Analysis of the amount of rat HEV-RNA in organs of wild rats indicated a liver tropism of the virus (Johne *et al.*, 2010b). The hepatotropism of rat HEV was also confirmed with laboratory rats intravenously inoculated with rat HEV-containing organ homogenates (Li *et al.*, 2013b). Immunodeficient nude rats inoculated with rat HEV developed persistent infections with prolonged shedding of high amounts of virus (Li *et al.*, 2013b). Experimentally infected Wistar rats showed transient virus shedding and developed anti-rat HEV-specific antibodies (Li *et al.*, 2013b). Clinical signs were not recorded after experimental infection of rats (Li *et al.*, 2013b; Purcell *et al.*, 2011). Preliminary infection trials with rat-derived cell cultures did not result in rat HEV replication (Johne *et al.*, 2010a). However, rat HEV was recently propagated successfully in the human hepatoma cell lines PLC/PRF/5, HuH-7 and HepG2 (Jirintai *et al.*, 2014).

The zoonotic potential of rat HEV is not clear. Experimental infections of rhesus monkeys and pigs with rat HEV did not result in signs of virus replication (Cossaboom *et al.*, 2012; Purcell *et al.*, 2011). Serological analysis of human and porcine sera identified only a few sera showing antibodies with higher reactivity to rat HEV as compared with human HEV, indicating a very rare transmission of rat HEV-related viruses (Dremsek *et al.*, 2012; Krumbholz *et al.*, 2013).

Reverse genetics systems, which enable the generation of infectious virus from cloned cDNA, have been widely used for site-directed mutagenesis of viral RNA genomes following various applications in basic and applied virology (Hoenen *et al.*, 2011; Stobart & Moore, 2014; Ye *et al.*, 2014). A reverse genetics system for human HEV was first developed by Panda *et al.* (2000). In this system, the cDNA of the genome of a genotype 1 strain was cloned downstream of the T7 RNA polymerase promoter. The linearized cDNA clone was transcribed *in vitro* and thereafter transfected into cell cultures. The cell culture supernatant was shown to be infectious for rhesus monkeys; however, direct inoculation of the RNA into the monkeys did not result in generation of the virus. Emerson *et al.* (2001) showed that a capping step during *in vitro* transcription enabled the generation of infectious virus by intrahepatic inoculation into rhesus monkeys and chimpanzees. Similar systems have been developed for HEV genotypes 3 and 4, rabbit HEV, and avian HEV, some of them using transfection of RNA into cell cultures for the generation of infectious virus, others using direct inoculation into the liver of laboratory animals (Córdoba *et al.*, 2012; Cossaboom *et al.*, 2014; Huang *et al.*, 2005a, b; Kwon *et al.*, 2011; Yamada *et al.*, 2009).

Here, in order to develop a reverse genetics system for rat HEV, its complete genome was cloned under control of the T7 RNA polymerase promoter. Capped *in vitro* transcribed rat HEV RNA was thereafter used to inoculate laboratory rats and the generation of infectious rat HEV was monitored. Generated virus was characterized by infectivity tests in rats and cell culture as well as by genome sequence analysis. The results should contribute to the development of a reliable reverse genetics system for rat HEV, enabling site-directed mutagenesis and subsequent phenotypic studies on the virus.

RESULTS

Construction of a genomic clone of rat HEV

The whole genome of the rat HEV prototype strain R63/DEU/2009 was amplified by reverse transcription (RT)-PCR in four overlapping fragments and cloned. The final genomic clone contained a T7 RNA polymerase promoter sequence at the 5′ end and a poly(A) sequence at the 3′ end of the rat HEV genome followed by a unique *Xba*I restriction site. A schematic map of the plasmid is shown in Fig. 1(a). Restriction analysis of the plasmid resulted in the expected DNA fragments (Fig. 1b). Sequencing of the cloned genome identified 23 point mutations as compared with the original sequence of strain R63/DEU/2009 (GenBank accession number GU345042). Most of the mutations were either synonymous or led to amino acids that have also been detected in other rat HEV strains at the respective positions (Table S1, available in the online Supplementary Material). Only four unique amino acid exchanges were detected, which were located exclusively within the hypervariable region of ORF1.

Recovery of rat HEV by inoculation of nude rats with *in vitro* transcribed RNA

The *Xba*I-linearized plasmid was transcribed *in vitro*, resulting in capped rat HEV genome-length RNA. A total of 500 µl *in vitro* transcribed RNA (2.6×10^{11} copies μl^{-1}) was intrahepatically inoculated in two nude rats LR1 (6 weeks old, female) and LR2 (10 weeks old, male). In addition, two nude rats VR1 (6 weeks old, female) and VR2 (10 weeks old, male) were intravenously inoculated through the tail vein with the same dose of *in vitro* transcribed RNA. Rat HEV RNA was detected in the stool specimens from both LR1 and LR2 on day 14 post-inoculation (p.i.) at 4.65×10^7 and 1.15×10^8 copies g^{-1}, reaching a plateau on day 30 p.i. at 1.08×10^{11} and 1.94×10^{11} copies g^{-1}, respectively (Fig. 2). Rat HEV RNA was detected in sera of LR1 and LR2 on day 49 p.i. at 3.84×10^7 and 4.75×10^7 copies ml^{-1}, respectively. In contrast, rat HEV RNA could not be detected in stool specimens or serum of VR1 and VR2.

Demonstration of infectivity of rat HEV recovered from inoculated nude rats

Stool samples taken from LR1 and LR2 on day 30 p.i. were used to prepare sterile-filtered 10 % suspensions. Two nude rats RR1 (13 week sold, female) and RR2 (17 weeks old, male) and two 15-week-old female Wistar rats WR1 and WR2 were intravenously inoculated with 300 µl stool suspension from LR1 or LR2 (RR1 and WR1 inoculated

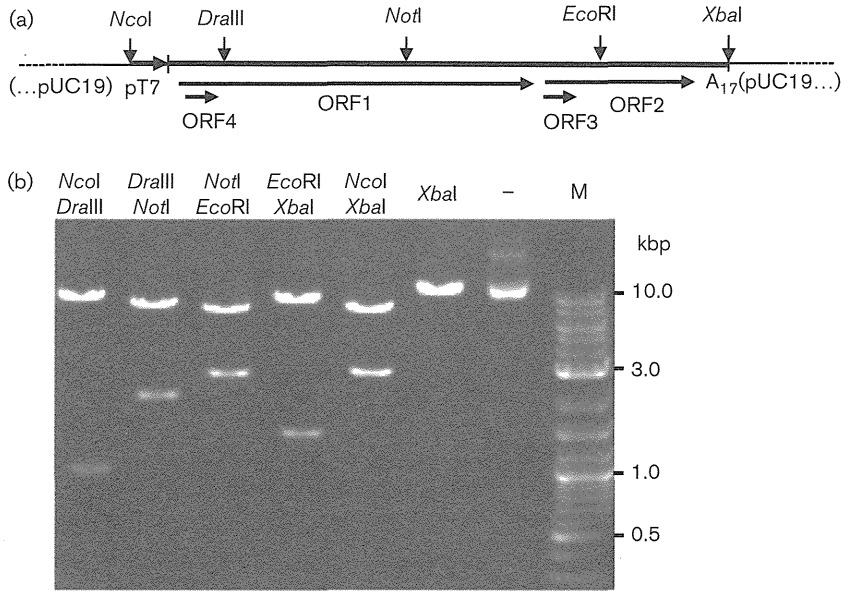

Fig. 1. Genomic clone of rat HEV. (a) Schematic presentation (not to scale). The T7 RNA polymerase promoter (pT7), rat HEV genome ORF1–4, the polyadenylation site (A_{17}), the flanking vector sequences (pUC19) and selected restriction enzyme sites are indicated. (b) Restriction enzyme analysis of the genomic clone of rat HEV. The plasmid DNA was digested with the indicated restriction enzymes and subjected to electrophoresis on ethidium bromide-stained agarose gel: –, without enzyme; M, molecular mass markers (Quick-Load 2-Log DNA Ladder; New England Biolabs; sizes of selected bands are indicated).

with suspension from LR1; RR2 and WR2 inoculated with suspension from LR2). Rat HEV RNA was detected in the stools of RR1 and RR2, starting on day 14 p.i. at 3.89×10^{10} and 4.73×10^{10} copies g^{-1}, and thereafter at $>10^{10}$ copies g^{-1} (Fig. 3a). Rat HEV RNA was also detected in the sera of RR1 and RR2 at day 28 p.i. at 7.04×10^{7} and 1.11×10^{8} copies ml^{-1}, respectively. In WR1 and WR2, anti-rat HEV IgG was detected beginning at day 14 p.i. and reached a plateau at day 28 p.i. (Fig. 3b). The alanine aminotransferase (ALT) levels of RR1, RR2, WR1 and WR2 were <40 IU l^{-1} throughout the whole experiment.

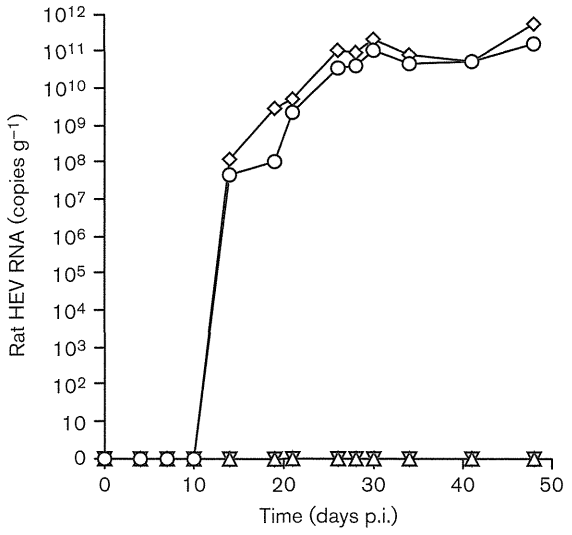

Fig. 2. Kinetics of rat HEV stool excretion after inoculation of nude rats with *in vitro* transcribed and capped rat HEV RNA. Two nude rats (Long-Evans *rnu/rnu*) LR1 (○) and LR2 (◇) were intrahepatically inoculated and two nude rats VR1 (△) and VR2 (▽) were intravenously inoculated with *in vitro* transcribed and capped rat HEV RNA. The rat HEV RNA copy numbers were determined by quantitative real-time RT-PCR.

