ORIGINAL RESEARCH PEER REVIEWED

Porcine rotavirus groups A, B, and C identified by polymerase chain reaction in a fecal sample collection with inconclusive results by polyacrylamide gel electrophoresis

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Summary

Objective: To evaluate the frequency of groups A, B, and C rotaviruses in diarrheic pigs from Brazilian pig herds.

Materials and methods: Fecal samples with inconclusive results when tested by the silver-stained polyacrylamide gel electrophoresis (ss-PAGE) technique were selected for this study (n = 144). Rotavirus A (VP4 and VP7 genes), rotavirus B (NSP2 gene), and rotavirus C (VP6 gene) were identified by reverse transcription-polymerase chain reaction (RT-PCR).

Results: Of the 144 fecal specimens analyzed by RT-PCR assays, 103 (71.5%) were positive for rotavirus infection. Single infections were detected in 58 samples (40.3%), with 34 (23.6%), 19 (13.2%), and 5 (3.5%) identified as rotavirus groups A, B, and C, respectively. Mixed infections with two and even three rotavirus groups were identified in 45 fecal samples (31.2%). Rotaviruses B and C were more frequently identified in mixed (65.2%) than in single infections.

Implications: The inclusion criteria for sample selection and use of RT-PCR assays for diagnosis in this study contributed to the higher frequencies of rotaviruses B and C, which are sporadically implicated in porcine neonatal diarrhea. The high rate of diagnosis of atypical rotavirus showed that rotaviruses B and C, as well as rotavirus A, are disseminated in Brazilian pig herds. These results suggest that failure to identify porcine rotaviruses B and C in diarrheic fecal samples is primarily due to use of diagnostic methods of low sensitivity, and not to low prevalence of infection.

Keywords: swine, neonatal diarrhea, group A rotavirus, atypical rotaviruses, reverse transcription-polymerase chain reaction

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Resumen - Rotavirus porcinos de los grupos A, B, y C identificados por medio de la reacción en cadena de polimerasa en muestras fecales con resultados inconclusos probados mediante electroforesis de gel de poliacrilamida

Objetivo: Evaluar la frecuencia de rotavirus de los grupos A, B, y C en cerdos diarreicos de hatos brasileños.

Materiales y métodos: Para este estudio se seleccionaron muestras fecales con resultados inconclusos (n = 144) bajo la técnica de tinción de plata de electroforesis de gel de poliacrilamida (ss-PAGE por sus siglas en inglés). Al probar las mismas muestras mediante la prueba de reacción en cadena de la polimerasa de transcriptasa reversa (RT-PCR por sus siglas en inglés), se identificaron rotavirus A (genes VP4 y VP7), rotavirus B (gen NSP2), y el rotavirus C (gen VP6).

Resultados: De los 144 especímenes fecales analizados con la prueba de RT-PCR, 103 (71.5%) resultaron positivos a la infección de rotavirus. Se detectaron infecciones individuales en 58 muestras (40.3%), con 34 (23.6%), 19 (13.2%), y 5 (3.5%) de los grupos A, B, y C de rotavirus, respectivamente. En 45 muestras fecales (31.2%), se identificaron infecciones mixtas con dos y hasta tres grupos de rotavirus. Los rotavirus B y C se identificaron más frecuentemente en infecciones mixtas (65.2%) que en individuales.

Implicaciones: El criterio de inclusión para la selección de muestras y el uso de la prueba de RT-PCR para diagnóstico en este estudio, contribuyeron a obtener frecuencias más altas de rotavirus B y C los cuales se implican esporádicamente en la diarrea neonatal porcina. El alto índice de diagnósticos de rotavirus atípicos mostró que los rotavirus B y C, así como el rotavirus A, están diseminados en los hatos de cerdos brasileños. Estos resultados sugieren que la falta de identificación de rotavirus porcinos B y C en muestras fecales diarreicas se debe básicamente a la utilización de métodos de diagnóstico de baja sensibilidad, y no a la baja prevalencia de la infección.

Résumé - Rotavirus porcins des groupes A, B, et C identifiés par réaction d'amplification en chaîne par la polymérase dans une série d'échantillons fécaux avec des résultats non concluants par électrophorèse en gel de polyacrylamide

Objectif: Évaluer la fréquence des rotavirus des groupes A, B, et C chez des porcs diarrhéiques dans des troupeaux brésiliens.

