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Identification of rotavirus G and P genotypes in nursing and weaned piglets in the metropolitan region of Belém, Pará State, Northern Brazil

Identificação de genótipos G e P de rotavírus em leitões em fase de amamentação e desmamados na região metropolitana de Belém, Estado do Pará, Região Norte do Brasil

Identificación de genotipos G y P de rotavirus en lechones en fase de amamantamiento y destetados en la región metropolitana de Belém, Estado de Pará, Región Norte de Brasil

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ABSTRACT

Rotaviruses are an important agent of diarrhea in piglets, primarily during nursing and post-weaning with severity. The aim of this study was to identify rotavirus G and P genotypes obtained from piglets reared at five piggeries in Belém, Pará State, Northern Brazil. Fecal specimens were collected from nursing and weaned piglets. A total of 172 samples were tested, of which 17 (9.9%) were positive for group A rotaviruses all of them from piglets kept at nursing and then were sequenced for the of VP7 and VP4 genotyping. The consistency of positive fecal samples were 53% (9/17) diarrheic, 23.5% (4/17) pasty and 23.5% (4/17) normal. The most common G genotype was G3 representing 53% (9/17), followed by G5 genotype (17%, 3/17). The P genotype recorded was P[23] corresponding to 23.5% (4/17). This study showed that rotaviruses circulated in swine herds in the metropolitan region of Belém, in Northern region. G3P[23] combination was recorded for the first time in Brazil.

Keywords: Rotavirus; Piglets, Weaning; Herds, G3P[23] genotype.

INTRODUCTION

Enteric illness is the major cause of mortality in weaned and post-weaned piglets and diarrhea is the main clinical symptom in infected animals, with economic losses considerably stronger for the producer[1]. Multifactorial disease and virus are the most common illnesses that pig herds tend to have, causing immune depression, and resulting in high morbidity and mortality[2]. A number of different agents can cause diarrhea in growing pigs, including *Brachyspira* spp., *Campylobacter* spp., *Clostridium perfringens* type A, enterotoxigenic lineages of *Escherichia coli*, *Lawsonia intracellularis*, *Salmonella* spp., *Yersinia* spp., *Coronavirus*, *Rotavirus, Norovirus, Sapovirus, Calicivirus, Isospora suis*, and *Trichuris suis*[3,4,5,6,7].

Rotaviruses are an important (primary or secondary) cause of diarrhea in weaned and post-weaned piglets, contributing (or predisposing animals) to outbreaks

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of enteric diseases, with knock-on effects for the whole herd. Mortality in piglets in the weaning and post-weaning phases during rotavirus outbreaks may be as high as 10%[8]. These viruses can induce neonatal diarrhea, causing dehydration, stress, weight loss, develop mental retardation and loss of earnings, predisposition to other illnesses, and death[3].

In Southern Brazil, estimates of the prevalence of rotaviruses in piglets herds affected by frequent episodes of gastroenteritis ranged from 20.5% to 90%[9]. In addition to their effects on these farm animals, a number of studies have indicated that porcine rotavirus may constitute a potential source of interspecies transmission involving humans[10,11,12,13].

Rotaviruses belong to the *Rotavirus* genus, which is a member of the *Reoviridae* family. These viruses measure approximately 70 nm, they have icosahedral symmetry, non-enveloped surrounded by a double-shelled capsid with three layers: Core, enclosing 11 double-stranded (ds) RNA segments; middle layer (VP6) and outer layer (VP7 and VP4). The inner shell consists of VP6 and the outer shell is composed of VP4 and VP7, which are independent neutralization antigens. They are classified in serogroups based on the serological cross-reactivity of VP6 protein or migration pattern of the genomic segments by polyacrylamide gel electrophoresis (electropherotype). Eight groups are recognized, and are denominated A to H. Group A, B, and C rotaviruses have been associated with diseases in humans and animals[14,15,16]. Groups D to G have been detected only in animals and recently the group H was associated with infection in humans[16,17]. The two outer capsid proteins, VP4 and VP7, which independently elicit neutralizing antibodies and induce protective immunity thus rotavirus strains were classified as P (for protease-sensitive) and G (for glycoprotein) genotypes. So far, 27 G and 37 P genotypes have been identified in humans and animals. More recently, novel VP7 genotypes were discovered (G20–G27), VP4 (P[28]–P[37]), VP6 (I12–I16), and other animals have been identified with RV strains like South american camelids (guanaco and vicuña)[18,19].

