NSP1 amino acid sequences were considered to be highly divergent among strains both within and between species, all porcine RVB strains retained conserved serine residues in peptide 1 and a cysteine- and histidine-rich motif, as in bovine and human RVB strains, in peptide 2 (Supplementary Fig. S1).

Phylogenetic analysis of RVB NSP1

A genetic classification of RVB NSP1 was performed on the basis of a cut-off value that was estimated from frequency distribution of pairwise sequence identities according to the definition recommended by the Rotavirus Classification Working Group (Matthijnssens *et al.*, 2008b). The cut-off values for the division of genotypes were defined as 72 % in peptide 1 and as 80 % in peptide 2 (Fig. 1). Consequently, multiple alignments of NSP1 amino acid sequences (peptides 1 and 2) of RVB strains could classify them genetically into seven genotypes according to host species. Although the functions of the two proteins from the NSP1 gene of RVBs are still unknown and might be different from those of RVAs and RVCs, we would provisionally define the new NSP1 genotypes with the argument of having a concordant nomenclature as follows: murine (A1), human (A2), ovine (A3), bovine (A4) and porcine (A5–7). Surprisingly, porcine RVB strains were divided broadly into multiple clusters distinct from other RVBs. In addition, a phylogenetic tree of RVB NSP1 nucleotide sequences showed the same clustering with a cut-off value of 76 % (data not shown).

Nine porcine RVB strains, common to our previous study (Kuga *et al.*, 2009), were classified into genotype A7 by analysis of RVB NSP1, whilst only strain PB-S43-17 was distinguished in a different cluster (G3) from other strains belonging to the G5 cluster in the analysis of RVB VP7.

DISCUSSION

Although sequence information on most RNA segments has been elucidated in human, bovine and murine RVBs, little is known about RNA segments other than those encoding the VP7 and NSP2 genes in porcine RVBs (Eiden *et al.*, 1992; Ghosh *et al.*, 2010; Yamamoto *et al.*, 2010). Without prior knowledge of any sequence information, the single-primer amplification method is the method of choice to understand the molecular characteristics of an unknown target (Lambden *et al.*, 1992; Potgieter *et al.*, 2002; Wakuda *et al.*, 2005; Maan *et al.*, 2007). In the analysis using this method, we firstly determined the NSP1 nucleotide sequences from two porcine RVB strains, and thereafter developed RT-PCR to amplify the NSP1 gene with primers based on the determined terminal sequences. Consequently, this study revealed the NSP1 nucleotide and deduced amino acid sequences of 15 porcine RVB strains, including PB-93-I5 and PB-107-G16. Their nucleotide sequences contained two ORFs, as in human, bovine and murine RVBs but not ovine RVBs, whilst the NSP1 genes of RVAs and RVCs had only one ORF (Eiden *et al.*, 1992; Shen *et al.*, 1999; Ghosh *et al.*, 2010; Yamamoto *et al.*, 2010). Alignment of NSP1 amino acid sequences of porcine RVBs with those of other RVBs indicated that a highly conserved cysteine-rich motif was present especially on peptide 2 among RVBs, instead of a cysteine-rich zinc-finger motif in the amino terminus of NSP1 of RVAs and RVCs (James *et al.*, 1999; Kobayashi *et al.*, 2001). Therefore, these discrepancies between the NSP1 of RVBs and those of RVAs and RVCs suggest that the mechanism of action of NSP1 may be different among these three groups of viruses. Comparing NSP1 between RVBs and novel rotaviruses (ADRV-N, B219 and J19), we interestingly found that B219 and J19, as well as ADRV-N, retained the highly conserved cysteine and histidine motif instead of the zinc-finger motif (data not shown). These findings support the hypothesis that ADRV-N, B219 and J19 would correlate closely with the evolution and origin of RVBs (Yang *et al.*, 2004; Alam *et al.*, 2007; Jiang *et al.*, 2008). Moreover, phylogenetic analyses provided evidence that NSP1 is highly divergent among strains both within and between species in RVBs, as in RVAs, and clustering is clearly distinct from clusters according to the species of origin (Hua *et al.*, 1993; Dunn *et al.*, 1994; Kojima *et al.*, 1996; Kobayashi *et al.*, 2003; Matthijnssens *et al.*, 2008a). These patterns imply that rotavirus strains infecting different animal species may have diverged genetically as the proteins evolved to better counter the antiviral pathways inherent to each host (Dunn *et al.*, 1994; Arnold & Patton, 2011). Thus, our data support the hypothesis that NSP1 could also be significant as a host-range restriction factor.

In this study, nine porcine RVB strains were common to those used in our previous analysis (Kuga *et al.*, 2009). These strains were divided into one genotype (A7) in the genotyping of NSP1. On the other hand, strain PB-S43-17 was classified into a genotype distinct from the other strains in the phylogenetic clustering of VP7. In addition, several other strains (e.g. PB-S43-2 and PB-S43-11) were detected from the same pig farm at the same time. Thus, the present data suggest the possibility of gene reassortment between porcine RVBs within the farm. Further genetic analysis of the remaining RNA segments is essential to confirm this hypothesis.

In RVA classification, the Rotavirus Classification Working Group offered a suggestion of the sequence identity cut-off value of RVA NSP1 genes in the definition of genotypes as 79 % in nucleotide sequence (Matthijnssens *et al.*, 2008a, b). In the study presented here, the cut-off values based on the distribution of RVB NSP1 were 76 % identity in nucleotide sequence, and 72 and 80 % amino acid sequence identity in peptides 1 and 2, respectively, which were relatively lower than those for RVA as described above. In addition, we reported previously that the sequence identity cut-off value for VP7 classification among RVBs from different host species was considerably lower than those among RVAs and RVCs (Tsunemitsu *et al.*, 1992, 1999; Kuga *et al.*, 2009). Thus, these data also support the hypothesis that RVB strains from different hosts may have diverged from one another over a longer period of time compared with RVAs and RVCs (Eiden

et al., 1992; Petric *et al.*, 1991; Tsunemitsu *et al.*, 1999). Interestingly, NSP1s from porcine RVBs were classified into three different genotypes, in contrast to NSP1 from other species, which belonged to monophyletic genotypes. These facts suggest that pigs could be the original host of RVB infections and that there are different serotypes within porcine RVBs.

In conclusion, the present study elucidated the full-length nucleotide sequences of NSP1 genes from two porcine RVB strains, PB-93-I5 and PB-107-G16, and NSP1 ORFs from 13 porcine RVB strains. Moreover, RVB NSP1 genes could be classified into seven genotypes according to genetic relatedness and the species of origin. The NSP1 genes of porcine RVBs were notably divided into three clusters. Our findings provide new insights into the understanding of molecular characteristics and evolution of porcine RVBs. Further genetic data will be helpful to elucidate the patterns of evolution of RVBs, overall and in different host species.

METHODS

Viruses. The 15 faecal samples used in this study, from suckling and weaned pigs collected at nine farms around Japan from 2002 to 2009, are summarized in Supplementary Table S1 (available in JGV Online). Of these, nine strains (PB-S22-3, PB-S24-11, PB-S40-1, PB-S43-17, PB-68-C17, PB-68-D5, PB-70-H3, PB-71-H5 and PB-72-H3) were also used in our previous genetic analysis of porcine VP7 genes (Kuga *et al.*, 2009). Viral RNA was extracted from 10 % faecal suspensions in minimum essential medium using TRIzol LS (Invitrogen) according to the manufacturer's instructions.

