Genetic divergence and classification of non-structural protein 1 among porcine rotaviruses of species B

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Porcine rotavirus B (RVB) has frequently been detected in diarrhoea of suckling and weaned pigs. Moreover, epidemiological studies using ELISA have demonstrated high antibody prevalence in sera from sows, indicating that RVB infections are widespread. Because it is difficult to propagate RVBs serially in cell culture, genetic analysis of RNA segments of porcine RVBs other than those encoding VP7 and NSP2 has been scarcely performed. We conducted sequence and phylogenetic analyses focusing on non-structural protein 1 (NSP1), using 15 porcine RVB strains isolated from diarrhoeic faeces collected around Japan. Sequence analysis showed that the porcine NSP1 gene contains two overlapping ORFs. Especially, peptide 2 of NSP1 retains highly conserved cysteine and histidine residues among RVBs. Comparison of NSP1 nucleotide and deduced amino acid sequences from porcine RVB strains demonstrated low identities to those from other RVB strains. Phylogenetic analysis of RVB NSP1 revealed the presence of murine, human, ovine, bovine and porcine clusters. Furthermore, the NSP1 genes of porcine RVBs were divided into three genotypes, suggesting the possibility that porcine species might be an original host of RVB infection. Of nine strains common to those used in our previous study, only one strain was classified into a different genotype from the others in the analysis of VP7, in contrast to the analysis of NSP1, where all belonged to the same cluster. This fact suggests the occurrence of gene reassortment among porcine RVBs. These findings should provide more beneficent information to understand the evolution and functions of RVBs.

INTRODUCTION

Rotaviruses are a major cause of severe gastroenteritis in humans. These viruses are also ubiquitous and responsible for a significant proportion of neonatal diarrhoeal illness in domestic animals, particularly in cattle and pigs. These viruses belong to the family Reoviridae, with their genome containing 11 segments of dsRNA. Six structural proteins (VP1–4 and VP6–7) encapsidate the dsRNA to assemble infectious triple-layered particles. Five (sometimes six) non-structural proteins (NSP1–6) are associated primarily with rotavirus dsRNA replication, transcription, cellular pathology and virus-particle maturation (Pesavento et al., 2006).

On the basis of genetic and antigenic studies, rotaviruses are classified into seven species, commonly termed groups (A–G). In addition, novel rotaviruses (ADRV-N, B219 and J19) distinct from known rotaviruses A (RVA), B (RVB) or C (RVC) were recently identified as the cause of an outbreak of sporadic diarrhoea among adults (Yang et al., 2004; Alam et al., 2007; Jiang et al., 2008). RVB infections in humans have been reported in China, India, Bangladesh and Myanmar (Hung et al., 1984; Chen et al., 1985; Krishnan et al., 1999; Sanekata et al., 2003; Kelkar & Zade, 2004; Aung et al., 2009). These viruses have been also isolated from a variety of animal species, including cattle, pigs, rats and lambs (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 diarrhoea in calves and adult cows from India, Japan and the USA (Chang et al., 1997; Tsunemitsu et al., 1999; Ghosh et al., 2007). On the other hand, porcine RVBs have been identified in gastrointestinal diseases of suckling and weaned pigs, and shown to cause acute, transitory diarrhoea in experimentally inoculated gnotobiotic pigs (Theil et al., 1985; Janke et al., 1990). Furthermore, seroepidemiological surveys of RVB infections using ELISA in cattle and pig farms in Japan and the UK demonstrated high antibody prevalence in sera (Brown et al., 1987; Tsunemitsu et al., 2005). RVBs are shed at a low level,
and serial propagation of RVBs in cell culture is rarely successful, with the exception of one porcine RVB strain (Sanekata et al., 1996). These facts might be an obstacle to studying the molecular biology of RVBs.

