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ORIGINAL ARTICLE

A549 and PLC/PRF/5 cells can support the efficient propagation of swine and wild boar hepatitis E virus (HEV) strains: demonstration of HEV infectivity of porcine liver sold as food

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Abstract Recent evidence has indicated the cross-species transmission of hepatitis E virus (HEV) from pigs and wild boars to humans, causing zoonosis, mostly via consumption of uncooked or undercooked animal meat/viscera. However, no efficient cell culture system for swine and boar HEV strains has been established. We inoculated A549 cells with 12 swine and boar HEV strains of liver, feces, or serum origin at an HEV load of $\geq 2.0 \times 10^4$ copies per well and found that the HEV progeny replicated as efficiently as human HEV strains, with a maximum load of $\sim 10^8$ copies/ml. However, the HEV load in the culture medium at 30 days post-inoculation differed markedly by inoculum, ranging from 1.0×10^2 to 1.1×10^7 copies/ml upon inoculation at a lower load of approximately 10^5 copies per well. All progeny were passaged successfully onto A549 and PLC/PRF/5 cells. In sharp contrast, no progeny viruses were detectable in the culture supernatant upon inoculation with 13 swine and boar HEV strains at an HEV load of $<1.8 \times 10^4$ copies per well. The present study also demonstrates that swine liver sold as food can be infectious, supporting the risk of zoonotic food-borne HEV infection.

The nucleotide sequences of HEV isolates reported herein have been assigned DDBJ/EMBL/GenBank Accession Nos. AB668379-AB668393.

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Introduction

Hepatitis E virus (HEV) is a major cause of epidemics and sporadic cases of acute hepatitis in many developing countries where the sanitation conditions are suboptimal, with occasional travel-related cases of hepatitis E being diagnosed in industrialized countries [9]. Since its discovery in 1983 [3], documented HEV transmission was linked almost exclusively to contaminated water. However, that association changed abruptly with the discovery of HEV infection following ingestion of uncooked meat or viscera of pigs, wild boars, and deer [17, 42, 48]. Hepatitis E now is recognized as being not only a water-borne disease of developing countries but also an emerging food-borne disease of numerous industrialized countries, including the United States, European countries and Japan [1, 5, 14, 16, 19, 23, 24]. Hepatitis E is typically a self-limiting disease with variable severity, presenting as acute icteric hepatitis with clinical symptoms similar to those of hepatitis A [9]. Recently, chronic or persistent HEV infection has been documented in immunocompromised solid-organ transplant recipients and human immunodeficiency virus–infected patients [6, 13, 15].

HEV is a non-enveloped virus with a single-stranded, positive-sense RNA genome of 7.2 kb [9] and is classified as a member of the genus *Hepevirus* of the family

Hepeviridae [8]. The genome is capped and polyadenylated and contains a short 5′ untranslated region (UTR) followed by three open reading frames (ORFs: ORF1, ORF2 and ORF3) and then a short 3′UTR [36]. The 5′ two-thirds of the genome contains ORF1, which encodes non-structural proteins involved in viral replication and viral protein processing. ORF2 occupies the 3′-terminal part of the genome and encodes a 660-amino-acid (aa) capsid protein. ORF3 mostly overlaps ORF2 and encodes a small protein of 113–114 aa that is required for virion egress from cells and is associated with numerous cellular pathways [4, 10, 46]. The ORF2 and ORF3 proteins are encoded by a bicistronic subgenomic RNA [12].

There are four recognized genotypes of HEV that infect humans: genotype 1 and 2 infections have been identified exclusively in humans, whereas genotype 3 and 4 viruses have been isolated from humans as well as pigs, wild boars, a deer, a mongoose, and rabbits [22, 25, 26]. Genotype 1 is responsible for the majority of HEV infections in developing countries in Asia and Africa; genotype 2 consists of strains not only detected in Mexico but also in African countries; genotype 3 is widely distributed throughout the world; and genotype 4 is distributed exclusively in Asian countries [18, 25]. The ubiquitous nature of genotype 3 and 4 viruses in domestic pigs and wild boars raises public-health concerns about zoonotic infection through direct contact with infected animals or, more likely, through the consumption of contaminated animal meat and viscera [22, 26, 29, 31].

Recently, using fecal specimens from patients with sporadic acute hepatitis E, genotype 3 and 4 HEVs have been grown efficiently in a human hepatocarcinoma cell line (PLC/PRF/5) and a human lung cancer cell line (A549) [40, 41]. In addition, various HEV strains of genotypes 1, 3, and 4 isolated from circulating blood replicated efficiently in cultured PLC/PRF/5 and A549 cells, even in the presence of HEV antibodies [35]. More recently, it was reported that A549 cells could also support the replication of swine HEV of genotype 4 derived from a fecal specimen [49]. However, it remains unclear whether swine HEV strains of genotypes 3 and 4 recovered from domestic pigs and wild boars can grow as efficiently as human HEV strains in human cultured cells, irrespective of the source of the inoculum virus. Therefore, in the present study, using liver homogenate and serum and fecal specimens obtained from HEV-infected domestic pigs and wild boars as inocula, various swine and boar HEV strains were cultivated in A549 and PLC/PRF/5 cells, and their replication efficiency was evaluated in relation to the viral load and origin of the inoculum. This study indicated that HEVs in raw porcine liver sold as food in grocery stores remain infectious, as determined using the established cell culture system.

Materials and methods

Liver tissues and serum and fecal specimens

Tissue specimens obtained from seven packages of raw porcine liver that had been purchased from grocery stores in Japan from 2002 to 2003 and found to be positive for HEV RNA [48] were stored at −80°C and used in the present study (samples 1–7 in Table 1). Liver specimens with a high viral load obtained from four (samples 20–23) of the 19 HEV-infected wild boars that had been captured in Japan from 2003–2010 [27] were also kept at −80°C and used in the present study. Liver tissues (200 mg) were minced with a razor blade and homogenized with an Eppendorf micropestle (SIGMA-ALDRICH Inc., St. Louis, MO) in the presence of 2 ml of phosphate-buffered saline (PBS, pH 7.5) and clarified by centrifugation in a high-speed refrigerated microcentrifuge (Tomy Seiko, Tokyo, Japan) at 7900×*g* at 4°C for 10 min, and a clear supernatant was obtained. The 3% (wt/vol) homogenates were used as the inoculum. Fecal specimens (2 to 5 g) obtained from domestic pigs were suspended at 10% (wt/vol) in Tris-HCl buffer (0.01 M, pH 7.5) and centrifuged in a Hitachi Refrigerated Centrifuge (Hitachi High-Technologies Corp., Tokyo, Japan) at 1600×*g* at 4°C for 30 min, and the supernatant was recovered. Cell debris was spun down in a refrigerated centrifuge (Tomy Seiko) at 6200×*g* at 4°C for 10 min, and a clear supernatant was obtained. Aliquots of the supernatant were used as the inoculum (samples 8–10). Serum samples with a high HEV load, obtained from domestic pigs [32, 39] and wild boars [27], that had been kept at −80°C were used as the serum-based inoculum (samples 11–19, 24, and 25). Prior to inoculation with liver homogenate, fecal, and serum samples, the virus stocks were subjected to purification by passaging them through microfilters with pore sizes of 0.45 and 0.22 μm (Millex-GV; Millipore Corp., Billerica, MA).

ELISA for detecting swine anti-HEV IgG

To detect anti-HEV IgG in the serum samples from pigs and wild boars, an enzyme-linked immunosorbent assay (ELISA) was performed using purified recombinant ORF2 protein from HE-J1 virus (genotype 4) that had been expressed in silkworm pupae [23] as described previously [32]. An OD value of 0.274 was used as the cutoff value for the swine anti-HEV IgG assay [32].

Quantitation of HEV RNA

HEV RNA was quantitated by real-time detection via reverse transcription (RT)-PCR according to a method described previously [33] with slight modifications, using a

Table 1 Liver tissue and fecal and serum samples, obtained from swine and wild boars, used to inoculate cultured cells

Sample no.	Animal species	Specimen for virus inoculation [OD_{450} value of anti-HEV IgG in serum sample]	HEV RNA titer (copies/ml)[a]	HEV genotype	Isolate name
1	Swine	Liver tissue (3% homogenate)	2.1×10^5	3	swJL82[b]
2	Swine	Liver tissue (3% homogenate)	1.5×10^6	3	swJL97[b]
3	Swine	Liver tissue (3% homogenate)	3.5×10^6	3	swJL98[b]
4	Swine	Liver tissue (3% homogenate)	$<1.0 \times 10^3$	3	swJL131[b]
5	Swine	Liver tissue (3% homogenate)	$<1.0 \times 10^3$	4	swJL145[b]
6	Swine	Liver tissue (3% homogenate)	3.1×10^3	3	swJL234[b]
7	Swine	Liver tissue (3% homogenate)	$<1.0 \times 10^3$	3	swJL325[b]
8	Swine	Feces (2% suspension)	5.8×10^5	3	swJF04-2548
9	Swine	Feces (2% suspension)	4.4×10^5	3	swJF04-2555
10	Swine	Feces (2% suspension)	1.2×10^5	3	swJF05-2307
11	Swine	Serum (2x dilution) [0.182 (-)]	2.6×10^5	3	swJMS9[b]
12	Swine	Serum (2x dilution) [0.254 (-)]	1.3×10^5	3	swJMS15[b]
13	Swine	Serum (2x dilution) [2.060 (+)]	9.1×10^3	3	swJMS13[b]
14	Swine	Serum (2x dilution) [0.118 (-)]	2.4×10^4	3	swJIW1-231
15	Swine	Serum (2x dilution) [>3.000 (+)]	3.6×10^4	3	swJIW1-1[b]
16	Swine	Serum (2x dilution) [>3.000 (+)]	4.7×10^4	3	swJIW1-2[b]
17	Swine	Serum (2x dilution) [1.039 (+)]	4.2×10^4	4	swJIB1-3[c]
18	Swine	Serum (2x dilution) [2.293 (+)]	4.5×10^4	3	swJAK1-2[c]
19	Swine	Serum (2x dilution) [0.167 (-)]	1.1×10^4	3	swJAK3-3[c]
20	Wild boar	Liver tissue (3% homogenate)	8.1×10^7	3	wbJYG_05[d]
21	Wild boar	Liver tissue (3% homogenate)	4.8×10^6	3	wbJSO_05-2[d]
22	Wild boar	Liver tissue (3% homogenate)	2.8×10^7	4	wbJGF_08-1[d]
23	Wild boar	Liver tissue (3% homogenate)	3.4×10^7	3	wbJNR_10[d]
24	Wild boar	Serum (2x dilution) [0.056 (-)]	1.9×10^4	3	wbJGF_08-1[d]
25	Wild boar	Serum (2x dilution) [0.986 (+)]	4.0×10^4	3	wbJFI_09[d]