Cloning and sequencing of the NSP1 gene from two porcine RVB strains, PB-93-I5 and PB-107-G16. The full-length nucleotide sequences of NSP1 genes from two porcine RVB strains, PB-93-I5 and PB-107-G16, were determined using the single-primer amplification method as described by Wakuda *et al.* (2005). Briefly, ligation of a single amino-group-linked oligonucleotide primer to the 3′ ends of both strands of the viral dsRNA, column-based purification and concentration of the ligated RNA, and RT-PCRs were carried out. The PCR products were cloned into the pCR2.1 TOPO vector (Invitrogen) and sequenced.

RT-PCR for amplification of NSP1 genes from several porcine RVB strains. Oligonucleotide primers NSP1-F (5′-AAGTCAGGGA-AACCTATGGGA-3′; nucleotide position 28–48 of PB-107-G16) and NSP1-R (5′-CTGCGTCAGGTTAGAGATCTGG-3′; nucleotide position 1233–1254 of PB-107-G16) were designed to determine ORFs of porcine RVB NSP1 genes. RT-PCR was conducted by using a OneStep RT-PCR kit (Qiagen). Both terminal sequences were confirmed by using a 5′ RACE kit (Invitrogen). The reaction was performed at 50 °C for 30 min and 95 °C for 15 min, followed by 35 cycles of 94 °C for 1 min, 48 °C for 1 min and 72 °C for 2 min, and then a final extension at 72 °C for 10 min. The products were sequenced using a BigDye Terminator v3.1 cycle sequencing kit on an automated ABI Prism 3130 Genetic Analyzer (Applied Biosystems).

Sequence and phylogenetic analysis. Sequence data were aligned by the CLUSTAL W method using the MEGALIGN program of the Lasergene software (DNASTAR). Phylogenetic analysis was performed using the MEGA 5 program (Tamura *et al.*, 2011). Genetic distances were calculated by the Poisson correction parameter at the amino acid level and the Kimura two-parameter correction at the nucleotide level (Matthijnssens *et al.*, 2008a). Phylogenetic trees were constructed by using the neighbour-joining algorithm with bootstrapping with 1000 replicates (Saitou & Nei, 1987).

Nucleotide sequence accession numbers. The nucleotide sequences of 15 porcine RVB strains determined in this study were submitted to GenBank and assigned the following consecutive accession numbers: PB-S22-3 (AB646350); PB-S24-11 (AB646351); PB-S40-1 (AB646352); PB-S43-17 (AB646353); PB-68-C17 (AB646354); PB-68-D5 (AB646355); PB-70-H3 (AB646356); PB-71-H5 (AB646357); PB-72-H3 (AB646358); PB-85-I3 (AB646359); PB-91-H1 (AB646360); PB-93-H2 (AB646361); PB-87-Z2 (AB646362); PB-93-I5 (AB646363); PB-107-G16 (AB646364).

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Sequence and phylogenetic analyses of nonstructural protein 2 genes of species B porcine rotaviruses detected in Japan during 2001–2009☆

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ABSTRACT

Porcine rotavirus B (RVB) has been often detected in diarrhea of suckling and weaned pigs. Because it is difficult to serially cultivate RVBs in cell culture, the number of available sequence data for RNA segments other than VP7 and NSP1 in especially porcine RVBs is still limited. We performed genetic analysis focusing on nonstructural protein 2 (NSP2) using several porcine RVB strains, which were detected in diarrheic feces collected around Japan during 2001–2009. Comparison of NSP2 nucleotide and deduced amino acid sequences from porcine RVB strains exhibited low identities (64.0–99.9% in nt and 66.7–100.0% in aa) to those of other RVB strains. Phylogenetic analysis of RVB NSP2 revealed the presence of four clusters (N1–N4) including human plus murine, bovine and porcine clusters with cut-off values of 75% at the nt and 85% at the aa level. Furthermore, the NSP2 genes of porcine RVBs were divided into three genotypes, of which some porcine RVBs belonged into bovine-cluster. PB-70-H5 and PB-70-H3, which belonged to same pig farm, might be identical in NSP2 gene as shown sequence identity of 99.9%, nevertheless both had different VP7 genes each other. Thus, this data demonstrates the occurrence of gene reassortment among porcine RVBs derived from same pig farm. Our findings presented here would provide more valuable information to elucidate evolution of RVBs.

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1. Introduction

Rotaviruses, member of the family *Reoviridae*, are a major causative pathogen of severe gastroenteritis in humans and animals. These viruses have a genome that consists of 11 segments of double-stranded RNA (dsRNA) encoding six structural proteins (VP1–4, VP6–7) and five (sometimes six) nonstructural proteins (NSP1–6). On the basis of their antigenic relationships and genomic characteristics, rotaviruses are classified into seven species (A–G), commonly termed groups (Matthijnssens et al., 2011). Rotaviruses A (RVA) and C (RVC) cause diarrhea mostly in infants and children, while rotavirus B (RVB) has been primarily associated with diarrhea in adults in China, and then widespread in India, Bangladesh and Myanmar (Hung et al., 1984; Chen et al., 1985; Krishnan et al., 1999; Sanekata et al., 2003; Kelkar and Zade, 2004; Aung et al., 2009). Apart from humans, RVBs have been detected in rodents, cows, pigs and sheep (Eiden et al., 1992; Chang et al., 1997; Shen et al., 1999; Tsunemitsu et al., 1999; Barman et al., 2004; Ghosh et al., 2007; Kuga et al., 2009). Bovine RVBs have been detected in sporadic cases and outbreaks of diarrhea in calves and adult cows from India, Japan and USA. On the other hand, porcine RVBs have been identified in gastrointestinal diseases of suckling and weaned pigs, and shown to cause acute, transitory diarrhea in experimentally inoculated gnotobiotic pigs (Theil et al., 1985; Janke et al., 1990). Furthermore, epidemiological surveys of RVB infections with ELISA in cattle and pig herds from Japan and UK demonstrated high antibody prevalence in sera (Brown et al., 1987; Tsunemitsu et al., 2005). RVBs are shed at low level and could not be propagated in cell culture, preventing progress in molecular study of RVBs (Saif, 1990).

NSP2 gene is known to have multiple essential roles in replication cycle, according to the functional data have been obtained in RVAs. NSP2 is a conserved basic protein, one of the earliest and most abundant viral proteins produced during infection. NSP2 of RVA self-assembles to form stable octamers, which have nonspecific affinity for single stranded RNA (Taraporewala et al., 1999; Schuck et al., 2001). NSP2 along with NSP5 forms cytoplasmic inclusion bodies termed viroplasms where viral RNA replication

☆ The GenBank accession numbers for the nucleotide sequences of 19 porcine RVB strains determined in this study are AB673215–AB673233.