Full-genome analysis is useful for understanding the ecology and evolution of pathogens. Recently, the full genome of human RVBs was analysed (Yamamoto et al., 2010). Our knowledge of the genome of porcine RVBs has not reached further than analyses of the VP7 and NSP2 genes. Further information on the remaining segments is required to understand the origin and genetic relatedness of porcine RVBs. Moreover, the accumulation of such genome information might be important for investigation of gene transmission and/or reassortment among RVBs.

NSP1 has several interesting properties that warrant investigation, particularly in RVAs. Immunofluorescent staining showed that NSP1 is distributed throughout the cytoplasm, in contrast to most other viral proteins, which are localized in viroplasms (Hua & Patton, 1994). NSP1 is not required for virus replication because mutant viruses with truncated NSP1 can grow in cell culture (Hundley et al., 1985; Taniguchi et al., 1996). Notably, NSP1 can interact with interferon (IFN) regulatory factor 3 (IRF3), IRF5 and IRF7, host key transcription factors that might be essential for early IFN secretion in response to virus infection, and target them for proteasome degradation (Graff et al., 2002, 2007; Barro & Patton, 2005, 2007). Moreover, NSP1 also inhibits activation of NF-κB by inducing proteasome-dependent degradation (Graff et al., 2009; Arnold & Patton, 2011). These findings suggest that NSP1 plays a role in modulating host immune responses in order to replicate viruses successfully.

Identification of genes in RVBs depends largely on sequence comparison with RVAs and between RVBs. In contrast to the NSP1 genes of RVAs and RVCs, the equivalent gene of RVBs contains two ORFs, except for that of ovine RVB, which has three ORFs (Shen et al. 1999). The deduced amino acid sequences encoded by ORFs 1 and 2 appear not to contain a zinc-finger motif, commonly present in NSP1 of RVAs and RVCs and assumed to be essential for the function of the protein (James et al., 1999). Instead of a zinc-finger motif, a cysteine- and/or histidine-rich region is highly conserved in NSP1 peptides, particularly peptide 2 (Kobayashi et al., 2001; Ghosh et al., 2010). Interestingly, the conserved cysteine and histidine region is also conserved in ADRV-N, but the NSP1 gene has only one ORF (Yang et al., 2004). In RVAs, the NSP1 protein is considered to be highly divergent among strains both within and between species (Hua et al., 1993; Kobayashi et al., 2003). Despite this high divergence, previous studies have shown the presence of clusters according to the species of origin (Dunn et al., 1994; Kojima et al., 1996). Similarly, the NSP1 sequences of bovine RVBs exhibited low similarities to those of human and murine RVBs, but not ovine RVB, and phylogenetic analysis also suggested the presence of species-specific clusters (Ghosh et al., 2010; Yamamoto et al., 2010). On the other hand, we previously found that some porcine and bovine RVBs clustered into the bovine genotype (tentatively defined as G3) by VP7 genotyping (Kuga et al., 2009). Therefore, the genetic relationship of porcine RVBs with other RVBs in NSP1 remains unclear.

In the present study, we determined the nucleotide sequences of several porcine RVB NSP1 genes in order to investigate their evolution. Moreover, sequence comparison of NSP1 of