Isolation of rat HEV from inoculated nude rats in PLC/PRF/5 cells

The sterile-filtered 10 % rat stool suspension taken from LR1 on day 30 p.i. was inoculated onto PLC/PRF/5 cells. Rat HEV RNA was detected in PLC/PRF/5 cell culture supernatant on day 30 p.i.at 9.27×10^{6} copies ml^{-1} and reached a plateau on day 60 p.i. at 2.59×10^{9} copies ml^{-1}. Thereafter, high amounts of rat HEV RNA were constantly detected ($>10^{9}$ copies ml^{-1}) until day 116 p.i. (Fig. 4a). The capsid protein of rat HEV was detected in culture supernatant beginning on day 44 p.i. and reached a plateau starting at day 64 p.i. until the end of the experiment (Fig. 4b). Inoculation of fresh PLC/PRF/5 cells with the cell culture supernatant taken at day 68 p.i. resulted in detection of rat HEV RNA as early as day 16 p.i. and capsid protein at day 24 p.i. In addition, the amounts of rat HEV RNA reached (1.87×10^{10}) were considerably higher compared with the first passage of the virus (Fig. 4a, b). No cytopathic effect was observed during the whole experiment.

Demonstration of infectivity of cell culture-derived rat HEV for nude rats

Two 20-week-old female nude rats CR1 and CR2 were each intravenously inoculated with 0.5 ml cell culture supernatant from day 68 p.i. of the first passage. Rat HEV RNA was detected in stools beginning at day 12 p.i. at 1.35×10^{7} and 9.97×10^{7} copies g^{-1}, and rose to 6.92×10^{10} and 9.21×10^{9} copies g^{-1} on day 18 p.i. in CR1 and CR2, respectively (Fig. 5). Rat HEV RNA was detected at day 21 p.i. in sera of CR1 and CR2 at 8.50×10^{5} and 9.20×10^{5} copies ml^{-1}, respectively.

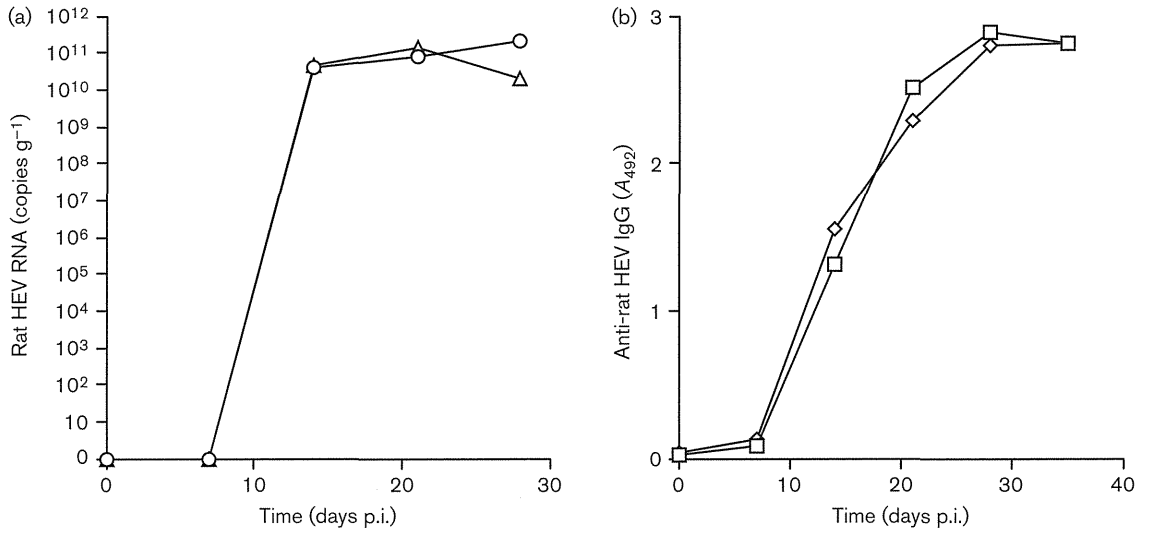

Fig. 3. Infectivity testing of rat HEV recovered from stool suspensions derived from rats LR1 and LR2. Two nude rats (Long-Evans *rnu/rnu*) RR1 (○) and RR2 (△) and two Wistar rats WR1 (□) and WR2 (◇) were intravenously inoculated with the stool suspension from rats LR1 and LR2, respectively. (a) Stool samples were collected weekly from nude rats for detection of rat HEV RNA using quantitative real-time RT-PCR. (b) Serum samples were collected weekly from Wistar rats for detection of anti-rat HEV IgG using ELISA.

Genome sequence comparison of rat HEV recovered from nude rats and cell culture

The entire genome sequences of rat HEV recovered from nude rat faeces (sample taken from LR1 on day 30 p.i.) and cell culture supernatant (sample collected from day 68 p.i. of the first passage) were analysed by next-generation sequence analysis and compared with the sequence of the cDNA clone. The identified mutations and the deduced amino acid exchanges are shown in Table 1. The genome sequences of the cDNA clone and the rat HEV recovered from the intrahepatically inoculated nude rat were identical. In contrast, nine mutations were found in rat HEV recovered from the cell culture, which resulted in four

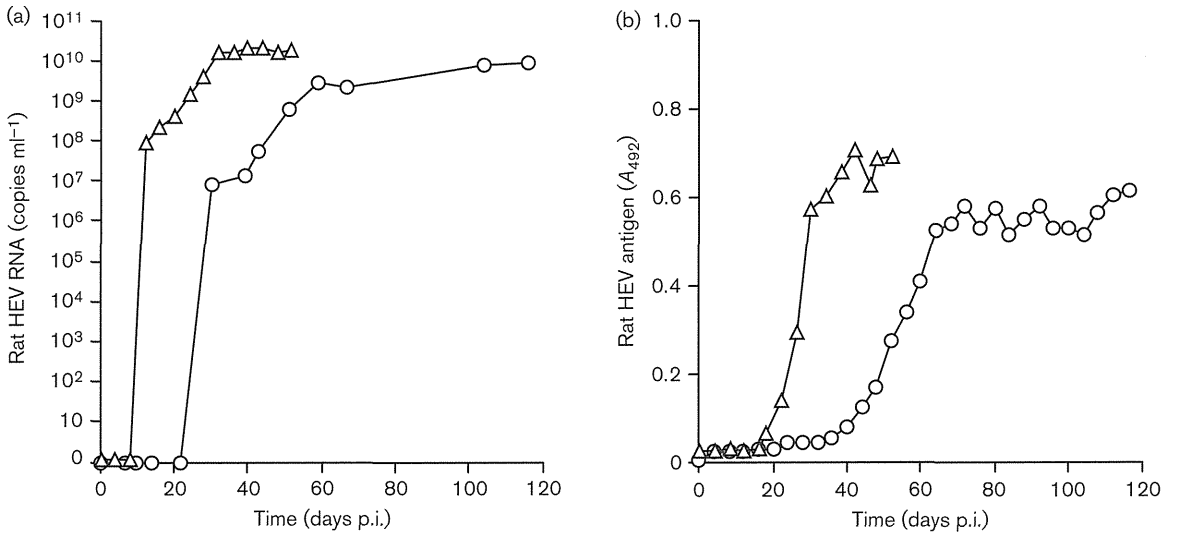

Fig. 4. Replication of rat HEV in a human hepatocarcinoma cell line. PLC/PRF/5 cells were inoculated with the rat (LR1) faecal specimen (○) or with the resulting rat HEV-containing culture supernatant (△). The culture supernatants were collected every 4 days. (a) Rat HEV RNA detection by quantitative real-time RT-PCR. (b) Rat HEV antigen detection by rat HEV-specific antigen ELISA.

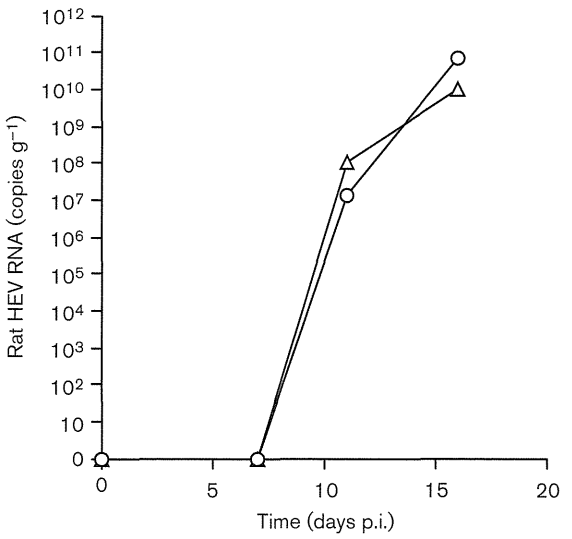

Fig. 5. Infectivity testing of rat HEV recovered from cell culture for rats. Two nude rats (Long-Evans *rnu/rnu*) CR1 (○) and CR2 (△) were intravenously inoculated with a rat HEV-containing cell culture supernatant. The rat HEV RNA copy numbers present in the faeces of the animals were determined by quantitative real-time RT-PCR.

synonymous and five non-synonymous mutations in ORF1 and ORF2. The mutations at positions 295 and 343 were synonymous for ORF1, but non-synonymous for the overlapping ORF4.

DISCUSSION

RatHEV is a recently discovered virus related to, but distinct from, human HEV (Johne *et al.*, 2014a). As laboratory rats can be reproducibly infected with rat HEV and a cell culture propagation system is available for rat HEV, this virus may be used in the future as a surrogate virus for human HEV, enabling studies on various aspects of virus replication and the immune response. However, a system for genetic manipulation of rat HEV has been missing. The results presented here indicate that the described genomic rat HEV cDNA clone is capable of generating rat HEV infectious for laboratory rats and cell culture.