[a] The HEV RNA titer without prior filtration

[b] The HEV isolate was described by Yazaki et al. [48]

[c] The HEV isolate was described by Tanaka et al. [39]

[d] The HEV isolate was described by Takahashi et al. [32]

[e] The HEV isolate was described by Sato et al. [27]

culture supernatant containing a known amount of HEV progeny (genotype 3; 1.2×10^7 copies/ml) as a standard. The load of the standard HEV was determined using an *in vitro*-transcribed RNA standard [47]. In brief, total RNA was extracted from 2 to 100 μl of serum or fecal sample, liver homogenate, or culture supernatant using TRIzol-LS Reagent (Invitrogen, Tokyo, Japan) and was subjected to real-time RT-PCR with a QuantiTect Probe RT-PCR Kit (QIAGEN, Tokyo, Japan), using the sense primer HE311 (5′-GGT GGT TTC TGG GGT GAC-3′), antisense primer HE312 (5′-AGG GGT TGG TTG GAT GAA-3′), and a probe (HE313-P) consisting of an oligonucleotide with a 5′-reporter dye (FAM) and a 3′-quencher dye (TAMRA) (5′-FAM-TGA TTC TCA GCC CTT CGC-TAMRA-3′) on a LightCycler apparatus (Roche Diagnostics K.K., Tokyo, Japan). The thermal cycler conditions were 50°C for 20 min during stage 1, 95°C for 15 min during stage 2, and 45 cycles of 95°C for 1 s and 60°C for 60 s during stage 3. Otherwise, an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosytems Japan Ltd., Tokyo, Japan) was used with thermal cycler conditions of 50°C for 30 min during stage 1, 95°C for 15 min during stage 2, and 50 cycles of 94°C for 15 s, 56°C for 30 s and 76°C for 30 s during stage 3. The reproducibility of the quantitative assay was assessed by testing each sample in duplicate, and the mean value was adopted.

Cell culture and virus inoculation

Human lung cancer cells (A549, No. RCB0098, RIKEN BRC Cell Bank, Tsukuba, Japan) or human hepatocarcinoma cells (PLC/PRF/5, ATCC No. CRL-8024, American

Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle medium (DMEM; GIBCO Cat. No. 12800-058) (growth medium), supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; HANA-NESCO BIO, Tokyo, Japan), 100 U/ml of penicillin G, 100 μg/ml of streptomycin and 2.5 μg/ml of amphotericin B at 37°C in a humidified 5% CO_2 atmosphere as described previously [40]. For virus infection, confluent cells in a 75-cm^2 flask (IWAKI, Tsukuba, Japan) were trypsinized and diluted in the growth medium, and 2.0 ml of the resulting dilution (adjusted to 5×10^5 cells) was added to each well (diameter of 3.5 cm) of a six-well microplate (IWAKI) two days before virus infection.

Monolayers of cultured cells (nearly 100% confluent, $\sim 10^6$ cells/well) in the six-well microplates were washed three times with 1 ml of PBS (pH 7.5) without Ca^{2+} and Mg^{2+} [PBS(-)], and 0.2 ml of the filtered virus stock or culture supernatant that had been diluted 1:2 to 1:20 in PBS(-) containing 0.2% (wt/vol) bovine serum albumin (BSA; SIGMA-ALDRICH Inc.) was added each well. One hour after inoculation at room temperature, the solution was removed, and 2 ml of maintenance medium was added. The maintenance medium used for virus culturing consisted of 50% DMEM and 50% medium 199 (GIBCO Cat. No. 31100-027) containing 2% (vol/vol) heat-inactivated FBS and 30 mM $MgCl_2$; other supplements were the same as those in the growth medium. The culture was carried out at 35.5°C in a humidified 5% CO_2 atmosphere.

On the day following inoculation, the inoculated cells were washed five times with 1 ml of PBS(-) and then 2 ml of maintenance medium was added. Then, every other day, one-half (1 ml) of the culture medium was replaced with fresh maintenance medium, and the collected media were stored at −80°C until virus titrations were performed. In this study, triplicate sets of inocula were inoculated in parallel on the cultivated cells in six-well plates. The HEV load was determined for all or selected series of culture supernatants from the inoculated wells, and representative data were used.

Amplification and sequence analysis of the HEV genome

For amplification of the HEV genome, total RNA was extracted from 10–100 μl of inocula or culture supernatants with the TRIzol-LS Reagent (Invitrogen) and subjected to nested RT-PCR to amplify a 457-nulceotide (nt) sequence in ORF2 as described previously [23]. The amplification product was sequenced directly on both strands, using a BigDye Terminator v 3.1 Cycle Sequencing Kit on an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems Japan Ltd.). Sequence analysis was performed using the Genetyx software program version 10.1.5 (Genetyx Corp., Tokyo, Japan), and multiple alignments were created using the CLUSTAL W software program version 1.8 [43]. Phylogenetic analysis was conducted by the neighbor-joining method based on the 412-nt ORF2 sequence with 1,000 bootstrapping replicates, using the MEGA version 5.0.5 software program [38].

Immunofluorescence assay

A549 or PLC/PRF/5 cells inoculated with cell-culture-produced HEV of swine liver origin at 20 dpi were subjected to immunofluorescence microscopy using an anti-ORF2 mAb (H6225) [33], an anti-ORF3 mAb (TA0536) [34], or an irrelevant mAb, No. 905 [30], as described previously [46]. The cultured cells were stained with Alexa Fluor 488–conjugated anti-mouse IgG (Invitrogen), and nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Roche Diagnostics). The stained cells were viewed under a FV1000 confocal laser microscope (OLYMPUS, Tokyo, Japan).

Results

Inoculation of A549 cells with liver, fecal and serum HEV from pigs and wild boars

Seven HEV-RNA-positive liver homogenate samples (1–7) obtained from raw porcine liver that had been purchased from grocery stores [48] were inoculated onto fresh monolayers of A549 cells at various HEV RNA titers (Table 2). HEV progeny viruses were released into the culture medium upon inoculation with three samples (1–3) with a higher HEV load (4.0×10^4 to 6.6×10^5 copies per well). The HEV RNA was first detected in the culture medium of A549 cells on the second to sixth day post-inoculation (dpi), with a viral load of 40–220 copies/ml, and continued to increase thereafter, with the highest titer reaching $1.1–7.3 \times 10^7$ copies/ml at 30 dpi (Fig. 1A). In contrast, when the four other liver homogenate samples (4–7) with a lower HEV load ($\leq 6.0 \times 10^2$ copies per well) were used for the inoculation, no progeny were detectable in the culture medium throughout the observation period of 30 days.

HEV progeny viruses were released into the culture medium upon inoculation with three fecal samples (8–10) containing swine HEV at a viral load of 2.0×10^4 to 1.1×10^5 copies per well (Table 2), reaching $1.0–7.2 \times 10^5$ copies/ml at 60 dpi (Fig. 1B). When two serum samples containing swine HEV (11 and 12) were used for inoculation at a viral load of $5.0–6.0 \times 10^4$ copies per well, HEV RNA was detectable in the culture medium, with a maximum load of 2.1×10^4 to 2.3×10^5 copies/ml at

Table 2 Detection of HEV RNA in the culture supernatants of A549 cells inoculated with liver tissue, fecal suspension, or serum samples at the indicated HEV RNA titers

Sample no.	Viral load of HEV inoculated in each well (copies per well)[a]	Detectability of HEV RNA	HEV RNA titer at 30 dpi (copies/ml)	Highest HEV RNA titer during 30–60 dpi (copies/ml)	HEV isolate in culture medium
1	4.0×10^4	+	1.1×10^7	1.1×10^7	swJL82_p0
2	2.8×10^5	+	1.1×10^7	1.1×10^7	swJL97_p0
3	6.6×10^5	+	5.4×10^7	5.4×10^7	swJL98_p0
4	$<2.0 \times 10^2$	−	<20	<20	
5	$<2.0 \times 10^2$	−	<20	<20	
6	6.0×10^2	−	<20	<20	
7	$<2.0 \times 10^2$	−	<20	<20	
8	1.1×10^5	+	1.3×10^4	1.7×10^5	swJF04-2548_p0
9	8.4×10^4	+	4.4×10^3	1.0×10^5	swJF04-2555_p0
10	2.0×10^4	+	7.7×10^3	7.2×10^5	swJF05-2307_p0
11	5.0×10^4	+	1.0×10^2	2.1×10^4	swJMS9_p0
12	6.0×10^4	+	1.1×10^4	2.3×10^5	swJMS15_p0
13	1.8×10^4	−	<20	<20	
14	2.4×10^2	−	<20	<20	
15	7.0×10^3	−	<20	<20	
16	9.4×10^3	−	<20	<20	
17	8.4×10^3	−	<20	<20	
18	8.8×10^3	−	<20	<20	
19	2.0×10^3	−	<20	<20	
20	1.6×10^6	+	1.1×10^7	1.2×10^8	wbJYG_05_p0
21	9.8×10^5	+	6.6×10^5	9.8×10^7	wbJSO_05-2_p0
22	5.4×10^6	+	9.1×10^5	3.5×10^8	wbJGF_08-1_p0
23	6.6×10^6	+	5.7×10^5	9.9×10^7	wbJNR_10_p0
24	3.8×10^3	−	<20	<20	
25	8.0×10^3	−	<20	<20	

[a] The HEV RNA titer after purification by passage through microfilters

60 dpi (Fig. 1C). However, no progeny were detected throughout the observation period of 60 days upon inoculation of the remaining seven serum samples (13–19) with swine HEV at an HEV load of 2.4×10^2 to 1.8×10^4 copies per well (Table 2).

Regarding wild boar HEV, efficient viral replication was observed upon inoculation with four liver homogenate samples (20–23) at an HEV load of 9.8×10^5 to 6.6×10^6 copies per well. HEV RNA was initially detected on day 2 and reached the highest titer of 9.8×10^7 to 3.5×10^8 copies/ml on day 50 (Fig. 1D). However, no progeny viruses were detected in the culture medium throughout the observation period of 50 days upon inoculation with serum samples (24 and 25) with a lower HEV RNA titer of $3.8–8.0 \times 10^3$ copies per well.