Matériels et méthodes: Des échantillons de fèces avec des résultats non concluants lorsque testés par électrophorèse en gel de polyacrylamide avec coloration à l'argent (ss-PAGE) ont été sélectionnés pour la présente étude (n = 144). Les rotavirus A (gènes VP4 et VP7), rotavirus B (gène NSP2), et rotavirus C (gène VP6) ont été identifiés par réaction

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d'amplification en chaîne par la polymérase à l'aide de la transcriptase réverse (RT-PCR).

Résultats: Parmi les 144 échantillons de fèces analysés par épreuves RT-PCR, 103 (71.5%) étaient positifs pour l'infection par le rotavirus. Des infections uniques ont été détectées dans 58 échantillons (40.3%), et ont été identifiées comme étant associées à des rotavirus du groupe A, B, et C dans respectivement 34 (23.6%), 19 (13.2%), et 5 (3.5%) échantillons. Des infections mixtes avec deux et même trois groupes de rotavirus ont été identifiées dans 45 échantillons de fèces (31.2%). Les rotavirus B et C étaient plus fréquemment identifiés dans les infections mixtes (65.2%) que dans les infections uniques.

Implications: Dans la présente étude, les critères d'inclusion pour la sélection des échantillons et l'utilisation d'épreuves de RT-PCR pour le diagnostic ont contribué au fait de trouver des fréquences plus élevées de rotavirus B et C, virus qui sont impliqués sporadiquement lors d'épisodes de diarrhée néonatale porcine. Le taux élevé de diagnostic de rotavirus atypiques a démontré que les rotavirus B et C, de même que les rotavirus A, sont disséminés dans les troupeaux porcins brésiliens. Ces résultats suggèrent que le fait de ne pas identifier les rotavirus porcins B et C dans des échantillons de fèces diarrhéiques est dû principalement à l'utilisation de méthodes diagnostiques de faible sensibilité, et non à une prévalence basse de l'infection.

Neonatal diarrhea is a major endemic health problem for pig herds worldwide due to its economic impact.[1,2] Diarrhea in piglets is a multifactorial and multi-etiological disease. Management failure might play an important role in the development of the disease, as well as infection by microorganisms such as bacteria, protozoa, and viruses.[3-5]

Worldwide, rotavirus is considered one of the main causes of diarrhea in young animals of several species.[6] Rotavirus, a member of the Reoviridae family, is characterized by double-stranded RNA (dsRNA) with 11 genomic segments, a capsid composed of three concentric protein layers, and the absence of an envelope.[7] Classification of rotaviruses into seven distinct serologic groups designated A to G is based on the main protein (VP6) of the intermediary capsid layer.[6] Group A rotaviruses are most often responsible for diarrhea in piglets.[8-11] Due to the difficulty in diagnosis of diarrhea caused by other rotavirus groups, nongroup A rotaviruses are rarely reported.[12-14] Atypical rotaviruses, such as groups B and C, may also be responsible for episodes of diarrhea in piglets.[15,16] Serological evidence indicates that atypical rotavirus infections in pigs are frequent. In adult animals, seroconversion rates of 80% to 100% for rotavirus C have been reported. However, only a few studies include identification of rotaviruses B and C in episodes of diarrhea in piglets.[15,17,18]

Several techniques have been developed to diagnose rotavirus infection, identifying the viral particle (cell culture, electron microscopy, immune electron microscopy), antigens (enzyme-linked immunosorbent assay, latex agglutination, direct immunofluorescence), and the viral genome (silver-stained polyacrylamide gel electrophoresis [ss-PAGE] and reverse transcription-polymerase chain reaction [RT-PCR]).[19-24] The ss-PAGE technique identifies rotavirus groups by analyzing the migration pattern of the 11 dsRNA segments.[24,25]

It is more difficult to identify rotavirus groups B and C than group A in fecal samples because of the lower quantity of group B and C viral particles shed during the acute period of the infection.[26,27] In addition, commercial kits are not available for diagnosis of groups B and C. Techniques of low sensitivity, such as ss-PAGE, may generate false-negative results, and the low intensity of dsRNA segments after silver staining may not allow analysis of the electrophoretic profile and identification of the electropherotype, thus giving inconclusive results.[12]

More sensitive diagnostic techniques, such as RT-PCR, can detect rotavirus groups B and C more efficiently in single or mixed infections, reflecting more precisely the real infection rate of nongroup A rotavirus and furthering knowledge of the epidemiology of infection.[12]

The aim of this study was to determine the frequency of diagnosis of rotavirus groups A, B, and C in a collection of pig fecal samples previously analyzed by ss-PAGE with inconclusive results.