Twelve G genotypes (G1 to G6, G8 to G12, and G26) and 13 P genotypes (P[1], P[5] to P[8], P[11], P[13], P[19], P[23], P[26], P[27], P[32], and P[34]) have been associated with pigs[18,20,21,22]. The most common G genotypes identified in swine are G3, G4, G5, and G11 associated with P[6] and P[7][23].

The porcine group A rotaviruses (PoRV-A) cause endemic or epidemic enteritis in nursing and weaned piglets, and are a major reservoir of genetic material for the diversification of human RV-A[22]. There are some indirect evidences that PoRV-A are able to cross the interspecies barrier and infect humans and other domesticated animals[24,25,26]. Data accumulation on genetic heterogeneity of porcine rotaviruses will be fundamental to identify uncommon human strains[22].

The aim of this study was to identify rotavirus G and P genotypes obtained from piglets reared at five places in the metropolitan region of Belém, in Northern Brazil.

MATERIALS AND METHODS

STUDY AREA

Samples were collected from five piggeries in the metropolitan region of Belém, Pará State. The present study was approved by the Animal Research Ethics Committee (CEPAN) of Instituto Evandro Chagas under registration number 0022/2008. Fecal samples were collected from at least 30% of the piglets in each herd. None of the piglets were vaccinated against rotaviruses. Samples were obtained from animals of two age groups – nursing (0-28 days) and weaning (29-56 days).

The piggeries analyzed in the present study were located in different areas from metropolitan region of Belém, and each one presented specific characteristics. Piggery 1 is located in a rural university in the urban area. Piggery 2 is on the suburb of the urban area, in a federal rural institution. Piggery 3 is on a farm in a rural zone where the installations are in a satisfactory condition: a large space between pigs allocation, a designed area arranged the animals by age, proper food and hygiene practices to get an intensive swine production, vaccines and supplementary medicines according to the Brazilian laws. In that farm there was a close contact between animals and humans. Piggery 4 is also located in the rural zone, presented the worst conditions of hygiene. Piggery 5 is located in another rural college, has the best installations and hygiene.

SAMPLES

The fecal samples were collected by anal stimulation using a silicone urethral sound - size number 10 (Mark Med, Bragança Paulista, Brazil) lubricated with vaseline, which was used for anal massage, following the procedure described by Kroeff[9]. The samples were placed in a sterile container without device for conservation. The vial was kept in a styrofoam-box with ice until being received at laboratory. The consistency of the feces was determined in one of these three categories – diarrheic, pasty or normal. The samples were collected between April, 2008, and May, 2009, and each farm was visited twice during that period. Fecal specimens were collected from a number of other domestic animals (goats and poultry).

IMMUNOCHROMATOGRAPHY

The fecal samples were screened for human RV-A using an immunochromatography kit (Rota-Strip, BioConcept, Belgium) according to the protocol specified by the manufacturing company.

VIRAL NUCLEIC ACID EXTRACTION AND POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

The suspensions were prepared in 20% Tris/Ca^{++} and clarified by centrifugation at 10,500 × g for 20 min at 4° C. The supernatant was retrieved for the extraction of nucleic acid (dsRNA), which was obtained by using the technique described by Boom et al[27]. Then the rotavirus groups were identified using PAGE[8,9].

DETERMINATION OF THE G AND P ROTAVIRUS GENOTYPES BY NUCLEOTIDE SEQUENCING

The RV-A were amplified by RT-PCR using specific primers for the VP4 and VP7 genes. The primer sets were Con2/Con3[28] and Beg 9/End 9[29]. Reverse transcription was conducted with SuperScriptTM (Invitrogen, Carlsbad, CA), and recombinant Taq DNA polymerase (Invitrogen) was used to amplify the cDNA, resulting in sequences of 876 bp and 1,062 bp, corresponding to genes encoding the VP4 and VP7 proteins, respectively. All PCR products were purified using a QIAquick® PCR purification kit (Qiagen, Hilden, Germany).