Overall, HEV progeny were released into the culture medium when swine and boar HEV strains were inoculated at an HEV load of $\geq 2.0 \times 10^4$ copies per well, irrespective of the inoculum source, although the HEV RNA titers in the culture medium at 30 dpi differed markedly by inoculum, ranging from 1.0×10^2 to 1.1×10^7 copies/ml, when cultures were inoculated at viral loads ranging from 2.0×10^4 to 1.1×10^5 copies per well (Fig. 2). In sharp contrast, no progeny viruses were detectable in the culture supernatant upon inoculation with swine and boar HEV strains at an HEV load of $<1.8 \times 10^4$ copies per well. The continuous increase in virus titers up to 30–60 days may be attributable to the low multiplicity of infection, and significant spreading was involved over the long period of culture. During these long-term cultures, cell damage was seen in the A549 cells, similar to that observed in the cultured cells without virus inoculation. However, no direct cytopathic effect (CPE) was observed in the A549 cells, which continued to produce HEV progeny for at least 60 days.

Comparison of nucleotide sequences between HEV strains used as the inoculum and their cell-culture-generated variants

The wild-type HEV and its cell-culture-generated variants were 99.5–100% identical to each other in a

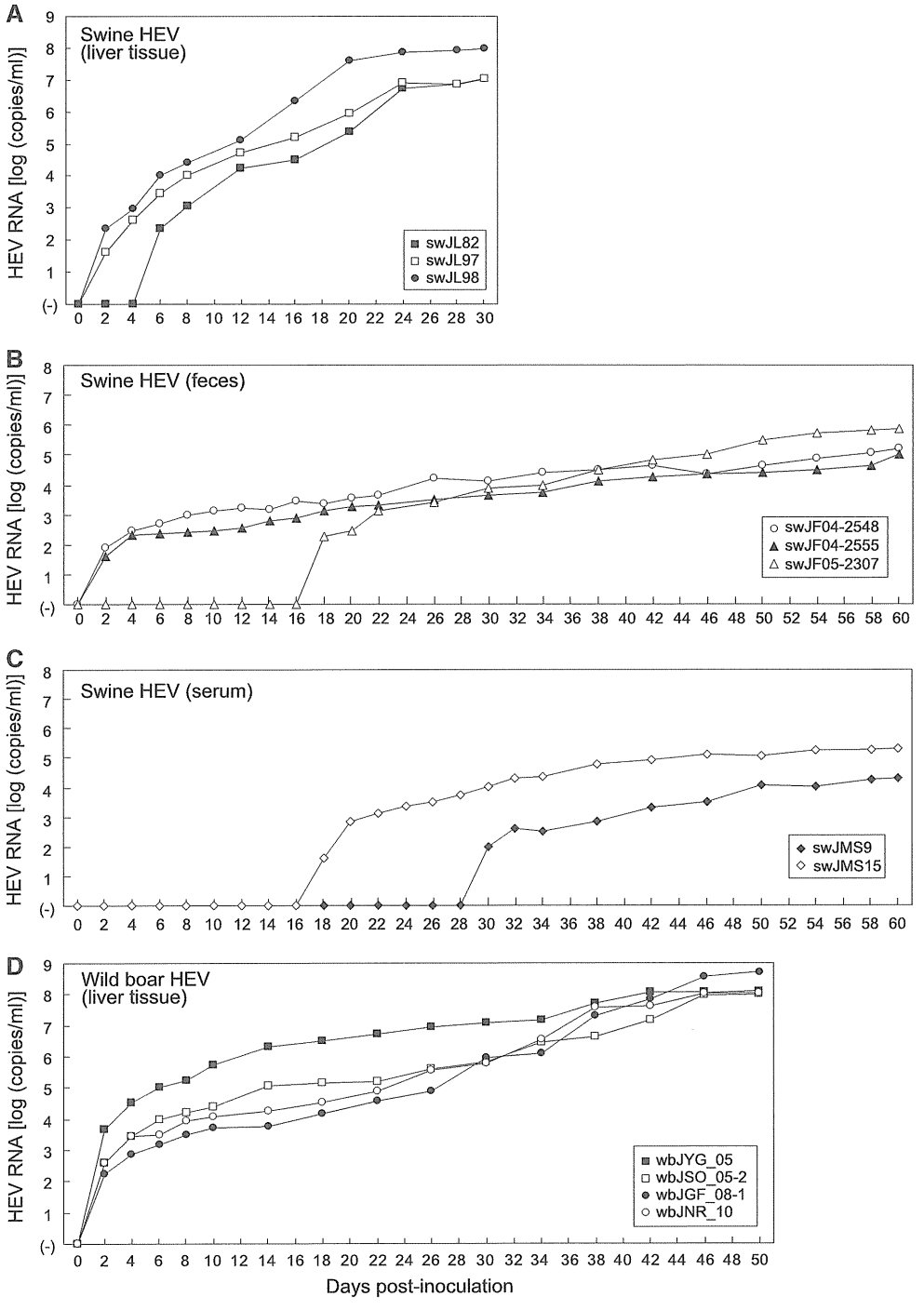


Fig. 1 Quantitation of HEV RNA in the culture supernatants of A549 cells inoculated with homogenate samples of swine liver tissues (**A**), swine fecal samples (**B**), swine serum samples (**C**), or homogenate samples of wild boar liver tissue samples (**D**) at various HEV loads (10^4 to 10^6 copies per well) as indicated in Table 2. The data are shown only for samples whose progeny viruses were detectable in the culture medium during the observation period of 30–60 days after inoculation

nucleotide sequence of 412 nt within the ORF2 gene in all 12 pairs (Fig. 3). A phylogenetic tree constructed based on the 412-nt ORF2 sequence showed that each pair of wild-type virus and its cell culture-produced variant segregated into a cluster within genotype 3 or 4, with a bootstrap value of 100% (Fig. 3), thereby confirming that various swine HEV strains of liver, feces and serum origin as well as wild boar HEV strains of liver origin were successfully propagated in cultured cells.

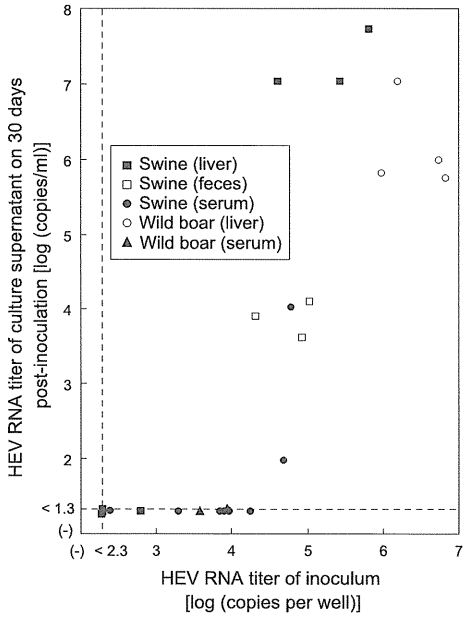


Fig. 2 A comparison of the HEV RNA titer of the seed virus with that of the progeny viruses released into the culture supernatant at 30 days post-inoculation, in relation to the source of inoculum

Supernatant passage of HEV in A549 cells and PLC/PRF/5 cells

All HEV progeny viruses (swJL82_p0, swJL97_p0, swJL98_p0, swJF04-2548_p0, swJF04-2555_p0, swJF05-2307_p0, swJMS9_p0, swJMS15_p0, wbJYG_05_p0, wbJSO_05-2_p0, wbJGF_08-1_p0, and wbJNR_10_p0) released in the culture medium of A549 cells that had been inoculated with 12 samples containing high-titer HEV of $>2.0 \times 10^4$ copies per well (samples 1–3, 8–12, and 20–23) (Table 2), were successfully passaged in A549 cells, as depicted in Fig. 4. The load of HEV detectable on the initial day of appearance (2 dpi) and the maximum load at 20 dpi were both dependent on the titer of the seed virus in the inoculum, reaching 2.5×10^6 copies/ml (swJL82_p0), 4.0×10^7 copies/ml (swJL97_p0), and 6.1×10^7 copies/ml (swJL98_p0) after inoculation with HEV at 10^6 copies per well (Fig. 4A).

Reflecting the efficiency of multiplication in the primary culture (p0), the HEV load increased gradually and reached a maximum load of only 1.1×10^5 to 1.5×10^6 copies/ml at 44–60 dpi in the culture medium of A549 cells after passage of feces- and serum-derived swine HEV progeny (Fig. 4B and C), while HEV grew rapidly and reached the highest load of $1.6–7.5 \times 10^8$ copies/ml at 40 dpi in the culture medium of A549 cells during passage of HEV progeny of wild boar liver origin (Fig. 4D).

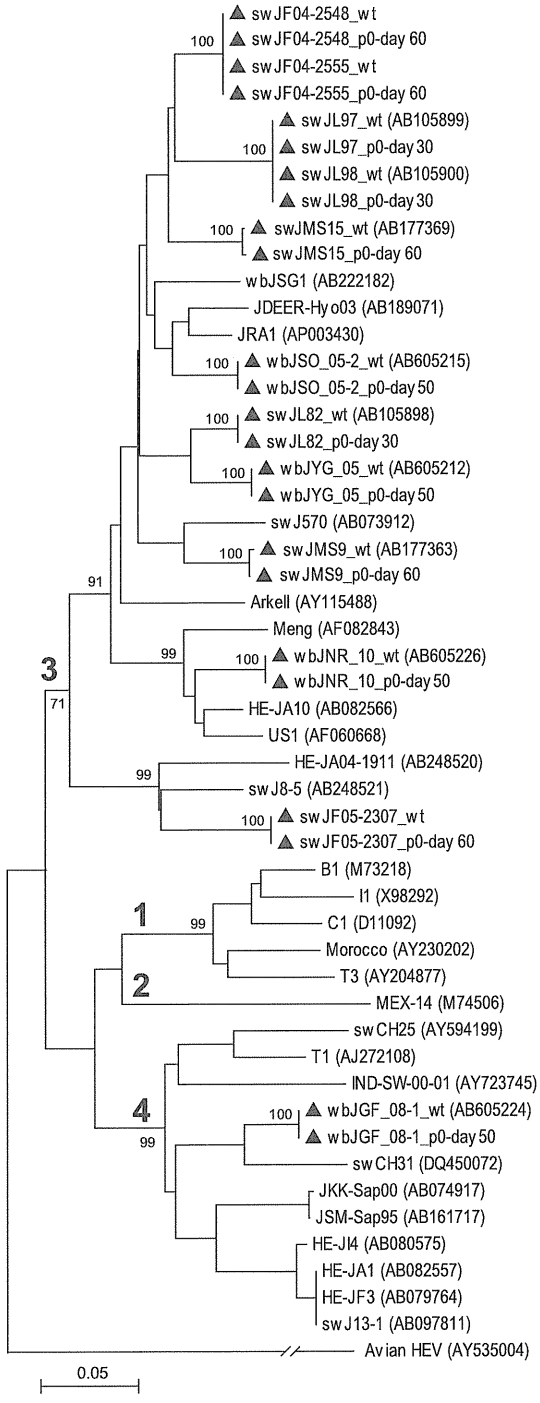
When the progeny viruses in the culture supernatants of A549 cells that had been inoculated with swine-liver- or wild-boar-liver-derived HEV were passaged onto PLC/PRF/5 cells at a load of 1.0×10^6 copies per well, the PLC/PRF/5 cells supported efficient multiplication of HEV, with maximum loads of 10^8 copies/ml at 30 dpi (data not shown). During these passages, no CPE was observed in either A549 or PLC/PRF/5 cells.

