

Detection and phylogenetic analysis of porcine enteric calicivirus, genetically related to the Cowden strain of sapovirus genogroup III, in Brazilian swine herds¹

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ABSTRACT.- Barry A.F, Alfieri A.F. & Alfieri A.A. 2008. **Detection and phylogenetic analysis of porcine enteric calicivirus, genetically related to the Cowden strain of sapovirus genogroup III, in Brazilian swine herds.** *Pesquisa Veterinária Brasileira* 28(1):82-86. Laboratório de Virologia Animal, Departamento de Medicina Veterinária Preventiva, Centro de Ciências Agrárias, Universidade Estadual de Londrina, Campus Universitário, Londrina, PR 86051-990, Brazil. E-mail: alinebarry@uol.com.br

Sapovirus of the *Caliciviridae* family is an important agent of acute gastroenteritis in children and piglets. The *Sapovirus* genus is divided into seven genogroups (G), and strains from the GIII, GVI and GVII are associated with infections in swine. Despite the high prevalence in some countries, there are no studies related to the presence of porcine enteric sapovirus infections in piglets in Brazil. In the present study, 18 fecal specimens from piglets up to 28 days were examined to determine the presence of sapovirus genome by RT-PCR assay, using primers designed to amplify a 331 bp segment of the RNA polymerase gene. In 44.4% (8/18) of fecal samples, an amplified DNA fragment was obtained. One of these fragments was sequenced and submitted to molecular and phylogenetic analysis. This analysis revealed high similarity, with nucleotides (87%) and amino acids (97.8%), to the Cowden strain, the GIII prototype of porcine enteric calicivirus. This is the first description of sapovirus in Brazilian swine herds.

INDEX TERMS: Swine; piglets, diarrhea, calicivirus, *Sapovirus*.

RESUMO.- [Detecção e análise filogenética do calicivírus entérico suíno, geneticamente relacionado com a estirpe Cowden do genogrupo III do sapovírus, em rebanhos brasileiros.] O sapovírus classificado na família *Caliciviridae* é um importante causador de gastroenterite aguda em crianças e leitões. O gênero *Sapovirus* é dividido em sete genogrupos (G), sendo que as estirpes dos GIII, GVI e GVII estão associadas com infecção em suínos. Apesar da alta prevalência da infecção em alguns países, ainda não existem estudos referentes à presença do calicivírus entérico suíno nos rebanhos brasileiros. No presente estudo 18 amostras de fezes de leitões com até 28 dias foram avaliadas pela RT-PCR para a presença do

genoma do sapovírus, utilizando os primers desenvolvidos para amplificar um segmento de 331 pb do gene da RNA polimerase viral. Em 44,4% (8/18) das amostras foi amplificado um fragmento de DNA. Um desses amplicons foi seqüenciado e pela análise molecular e filogenética foi verificada similaridade de 87% em nucleotídeos e 97,8% em aminoácidos com a estirpe Cowden, protótipo do GIII. Esta é a primeira descrição do sapovírus em rebanhos suínos brasileiros.

TERMOS DE INDEXAÇÃO: Suínos, leitões, diarreia, calicivírus, *Sapovirus*.

INTRODUCTION

Caliciviruses are small, non-enveloped viruses with 27-35nm in diameter. Their genome consists of a single-stranded poly-adenylated RNA molecule with positive polarity of 7.4 to 8.3 Kb, that encodes a major (VP1) and a minor (VP2) structural protein, and nonstructural proteins including RNA dependent RNA polymerase (RdRp) (ICTVdB). Four genera have been established in the

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Caliciviridae family: *Vesivirus*, *Lagovirus*, *Norovirus*, and *Sapovirus* (Green et al. 2000). In animals, lagoviruses and vesiviruses are associated with a number of important hemorrhagic, respiratory, and vesicular diseases. Norovirus (NoV) and sapovirus (SaV) are associated with gastroenteritis in humans and animals (Kapikian et al. 1972, Madeley & Cosgrove 1976, Flynn et al. 1988).