The PCR products were purified, resuspended in formamide and sequenced automatically using the same primers as in RT-PCR. Sequencing was carried out by the dideoxynucleotide chain termination method on an ABI Prism 3100 automatic sequencer (Applied Biosystems, Foster City, USA), using the ABI Prism Big Dye Terminator cycle sequencing Ready Reaction Kit (Applied Biosystems). The sequences obtained for the VP4 and VP7 genes were assembled and analyzed with the BioEdit Sequence Alignment Editor (version 7.2.5). Phylogenetic analysis was performed in MEGA (version 6.0.6). Distances between sequences were analyzed using the neighbor-joining algorithm based on the Kimura two-parameter distance method to estimate the number of nucleotides[30]. The second approach was the maximum parsimony method with heuristic or branch-and-bound searches. Bootstrap resampling (over 1,000 replicates) was performed for the neighbor-joining analyses of the sequences of both genes. Comparative analyses with the prototype strains for the VP4 and VP7 genes were conducted out using the GenBank at the National Center for Biotechnology Information, USA (www.ncbi.nlm.nih.gov), by using BLAST and nucleotide searches.

NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

The nucleotide sequence data reported in this paper were submitted to GenBank with the following accession numbers (VP7 gene): JX103948, JX103949, JX103950, JX103951, JX103952, JX103953, JX103954, JX103955, JX103956, JX103957, JX103958, and JX103959, and (VP4 gene) JX103944, JX103945, JX103946 and JX103947.

RESULTS

DETECTION OF ROTAVIRUSES

A total of 172 fecal samples were collected from the piglets of five sample herds, which 17 (9.9%) were positive for rotaviruses in immunochromatography and PAGE techniques (Table 1). All positives samples showed long electropherotype pattern, came from piggery 5 and collected in two different periods from nursing piglets (< 30 days of age) fed on gilt's milk.

Most of the samples (93/172; 54.7%) were diarrheic in consistency, while just over a quarter (49/172; 27.9%) was normal, and the remainder (30/172; 17.4%), pasty. The consistency of the positive samples followed virtually the same pattern, with 53% diarrheic (9/17), 23.5% (4/17) pasty, and the same proportion to normal consistency (Table 2). The most common symptoms presented by the positive piglets were fever and prostration. Nine samples were obtained from other animals (eight goats at piggery 5 and a chicken from site 3), although PoRV-A was not detected in any of these specimens.

Table 1 – PoRV-A detected in fecal specimens collected from five piggeries in metropolitan area of Belém, Pará State, Brazil

Piggeries	Collection	Number of samples	Total samples	Positive %	Negative
1	1st collection	7	14 (8.1%)	–	7
	2nd collection	7		–	7
2	1st collection	10	20 (11.6%)	–	10
	2nd collection	10		–	10
3	1st collection	33	47 (27.3%)	–	33
	2nd collection	14		–	14
4	1st collection	15	23 (13.4%)	–	15
	2nd collection	8		–	8
5	1st collection	26	68 (39.5%)	5 (19.2%)	21
	2nd collection	42		12 (28.6%)	30
Total			172 (100%)	17 (9.9%)	155

–: Numeric data are not equal to zero due to rounding.

Table 2 – Consistency of fecal samples and animals ages according to the presence of PoRV-A

SAMPLES	WEANING			Total	NURSING			Total
	Diarrheic	Pasty	Normal		Diarrheic	Pasty	Normal	
Positives	–	–	–	–	9 (53%)	4 (23.5%)	4 (23.5%)	17
Negatives	22 (32.3%)	27 (39.7%)	19 (28%)	68	24 (27.9%)	29 (33.7%)	34 (38.4%)	87
Total	22 (32.3%)	27 (39.7%)	19 (28%)	68	33 (31.7%)	33 (31.7%)	38 (36.6%)	104

–: Numeric data are not equal to zero due to rounding.

GENOTYPING OF POSITIVE SAMPLES BY NUCLEOTIDE SEQUENCING

Twelve of the seventeen positive samples (70.5%) yielded an adequate amplicon for G genotype sequencing. Based on these sequences, the most frequent genotype was G3, that accounted for 53% (9/17) of the positive isolates and had amino acid sequences 96.4% to 96.5% similar to TJ3-2 strain, a G3 type PoRV-A found in Japanese pigs[31]. The remainder of these positive samples (3/17; 17%) presented G5 genotype, and VP7 analysis revealed a 96.9% amino acid sequence similar to the human G5 strain IAL-28, and a 96.5% match with the rj40644/90 strain, a second human G5 strain found in Brazil (Figure 1).

