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.

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., 2012).
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.
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 × 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 × 0.037) (Fig. 5b). Based on this cut-off value, no serum sample was positive for anti-ferret HEV.
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
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).
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

![Graph](image_url)

**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.
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 antibodies, 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 are genetically different from the HEV-like viruses from different genera. 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 BamHI site before the start codon and the XhoI site after the stop codon was synthesized based on the ferret HEV sequence deposited in GenBank (JN998607). 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 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 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 BamHI and XhoI and ligated with a baculovirus transfer vector, pVL1393 (Pharmingen), to yield plasmids pVL1393-ORF2, pVL1393-n6c47-ORF2, pVL1393-n18c47-ORF2, pVL1393-n112c47-ORF2, pVL1393-n47-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

<table>
<thead>
<tr>
<th>Recombinant baculovirus</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product</th>
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<tr>
<td>Ac[n6c0-ORF2]</td>
<td>FN6 (5′-AGGATCCATGCGTTTTGTTC-CTTGGTCTCC-3′)</td>
<td>CR (5′-ATCTAGATTAGACATCCTCAGA- ACGGACAAA-3′)</td>
<td>7–654 aa</td>
</tr>
<tr>
<td>Ac[n18c0-ORF2]</td>
<td>FN18 (5′-AGGATCCATGGTGGCTGGTGGCTG- TGCGGCGCCCA-3′)</td>
<td>CR (5′-ATCTAGATTAGACATCCTCAGA- ACGGACAAA-3′)</td>
<td>19–654 aa</td>
</tr>
<tr>
<td>Ac[n112c0-ORF2]</td>
<td>FN112 (5′-AGGATCCATGGCGGTTCGGCAGG- CGCCCGCACA-3′)</td>
<td>CR (5′-ATCTAGATTAGACATCCTCAGA- ACGGACAAA-3′)</td>
<td>113–654 aa</td>
</tr>
<tr>
<td>Ac[n6c47-ORF2]</td>
<td>FN7 (5′-AGGATCCATGGCTTTTCGT- TCTGCTCTCC-3′)</td>
<td>CR47 (5′-TTCTAGATTAGTGCGGCA- CAGCCAACC-3′)</td>
<td>7–607 aa</td>
</tr>
<tr>
<td>Ac[n18c47-ORF2]</td>
<td>FN18 (5′-AGGATCCATGGCTTTTCGT- TCTGCTCTCC-3′)</td>
<td>CR47 (5′-TTCTAGATTAGTGCGGCA- CAGCCAACC-3′)</td>
<td>19–607 aa</td>
</tr>
<tr>
<td>Ac[n112c47-ORF2]</td>
<td>FN112 (5′-AGGATCCATGGCGGTTCGGCAGG- CGCCCGCACA-3′)</td>
<td>CR47 (5′-TTCTAGATTAGTGCGGCA- CAGCCAACC-3′)</td>
<td>113–607 aa</td>
</tr>
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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 S99 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* (Invtrogen), 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 (Gu 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 SW28Ti rotor and the resulting pellett 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 weighted 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 TLS55 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-termina asequence 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 milliliter of the solution containing 2 × 10⁵ 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 × 10⁵ cells per well) with 5 ml Dulbecco’s modified Eagle’s medium (DMEM; Wako) containing 10% (v/v) heat-inactivated FCS. One milliliter 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 MgCl₂. 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. from the breeding colony in Japan was immunized with the ferret HEV-LPs as described above.

**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.
Group B comprised a total of 90 laboratory ferret sera collected from a breeding colony in the USA. All ferrets in group B were female, with ages ranging from 13 to 15 weeks. All serum samples were determined to be negative for anti-influenza virus antibodies, and were stored at −80°C until use.

Detection of IgG and IgM antibodies. Flat-bottom 96-well polystyrene microplates (Immulon 2; Dynex Technologies) were coated with the purified ferret HEV-LPs (1 μg ml⁻¹, 100 μl per well) and incubated overnight at 4°C. The unbound V-LPs were removed, and the plates were washed twice with 10 mM PBS containing 0.05% Tween 20 (PBS-T) and then blocked with 200 μl of 5% skimmed milk (Difco) dissolved in PBS-T for 1 h at 37°C. After the plates were washed four times with PBS-T, diluted ferret (100 μl per well) serum samples were added in duplicate. The plates were incubated at 37°C for 1 h and washed three times as described above. The wells were incubated with 100 μl horseradish peroxidase-labelled goat anti-ferret IgG (H+L) (KPL) (1:2000 dilution) or horseradish peroxidase-conjugated goat anti-ferret IgM (ROCKLAND Antibodies & Assays) (1:10 000 dilution), and diluted with PBS-T containing 1% skimmed milk. The plates were incubated at 37°C for 1 h and washed four times with PBS-T. The substrate orthophenylenediamine (100 μl) (Sigma) with H₂O₂ was added to each well. The plates were incubated in a dark room at room temperature for 30 min, and then 50 μl of 2 M H₂SO₄ was added to each well. OD values were measured at 492 nm. The pre-immunized and ferret HEV-LPs-immunized ferret sera were used as negative and positive controls, respectively. A sample was considered to be positive when the absorbance exceeded the cut-off value.

Nested broad-spectrum RT-PCR for detection of ferret HEV RNA. The RNA was extracted using the MagNA Pure LC system with MagNA Pure LC Total Nucleic Acid isolation kit (Roche Applied Science) according to the manufacturer’s recommendations. RT was performed with a high-capacity cDNA reverse transcription kit (ABI Applied Biosystems) at 25°C for 10 min, 37°C for 120 min followed by 85°C for 5 min in a 20 μl reaction mixture containing 1μl reverse transcriptase, 2 μl of the random primer, 1 μl RNase inhibitor, 2 μl 10× RT buffer, 0.8 μl 10 mM deoxynucleoside triphosphates, 8 μl RNA and 5.2 μl distilled water.

A nested broad-spectrum RT-PCR analysis was performed to amplify a portion of the ORF1 genome, based on the method described previously with slight modification (Johne et al., 2010b). Five microlitres of cDNA was used for the first PCR in 50 μl of reaction mixture containing an external forward primer, HEV-cs (5'-TC-GCGCATACMTCYTTCGARAA-3') and an external reverse primer, HEV-cas (5'-GCGATTTCCACGACGDRTTCTCCA-3'). Each cycle consisted of denaturation at 95°C for 30 s, primer annealing at 52°C for 45 s and an extension reaction at 72°C for 60 s, followed by final extension at 72°C for 7 min. Two microlitres of the first PCR product were used for nested PCR with an internal forward primer, HEV-csn (5'-TGCTGTCTGTTTGGCCCGCTTGGTYCDG-3'), and an internal reverse primer, HEV-casn (5'-CACGGCTCACCRGARTGYTCTCCCA-3'). Each cycle consisted of denaturation at 95°C for 30 s, primer annealing at 55°C for 45 s and an extension reaction at 72°C for 60 s, followed by final extension at 72°C for 7 min. The nested PCR products were separated by electrophoresis on 2% agarose gels.

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