Based on the sequence of the capsid gene, the *Sapovirus* genus can be classified into seven genogroups: GI, GII, GIV, and GV that infect humans; and GIII, GVI and GVII that infect pigs. The Cowden strain causes diarrhea and intestinal lesions in pigs, and is considered the prototype of the GIII, while the other porcine genogroups have JJ681 and K7/JP as main strains, respectively (Guo et al. 1999, Farkas et al. 2004, Wang et al. 2005, Yin et al. 2006). In addition to its putative importance as a porcine pathogen, there is the possibility of the zoonotic potential for the virus. Despite the high genetic variability of the SaV strains, intragenogroups and intergenogroups recombination have already been described, suggesting that recombination between porcine and human strains may also occur (Katayama et al. 2004, Hansman et al. 2005, Wang et al. 2005).

Porcine enteric caliciviruses were detected in pigs stool samples only in a few countries from Europe (Reuter et al. 2007), America (Wang et al. 2005, Martínez et al. 2006) and Asia (Kim et al. 2006, Yin et al. 2006). However, the association between infection and diarrhea was not verified in all studies, possibly due the high genetic variability of the SaV strains, in which not all strains lead to the development of enteric disease. In this study, we describe the detection of SaV in stool samples from piglets in Brazilian swine farms by RT-PCR assay. Additionally, comparing by phylogenetic analysis with other published SaV strains, we classify the partial RdRp nucleotide sequence obtained from the sequencing of one sample.

MATERIALS AND METHODS

Eighteen stool samples were collected from piglets aging up to 28 days from three Brazilian swine farms (G, L, M) located in Mato Grosso do Sul (MS) State (S 19° 23' 43" W 54° 33' 59"), during 2004. Four age groups were included: 1 to 7 ($n=3$), 8 to 14 ($n=2$), 15 to 21 ($n=6$), and 22 to 28 ($n=7$) days old. Thirteen animals had diarrhea and five were asymptomatic.

Viral RNA was extracted from 10% to 20% (w/v) of fecal suspensions in PBS using a combination of phenol/chloroform/isoamyl alcohol and silica/guanidinium isothiocyanate method according to Alfieri et al. (2006). The primer pair used (p289/290) was designed by Jiang et al. (1999) to amplify a 319 or 331 bp fragment from RdRp gene of NoV or SaV, respectively. Reverse transcription reaction was performed in a 20 μ l final volume containing 5 μ l of extracted RNA, 1 μ l (20pmol) of p289 reverse primer and 4 μ l of ultra pure RNase free water, that was denaturated at 97°C for 5min. After, it was placed on ice for 5min, and 10 μ l of the RT mix (1 \times RT buffer, 0.1mM of dNTP, 10mM DTT, 100 U of M-MLV reverse transcriptase and ultrapure sterile water) was added and incubated at 42°C for 30min followed by enzyme inactivation at 95°C for 5min. The PCR had

a total of 50 μ l [8 μ l of the RT reaction, 1.5 \times PCR buffer, 2mM MgCl₂, 0.2mM of each dNTP, 1 μ l (20pmol) of each primer, 2.5 units *Platinum Taq* DNA polymerase and ultrapure RNase free water]. Thermocycler temperatures included one step at 94°C for 3min, 40 cycles at 94°C for 30s, 49°C for 1min 20s, and 72°C for 1min, and a final step at 72°C for 10min. The RT-PCR products were analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide and visualized under UV light.

A stool sample from a child with NoV infection detected by RT-PCR assay and electron microscopy was used as positive control and ultrapure water as negative control.

All samples included in the study were previously tested by silver stained polyacrylamide gel electrophoresis (ssPAGE) to the presence of rotaviruses, being only one positive to group A rotaviruses (GAR).

The PCR amplicon was purified using GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, USA), and sequenced in MegaBACE™ 1000 and Thermo Sequenase™ II DNA Polymerase, DYEnamic™ ET Dye Terminator Kit (GE Healthcare, USA) using the forward and reverse primers.

Sequence editing was performed with Phred and CAP3 softwares (<http://adenina.biomol.unb.br/phph/>). The translation and verification of conserved motifs was done by using Gene Runner version 3.05. Similarity searches were carried out by using BLAST (<http://www.ncbi.nlm.nih.gov/>) followed by multiple alignments by CLUSTAL W (version 1.4) and sequence identity matrix using BioEdit v7.0.5.3. Phylogenetic distances and tree were generated by using MEGA v3.1 software.