The cDNA clone was constructed in a similar manner to recently described infectious cDNA clones from human, rabbit and avian HEVs, using the T7 RNA polymerase promoter to generate capped *in vitro* transcribed genomic virus RNA (Córdoba *et al.*, 2012; Cossaboom *et al.*, 2014; Huang *et al.*, 2005a, b; Kwon *et al.*, 2011; Yamada *et al.*, 2009). Several point mutations were detected in the cDNA clone when compared with the original genome sequence of the rat HEV isolate used as deposited at GenBank. Errors during RT-PCR amplification or the presence of viral quasispecies in the original sample may explain the mutations. As the nucleotide exchanges were either silent or resulted in amino acids that were also present at the same positions in other rat HEV strains, it was assumed that they did not affect infectivity of the cDNA clone.

For recovery of infectious virus from the *in vitro* transcribed RNA, it was directly injected into the liver of nude rats. Similar procedures have been described for human, pig, rabbit and avian HEV cDNA clones (Emerson *et al.*, 2001; Cossaboom *et al.*, 2014; Huang *et al.*, 2005a, b). Nude rats were selected for inoculation as they have been shown to be highly susceptible to rat HEV infection, leading to shedding of high titres of rat HEV for a prolonged time (Li *et al.*, 2013b). The detection of rat HEV RNA in serum and faeces after inoculation of the RNA into the liver of the nude rats was the first indication of the successful generation of infectious virus. In contrast, intravenous inoculation of rat HEV RNA was not able to initiate virus replication, presumably because of the lack of its internalization into permissive cells. The virus excreted by intrahepatically inoculated nude rats was shown to be infectious for nude rats and immunocompetent Wistar rats by intravenous inoculation. Virus shedding, antibody production and (no) clinical signs were similar to those described for rats infected with an organ homogenate of a rat HEV-infected wild rat (Li *et al.*, 2013b). It can be therefore concluded that the developed system is suitable for the generation of infectious rat HEV from the cloned genomic cDNA.

The recent publication of a cell culture system for rat HEV (Jirintai *et al.*, 2014) prompted us to analyse the infectivity of the generated rat HEV for cell culture. The results of the experiments showed that the virus replicated – after a lag phase of 30 days – with high titres in PLC/PRF/5 cells. After a passage in this cell culture system, virus growth was observed earlier and with even higher titres. Notably, the obtained titres of $>10^9$ genome copies ml^{-1} are considerably higher than those described for other rat HEV strains [10^7 genome copies ml^{-1} (Jirintai *et al.*, 2014)] or for human HEV strains [10^7 genome copies ml^{-1} (Johne *et al.*, 2014b); 10^8 genome copies ml^{-1} (Okamoto, 2011)]. Genome analysis identified several point mutations in the recovered virus, which may be linked to cell culture adaptation. Although infectivity of the recovered virus for nude rats could still be demonstrated, the biological significance of these mutations has to be assessed in future studies. Direct transfection experiments of cell cultures with in *vitro* transcribed RNA of the original clone compared with a clone containing the mutations may be performed in order to assess the significance of the mutations for efficient cell culture growth. A direct transfection system would generally be useful for cell culture studies on rat HEV without the need for nude rat inoculation.

In summary, the generation of a versatile reverse genetics system for rat HEV has been shown here. After introduction of specific mutations into the cloned cDNA, the system may be useful to study the phenotypic effects of the mutations. As rats are widely used and well-characterized

Table 1. Comparison of the genome sequences of rat HEV derived from cDNA, faeces of nude rats and cell culture

Nucleotide and amino acid numbering according to rat HEV strain R63/DEU/2009 (GenBank accession number GU345042). A dash indicates a synonymous exchange.

Nucleotide position	Region	Nucleotide				Amino acid			
		Position	cDNA	Nude rat	Culture	Position	cDNA	Nude rat	Culture
295	ORF1	285	T	T	C	95	–	–	–
343	ORF1	333	C	C	T	111	–	–	–
1552	ORF1	1542	A	A	G	514	–	–	–
1686	ORF1	1676	A	A	T	559	Y	Y	F
1889	ORF1	1879	C	C	T	627	P	P	S
4599	ORF1	4589	A	A	G	1530	D	D	G
5939	ORF2	991	G	G	A	331	A	A	T
6338	ORF2	1390	T	T	C	464	–	–	–
6411	ORF2	1463	C	C	T	488	T	T	I
295	ORF4	269	T	T	C	90	L	L	P
343	ORF4	317	C	C	T	106	T	T	M

laboratory animals, studies on genetically engineered rat HEV may provide novel insights into organ tropism, replication and excretion kinetics as well as immunological changes induced by hepeviruses.

METHODS

Generation of a genomic clone of rat HEV. The complete genome of rat HEV was amplified in four fragments by RT-PCR using RNA isolated from the liver of a wild rat from Germany containing the rat HEV prototype strain R63/DEU/2009 (GenBank accession number GU345042). RT-PCR was performed with a LongRange 2Step RT-PCR kit (Qiagen) and primers listed in Table S2. The primer binding sites overlapped with unique binding sites for restriction enzymes within the rat HEV genome, thus enabling subsequent cloning of the RT-PCR products. The 5′ end primer contained a T7 RNA polymerase promoter sequence and the 3′ end primer contained an extension of 17 thymidine residues followed by a unique *Xba*I site (Fig. 1a). The RT-PCR products were cloned successively into a pUC19 vector derivative, which contained a multiple cloning site adapted to the restriction sites used. The sequence of the resulting genomic clone was determined by Sanger sequencing using the PCR primers and additional primers.

***In vitro* transcription for the generation of capped RNA.** The genomic plasmid was purified using a Plasmid Maxi kit (Qiagen) and subsequently linearized with *Xba*I. The preparation was further purified by phenol/chloroform extraction and resuspended in RNase-free water. *In vitro* transcription was performed using a mMESSAGE mMACHINE T7 kit (Ambion), which included adding a cap analogue to the 5′ end of the synthesized RNA, according to the protocol of the supplier. RNA purification was carried out by lithium chloride precipitation.

Inoculation of rats and sample collection. Eight nude rats (Long-Evans *rnu/rnu*; Japan SLC) and two specific-pathogen-free rats (Wistar; Japan SLC) were used in this study. The rats were individually housed in Biosafety Level 2 facilities. All rats were negative for rat HEV RNA and anti-rat HEV antibodies, as determined by nested broad-spectrum RT-PCR (Johne *et al.*, 2010a) and rat HEV-specific ELISA (Li *et al.*, 2013b), respectively. To analyse the infectivity of the *in vitro* transcribed RNA, intravenous injection and intrahepatic inoculation were carried out. The RNA (2.6×10^{11} copies μl^{-1}) was injected through the tail vein or through a percutaneous inoculation procedure into five different sites of the liver with ~100 µl per injection site. The rats were injected under xylazine/ketamine hydrochloride anaesthesia. To examine the infectivity of recovered rat HEV, stool specimens were diluted in PBS to prepare 10 % suspensions by shaking at 4 °C for 1 h. Both the cell culture supernatants and 10 % stool suspensions were clarified by centrifugation at 10 000 ***g*** for 30 min, and then passed through a 0.45 µm membrane filter (Millipore). The rats were intravenously inoculated with the stool suspension or with cell culture supernatant through the tail vein. Serum samples from the nude rats were collected at the end of the experiment for detection of rat HEV RNA. Serum samples from the Wistar rats were collected weekly for examination of rat HEV-specific IgG antibodies and ALT values. Stool samples were collected one to two times per week. The experiments were reviewed by the ethics committee of the National Institute of Infectious Diseases, and carried out according to the 'Guides for Animal Experiments Performed at the National Institute of Infectious Diseases' under codes 113029 and 114012.

Inoculation of cell culture. The human hepatocarcinoma cell line PLC/PRF/5 (JCRB0406) from the Health Science Research Resources Bank (Osaka, Japan) was used. Cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10 % (v/v) heat-inactivated FBS (Nichirei), 100 U penicillin ml^{-1} and 100 µg streptomycin ml^{-1} (Gibco) at 37 °C in a humidified 5 % CO_2 atmosphere. For virus inoculation, confluent cells were trypsinized, diluted 1 : 3 and cultured in a 25 cm^2 tissue culture flask. On the next day, the medium was removed and the cells were washed with PBS. A total of 1 ml sample was inoculated onto PLC/PRF/5 cells. After adsorption at 37 °C for 1 h and washing of the cells two times with PBS, the suspension was removed and then replaced by 10 ml maintenance medium consisting of medium 199 (Invitrogen) containing 2 % (v/v) heat-inactivated FBS and 10 mM $MgCl_2$. Further incubation was at 36 °C. The culture maintenance medium was replaced with new medium every 4 days, and used for the detection of rat HEV RNA and capsid antigen. Cells were observed daily by light microscopy for the occurrence of a cytopathic effect.

Quantitative real-time RT-PCR for detection of rat HEV. The RNA was extracted using the MagNA Pre LC system with a MagNA Pre LC Total Nucleic Acid isolation kit (Roche Applied Science)

according to the manufacturer's recommendations. To determine rat HEV RNA copy numbers, a TaqMan assay was performed in the 7500 FAST Real-Time PCR system (Applied Biosystems) using TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems). Forward primer 5′-GTGGTGCTTTTATGGTGACTG-3′ (nt 4123–4143; 900 nmol l^{-1}), reverse primer 5′-CAAACTCACTAAAATCATTCTCAAACAC-3′ (nt 4196–4223; 900 nmol l^{-1}) and probe 5′-6FAM-GTTCAGGAGA-AGTTCGAGGCCGCCGT-TAMRA-3′ (nt 4148–4173; 250 nmol l^{-1}) were used. One-step quantitative RT-PCR cycling conditions were 15 min at 48 °C, 10 min incubation at 95 °C, and 50 cycles for 15 s at 95 °C and 1 min at 60 °C (Li *et al.*, 2013b). The capped *in vitro* transcribed RNA of R63/DEU/2009 was used as standard for calculation of the RNA molecule number. A 10-fold serial dilution of the RNA standards (from 10^7 to 10^1 copies) was used for the quantification of viral genome copy numbers in reaction tubes. Amplification data were collected and analysed with Sequence Detector software version 1.3 (Applied Biosystems).

