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VP6 genetic diversity, reassortment, intragenic recombination and classification of rotavirus B in American and Japanese pigs

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ABSTRACT

Rotavirus B (RVB) has been identified as a causative agent of diarrhea in rats, humans, cattle, lambs, and swine. Recently, 20 RVB VP7 genotypes were determined based on an 80% nucleotide percent cut-off value. In this study, we sequenced the RVB VP6 gene segment from 80 RVB positive swine samples from the United States and Japan. Phylogenetic analyses, using the 30 available RVB VP6 sequences from GenBank and our 80 novel RVB VP6 sequences, revealed a large genetic diversity of RVB strains, mainly in pigs. For classification purposes, pairwise identity frequency analyses suggested an 81% nucleotide percent cut-off value, resulting in 13 RVB VP6 (I) genotypes. In addition, an intragenic recombinant RVB VP6 segment was identified from Japan. Furthermore, the data indicates frequent reassortment events occurred between the porcine RVB VP7 and VP6 gene segments.

1. Introduction

Rotaviruses (RVs) are a significant cause of diarrhea in animals. RVs belong to the family *Reoviridae* and contain an eleven segmented, double strand RNA genome. RVs are classified into eight groups or species (A–H) based on antigenic properties and sequencing of the viral protein 6 (VP6) (Bohl et al., 1982; Estes and Kapikian, 2007; Trojnar et al., 2009; Matthijnssens et al., 2012). The RV triple-layered icosahedral capsid is composed of an outer layer (VP7 and VP4), an intermediate layer (VP6), and the inner core (VP1, VP2 and VP3) (Estes and Kapikian, 2007). Due to the increased use of sequencing, sequence based classification systems have complemented serotyping and are now widely used (Matthijnssens et al., 2008a; Marthaler et al., 2012, 2013). In 2008, a RVA nucleotide sequenced based classification system was developed for all eleven gene segments, which is maintained by the Rotavirus Classification Working Group (RCWG) (Matthijnssens et al., 2008a, 2011). The 11 RVA genome segments VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 are referred to as the Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx genotypes, respectively (Matthijnssens et al., 2008b). Following the identification of multiple genetically divergent swine RVB

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VP7 sequences in 2012 in the United States, a RVB VP7 genotyping classification system was developed based on an 80% nucleotide cut-off value, yielding 20 RVB G genotypes (Marthaler et al., 2012).

RVB strains were initially associated with human adult diarrhea in southeast Asia, and RVB infections have also been documented sporadically in rats, cattle, and lambs (Hung et al., 1984; Dai et al., 1987; Fang et al., 1989; Eiden et al., 1992; Chang et al., 1997; Shen et al., 1999; Kelkar and Zade, 2004; Rahman et al., 2007; Ghosh et al., 2007; Chitambar et al., 2011). Furthermore, RVBs have been identified as a significant cause of porcine diarrhea (Bridger and Brown, 1985; Kuga et al., 2009; Marthaler et al., 2012). The difficulty in adapting RVB to cell culture has hindered serological analyses, and RVB strains have only been characterized at the genetic level (Bridger, 1994; Ghosh et al., 2007; Kuga et al., 2009; Suzuki et al., 2011, 2012a, 2012b).

Because the swine RVB VP6 genetic diversity has been studied to a limited extent, we sequenced the RVB VP6 gene segment from 80 American and Japanese pigs to understand the genetic diversity and reassortment dynamics within and between host species, with future plans to develop a RVB VP6 enzyme-linked immunosorbent assay (ELISA) to assess immunity against RVB in the swine population.

