolated from river water samples in Manila City, the Philippines. Nucleic acid sequence alignment was performed using Clustal X 1.81 (www.clustal.org). The genetic distance was calculated by Kimura's two-parameter method. A phylogenetic tree with 1,000 bootstrap replicates was generated by the neighbor-joining method based on the partial genome (338nt) of HEV ORF2 of the genotypes 1-4 and avian HEV isolates. The scale bar indicates nucleotide substitutions per site.

230 Fig. 1


Pasig River
Sampling site 3
Sampling site 2
Pasig River
Sampling site 1
Paranaque River
Sampling site 4
Las Pinas River
Sampling site 5
Sampling site 6
4km

234 Fig.2

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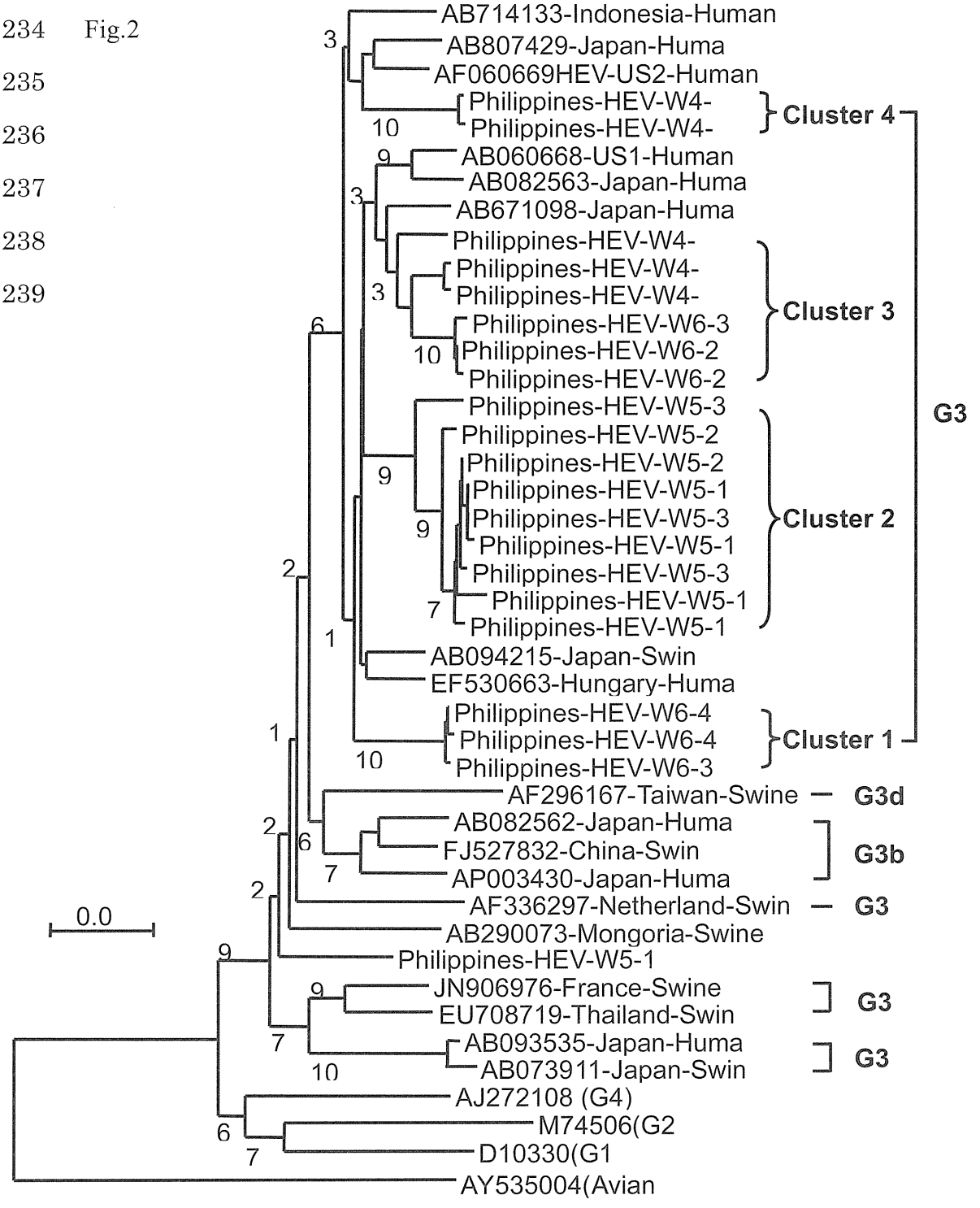

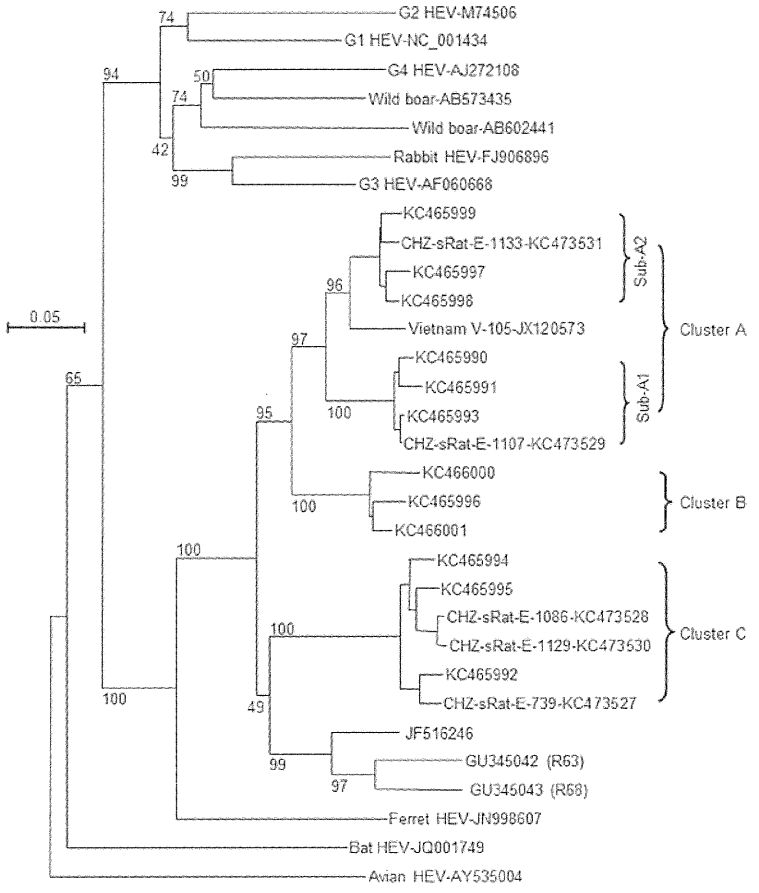


Figure. Phylogenetic analysis of rat hepatitis E virus (HEV) isolated from Asian musk shrews (*Suncus murinus*) in Zhanjiang City, China. Nucleic acid sequence alignment was performed by using ClustalX 1.81 (www.clustal.org). The genetic distance was calculated by using the Kimura 2-parameter method. The phylogenetic tree, with 1,000 bootstrap replicates, was generated by the neighbor-joining method based on the partial sequence (281 nt) of HEV open reading frame 1 of genotype 1–4, wild boar, rabbit, ferret, bat, avian, and rat HEV isolates. The scale bar indicates nucleotide substitutions per site.


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# No Evidence for Hepatitis E Virus Genotype 3 Susceptibility in Rats

**To the editor:** Hepatitis E virus (HEV) is a positive-sense single-stranded RNA virus (genus *Hepevirus*, family *Hepeviridae*) (*1*). In humans, acute hepatitis infection caused by HEV is a serious public health concern in developing countries. Four HEV genotypes, G1–4, have been isolated from humans (*2*). G3 and G4 HEV have also been isolated from swine, wild boars, wild deer, and mongooses; these animals are thought to be the reservoirs of HEV (*3*). Direct evidence has indicated that HEV is transmitted from pigs or wild boars to humans; therefore, hepatitis E caused by G3 and G4 HEV infection is recognized as a zoonosis (*3*).

Although rats have long been suspected to be a potential reservoir for human HEV, no direct evidence has been found. The susceptibility of rats to human HEV genotypes is

controversial. For example, anti-HEV IgG has been detected in various rat species, including Norway (*Rattus norvegicus*), black (*Rattus rattus*), and cotton (*Sigmodon hispidus*) rats, by using ELISA with antigens derived from G1 HEV. These results suggest that HEV or HEV-like virus infections occur in wild rats. However, the virus genome has not been detected, and the source of the infection was confirmed in few cases; thus far, it is not clear whether the anti-HEV IgG was induced by HEV or other HEV-like viruses. The detection of a partial genome of G1 HEV from wild rats in Nepal was reported in 2002 (*4*); however, this report was retracted in 2006 because the isolated strain was determined to be a result of laboratory contamination. Recently, Lack et al. isolated strains of G3 HEV from a variety species of wild rats in the United States (*5*); this finding suggests that wild rats are hosts for G3 HEV. Maneerat et al. also reported that human HEV (presumably G1) was transmissible to Wistar laboratory rats (*6*). However, Purcell et al. recently reported that G1, G2, and G3 do not infect laboratory rats (*7*), and we found in a previous study that laboratory rats are not susceptible to G1, G3, or G4 HEV (*8*).

