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# VP6 gene diversity in Brazilian strains of porcine group C rotavirus

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**ABSTRACT.** Group C rotavirus (RV-C) has been found in Brazilian pig herds; however, wild-type strains have not yet been characterized. We made a molecular analysis of a region of gene 5 in Brazilian RV-C strains. Stool samples from 11 piglets (diarrheic and with normal consistency) positive for the RV-C VP6 gene in an RT-PCR assay were sequenced. A 270-bp amplicon of nine sequences was analyzed. All sequences showed high identity to the Cowden strain of the porcine RV-C prototype and 81.3 to 94.3% to each other (230 nucleotide fragment). Three Brazilian strains were classified in the Cowden group, while the other six showed higher heterogeneity (84.3 to 87.3%) with the prototype strain. Four clusters were formed in the dendrogram, including one human, one bovine, and two porcine clusters; one of these was formed by the six Brazilian strains described in this study. The Brazilian RV-C strains described here did not show any association with the year of collection, the presence of diarrhea, the age of the pig, or the geographical region of herd origin. This strongly suggests that these heterogeneous strains are widely spread in Brazilian pig herds. We conclude that there is genetic polymorphism in the VP6 gene of porcine RV-C strains in Brazil.

**Key words:** Piglets; Diarrhea; Porcine group C rotavirus; VP6 gene; Phylogenetic analysis

 

## INTRODUCTION

Rotavirus infections are the major cause of viral diarrhea in young animals and children all over the world (Kapikian et al., 2001). The viruses belong to the family Reoviridae, and the rotavirus genome consists of 11 segments of double-stranded RNA. Rotaviruses may be classified into seven groups (A to G) on the basis of distinct antigenicity, as determined by VP6 (middle layer capsid protein), and by electropherotype patterns (Pedley et al., 1983; Snodgrass et al., 1984).

Group A rotaviruses (RV-A) are a well-established cause of diarrhea in piglets worldwide. The disease can be endemic or epidemic, resulting in serious economic losses, mainly in suckling and recently weaned piglets. In addition to the VP6 classification, strains may be classified into serotypes based on the VP4 (P) and VP7 (G) structural proteins of the outer shell, which induce neutralizing antibodies (Offit and Blavat, 1986). The group B rotaviruses (RV-B) have been described in humans only in China, India, and Bangladesh, in which most of the infections occurred in adults (Hung et al., 1983; Krishnan et al., 1999; Sanekata et al., 2003). The virus was also detected in pigs, cattle, lambs, and rats (Snodgrass et al., 1984). Seroprevalence studies demonstrate that RV-B is widespread in the pig population, although the epidemiology of the disease is not yet well known (Brown et al., 1987).

Group C rotaviruses (RV-C) were first described in a diarrheic stool sample from a nursing piglet in 1980 (Saif et al., 1980), and they were later identified as a cause of enzootic diarrhea in neonates and older pigs (Morin et al., 1990; Kim et al., 1999). The virus was also detected in fecal specimens from humans, dogs, ferrets, and cattle (Rodger et al., 1982; Torres-Medina, 1987; Tsunemitsu et al., 1991; Otto et al., 1999). A previous study reported a 59 to 100% seroprevalence of RV-C infection in pigs of all ages, with the antibody titer being higher in older pigs (Terrett et al., 1987). Serologic studies have indicated that the virus is circulating worldwide (Bridger and Brown, 1985; Nagesha et al., 1988; Tsunemitsu et al., 1992).

Because of the requirements for serial propagation of RV-C in tissue culture, many attempts have failed to replicate the virus. The exceptions are one porcine (Cowden), one bovine (Shintoku), and one human (Ehime 9301) strain (Saif et al., 1988; Tsunemitsu et al., 1991; Shinozaki et al., 1996).

In spite of what happens in RV-A, there has been no formal genotyping classification of RV-C based on P and G types. However, sequence comparison suggests that there is significant genetic diversity among the RV-C. Analyses revealed that the Cowden, Shintoku, HF (porcine), and other human strains each belong to different G serotypes (Tsunemitsu et al., 1991; Jiang et al., 1996). There was also a proposed classification based on P types, with porcine, bovine, and human strains constituting three distinct groups (Jiang et al., 1999).