2. Material and methods

2.1. Generation of RVB VP6 sequences

A VP6 specific RT-PCR was performed on the RNA extracts from 64 RVB USA strains, from which the VP7 gene segment was analyzed previously, using the Qiagen OneStep RT-PCR Kit (Qiagen/Westburg) with previously described thermal cycling conditions and published primers GB6-1 and GB6-3 (Ahmed et al., 2004; Marthaler et al., 2012). RT-PCR products were run in a 2% ethidium bromide gel, bands of ~1200 nucleotides were visualized under UV light, and the RT-PCR products were purified using the Qiagen QIAquick PCR kit (Qiagen/Westburg). The published primers GB6-1 and GB6-3 were submitted to the University of Minnesota Genomics Center (UMGC) for Sanger sequencing on a ABI 3730xl DNA Analyzer (Perkin-Elmer) using the ABI 3.1 BigDye Terminator v3.1 kit (Perkin-Elmer) (Ahmed et al., 2004). Additional primers were designed to obtain a minimal coverage of 2X across the VP6 gene segment. The chromatograms were analyzed using Lasergene Seqman 10.0 software (DNASTAR, Madison, WI). The 16 Japanese RVB strains were identified, extracted, and sequenced using a single primer amplification method as described previously (Wakuda et al., 2005; Suzuki et al., 2012a, 2012b).

2.2. Sequence alignment and phylogenetic tree creation

The novel porcine ($n = 80$) and the available GenBank ($n = 30$) RVB VP6 sequences were aligned using Clustal W in Geneious Pro (Thompson et al., 1994; Drummond et al., 2011) (Supplemental Data 1). The JModelTest program determined the general time-reverse substitution model (GTR) with a proportion of invariable sites (+I) and rate variation among sites (+G) as appropriate nucleotide substitution model using Maximum Likelihood (PHYML) (Guindon and Gascuel, 2003; Guindon et al., 2010; Darriba et al., 2012). The Prottest program determined the LG (+G) substitution model as the appropriate amino acid substitution model (Drummond and Strimmer, 2001; Guindon and Gascuel, 2003; Abascal et al., 2005). The porcine RVB VP7 (G) sequences available from GenBank were used to construct phylogenetic trees (Supplemental Data 1). All phylogenetic trees were built in Geneious Pro.

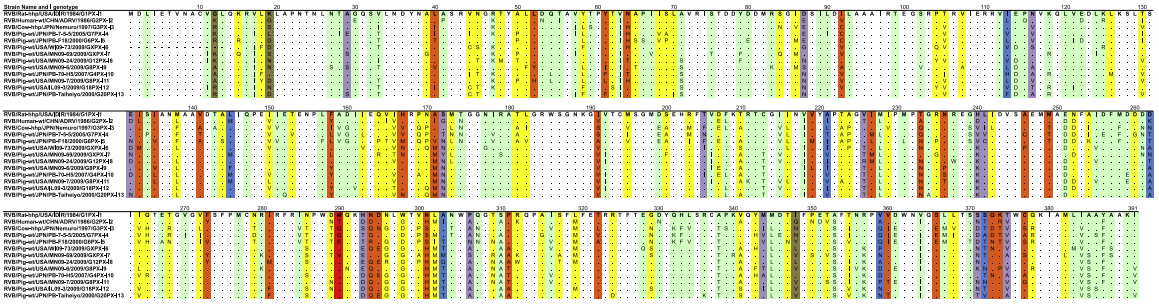
2.3. Pairwise identity frequency graphs

Nucleotide and amino acid pairwise identity frequency graphs were constructed with the identity on the *x*-axis and the frequency on the *y*-axis to determine the appropriate nucleotide and amino acid cut-off values (Ball, 2005).

2.4. Intragenic recombination

In addition, the porcine RVB VP6 sequence alignment was tested with the Recombination Detection Program (RDP) under default setting to search for intragenic recombinant RVB VP6 sequences (Martin et al., 2010). To confirm the RDP results, the sequences were split at the

Table 1
Amino acid alignment of 13 representative RVB I genotypes. Dots represent the same amino acid as the reference strain, IDIR. Colors represent number of amino acid per position from the 110 RVB VP6 aligment: white = 1 ($n = 150$), green = 2 ($n = 110$), yellow = 3 ($n = 76$), orange = 4 ($n = 30$), purple = 5 ($n = 14$), blue = 6 ($n = 7$), brown = 7 ($n = 3$), and red = 8 ($n = 1$).



suggested intragenic recombinant position, and separate nucleotide phylogenetic tree were constructed.