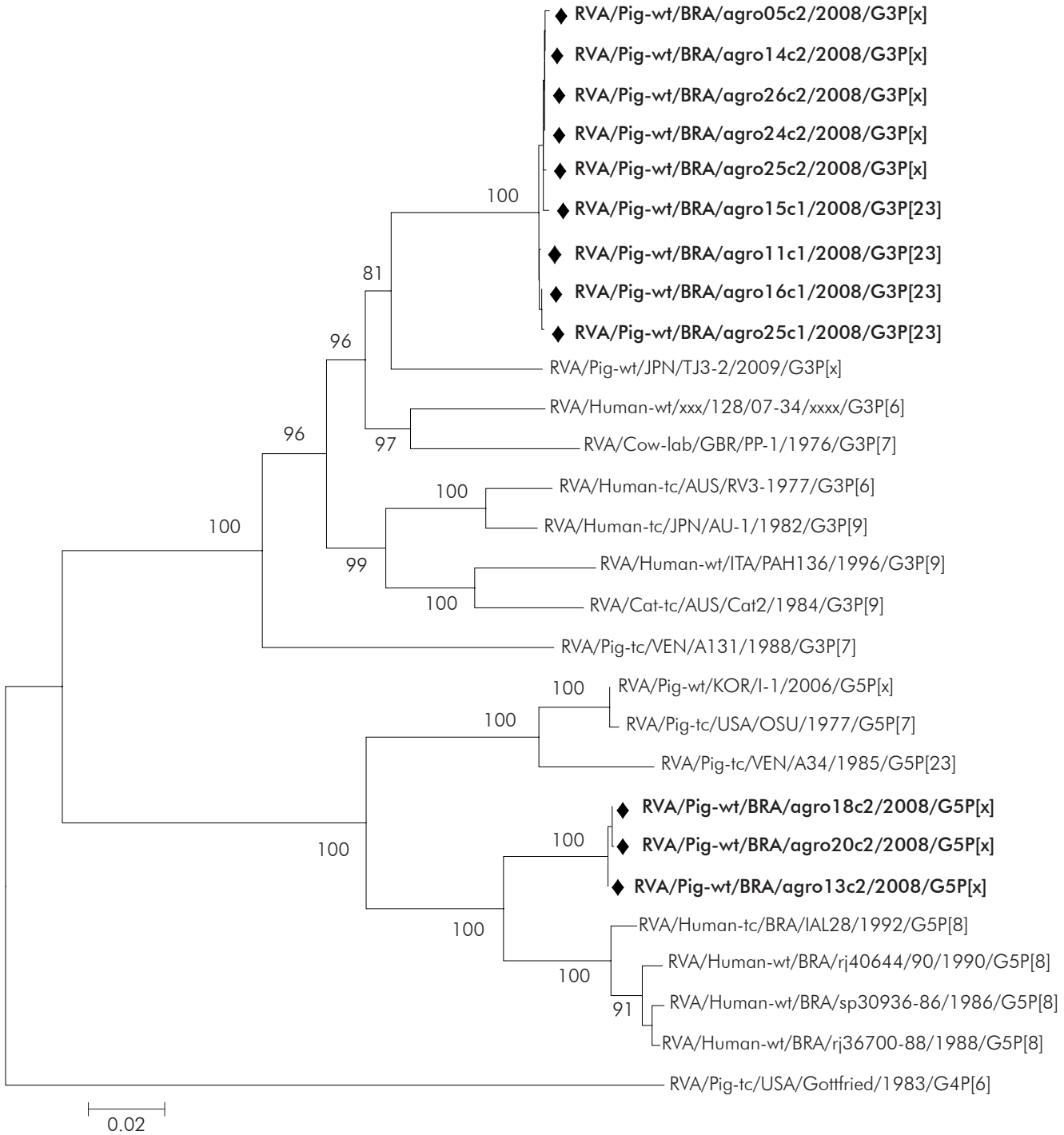

Figure 1 – Phylogenetic tree of the full-lenght VP7 amino acid sequences displaying the relationship between PoRV-A Belém's strains and representative strains of all VP7 genotype recognized to date. Rotavirus strains analyzed in this study are in bold