Since RV-C is distinguished from other rotavirus groups essentially by the features of VP6, the molecular detection of this protein constitutes a frequent target of diagnostic assays to test for the presence of RV-C in stool samples. VP6 is encoded by gene 5 of RV-C and is highly immunogenic and antigenic, although it does not induce the production of neutralizing antibodies (Estes and Cohen, 1989; Kapikian et al., 2001). Recently, a high level of genetic variation within the VP6 gene among porcine RV-C strains from Italy was described, a finding that was unexpected (Martella et al., 2007).

Previous studies have described a high incidence of RV-C infection in Brazilian swine herds; however, wild-type Brazilian RV-C strains have not yet been characterized (Alfieri et al., 1999). The present study describes molecular and phylogenetic analyses of one region of gene 5 from several RV-C strains identified in Brazilian pig herds from distinct geographical regions.

## MATERIAL AND METHODS

### RV-C-positive samples

Eleven stool samples from different piglets were selected to be included in the analysis. These samples showed an atypical electropherotype pattern in polyacrylamide gel electrophoresis (PAGE) analysis (Herring et al., 1982). In the reverse transcription-polymerase chain reaction (RT-PCR) assay, the samples were positive for RV-C by amplifying a 270-bp fragment of gene 5 (VP6). RNA extraction was performed with a combination of the phenol/chloroform/isoamyl alcohol and silica/guanidinium isothiocyanate methods as described by Alfieri et al. (2006). RT-PCR was performed using the forward BMJ41 (5' GGC TTT AAA AAT CTC ATT CA 3', [nt] 1-20) and reverse BMJ42 (5' CCT CTA GTT GAT TGA ACA TA 3', [nt] 251-270) primers (Alfieri et al., 1999). Electrophoresis analysis was carried out using an ethidium bromide-stained 2% agarose gel that was visualized under UV light.

The specimens were collected between 2004 and 2006 and were obtained from four Brazilian States: Mato Grosso do Sul (N = 1); Paraná (N = 2); Santa Catarina (N = 2), and Rio Grande do Sul (N = 6), which are located in the South and Central West geographical regions in Brazil. Eight fecal specimens were diarrheic, and three (one from Santa Catarina and two from Rio Grande do Sul) had a normal consistency. The age of the animals ranged from 1 to 4 weeks.

All samples were previously shown to be negative for RV-A and RV-B by PAGE and RT-PCR analysis, respectively (Herring et al., 1982; Gouvea et al., 1991).

### Purification and sequencing

The PCR products were purified using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, UK) and quantified with Low DNA Mass Ladder (Invitrogen™ Life Technologies, USA). The sequences were obtained using the DYEnamic ET Dye Terminator Kit (GE Healthcare) with a MegaBACE 1000/automated 96 capillary DNA sequencer. The amplicons were sequenced directly using forward and reverse primers.

### Sequence analysis

Sequence quality analysis was carried out using Phred and CAP3 softwares (http://genoma.cenargen.embrapa.br/phph/). Sequence similarity searches were performed using the BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/), and multiple alignment and identity matrix analyses were performed using BioEdit version 7.0.5.3. Phylogenetic trees were generated using the neighbor-joining method based on the

Tamura-Nei model using MEGA package (version 4.0), with 1000 bootstrap replicates (Tamura and Nei, 1993). The sequences included in the study were acquired from the National Center for Biotechnology Information, USA (GenBank) (http://www.ncbi.nlm.nih.gov/GenBank/) (Table 1).

**Table 1.** Rotavirus strains with available VP6 gene sequences used in the study, and their original hosts.

| Rotavirus group | Strain | Original host | GenBank accession number |
|---|---|---|---|
| A | WA | Human | K02086 |
| | NCDV | Bovine | AF317127 |
| | OSU | Porcine | AF317123 |
| B | WH1 | Human | AY215071 |
| | Nemuro | Bovine | AB106542 |
| C | Bristol | Human | X59843 |
| | Modunagari | Human | AF325806 |
| | Belem | Human | M94155 |
| | Yamagata | Bovine | AB108680 |
| | Shintoku | Bovine | M88768 |
| | WD534tc | Bovine | AF162434 |
| | Cowden | Porcine | M94157 |

## RESULTS

Nine sequences could be analyzed of the 11 amplicons that were purified and sequenced. The other two were not of high enough quality and were excluded. When a BLAST search was performed, all 9 sequences showed high similarity to published RV-C sequences. The study was done using a 230-bp fragment of gene 5 (VP6) that resulted from the multiple alignments of the 9 Brazilian sequences.