To further investigate the potential susceptibility of rats to infection with human HEV, we experimentally injected nude rats with G3 HEV and monitored virus growth. We used 2 samples of G3 HEV for the infection experiments, 1 derived from fecal specimens collected from a pig farm in Japan (GenBank accession no. DQ079632) and 1 derived from the supernatant of a hepatocarcinoma cell line, PLC/PRF/5, that was injected with the pig specimen. The infectivity of these samples was confirmed by experimental infections of cynomolgus monkeys (*9*; data not shown).

Six 15-week-old female nude rats (athymic rats, Long-Evans-run/run; Japan SLC, Inc., Hamamatsu, Japan) were used in this study. These rats, which are bred to be immunodeficient, are known to be susceptible to rat HEV, but it is unknown if they are susceptible to other types of HEV. All rats were negative for G3 HEV RNA and anti-HEV antibodies, as determined by nested reverse transcription PCR (*10*) and ELISA (*8*), respectively. Rats were housed individually in biosafety level 2 facilities. Experiments were reviewed by the ethics committee of the National Institute of Infectious Diseases (NIID) Japan and carried out according to the "Guidelines for animal experiments performed at NIID" under code 113060.

The 6 rats were randomly assigned to 2 groups, injected intravenously with 500 μL of an HEV sample suspension through the tail vein, and monitored for 3 months. The 3 rats in group 1 were injected with the sample derived from pig feces, which contained $5 \times 10^4$ copies of G3 HEV; the 3 rats in group 2 were injected with the cell culture supernatant sample, which contained $4 \times 10^6$ copies of G3 HEV. Serum samples were collected weekly for examination of HEV RNA and anti-HEV IgG and IgM and were also used to determine alanine aminotransferase values. Fecal samples were collected every 3 days to detect HEV RNA. The animals were humanly killed by exsanguination 91 days postinjection, liver tissues were collected, and a 10% tissue suspension was prepared as described (*8*).

For groups 1 and 2, all serum samples collected 1–13 weeks postinjection were negative for HEV RNA and anti-HEV IgG and IgM. HEV RNA also was not detected in fecal samples or liver tissues (Table). Alanine aminotransferase elevation was not observed in any serum samples.

In conclusion, even by using samples with high titers of HEV RNA in injection experiments, we were unable to cause infection with G3 HEV in immunodeficient nude rats. We found no evidence that rats are susceptible to infection with G3 HEV.

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## Fatal Case of Enterovirus 71 Infection and Rituximab Therapy, France, 2012

**To the Editor:** Enterovirus 71 (EV-71) causes primarily asymptomatic or benign infections in children <5 years of age. However, it may cause severe and sometimes fatal neurologic complications, such as brainstem encephalitis and polio-like paralysis (*1*). Over the last 15 years, large outbreaks of EV-71 infection have been described in the Asia–Pacific region, associated with the regular emergence of new genetic lineages (*2*). Since the 1978 outbreak in Hungary, rare sporadic cases have been reported in Europe (*1*). In France, during 2000–2009, a total of 81 hospitalized patients with EV–17 infection were reported by the sentinel surveillance system, including 2 child deaths, 1 due to proven rhombencephalitis (*3*,*4*).

We report here a fatal case of EV-71 rhombencephalitis in an immunocompromised adult who was receiving rituximab therapy. Rituximab is a chimeric anti-CD20 monoclonal antibody that is widely used for treating B-cell lymphoma and an increasing number of autoimmune diseases. Since rituximab became commercially available, several infectious side-effects for the drug have been reported, including hepatitis B reactivation, progressive multifocal leukoencephalopathy, and enteroviral meningoencephalitis (*5*). The first 2 cases of rituximab-associated enteroviral meningoencephalitis were reported in 2003 (*6*), and 5 additional cases have been reported to date (*7*,*8*).

In May 2012, a 66-year-old woman was hospitalized in the neurology unit of Bordeaux University Hospital with a 10-day history of fever, asthenia, and psychomotor retardation. She had no history of travel and had not been in close contact with sick persons. She had received a diagnosis of grade I follicular lymphoma 3 years earlier, and it had been treated with 6 cycles of R-CHOP (rituximab, cyclophosphamide, hydroxydaunorubicin, oncovin, prednisolone). Since July 2010, the lymphoma had been in remission, and she had been receiving maintenance therapy with rituximab since that time. The most recent rituximab infusion had been administered in March 2012. Her condition was treated initially with broad-spectrum antibiotics and acyclovir. Still, aphasia, facial paralysis, spastic movements, and consciousness disorders rapidly developed. On day 6, she was transferred to the intensive care unit for ventilatory support.

On patient's admission, blood samples showed lymphopenia ($0.64 \times 10^3$ cells/mm$^3$) and low immunoglobulin levels, i.e., IgG 4.5 g/L (reference range 6.75–12.8 g/L) and IgM 0.33 g/L (reference range 0.56–1.9 g/L). Three cerebrospinal fluid (CSF) samples were collected on days 1, 4, and 6. CSF leukocyte count rose from 5 to 89 cells/mm$^3$, with lymphocytes from 24% to 95%, and protein levels rose from 0.68 to 1.03 g/L (reference range 0.15–0.45 g/L). CSF glucose level varied from 3.5 to 4.5 mmol/L (reference range 2.7–3.9 mmol/L). Enterovirus RNA was detected in the patient's first 3 CSF samples and in CSF, stool specimens, and blood until 4 weeks after admission (online Technical Appendix Table 1, wwwnc.cdc.gov/EID/article/19/8/13-0202-Techapp1.pdf). PCR assays of the first 3 CSF samples were negative for JC polyomavirus, herpes simplex virus, varicella-zoster virus, cytomegalovirus, Epstein-Barr virus, human herpesvirus 6, adenovirus, and *Toxoplasma gondii*. Serologic tests for parvovirus B19, mumps virus, and measles virus were IgM negative. Samples were also negative for antibodies against Hu, Ri, Yo, and voltage-gated potassium channel antigens. All bacterial cultures were negative. No evidence for central nervous system infiltration by lymphoma cells was found, on the basis of CSF cytology.

Results of brain magnetic resonance imaging (MRI) scans performed on days 2 and 6 were normal, despite the patient's consciousness disorders (Figure, panel A). However, on day 13, MRI scans showed bilateral and symmetric T2 and FLAIR hypersignals in the medulla, the pons, and the mesencephalon, compatible with rhomboencephalitis (Figure, panel B). On day 24, the MRI scan showed a supratentorial extension involving white matter, the insular cortex, and basal ganglia (Figure, panel C). The patient's neurologic condition deteriorated progressively, and she died of enteroviral rhomboencephalitis 32 days after admission.

The EV associated with the rhomboencephalitis was identified as an EV-71 genogroup C2 isolate by 1D gene complete sequencing and phylogenetic analysis (online Technical Appendix Figure; online Technical Appendix Table 2). The 1D gene sequences determined from cerebrospinal fluid and fecal specimens from the patient showed 95%–97% nucleotide

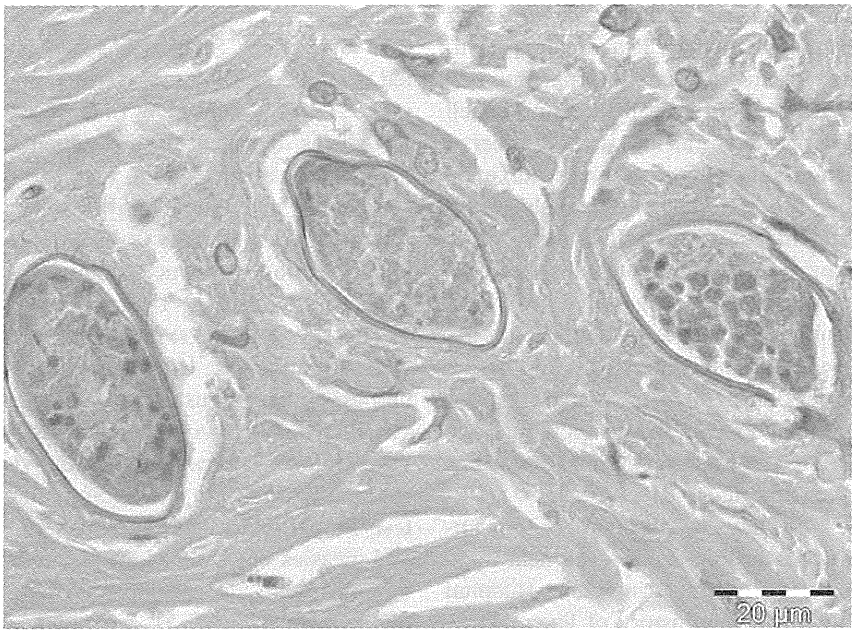


Figure. Close-up of liver granuloma with section through 3 *Schistosoma malayensis*–like ova embedded in dense fibrous tissue. The thin-walled, nonstriated helminth ova are not operculated and contain nonvital miracidial cells. Hematoxylin and eosin stain; original magnification ×100.

because it was obtained from a relative, and possible unreported drug-related travel by the patient to neighboring countries cannot be fully excluded. *R. muelleri*, the jungle rat and definitive host for *S. malayensis*, is often seen at river banks (*4*), and rodent feces could have contaminated the water with schistosome eggs.

Future field studies are needed to identify focal hot spots of sylvatic transmission by snail examination and seroprevalence studies of persons living in rural areas, especially the Orang Asli population. Moreover, in light of growing ecotourism, which also encompasses stays at remote Orang Asli villages and canoeing on small streams (*10*), appropriate public health measures, such as rodent and snail control near tourist sites, should be implemented.