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and early particle assembly take place. In addition, NSP2 interacts with other structure protein, VP2, which forms the inner capsid layer of the virion and VP1, which is the viral RNA-dependent RNA polymerase (Kattoura et al., 1994; Fabbretti et al., 1999; Berois et al., 2003; Patton et al., 2006). NSP2 octamers possess helix-destabilizing activity and hydrolysis activities serving as nucleoside triphosphatase (NTPase) and 5′-RNA triphosphatase (RTPase) via histidine triad (HIT)-like motif, suggesting a possible role in the homeostasis of nucleotides within the viral factories involved in unwinding, packaging and replication of the viral RNA (Taraporewala and Patton, 2001; Jayaram et al., 2002; Vasquez-Del Carpio et al., 2006; Kumar et al., 2007). The X-ray structure of NSP2 from human RVC Bristol strain shows extensive conservation of octamer architecture similar to those of RVAs (Taraporewala et al., 2006). Additionally the HIT-like motif, the residues important for catalytic activity are obviously conserved among RVAs, RVBs and RVCs, despite some variation in the overall sequences of those NSP2 proteins (Taraporewala and Patton, 2004; Kumar et al., 2007). Thus, these findings suggest that the NTPase and RTPase in the C-terminal domain are evolutionarily conserved in rotaviruses.

Genetic characterization in RVBs depends largely on sequence comparison with RVAs, which have been well characterized. The RVA NSP2 gene has been highly conserved among strains within and between species (Matthijnssens et al., 2008a). The NSP2 sequences of human RVBs indicated much less diversity than other genes (Yamamoto et al., 2010). The NSP2 sequence identities of bovine RVBs to other host species were the highest among different bovine RVB genes sequenced so far, but bovine RVB NSP2 sequences exhibited high genetic diversity to those of human and murine RVBs (Ghosh et al., 2010). These facts suggest that the NSP2 genes might be more strongly conserved than other genes in RVBs as well as RVAs. However, sequence and phylogenetic analyses for partial NSP2 genes of porcine RVBs exhibited low identities to human, bovine and murine RVB NSP2 genes, and the presence of species-specific cluster, similar to those described for NSP1 genes (Medici et al., 2010). Therefore, the genetic relationship of porcine RVB NSP2 with those of other RVBs remains unclear.

In the present study, we determined the nucleotide sequences of several porcine RVB NSP2 genes in order to obtain genome information might be necessary to not only elucidate mechanisms for the generation of genetic diversity such as gene reassortment and/or crossing of the host-species barrier but also establish their classification. Moreover, comparative analyses between NSP2 genes of RVBs and those of RVAs and RVCs would provide important information to our understanding of diversity and function of the viral protein.

2. Materials and methods

2.1. Origin of the sample

Nineteen fecal samples, which were used in this study, from suckling and weaned pigs that collected at eleven farms around Japan from 2001 to 2009 were summarized in Table 1. Of them, ten strains (PB-S22-3, PB-S26-1, PB-S40-1, PB-68-C17, PB-70-H3, PB-70-H5, PB-71-H5, PB-72-H3, PB-91-H1 and PB-72-I2) and nine strains (PB-S22-3, PB-S40-1, PB-68-C17, PB-70-H3, PB-71-H5, PB-72-H3, PB-91-H1, PB-87-Z2 and PB-93-I5) were identical with those used in previous genetic analyses of porcine VP7 and NSP1 genes, respectively (Kuga et al., 2009; Suzuki et al., 2011). Viral RNA was extracted from 10% fecal suspensions in minimum essential medium (MEM) using TRIsol LS (Invitrogen, CA, USA) according to manufacturer's instructions.

2.2. Cloning and sequencing of the NSP2 gene from three porcine RVB strains, PB-Kyushu, PB-107-G16 and PB-93-I5

The full-length nucleotide sequences of NSP2 genes from three porcine RVB strains, PB-Kyushu, PB-107-G16 and PB-93-I5 were determined using the single primer amplification method as mentioned by Wakuda et al. (2005). Briefly, ligation of the single amino-group linked oligonucleotide primer to the 3′-ends of both strands of the viral dsRNA, column-based purification and concentration of the ligated RNA and RT-PCR reactions were carried out. The PCR products were cloned into the pCR2.1 TOPO vector (Invitrogen, CA, USA) and sequenced using BigDye Terminator v3.1 cycle sequencing kit on an automated ABI Prism 3130 Genetic Analyzer (Applied Biosystems, CA, USA).

2.3. RT-PCR for amplification of NSP2 genes from several porcine RVB strains

In order to determine ORFs of NSP2 genes from other porcine RVB strains, two pairs of oligonucleotide primers, NSP2-1F [5′-TTGGTGTAGTGCGTCGTGAGA-3′; nucleotide (nt) position 14–34 of the PB-107-G16] and NSP2-1R [5′-GTATAGCGGATTTATCACAG-3′: nt position 988–1007 of the PB-107-G16], and NSP2-2F [5′-TCACCCATCCCCTAGGTCA-3′; nt position 36–54 of the PB-93-I5] and NSP2-2R [5′-GGGGTTTATCACAGTCTAC-3′; nt 966–974 of the PB-93-I5] were designed by reference to full genome of those from PB-107-G16 and PB-93-I5 strains, respectively. RT-PCR was conducted using Onestep RT-PCR kit (Qiagen, CA, USA). Both terminal sequences were confirmed using 5′ RACE kit (Invitrogen, CA, USA). The reaction was performed at 50 °C for 30 min and 95 °C for 15 min, followed by 35 cycles of 94 °C for 1 min, 48 °C for 1 min and 72 °C for 2 min, and then a final extension at 72 °C for 10 min. The products were sequenced using BigDye Terminator v3.1 cycle sequencing kit on an automated ABI Prism 3130 Genetic Analyzer (Applied Biosystems, CA, USA).

2.4. Sequence and phylogenetic analyses

Sequence data were aligned by the Clustal W method using MegAlign program of the Lasergene software (DNASTAR, WI, USA). Phylogenetic analysis was performed using MEGA 5 program (Tamura et al., 2011). Genetic distances were calculated by the Poisson correction parameter at amino acid level and the Kimura 2-parameter correction at the nucleotide level (Matthijnssens et al., 2008a). A cut-off percentage for genetic classification is estimated as the percentage separating the intragenotype identities, the nucleotide/amino acid identities between strains belonging to the same genotype and the intergenotype identities, the nucleotide/amino acid identities between strains belonging to different genotypes according to the definition recommended by Matthijnssens et al. (2008a,b). The phylogenetic trees were constructed using neighbor-joining algorithm with bootstrapping with 1000 replicates (Saitou and Nei, 1987).

3. Results

3.1. Sequence analysis of NSP2 genes from three porcine RVB strains, PB-Kyushu, PB-107-G16 and PB-93-I5

The full-length NSP2 genes of PB-Kyushu, PB-107-G16 and PB-93-I5 porcine RVB strains were variable among three strains and other RVBs including human, bovine and murine strains (Table 2). In addition, sequences in 5′ and 3′ terminals of NSP2 genes were distinctively different between PB-Kyushu and/or PB-107-G16 and PB-93-I5 strains. They were suspected to encode one ORF consisting of 300 or 301 amino acids as well as other RVBs. Despite some

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Table 1
The origin of nineteen porcine RVBs in fecal samples from suckling and weaned pigs.