The nucleotide identity among the Brazilian porcine RV-C strains ranged from 81.3 to 94.3%. All 9 sequences had the highest nucleotide identity (84.3 to 93.9%) with the porcine prototype Cowden strain. However, the percent identity of these sequences when compared to the porcine prototype was lower than the values observed among human RV-C strains, which varied from 96.9 to 100% identity (Bristol and Preston strains were identical), or between the two original bovine strains, which were 98.2% identical (excluding WD534tc - a bovine strain previously described with high similarity with the porcine prototype Cowden). The percent identities of the Brazilian porcine RV-C strains with RV-A and RV-B strains were 60.4 to 67.3% and 33.7 to 40.5%, respectively, while the lowest similarity between the Brazilian sequences and RV-C prototypes was 78.2% (for the Shintoku and BRA30-UEL strains).

In the dendrogram reconstruction, four distinct clusters were formed, designated Porcine I, Porcine II, Human, and Bovine. The Porcine I group included two subclusters where the BRA21-UEL, BRA22-UEL, and BRA29-UEL sequences were grouped together but were segregated from the Cowden and WD534tc subcluster. The other Brazilian strains, BRA23-UEL, BRA25-UEL, BRA26-UEL, BRA27-UEL, BRA28-UEL, and BRA30-UEL, formed the Porcine II cluster, which also formed two subclusters with recent published porcine RV-C sequences from Korea (Jeong et al., 2009). The Human and Bovine clusters included exclusively human and bovine strains, respectively (Figure 1).

 

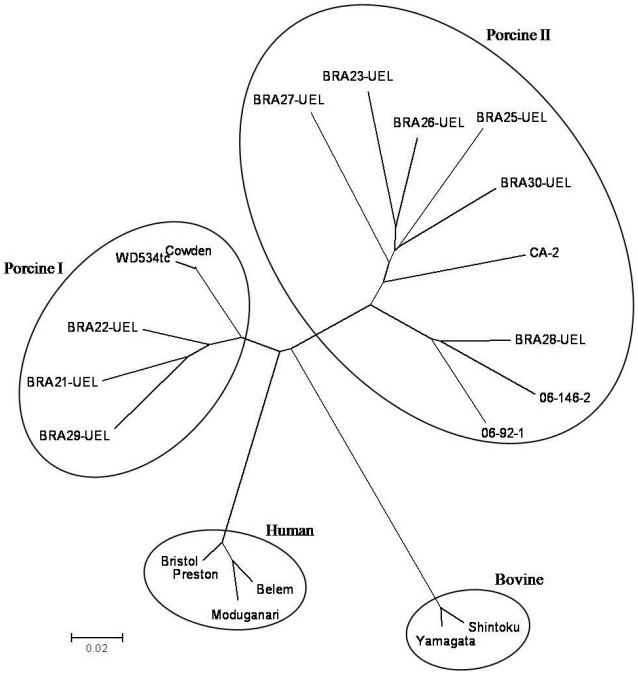


**Figure 1.** Phylogenetic tree reconstructed by the neighbor-joining method (Tamura-Nei model), and using 1000 replicates among human, bovine, and porcine strains of RV-C based on one region (230 nucleotides) of the VP6 gene. The sequences were acquired from GenBank: BRA27-UEL (EU002783), BRA23-UEL (EU002780), BRA26-UEL (EU002782), BRA25-UEL (EU002781), BRA30-UEL (EU002786), CA-2 (GQ925781), BRA28-UEL (EU002784), 06-146-2 (FJ494692), 06-92-1 (FJ494690), Shintoku (M88768), Yamagata (AB108680), Belem (M94155), Moduganari (AF325806), Preston (M94156), Bristol (X59843), BRA29-UEL (EU002785), BRA21-UEL (EU002778), BRA22-UEL (EU002779), WD534tc (AF162434), and Cowden (M94157).

## DISCUSSION

RV-C has already been described as a cause of outbreaks either in young or older pigs, in some cases leading to substantial mortality rates (Morin et al., 1990; Kim et al., 1999). Studies of the seroprevalence of RV-C also reveal high rates of infection and indicate that the virus is widespread (Terrett et al., 1987; Tsunemitsu et al., 1992). In spite of these data, epidemiologic characterization of RV-C infection is very limited. The molecular characterization of RV-C strains is still not extensively performed, which restricts the study of these circulating strains and may hamper the implementation of control measures specific to RV-C in the future.