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## Asian Musk Shrew as a Reservoir of Rat Hepatitis E Virus, China

**To the Editor:** Rat hepatitis E virus (HEV), a member of genus *Hepevirus* in the family *Hepeviridae*, was first detected in Norway rats in Germany in 2010 (*1, 2*). Since then, this rat HEVhas been detected in multiple wild rat species in the United States, Vietnam, Germany, and Indonesia (*3–7*). Studies have shown that rat HEV failed to infect rhesus monkeys and pigs,

suggesting that rat HEV is restricted to its natural host (*6, 8*). However, it is not known whether animals other than rats are susceptible to rat HEV.

The Asian musk shrew (*Suncus murinus*), also called the Asian house shrew, is a small mole-like mammal belonging to the family *Soricidae* (order Soricomorpha), and wild rats are classified in the family *Muridae* (order Rodentia). Musk shrews originated from the Indian subcontinent and are now found from southern Asia and Afghanistan to the Malay Archipelago and southern Japan. These shrews are commensal rodents, commonly found living in human households. We previously showed that rat HEV infection frequently occurs in wild rats in Zhanjiang City, Guangdong Province, China (*9*). Asian musk shrews share this same environment; thus, they can be exposed to rat HEV derived from wild rats.

To determine whether Asian musk shrews are a reservoir for rat HEV, we examined 260 shrews (112 males, 148 females) that were trapped in Zhanjiang City during December 2011–September 2012. Of the 260 trapped shrews, 147 were from Mazhang District (23 from a pig farm and 124 from the villages of Chiling, Chofa, Beigou, Huangwai, Houyang, and Nanpan) and 113 were from Chikan District.

Blood samples were collected from the shrews, and serum was separated by centrifugation (2,500 × *g* for 20 min at 4°C), and stored at −80°C until use. We tested the serum samples for the presence of HEV IgG and IgM antibodies by using an ELISA based on rat HEV-like particles, as described (*3*). Of the 260 samples, 27 (10.4%) were HEV IgG positive and 12 (4.6%) were HEV IgM positive. Of these, 3 IgG-positive and 1 IgM-positive samples were among the 113 samples (2.7% and 1.0%, respectively) collected from shrews in Chikan District, and 24 IgG-positive and 11 IgM-positive samples were among the 147 samples (16.3% and 7.5%, respectively) collected from shrews in 6 villages (124 total samples) and the pig farm (23 total samples) in Mazhang District. The IgG-positive rate was higher for shrews from Mazhang District than for those from Chikan District ($p<0.05$); the rates of IgM-positivity did not differ significantly. The IgG-positive rate among the 6 villages varied substantially (8.3%–71.4%) (online Technical Appendix Table 1, wwwnc.cdc.gov/EID/article/19/8/13-0069-Techapp1.pdf). The IgG-positive rates were 11.6% (13/112) in male and 9.5% (14/148) in female shrews, respectively; the difference in rates between the sexes was not statistically significant.

A total of 12 IgM-positive serum samples were selected for HEV RNA testing by nested broad-spectrum reverse transcription PCR (*2*); results for 5 were positive (online Technical Appendix Table 2). The length of the nested reverse transcription PCR products was 334 nt. After the primer sequences were removed, we sequenced the remaining 281 nt corresponding to nt 4107–4387 in the C-terminal open reading frame 1 of the rat HEV genome (GU345042) (GenBank accession nos. KC473527–KC473531). Phylogenetic analysis indicated that the 5 HEV isolates were all classified into the same group as rat HEV and clearly separated into 2 clusters, A and C. Cluster A isolates were further divided into 2 subclusters, sub-A1 (CHZ-sRat-E-1107) and sub-A2 (CHZ-sRat-E-1133) (Figure). Strains CHZ-sRat-E-739, CHZ-sRat-E-1086, and CHZ-sRat-E-1129 belong to cluster C. These findings are of limited precision because of the shot sequence that was analyzed, and, thus, they may not be predictive of results obtained with complete genomes.

Rat HEV isolated from the *S. murinus* shrews shared 77.4%–99.6% nt sequence identity with other rat HEV strains; the sequences were especially similar to those of HEV isolates from wild rats in this area (GenBank accession nos. KC465990–KC466001) (online Technical Appendix Table 3). In addition, nucleotide sequences from subcluster A1 and A2 and cluster C rat and shrew strains shared 97.5%–99.6%, 96.8%–97.2%, and 94.0%–97.5% identity, respectively (online Technical Appendix Table 3). These results indicate that rat HEV infection occurs in *S. murinus* shrews and that these rodents are a reservoir for rat HEV.

Evidence indicates that rat HEV may be capable of inducing an immune response in humans; thus, this virus may be relevant to the epidemiology of HEV in humans (*10*). A key step in understanding this epidemiology is to know the reservoirs of rat HEV, especially reservoirs like *S. murinus* shrews, which live in close proximity to humans.

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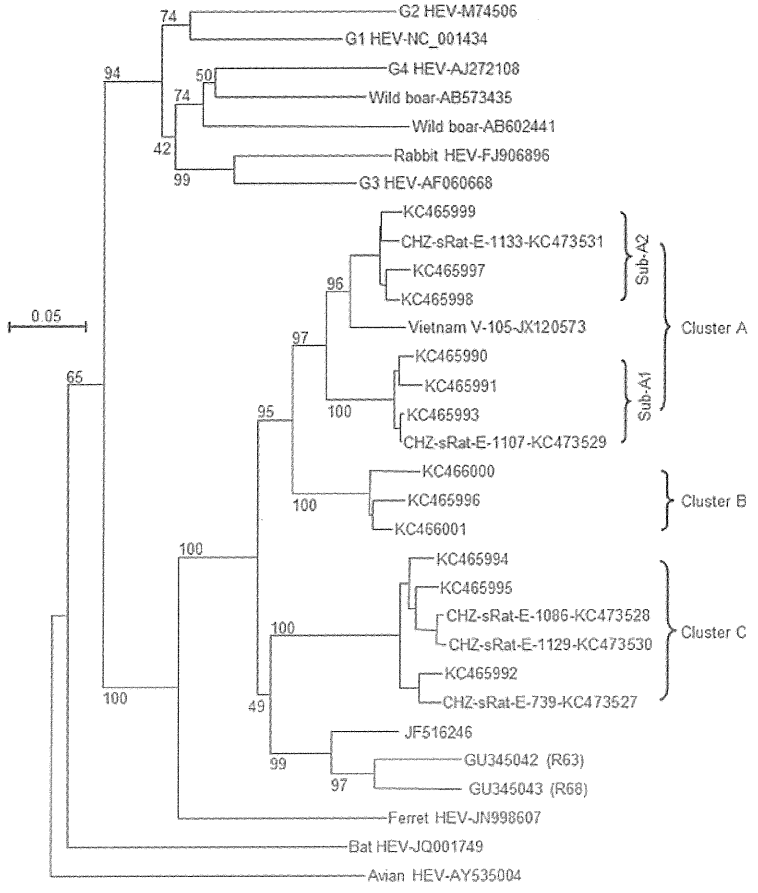


Figure. Phylogenetic analysis of rat hepatitis E virus (HEV) isolated from Asian musk shrews (*Suncus murinus*) in Zhanjiang City, China. Nucleic acid sequence alignment was performed by using ClustalX 1.81 (www.clustal.org). The genetic distance was calculated by using the Kimura 2-parameter method. The phylogenetic tree, with 1,000 bootstrap replicates, was generated by the neighbor-joining method based on the partial sequence (281 nt) of HEV open reading frame 1 of genotype 1–4, wild boar, rabbit, ferret, bat, avian, and rat HEV isolates. The scale bar indicates nucleotide substitutions per site.


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# No Evidence for Hepatitis E Virus Genotype 3 Susceptibility in Rats

**To the editor:** Hepatitis E virus (HEV) is a positive-sense single-stranded RNA virus (genus *Hepevirus*, family *Hepeviridae*) (*1*). In humans, acute hepatitis infection caused by HEV is a serious public health concern in developing countries. Four HEV genotypes, G1–4, have been isolated from humans (*2*). G3 and G4 HEV have also been isolated from swine, wild boars, wild deer, and mongooses; these animals are thought to be the reservoirs of HEV (*3*). Direct evidence has indicated that HEV is transmitted from pigs or wild boars to humans; therefore, hepatitis E caused by G3 and G4 HEV infection is recognized as a zoonosis (*3*).

Although rats have long been suspected to be a potential reservoir for human HEV, no direct evidence has been found. The susceptibility of rats to human HEV genotypes is

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# Characterization of self-assembled virus-like particles of ferret hepatitis E virus generated by recombinant baculoviruses

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Ferret hepatitis E virus (HEV), a novel hepatitis E-like virus, has been identified in ferrets in The Netherlands. Due to the lack of a cell-culture system for ferret HEV, the antigenicity, pathogenicity and epidemiology of this virus have remained unclear. In the present study, we used a recombinant baculovirus expression system to express the 112-N-terminus and 47-C-terminus-amino-acid-truncated ferret HEV ORF2 protein in insect Tn5 cells, and found that a large amount of a 53 kDa protein (F-p53) was expressed and efficiently released into the supernatant. Electron microscopic analysis revealed that F-p53 was self-assembled into virus-like particles (ferret HEV-LPs). These ferret HEV-LPs were estimated to be 24 nm in diameter, which is similar to the size of G1, G3, G4 and rat HEV-LPs derived from both the N-terminus- and C-terminus-truncated constructs. Antigenic analysis demonstrated that ferret HEV-LPs were cross-reactive with G1, G3, G4 and rat HEVs, and rat HEV and ferret HEV showed a stronger cross-reactivity to each other than either did to human HEV genotypes. However, the antibody against ferret HEV-LPs does not neutralize G3 HEV, suggesting that the serotypes of these two HEVs are different. An ELISA for detection of anti-ferret HEV IgG and IgM antibodies was established using ferret HEV-LPs as antigen, and this assay system will be useful for monitoring ferret HEV infection in ferrets as well as other animals. In addition, analysis of ferret HEV RNA detected in ferret sera collected from a breeding colony in the USA revealed the genetic diversity of ferret HEV.