RVB strain	GenBank ID	Prefecture	Farm	Time-point of sampling	Age or stage[a]	Diarrhea[b]
PB-Kyushu	AB673231	Kagoshima	A	April 2001	5	+
PB-S22-3	AB673215	Aomori	B	August 2002	8	+
PBS26-1	AB673216	Aomori	C	September 2002	15	+
PB-S40-1	AB673217	Miyagi	D	May 2003	15	+
PB-S43-11	AB673229	Aomori	E	July 2003	28	–
PB-68-C17	AB673218	Gifu	F	August 2007	Suckling	+
PB-70-H3	AB673219	Chiba	G	August 2007	30	–
PB-70-H5	AB673220	Chiba	G	August 2007	30	+
PB-71-H5	AB673221	Chiba	G	August 2007	30	–
PB-72-H3	AB673222	Ibaraki	H	August 2007	30	–
PB-72-I2	AB673226	Ibaraki	H	August 2007	60	–
PB-87-Z2	AB673227	Ibaraki	H	September 2008	Weaned	–
PB-93-I5	AB673233	Gifu	F	October 2008	Weaned	–
PB-91-H1	AB673223	Chiba	I	October 2008	Weaned	–
PB-91-Z4	AB673230	Chiba	I	October 2008	Weaned	–
PB-104-3	AB673225	Chiba	I	March 2009	3	–
PB-98-3	AB673228	Miyazaki	J	February 2009	5	+
PB-103-1	AB673224	Miyazaki	J	March 2009	9	+
PB-107-G16	AB673232	Tochigi	K	April 2009	6	+

[a] The number is days of age. The sample with uncertain age is shown in the breeding stage: suckling; 0–34 days old, weaned; 30–90 days old.
[b] The presence of diarrhea when the sample is collected is shown.

Table 2
Full-length and deduced amino acids of NSP2 gene from RVB strains published previously including three porcine strains, PB-Kyushu, PB-107-G16 and PB-93-I5.

RVB strain	GenBank ID	Full-length	Amino acids
IDIR (murine)	U03558	1005	301
ADRV (human)	AJ867609	975	301
Bang117 (human)	GU391308	1007	301
Bang373 (human)	AY238393	1007	301
CAL-1 (human)	AY238383	1007	301
IC-008 (human)	GU377220	1007	301
IDH-084 (human)	GU377231	1007	301
MMR-B1 (human)	GU370058	1007	301
WH-1 (human)	AY539861	1007	301
PB-Kyushu (porcine)	AB673231	1029	301
PB-107-G16 (porcine)	AB673232	1029	301
PB-93-I5 (porcine)	AB673233	1004	300
DB176 (bovine)	GQ358725	1001	300
RUBV226 (bovine)	GQ358726	1001	300
RUBV282 (bovine)	GQ358727	1001	300

variation in the overall sequences of NSP2 proteins among three strains, the histidine triad (HIT) motif, residues important for catalytic activity were highly conserved among them (Fig. 1). Sequence identities of NSP2 gene between three porcine RVB strains were 65.2–91.9% in nucleotide (nt), and 69.2–99.1% in deduced amino acids (aa), respectively. Comparison NSP2 genes of porcine RVB strains with those of human, bovine, murine RVB strains indicated low identities of 65.2–75.5% in nt, 67.3–87.0% in aa.

3.2. Sequence identification of NSP2 genes from several porcine RVB strains

There were distinctive differences of sequence in both terminals of NSP2 genes between PB-107-G16 and PB-93-I5 strains, and hence two pairs of oligonucleotide primers were designed on the basis of each sequence. The ORFs of NSP2 genes from sixteen porcine RVB strains were amplified and sequenced by RT-PCR reactions using either primer. Substantial diversity in NSP2 nucleotide sequences was shown among nineteen porcine RVB strains including three porcine strains described above (64.0–99.9%), which were larger than those among human (93.0–99.3%) and bovine RVB strains (95.1–99.9%). Percent identities of NSP2 amino acid sequences among porcine RVBs also exhibited relatively variable (66.7–100.0%) as compared with those among human and bovine strains (96.0–99.7% and 98.0–100.0%). Moreover, comparison of NSP2 nucleotide and amino acids sequences among RVBs including

Fig. 1. Alignment of deduced amino acids sequences in the C-terminal of NSP2 genes from representative RVA, RVB and RVC strains. The histidine triad motif (HϕHϕHϕϕ) indicated by dashed box is highly conserved among those strains. The GenBank accession numbers are as follows: RVB strains, shown in Table 2; RVA strains, Wa (human) [AAA47301]; SA11-P (simian) [AAA47298]; RVC strains, Bristol (human) [AJ132205]; Cowden (porcine) [CAA46742].

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Table 3
NSP2 sequence identities (%) in nucleotide (upper right) and amino acids (lower left) among genotypes grouped by phylogenetic analysis.

Genotype [a]		N1		N2		N3	N4
		8 human RVBs	1 Murine RVB (IDIR)	3 bovine RVBs	4 porcine RVBs	1 Porcine RVB (PB-91-Z4)	17 porcine RVBs
N1	8 human RVBs	93.0-99.3 / 96.0-99.7	78.0-79.1	68.2-69.0	67.0-68.7	67.0-68.0	67.0-74.1
	1 Murine RVB (IDIR)	87.0-88.4	- / -	68.2-68.4	68.2-69.0	67.1	67.9-73.7
N2	3 bovine RVBs	70.0-71.3	74.3	95.1-99.9 / 98.0-100	74.3-75.5	74.5-74.8	64.3-69.5
	4 porcine RVBs	66.3-68.7	69.0-71.7	85.7-88.7	84.9-98.2 / 92.3-98.0	74.6-76.3	64.8-70.7
N3	1 Porcine RVB (PB-91-Z4)	66.3-67.7	69.0	81.3-81.7	82.7-84.3	- / -	64.0-69.1
N4	17 porcine RVBs	69.2-79.7	72.5-82.4	71.3-74.7	66.7-73.0	66.7-71.0	76.7-99.9 / 88.0-100

[a] Each genotype includes in strains as shown in Fig. 2.

human, bovine and murine strains indicated that porcine RVBs had a high diversity distinct from other RVBs (Table 3). All porcine RVB strains retained the conserved HIT motif as human, bovine and murine RVB strains (Fig. 1).

3.3. Phylogenetic analysis of RVB NSP2

The genetic classification of RVB NSP2 was performed on the basis of a value that was estimated from frequency distribution of pairwise sequence identities according to the definition recommended by the Rotavirus Classification Working Group (Matthijnssens et al., 2008a,b). The cut-off values for the division of genotypes were defined as 75% at the nt and as 85% at the aa level, respectively. Phylogenetic dendrogram of NSP2 amino acids sequences on RVB strains showed to be genetically classified into four genotypes (Fig. 2). Although the functions of RVB NSP2 genes are still unknown, we would be provisionally defined the new NSP2 genotypes with the argument of having a concordant nomenclature as follows: human-cluster including murine strain (N1), bovine-cluster including some porcine strains (N2) and remaining porcine-clusters (N3–N4). Surprisingly, porcine RVB strains were widely divided into three clusters close to human- and bovine-clusters. Three porcine RVB strains from Brazilian wild pigs were grouped into N4 genotype in the present study (Medici et al., 2010). In addition, the phylogenetic tree of RVB NSP2 nucleotide sequences showed the same clustering with cut-off value of 75% (data not shown).