With regards to the VP4 gene, it was identified P[23] genotype in four positive samples (4/17; 23.5%) with amino acid sequences most closely related (95.9-96.5%) to the Hokkaido-14 strain (Japan), also found in pigs (Figure 2). In the remainder of samples (13/17; 76.5%) it was not possible to determine the VP4 gene sequence due to inadequate amplicon. Of note, further studies will be carried out aiming to identify the untyped genotypes. All four (23.5%) samples were G3P[23] combination.

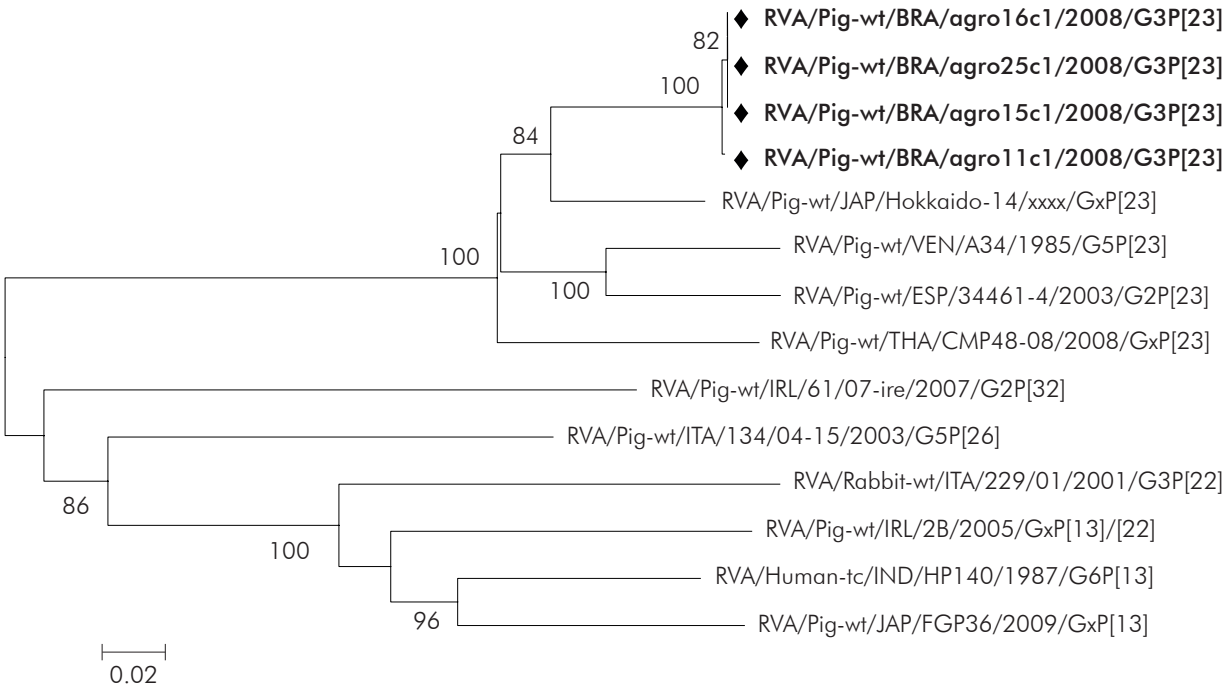

Figure 2 – Phylogenetic tree of the partial VP4 amino acid sequences displaying the relationship between PoRV-A Belém's strains and representative strains of all VP4 genotype recognized to date. Rotavirus strains analyzed in this study are in bold

DISCUSSION

It was the first study conducted in Northern Brazil about swine where the occurrence of rotaviruses was confirmed with an infection rate of 9.9%. In Southern Brazil, Kroeff[9] has recovered PoRV-A from 35.3% of the nursing/weaning piglets sampled. This comparatively high infection rate was found even with the best management and hygiene conditions, and productivity in compared to the facilities analyzed in the present study. The low rate of infection with PoRV-A (9.9%) recorded in this study could be related to the hot weather, management practices or, probably, the small number of piggeries sampled.