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## INTRODUCTION

Hepatitis E virus (HEV) is a single-stranded positive-sense RNA virus that belongs to the genus *Hepevirus* in the family *Hepeviridae* (Meng *et al.*, 2012). HEV is a causative agent of hepatitis E, a viral disease that manifests as acute hepatitis (Emerson & Purcell, 2003). The disease is known as an important public health problem not only in developing countries but also in industrialized countries (Meng, 2010). Hepatitis E is primarily transmitted by the faecal–oral route (Balayan *et al.*, 1983). Recently, a number of sporadic cases have been transmitted in a zoonotic fashion, with zoonotic hepatitis E being mainly associated with genotype 3 and 4 HEV infection (Li *et al.*, 2005a; Meng, 2010; Tei *et al.*, 2003). Many animals such as monkeys, rabbits, mongooses, rats, ferrets, chickens and bats are known to harbour HEV or HEV-like viruses (Drexler *et al.*, 2012; Haqshenas *et al.*, 2001; Johne *et al.*, 2010a; Raj *et al.*, 2012; Yamamoto *et al.*, 2012; Zhao *et al.*,

The GenBank/EMBL/DDBJ accession numbers for the C-terminal ORF1 fragment sequences of the ferret HEV genome generated in this study are KF268376 to KF268393.

2009). However, whether these HEVs can be transmitted to humans is still not clear.

The ferret HEV sequence was first detected in ferrets (*Mustela putorius*) from The Netherlands (Raj *et al.*, 2012). The ferret HEV genome contains three open reading frames (ORF1–3). ORF1 encodes a nonstructural protein of 1596 aa, ORF2 encodes a capsid protein of 654 aa and ORF3 encodes a phosphoprotein of 108 aa. A putative ORF4 observed in the rat HEV genome was also found in the ferret HEV genome. Sequence analyses indicated that the ferret HEV genome shared the highest nucleotide sequence identity (72.3 %) with rat HEV (GU345042). In contrast, the nucleotide sequence identity with HEV genotypes 1–4 (G1–4), rabbit and avian HEVs ranged from 54.5 to 60.5 % (Raj *et al.*, 2012). While the genome has been sequenced, the antigenicity, pathogenicity and epidemiology of ferret HEV have remained unclear.

In this study, we describe the efficient expression of full-length, N-terminal-truncated, and both N- and C-terminal-truncated ferret HEV ORF2 proteins on the basis of the nucleotide sequence derived from a ferret HEV strain (JN998607) identified in The Netherlands in 2012 (Raj *et al.*, 2012). When both 112-N-terminus and 47-C-terminus-aa-deleted ORF2 proteins were expressed by a recombinant baculovirus in insect Tn5 cells, the proteins were found to self-assemble into virus-like particles (V-LPs). The V-LPs exhibited antigenic cross-reactivity against rat, G1, G3 and G4 HEVs. However, the antibody against ferret HEV-LPs did not neutralize the G3 HEV. An ELISA was developed using ferret HEV-LPs as antigen to examine ferret HEV-specific IgG and IgM responses.

## RESULTS

### Expression of ferret HEV ORF2 and formation of V-LPs

Seven recombinant baculoviruses, Ac[ORF2], Ac[n6c0-ORF2], Ac[n18c0-ORF2], Ac[n112c0-ORF2], Ac[n6c47-ORF2], Ac [n18c47-ORF2] and Ac[n112c47-ORF2], comprising the full-length, N-terminal-truncated, or both N- and C-terminal-truncated ferret HEV ORF2, were prepared as described in Methods. Tn5 cells were infected with the recombinant baculoviruses at an m.o.i. of 10, incubated at 26.5 °C and harvested every day up to day 9 post-infection (p.i.). The proteins generated in the infected cells and supernatant were analysed by SDS-PAGE. In Ac[ORF2], Ac[n6c0-ORF2], Ac[n18c0-ORF2], Ac[n112c0-ORF2], Ac[n6c47-ORF2] and Ac[n18c47-ORF2]-infected Tn5 cells, major protein bands with molecular masses of 58 to 70 kDa corresponding to the full-length or truncated ORF2 proteins were detected at 3 days p.i. However, those proteins were not detected in the supernatant and no V-LPs were observed in either the cells or their supernatants (data not shown).

In contrast, a major protein with a molecular mass of 53 kDa (F-p53) was detected in the cells on day 2 p.i. in the Ac[n112c47-ORF2]-infected Tn5 cells, and the expression levels reached a peak on days 3 to 4 p.i. (Fig. 1). The F-p53 protein was found in the supernatant on day 4 p.i., and reached a peak from day 6 to day 9 p.i. The F-p53 protein was synthesized only in the Ac[n112c47-ORF2]-infected cells, but not in the mock-infected or wild-type baculovirus-infected cells. The F-p53 protein reacted with the anti-rat HEV-LPs antibody in Western blotting (Fig. 1).

To examine whether the F-p53 would form into virus-like particles, the cell-culture supernatants of the Ac[n112c47-ORF2]-infected Tn5 cells were harvested at 7 days p.i. and F-p53 was purified by CsCl gradient centrifugation as described in Methods. The F-p53 protein was primarily distributed in fractions 12, 13 and 14, with densities ranging from 1.295 g $cm^{-3}$ to 1.287 g $cm^{-3}$ (Fig. 2a). The N-terminal aa sequence was determined by microsequencing to identify the F-p53 and Alanine-Valine-Alanine-Proline-Alanine (AVAPA) was obtained. This sequence is identical to the 113–117 aa residues of ferret HEV ORF2, indicating that F-p53 was derived from the ferret HEV ORF2 protein. Observation of fractions 12–14 by electron microscopy revealed many spherical particles of diameter 24 nm (Fig. 2b). The size of ferret HEV-LPs was similar to those of the G1, G3 G4 and rat HEV-LPs, produced by recombinant baculoviruses harbouring N-terminal 111 aa- or 100 aa-deleted HEV ORF2 (Guu *et al.*, 2009; Li *et al.*, 2011b; Xing *et al.*, 2010; Yamashita *et al.*, 2009). To determine whether nucleic acids were packaged into the ferret HEV-LPs, the nucleic acid was extracted from purified ferret HEV-LPs and analysed by agarose gel electrophoresis. However, no nucleic acid was found in ferret HEV-LPs (data not shown). The yield of the purified ferret HEV-LPs reached 0.5 mg per $10^7$ Tn5 cells. These results indicated that the F-p53 self-assembled into V-LPs, and demonstrated that the ORF2 gene encodes the ferret HEV capsid protein.

### Antigenic cross-reactivity among ferret, rat, G1, G3 and G4 HEVs

Western blot analysis indicated that the ferret HEV capsid protein F-p53 reacted with the antibody elicited in response to rat HEV, suggesting that ferret HEV has similar antigenicity to rat HEV. To explore the antigenic cross-reactivity among ferret, rat, G1, G3 and G4 HEVs, rats were immunized with ferret, rat, G1, G3 or G4 HEV-LPs, respectively. After three injections, all of the rats elicited a high level of IgG antibodies against each homologous antigen and IgG titres by antibody ELISA reached as high as 1 : 1 638 400 (ferret), 1 : 409 600 (rat), 1 : 409 600 (G1), 1 : 1 638 400 (G3) and 1 : 204 800 (G4), respectively (Fig. 3). The antibody against ferret HEV-LPs reacted not only with homologous HEV-LPs, but also with heterologous rat, G1, G3 and G4 HEV-LPs with titres of 1 : 204 800, 1 : 51 200, 1 : 51 200 and 1 : 51 200, respectively. Conversely, the antibodies against rat, G1, G3 and G4 HEV-LPs were cross-reactive to ferret HEV-LPs with titres of 1 : 51 200, 1 : 6400, 1 : 409 600 and 1 : 51 200, respectively (Fig. 3). These results indicated that ferret HEV has antigenic epitope(s) common to those of rat G1, G3 and G4 HEVs.