Immunofluorescence assay

A549 and PLC/PRF5 cells were inoculated with cell-culture-generated HEV of swine liver origin at an HEV load of 10^5 copies per 10^6 cells, and 20 days later, the cells were stained for evaluation by immunofluorescence microscopy using antibodies to the ORF2 capsid protein and to the ORF3 protein. Because these viral proteins are translated from a subgenomic mRNA, their presence indicates the occurrence of viral RNA synthesis. Infected foci were found in abundance in A549 cells (Fig. 5), detectable by both the anti-ORF2 and anti-ORF3 mAbs. Similarly, PLC/PRF/5 cells inoculated with cell-culture-produced swine HEV had detectable ORF2 and ORF3 proteins (data not shown), confirming that these two cell lines are permissive for the culture of swine and wild boar HEV strains.

Discussion

The present study revealed that swine and wild boar HEV strains can replicate as efficiently as human HEV strains in human cultured cells, including A549 lung cancer cells and PLC/PRF/5 hepatocarcinoma cells, with a maximum load of HEV progeny in the culture supernatant reaching $\sim 10^8$ copies/ml. Of note, swine HEV strains were able to replicate successfully in cultured cells, irrespective of the inoculum sources, such as feces and serum, similar to human HEV strains [35, 40]. Corroborating our previous studies on culturing human HEV strains, reporting that virtually any serum with a high HEV titer can infect cultured cells [35], the present study suggested that the HEV load of the seed virus is an important factor for determining whether a swine/wild boar HEV strain can propagate successfully in cultured cells and that $\sim 10^4$ copies per well (in six-well plates) is the minimum HEV load required for successful replication of the seeded virus in our cell culture system for HEV. In the present study, all 12 HEV strains inoculated at $\geq 2.0 \times 10^4$ copies per well were cultivated successfully, while those inoculated at $\leq 1.8 \times 10^4$ copies per well were not able to produce progeny in the culture supernatant, irrespective of the inoculum sources, including liver homogenate, fecal, and serum samples (Fig. 2). While the HEV RNA determined by RT-PCR in serum or feces samples are mainly virion-associated, liver samples contain not only mature virions but also replication intermediates that may not be able to initiate replication.



◀ **Fig. 3** The phylogenetic tree constructed by the neighbor-joining method based on the partial nucleotide sequence of the ORF2 region (412 nt) of 50 HEV isolates, using an avian HEV (AY535004) as an outgroup. In addition to the 24 HEV isolates used as the inocula or recovered from the culture medium in the present study (highlighted by a closed triangle), 26 representative HEV isolates of genotypes 1 to 4 whose common 412-nt sequence is known were included for comparison, and their accession numbers are indicated in parentheses. The "wt" denotes an HEV isolate recovered from a liver, fecal or serum sample, and the accession numbers are indicated in parentheses for the reported wt isolates [27, 32, 39]. The term "p0" represents an HEV isolate obtained from the culture supernatant harvested 30–60 days after inoculation with the corresponding sample (see Fig. 1). Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1,000 resamplings. Bar 0.05 nucleotide substitutions per site

Therefore, this difference has to be taken into consideration when directly comparing the titers of samples from various sources. However, since the diluent of the liver homogenates was not RNase-free, the load of replication intermediates in the filtered liver samples seems to be negligible in the present study.

Since RNA viruses exist as quasispecies [7], it is likely that a sample with a high viral load has an increased probability of containing variant(s) with the non-defective genomic sequence, or mutations needed to permit the infection of cultured cells. In addition, the replicative ability intrinsic to each swine/boar HEV strain seems to be a pivotal factor for the higher multiplication efficiency, based on the results showing that the HEV RNA titer in the culture medium at 30 dpi differed markedly by inoculum, ranging from 1.0×10^2 to 1.1×10^7 copies/ml, even when the wells were inoculated at a similar viral load (Fig. 2).

It has recently been reported that zoonotic food-borne transmission of HEV from domestic pigs, wild boars and wild deer to humans plays an important role in the occurrence of cryptic hepatitis E in industrialized countries including Japan and France, where people frequently eat raw or undercooked meat (including the liver and colon/intestine of animals) [5, 20, 37, 42, 48]. The ubiquitous nature of HEV infection in pigs suggests that contamination of organ meats by HEV may be unavoidable. Our previous study showed that approximately 2% of the pig livers sold in grocery stores in Japan were contaminated with HEV [48]. The virus sequences of genotypes 3 and 4 recovered from commercial pig livers were closely related or identical to the viruses recovered from hepatitis E patients in the same area where the pig livers were being sold in Japan [48], supporting the notion that genotype 3 and 4 HEVs cause zoonosis. Porcine liver being sold as food also tested positive for HEV RNA in 11% of the livers tested in the United States [11] and 4% of those tested in Germany [44]. In a recent case-control study in France, 7 of 13 individuals from three families who ate raw figatelli pig liver sausages became infected with HEV, whereas five other individuals from the same families who did not eat figatelli sausages were not infected [5]. Genotype 3 HEV RNA was detected from 7 of 12 figatelli sausages purchased from supermarkets [5]. These results indicate that consumption of undercooked or raw meat derived from pigs could potentially pose a risk of food-borne zoonotic HEV infection.

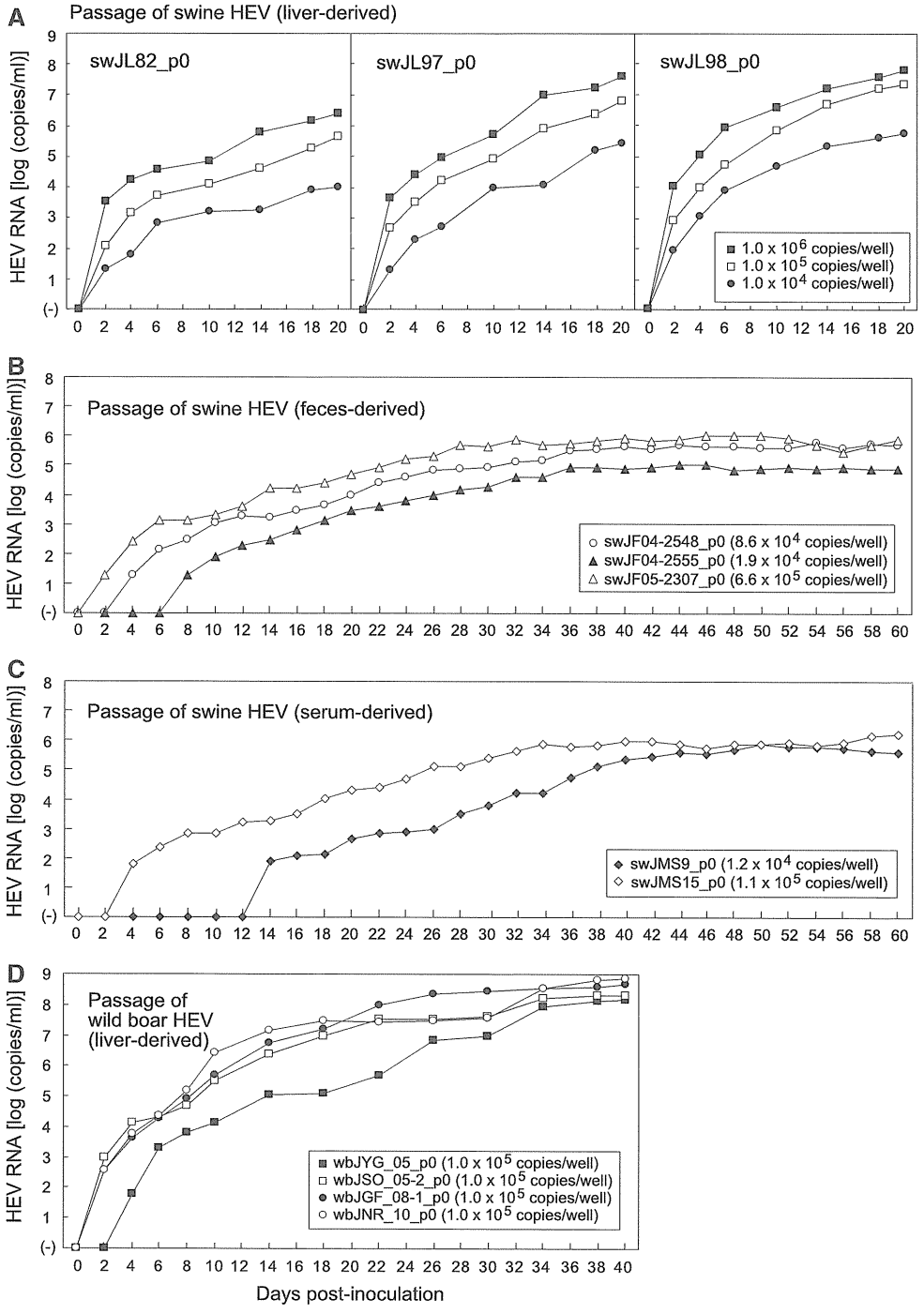


Fig. 4 Quantitation of HEV RNA in culture supernatants of A549 cells passaged with progeny viruses (passage 0) in the culture supernatants of A549 cells inoculated with swine HEV (liver-derived) (**A**), swine HEV (feces-derived) (**B**), swine HEV (serum-derived) (**C**), or wild boar HEV (liver-derived) at the indicated viral loads. The harvested culture supernatants from passage 0 were purified by passing them through a microfilter with a pore size of 0.22 μm (see "Materials and methods") and then applied to fresh A549 cells

It has been shown that non-human primates such as rhesus monkeys and chimpanzees, which are experimental surrogates for humans, can be experimentally infected with genotype 3 and genotype 4 swine HEV, thereby crossing the species barrier [2, 21]. Feagins et al. [11] demonstrated the infectivity of the contaminating virus in pig livers sold in grocery stores in the United States by intravenous injection of liver homogenates into infection-naïve pigs. The pigs inoculated with two of the three PCR-positive pig-liver homogenates became infected, as evidenced by the detection of fecal virus shedding, viremia and seroconversion. However, this experimental transmission of swine HEV was conducted from pig to pig, not through cross-species infection of human cells. In this context, our present study may be the first to indicate that the contaminating HEV present in pig livers sold in grocery stores remains fully infectious to human cells, demonstrating that transmission is possible across the species barrier.