3. Results

3.1. Sequence alignment

The genome segment 6 (encoding VP6) of 80 novel porcine RVB VP6 strains were sequenced. The gene 6 segments were between 1271 and 1273 nucleotides in length, excluding the primer binding sites. The 5′ UTR contained no INDELs (INnsertions/DELetions), while the 3′ untranslated region (UTR) contained multiple INDELs (Supplemental Data 2). The single rat, 4 bovine, 25 human, and 80 porcine strains RVB VP6 open reading frames (ORF) were aligned ($n = 110$), yielding 23 extremely diverse nucleotide positions (containing A, C, G, and T) and 434 (36.9%) conserved positions within the RVB VP6 ORF. Porcine samples CO09-12, IL09-16, NE09-26, MO09-36, KS09-44, NC09-61, MN09-65, MO09-66, and IA09-67,

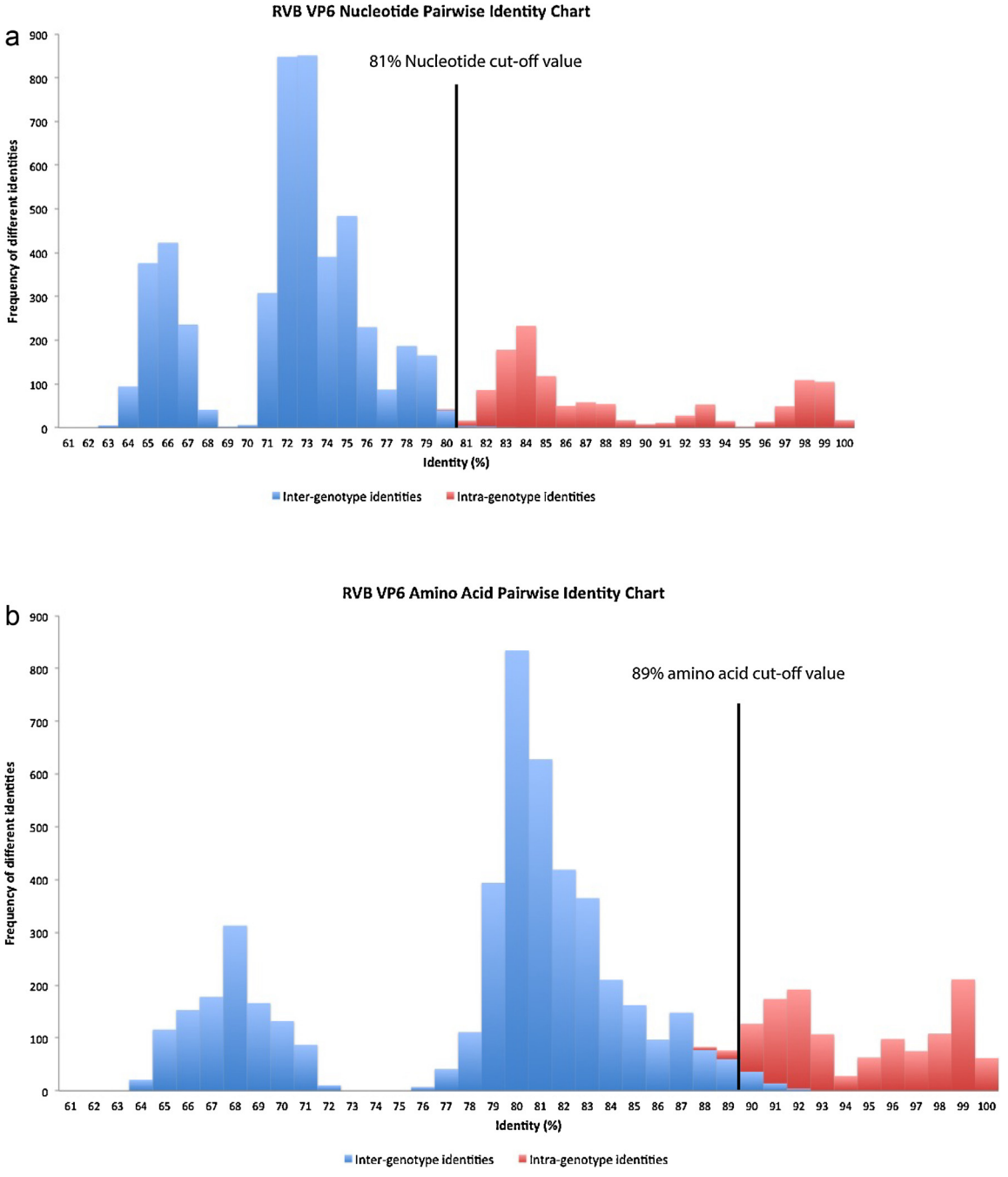


Fig. 1. The RVB nucleotide (a) and amino acid (b) pairwise identity histograms illustrating the proposed 81% and 89% cut-off values, respectively.

isolated in the USA from 2009, contained more than one RVB strain as indicated by degenerate bases within the nucleotide sequence.