PB-70-H5 strain, which belonged to G4 genotype in the analysis of RVB VP7, was classified into N4 in the analysis of RVB NSP2 (Kuga et al., 2009). PB-93-I5 and PB-87-Z2 strains, which grouped into A5 and A6 genotype in the RVB NSP1 genotyping, were classified into N2 genotype in the NSP2 genotyping (Suzuki et al., 2011).

4. Discussion

In contrast with human, bovine and murine RVBs, available genome information except for VP7, NSP1 and partial NSP2 genes remain to be limited particularly in porcine RVBs (Eiden et al., 1992; Kuga et al., 2009; Yamamoto et al., 2010; Ghosh et al., 2010; Matthijnssens et al., 2010; Suzuki et al., 2011). In order to obtain further genome information of unknown target, the single primer amplification method is one of valid method (Lambden et al., 1992; Potgieter et al., 2002; Wakuda et al., 2005; Maan et al., 2007; Potgiter et al., 2009). In the present study, we firstly determined full-length nucleotide sequences of NSP2 genes from three porcine RVB strains using this method, and moreover NSP2 ORFs from sixteen porcine RVB strains with two sets of original primers designed from the terminal sequences of them. The full-length nucleotide sequences of NSP2 genes were variable among three strains and other RVBs, whilst their deduced amino acid sequences were almost identical (Eiden et al., 1992; Shen et al., 1999; Yamamoto et al., 2010; Ghosh et al., 2010). Alignment of NSP2 amino acid sequences among porcine and other RVBs indicated the conserved histidine triad motif, the residues important for catalytic activity in the homeostasis of nucleotides was present in RVBs as well as RVAs and RVCs (Taraporewala and Patton, 2004; Kumar et al., 2007). Moreover, the NSP2 genes showed much less diversity than other genes in RVBs like in RVAs (Matthijnssens et al., 2008a; Yamamoto et al., 2010; Ghosh et al., 2010). Therefore, these facts suggest that the function of NSP2 may be common among these three groups of viruses, despite some variation in the overall sequences of those NSP2 proteins. The structural analysis of RVB NSP2 by X-ray crystallography is awaited to confirm possible functional similarities.

The Rotavirus Classification Working Group offered a suggestion of the sequence identity cutoff value on RVA NSP2 genes in the definition of genotypes as 85% in nt (Matthijnssens et al., 2008a,b). In the present study, the cutoff values based on the distribution of RVB NSP2 were 75% identity in nt and 85% identities in aa, respectively. In addition, we previously reported that the nucleotide sequence identity cutoff value on VP7 (76%) and NSP1 (76%) classification among RVBs from different host species was lower than those among RVAs and RVCs (Tsunemitsu et al., 1992, 1999; Kuga et al., 2009). Thus, these data support that RVB strains from different host may have diverged from one another through a longer period of time than RVAs and RVCs (Eiden et al., 1992; Petric et al., 1991; Tsunemitsu et al., 1999). Interestingly, NSP2 from porcine RVB was classified into three different genotypes, in contrast to NSP2 from other species, which belonged to monophyletic genotypes. Furthermore, some porcine RVB strains were assigned to a bovine

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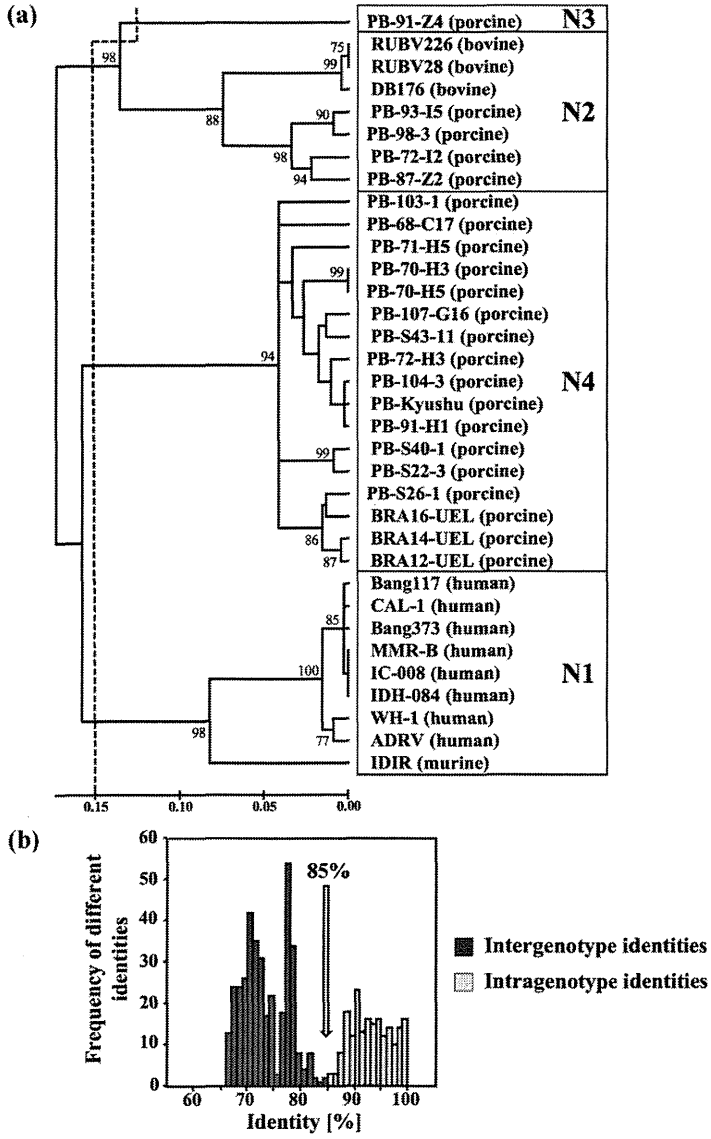

Fig. 2. Phylogenetic tree (a) and distribution of pairwise identity frequencies (b) in RVB NSP2 amino acid sequences. Phylogenetic dendrogram is constructed by the neighbor-joining method with the MEGA 5 program. The percentage bootstrap support (per 1000 replicates) is indicated by the values at each node; bootstrap values of less than 70% are not indicated. The dotted line represents the division of genotypes with cutoff value of 85% at amino acids level. NSP2 genotypes (N1–N4) of RVB strains are indicated on the right.

cluster (tentatively defined as N2) as previously reported in RVB VP7 classification (Kuga et al., 2009). This finding suggests that porcine RVBs originally have genetic diversity, and some porcine and bovine RVBs might be derived from a common ancestor. In addition, three RVB strains from Brazilian wild pigs were grouped into N4 genotype in the present study (Medici et al., 2010). These data also support the notion that common porcine RVB strains were widespread throughout worldwide not just in Japan and UK (Brown et al., 1987; Tsunemitsu et al., 2005).

Ten and nine strains were common as those used in our previous genetic analysis of RVB VP7 and NSP1 genes, respectively (Kuga et al., 2009; Suzuki et al., 2011). Although PB-93-I5 and PB-68-C17 strains originated from the same farm, the NSP2 of these strains were classified as N2 and N4 genotype, respectively. Similarly, PB-72-H3, PB-72-I2 and PB-87-Z2 strains derived from the same farm also carried different NSP2 genes, namely N2 and N4 genotype. These data means that two or more different viral strains in connection with collected time of materials and host growth stage would be generally intermingled within the same farm. On the other hand, PB-70-H5 and PB-70-H3 strains from same pig farm were shown to be closely related (sequence identity as 99.9%) and were classified as N4 genotype. However, PB-70-H5 strain belonged to G4 genotype distinct from PB-70-H3 strain in our previous analysis of RVB VP7 (Kuga et al., 2009). These findings suggest the possibility of gene reassortment between different porcine RVBs within the same farm. Further genetic analysis of the remaining segments would be essential to strengthen this hypothesis.

