ARS VETERINARIA, Jaboticabal, SP, v.37, n.1, 015-020, 2021. ISSN 2175-0106
DOI: http://dx.doi.org/10.15361/2175-0106.2021v37n1p15-20

DETECTION OF SAPOVIRUS IN BRAZILIAN PIG FARMS

DETECÇÃO DE SAPOVÍRUS EM CRIAÇÕES DE SUÍNOS BRASILEIRAS

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SUMMARY

Sapoviruses (*Caliciviridae*) are considered important agents of gastroenteritis worldwide affecting animals and humans. In pig farming, the epidemiology is not completely understood because it can affect all stages of production, with symptomatic (diarrhea) or asymptomatic pigs. The aim of our study was to investigate Sapovirus occurrence in Brazilian pig farms. A total of 166 fecal samples of pigs, with different ages, from Minas Gerais, São Paulo, and Mato Grosso States were submitted to RT-PCR reactions and confirmed with nucleotide sequencing of Sapovirus RdRp gene. Six (3.61%) samples were positive and four had partial RdRp gene sequenced, putatively belonging to GVII.1 genogroup, also reported in swine herds in Brazil.

KEY-WORDS: Sapoviruses. Calicivirus. Swine. RdRp gene. Genogroup.

RESUMO

Sapovírus (*Caliciviridae*) são considerados importantes agentes causadores de gastroenterites em todo o mundo, afetando animais e humanos. Na suinocultura, sua epidemiologia ainda não foi totalmente esclarecida, pois pode afetar todas as fases da produção, com suínos sintomáticos (diarreia) ou assintomáticos. O objetivo do nosso estudo foi investigar a ocorrência de Sapovírus em granjas de suínos brasileiras. Um total de 166 amostras fecais de suínos, com diferentes idades, dos estados de Minas Gerais, São Paulo e Mato Grosso foram submetidas a reações de RT-PCR e confirmadas com sequenciamento de nucleotídeos do gene RdRp do Sapovírus. Seis (3,61%) amostras foram positivas e quatro delas tinham sequenciamento parcial do gene RdRp, supostamente pertencente ao genogrupo GVII.1, previamente relatado em rebanhos suínos no Brasil.

PALAVRAS-CHAVE: Sapoviroses. Calicivirus. Suínos. RdRp gene. Genogrupo.

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Submetido: 08/02/2021 Aceito: 23/03/2021

INTRODUCTION

Sapovirus (SaV) is a non-enveloped linear RNA (+) virus, belonging to *Caliciviridae* family, presenting *Sapporo virus* as unique viral species, classified in nineteen genogroups (GI to GXIX), additionally subdivided into different genotypes (ICTV, 2020). Besides, these viruses can be found in a wide variety of hosts: humans (GI, GII, GIV, GV), pigs (GIII, GV to GXI), sea lion (GV), mink (GXII), dogs (GXIII), bats (GXIV, GXVI to GXIX), rodents (GXV) (Yinda et al., 2017; Diez-Valcarce et al., 2018).

Although SaV are considered important cause of childhood gastroenteritis worldwide (by fecal-oral route), as well as Rotaviruses (*Reoviridae*) and Noroviruses (*Caliciviridae*) in terms of occurrence (Gutiérrez et al., 2011; Becker-Dreps et al.,2020), in pig farming, the virus epidemiology has not completely understood (Lauristen et al., 2015; Kuroda et al., 2017; Desselberger, 2019). SaV may cause economic losses in all stages of production, especially in newly weaned piglets, where animals may present diarrhea with viral elimination and small intestine lesions (Guo et al., 2001; Martella et al., 2008; Lu et al., 2016). Meanwhile, SaV detection was reported in asymptomatic swine (Chao et al., 2012; Valente et al., 2016).

So far, porcine SaVs are classified into eight genogroups, presenting 21 genotypes among them (Li et al. 2018). Molecular analyzes (genogroup and genotype) are important for understanding the dynamics and evolution of SaVs, given their remarkable genetic variability (Oka et al., 2016). Although transmission of the same genotype between humans and pigs has not been reported, it is possible to identify genetic proximity mainly in the GV genogroup (pig GV.3 / GV.5 and human GV.1-2) (Nagai et al., 2020).