The RVB VP6 protein (391 amino acids) contained 150 conserved amino acids positions and 24 hypervariable positions containing five or more amino acids variants (Table 1). Fourteen hypervariable amino acid positions (29, 87, 116, 130, 172, 205, 226, 240, 241, 293, 306, 344, 371, and 375) contained five different amino acids, while seven hypervariable positions contained six different amino acids (113, 145, 221, 262, 303, 359, and 372). Hypervariable positions 12, 19, and 348 contained seven different amino acids while position 290 was the most hypervariable site with eight different amino acids ($M = 69$, $N = 12$, $S = 11$, $E = 6$, $Q = 6$, $V = 3$, $T = 2$, and $I = 1$).

3.2. Phylogenetic analysis

Nucleotide and amino acid pairwise identity frequency charts of the 110 RVB sequences were constructed (Fig. 1). Based on the pairwise identity charts and the phylogenetic trees, an 81% nucleotide percent identity cut-off value and an 89% amino acid cut-off value were appropriate and yielded 13 RVB VP6 (I) genotypes (Fig. 2a). A single I genotype each was detected for the rat (I1), human (I2), and bovine (I3) RVB strains, while the 10 additional I genotypes were detected among porcine RVB strains. The large RVB I13 genotype contained half ($n = 40$) of the porcine strains. With nucleotide and amino acid percent values slightly below the proposed cut-off value, the RVB I13 genotypes had the highest nucleotide and amino acid intra-genotype diversity of 80.7% and 88.5%, respectively (Table 2). In addition, the RVB genotypes I10 and I13 had the highest nucleotide and amino acid inter-genotype similarities (83.4% and 92.3%, respectively), which is slightly higher than the proposed nucleotide and amino acid percent cut-off value.

3.3. Intragenic recombination

The porcine RVB VP6 sequence alignment was tested with the RDP. At approximately nucleotide position 555, the Japanese strain PB-85-I3 from 2008 was indicated as an intragenic recombinant of Japanese RVB strains closely related to PB-70-H5 and PB-Taiheiyo strains isolated in 2007 and 2000, respectively. The PB-85-I3 sequence clustered in different branches when different regions of the ORF (nucleotides 1–554 and 555–1176) were used, indicating recombination (Fig. 3).

3.4. Reassortment events among VP7 and VP6 of RVB strains

To investigate the frequency of reassortment events between VP7 and VP6 gene segments of porcine RVB strains, we constructed a phylogenetic tree for the VP6 gene segment and color-coded the strains according to their VP7 G genotype (if known) (Fig. 2a). In addition, a RVB VP7 phylogenetic tree was color-coded according to the VP6 I genotype (Fig. 2b) to investigate a potential linkage between these two gene segments.

The RVB I genotypes from the rat (I1) and human (I2) strains were associated with a single G genotype, G1 and G2, respectively. The bovine RVB I3 genotype contained the G genotypes G3 and G5. The porcine RVB I6 genotype, which contained a single sequence, has an unknown RVB G genotype. Representing a limited number of strains, the porcine RVB I4, I7, I8, I9, and I10 genotypes were only found in combination with a solitary G genotype (G7, G8,

Table 2
RVB VP6 nucleotide (top) and amino acid (bottom) percent identities.