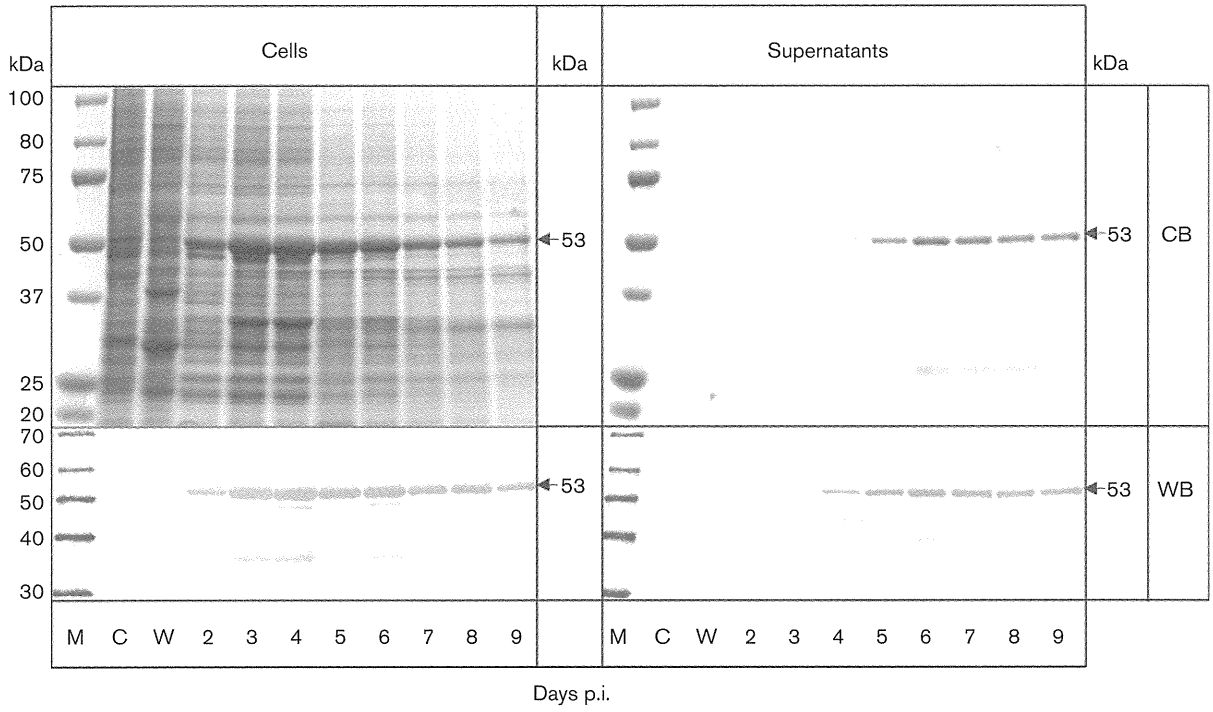


**Fig. 1.** Time course of the expression of 112-N-terminal aa and 47-C-terminal aa-truncated ferret HEV ORF2. Insect Tn5 cells were infected with a recombinant baculovirus, Ac[n112c47-ORF2], incubated at 26.5 °C and harvested on the indicated days (2 to 9). Five microlitres of the culture medium and the lysate from $10^5$ cells were analysed by SDS-PAGE. Protein bands were visualized by Coomassie blue staining (CB) and Western blot assay with anti-rat HEV-LP rabbit serum (WB). M, molecular mass marker; C, mock-infected Tn5 cells; W, wild-type baculovirus-infected Tn5 cells; lanes 2 to 9, Ac[n112c47-ORF2]-infected Tn5 cells harvested on days 2 to 9 p.i.

## Cross-neutralization of G3 HEV with various anti-HEV-LPs antibodies

To examine the neutralization activity of anti-HEV-LPs antibodies, antisera against ferret, rat, G1, G3 or G4 HEV-LPs or rat HEV (V-105) (Li *et al.*, 2013) was mixed with G3 HEV and incubated for neutralization, and then inoculated to PLC/PRF/5 cells. This is because there is no cell-culture system to grow rat and ferret HEV and although G1 and G4 HEV grow in PLC/PRF/5 cells it is well known that G1–4 HEVs form a single serotype. Pre-immunized rat serum was used as the negative control. As shown in Fig. 4, G3 HEV Ag (capsid protein) was detected in the samples inoculated with anti-rat and anti-ferret HEV-LPs, anti-rat HEV serum and pre-immunized rat serum, with OD values ranging from 1.126 to 1.583. In contrast, HEV Ag was not detected in samples incubated with rat sera against G1, G3 and G4 HEV-LPs. These results indicated that G3 HEV was cross-reactive to G1, G3 and G4 HEVs, but not to ferret and rat HEVs. In other words, the antigenicities of ferret and rat HEVs were different from those of G1, G3 and G4 HEVs.

## ELISA for detection of anti-ferret HEV IgG and IgM antibodies

An ELISA for detection of anti-ferret HEV antibodies was developed as described in Methods. A positive control for anti-ferret IgG and IgM was produced by immunizing a ferret with ferret HEV-LPs. The pre-immunized ferret serum was used as negative control. As shown in Fig. 5(a), anti-ferret HEV IgG and IgM were not detected in the pre-immune serum, with OD values lower than 0.05. IgG and IgM were detected in the post-immunized ferret serum with titres of 1 : 1 024 000 and 1 : 6400, respectively.

A total of 163 serum samples from two separate breeding groups of laboratory ferrets were used to detect anti-ferret HEV IgG and IgM antibodies at a dilution of 1 : 200. The distributions of the OD values of the two ferret groups are shown in Fig. 5(b) (group A) and Fig. 5(c) (group B). Because no significantly high OD value samples were detected in group A, we used these 73 laboratory ferret sera as the negative samples to set the cut-off value for the ELISA on the basis of the mean OD values plus three SD. The OD values of anti-ferret HEV IgG and IgM ranged from 0.008 to 0.146, and from 0.011 to 0.121, respectively. The mean OD value of anti-ferret HEV IgG in these serum samples was 0.045 with an SD of 0.039. The cut-off value for IgG was set at 0.162 ($0.045 + 3 \times 0.039$). Similarly, the mean value of anti-ferret HEV IgM was 0.041 with an SD of 0.037. The cut-off value for IgM was set at 0.152 ($0.041 + 3 \times 0.037$) (Fig. 5b). Based on this cut-off value, no serum sample was positive for anti-ferret HEV

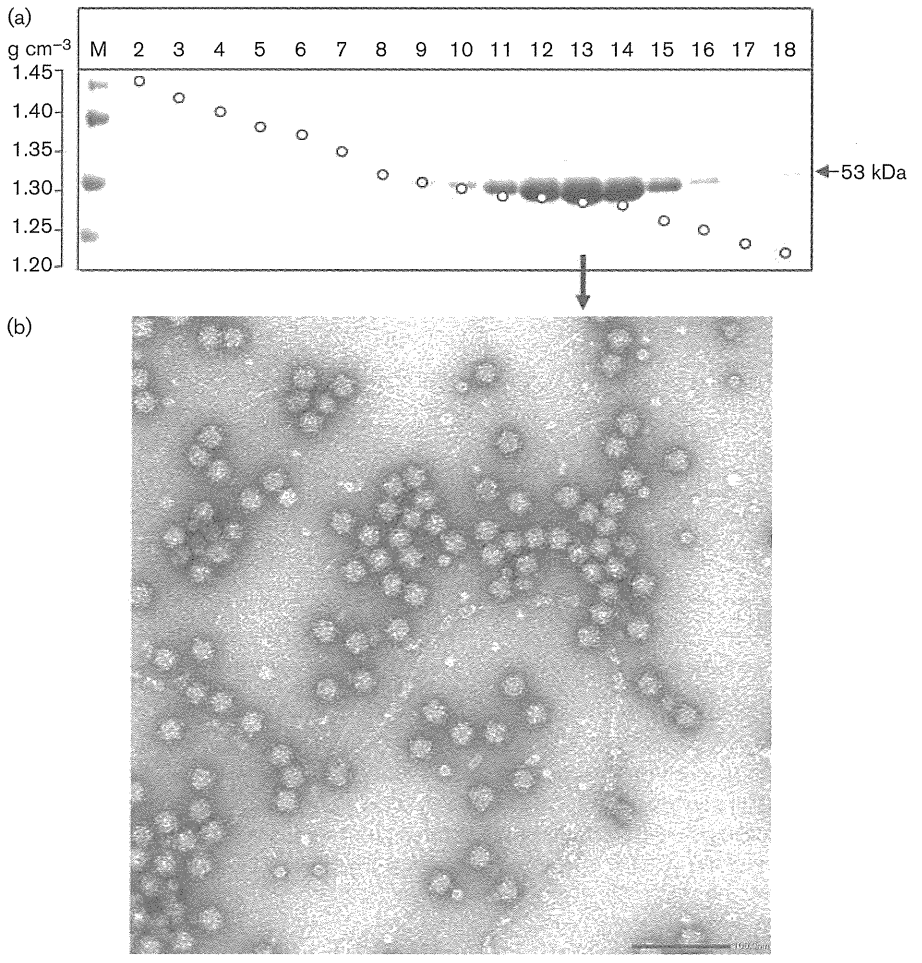


**Fig. 2.** Purification of ferret HEV-LPs. The supernatant of the recombinant baculovirus Ac[n112c47-ORF2]-infected Tn5 cells was centrifuged for 3 h at 32 000 r.p.m. in a Beckman SW32Ti rotor. The pellet was resuspended in 500 µl EX-CELL 405 and then purified by CsCl equilibrium density-gradient centrifugation. Aliquots from each fraction were analysed by electrophoresis on 5 to 20 % polyacrylamide gel, and stained with Coomassie blue (a). The density of each fraction is shown (○). To examine the ferret HEV-LPs, fraction 13 containing F-p53 protein was stained with 2 % uranyl acetate and observed by electron microscopy (b). Bar, 100 nm.

antibodies, indicating the lack of ferret HEV infection in group A. In contrast, the OD values of anti-ferret HEV IgG and IgM ranged from 0.005 to 3.273, and from 0.010 to 3.132, respectively, in group B, showing that 23.3 % (21/90) were positive for anti-ferret HEV IgG and 24.4 % (22/90) were positive for IgM antibody. Except for one sample positive for IgM but negative for IgG, the remaining 21 samples were all positive for both IgM and IgG, suggesting that ferret HEV infection occurred in this group.