In Brazil, the first description of SaV occurred in 1992 in a diarrheal child in the State of Pará (Nakamura et al., 2006), while in pigs it was described by Barry et al. (2008a) in the State of Mato Grosso do Sul, similar to the *Cowden* prototype (strain) - genogroup III. Since then, it has been described in pig herds from different States: Mato Grosso do Sul (Genogroup GIII) (Barry et al., 2008a), Minas Gerais, Paraná, Santa Catarina, Rio Grande do Sul (GIII and GVIII) (Barry et al., 2008b), Rio de Janeiro (GVII) (Cunha et al., 2010) and the Amazon region with a higher prevalence of the GIII, GVII and GVIII genogroups (Hernandez et al., 2014). In addition, in Paraná, was the circulation of the probable 'GIX?' group was also evident (Valente et al., 2016).

Due to the scarcity of data available on Brazilian pig herds, the aim of our study was to investigate SaV infection in animals raised in farms from three states, providing a better understanding on virus occurrence.

MATERIAL AND METHODS

Study design

This is a cross-sectional descriptive study that aimed at detecting the occurrence and respective frequency, from convenience sampling of swine fecal samples.

Sample collection

Here, 166 fecal samples of pigs with different ages (maternity to adult) diarrheal or not, in multiple Brazilian commercial farms in Minas Gerais, São Paulo and Mato Grosso States were collected between 2017/2018 and tested for SaV. The samples were collected directly from the animals when they were defecating using RNAse free tubes (falcon type) and transported under refrigeration, and stored at -20ºC until processing. This study was authorized by the Ethics Committee on the Use of Animals of the Faculty of Veterinary Medicine and Zootechnics under the protocol 4416230217.

RNA extraction protocol

Viral RNA was extracted from fecal samples diluted in ultrapure water, proportion of 50% (w/v) and clarified by centrifugation at 2,000 g/15 minutes, using the TRIzol reagent (Invitrogen™) according to manufacturer's instructions. The last step was done with the resuspension of the final product in 15 µL of ultrapure water.

For the DNA and PCR synthesis reactions, we developed new primers (upCavF and upCavR) to detect a region (4327 to 4657 nt) of the SaV RdRp gene, referring to the Po/SaV/GIII/Cowden sample (accession number AF182760). Briefly, from GenBank database, 122 sequences encoding the SaV RdRp, were retrieved and aligned using the ClustalW 1.82 software (© 1994, Thompson & Gibson). Primers were selected visually from conserved regions of the alignment, being designated upCavF (5´-TACTCCARGTGGGAYTCCAC 3 ') and upCavR (5´-TGACAATGTAATCATCMCCRT 3'), targeting a 328 nt-long fragment. These sequences were submitted to BLAST/n (https://blast.ncbi.nlm.nih.gov/) to verify its analytical specificity.

cDNA synthesis, diagnostics by PCR and Sequencing analysis protocols

Reverse transcription reactions were used according to the following protocol, provided by the enzyme manufacturer: 1× First Strand Buffer, 0.5 mM of each dNTP (Invitrogen™), 5.0 mM of DTT, 0.25 µM of forward and reverse primers each, 200U of Superscript Reverse Transcriptase III (Invitrogen™) and ultrapure water q.s.p. to 20µL. This was incubated at 55°C for 60 min followed by 70°C for 15 min.

For the PCR reaction, we adopted the Taq Polymerase manufacturer protocol, as follows: 5.0 µL cDNA added to the mix of 1× PCR Buffer™ (Invitrogen™), 0.2 mM of each dNTP (Invitrogen™), 0.5 µM of forward and reverse primer, 1.5 mM $MgCl_2$, and 2.0 U Platinum Taq Polymerase (Invitrogen™) and nuclease-free water q.s.p. to 25 µL. Thermocycling conditions were: initial denaturation at 94°C for 2 min, followed by 40 cycles at 94°C for 30 sec, 48°C for 40 sec and 72°C for 1 min, followed by final extension at 72°C for 10 minutes.

Finally, 5 µL of the products from the reactions were analyzed by electrophoresis on 1.5% (w/v)

agarose gel in 0.045 M TRIS-borate buffer; EDTA 0.001 M pH 8.0, making the gel stain through applying SYBR Safe DNA gel stain (Invitrogen™) and visualized in UV transilluminator (InGenius, Syngene®).

Amplicons were purified using ExoSAP-IT® PCR Product Cleanup (USB® Products Affymetrix) and subjected to bidirectional Sanger sequencing using BigDye Terminator 3.1™ kit (Applied Biosystems®), according to manufacturer's protocol and a 3500™ Genetic Analyzer system (Applied Biosystems®). These confirmed SaV sequenced samples were considered as positive controls in all reactions, while ultrapure water was used as negative control.