Regarding HEV from wild boars, HEV RNA and antibodies have been detected in several European countries,

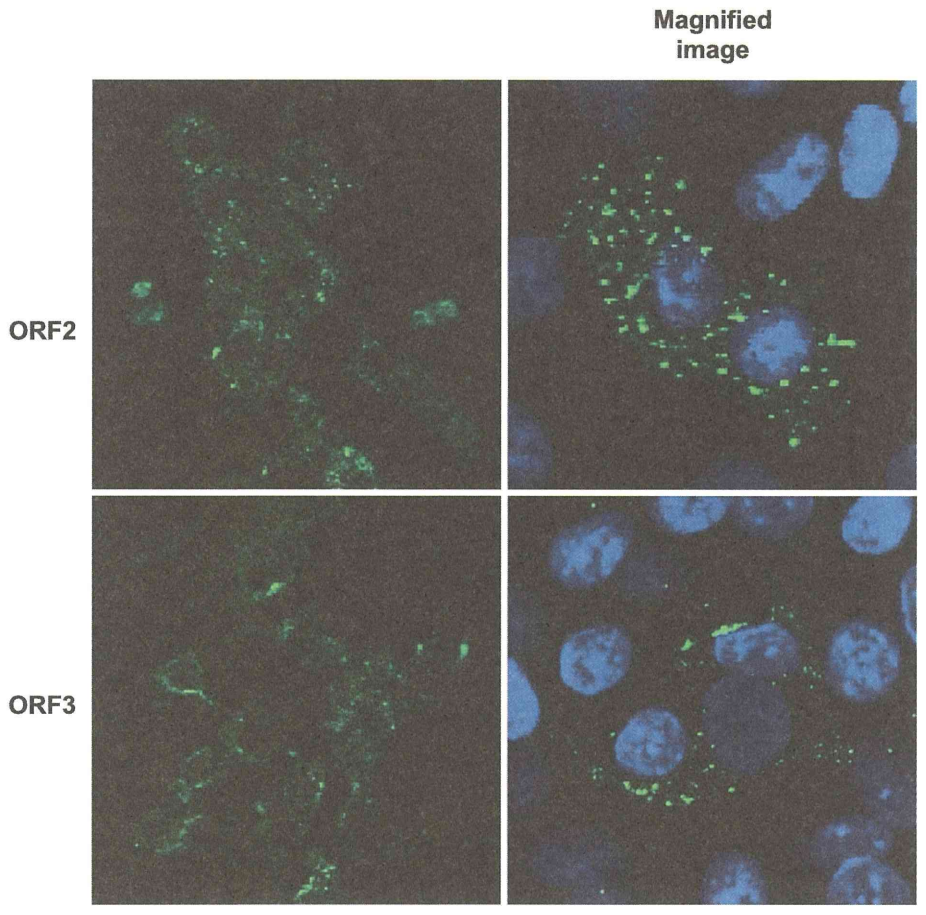


Fig. 5 Indirect immunofluorescence staining of ORF2 and ORF3 proteins in A549 cells with anti-ORF2 and anti-ORF3 mAbs, respectively. A549 cells inoculated with cell-culture-generated swine HEV were incubated with an anti-ORF2 mAb (H6225), anti-ORF3 mAb (TA0536), or an irrelevant mAb (No. 905) and then stained with AlexaFluor 488–conjugated anti-mouse IgG (data not shown for No. 905, which gave no signal). Nuclei were stained with DAPI (*Right panel*). The images of stained cells were obtained using a FV1000 confocal laser microscope (OLYMPUS)

Australia, and Japan, with a seroprevalence rate of 9–43% and an HEV RNA detection rate of 2–25% [26, 27, 29]. The transmission of HEV from wild boars to humans via consumption of uncooked or undercooked meat or liver has been reported, particularly from Japan [17, 20, 37]. The current study indicates that the HEV in liver homogenates from wild boars was also infectious in A549 and PLC/PRF/5 cells, thus supporting public-health concerns about food-borne HEV infection from wild boars to humans and indicating that cross-species infections of human cultured cells by wild boar HEV are possible.

HEV has been shown to replicate not only in the liver, but also in the lymph nodes, spleen, and even in the colon and small intestine *in vivo* [45]. The present study proved that swine and wild boar HEV strains can propagate efficiently in non-hepatocytes, consistent with a recent report by Zhang et al. [49], thus showing that a swine HEV strain (HB-3) of genotype 4 (feces origin) could replicate successfully in A549 cells. However, the RNA titer of the progeny viruses released into the culture medium upon primary propagation and passaging of the HB-3 strain was not detected quantitatively, and the replication efficiency of the swine virus in their cell culture system was unclear.

In conclusion, the present study revealed that both swine and wild boar HEV strains are able to replicate as efficiently as human HEV strains in human cultured cells, including A549 cells and PLC/PRF/5 cells, thus indicating that infection is possible across the species barrier and demonstrating that the contaminating HEV in pig livers sold in grocery stores remain fully infectious to human cells. It has recently been reported that genotype 3 strains (Kernow-C1, from a chronically infected patient, and US-2) can infect swine kidney cells (LLC-PK1) [28]. Therefore, further studies are warranted to examine whether swine and wild boar HEV strains can replicate more efficiently in non-human cells, including swine kidney cells, than in human cells and to establish more efficient cell culture systems for HEV using a lower viral load. Such a system would be useful for evaluation of the infectivity of contaminating virus in animal meat/viscera intended as food.

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

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Hepatitis E virus (HEV) is a causative agent of hepatitis E. Recently, a novel hepatitis E-like virus was isolated from Norway rats in Germany. However, the antigenicity, pathogenicity and epidemiology of this virus are unclear because of the lack of a cell-culture system in which to grow it. In this study, an N-terminally truncated ORF2 protein was expressed in insect Tn5 cells using a recombinant baculovirus expression system and a large amount of 53 kDa protein was expressed and efficiently released into the supernatant. Electron microscopic analyses of the purified 53 kDa protein revealed that the protein self-assembled into two types of empty HEV-like particles (rat HEVLPs). The smaller rat HEVLPs were estimated to be 24 nm in diameter, which is similar to the size of genotype G1, G3 and G4 HEVLPs. The larger rat HEVLPs were estimated to measure 35 nm in diameter, which is similar to the size of native rat HEV particles. An ELISA to detect antibodies was established using rat HEVLPs as the antigens, which demonstrated that rat HEVLPs were cross-reactive with G1, G3 and G4 HEVs. Detection of IgG and IgM antibodies was performed by examination of 139 serum samples from wild rats trapped in Vietnam, and it was found that 20.9 % (29/139) and 3.6 % (5/139) of the samples were positive for IgG and IgM, respectively. In addition, rat HEV RNA was detected in one rat serum sample that was positive for IgM. These results indicated that rat HEV is widespread and is transmitted among wild rats.

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INTRODUCTION

Hepatitis E virus (HEV) is the causative agent of hepatitis E, a viral disease that manifests as acute hepatitis (Emerson & Purcell, 2003). The disease represents an important

The GenBank/EMBL/DDBJ accession number for the 901 nt region of Vietnamese rat HEV strain 105 sequence determined in this study is JN040433.

public health problem in developing countries and is transmitted primarily by the faecal–oral route (Balayan *et al.*, 1983). In developed countries, a number of sporadic cases have been described, and the disease is primarily transmitted in a zoonotic fashion (Meng, 2010). HEV is a positive-sense ssRNA virus that belongs to the genus *Hepevirus* in the family *Hepeviridae* (Emerson *et al.*, 2005). The HEV genome is approximately 7.2 kb, containing a 5′ non-coding region (27–35 nt) followed by three overlapping ORFs and a 3′ non-coding region of approximately 65–74 nt followed by a poly(A) tail. ORF1 at the 5′ end of the genome encodes several non-structural proteins, whilst ORF2 encodes an immunodominant capsid protein (Jameel, 1999). ORF3, which partially overlaps with ORF2, encodes a cytoskeleton-associated phosphoprotein with multiple functions (Korkaya *et al.*, 2001; Meng *et al.*, 1997; Zafrullah *et al.*, 1997).

To date, at least four genotypes of HEV, G1–G4, have been isolated from humans, and G3 and G4 HEVs have been isolated from pigs, wild boar and wild deer (Bradley & Balayan, 1988; Huang *et al.*, 1992; Meng *et al.*, 1997; Takahashi *et al.*, 2001, 2004; Wang *et al.*, 2000). Recent evidence has indicated that G3 and G4 HEVs are transmitted from wild boar and wild deer to humans by zoonosis (Li *et al.*, 2005a; Tei *et al.*, 2003). Even more recently, new HEV strains (G5 and G6 HEVs) have been identified in wild boar, and other HEV-like viruses have been identified in rabbits and rats (Johne *et al.*, 2010a; Zhao *et al.*, 2009). Rat HEV shares little sequence identity with G1–G4 HEVs discovered thus far, suggesting that there are additional HEV-like viruses in other animal species (Johne *et al.*, 2010b).

To date, the entire rat genome sequence has been determined using two rat HEVs and it has been demonstrated that the genome contains three major ORFs, ORF1–3, similar to the genomes of G1–G4 HEVs (Johne *et al.*, 2010a). However, the antigenicity, pathogenicity and epidemiology of this virus remain unclear because of the lack of a viable cell-culture system in which to grow the virus.

In this study, we describe the efficient expression of N-terminally truncated rat HEV ORF2 protein with a synthetic gene derived from a German rat HEV strain isolated in 2010 (Johne *et al.*, 2010a). The viral protein, expressed by a recombinant baculovirus in insect Tn5 cells, was found to self-assemble into virus-like particles (VLPs), which were then efficiently released into the culture medium. The VLPs exhibited antigenic cross-reactivity with G1, G3 and G4 HEVs. An ELISA was developed using rat HEV-like particles (HEVLPs) as antigen and used to examine rat HEV-specific IgG and IgM responses. The antibody prevalence indicated that rat HEV is widespread among wild rats in Vietnam.

RESULTS

Expression of rat HEV ORF2 in insect cells

BTL-Tn-5B1-4 (Tn5) cells were infected at an m.o.i. of 10 with recombinant baculoviruses Ac[ORF2] and Ac[ΔORF2] containing the full-length and N-terminal 100 aa-deleted ORF2 of rat HEV, and the infected cells were incubated at 26.5 °C to express the full-length ORF2 and N-terminally truncated ORF2. The cells were harvested daily up to day 10 post-infection (p.i.), and the proteins generated in the infected cells and supernatant were analysed by Western blotting. In Ac[ORF2]-infected Tn5 cells, a protein band with a molecular mass of 69 kDa (p69) appeared at 2 days p.i., and reached a peak on day 3 p.i. The molecular mass of p69 was in agreement with that calculated for the full-length rat ORF2; however, p69 was not detected in the supernatant (data not shown).