In conclusion, the present study firstly elucidated the full-length nucleotide sequences on NSP2 genes from three porcine RVB strains, PB-Kyushu, PB-107-G16 and PB-95-I3 and ORFs on NSP2 from sixteen porcine RVB strains. Moreover, RVB NSP2 genes could be classified into four genotypes according to genetic relatedness and the species of origin. In particular, the NSP2 genes of porcine RVBs were divided into three clusters. Furthermore, some porcine RVBs were classified into the cluster to which bovine RVBs belonged. The findings presented here provide new insights into the evolution of viral genes of porcine RVB. Further accumulation of genetic data will be helpful to uncover the full process of evolution of these viruses.

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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.

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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

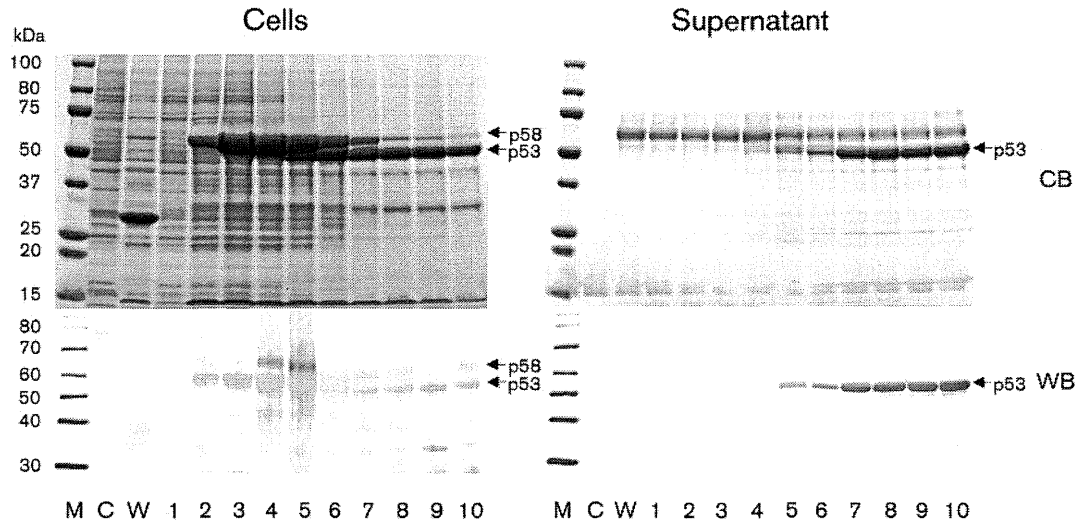

Fig. 1. Time course of the expression of N-terminal 100 aa-truncated rat HEV ORF2. Tn5 cells were infected with recombinant baculovirus Ac[ΔORF2], incubated at 26.5 °C and harvested on days 1–10 p.i. (lanes 1–10). Five microlitres of the culture medium or lysate from 10^5 cells was analysed by SDS-PAGE. 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

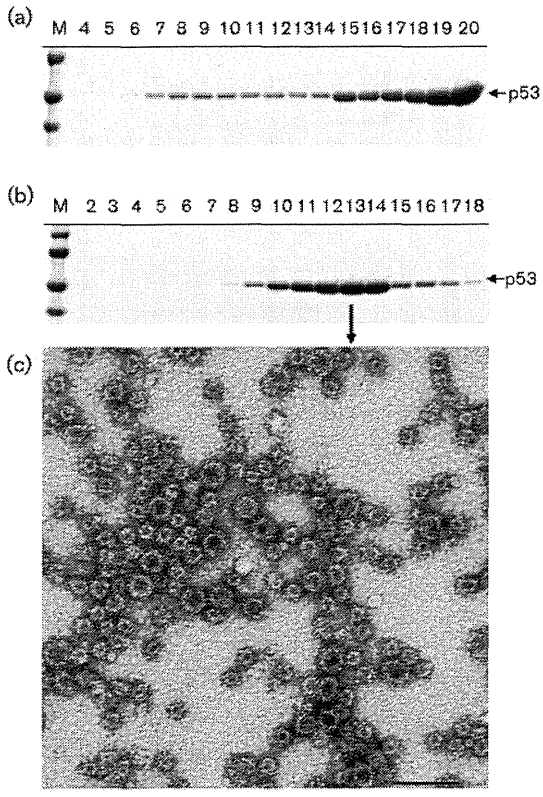

Fig. 2. Purification of rat HEVLPs. (a, b) The supernatant of recombinant baculovirus-infected Tn5 cells was centrifuged for 3 h at 32 000 r.p.m. in a Beckman SW32Ti rotor. The pellet was resuspended in 4.5 ml or 100 µl EX-CELL 405 and purified by CsCl-gradient (a) or sucrose-gradient (b) centrifugation, 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.

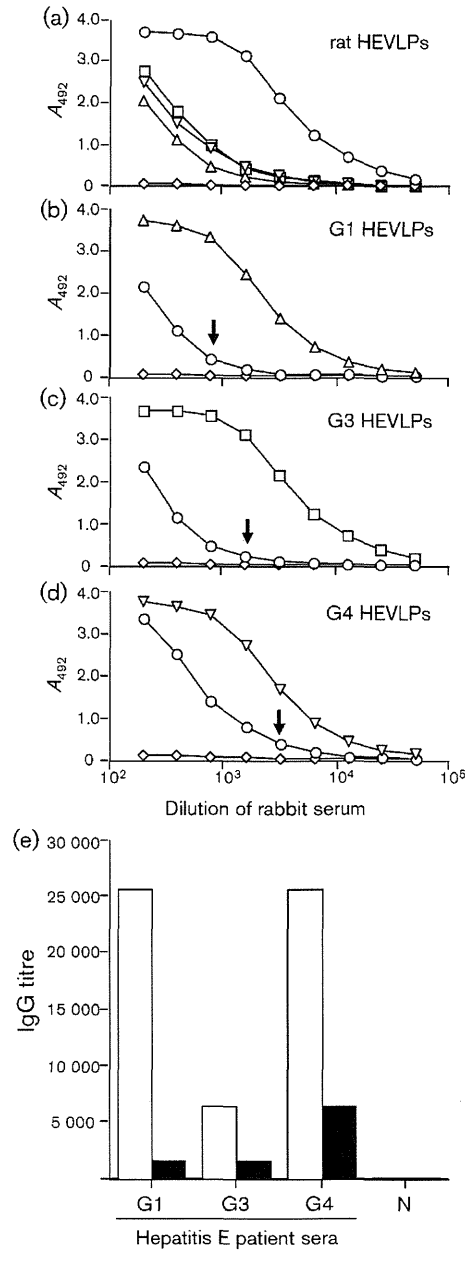

Fig. 3. Antigenic cross-reactivity among rat, G1, G3 and G4 HEVLPs. (a–d) The A_{492} values of hyperimmune sera from rabbits immunized with rat (○; a), G1 (△; b), G3 (□; c) or G4 (▽; d) HEV and of pre-immunized rabbit serum (◇) were determined by antibody ELISA using the four VLP antigens indicated. 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