Materials and methods

Fecal samples and inclusion criteria

In this study, 144 fecal samples from pigs with diarrhea were evaluated. These samples were part of a pig fecal sample collection, comprising 588 samples from pigs 1 to 4 weeks old, that had been included in a neonatal-diarrhea epidemiological study. The 588 fecal samples were collected between June 2005 and June 2007 in 115 pig herds situated in four states (Paraná, Santa Catarina, Rio Grande do Sul, and Mato Grosso do Sul) in two geographical regions in Brazil (south and central-west), considered to be the main Brazilian pig-producing regions (Table 1). In all herds, good management practices were used, including multi-site production and all-in, all-out management. However, at the time when the fecal samples were collected, none of the evaluated farms used a rotavirus group A vaccination program for prophylaxis of porcine neonatal diarrhea.

Pooled fecal samples were collected from each pen, with each pool representing a litter in the farrowing unit (≤ 3 weeks old) or a group of weaned pigs in the nursery (4 weeks old). The samples were transported to the laboratory on ice and stored at -80°C. Porcine rotavirus was diagnosed using the ss-PAGE technique as described by Herring et al[24] and Pereira et al.[25] This technique identified 155 positive results (26.36%), 289 negative results, (49.15%), and 144 inconclusive results (24.49%). Polyacrylamide gel bands (dsRNA segments) for samples with inconclusive results were of low intensity or in anomalous positions or there were extra bands, and some samples were of undefined electropherotype. These 144 diarrheic fecal samples were included in the present study to evaluate the presence of rotavirus groups A, B, and C by RT-PCR assays.

RNA extraction

Fecal suspensions of 20% (w/v) in Tris-Ca^{++} buffer (50 mM Tris-HCl; 10 mM NaCl; 1.5 mM 2-mercaptoethanol; 3 mM $CaCl_2$) were centrifuged at 3000*g* for 15 minutes at 4°C. Aliquots of 450 µL of the supernatant were treated with sodium dodecyl sulfate to a final concentration of 1% and were incubated for 30 minutes at 56°C. The nucleic acid extraction was performed by a combination of phenol, chloroform, isoamyl alcohol (25:24:1) and silica-guanidine isothiocyanate methods,[10] and RNA was eluted in 50 µL of ultrapure sterile water treated with diethyl pyrocarbonate (Invitrogen Life Technologies, Carlsbad, California) and used for RT-PCR.

Detection of group A, B, and C rotaviruses

Detection of rotavirus group A was performed by RT-PCR assay with consensus primers for amplification of an 876-bp

Table 1: Origin of fecal samples collected from pigs either in the farrowing unit (≤ 3 weeks old) or in the nursery (4 weeks old) and analyzed by the ss-PAGE technique for porcine rotavirus diagnosis*

State	Counties	Herds	Samples
Paraná†	23	51	234
Santa Catarina†	13	20	143
Rio Grande do Sul†	31	41	187
Mato Grosso do Sul‡	1	3	24
Total	68	115	588

* The 588 samples were collected in 115 commercial pig herds situated in four states (Paraná, Santa Catarina, Rio Grande do Sul, and Mato Grosso do Sul) in the main pig-producing regions of Brazil. Pooled fecal samples represented either one litter in the farrowing unit or one pen in the nursery.

† South area of Brazil.