In the Ac[ΔORF2]-infected Tn5 cells, a major protein with a molecular mass of 58 kDa (p58) was detected in the cells on day 2 p.i., and expression levels reached a peak at day 4 p.i. (Fig. 1). A protein migrating with a molecular mass of 53 kDa (p53) was found in the cells on day 5 p.i., and reached a peak on days 7–10 p.i. in the supernatant. These p58 and p53 proteins were synthesized only in Ac[ΔORF2]-infected cells, and not in the mock-infected or wild-type baculovirus-infected cells. The p58 and p53 proteins reacted with anti-G1 HEVLP antibody in Western blots (Fig. 1).

Self-assembly of the recombinant N-terminal 100 aa-deleted ORF2 protein

The culture medium of Ac[ΔORF2]-infected Tn5 cells was harvested at 7 days p.i., and p53 was purified by CsCl-gradient centrifugation as described in Methods. The p53 protein was broadly distributed in fractions 5–20, but mainly in fractions 19 and 20 (Fig. 2a). However, no HEVLPs were observed by electron microscopy (data not shown). When separated by a sucrose gradient, the p53 protein was distributed primarily in fractions 12–14, all of which showed a mean density of 1.100 g ml^{-3} (Fig. 2b). To identify the p53 protein, the N-terminal amino acid sequence was determined by microsequencing and the sequence AQAPAPNTAP was obtained. This sequence is identical to aa 101–110 of rat HEV ORF2, indicating that p53 was derived from the rat HEV ORF2 protein. Because the molecular mass of the 100 aa-deleted rat HEV ORF2 protein was 58 kDa, the p53 protein is processed from p58, presumably by a deletion at the C terminus. Observation of fraction 13 by electron microscopy revealed two sizes of spherical particles with respective diameters of 24 and 35 nm (Fig. 2c). The morphology of these small particles was similar to that of G1, G3 and G4 HEVLPs produced by recombinant baculoviruses harbouring N-terminal 111 aa-deleted HEV ORF2 (Guu *et al.*, 2009; Li *et al.*, 1997; Yamashita *et al.*, 2009). The size of the 35 nm particles was the same as that of the native rat HEV particles. The yield of the purified rat HEVLPs reached 1.5 mg per 10^7 Tn5 cells. To determine whether nucleic acids were packaged into rat HEVLPs, nucleic acids were extracted from purified rat HEVLPs and analysed by agarose gel electrophoresis. However, we could not detect any nucleic acids in rat

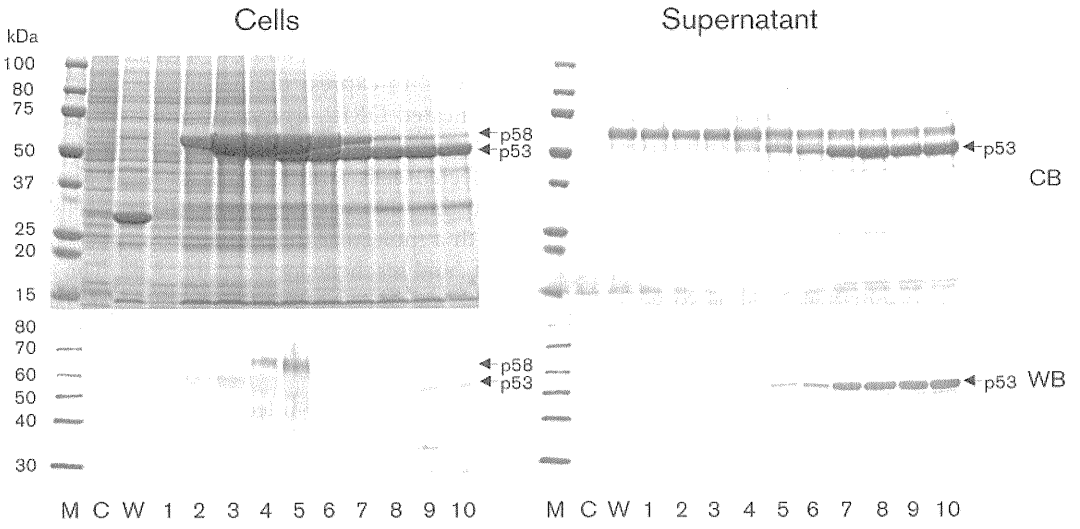

Fig. 1. Time course of the expression of N-terminal 100 aa-truncated rat HEV ORF2. Tn5 cells were infected with recombinant baculovirus Ac[ΔORF2], incubated at 26.5 °C and harvested on days 1–10 p.i. (lanes 1–10). Five microlitres of the culture medium or lysate from 10^5 cells was analysed by SDS-PAGE. Protein bands were visualized by Coomassie blue staining (CB) and Western blotting with anti-G1 HEVLP rabbit serum (WB). M, Molecular mass marker; C, mock-infected control; W, wild-type baculovirus-infected cells.

HEVLPs (data not shown). These results indicated that the p53 protein self-assembled into VLPs and demonstrated that the ORF2 gene encoded the rat HEV capsid protein. No HEVLPs were obtained from either Ac[ΔORF2]-infected or Ac[ORF2]-infected *Spodoptera frugiperda*-derived (Sf9) cells (data not shown).

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

The rat HEV capsid protein p53 reacted with anti-G1 HEV antibody, as determined by Western blotting (Fig. 2), which suggested that the rat HEV had a similar antigenicity to G1 HEV. The antigenic cross-reactivity among rat, G1, G3 and G4 HEVs was examined by ELISA. For this purpose, rabbits were immunized with rat, G1, G3 or G4 HEVLPs. Three weeks after injection, all of the rabbits elicited high levels of IgG antibodies against the homologous antigen (HEVLPs), with ELISA titres reaching 1:25 600 (rat), 1:12 800 (G1), 1:12 800 (G3) and 1:25 600 (G4) (Fig. 3a–d). The anti-rat HEVLP antibody reacted with not only homologous rat HEVLPs (Fig. 3a) but also with heterologous G1, G3 and G4 HEVLPs with titres of 1:800, 1:1600 and 1:3200, respectively (Fig. 3b–d, arrows). Conversely, the antibodies against G1, G3 and G4 HEVLPs were cross-reactive with rat HEVLPs (Fig. 3a). The antigenic cross-reactivity was confirmed by an antibody ELISA using rat HEVLPs and serum from convalescent hepatitis E patients. As depicted in Fig. 3(e), rat HEVLPs showed cross-reactivity with sera from G1, G3 and G4 hepatitis E patients, although the titres were lower than those detected using G1 HEVLPs as antigen. These results indicated that rat HEV has antigenic epitope(s) in common with those of G1, G3 and G4 HEVs.

Prevalence of IgG and IgM antibodies in wild rats

In order to detect IgG and IgM antibodies against rat HEV, ELISAs were developed as follows. A total of 130 serum samples from laboratory rats were used at a dilution of 1:200 to determine the cut-off value for the ELISA. The absorbance values at 492 nm (A_{492}) of the IgG of these serum samples were between 0.016 and 0.147, with a mean value ± SD of 0.052 ± 0.043. The cut-off value for IgG was set at 0.181, 3 SD above the mean A_{492} value (Fig. 4a). Similarly, the A_{492} values of the IgM of these serum samples were between 0.021 and 0.178, with a mean of 0.061 ± 0.050. The cut-off value for IgM was set at 0.211, 3 SD above the mean A_{492} value (Fig. 4b).

A total of 139 serum samples collected from wild rats in Vietnam were examined, and 20.9 % (29/139) of the samples were found to be positive for IgG antibody, whereas 3.6 % (5/139) were positive for IgM antibody (Fig. 4). All five of the IgM-positive serum samples were also positive for IgG. Among the animals trapped in Vietnam, 75 were from Haiphong and 64 were from Hanoi. The rate of positivity for anti-rat HEV IgG was 22.7 % (17/75) for the samples from Haiphong, and 18.8 % (12/64) for the samples from Hanoi. The rate of positivity did not differ significantly between samples from these two areas ($P>0.05$). Among the 139 rat serum samples, 16 were from *Rattus tanezumi* and 123 from *Rattus norvegicus*; the IgG-positive rates were 25.0 % (4/16) and 20.3 % (25/123), respectively. The IgG-positive rates were not significantly

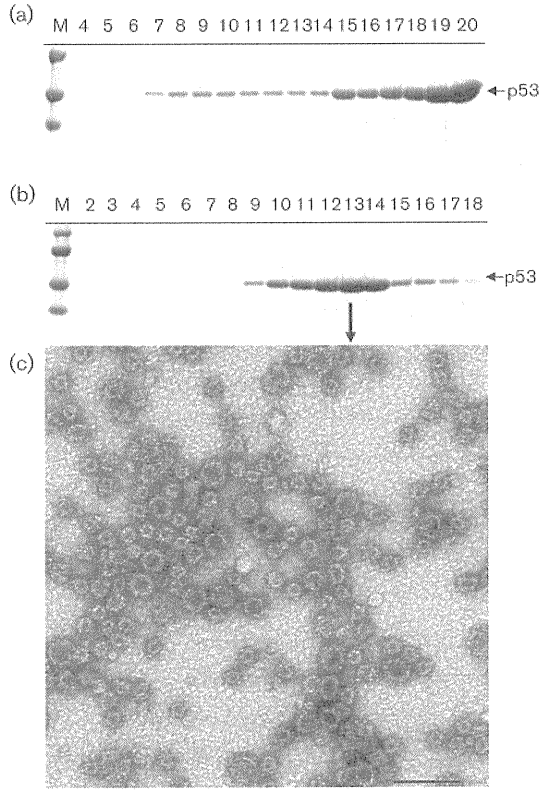

Fig. 2. Purification of rat HEVLPs. (a, b) The supernatant of recombinant baculovirus-infected Tn5 cells was centrifuged for 3 h at 32 000 r.p.m. in a Beckman SW32Ti rotor. The pellet was resuspended in 4.5 ml or 100 µl EX-CELL 405 and purified by CsCl-gradient (a) or sucrose-gradient (b) centrifugation, respectively. Aliquots from the gradient were analysed by SDS-PAGE (5–20 % acrylamide gradient) and stained with Coomassie blue. (c) To examine the HEVLPs, each fraction containing p58 protein was stained with 2 % uranyl acetate and observed by electron microscopy. Bar, 100 nm.