	I1	I2	I3	I4	I5	I6	I7	I8	I9	I10	I11	I12	I13
I1	NA NA	71.9-73.6	67-68.4	65.3-66.4	64.7-66.4	72.4	71-71.3	73.9-74	72.1	72.1-73.2	70.5-72.8	70.6-73.6	69.9-73.7
I2	83.4-84.4	92.4-100 96.7-100	65.6-66.8	66.5-67.9	63.9-66.2	72.2-73.2	71.5-72.2	72.3-73.7	71.3-72.4	71-72.9	71.1-74.2	71.2-73.8	71.3-74.7
I3	72.1-72.6	69.6-71.6	81.5-100 96.7-100	74.3-75.9	73.6-75.3	68.3-68.9	66.3-67.3	67.2-68.2	65.5-66.2	66.7-68.5	65.4-68	66-68.4	64.8-68.3
I4	69.3-69.6	68.5-70.6	83.9-85.4	83.2 95.9	72.5-74.3	68.6-69.1	66.6-66.8	65.7-66.9	64.1-64.6	63.8-65.7	63.9-66.5	66.2-68.3	63.4-67.1
I5	67.1-68.5	67.1-69.3	81.3-82.4	78.2-81.3	84.1-97.2 96.9-99.2	67.1-68.7	65.7-66.8	65.7-66.8	64.9-65.5	64.7-67	65.4-67.8	64.5-66.4	64.2-68.5
I6	79.6	80.2-81.7	69.6-70.9	69.6-69.8	66.9-67.5	NA NA	73.8-74.5	75.5	72	73.9-74	74.9-76.4	76-76.6	73.4-76.2
I7	78-78.3	78.3-79.8	66.8-67.5	65.2-66.8	64-65	77.5-77.8	97.6-99.1 98.2-98.7	73.1-73.7	72.1-72.5	71.6-73.1	71-73.3	73.2-75.6	72-74.9
I8	81.1	80.6-81.8	68.8-69.1	68.8-69.8	66.5-67.1	84.4	81.9-82.4	99.9-100 100	75.7	76-77.1	74.6-76.4	77-78	73.7-76.7
I9	79.8	78.5-79.8	66.2-67	66.8-67.5	66.5-67.3	79.4	76.7-77.2	82.1	NA NA	75.5-77.2	75-76.5	74.6-76.3	73.6-76.1
I10	79.3-79.5	79.5-81.6	68.5-71.1	67-68.8	65-68.5	82.3-83.9	76.7-77.7	84.1-84.9	82.6-85.2	82.7-87.8 91.6-95.9	76.6-79.8	75.2-79.8	74.2-83.4
I11	77.7-79.8	77.8-81.3	67.9-70.8	68.1-70.8	66.8-69.9	81.5-83.9	76.2-79.3	81.9-85.4	82.4-85.7	83.1-88	83.6-100 90.9-100	73.5-76.6	73.4-77.5
I12	80.6-82.4	81.1-83.6	70.2-72.9	70.3-71.9	67.5-68.9	85.2-86.4	79.5-81.1	85.4-87.2	81.9-83.4	84.7-89	82.6-86.2	83.3-100 95.2-100	76.7-80.8
I13	78.8-81.6	78.3-82.9	66.5-71.4	65.2-69.1	64.5-68.5	81.7-85.6	77.2-80.8	81.5-85.9	82.4-86.7	83.4-92.3	79.6-85.7	84.7-92.1	80.7-100 88.5-100