In Southern and Southeastern Brazil, an extensive study of nursing piglets recorded a prevalence of 28.3% PoRV-A in stool with normal to diarrheic consistency (81.3% diarrheic)[32]. Gatti et al[1] also found a high rate of PoRV-A (84.6%) in diarrheic episodes. Alfieri et al[33] observed PoRV-A in 96.5% of diarrheic pigs. The consistency of the fecal samples collected during the present study varied from normal (23.5%) to pasty (23.5%), although most of the samples (52.3%) were diarrheic ones.

Jacobson et al[3] detected symptoms such as diarrhea, vomiting, dehydration, and anorexia in pigs infected naturally by PoRV-A. The main symptoms presented by piglets from Belém were diarrhea, fever, prostration, anorexia and dehydration, but vomiting was not observed.

In Venezuela, Liprandi et al[34] found PoRV-A in 4-6 week old piglets. In Denmark, PoRV-A was predominant in 60% of 4-week old piglets[35]. Alfieri et al[33] demonstrated that porcine rotaviruses are amply distributed in Brazilian swine herds of 3-5 weeks of age. A similar pattern was observed in the present study with PoRV-A recorded in piglets of 0-4 weeks of age. The mother's milk plays an important role in protecting the piglet against infections such as rotaviruses, which tend to occur at higher rates in weaned piglets[33,34,36].

In Ireland, Collins et al[22] identified PoRV-A in 6.5% of asymptomatic piglets (no diarrhea) of over 4 weeks of age and Lorenzetti et al[37] recorded an outbreak of PoRV-A in piglets of suckling and weaned ages in a vaccinated herd, with a frequency of 27.3% from asymptomatic individuals. Asymptomatic pigs of this study represented 23.5% of positive samples and 2.32% of the total samples, there was no significant difference ($p = 0.41$) when it compared rates of PoRV-A frequency between asymptomatic or symptomatic pigs. Age differences should also be taken into account, however, because the animals may develop PoRV-A antibodies as they age, so symptoms such as diarrhea may be less frequent in older animals.

Miyazaki et al[38] have reported outbreaks of PoRV-A in a large farrow-to-finish piggery in Japan during 2009 and 2010. P[23] genotype occurred at a higher rate (61.5%) when comparing with the present study (23.5%). In the case of G genotypes, G3 – the most common in Belém (53%) – was recorded in only 13.3% of the cases from Japan, while G5 was recorded only once (6.6%). While P[23] genotype was combined with G3 was in this study, all the cases of P[23] from Japan were combined with G9 which was not recorded in the present study. In China, a rare P[23] genotype was also found in association with G9 in 2008, showing a high degree of nucleotide homology (98.4%) when compared with Brazilian PoRV-A strains (data not shown)[31].

Lorenzetti et al[37] registered an outbreak of PoRV-A in suckling and weaned piglets from a vaccinated pig herd against G4 and G5 genotypes. The frequency of infection was 72.7% in diarrheic samples and 27.3% in those of asymptomatic piglets. The main P genotype was P[6], which is of human origin. While the vaccine protected against the principal symptoms of the rotaviruses, considerable antigenic diversity of P[6] may reduced the vaccine effectiveness. This effect can be associated with immune pressure produced by developing of mass vaccination and not specifically to failure vaccination[39]. The piglets in the present study in Belém were infected by G3P[23] and G5[not typed] genotypes and did not receive the rotavirus vaccine for animals. As in the study of Lorenzetti et al[37], the positive cases recorded in the present study were collected from asymptomatic animals.

In Southern and Southeastern Brazil several studies have shown the diversity PoRV-A genotype which are relatively common in pigs. Barreiros et al[40] isolated G3P[7] and G5P[7] strains. Rácz et al[41] identified the G3, G4, G5, P[6], and P[7] types. Gregori et al[42] reported the G5P[6] genotype as the most frequent type and Kroeff[9] recorded G5, G3, G4, G10 and G9 strains. While G9 was not recorded in studies from Belém, G3 and G5 were found to be common genotypes in piglets. The P[6] genotype was predominant in all the studies from Southern Brazil[9,35,42]. Tonietti et al[43] related the first detection of P[23] genotype in Brazil associated with G5 in samples that were collected in 2011, however P[23] genotype of this present study were collected in 2008, showing that this P-type has circulated before in Northern Region, furthermore this is the first reported case of G3P[23] combination in Brazil.