### Detection of ferret HEV RNA

All 90 serum samples in group B were tested for HEV RNA by a nested broad-spectrum reverse transcription polymerase chain reaction (RT-PCR) (Johne *et al.*, 2010b) and 18 samples tested positive, including 14 samples positive for both IgG and IgM and four samples negative for both antibodies. Using nested RT-PCR products (333 nt), 280 nt corresponding to nucleotides 3993–4272 in the C-terminal ORF1 of the ferret HEV genome (JN998607) were sequenced and the 18 positive samples were deposited in GenBank under accession nos KF268376 to KF268393. A phylogenetic analysis indicated that all 18 HEV isolates were classified into the same ferret HEV group (Fig. 6) with 98.6 to 100 % nucleotide identities, suggesting that the same strain was circulated in the group B ferrets. However, these HEV shared 81.4 to 82.9 % nucleotide identity with the ferret HEV isolated in The Netherlands, suggesting that ferret HEV is genetically diverse as has also been observed in rat HEV.

## DISCUSSION

Ferret HEV is a new member of the *Hepevirus* genus sharing 53.6 to 54.5 % nucleotide sequence identities with

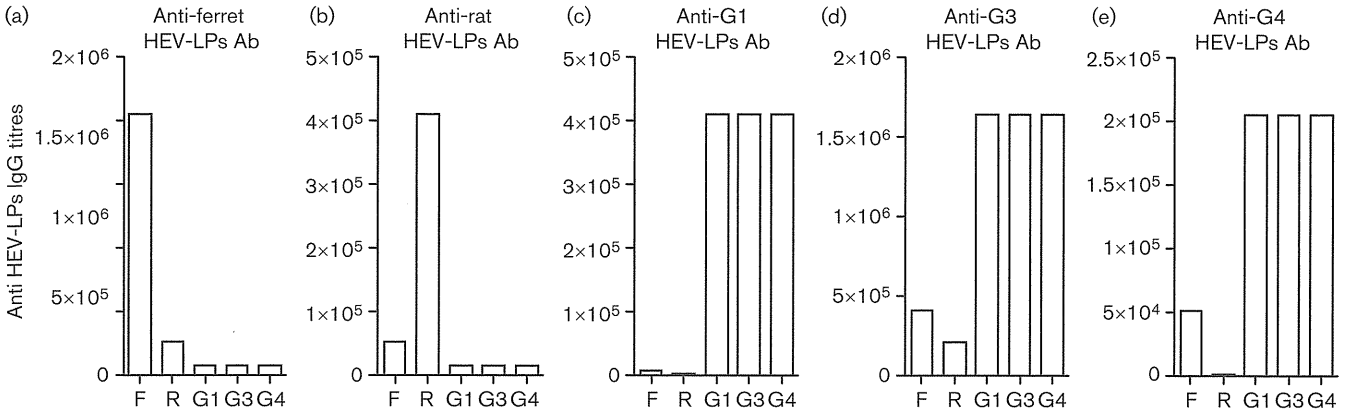


**Fig. 3.** Antigenic cross-reactivity among ferret, rat, G1, G3 and G4 HEV-LPs. IgG titres against ferret, rat, G1, G3 and G4 HEV-LPs determined by ELISA are shown. The ELISA was performed using ferret HEV-LPs (F), rat HEV-LPs (R), G1 HEV-LPs (G1), G3 HEV-LPs (G3) and G4 HEV-LPs (G4) as antigen, respectively.

G1–4 HEV and 61.0 to 68.7 % with rat HEVs. Because no cell-culture system has yet been developed for ferret HEV, it remains necessary to express the capsid protein and generate V-LPs in order to analyse the antigenicity and immunogenicity.

The full-length ferret HEV ORF2 was initially expressed by a recombinant baculovirus; however, the recombinant proteins derived from this gene were neither released into the culture supernatant nor formed into V-LPs. The alignment of ORF2 indicated six extra aa at the N terminus when compared with other HEVs. However, deletion of the six N-terminal aa did not allow us to produce the ferret HEV-LPs.

In the case of G1, G3 and G4 HEV, the 111-N-terminus-aa-deleted ORF2 protein was efficiently released into the supernatant and self-assembled into V-LPs (Li *et al.*, 1997). Based on this information we produced rat HEV-LPs by expressing the 100-N-terminus-aa-deleted rat HEV ORF2 (Li *et al.*, 2011b). Furthermore, native-size V-LPs were produced with the 13-N-terminus-aa-truncated G3 HEV ORF2 (Xing *et al.*, 2010). Therefore, we employed the same strategy in this study. When the deduced aa sequence of the ferret HEV ORF2 was aligned with that of the representatives of HEV G1, G3 and G4 (GenBank accession nos DQ079624, DQ079627 and DQ079631), we found that aa positions 19 to 113 in the ferret HEV ORF2 correspond to aa positions 14 to 112 in the G1, G3 and G4 HEVs. Once again, however, deletion of the 18 or 112 N-terminus aa did not allow us to produce the ferret HEV-LPs.

Our previous experiments indicated that G1, G3 and G4 HEV-LPs are composed of 497 aa (112–608 aa) and the native-size V-LPs are composed of 595 aa (14–608 aa). In

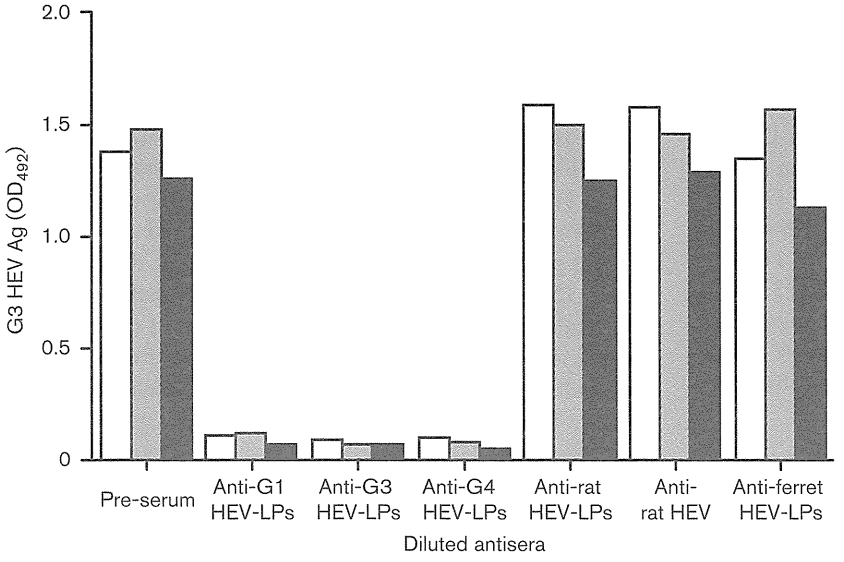


**Fig. 4.** Neutralizing activity of antibodies against G3 HEV. Neutralization of G3 HEV by anti-HEV-LPs or rat HEV sera was carried out by a cell-culture-based neutralization test. Bars indicate the OD values of G3 HEV Ag. Pre-serum, pre-immunized rat serum; anti-G1 HEV-LPs, rat anti-G1 HEV-LPs serum; anti-G3 HEV-LPs, rat anti-G3 HEV-LPs serum; anti-G4 HEV-LPs, rat anti-G4 HEV-LPs serum; anti-rat HEV-LPs, rat anti-rat HEV-LPs serum; anti-rat HEV, rat anti-rat HEV serum; anti-ferret HEV-LPs, rat anti-ferret HEV-LPs serum. Rat serum was diluted at 1 : 10 (white bar), 1 : 20 (grey bar) and 1 : 40 (black bar).