The nucleotide sequences obtained were compared with BLAST/n results to check identity with the expected viruses, using the query default parameters, and returning 250 maximum target sequences. They were also translated into amino acids and aligned with ClustalW 1.82 software (© 1994, Thompson & Gibson), to observe amino acid diversity.

SaV RdRp gene neighbor joining phylogenetic trees were constructed with MEGA®X software (Kumar et al., 2018), considering 236 positions in final dataset, using maximum composite likelihood as substitution model and 1,000 bootstrap replicates, including representatives from genogroups GI to GXI. There is no public RdRp sequence data available for SaV genogroups GXII-GXIX, which have been found in species such as mink, dogs, bats and rodents, and hence, not present in the tree.

RESULTS AND DISCUSSION

In total, 3.61% (6/166) samples were positive for Sapovirus by PCR, originated from: Minas Gerais - MG (2), Jaguariúna - SP (3) and Itu - SP (1) (Table 1). Out of six samples with positive results, four had partial RdRp gene sequenced (Genbank accession numbers MW086610, MW086611, MW086612, and MW086613). We assumed that these two PCR positive samples from Minas Gerais State are SaV suspect, due to lack of confirmation by genetic sequencing.

Table 1 - Distribution and positivity by location of Brazilian swine production and the age of fecal samples used to detect porcine Sapovirus.

	Maternity	Nursery	Maternity/ Nursery	Adults	No record	Total
Minas Gerais (MG)	11	30 (2)	-	-	8	**49**
São Paulo (SP)						
Jaguariúna	-	-	38 (3)*	-	-	**38**
Itu	19 (1)*	-	-	-	-	**19**
Pereiras	4	-	-	-	-	**4**
Holambra	8	-	-	-	-	**8**
Cunha	3	1	-	-	-	**4**
Fartura	2	-	-	-	-	**2**
Bragança Paulista	11	-	-	-	-	**11**
Jundiaí		2		3	-	**5**
São Roque			2	10	4	**16**
Mato Grosso (MT)						
Sorriso	3	-	-	-	-	**3**
Castanheira	3	-	-	-	1	**4**
No record[4]	2	1				**3**
TOTAL	**66**	**34**	**40**	**13**	**13**	**166**

[1]: Maternity, pig up to 30 days;
[2]: Nursery, pig between 31 to 70 days;
[3]: Adults, pig over 70 days;
[4]: No record in our database about the location of the sample.
( ): number of positive animal for Sapovirus;
*: sequenced positive

This study samples clustered with GVII.1 genogroup (Figure 1), considering the RdRp gene. Sapovirus sequences obtained come from animals aged between 0 to 70 days. However, Kuroda et al. (2017) describe the SaV infection as being common in nursery animals (31 to 70 days) going against the highest frequency in maternity animals (0 to 30 days) (Valente et al., 2016; Barry et al., 2008b).

While the prevalence of SaV in the Brazilian swine herd is not known, surveys of this viral disease present the GIII genogroup as prevalent in all regions of the country already addressed in previous studies (Barry et al., 2008a; Barry et al., 2008b; Cunha et al., 2010; Hernandez et al., 2014; Valente et al., 2016). Our results, given the limited fragment length under analysis and the absence of genogroups GXII-GXIX representatives, can putatively be defined as GVII.1 genogroup by RdRp gene. If so, it corroborates the findings of Hernandez et al. (2014) and Cunha et al. (2010), which describe this genogroup in the Amazon Region and Rio de Janeiro State, respectively. In addition, GVII.1 genogroups are commonly demonstrated in pig farming in different countries, like Belgium (Mauroy et al., 2008), Canada (L'Homme et al., 2009), Denmark (Reuter et al., 2010), and Japan (Nakamura et al., 2010).