RESULTS AND DISCUSSION

SaVs were detected by RT-PCR assay in 44.4% (8/18) of stool samples analyzed (Fig.1). Two samples were from farm G (both from 22 to 28-day-old diarrheic piglets), one from farm L (22 to 28-day-old asymptomatic piglet) and the other five from farm M (two from 15 to 21-day-old diarrheic piglets and three from 22 to 28-day-old piglets, one of them diarrheic). In summary, from the eight RT-PCR amplicons with the 331 bp expected size, five (62.5%) were from diarrheic piglets. One diarrheic stool sample from farm G that was positive to SaV, was also positive to GAR in ssPAGE.

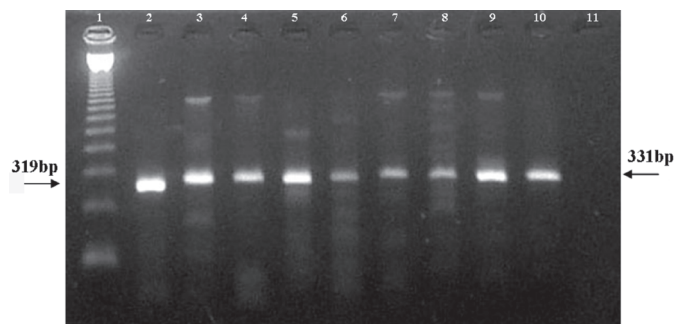


Fig.1. Ethidium bromide stained agarose gel electrophoresis of sapovirus polymerase gene amplified with primer pair p289/p290 by RT-PCR assay, from piglet stool samples. Lane 1, 123 bp ladder; lane 2 human norovirus with 319 bp; lanes 3 to 10 porcine sapovirus with 331 bp; lane 11 negative control (ultrapure water).

The number of stool samples tested was relatively low to perform statistical tests in order to associate the presence of SaV and clinical signs of diarrhea. In addition, piglets in the beginning and in the end of the infection can show viral shedding without clinical signs. Stool samples from these piglets can increase the number of SaV positive samples in asymptomatic animals leading to wrong conclusions.

One sample (BRA29-MS/04, GenBank accession number EF183480), from a 22 to 28-day-old diarrheic piglet from farm G, was sequenced. When the obtained sequence was compared to other sequences by BLAST, it was observed a high similarity with porcine SaVs. Before proceeding with the analysis, the sequence was translated and the presence of GLPSG and YGDD RdRp conserved motifs was verified.

To classify the Brazilian sequence, BRA29-MS/04 was aligned with 17 GenBank published SaV sequences including members of all clusters from the seven genogroups, and with 13 strains, from other countries in which the porcine SaV were described. To perform the molecular analysis, the primers sequences were excluded, resulting in a 286 nucleotides fragment. The intra-genogroup distance (overall average of 0.36) was compared to investigate whether BRA29-MS/04 was a GIII strain. Phylogenetic distances based on nucleotides alignments in that partial RdRp gene ranged from 0.03 to 0.33 between members of the same genogroup. Houston7-1181/90 strain, the GIV prototype, had lower distance with GII members and was not included in this analysis. BRA29-MS/04 had 0.13 of nucleotide distance with Cowden strain. The distance between the Brazilian strain and new porcine genogroups (GVI and GVII) ranged from 0.45 to 0.50 (data not shown), being above the distance of members from the same genogroup.

Schuffenecker et al. (2001) comparing distances between 11 SaVs strains in five different regions of the

genome (RdRp, 3'ORF, capsid overlap, 3'UTR, and capsid) verified that the same genogroups and genetic clusters organization were maintained throughout the genomic regions examined. A similar study was performed in which 17 sequences were classified in nine genetic clusters and five genogroups according to the capsid gene sequence. However, the same organization was not observed to all strains analyzed in the RdRp gene (Farkas et al. 2004). Despite these different results on classifying based on the RdRp gene, the GIII Cowden strain was identified in these two studies as one clearly distinct genogroup either in RdRp partial gene or in capsid gene, being the difference in organization limited to a few human SaV strains. In this way, the classification based on this 286 bp of RdRp gene was enough to classify, at least in genogroup, a SaV strain.