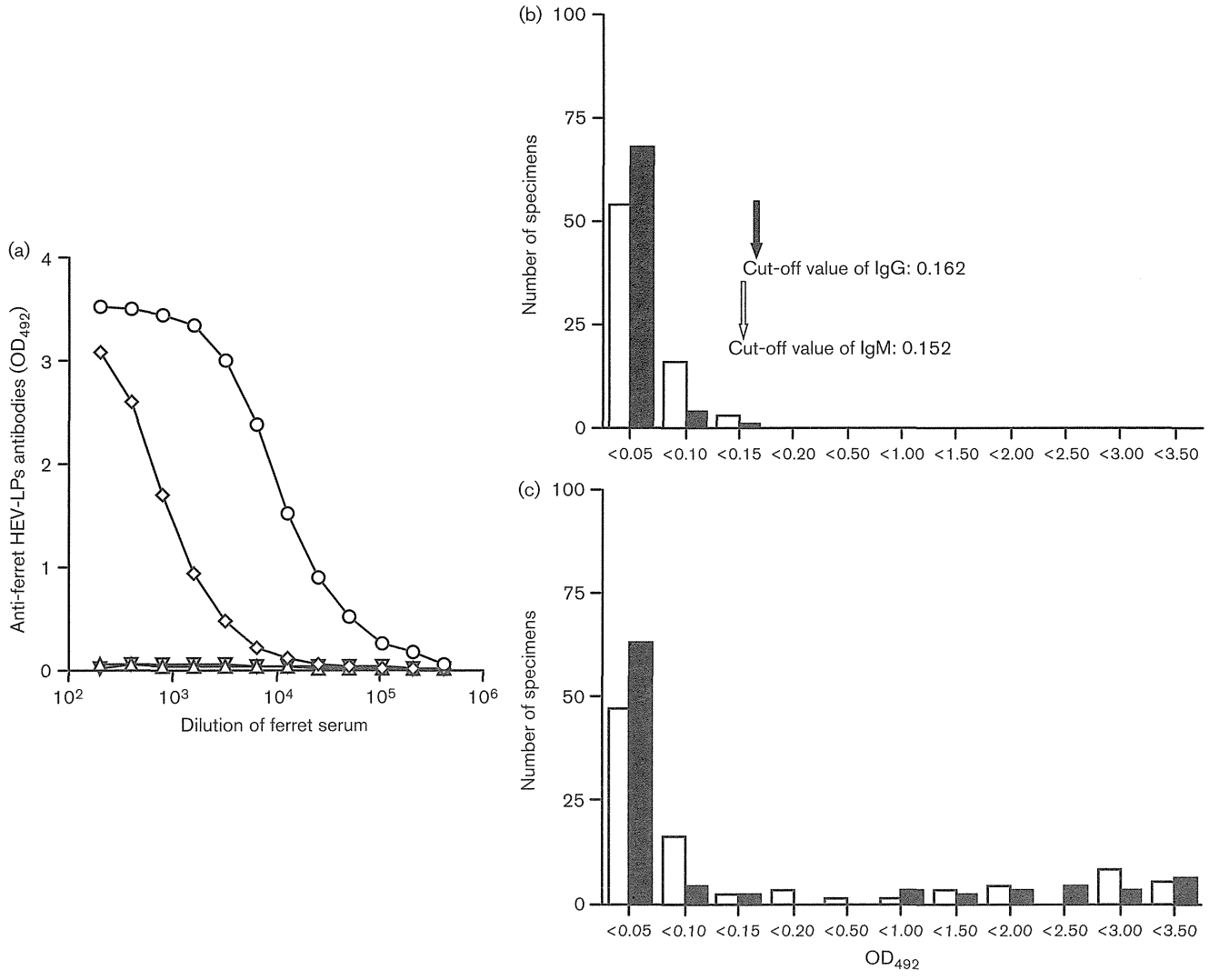


**Fig. 5.** Detection of anti-ferret HEV IgG and IgM antibodies of laboratory ferret. (a) Twofold dilutions of pre-immunized and ferret HEV-LPs-immunized ferret serum were used to detect anti-ferret HEV IgG and IgM antibodies. Anti-ferret HEV IgG (△) and IgM (▽) detected in pre-immunized serum; anti-ferret IgG (○) and IgM (◇) in post-immunized serum. (b, c) Distribution of OD values of IgG (black bar) and IgM (white bar) antibodies at 492 nm. Ferret serum samples from group A (b), and group B (c) were tested and the OD values were plotted as a frequency distribution. Arrows indicate the cut-off values.

addition, both V-LPs lack the 52 C-terminus aa of ORF2, suggesting that the 52 C-terminus aa are not essential in the formation of the particle. The aa position 608 in the G1, G3 and G4 HEV ORF2s corresponds to aa position 596 in the ferret HEV ORF2. We finally expressed the 112-N-terminus-aa- and 47-C-terminus-aa-deleted ORF2, and the recombinant protein F-p53 was released into the supernatant and formed V-LPs (Fig. 1). However, we failed to obtain V-LPs by expressing the 18-N-terminus-aa-deleted and 47-C-terminus-aa-deleted ORF2.

When rats were immunized with ferret HEV-LPs, a strong immune response was induced with high IgG titres in the absence of any adjuvant, suggesting that ferret HEV-LPs are highly immunogenic in rats. The antibody induced by the ferret HEV-LPs was cross-reactive with rat, G1, G3 and G4 HEV-LPs. These results clearly demonstrated that the ferret HEV and rat, G1, G3 and G4 HEVs share at least one common epitope. HEV-LPs are composed of a single capsid protein, which folds into three major domains: the shell (S) domain, the middle (M) domain and the protruding (P) domain. The outer surface of the particles, which is a target for antibodies, is primarily formed by the M and P domains (Xing *et al.*, 2010; Yamashita *et al.*, 2009). The aa identities of the full-length capsid proteins between ferret HEV (JN998607) and G1, G3 and G4 HEVs were found to be 55.2 to 57.2 %; however, those of the partial P domains (538–564 aa) of ferret HEV ORF2 were as high as 85.1 to 85.9 %, suggesting that common

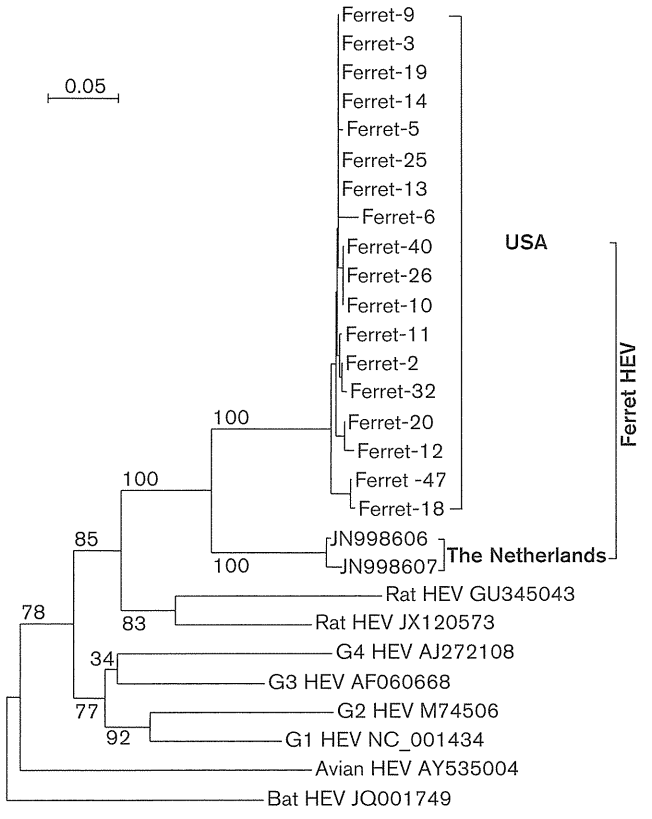


**Fig. 6.** Phylogenetic analysis of ferret hepatitis E virus based on partial nucleotide sequences of the ferret HEV ORF1 (280 nt) using bat HEV as an outgroup. The phylogenetic tree was generated by the neighbour-joining method. Bootstrap values were determined based on 1000 resamplings of the datasets. Bar, 0.05 substitutions per site.

epitope(s) may be present in the P domains of HEV ORF2. The rat HEV capsid protein shared higher aa identity (78.6–79.4 %) with ferret HEV than did G1, G3 and G4 HEVs (55.2–56.4 %), and the rat HEV-LPs showed stronger cross-reactivity with anti-ferret HEV-LPs serum (Fig. 3).

G1–G4 HEVs are known to represent a single serotype (Emerson *et al.*, 2006; Engle *et al.*, 2002), and we found that ferret and rat HEV shared 55.2 to 57.2 % and 54.6 to 57.1 % aa sequence identities with G1–G4 HEV, respectively. Furthermore, we found that G3 HEV was neutralized by anti-G1, -G3 and -G4 HEV antibodies, but not by anti-rat or -ferret HEV antibody, indicating clearly that the antigenicities of rat HEV, ferret HEV and G1, G3 and G4 HEVs are different from each other, and that the serotype of HEV varies in a host-dependent manner. The genomic sequences of the HEV-like viruses including rat, bat and ferret HEVs showed a high degree of divergence (66.5–83.0 %) when compared to G1–G4 HEV, suggesting that the HEV-like viruses as well as avian HEV should be considered as new genera in the *Hepeviridae* family (Oliveira-Filho *et al.*, 2013). Taken together, the serotype of HEV-like viruses from different genera might be different.

At present, data other than the nucleotide sequences of the two strains of ferret HEV from The Netherlands remain unknown, including the epidemiology, virology and pathology of this virus. ELISA based on ferret HEV-LPs for detection of anti-ferret IgG and IgM proved useful to understanding the transmission of ferret HEV. In this study, anti-ferret IgG and IgM antibodies were detected by this ELISA with positive rates of 23.3 % and 24.4 %, respectively. Furthermore, 63.6 % (14/22) of the IgM-positive serum samples were also positive for HEV RNA, indicating that ferret HEV infection occurred consistently in laboratory ferrets, and that ELISA is useful for the epidemiological study of ferret HEV. Although the pathogenicity of ferret HEV to humans is not clear at this moment, it is important to be aware of the risk of ferret HEV infection when ferrets are used as laboratory animals or kept as pets. Due to the lack of an authentic method for detection of ferret HEV RNA, we tried to use nested broad-spectrum RT-PCR, and a part of the ferret HEV genome was amplified. Our results indicated that the nested broad-spectrum RT-PCR is universal for amplifying HEV RNA.

Phylogenetic analysis showed that the ferret HEV sequences detected in this study are genetically different from the prototypic strains from The Netherlands, suggesting the ferret HEV genome is genetically diverse. More serological as well as genetic studies are needed to understand the epidemiology of ferret HEV.