The phylogenetic analysis of the full amino acid sequences of the VP7 genes of the three G5 samples identified in the present study clustered closely (96.5-96.9% similarity) with the human IAL-28 and rj40644/90 strains[44,45]. The amino acid sequences of the nine G3 samples were also very close (96.4-96.5%) to the TJ3-2 strain found in Japanese pigs.

Four of the five piggeries investigated in the present study in Belém were in a technically satisfactory conditions and the only positive test results were obtained from these conditions of rearing pigs. The distances between the sampled places (or in any other place) in this present study, probably did not have influence in the results. Vaccination of piglets and gilts is the most effective way of protecting those animals against rotavirus because gilts can produce antibodies should pass on to the piglets by the colostrums[46]. Dewey et al[2] has reported that changes in management practices, such as the expansion of the installations, early weaning, and all-in all-out production, modified the frequency of PoRV-A in a herd in Ontario, Canada. Other factors may contribute to an increased incidence of rotaviruses, such as low relative humidity, high population density, and the density of primiparous females[35].

CONCLUSION

This study showed the circulation of rotaviruses in swine herds in the metropolitan region of Belém, in Northern Region, and to our knowledge. G3P[23] combination was recorded for the first time in Brazil.

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Identificação de genótipos G e P de rotavírus em leitões em fase de amamentação e desmamados na região metropolitana de Belém, Estado do Pará, Região Norte do Brasil

RESUMO

Os rotavírus são um importante agente de diarreia em leitões, principalmente durante a amamentação e pós-desmame com gravidade. O objetivo deste estudo foi identificar os genótipos G e P de rotavírus obtidos de leitões de cinco criadouros em Belém, Estado do Pará, Norte do Brasil. Espécimes fecais foram coletados de leitões em fase de amamentação e desmamados. Foi testado um total de 172 amostras, das quais 17 (9,9%) foram positivas para rotavírus do grupo A, a partir de leitões amamentados e, em seguida, foram sequenciadas pela genotipagem do VP7 e VP4. A consistência das amostras de fezes positivas foi de 53% (9/17) diarreico, 23,5% (4/17) pastosa e 23,5% (4/17) normal. O genótipo G mais comum foi o G3 com 53% (9/17), seguido pelo genótipo G5 (17%, 3/17). O genótipo P registrado foi de P[23] que corresponde a 23,5% (4/17). Este estudo mostrou a circulação dos rotavírus em rebanhos suínos na região metropolitana de Belém, na Região Norte. A combinação de G3P[23] foi registrada pela primeira vez no Brasil.

Palavras-chave: Rotavirus; Leitões Desmamados; Rebanhos e genótipo G3P[23].

Identificación de genotipos G y P de rotavirus en lechones en fase de amamantamiento y destetados en la región metropolitana de Belém, Estado de Pará, Región Norte de Brasil

RESUMEN

Los rotavirus son un importante agente de diarrea en lechones, principalmente durante el amamantamiento y el pos destete con gravedad. El objetivo de este estudio fue el de identificar los genotipos G y P de rotavirus obtenidos de lechones de cinco criaderos en Belém, Estado de Pará, Norte de Brasil. Fueron colectados especímenes fecales de lechones en fase de amamantamiento y destetados. Se hicieron pruebas a un total de 172 muestras, de las cuales 17 (9,9%) fueron positivas para rotavirus del grupo A, a partir de lechones amamantados y, enseguida, se secuenciaron por el genotipado del VP7 y VP4. La consistencia de las muestras de heces positivas fue de 53% (9/17) diarreico, 23,5% (4/17) pastoso y 23,5% (4/17) normal. El genotipo G más común fue el G3 con 53% (9/17), seguido por el genotipo G5 (17%, 3/17). El genotipo P registrado fue de P[23] que corresponde a 23,5% (4/17). Este estudio mostró la circulación de los rotavirus en rebaños porcinos en la región metropolitana de Belém, en la Región Norte. La combinación de G3P[23] se registró por primera vez en Brasil.

Palabras clave: Rotavirus; Lechones Destetados; Rebaños y genotipo G3P[23].

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