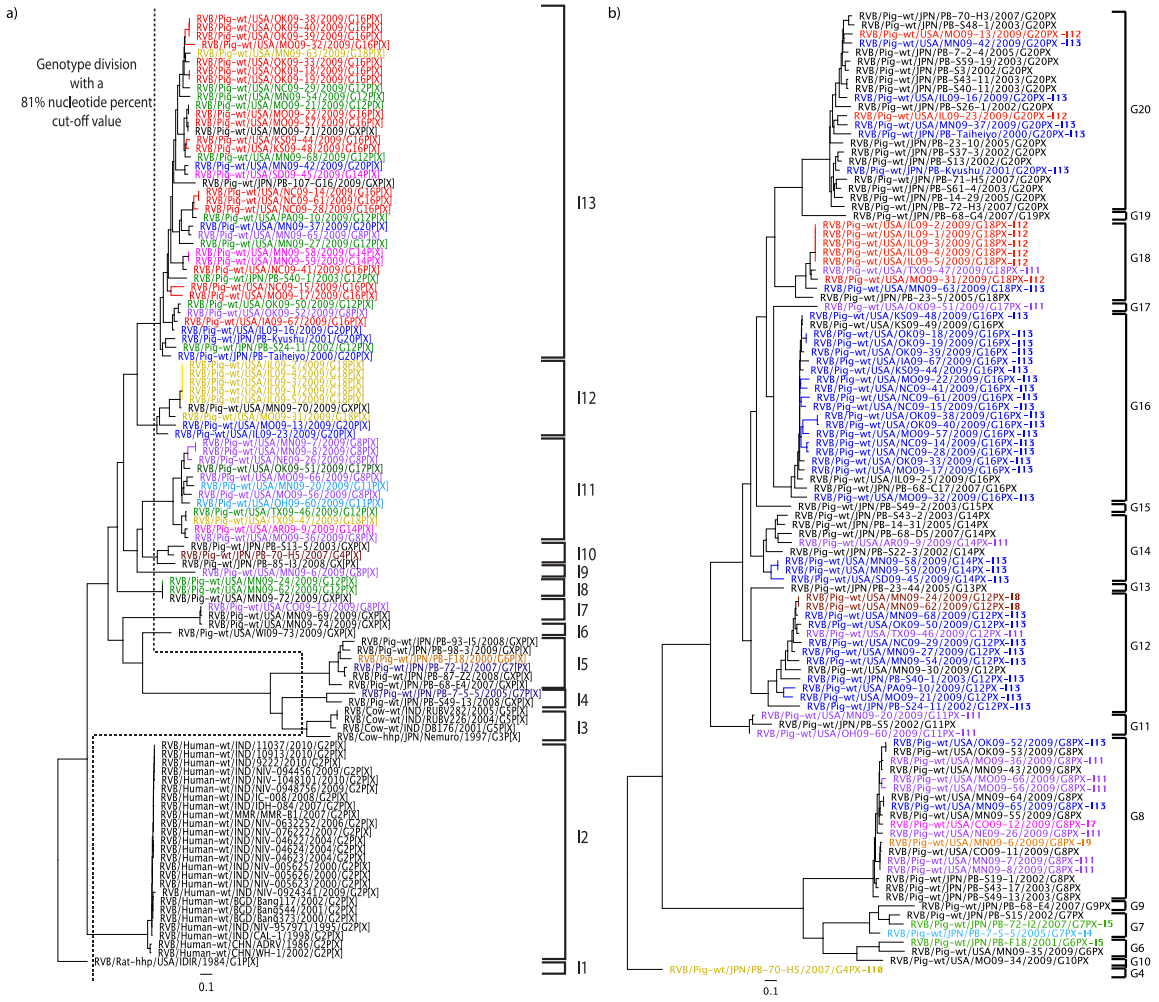


Fig. 2. RVB VP6 phylogenetic tree with the proposed 81% nucleotide cut-off value, yielding 13 I-genotypes (A). The porcine strains with an available VP7 sequence have been colored according their VP7 genotype. The RVB G genotypes were unavailable for 13 porcine RVB strains due to the lack of original RV infected fecal or intestinal material for VP7 sequencing. The RVB VP7 phylogenetic tree was colored according to the available VP6 genotypes (B).

G12, G8, and G4, respectively). The porcine RVB I5 genotype encompassed two G genotypes (G6 and G7). In addition, porcine RVB I12 encompassed two genotypes (G18 and G20). The porcine RVB I11 genotype was naturally associated with 6 RVB G genotypes (G8, G11, G12, G14, G17, and G18). Moreover, the widely circulating RVB I13 genotype ($n = 40$) was also associated with 6 RVB G genotypes (G8, G12, G14, G16, G18 and G20).

Inversely, the RVB G4, G6, G11, G16 and G17 genotypes were associated with a solitary RVB I genotype (I10, I5, I11, I13, and I11, respectively). The RVB G7, G14, and G20 genotypes were associated with two I genotypes (I4 and I5, I11 and I13, and I12 and I13, respectively). The RVB G12 and G18 genotypes encompassed 3 I genotypes (I8, I11, and I13; and I11, I12, and I13, respectively). The RVB G8 genotype was found in combination with the greatest number of I genotypes (I7, I9, I11, and I13) while the RVB I genotypes were unavailable for the G9, G10, G13, G15, and G19 genotypes.