Detection of anti-rat HEV IgG and rat HEV capsid antigen. Anti-rat HEV IgG was detected by ELISA as described previously (Li *et al.*, 2011). An antigen capture ELISA was used to detect rat HEV antigen. Briefly, duplicate wells of flat-bottom 96-well polystyrene microplates (Dynex) were coated with 100 µl coating buffer (0.1 M carbonate/bicarbonate buffer, pH 9.6) containing a 1 : 1000 dilution of a hyperimmune serum elicited in a rabbit with rat HEV-like particles (Li *et al.*, 2011). The coating was performed at 4 °C overnight. Unbound antibodies were removed and the wells were washed twice with 10 mM PBS containing 0.05 % Tween 20 (PBS-T), and then the blocking was carried out at 37 °C for 1 h with 150 µl 5 % skim milk (Difco) in PBS-T. Aliquots of 100 µl cell culture supernatants were added to the wells and incubated for 1 h at 37 °C. After the wells were washed three times with PBS-T, 100 µl guinea pig rat HEV-like particle hyperimmune serum (1 : 1000 dilution with PBS-T containing 1 % skim milk) was added to the wells and the plate was incubated for 1 h at 37 °C. The plate was washed three times with PBS-T and then HRP-conjugated goat anti-guinea pig IgG antibody (Cappel) (1 : 1000 in PBS-T containing 1 % skim milk.) was added to each well. After incubation for 1 h at 37 °C, the plate was washed three times with PBS-T, and 100 µl substrate *o*-phenylenediamine and H_2O_2 was added. The plate was left for 30 min at room temperature and then the reaction was stopped with 50 µl 4 N H_2SO_4. A_{492} was measured with a microplate reader (Molecular Devices). The cut-off was defined using supernatants of non-infected cell cultures and set as 0.150. Test samples were considered positive when the A_{492} was above the cut-off value.

Viral genome sequencing. The whole-genome sequence of rat HEV present in selected samples was analysed by next-generation sequencing. Virus particles were enriched from the samples by caesium chloride density-gradient centrifugation (Li *et al.*, 2011). A 200 bp fragment library was constructed for each sample preparation using a NEBNext Ultra RNA Library Prep kit for Illumina version 2.0 (New England Biolabs) according to the manufacturer's instructions. Samples were bar-coded for multiplexing using NEBNext Multiplex Oligos for Illumina, Index Primer Sets 1 and 2 (New England Biolabs). Library purification was done using Agencourt AMPure XP magnetic beads (Beckman Coulter) as recommended in the NEBNext protocol. The quality of the purified libraries was assessed on a MultiNA MCE-202 bioanalyser (Shimadzu) and the concentrations were determined on a Qubit 2.0 flourometer using the Qubit HS DNA assay (Invitrogen). A 151-cycle paired-end read sequencing run was carried out on a MiSeq desktop sequencer (Illumina) using MiSeq Reagent kit version 2 (300 cycles). Following preliminary analysis, the MiSeq reporter programme was used to generate FASTQ formatted sequence data for each sample. Sequence data were analysed using CLC Genomics Workbench Software version 7.5.1 (CLC Bio). Contigs were assembled from the obtained sequence reads by *de novo* assembly. Missing sequences of the 5′-terminal non-coding regions of the genomes were determined using the a Rapid Amplification of cDNA Ends kit (Invitrogen) according to the manufacturer's instructions. In order to identify point mutations, the generated sequences were compared with that derived from the cDNA clone using DNASIS-Mac version 3.0 (Hitachi Solutions).

Liver enzyme level. ALT values in rat sera were monitored weekly by a Fuji Dri-Chem Slide GPT/ALT-PIII kit (Fujifilm). The geometric mean of ALT values during the pre-inoculation period of each animal was defined as the normal ALT value and a twofold or greater increase at the peak was considered as a sign of hepatitis.

ACKNOWLEDGEMENTS

We thank Paul Becher (University of Veterinary Medicine Hannover, Foundation, Germany) for critical discussions. This study was supported in part by grants for Research on Emerging and Re-emerging Infectious Diseases and for Research on Hepatitis from the Ministry of Health, Labour, and Welfare of Japan, and by an intramural research grant of the Federal Institute for Risk Assessment, Germany (SFP 1322-444).

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Supplementary Table S1: Sequences of primers used for amplification and cloning of the ratHEV genome.

Designation	Sequence (5'-3')	Notes
63inf-1s	TTGCCATGG**TAATACGACTCACTATA**GCAACCCCCGATGGAGACCCATC	*Nco*I-site, **T7 promotor**
63inf-1as	TAGGCACAGAGTGGAACGTAGAC	*Dra*III-site
63inf-2s	GTTCCACTCTGTGCCTACAGATAT	*Dra*III-site
63inf-2as	ATCCGCGGCCGCAGTCATGTAGC	*Not*I-site
63inf-3s	GACTGCGGCCGCGGATGTAGTTC	*Not*I-site
63inf-3as	GATGGAATTCATATCCACCGACG	*Eco*RI-site
63inf-4s	ATATGAATTCCATCACTTCCACCG	*Eco*RI-site
63inf-4as	CTTTCTAGA**TTTTTTTTTTTTTTTTTTT**GTCTTGCGGGGA	*Xba*I-site, **poly A**

Supplementary Table S2: Nucleotide and amino acid exchanges present in the ratHEV genomic clone as compared to the original sequence GU345042.

Nucleotide position	Nucleotide		Amino acid				Notes
	GU345042	Genomic clone	ORF	Position	GU345042	Genomic clone	
889	T	C	-*	-	-	-	-
1549	A	G	-	-	-	-	-
1588	C	T	-	-	-	-	-
2516	C	A	ORF1	836	Q	K	R in JN167537
2520	A	G	ORF1	837	D	G	G in JN167537
2528	C	T	ORF1	840	P	S	hypervariable region
2534	T	C	ORF1	842	S	P	hypervariable region
2540	C	T	ORF1	844	P	S	hypervariable region
2546	C	T	ORF1	846	P	S	hypervariable region
2903	C	T	-	-	-	-	-
3808	G	A	-	-	-	-	-
4078	C	T	-	-	-	-	-
4079	T	C	ORF1	1357	F	L	L in JN167537 and GU345043
4087	T	C	-	-	-	-	-
4088	C	T	-	-	-	-	-
4090	G	A	-	-	-	-	-
4150	T	C	-	-	-	-	-
4347	G	C	ORF1	1446	G	A	A in JN167537 and GU345043
5386	A	G	-	-	-	-	-
5493	A	G	ORF2	182	H	R	R in JN167537 and GU345043
5503	G	C	-	-	-	-	-
5656	T	G	-	-	-	-	-
5950	C	T	-	-	-	-	-

*- synonymous exchange

Liu et al. BMC Veterinary Research (2015) 11:11
DOI 10.1186/s12917-015-0322-z

BMC Veterinary Research

RESEARCH ARTICLE Open Access

Seroprevalence and molecular characteristics of hepatitis E virus in household-raised pig population in the Philippines

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Abstract

Background: Hepatitis E virus (HEV) infection is a significant public health concern in Asia, and swine is an important source of sporadic HEV infection in human. However, no epidemiological data are available regarding HEV infection among the swine or human population in the Philippines. To assess the HEV infection status among pigs in rural areas, we investigated the molecular characteristics and seroprevalence of HEV among household-raised pigs in San Jose, Tarlac Province, the Philippines.

Result: Serum and rectal swab samples were collected from 299 pigs aged 2–24 months from 155 households in four barangays (villages) between July 2010 and June 2011. Enzyme-linked immunosorbent assay (ELISA) revealed that 50.3% [95% confidence interval (CI) 44.5–56.2%] and 22.9% (95% CI 18.2–28.1%) of pigs tested positive for anti-HEV IgG and IgM, respectively. HEV RNA was detected in the feces of 22 pigs (7.4%, 95% CI 4.7–10.9%). A total of 103 households (66.5%, 95% CI 58.4–73.8%) had at least one pig that tested positive for anti-HEV IgG or IgM or HEV RNA. The prevalence of anti-HEV IgG and IgM in breeding pig (8–24 months) were higher than that in growing pigs (2–4 months) ($p < 0.0001$ and $p = 0.008$, respectively). HEV RNA was more frequently detected in 2–4-month-old pigs (9.2%, 95% CI 5.4–14.6%) than in ≥5-month-old pigs (4.8%, 95% CI 1.1–8.5%) without statistical significance ($p = 0.142$). HEV RNA showed 0–27.6% nucleotide difference at the partial ORF2 gene among the detected viruses, and a majority of them belonged to subtype 3a (20/22, 90.9%).

Conclusion: We found a high prevalence of HEV antibodies in the household-raised pig population in rural areas of the Philippines, which indicates the potential risk of HEV infection among local residents. Only genotype 3 of HEV was observed, and genetically diverse strains of HEV were found to be circulating in pigs in this study.

Keywords: Hepatitis E virus, Household-raised pig, Seroprevalence, Genotype 3, Philippines

Background

Hepatitis E was first documented as a unique clinical entity distinct from hepatitis A and B in water-borne epidemic hepatitis in India in 1978 [1]. Hepatitis E virus (HEV), the sole member of genus *Hepevirus* in the *Hepeviridae* family, is the causative agent of self-limited or fulminant hepatitis [2]. The virion of HEV is spherical, nonenveloped, 27–34 nm in diameter, with a single-stranded, positive sense RNA genome. The RNA is approximately 7300 nucleotides in length and contains three open reading frames (ORFs). ORF1 encodes nonstructural proteins, while ORF2 encodes capsid proteins and ORF3 encodes a small protein of unknown function [3]. Mammalian HEV falls into four major genetically distinct genotypes based on nucleotide differences [4-6]. Genotypes 1 and 2 are the most common causes of epidemic hepatitis in humans in tropical and subtropical countries with poor sanitation and unsafe water supply [1,7]. Genotypes 3 and 4 are considered to be of zoonotic origin and are together recognized as an important cause of sporadic hepatitis cases in humans both in developing and industrialized countries [6,8,9].