In this study, 9 partial VP6 gene sequences from porcine RV-C strains were analyzed. The strains were obtained from piglet stool samples from different swine herds in four Brazilian states. The molecular analyses revealed that all strains had a high identity with the porcine RV-C prototype Cowden strain, in contrast to the lower identity with human and bovine strains; however, the similarity rates were variable (84.3 to 93.9%). Among the Brazilian sequences, the nucleotide identity showed a wide range of values, with a surprising minimum

similarity of 81.3% between two sequences. The comparison among porcine strains, including the prototype Cowden strain and the Brazilian strains, reveals the genetic heterogeneity in the gene fragment (230 nucleotides) analyzed. Recently, the genetic variability of the same gene was described in porcine RV-C strains in Italy, with nucleotide identity diverging in almost 12% (Martella et al., 2007). The three Brazilian strains (BRA21-UEL, BRA22-UEL, and BRA29-UEL) that were grouped with the Cowden strain in the dendrogram did not display such high variability; identity with the prototype ranged from 91.3 to 93.9%. However, the other 6 sequences showed important differences (12.7 to 15.7%) with the Cowden strain in this 230-bp fragment. Even with representative divergence in similarity (identity from 86.9 to 92.1%), these 6 strains were all grouped together.

The analysis performed in the present study was based on the 5' region of gene 5. This is in contrast to the study from Italy, which included the 3' region of the gene. This different target in the PCR assay, even though it is located in the same gene, does not allow for direct comparison of the Italian and Brazilian strains, and, consequently, it is currently impossible to use phylogenetic analysis to classify the RV-C strains from different continents.

In the phylogenetic tree, the three Brazilian strains that were placed in the same cluster as the Cowden strain (Porcine I cluster) formed a distinct subcluster. These results are in accordance with Martella et al. (2007) who showed that Cowden-like strains grouped in a large common branch with the prototype but in a different subcluster, also demonstrating that the porcine RV-C VP6 gene has a higher genetic heterogeneity than do the human and bovine groups.

The large branch formed by the other Brazilian sequences that have a low VP6 gene identity with other RV-C strains was designated the Porcine II cluster. This analysis indicates that the genetic variability is higher than what had been described previously. Furthermore, this cluster was placed farther from the Porcine I cluster (Cowden) than the Human cluster. Whether the proximity of the Porcine I and Human clusters is relevant to the ease of interspecies transmission needs to be investigated.

The genetic variation presented herein, in a gene that is supposedly conserved, opens up the possibility that RV-C is more variable than previously thought and that there is more than one porcine genogroup based on VP6 gene classification. These strains were more similar to the porcine strains already described, although their classification in the same genogroup is impossible. With the results from the Brazilian and Italian studies, it is clear that not only are there new genogroups but also new sub-genogroups of porcine RV-C strains.

None of the three Brazilian strains from the Porcine I cluster or the 6 Brazilian strains from the Porcine II cluster showed common features that correlated with the year of collection, the presence of diarrhea, the age of the piglets, or the geographic origin of the pig herds. This evidence strongly suggests that heterogeneous strains are widespread in Brazilian pig herds.

In the present study, it was shown that the heterogeneity in this VP6 gene fragment from porcine RV-C was higher than expected. Previous studies have indicated that genetic similarity might occur between strains from different host species, where porcine/bovine and porcine/human strains demonstrated high identity (Chang et al., 1999; Gabbay et al., 2008). With the description of more RV-C sequences from different host species, the molecular features of VP6 will be more clearly defined. This description of the genetic variability in porcine strains increases the possibility that there is considerable variation in gene 5 of RV-C, supposedly a more conserved gene, which contrasts with the hypothesis that only RV-A strains have high variability (Rahman et al., 2005). Furthermore, divergent Brazilian strains were detected

in different pig herds at different time periods (2004 to 2006), which suggests that the variability of wild-type strains occurred frequently and that divergent strains are widely spread in the Brazilian pig herds evaluated. If further epidemiological studies show RV-C to be important either in the development of severe intestinal lesions or in diarrhea, more molecular studies will be necessary to develop and implement prophylactic measures based on efficient vaccines that provide protection against the main circulating strains.

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