## METHODS

**Construction of a transfer vector.** The full-length ORF2 of the ferret HEV containing the *Bam*HI site before the start codon and the *Xba*I site after the stop codon was synthesized based on the ferret HEV sequence deposited in GenBank (JN9908607). The full-length ORF2 was cloned into a vector, pUC57, to generate a plasmid, pUC57-ferret-ORF2 (GeneScript). DNA fragments encoding the N-terminus-truncated, or both N- and C-terminus-truncated ferret HEV ORF2s were amplified by PCR using plasmid pUC57-ferret-ORF2 as a template. The DNA fragments encoding 6-, 18- and 112-N-terminal-aa-truncated ferret HEV ORF2s were amplified by PCR with the primers FN6/CR, FN18/CR and FN112/CR, respectively (Table 1). The 6-, 18- and 112-N-terminal-aa- and 47-C-terminal-aa-truncated ferret HEV ORF2s were amplified by PCR using primers FN6 /CR47, FN18/CR47 and FN112/CR47, respectively (Table 1). The amplified DNA fragments were purified with a Qiagen Gel purification kit and cloned into TA 2.1 cloning vector (Invitrogen). The full-length and truncated ORF2s were digested with *Bam*HI and *Xba*I, and ligated with a baculovirus transfer vector, pVL1393 (Pharmingen), to yield plasmids pVL1393-ORF2, pVL1393-n6c0-ORF2, pVL1393-n18c0-ORF2, pVL1393-n112c0-ORF2, pVL1393-n6c47-ORF2, pVL1393-n18c47-ORF2 and pVL1393-n112c47-ORF2.

**Construction of a recombinant baculovirus and expression of capsid proteins.** Sf9 cells (RIKEN Cell Bank, Tsukuba, Japan), derived from an insect, *Spodoptera frugiperda*, were co-transfected with a linearized wild-type *Autographa californica* nuclear polyhedrosis virus DNA (BaculoGold 21100D; Pharmingen) and transfer plasmids by a Lipofectin-mediated method as specified by the manufacturer (Gibco-BRL). The cells were incubated at 26.5 °C in

**Table 1.** Recombinant baculoviruses, primers and ferret HEV ORF2 proteins

| Recombinant baculovirus | Forward primer | Reverse primer | Product |
|---|---|---|---|
| Ac[n6c0-ORF2] | FN6 (5′-AGGATCCATGCGTTTTGTT-CTCTTGCTCCT-3′) | CR (5′-ATCTAGATTAGACATCCTCAGA-AACGGACAAA-3′) | 7–654 aa |
| Ac[n18c0-ORF2] | FN18 (5′-AGGATCCATGGTGTCGGTGTTGC-TCGCCGCCCCA-3′) | CR (5′-ATCTAGATTAGACATCCTCAGA-AACGGACAAA-3′) | 19–654 aa |
| Ac[n112c0-ORF2] | FN112 (5′-AGGATCCATGGCGGTTGCGCCCG-CCCCGAACA-3′) | CR (5′-ATCTAGATTAGACATCCTCAG-AAACGGACAAA-3′) | 113–654 aa |
| Ac[n6c47-ORF2] | FN7 (5′-AGGATCCATGCGTTTTGTTC-TCTTGCTCCT-3′) | CR47 (5′-TTCTAGATTAAGTGGGGCCAA-CAGCACCCACT-3′) | 7–607 aa |
| Ac[n18c47-ORF2] | FN18 (5′-AGGATCCATGGTGTCGGTGTTGC-TCGCCGCCCCA-3′) | CR47 (5′-TTCTAGATTAAGTGGGGCCAAC-AGCACCCACT-3′) | 19–607 aa |
| Ac[n112c47-ORF2] | FN112 (5′-AGGATCCATGGCGGTTGCGCCC-GCCCCGAACA-3′) | CR47 (5′-TTCTAGATTAAGTGGGGCCAACA-GCACCCACT-3′) | 113–607 aa |

TC-100 medium (Gibco-BRL) supplemented with 8 % FBS and 0.26 % tryptose phosphate broth (Difco). The recombinant viruses were plaque-purified three times in Sf9 cells and designated Ac[ORF2], Ac[n6c0-ORF2], Ac[n18c0-ORF2], Ac[n112c0-ORF2], Ac[n6c47-ORF2], Ac[n18c47-ORF2] and Ac[n112c47-ORF2] (Table 1). To achieve large-scale expression, an insect cell line from *Trichoplusia ni*, BTL-Tn 5B1-4 (Tn5) (Invitrogen), was used (Wickham & Nemerow, 1993). Tn5 cells were infected with recombinant baculoviruses at an m.o.i. of 10, and the cells were cultured in EX-CELL 405 medium (JRH Biosciences) at 26.5 °C as described previously (Li *et al.*, 1997, 2005b). The V-LPs of G1, G3, G4 and rat HEVs were produced as described previously (Guu *et al.*, 2009; Li *et al.*, 1997, 2011b; Yamashita *et al.*, 2009).

**SDS-PAGE and Western blot analysis.** The proteins in the cell lysates and culture medium were separated by 5–20 % SDS-PAGE and stained with Coomassie blue (CB). For the Western blot analysis, proteins in the gel were electrophoretically transferred onto a nitrocellulose membrane. The membrane was then soaked with 5 % skimmed milk in 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, and incubated with rabbit anti-rat HEV-LPs polyclonal antibody as previously described (Li *et al.*, 2011b). Detection of the rabbit IgG antibody was achieved using alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (1 : 1000 dilution) (Chemicon International). Nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate P-toluidine were used as colouring agents (Bio-Rad).

**Purification of ferret HEV-LPs.** The recombinant baculovirus-infected Tn5 cells were harvested on day 7 p.i. The intact cells, cell debris and progeny baculoviruses were removed by centrifugation at 10 000 *g* for 60 min. The supernatant was then spun at 32 000 r.p.m. for 3 h in a Beckman SW32Ti rotor and the resulting pellet was resuspended in EX-CELL 405 medium at 4 °C overnight. For the CsCl gradient centrifugation, 4.5 ml of the samples were mixed with 2.1 g CsCl, and then centrifuged at 35 000 r.p.m. for 24 h at 10 °C in a Beckman SW55Ti rotor. The gradient was fractionated into 250 µl aliquots and each fraction was weighed in order to estimate the buoyant density and isopycnic point. Each fraction was diluted with EX-CELL 405 medium and centrifuged for 2 h at 50 000 r.p.m. in a Beckman TLA55 rotor to sediment HEV-LPs.

**Electron microscopy.** Purified HEV-LPs were placed on a carbon-coated grid for 45 s, rinsed with distilled water, stained with a 2 % uranyl acetate solution and examined with a JEOL TEM-1400 electron microscope operating at 80 kV.

**N-terminal aa sequence analysis.** The proteins were purified by CsCl gradient centrifugation. The N-terminal aa microsequencing was carried out using 100 pmol of protein by Edman automated degradation on an Applied Biosystems model 477 protein sequencer.

**Hyperimmune sera.** Rats (Wistar, 12 weeks old, female) were immunized with ferret, rat, G1, G3 and G4 HEV-LPs, respectively. The immunization was performed with thigh muscle injection of purified HEV-LPs with a dose of 200 µg per rat, and booster injections were carried out at 4 and 6 weeks after the first injection with half doses of HEV-LPs. All of the injections, including booster injections, were carried out without any adjuvant. Immunized animals were bled 1 week after the last injection. To generate a positive control for ferret anti-ferret HEV IgG and IgM antibodies, an 18-week-old female ferret (*Mustela putorius furo*) from the breeding colony in Japan was immunized with the ferret HEV-LPs as described above.

**Cell-culture-based neutralizing activity test.** Infectious G3 HEV strain (G3-HEV83-2-27, GenBank accession no. AB740232) was used to evaluate the neutralizing activity of anti-HEVs antibodies. G3-HEV83-2-27 grows in PLC/PRF/5 cells and is efficiently released to cell-culture supernatants (Li *et al.*, 2011a). Pre- and post-HEV-LPs-immunized rat serum and rat HEV-infected rat serum were heated at 56 °C for 30 min and then diluted to 1 : 10, 1 : 20 and 1 : 40 with medium 199 (Invitrogen). One mililitre of the solution containing $2 \times 10^6$ copies of G3-HEV83-2-27 was mixed with 1 ml of diluted antiserum and incubated at 37 °C for 1 h, and then at 4 °C for 3 h. A hepatocarcinoma cell line, PLC/PRF/5, was cultured in 6-well cell-culture plates ($5 \times 10^5$ cells per well) with 5 ml Dulbecco's modified Eagle's medium (DMEM; Wako) containing 10 % (v/v) heat-inactivated FCS. One mililitre of the virus/serum mixture was added to each well of the plates, after removing the cell-culture medium. After adsorption at 37 °C for 1 h, the cells were washed three times with PBS (-), and 4 ml maintenance medium consisting of medium 199, 2 % (v/v) heat-inactivated FCS and 10 mM $MgCl_2$. The culture medium was replaced with new medium every 3 days. Neutralizing activity was monitored by detection of HEV Ag (capsid protein) in the cell-culture supernatant at 3 weeks p.i. by ELISA (Yamamoto *et al.*, 2012). Detection of HEV Ag in the culture supernatant would mean that HEV was not neutralized by the antibody, whereas the lack of detection of HEV Ag would indicate that the antibody has neutralizing activity against G3 HEV.

**Ferret serum samples.** Two groups of ferret sera were collected to detect anti-ferret HEV antibodies. Group A comprised a total of 73 laboratory ferret sera collected from a breeding colony in Japan. All ferrets in group A were male, with ages ranging from 17 to 48 weeks.