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Some evidence indicates that pigs are an important source of zoonotic HEV genotypes 3 and 4. Case reports have shown that viruses recovered from clinical patients with hepatitis E and the consumed pork were genetically similar [8,10]. A cluster of human isolates from autochthonous hepatitis E cases were found to be genetically similar to the local swine strains by phylogenetic analysis [11]. Meta-analysis of 10 cross-sectional studies revealed greater chances of HEV seropositivity in people with occupational exposure to pigs than in the general human population [12].

HEV genotype 3, which was first isolated in 1997 [6] from domestic pigs in the United States, has been shown to be widely distributed in pigs in all continents. Genotype 4 was first reported in China [5,9], and it appears to be present in pigs and humans exclusively in Southeast Asia. Recently, however, genotype 4 has been detected in pigs and in human cases with more severe clinical manifestations than those with other HEV genotypes in Europe [13,14]. Genotypes 3 and 4 are quite diverse and can be further classified into 10 (3a–3j) and seven (4a–4 g) subtypes, respectively, on the basis of five different regions of HEV, including 5994–6294 nucleotide positions of ORF 2 (GenBank accession number M73218) [4].

The increasing documentation of zoonotic HEV in Asian countries such as China, Japan, Korea, Indonesia, Cambodia, Thailand, and Laos [15-17] suggests a significant health risk for the people. No epidemiological data are available regarding HEV infection among pigs or humans in the Philippines. However, recently, Li et al. reported that genotype 3 of HEV was found in the river water in Manila [18]. HEV infection in commercial pig farms were previously reported; however, there are very few reports on HEV infections in family-scale farms (backyard pig farms), where local people could be more frequently exposed to pigs or pig feces because of the open breeding system and poor sanitation of backyard pigs. The seroprevalence of HEV in family-scale pig farms was higher than that in large-scale pig farms as reported from Thailand [19] and China [20]. In rural areas of the Philippines, backyard pig farms are still quite common, and backyard pigs are an important source of income for pig owners. As a part of the project conducted in the Philippines to assess the prevalence of zoonotic pathogens, including Japanese encephalitis virus and Reston Ebola virus [21], we investigated the molecular characteristics and seroprevalence of HEV among household-raised pigs in four barangays (Villa Aglipay, Moriones, Pao, and Lubigan) in San Jose, Tarlac Province, the Philippines. Notably, San Jose is a third-class municipality and comprises mainly of rural areas in the Tarlac Province (Figure 1), where the density of household-raised pigs is quite high.

Results

Detection of anti-HEV IgG and IgM in pig sera and HEV RNA in stool swabs

Serum and rectal swab samples were collected from a total of 299 pigs aged 2–24 months (median age, 4 months) from 155 households in four barangays. The median numbers of pigs raised and numbers of samples per household were 2 [interquartile range 1–5] and 1[interquartile range 1–2], respectively. A majority of pigs were healthy, raised in simple piggeries in backyards,

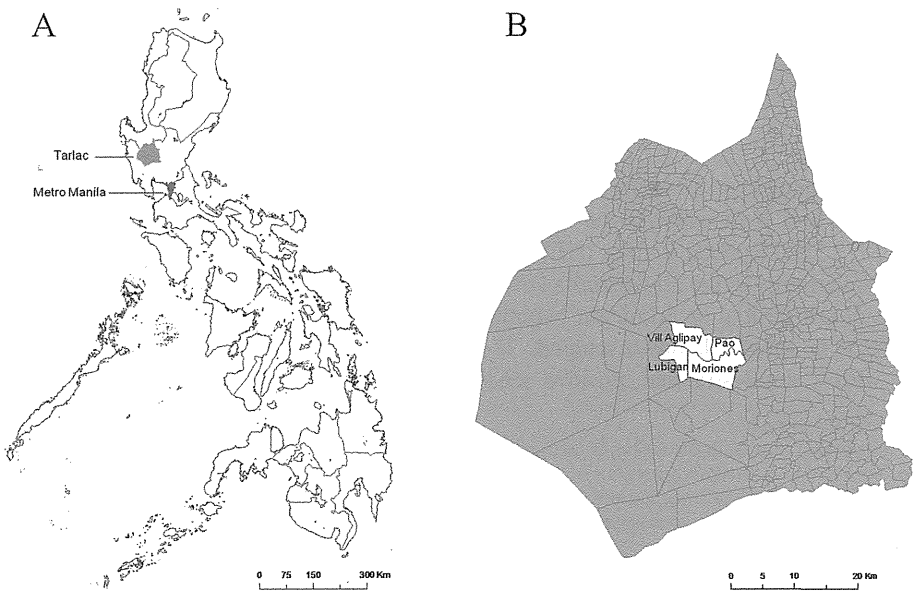

Figure 1 Maps of study sites in the Philippines. A. Tarlac Province (in green) is located north of the Philippines. **B**. Barangays of Pao, Villa Aglipay, Moriones, and Lubigan are located in the center of Tarlac Province.

fed with commercial feeding or kitchen residues, and living with other domestic animals such as chickens and ducks. Anti-HEV IgG was found in 150 serum samples [50.3%, 95% confidence interval (CI) 44.5–56.2%] from 93 households (60.0%), with the similar average prevalence of 43.4–55.1% among four barangays (Table 1). Anti-HEV IgM was detected in 68 serum samples (22.9%, 95% CI 18.2–28.1%) from 52 households (33.5%). On the other hand, a total of 22 rectal swabs (7.4%, 95% CI 4.7–10.9%) from 16 households (10.3%) were positive for HEV RNA (Table 1). The average prevalence (56.2%, 95% CI 50.4–61.9%) of any of the three markers (anti-HEV IgG, IgM, and RNA) in the pig population was observed at similar range between 47.0% and 62.5% among the four barangays. Overall, 66.5% households (103/155, 95% CI 58.4–73.8%) had pigs positive for either anti-HEV IgG, IgM, or viral RNA. Among the 22 RNA positive samples, six samples were positive for both anti-HEV IgM and IgG and 10 samples were only positive for anti-HEV IgG. The remaining six samples were negative for both anti-HEV IgM and IgG.

The presence of anti-HEV IgG, IgM, and HEV RNA in different age groups

The prevalence of anti-HEV IgG (37.6%, 95% CI 30.3–45.2%) was the lowest in growing pigs ($P < 0.0001$) and then increased in finishing pigs (64.1%, 95% CI 53.6–73.9%) and reached a peak of 78.8% (95% CI 61.1–91.0%) in breeding pigs (Table 2). Also, the prevalence of anti-HEV IgM was the lowest in growing pigs (16.9%, 95% CI 11.6–23.3%), comparing to that in finishing pigs (27.2%, 95% CI 18.4–37.4%) and breeding pigs (42.4%, 95% CI 25.5–60.8%) ($p = 0.05$ and $p = 0.0008$, respectively). Growing pigs had the highest prevalence of viral RNA (9.2%, 95% CI 5.4–14.6%), followed by finishing pigs (5.4%, 95% CI 1.8–12.1%), and breeding pigs (3.0%, 95% CI 0.1–15.8%), although it was not statistically significant ($p = 0.26$ and $p = 0.23$, respectively).

Genetic analysis of HEV strains from stool swabs

Phylogenetic analysis of 301 nucleotides corresponding to nucleotide positions 5994–6294 of M73218 in ORF2 revealed that 22 HEV strains in the Philippines belonged to genotype 3 (Figure 2). Pairwise comparison of 22 strains over 301 nucleotides revealed a 0–27.6% nucleotide difference. Compared with representative strains from river water in Manila, the strains in this study showed 10.0–24.0% nucleotide difference. With the exception of two strains (HEV_Vil_PHL_2011_Tjs-224_ORF2 and HEV_Vil_PHL_2010_Tjs-078_ORF2), the other 20 strains fell into a unique cluster within subtype 3a with a genetic distance of 0–4.9%. BLAST analysis revealed that these 20 strains shared less than 94% nucleotide similarities with any other sequence in GenBank. HEV_Vil_PHL_2011_Tjs-224_ORF2 shared the highest similarity (91%) with unclassified strains (JSW-Kyo-FH06L, AB291955) and subtype 3b strains (swJA11, AB082567) from Japan [22] and was also clustered with genotype 3b strains in the phylogenetic tree. The remaining strain HEV_Vil_PHL_2010_Tjs-078_ORF2 displayed 15.9–27.6% pairwise distance with other strains in this study. In the phylogenetic tree, it was clustered with unclassified reference strains from pigs (G3-HEV83-2-27 and G3-4531) and humans (HRC-HE200, HEJSB6151, and E088-STM04C) in Japan and shared 94–100% similarity with them. This cluster was genetically distant from other subtypes and may represent a novel subtype. Strains detected from the same household were closely clustered except two strains (HEV_Vil_PHL_2011_Tjs-223_ORF2 and HEV_Vil_PHL_2011_Tjs-224_ORF2). HEV_Vil_PHL_2011_Tjs-223_ORF2 and HEV_Vil_PHL_2011_Tjs-224_ORF2, which were collected in the same batch of pigs raised in the same household, genetically differed from each other by 15.4% and were grouped into subtype 3a and 3b in the phylogenetic tree, respectively.