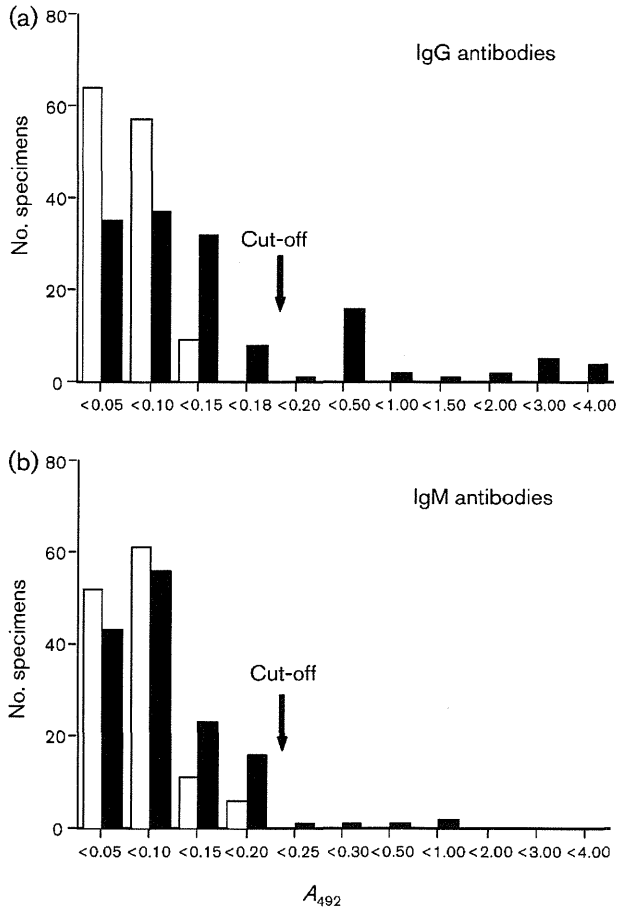

Fig. 4. Detection of anti-rat HEV IgG and IgM antibodies in laboratory and wild rats. Serum samples were collected from laboratory rats in Japan and from wild rats in Vietnam. Anti-HEV IgG and IgM antibodies were detected by antibody ELISA with 1 : 200-diluted sera. Open bars, laboratory rats; filled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

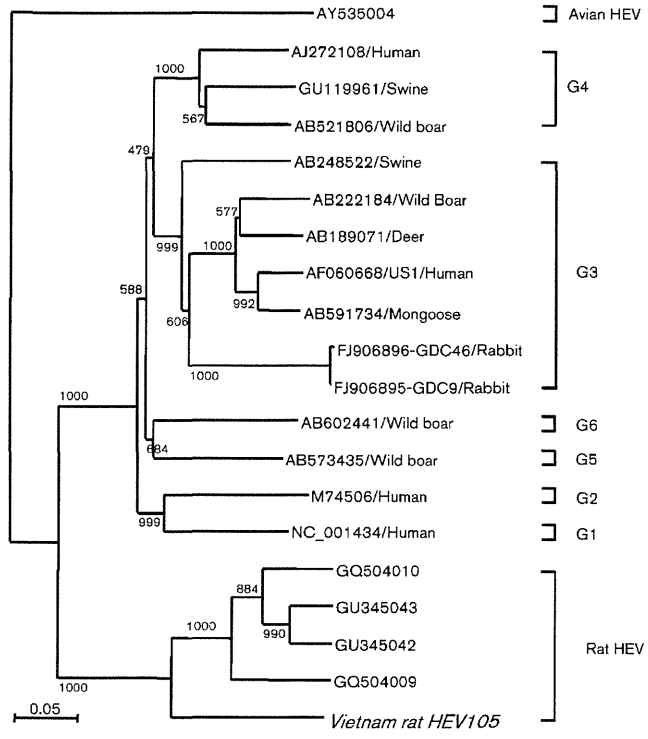

Fig. 5. Phylogenetic analysis of rat HEV based on the partial nucleotide sequence of the Vietnam rat HEV 105 strain (901 nt) using avian HEV as an outgroup. Bootstrap values were determined based on 1000 resamplings of the datasets and are shown at the nodes.

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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Epidemiological and genetic analyses of a diffuse outbreak of hepatitis A in Japan, 2010

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ABSTRACT

Background: Hepatitis A virus (HAV) is still one of the most common causative agents of acute hepatitis in Japan. Although a relatively small number of annual acute hepatitis A cases (approximately 100–150, 0.78–1.17 per million) were recently reported, a larger number of cases (346, 2.71 per million) were reported in 2010.
Objectives: To investigate the causes of the 2010 HAV resurgence in Japan by using molecular epidemiological and genetic analyses.
Study design: HAV specimens were obtained from 61 cases from 22 different prefectures. These viral specimens were genotyped by PCR amplification and sequencing of the VP1/2A region of HAV genome.
Results: Phylogenetic analysis revealed that 61 HAV strains could be divided into three genotypes: IA (44 cases), IB (1 case) and IIIA (16 cases). The IA genotype consisted of two genomic sub-lineages. The sequences of one of the two IA sub-lineages (corresponding to 31 cases) were very similar, 26 of these 31 isolates had 100% identity. The other IA sub-lineage corresponded to strains endemic to Japan. The sequences of Japanese IIIA strains were similar to those of strains that caused a large epidemic in the Republic of Korea from 2007 to 2009.
Conclusions: The resurgence of HAV in 2010 can be attributed to importation of two newly emerged HAV genotypes.

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1. Background

HAV is a member of the genus *Hepatovirus* within the family *Picornaviridae*, and contains a positive-sense, single-stranded RNA genome of approximately 7.5 kb in length. HAV strains isolated from different parts of the world have been classified into six genotypes (I–VI); genotypes I–III are found in humans, and each of these is further divided into subgenotypes A and B. Most of the human HAV strains belong to genotypes I and III.[1–3] Subgenotypes IA and IB are most often found in North and South America, Europe, China and Japan.[1,4,5] Subgenotype IA appears to be the predominant virus of hepatitis A cases worldwide, whereas subgenotype IB has been prevalent in the European and Mediterranean regions.[3,6–8] Subgenotype IIIA was recovered from various countries in Asia, Europe (especially in Roman ethnic population), Madagascar and the USA,[1,5,9–12] and subgenotype IIIB was responsible for some cases of HAV infection in Denmark and Japan.[1,10,11,13]

HAV infection has been a major public health problem in many countries worldwide. The annual incidence of hepatitis A is 1.5 million cases of clinical disease.[14] HAV is transmitted primarily via the fecal–oral route by contaminated food or water,[15–17] but also has been associated with outbreaks in injecting drug users and men who have sex with men (MSM).[18]

The number of acute hepatitis A patients in Japan has been steadily decreasing since the 1990s. Most of the infections that occurred in Japan were sporadic, with the exceptional occurrence of small-scale outbreaks. In 2007–2009, a relatively low number of annual cases (approximately 100–150, 0.78–1.17 per million) of acute hepatitis A were reported. In 2010, however, 346 cases (2.71 per million) were reported. To investigate the epidemiology of this 2010 HAV resurgence, we collaborated with 28 local institutes of health in Japan to obtain stool and plasma specimens from 98 acute hepatitis A patients. The DNA of these viral isolates was PCR-amplified and sequenced, and the sequences were used to perform phylogenetic analyses.