When compared with the prototypes strains from the seven SaV genogroups, BRA29-MS/04 showed the highest identity with the Cowden strain (87%) (Table 1). Nucleotide identity with strains from Venezuela (Aragua/1999, Miranda1/2001 and Yaracuy/1999), Hungary (Id3/2005), Korea (Korean6802 and Korean10802), The Netherlands (PECIVA20, PECIVA36 and SWECI/VA10) and North-America [LL14, JJ259/00 and MM280/03 (excluding QW19, classified as human GII)] ranged from 72.2 to 89.4% (Wang et al. 2005, Kim et al. 2006, Martínez et al. 2006, Reuter et al. 2007). It was not verified higher similarity among strains from near geographic areas included in the study, since the identity of BRA29-MS/04 with other American strains (USA and Venezuela) was not higher than with strains from other continents. The amino acid analysis (95aa) revealed 97.8% identity between BRA29-MS/04 and the Cowden strain, whereas the similarity with the others porcine genogroups were 41 and 45.2% to GVI and GVII, respectively (data not shown). Based on distance, identity and phylogenetic analysis BRA29-MS/04 was classified as a strain from GIII (Fig.2).

Table 1. Nucleotide sequence identity matrix of partial RdRp gene (286 nt) between BRA29-MS/04 wild-type sapovirus strain, and prototype sapovirus strains, representing all clusters into seven genogroups

Genogroup/ cluster	Strain	BRA29	Sapp	Park	Mex14917	Lond	Mex340	Cruise ship	Cowden	Hou7-1181	Arg39	JJ681	K7/JP
	BRA29-MS/04	ID	0.552	0.601	0.58	0.597	0.59	0.608	0.87	0.594	0.548	0.531	0.5
GI/1	Sapporo/82	0.552	ID	0.671	0.723	0.646	0.646	0.646	0.562	0.636	0.608	0.493	0.444
GI/2	Parkville/94	0.601	0.671	ID	0.73	0.657	0.678	0.678	0.604	0.65	0.583	0.531	0.468
GI/3	Mexico14917	0.58	0.723	0.73	ID	0.688	0.646	0.667	0.573	0.671	0.59	0.513	0.468
GII/1	London/92	0.597	0.646	0.657	0.688	ID	0.793	0.783	0.594	0.765	0.573	0.503	0.475
GII/2	Mexico340/90	0.59	0.646	0.678	0.646	0.793	ID	0.79	0.597	0.79	0.58	0.51	0.506
GII/3	Cruise ship/00	0.608	0.646	0.678	0.667	0.783	0.79	ID	0.594	0.769	0.58	0.534	0.5
GIII	Cowden	0.87	0.562	0.604	0.573	0.594	0.597	0.594	ID	0.587	0.566	0.538	0.496
GIV	Houston7-1181/90	0.594	0.636	0.65	0.671	0.765	0.79	0.769	0.587	ID	0.541	0.524	0.486
GV	Argentina39	0.548	0.608	0.583	0.59	0.573	0.58	0.58	0.566	0.541	ID	0.451	0.454
GVI	JJ681	0.531	0.493	0.531	0.513	0.503	0.51	0.534	0.538	0.524	0.451	ID	0.653
GVII	K7/JP	0.5	0.444	0.468	0.468	0.475	0.506	0.5	0.496	0.486	0.454	0.653	ID