Discussion

We used the recombinant antigen (112–660 amino acids of ORF2) of one of the prototype strains of genotype 1 (GenBank accession number D10330), which proved to be effective in detecting HEV antibodies in both human and pig serum [23,24]. Notably, numerous commercial or in-house enzyme immunoassays, which were developed to detect antibodies in human sera, were also adapted to detect anti-HEV in pigs since only one serotype has been described [3,6,25]. We found that the

Table 1 The detection of anti-HEV IgG, IgM, and HEV RNA in household-raised pigs in four barangays

Barangay	No. of swine	% RNA (95% CI)	% IgG (95% CI)	% IgM (95% CI)	% one of three markers (95% CI)	No. of household	% household positive for one of three markers (95% CI)
Pao	83	3.6 (0.8–10.2)	43.4(32.5–54.7)	22.0 (13.6–32.5)	47.0 (35.9–58.3)	49	53.1 (38.3–67.5)
Villa Aglipay	70	15.7 (8.1–26.4)	49.3 (37.0–61.6)	24.6 (15.1–36.5)	57.1 (44.7–68.9)	24	70.8 (48.9–87.4)
Moriones	98	7.1 (2.9–14.2)	55.1 (44.7–65.2)	21.4 (13.8–30.9)	60.2 (49.8–70.0)	52	73.1 (59.0–84.4)
Lubigan	48	2.1 (0.5–11.1)	54.2 (39.2–68.6)	25.0 (13.6–39.6)	62.5 (47.4–76.0)	30	73.3 (54.1–87.7)
Total	299	7.4 (4.7–10.9)	50.3 (44.5–56.2)	22.9 (18.2–28.1)	56.2 (50.4–61.9)	155	66.5 (58.4–73.8)

Table 2 The presence of anti-HEV IgG, IgM, and HEV RNA in pigs of different age groups

Age group of pigs	No. of pig	% RNA (95% CI)	% IgG (95% CI)	% IgM (95% CI)
Growing pigs (2–4 months)	173	9.2 (5.4–14.6)	37.6 (30.3–45.2)	16.9 (11.6–23.3)
Finishing pigs (5–7 months)	93	5.4 (1.8–12.1)	64.1 (53.5–73.9)	27.2 (18.4–37.4)
Breeding pigs (8–24 months)	33	3.0 (0.1–15.8)	78.8 (61.1–91.0)	42.4 (25.5–60.8)
Total	299	7.4 (4.7–10.9)	50.3 (44.5–56.2)	22.9 (18.2–28.1)

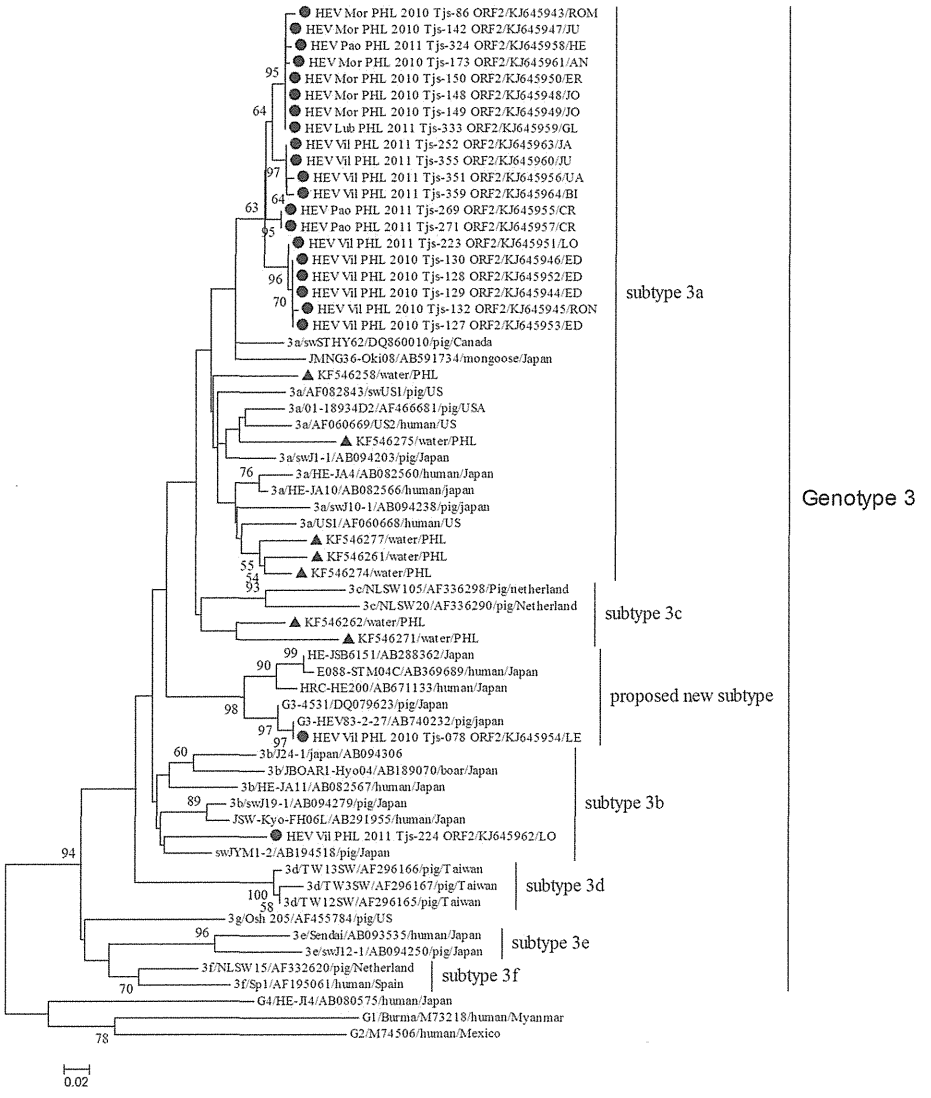

Figure 2 Phylogenetic analysis of HEV strains from pigs in San Jose, Tarlac Province, the Philippines. The phylogenetic tree was constructed using the neighbor-joining method (Kimura 2-parameter model) based on 301 nucleotides of ORF2 of HEV. Strains from this study were labeled with ● and tagged with household name (in capital letters) from where they originated. HEV genotype 3 strains from rivers in Manila, the Philippines, which were labeled with ▲, with GenBank accession numbers of KF546258, 546261, 546262, 546271, 546274, and 546277, were also included. Other reference strains were representatives of genotypes 1, 2, and 4 and subtypes 3a–3 g of genotype 3 in other countries. Reference strains were indicated as genotypes or subtypes, name, country, and GenBank accession number. The numbers on branches were bootstrap values (1,000 replicates; values less than 50% were not shown).

anti-HEV IgG prevalence (50.3%) in pigs from rural communities of the Philippines was much higher than that in a similar study conducted in smallholder-raised pigs in rural villages of Laos (15.3%) [26] and comparable to that in large-scale surveys of pigs of all age groups from commercial pig farms in Japan (56%) [15], Germany (46.9%) [27] and Italy (50.2%) [28]. It is worthwhile to mention that in several other studies, discrepant results have been reported when comparing ELISAs using different antigens of HEV with human or porcine origin for detecting anti-HEV in pigs [29,30]. Compared with the prevalence of anti-HEV IgG, there are limited data on the seroprevalence of anti-HEV IgM in domestic pigs. The seroprevalence of IgM in this study (22.9%) was much higher than that in large-scale surveys in Japan (3%) [15] and similar to that in Spain (28.2%) [31]. Furthermore, 66.5% of households had at least one pig positive for anti-HEV IgG, IgM, or HEV RNA. In the present study, the piggeries of pigs were simple, in poor sanitary conditions and located at the household or near to the household. Such a high level of HEV infection in household-raised pigs, frequent exposure to pigs or pig waste, and poor sanitation in rural areas in the Philippines indicate potential risks of HEV transmission from pigs to local residents. Besides, the local people commonly consume the cooked pig livers, pork and sausages made by local manufactures. Therefore, workers in slaughterhouses and pork handlers in the local area are at potential risk of getting HEV infection. However, there are no available data on viral hepatitis incidence due to HEV in the Philippines. The human health impact of HEV should be properly defined to establish appropriate interventions.

Our data revealed that the seroprevalence of anti-IgG increased with age from 2–4-month-old pigs to 8–24-month-old pigs. A higher seroprevalence of IgG in adult pigs than in young pigs has also been documented in other studies [25,32]. However, according to antibody dynamics studies, there may be two seroprevalence peaks of anti-HEV IgG at less than one month old pigs due to maternal antibody and adult pigs in commercial pig herds [6,33-35]. In the present study, we did not observe the first peak of anti-IgG because the pigs in the present study were ≥2 months old and the maternal anti-IgG could persist up to 8–9 weeks of age in young pigs depending on the titers in breeding pigs [6,34,35]. It has been reported that seroconversion of IgM occurs in pigs aged 2–3 months and its duration varies from 4 to 7 weeks in commercial herds [6,34,35]. However, in some commercial herds, the peak prevalence of IgM was reported in pigs aged 25 weeks, which could be slaughtered, and IgM were also frequently detected in sows (up to 40%) [33]. We observed that the prevalence of IgM increased from 2–4-month-old pigs (16.9%) and reached a peak in 8–24-month-old pigs (42.4%); however, no infectious RNA was detected in rectal swabs of these breeding pigs except one. All these pigs were raised under poor sanitary condition, and breeding pigs usually lived with young pigs in rural communities. The high seroprevalence of IgM in breeding pigs was probably caused by the secondary immune response to frequent HEV exposure as reported among the vaccinated population exposed to measles virus [36]. This quick secondary immune response could prevent viral proliferation in the early phase; therefore, no RNA was detected. In the current study, we have provided important information about HEV infection status of pigs aged above 6 months while a majority of prevalence studies have been performed among pig aged less than 6 months. Six pigs with age ranging from 2–4 months were found negative for both IgG and IgM antibodies in sera but positive for HEV RNA in feces probably because of a recent infection [37]. On the other hand, in 10 pigs with age ranging from 2 to 8 months, RNA was detected in feces and IgG was found positive, but IgM was found negative. Detected IgG antibody might be due to persistent maternal antibody among 2–3 months old pigs (n = 5) and existed antibody from past infection in other five pigs aged 4–8 months. IgM negative results were probably because of a recent recurrent viral infection which could result in a low IgM immune response to HEV and a false negative result of IgM serological test. Moreover, it is possible that positive HEV in stools might reflect transient exposure to the virus through ingestion of contaminated food or water.