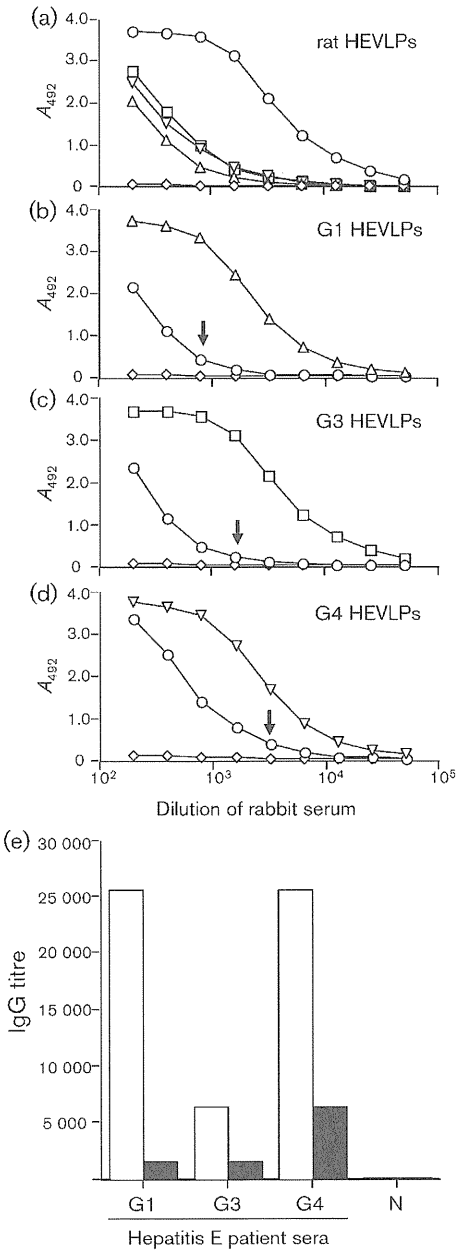

Fig. 3. Antigenic cross-reactivity among rat, G1, G3 and G4 HEVLPs. (a–d) The A_{492} values of hyperimmune sera from rabbits immunized with rat (○; a), G1 (△; b), G3 (□; c) or G4 (▽; d) HEV and of pre-immunized rabbit serum (◇) were determined by antibody ELISA using the four VLP antigens indicated. Arrows indicate the end-point titres against anti-rat HEVLP serum. (e) Antigenicity of rat HEVLPs. The IgG titres in serum samples from G1, G3 and G4 hepatitis E patients or serum from a healthy individual (N) were determined by antibody ELISA using rat HEVLPs (filled bars) or G1 HEVLPs (open bars) as the antigen.

different between *R. tanezumi* and *R. norvegicus* ($P>0.05$). These results suggested that rat HEV infection is widespread and that transmission is ongoing among wild rats in Vietnam.

Detection of the rat HEV genome by RT-PCR

The IgM-positive serum samples were selected to detect rat HEV RNA using a nested broad-spectrum RT-PCR, and one serum sample was found to be positive for rat HEV. A total of 901 nt corresponding to nt 4108–5008 of the rat HEV genome (GenBank accession no. GU345042) comprising the C terminal ORF1 (814 nt), the junction region (27 nt) and the N terminal ORF2 (60 nt) were compared with the corresponding sequences of other HEVs. A phylogenetic analysis based on these 901 nt indicated that the Vietnamese rat strain formed a cluster with other rat HEVs (Fig. 5). This strain was designated Vietnam rat HEV 105. The nucleotide identity between Vietnam rat HEV 105 and the four German rat HEV strains was 78.18–79.43 %. The identity of the deduced 270 aa of the ORF1 C terminus was 94.07–92.96 %, demonstrating considerable differences among strains. Because the nucleotide identity between the

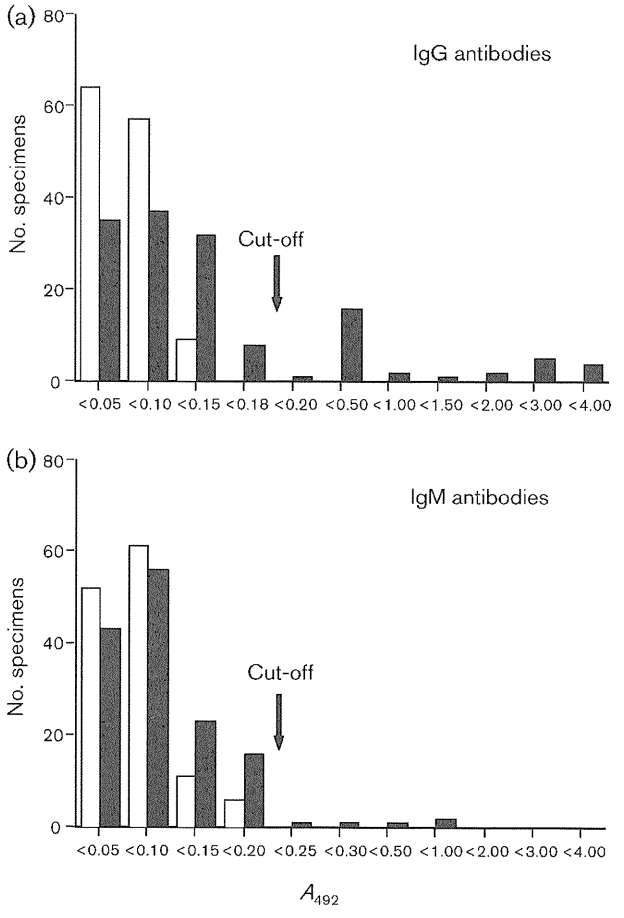

Fig. 4. Detection of anti-rat HEV IgG and IgM antibodies in laboratory and wild rats. Serum samples were collected from laboratory rats in Japan and from wild rats in Vietnam. Anti-HEV IgG and IgM antibodies were detected by antibody ELISA with 1 : 200-diluted sera. Open bars, laboratory rats; filled bars, wild rats.

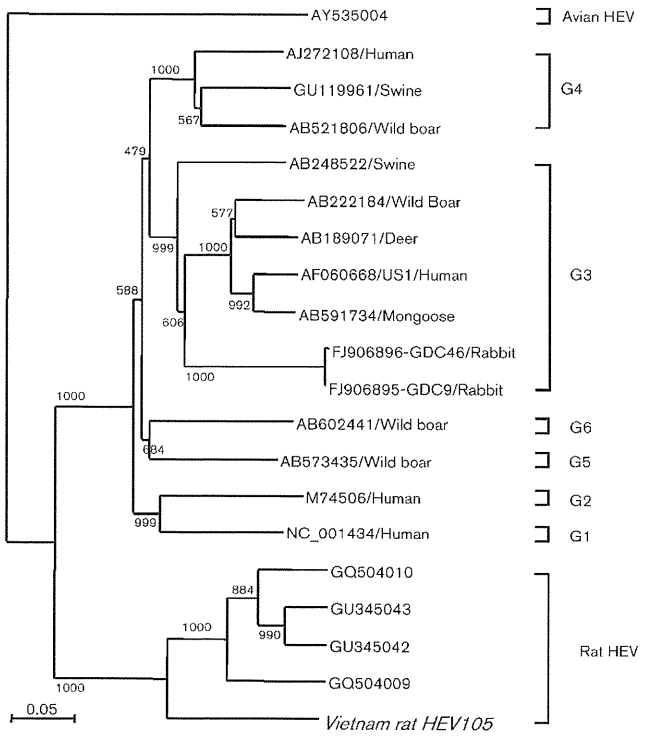

Fig. 5. Phylogenetic analysis of rat HEV based on the partial nucleotide sequence of the Vietnam rat HEV 105 strain (901 nt) using avian HEV as an outgroup. Bootstrap values were determined based on 1000 resamplings of the datasets and are shown at the nodes.

Vietnamese and German strains was <80 %, the Vietnam rat HEV 105 strain may belong to a new genotype of rat HEV.

DISCUSSION

Rat HEV is a new genotype of HEV, and nucleotide sequence identities with HEV G1–4 were 55.1–55.9 %. Because no cell-culture system has yet been developed for rat HEV, it remains necessary to express the capsid protein and generate VLPs in order to analyse the antigenicity and immunogenicity of this pathogen; these recombinant molecules are also extremely useful for seroepidemiological studies of rat HEV infection in wild rats.

For the production of VLPs, the full-length rat HEV ORF2 was initially expressed by a recombinant baculovirus; however, the recombinant protein derived from this gene was not released into the culture supernatant and did not form VLPs. In the case of G1, G3 and G4 HEVs, an N terminal 111 aa-deleted ORF2 protein has been found to be released efficiently into the supernatant and to self-assemble into VLPs. Therefore, we employed the same strategy for the current analysis. When the deduced amino acid sequence of the rat HEV ORF2 (GenBank accession no. GU345042) was aligned with that of representatives of HEV G1, G3 and G4 (GenBank accession nos DQ079624, DQ079627 and DQ079631, respectively), we found that aa 101 in the rat HEV ORF2 corresponded to aa 112 in the G1, G3 and G4 HEV ORF2. Therefore, we expressed the N-terminal 100 aa-deleted rat HEV ORF2 using a recombinant baculovirus expression system. As expected, the recombinant protein, p53, was released into the supernatant and formed VLPs (Fig. 1). Deletion of 100 aa from the N terminus of ORF2 was essential for the formation of rat HEV VLPs. Although we attempted to express the full-length and N-terminal 100 aa-deleted rat HEV ORF2 in Sf9 cells, another insect cell line, the recombinant protein was not released into the supernatant and no VLPs were detected. These characteristics are identical to those observed with G1, G3 and G4 HEVs, indicating that processing of the recombinant protein and the formation of VLPs are both cell-dependent events.

In previous studies of G1, G3 and G4 HEVLPs, the VLPs were purified by CsCl-gradient centrifugation and were

concentrated in the fraction with a density of 1.285 g cm^{-3} (Guu *et al.*, 2009; Li *et al.*, 1997; Yamashita *et al.*, 2009). In contrast, in the present study, the rat HEV p53 was broadly separated and no particles were visible after CsCl-gradient centrifugation. However, a large amount of purified rat HEVLPs was obtained by sucrose-gradient centrifugation, indicating that rat HEVLPs are unstable at high concentrations of CsCl. In previous studies, only small empty particles with a diameter of 24 nm were detected in the cells and supernatant when the N-terminal 111 aa-deleted G1, G3 and G4 HEV ORF2s were expressed in the recombinant baculovirus (Guu *et al.*, 2009; Li *et al.*, 1997; Yamashita *et al.*, 2009). When the N-terminal 100 aa-deleted rat HEV ORF2 was expressed, two VLPs with respective diameters of 24 and 35 nm were visible. At the present time, there is no explanation for this finding and therefore further studies including three-dimensional structural analysis are needed.