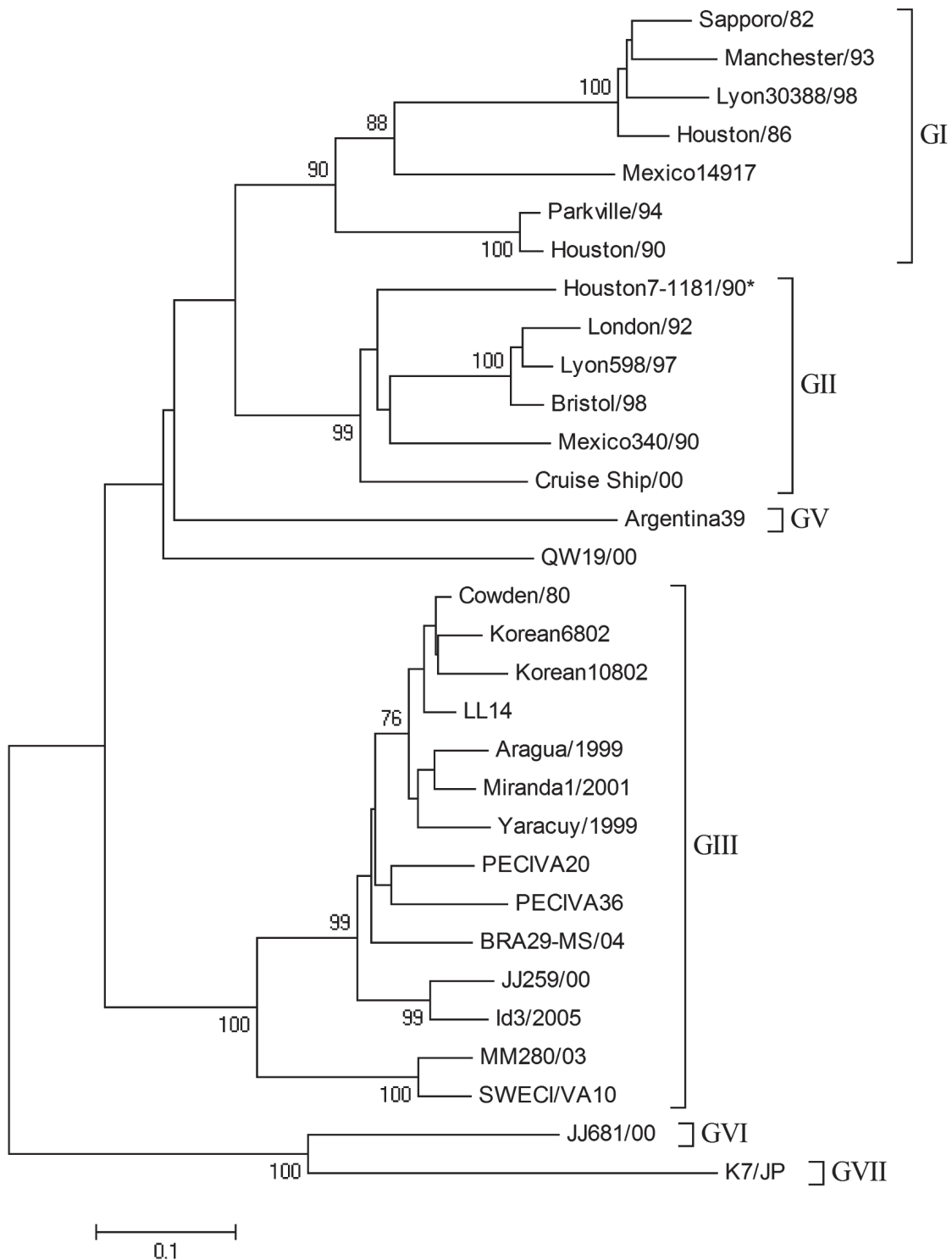


Fig.2. Phylogenetic tree reconstructed by neighbor-joining method (Kimura 2-parameter) and 1000 replicates between human, porcine and a Brazilian strain of SaV based on partial (286 nt) RdRp gene. Sequences used in the alignment were acquired from GenBank as follows accession numbers: Sapporo/82 (U65427), Manchester/93 (X86560), Lyon30388/98 (AJ251991), Houston/86 (U95643), Mexico14917 (AF435810), Parkville/94 (U73124), Houston/90 (U95644), Houston7-1181/90 (AF435811), London/92 (U95645), Lyon598/97 (AJ271056), Bristol/98 (AJ249939), Mexico340/90 (AF435809), Cruise ship/00 (AY157863), Argentina39 (AF405715), QW19/02 (AY826424), Cowden (AF182760), LL14 (AY425671), Korean6802 (AY289186), Korean10802 (AY289188), Aragua/1999 (AY633967), Miranda1/2001 (AY633964), Yaracuy/1999 (AY633966), BRA29-MS/04 (EF183480), PECIVA36 (AY615805), PECIVA20 (AY615804), JJ259/00 (AY826423), Id3/2005 (DQ383274), MM280/03 (AY823308), SWECI/VA10 (AY615807), JJ681/00 (AY974192) and K7/JP (AB221130). *Sapovirus* genogroups are indicated. *GIV strain

CONCLUSION

This is the first description of the presence of SaV in stool samples of piglets in Brazil. The detection of the virus in other countries, including South-American ones, is an important indicative that the virus is widespread. In this study, one Brazilian virus was classified as GIII member based on the similarity with Cowden strain, which causes small intestinal villous atrophy and clinical signs of enteric disease in experimental inoculated gnotobiotic pigs. In summary, these results increase the number of viruses potentially present in feces of piglets in Brazilian swine farms. Further studies are needed to understand the epidemiology of the SaV infection in Brazil, and the possible role of these infections in enteric disease and diarrhea in piglets.

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