The average RNA-positive rate in rectal swab samples in this study (7.4%) was similar to that in Japan (5%) [15], Laos (11.6%) [16] and Thailand (2.9–7.75%) [19,38]. In this study, the viral RNA-positive rate was higher in 2–4-month-old pigs than in adult pigs, however the difference was not found statistically significant. Our finding is in line with other reports which stated that the highest incidence of HEV infections occurs in young pigs (2–4 months old) [15,39]. In Southeast Asia, both HEV genotypes 3 and 4 are circulating in human and swine; however, the geographical distribution of genotypes of zoonotic HEV in pigs varies. In Cambodia [40] and Thailand [19,38], only HEV genotype 3 has been reported in local pigs, while only genotype 4 of HEV has been reported in pigs from Laos [16]. In China [41], Japan [42], Korea [43], Indonesia [17], and Taiwan [44], both genotypes 3 and 4 were circulating in domestic pigs or wild boars. In the present study, we only detected HEV genotype 3 strains and a majority of them (20/22) were classified into the existing subtype 3a. Subtype 3a was also the most frequently detected subtype in Japan, Korea, and North America [4,45,46] as well as in river water in Manila in 2012 [18]. Subtype 3a strains in San

Jose shared less than 94% nucleotide similarity with strains in GenBank (including strains from river water in Manila), which suggests that area-specific strains of HEV were circulating in the Philippines. HEV_Vil_PHL_2010_Tjs-078_ORF2 shares 100% sequence identity with the strain (G3-HEV83-2-27, AB740232) from a domestic pig in Japan in 2003; thus, HEV_Vil_PHL_2010_Tjs-078_ORF2 may have the same origin as the strain detected in a domestic pig in Japan. However, the exact transmission route of this virus remains unknown. HEV_Vil_PHL_2010_Tjs-078_ORF2 together with G3-HEV83-2-27 and some unclassified strains from Japan formed a distinct cluster from other subtypes. The full sequence of G3-HEV83-2-27 is available in GenBank (accession number AB740232), and it can also form a distinct cluster in a phylogenetic tree based on the full genome sequence (see Additional file 1: Figure S1). Therefore, this cluster, including HEV_Vil_PHL_2010_Tjs-078_ORF2, could represent a new subtype.

Conclusion

The present study is the first report on the seroprevalence and molecular characterization of HEV in pigs in the Philippines. We found a high proportion of IgG and IgM, and three different subtypes of HEV among household-raised pigs suggesting that the risk of HEV transmission to humans in this geographical area was substantial. Hepatitis E is not included as a notifiable disease in the Philippines, and laboratory testing for acute hepatitis is not routinely performed in the country. Since only pig population from a small geographic area were investigated in the present study, further studies are required to define the genotype distribution in other areas, genetic relationship between HEV strains from swine and human and human health impact of HEV in the Philippines.

Methods

Samples

Swine blood and rectal swab samples from previous cross-sectional survey for validation of an ELISA assay [21] were tested for prevalence of HEV at the research institute for tropical medicine in Manila, Philippines. The sample collection was divided in six phases between July 2010 and June 2011. Households known to have pigs in their backyard farms were visited and owners were asked for the participation into the study. Informed consent was obtained from the pig owners. The pigs were stratified by age in months and selected to have all age groups available in each household. Piglets less than two months old were excluded from the sampling. The sampling was not systematic or random. Up to 50 samples were collected per sampling phase. This study was approved by ethics committee of Research Institute for Tropical Medicine and Institutional Animal Care and Use Committee at the Animal Welfare Division of Bureau of Animal Industry, the Department of Agriculture.

Assessment of HEV infection by serology

To detect anti-HEV IgM and IgG, ELISA was performed. Virus-like particles (VLPs) were expressed by a recombinant HEV Burma strain (genotype 1) ORF2 (112–660 amino acids of D10330) using baculovirus in Tn5 cells, as described previously [47]. Flat-bottom 96-well polystyrene microplates (Becton, Dickinson and Company, NJ, USA) were coated with purified VLPs (1 μg/ml, 100 μl/well) and incubated at 4°C overnight. Unbound VLPs were washed out with 300 μl of 10 mM phosphate-buffered saline containing 0.05% Tween 20 (PBS-T). The wells were blocked for 1 h with 200 μl of 5% skim milk (Becton, Dickinson and Company, NJ, USA) in PBS-T at 37°C. After the plates were washed three times with PBS-T, swine serum samples (100 μl/well) were added in duplicate at a dilution of 1:200 in PBS-T containing 5% skim milk. The plates were then incubated for 1 h at 37°C. The plates were washed three times as described above and were administered 100 μl of horseradish peroxidase-conjugated goat anti-pig IgG (Bethyl, Laboratories, Inc., TX, USA) (1:10,000 dilution) or IgM (Kirkegaard & Perry Laboratories, Inc., MD, USA) (1:2,500 dilution) in PBS-T containing 1% skim milk. The plates were incubated for 1 h at 37°C and washed three times with PBS-T. Subsequently, 100 μl of o-phenylenediamine dihydrochloride (Sigma-Aldrich, Co., MO, USA) was added to each well. The plates were incubated in a dark room for 30 min at room temperature, following which 100 μl of 4 N H_2SO_4 was added to each well. The optical density was measured at 492 nm. Four standard deviation values above the mean OD value of negative controls (n = 4) were applied as the cut-off value for each plate.

Detection and genotyping of HEV infection

Rectal swabs were soaked in a viral transport medium containing Hank's balanced salt solution supplemented with gelatin, streptomycin, penicillin-G, and amphotericin B. RNA was extracted from 140 μl of sample using the QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany). Reverse transcription was performed using SuperScript III reverse transcriptase with a random hexamer (Life technologies, Carlsbad, CA, USA). A previously described nested polymerase chain reaction (PCR) [8] was performed to amplify part of ORF2, which corresponds to the nucleotide positions 5939–6316 of the genotype 1 HEV genome (GenBank accession number M73218). The PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen), sequenced using BigDye Terminator version 1.1 (Life technologies), and analyzed using Applied Biosystems 3700 Genetic Analyzer (Life technologies). HEV genotypes were determined by

phylogenetic analysis using MEGA (version 5) [48]. The pairwise distance was calculated by the neighbor-joining method, and the phylogenetic tree was constructed by the Kimura 2-parameter model, neighbor-joining method by MEGA 5. Strains in this study were named as HEV_ Barangay code_PHL _year_ID number of strain_ORF2 and were deposited in the GenBank database under the accession number of KJ645943–KJ645964. The GenBank accession numbers of reference strains are given as follows: DQ860010, AB082566, AB194492, AF060669, AB740232, DQ079632, AB671133, AB369689, AB288362, AB291955, AB094279, AB082567, AF296167, AF296165, AF336298, AF336290, AF336293, AB093535, AF332620, AF455784, AB080575, AF296166, AB094250, AF195061, AB591734, AB291955, AB194518, M73218, and M74506.

Availability of supporting data

The data set supporting the results of this article is included within the article and its additional file.

Statistical Analysis

Pigs in this study were classified into three age groups according to local pig production stage: growing pigs (2–4 months), finishing pig (5–7 months), and breeding pigs (8–24 months). The prevalence of anti-HEV IgG, IgM and HEV RNA between different age groups were compared by using Chi-square test in Stata software, version 12 (StataCorp, College Station, Texas), $p \leq 0.05$ was considered statistically significant.

Additional file

Additional file 1: Figure S1. Phylogenetic analysis of HEV based on complete genome. The phylogenetic tree was constructed by the neighbor-joining method with the Kimura 2-parameter model. The strain G3-HEV83-2-7 (sharing 100% similarity in 301 nucleotides with HEV_Vil_PHL_2010_Tjs-078_ORF2) with ♦ label could represent a new subtype under genotype 3 based on full genome sequence analysis.

Abbreviations

HEV: Hepatitis E virus; ORF: Open reading frame; nt: Nucleotide; ELISA: Enzyme-linked immunosorbent assay; PCR: Polymerase chain reaction; VLP: Virus-like particle; Ig: Immunoglobulin; PBS-T: Phosphate buffered saline containing 0.05% Tween 20.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

XFL carried out molecular analysis and draft the manuscript. MS participated in the design of the study, carried out the sample collection, immunological assay and involved to drafting the manuscript. YS participated in the design of the study, carried out the sample collection, immunological assay and molecular analysis. ES carried out the molecular analysis. FFM and HOG worked on the coordination with local government unit and carried out the sample collection. YF helped in phylogenetic analysis for the revised manuscript. MS carried out the epidemiological analysis and helped to draft the manuscript. TCL prepared the virus-like particle for immunological assay. AS carried out the sample collection and helped to draft the manuscript. HO conceived of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

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Acknowledgement

We are grateful to the Local Government Unit of San Jose Municipality of Tarlac Province, the Philippines for their considerable support. We especially thank Dr. Lorna Baculanta, Provincial Veterinarian of Provincial Veterinary Office in Tarlac and Ms. Cecille Lopez,Epidemiologist of Tarlac Provincial Hospital for their valuable assistance. We thank the staff of RITM, Department of Virology, Tohoku University Graduate School of Medicine, and Tohoku-RITM Collaborating Research Center on Emerging and Re-emerging Infectious Diseases for technical assistance and logistic support. This work received financial support from a grant-in-aid for the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Received: 6 June 2014 Accepted: 12 January 2015
Published online: 27 January 2015