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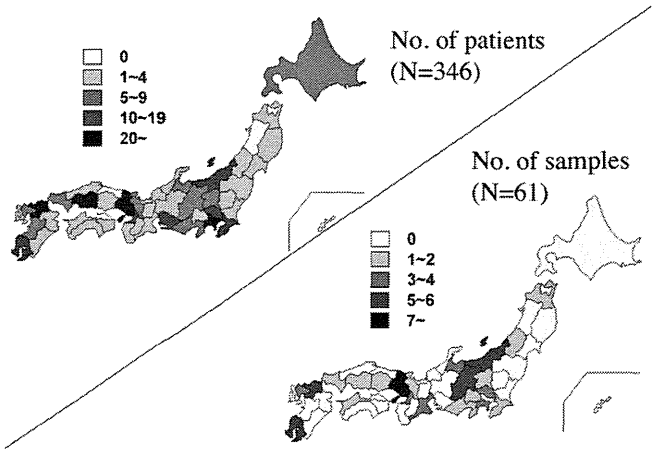

Fig. 1. Geographical distribution of acute hepatitis A patients and collected samples in Japan in 2010.

2. Objectives

The primary objective of this study was to investigate the causes of the 2010 HAV resurgence in Japan by using molecular epidemiological and genetic analyses. This study, performed in collaboration with local institutes of public health, is expected to provide insights useful for setting appropriate public health guidelines for HAV control.

3. Study design

3.1. Data collection

We collected stool and plasma specimens from 98 acute hepatitis A patients in collaboration with 28 local institutes of health in Japan. The collection sites were located at 22 different prefectures (regions in Japan) (Fig. 1).

3.2. RNA extraction, RT-PCR and phylogenetic analysis

A 10% fecal suspension (wt/vol) was prepared with phosphate-buffered saline (PBS; pH 7.2) and centrifuged at 10,000 × *g* for 10 min. Viral RNA was extracted from the fecal suspension or sera by using a QIAamp Viral RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription (RT) was performed with the SuperScript III cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). Seven microliters of the purified RNA was added to a reaction mixture (final volume, 20 μl) containing 50 pmol of random hexamer, 25 mM $MgCl_2$ buffer, 10 mM deoxynucleotide triphosphates, 10 × RT buffer, 0.1 M dithiothreitol, and 200 U SuperScript III RT. The mixture was incubated at 42 °C for 1 h, after which 10 U of RNase H was added at 37 °C for 20 min.

Four degenerate primers (P1 to P4) were used in PCR to amplify the VP1/2A region of the HAV genome.[1] The sequences of these primers were:

HAV-2799 (5′-ATTCAGATTAGACTGCCTTGGTA-3′)
HAV-2907 (5′-GCAAATTACAATCATTCTGATGA-3′)
HAV-3162 (5′-CTTCYTGAGCATACTTKARTCTTTG-3′)
HAV-3273 (5′-CCAAGAAACCTTCATTATTTCATG-3′)

PCR was carried out using the HAV-2799 and HAV-3273 primer pair, followed by nested PCR with the HAV-2907 and HAV-3162 primer pair. PCR was performed with EX-taq (Takara, Shiga, Japan) according to the manufacturer's instructions. Amplification was performed for 40 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min, and a final extension at 72 °C for 15 min. Three microliters of the PCR product was used as the template for a second round of PCR amplification under the same conditions. The PCR product was purified with the QIAquick PCR Purification Kit (Qiagen) and used as a template for direct sequencing.

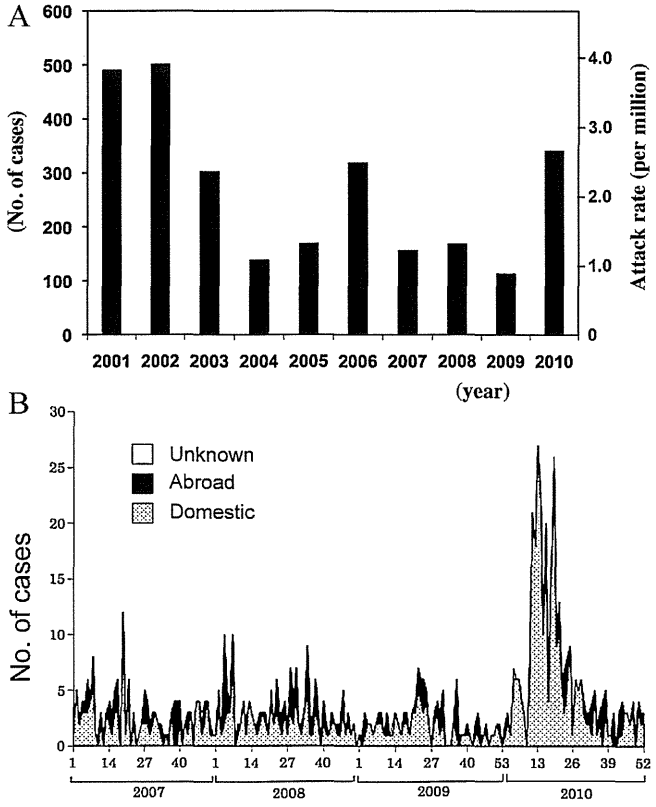

Fig. 2. (A) Reported number of acute hepatitis A patients in Japan from 2001 through 2010. The increase of the number in 2010 was statistically significant compared with the number in 2007 ($t = 5.4 \times 10^{-}$), 2008 ($t = 5.6 \times 10^{-5}$) and 2009 ($t = 1.8 \times 10^{-5}$). (B) Weekly acute hepatitis A cases from week 1 of 2007 to week 52 of 2010.

Phylogenetic trees were constructed with the MEGA software (DNA DATA Bank of Japan) by the neighbor-joining method from a Kimura two-parameter distance matrix, and bootstrap values were determined from 1000 bootstrap re-samplings of the original data.[19–22] All reference sequences used in this study were obtained from GenBank.

4. Results

In 2010, the number of acute hepatitis A cases increased to 346 (2.71 per million) (Fig. 2A) because of a diffuse outbreak that occurred from March through May (Fig. 2B). Most of the patients in this outbreak reflected domestic infection events (Fig. 2B). Clinical descriptions of these patients are summarized in Table 1.

Sera and fecal samples from 98 patients were available for PCR. Of these, 61 yielded a PCR product that could be used for sequencing. Among these 61 isolates, 44 were of genotype IA, one was of genotype IB and 16 were of genotype IIIA by phylogenetic analysis (Fig. 3). The genotype IA isolates could be sorted into two sub-lineages. One sub-lineage (referred to as IA-1 in this paper) grouped with several isolates found in 2006,[23–25] suggesting that the isolates in this lineage were endemic to Japan. In contrast, the sequences of most of the genotype IA isolates belonged to a second sub-lineage (referred to as IA-2 in this paper) with sequences almost identical to one another. Among the IA-2-infected patients, two had developed acute hepatitis shortly after returning from Philippines,

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