4. Discussion

To our knowledge, this is the first report of porcine RVB VP6 sequences, and the first RVB VP6 sequences to be released from the United States. The porcine RVB VP6 sequences contained several INDELs in the 3′ UTR. Unfortunately, human and bovine RVB VP6 3′ UTR sequences were not available for comparison. While the particular importance of the VP6 3′ UTR had not been determined for the RVB, some interesting difference may exists between host species. Future RVB sequencing efforts should include the ORF, 5′ and 3′ UTR.

We identified nine RVB VP6 strains that contained ambiguous bases, indicating multiple variants within these samples. Additionally, we indicated that the RVB VP6 gene segment of Japanese strain PB-85-I3 was an intragenic recombinant between closely related Japanese strains PB-70-H5 and PB-Taiheiyo. Both the PB-85-I3 and PB-70-H5 were from the same farm in the Chiba

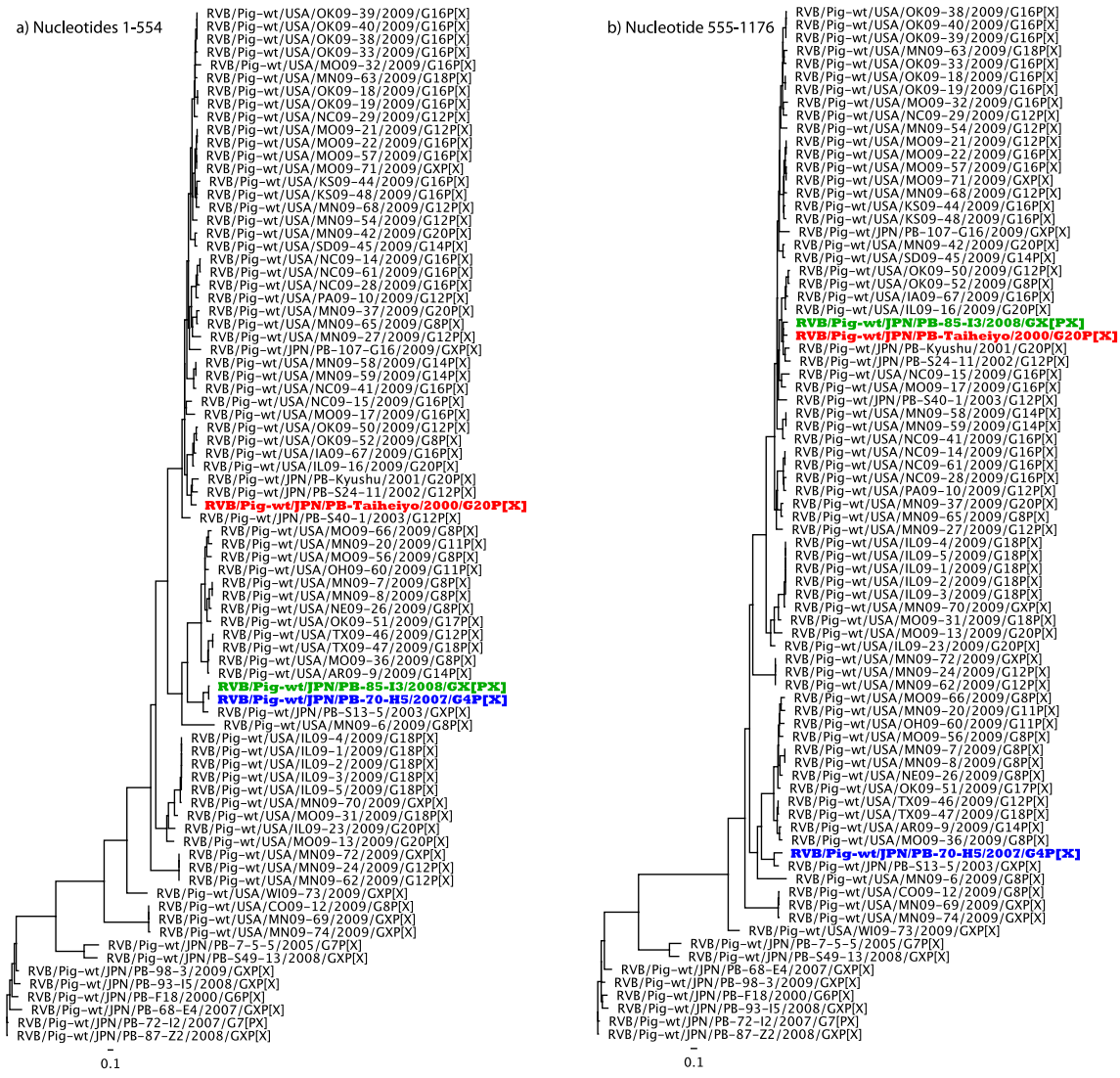


Fig. 3. Nucleotide phylogenetic trees illustrating the recombinant RVB strain PB-85-I3 (green) and the parental RVB strains PB-70-H5 (blue) and PB-Taiheiyo strain (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Prefecture, but the PB-Taiheiyo strain was from the Fukushima Prefecture. While a single RVC and several RVA intragenic recombinant strains have been identified, this is the first identification of a RVB intragenic recombinant strain (Parra et al., 2004; Martella et al., 2007; Phan et al., 2007; Martinez-Laso et al., 2009). Since RVA, RVB, and RVC intragenic recombinant strains have been identified, future RV studies should utilize intragenic recombinant software before starting in-depth phylogenetic analysis because these strains can generate inaccurate phylogenetic trees and hence incorrect conclusions.

The RVA and RVB G genotypes share the same nucleotide (80%) and amino acid (89%) cut-off value. However, an 85% nucleotide cut-off value for the RVA VP6 gene segment has identified 16 I genotypes from 11 host species, while an 81% nucleotide cut-off value for the RVB VP6 gene segment identified 13 I genotypes in only 4 host species (Matthijnssens et al., 2011). Thus far, only three porcine RVA VP6 genotypes (I1, I2, I5) have been recognized while ten porcine RVB VP6 genotypes (I4–I13) have been identified (Shi et al., 2012; Okitsu et al., 2013). Moreover, the RVB I genotypes from the RVB