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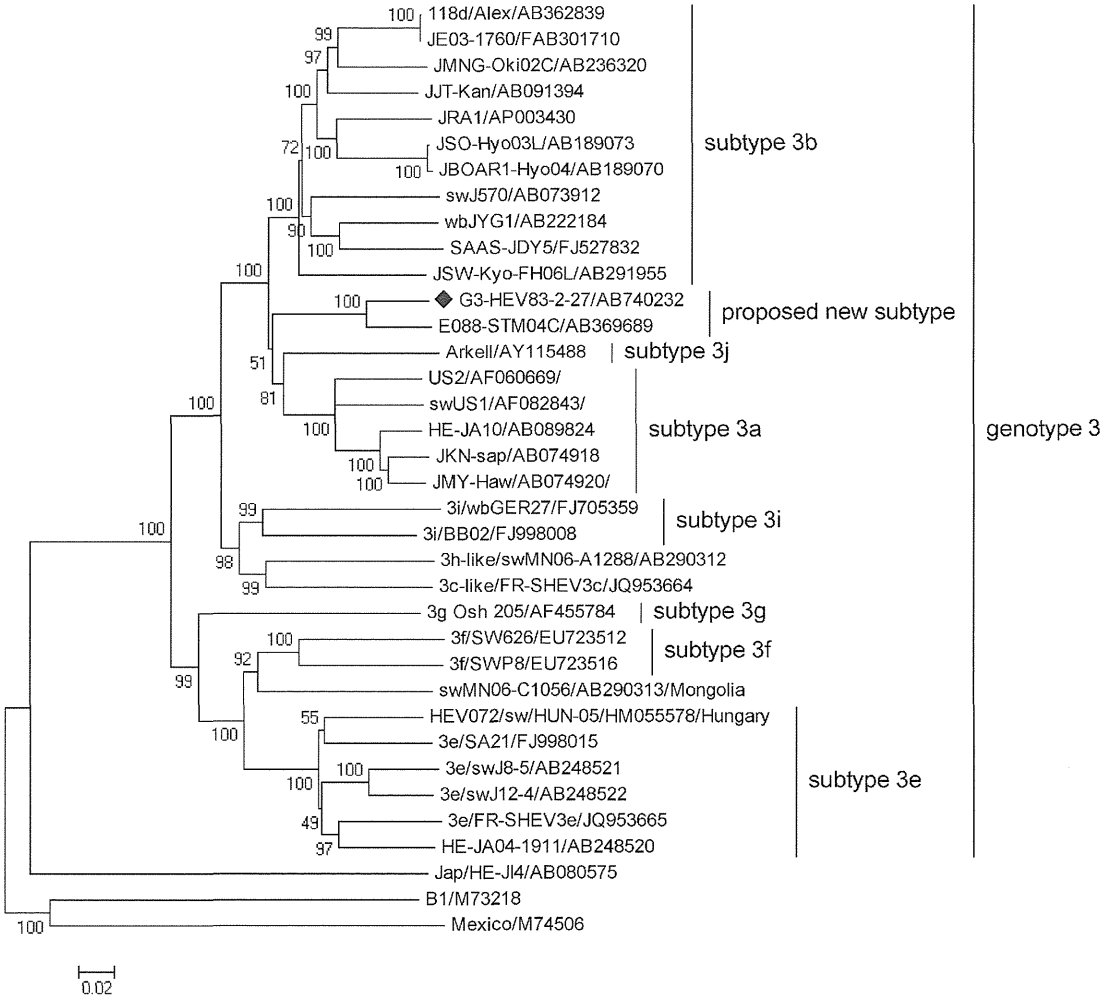

Supplementary Fig. S1

Microbiology and Immunology

Microbiol Immunol 2015; 59: 89–94
doi: 10.1111/1348-0421.12219

NOTE

Establishment of hepatitis E virus infection-permissive and -non-permissive human hepatoma PLC/PRF/5 subclones

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ABSTRACT

PLC/PRF/5 cells show limited permissiveness, meaning that almost all subclones are permissive; however, some subclones do not exhibit permissiveness for hepatitis E virus (HEV) infection. In this study, the single-cell cloning of PLC/PRF/5 was performed and heterogeneous subclones characterized. Notably, the efficiency of intracellular virus replication did not correlate with the permissiveness for HEV infection. However, as well as binding permissive subclones, virus-like particles bound non-permissive subclones on various levels, suggesting that these subclones have some deficiencies in the attachment and entry steps of infection. Our data would be useful for investigating the HEV life cycle.

Key words attachment, cloning cell lines, hepatitis E virus, non-permissive.

Hepatitis E virus is responsible for acute and enterically transmitted hepatitis in the developing world (1), accounts for more than 50% of acute viral hepatitides in young adults, and has a fatality of at least 1%, up to 20% in pregnant women (2–4). Recent epidemiological studies have revealed the widespread prevalence of HEV and anti-HEV antibodies in humans and several animal species worldwide, including in developed countries (5–9). HEV has only one serotype, but at least four genotypes: G1 and G2 exclusively infecting humans and G3 and G4 additionally infecting swine and several other mammalian species (5–7). Other genotypes have been reported as causative agents only in wild boar (10, 11).

Hepatitis E virus is a single-stranded positive-sense RNA virus that belongs to the genus *Hepevirus* in the family *Hepeviridae* (12). The virions exhibit distinct buoyant densities in feces (1.26–1.27 g/mL) and in circulating blood (1.15–1.16 g/mL), differences that might be associated with their cellular membrane composition (13). The 7.2 kb genome contains three partially overlapping ORFs (14). *ORF1* encodes a nonstructural polyprotein that provides RNA-dependent RNA polymerase activity and possibly other functions (15–17). The ORF2 and ORF3 proteins are believed to be encoded by individual subgenomic RNAs generated during replication (18). *ORF2* encodes the viral capsid protein (14, 19).

Before the establishment of high-efficiency HEV cell culture systems, *in vitro* generation of HEV-LPs from modified capsid proteins in insect cells (20, 21) or *in vivo* propagation in nonhuman primates were the most useful models for studying HEV (22).

In 2007, efficient HEV cell-culture propagation systems were established by using PLC/PRF/5, a hepatocarcinoma cell line, and A549, an adenocarcinomic human alveolar basal epithelial cell line (23). The replication efficiencies of these new systems are several orders of magnitude higher than those of previous culturing systems (23–33). The increased availability of HEV reagents provided by the new culturing systems has permitted many groups to construct HEV infectious clones, thereby facilitating multiple recent reverse genetic studies (34–38).

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Received 16 October 2014; revised 20 November 2014; accepted 28 November 2014.

List of Abbreviations: DMEM, Dulbecco's modified Eagle's medium; G, genotype; HEV, hepatitis E virus; HEV-LP, HEV virus-like particle; ORF, open reading frame; S-VLP, small virus-like particle.

PLC/PRF/5 cells obtained from the Japanese Collection of Research Bioresources (cell number, JCRB0406; lot number, 01272003; Osaka, Japan) were first characterized for HEV infection in our laboratory. The cells were grown in DMEM supplemented with 10% (vol/vol) heat-inactivated FCS, 100 U/mL of penicillin G, and 100 μg/mL of streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. Separate 35 mm^2 confluent cultures of PLC/PRF/5 cells on treated culture plates (BD Biosciences, Franklin Lakes, NJ, USA) were infected with G3-HEV83–2-27 strain. As a negative control, the same amount and number of cells were not infected but were cultured for the same duration. One set of plates from each group (infected and uninfected) was stained at weekly intervals to provide a time course for testing strength of HEV antigen expression as an indication of viral growth. Specifically, at each time interval, separate plates were fixed with 4% formamide, permeabilized with 0.5% Triton X-100 (Sigma, St Louis, MO, USA) in PBS, stained with rabbit polyclonal anti-G3-HEV-LPs antibody (21) and finally with Alexa488-conjugated secondary antibody (Life Technologies, Carlsbad, CA, USA). Immunofluorescent images of each plate were recorded using a BIOREVO BZ-9000 (Keyence, Osaka, Japan). In the HEV-infected cells, the intensities of immunofluorescence were observed to gradually increase, reflecting the gradual growth of HEV, and spread, indicating production of HEV progeny virion. However, in the infected cells, distinct distributions of intensities were observed with the passage of time (data not shown). To determine the unique permissiveness of each subclone, single-cell cloning of the host cell line was performed by a limiting dilution method.

To obtain subclones, PLC/PRF/5 cells were diluted with medium to a density of 10 cells/mL and seeded at 100 μL/well in 96 well plates. Ninety-eight clones were isolated from the original PLC/PRF/5 cell line. Each clone was designated based on the source plate number-well number (ex. 3–7). The infectious virus G3-HEV83–2-27 was used for experimental infection (38). The cells were infected after initial growth of the stocked subclones, then assayed for HEV antigen expression; spent media were tested by HEV antigen ELISA using rabbit polyclonal anti-G3-HEV-LP antibody (21), susceptibility being defined as OD at 492 nm exceeding 0.2 compared with a pre-immune serum. To determine the susceptibility of each subclone, cells were infected with G3-HEV83–2-27 strain at a concentration of 2.7×10^6 copies/well (six well cell culture plate; Corning-Costar, Cambridge, MA, USA). Three days after inoculation, the culture medium was replaced with fresh maintenance medium consisting of 50% DMEM and 50% Medium 199 (Life Technologies, Carlsbad, CA, USA) containing 2% FCS, 30 mM $MgCl_2$, 100 U/mL penicillin G and 100 μg/mL streptomycin. Subsequently, the spent media were collected and replaced with fresh maintenance media every three or four days. Antigen ELISA analysis was used to monitor virus production by infected subclones.

Subclones could be divided into the following three groups based on the pattern of antigen production: (A) early antigen secretion (secreted levels observed primarily in the first three weeks) (Fig. 1a); (B) antigen secreted from Day 21 to 63 (Fig. 1b); and (C) antigen not secreted by the end of this study (Fig. 1c). All the 73 Group A clones showed susceptibility; almost half of the Group A clones showing early growth within 2 weeks. In ELISA testing, the Group A clones yielded ODs of >0.2 within 2–3 weeks, indicating that these subclones were highly permissive for HEV infection (Fig. 1a). In the 13 Group B clones, antigen production yielded ODs of <0.2 through the first three weeks; all of these subclones showed slower growth than Group A clones, suggesting that the Group B subclones were moderately permissive for HEV infection. This intermediate permissiveness may have derived from the deletion or partial down-regulation of some non-essential host factors that promote growth efficiency of HEV infection (Fig. 1b). None of the 12 Group C lines supported HEV infection; these isolates were defined as non-permissive clones (Fig. 1c). To address the possibility that these properties changed during long-term culturing, representative Group C subclones were repeatedly re-subcloned and cultured. Even after three months, the Group C-derived clones still did not support HEV infection (data not shown), demonstrating that these lines remained non-permissive.

To confirm the non-permissiveness of Group-C PLC/PRF/5 subclones for HEV infection, the corresponding isolates were infected with the G3-HEV83–2-27 virus strain and cultured supernatants collected periodically. Two inoculated non-permissive clones (3–13 and 4–29) showed no antigen production (Fig. 1c under the horizontal black line, Fig. 2a under the horizontal black line). Two other clones, 4–9 belonging to Group B (moderately permissive) and 4–21 belonging to Group A (highly permissive), demonstrated permissiveness when assessed under the same conditions. Time-course quantification of Subclone 4–21 showed a slight increase in the amount of HEV capsid antigen in medium collected 7 days post-infection, whereas the amount of antigen produced by Subclone 4–9 started to increase on Day 21, that is, 14 days later than the start of antigen secretion by 4–21 (Fig. 2a over the horizontal black line).

To investigate the subclones' HEV replication capability after the viral cell entry step, an infectious clone,