<table>
<thead>
<tr>
<th>Genotype*</th>
<th>A1</th>
<th>A2</th>
<th>A3</th>
<th>A4</th>
<th>A5</th>
<th>A6</th>
<th>A7</th>
</tr>
</thead>
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<tr>
<td>A1</td>
<td>67.3–68.2</td>
<td>40.6</td>
<td>42.6</td>
<td>41.1</td>
<td>35.6</td>
<td>35.6–40.6</td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>62.5–63.8</td>
<td>97.2–100</td>
<td>36.6–37.6</td>
<td>35.6–37.6</td>
<td>35.8–36.8</td>
<td>38.6–40.6</td>
<td>38.6–45.5</td>
</tr>
<tr>
<td>A3</td>
<td>47.4</td>
<td>45.1–45.7</td>
<td>64.4–65.3</td>
<td>58.9</td>
<td>47.5</td>
<td>47.5–54.7</td>
<td></td>
</tr>
<tr>
<td>A4</td>
<td>44.5–44.8</td>
<td>43.4–44.4</td>
<td>64.0–64.6</td>
<td>99.0–100</td>
<td>56.8</td>
<td>44.6</td>
<td>42.6–49.5</td>
</tr>
<tr>
<td>A5</td>
<td>48.0</td>
<td>44.7–45.6</td>
<td>62.3</td>
<td>59.4</td>
<td>61.1</td>
<td>64.2–67.4</td>
<td></td>
</tr>
<tr>
<td>A6</td>
<td>48.0</td>
<td>43.8–44.7</td>
<td>57.7</td>
<td>56.2–56.6</td>
<td>73.8</td>
<td>62.4–68.4</td>
<td></td>
</tr>
<tr>
<td>A7</td>
<td>44.8–47.0</td>
<td>42.5–45.6</td>
<td>52.6–56.6</td>
<td>55.6–56.9</td>
<td>69.4–72.8</td>
<td>72.8–76.2</td>
<td>74.3–99.0</td>
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*Each genotype includes strains as shown in Fig. 1.
RVBs with those of RVAs, RVCs and novel rotaviruses including ADRV-N would provide useful information for our understanding of diversity and function.

**RESULTS**

**Sequence analysis of NSP1 genes from two porcine RVB strains, PB-93-I5 and PB-107-G16**

The full-length NSP1 genes of porcine RVB strains PB-93-I5 and PB-107-G16 were 1279 and 1278 nt longer, respectively, than those of other RVB strains. The 5’ and 3’ ends of both porcine RVB strains started with GG and ended with AAAAAACC, which are sequences common to other RVB strains (Kobayashi et al., 2003). They also contained two overlapping ORFs encoding peptides 1 and 2, the same as other RVBs except for an ovine RVB strain (KB63), in contrast to those of RVAs and RVCs, which have one ORF. The lengths of peptide 1 were variable between the two strains and other RVBs, including human, bovine, ovine and murine strains (Supplementary Fig. S1, available in JGV Online). In contrast, peptide 2 was almost the same length for all RVB strains, excluding KB63, and contained notably conserved cysteine and histidine residues. Sequence identities of the NSP1 gene between the two porcine RVB strains were 68.8 % in nucleotide sequence, and 66.3 and 72.2 % in deduced amino acid sequences of peptides 1 and 2, respectively. Comparison of NSP1 genes of porcine RVB strains with those of human, bovine, murine and ovine RVB strains indicated low sequence similarities of 53.8–64.4 % in nucleotide, 35.8–58.9 % in peptide 1 and 44.7–62.3 % in peptide 2 sequences (Table 1).

**Sequence identification of NSP1 genes from several porcine RVB strains**

The ORFs of NSP1 genes from 13 porcine RVB strains were amplified and sequenced by RT-PCR using the oligonucleotide primers described elsewhere (see Methods). The PCR products were 1190 nt in length, and contained two overlapping ORFs. Substantial diversity in NSP1 nucleotide sequences (67.4–98.9 % identity) was shown among the 13 porcine RVB strains, which were more divergent than human (91.1–99.6 %) and bovine (98.4–100.0 %) RVB strains. Moreover, comparison of the NSP1 nucleotide sequence among RVBs, including human, bovine, ovine and murine strains, indicated low sequence similarities of 53.8–64.4 % in nucleotide, 35.8–58.9 % in peptide 1 and 44.7–62.3 % in peptide 2 sequences (Table 1).
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; Jiangu 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., 2007).
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 IGV Online).

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) according to the manufacturer’s instructions.

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’-CTGGGTCAAGTTAGATCTGG-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 reaction 1233–1254 of PB-107-G16) were designed to determine ORFs of porcine RVB strains. More, 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.

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