G9, G10, G13, G15 and G19 genotypes have not determined, and more RVB I genotypes may exist within the porcine population. Overall, these observations suggest more genetic diversity within porcine RVB VP6 gene segment than in the porcine RVA VP6 gene segment. Furthermore, while the G3 and G5 bovine RVB strains from Japan and India, respectively, share the same I genotype (I3); an American bovine VP6 sequence has yet to be determined, potentially resulting in additional RVB I genotypes.

At first glance, the RVB I genotype diversity appears to be region and host specific. However, the RVB I13 genotype contains both Japanese and American strains, suggesting

the spread or prevalence of certain RVB genotypes worldwide. Also, our analyses also revealed a surprisingly high frequency of reassortment events involving the porcine RVB VP7 and VP6 gene segments, which further confirms that RVB co-infections must occur frequently. On the other hand, the RVB G4 and G16 genotypes were only associated with genotypes I10 and I13, respectively. While this may be a sampling artifact within the G4 genotype (only one strain has been identified), the RVB G16 genotype contained 18 strains, all belonging to the I10 genotype, suggesting that certain genotype combinations (i.e. G16/I10) avoid reassortment compared to other genotype combinations. Further complete genome analyses of porcine RVB strains will enhance our understanding on the reassortment dynamics of RVB strains.

In conclusion, the sequencing of 80 American and Japanese porcine RVB VP6 sequences increases our understanding of the genetic diversity and phylogenetic relationships of RVB strains. Based on nucleotide pairwise identity chart and the phylogenetic tree, we propose an 81% nucleotide percent cut-off, generating 13 RVB I genotypes. Moreover, rat, human, and bovine RVB strains consist of a single I genotype each, suggesting that the RVB I genotype may be species specific (although it may need to be confirmed by testing additional RVB strains from these species). In contrast, we identified 10 porcine RVB I genotypes, implying a large genetic diversity among RVB strains circulating in the swine population in the United States, Japan, and most likely in other pig populations worldwide.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2014.05.015.

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