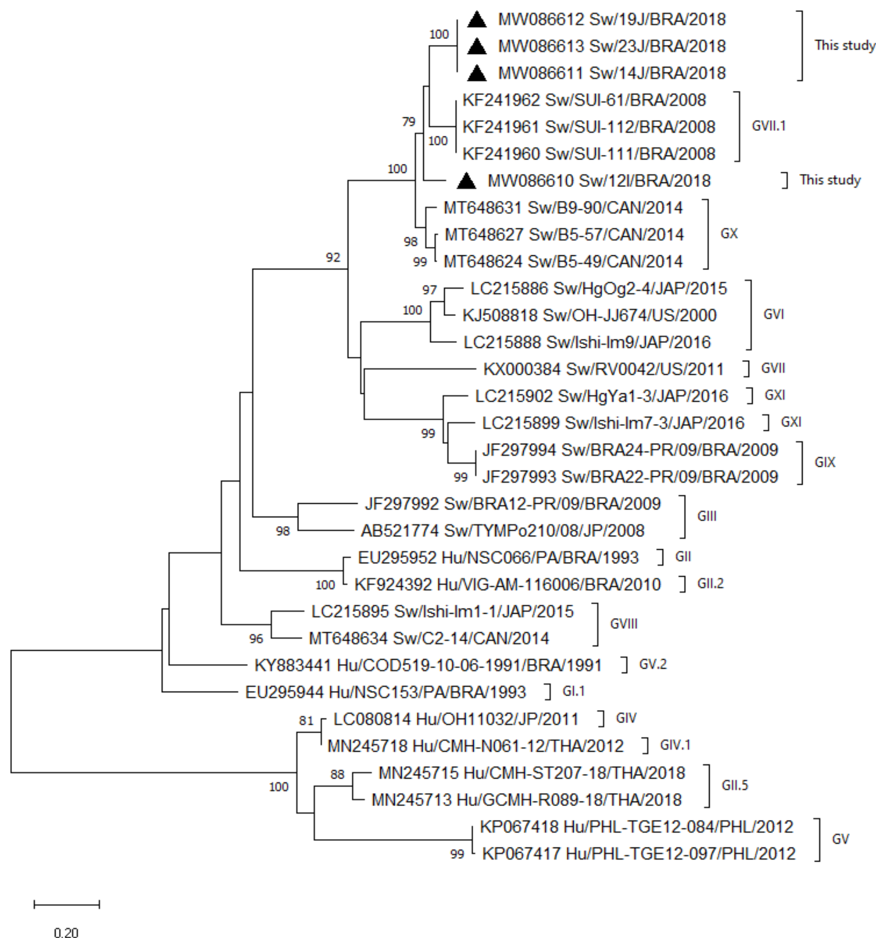

Figure 1 - Nucleotide neighbor-joining distance tree (maximum composite likelihood substitution model) for the partial RdRp Sapovirus gene showing the known genotypes. Strains detected in the present study are preceded by black triangles. The numbers at each node are bootstrap values greater than 75% from 1,000 replicates. The bar represents the number of substitutions per site.

Porcine SaV has been identified on every continent except Oceania (Reuter et al., 2010), presenting a wide range of frequency and genotypes. In most of these studies, no direct association was observed between diarrhea and SaV (Reuter et al., 2010; Salamunova et al., 2018; Nagai et al., 2020).

Although the viral classification is based on the VP1 gene, Schuffenecker et al. (2001) showed that, when comparing different strains of SaVs in the RdRp gene, the 3'ORF and ORF capsid overlapping sequences and 3'UTR (untranslated region) demonstrate the same classification. With this, it is possible to verify that most of the molecular detection studies use the RdRp gene and/or capsid junction region because it presents a more conserved region (Nagai et al., 2020).

Using as reference the RdRp gene of porcine Sapovirus GVII.1 (KF241961) (Hernandez et al., 2014), our samples presented five amino acids substitutions (13I→V; 20V→I; 23D→N; 31S→N; 65T→V), including the cluster of samples from the city of Jaguariúna-SP identical to each other and differed (4 aa / 4.65%) from those of Itú-SP, suggesting the detection of two viral variants, despite belonging to the same genotype.

The results with the RdRp detection take into consideration the fact this gene is highly conserved, given its biological role in viral replication, but can suffer genetic drift or shift contributing to viral fitness (Wang et al., 2020). Kuroda et al. (2017) analyzed complete genomes and the phylogenetic tree based on the RdRp region and found it was essentially similar to the trees obtained from the VP1 and VP2 regions.

In conclusion, this is the first report of SaV in pig production of São Paulo State. It must be considered that different physiological and nutritional factors, as well different bacterial, parasitic and viral agents can be present, and a more comprehensive study on etiology diarrhea is advisable.

ACKNOWLEDGMENTS

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code. PROEX 2016/1841.

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