When rabbits were immunized with rat HEVLPs, a strong immune response was induced, with high IgG titres and in the absence of any adjuvant or booster injections, suggesting that rat HEVLPs are highly immunogenic in rabbits. Rat HEVLPs were cross-reactive with antibodies elicited in acute hepatitis E patients; moreover, the antibodies induced by rat HEVLPs were cross-reactive with G1, G3 and G4 HEVLPs. These results clearly demonstrated that rat HEV and G1, G3 and G4 HEVs share at least one common epitope. HEVLPs 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 formed primarily by the M and P domains (Xing *et al.*, 2010; Yamashita *et al.*, 2009). The amino acid identities of the full-length capsid protein (1–660 aa), S domain (118–308 aa), M domain (309–444 aa) and partial P domain (528–556 aa) between rat HEV (GenBank accession no. GU354042) and the G1, G3 and G4 HEVs were found to be 50.5–51.2, 75.4–76.4, 66.2–67.6 and 75.9–79.3 %, respectively. The amino acid identities of the S, M and partial P domains of each group were clearly higher than those of the other capsid regions, which suggests that common epitope(s) may be present in the M and/or P domains on the surface of the particles.

A high prevalence of anti-HEV antibody has been reported in wild rats in the USA and Japan (Favorov *et al.*, 2000; Hirano *et al.*, 2003; Kabrane-Lazizi *et al.*, 1999). In samples from these countries, antibodies to rat HEV were detected by ELISA using antigens derived from G1 HEV isolated in Pakistan or Myanmar. As this is only indirect evidence of the cross-reactivity between rat and G1 HEVs, it will be necessary to examine the prevalence of anti-rat HEV antibodies in wild rats using homologous antigens, i.e. rat HEVLPs, which may exhibit stronger reactivity than the heterologous antigens, i.e. G1 HEVLPs. The ELISA method developed in this study will be useful for monitoring the circulation of rat HEV in wild rats.

METHODS

Construction of a transfer vector. The full-length ORF2 of rat HEV containing a *Bam*HI site before the start codon and an *Xba*I site after the stop codon was synthesized based on the rat HEV sequence deposited in GenBank (GenBank accession no. GU345042). The full-length ORF2 was then was cloned into the vector pUC57 (GeneScript) to generate the plasmid pUC57-rat-ORF2. A DNA fragment encoding the N-terminal 100 aa-truncated rat HEV ORF2 was amplified by PCR using pUC57-rat-ORF2 with forward primer rat-E-F2 (5′-AAGGATCCATGGCACAGGCACCGGCGCCTA-3′) and reverse primer rat-E-R1 (5′-ATCTAGATCAGACACTATCGGCGGCTGCTG-3′). The amplified DNA fragment was purified using a PCR purification kit (Qiagen). The full-length and N-terminal 100 aa-truncated ORF2 were digested with *Bam*HI and *Xba*I and ligated into a baculovirus transfer vector, pVL1393 (Pharmingen), to yield plasmids pVL1393-ORF2 and pVL1393-ΔORF2, respectively.

Construction of a recombinant baculovirus and expression of capsid proteins. Sf9 cells (RIKEN Cell Bank) were co-transfected with linearized wild-type *Autographa californica* nuclear polyhedrosis virus DNA (BaculoGold 21100D; Pharmingen) and either pVL1393-ORF2 or pVL1393-ΔORF2 by a Lipofectin-mediated method as specified by the manufacturer (Gibco-BRL). The cells were incubated at 26.5 °C in TC-100 medium (Gibco-BRL) supplemented with 8 % FBS and 0.26 % bactotryptose phosphate broth (Difco Laboratories). The recombinant virus was plaque purified three times in Sf9 cells and designated Ac[ORF2] and Ac[ΔORF2], respectively. To achieve large-scale expression, an insect cell line from *Trichoplusia ni*, Tn5 (Invitrogen), was used (Wickham & Nemerow, 1993). Tn5 cells were infected with Ac[ORF2] or Ac[ΔORF2] 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.*, 2005b, 1997). VLPs of G1, G3 and G4 HEVs were produced as described previously (Guu *et al.*, 2009; Li *et al.*, 1997; Yamashita *et al.*, 2009).

SDS-PAGE and Western blot analysis. The proteins in the cell lysates and culture medium were separated by SDS-PAGE with a 5–20 % acrylamide gradient gel and stained with Coomassie blue. For 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 a rabbit anti-G1 HEVLP polyclonal antibody as described previously (Li *et al.*, 1997). Detection of the rabbit IgG antibody was achieved using alkaline phosphatase-conjugated goat anti-rabbit antibody (1 : 1000 dilution; Chemicon International). Nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine were used for detection of antibody binding (Bio-Rad Laboratories).

Purification of rat HEVLPs. 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 sucrose-gradient centrifugation, 1 ml of each sample was laid on top of a 10–40 % (w/w) gradient and centrifuged at 32 000 r.p.m. for 2 h in a Beckman SW55Ti rotor. For CsCl-gradient centrifugation, 4.5 ml of each sample was mixed with 2.1 g CsCl and centrifuged at 35 000 r.p.m. for 24 h at 10 °C in the same rotor. The gradient was fractionated into 250 µl aliquots, and each fraction was weighed 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 the HEVLPs.

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

N-terminal amino acid sequence analysis. The proteins separated by SDS-PAGE were visualized by staining with GelCode Blue Staining Reagent (Pierce) and purified by sucrose-gradient centrifugation. N-terminal amino acid microsequencing was carried out using 100 pmol protein by Edman automated degradation on an Applied Biosystems Model 477 Protein Sequencer.

Hyperimmune sera. Rabbits were immunized with rat, G1, G3 and G4 HEVLPs. Immunization was performed by one percutaneous injection of purified HEVLPs with a dose of 500 μg per rabbit. Rats were immunized with the recombinant rat HEVLPs by intramuscular injection at 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 rat HEVLPs. All of the injections, including booster injections, were carried out without adjuvant. Immunized animals were bled 3 weeks after the last injection.

Rat serum samples. A total of 130 serum samples from laboratory rats (Wistar; Japan SLC) were collected at the Division for Experimental Animal Research of the National Institute of Infectious Diseases of Japan. A total of 139 serum samples from wild rats were collected in Vietnam (39 samples were collected in 2009 in Haiphong, and 64 and 36 sera were collected in Hanoi and Haiphong in 2011, respectively). With regard to the rat species sampled, 123 were identified as *R. norvegicus* and 16 were identified as *R. tanezumi*. All of the serum samples were stored at −80 °C until use.

Detection of IgG and IgM antibodies. Flat-bottomed 96-well polystyrene microplates (Immulon 2; Dynex Technologies) were coated with the purified rat HEVLPs (1 μg ml^{-1}, 100 μl per well) and incubated overnight at 4 °C. Unbound HEVLPs were removed and the plates washed twice with 10 mM PBS containing 0.05 % Tween 20 (PBS-T) and then blocked with 200 μl 5 % skimmed milk (Difco Laboratories) dissolved in PBS-T for 1 h at 37 °C. After the plates had been washed four times with PBS-T, diluted rat serum samples (100 μl per well) were added in duplicate. The plates were incubated at 37 °C for 1 h and then washed three times as described above. The wells were incubated with 100 μl HRP-conjugated goat anti-rat IgG (H+L) (1 : 10 000 dilution; Zymed Laboratories) or HRP-conjugated goat anti-rat IgM (1 : 100 000 dilution; Jackson ImmunoResearch Laboratories) diluted in PBS-T containing 1 % skimmed milk. The plates were incubated at 37 °C for 1 h and washed four times with PBS-T. One hundred microlitres of substrate orthophenylenediamine (0.4 mg ml^{-1}; Sigma Chemical) and 5 μl H_2O_2 (30 % in 12.5 ml substrate buffer) were added to each well. The plates were incubated in a dark room at room temperature for 30 min, and then 50 μl 2 M H_2SO_4 was added to each well. Absorbance was measured at 492 nm. The cut-off values for IgG and IgM were determined as described previously (Li *et al.*, 2000). A sample was considered to be positive when the absorbance exceeded the cut-off value. Pre-immunization and rat HEVLP-immunized rat sera were used as the negative and positive controls, respectively. Detection of human and rabbit anti-HEV IgG was performed as described previously (Li *et al.*, 1997).

RNA extraction and nested broad-spectrum RT-PCR. Total RNA was extracted using a QIAamp viral RNA mini kit (Qiagen) and resuspended in 20 μl DNase-, RNase- and proteinase-free water. Reverse transcription was performed at 42 °C for 50 min, followed by 70 °C for 15 min in a 20 μl reaction mixture containing 1 μl Superscript II RNase H^- reverse transcriptase (Invitrogen), 1 μl oligo(dT) primer, 1 μl RNaseOUT (Invitrogen), 2 μl 0.1 M DTT, 4 μl 5× RT buffer (Invitrogen), 1 μl 10 mM dNTPs, 5 μl RNA and 5 μl distilled water.

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

To amplify the Vietnam rat HEV genome, two pairs of primers were designed. The forward primers were designed according to the Vietnamese rat HEV strain (GenBank accession no. JN040433). The reverse primers were designed according to the German rat HEV strain (GenBank accession no. GU354042). The first PCR analysis was carried out in a 50 μl volume reaction mixture with an external forward primer, rat-HEV-F10 (5′-GAAGGCCATAGTCGCCAACCTG-3′, nt 4117–4138), and an external reverse primer, rat-HEV-R7 (5′-TCAGACA-CTATCGGCGGCTG-3′, nt 6864–6883). Each cycle consisted of 95 °C for 30 s, 55 °C for 60 s and 72 °C for 4 min, followed by 72 °C for 7 min. Two microlitres of the first PCR product was used for the nested PCR with an internal forward primer, HEV-F11 (5′-AAGGC-GTGCAGAGTGTTTGAGA-3′, nt 4205–4226), and an internal reverse primer, rat-HEV-R9 (5′-CGGGCTCCACCGGGGTACAT-3′, nt 5013–5032). Each cycle consisted of 95 °C for 30 s, 55 °C for 45 s and 72 °C for 60 s, followed by 72 °C for 7 min.

Nucleotide sequencing of the PCR products was carried out using primers HEV-csn, HEV-casn, HEV-F11 and HEV-R9 on an ABI 3130 Genetic Analyzer automated sequencer (Applied Biosystems) and a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) according to the manufacturer's instructions.

Statistical analysis. Comparisons of the rate of positivity between different areas and between *R. norvegicus* and *R. tanezumi* were performed with an unpaired Student's *t*-test.

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