‡ Central-west area of Brazil.

ss-PAGE = silver-stained polyacrylamide gel electrophoresis

product from the VP4 gene (P type), and a 1062-bp fragment from the VP7 gene (G type), as described by Gentsch et al[23] and Gouvea et al,[22] respectively. Atypical rotaviruses were identified by a semi-nested (SN)-PCR assay with amplification of a 434-bp product from gene 8 (NSP2) of rotavirus group B[12] and an RT-PCR assay for amplification of a 270-bp amplicon of rotavirus group C gene 5 (VP6).[28] All fecal samples were tested using the three PCR assays. The Ohio State University strain of porcine rotavirus amplified in MA104 cells was used as the positive control for rotavirus group A.[29] Due to the difficulty in adapting strains of rotavirus groups B and C in cell culture, two porcine fecal samples with electropherotype characteristics of group B or C were used as positive controls. The specificities of controls positive for rotavirus groups B and C were also confirmed by sequence analysis.[13,14] An aliquot of ultrapure water was included in each reaction as a negative control.

Analysis of amplified products

Aliquots of 10 µL from the products of RT-PCR (groups A and C) and SN-PCR (group B) reactions were subjected to ethidium bromide-stained (0.5 µg per mL) agarose gel electrophoresis at 2%, in Tris-borate-EDTA buffer, pH 8.4 (89 mM Tris; 89 mM boric acid; 2 mM EDTA), under constant voltage (100 V) for 30 minutes and visualized under UV light.

Sequencing

The specificities of three and nine products amplified in the reactions for rotavirus groups B and C, respectively, were evaluated by direct sequencing of amplicons in both directions (forward and reverse). Products were purified using commercial kits GFX PCR DNA and Band Purification (GE Healthcare, Little Chalfont, United Kingdom) and then sequenced with the kit DYEnamic ET Dye Terminator (GE Healthcare) in an automatic sequencer (MegaBACE 1000; GE Healthcare).

Sequence quality analysis was performed using Phred and CAP3 software (**http://genoma.cenargen.embrapa.br/phph/**). Similarity searches were performed with sequences deposited in GenBank using BLAST software (**http://www.ncbi.nlm.nih.gov/BLAST/**). A multiple alignment and an identity matrix were produced in BioEdit version 7.0.5.3 (Ibis Therapeutics, Carlsbad, California).

Statistical analysis

The association between rotavirus group and pig age (by week) was determined by Fisher exact test, with confidence limits of 95% and $P < .05$ considered statistically significant, in Epi Info version 3.5.2. (**http://wwwn.cdc.gov/epiinfo/html/downloads.htm**).

Results

The RT-PCR assays performed on the collection of 144 diarrheic fecal samples with inconclusive results by ss-PAGE analysis enabled identification of group A, B, and C rotaviruses from pigs of all geographical regions included in this study. Both single infections, where only rotavirus group A, B, or C was identified, and mixed infections, where more than one rotavirus group was identified in the same fecal sample, were detected. Forty-one samples were negative for the three rotavirus groups evaluated. Table 2 shows the distribution of groups A, B, and C rotaviruses identified in single and mixed infections. There was no association ($P > .05$) between pig age and prevalence of infection with a specific rotavirus group. Single and mixed group A, B, and C infections occurred in all age groups.

Sequence analyses from 12 amplified products (three from rotavirus group B and nine from group C) confirmed that the amplified products were specific for their respective rotavirus groups.

Discussion

In ss-PAGE analysis, it is common for some fecal samples to present extra bands and bands of low intensity or in anomalous positions or both.[12] In the fecal samples included in this study for which the ss-PAGE results were considered inconclusive, it was possible to identify rotavirus from groups B and C as well as group A. Of the 144 diarrheic fecal samples with inconclusive ss-PAGE results that were evaluated by molecular amplification techniques (RT-PCR and SN-PCR), only 41 (28.5%) were identified as negative. Identification of rotavirus groups A, B, and C both in single and mixed infections was possible in the remaining 103 (71.5%) fecal samples. The SN-PCR and RT-PCR assays used in this study have already been described as specific for rotavirus groups B and C, respectively. However, due to the low frequency of these porcine rotavirus groups in previous studies conducted in Brazil,[13,14] amplicons with high-intensity agarose gel electrophoresis bands and ethidium bromide visualization were selected to evaluate specificity.

Depending on the diagnosis technique used, a frequency of 0.5% for porcine rotavirus group B infection is reported in pig herds.[5] In this study, rotavirus group B was identified in 13.2% of samples in single infections and in 22.9% of samples in mixed infections (with groups A and C). These results show that the rotavirus group B infection rate in piglets may be higher than that reported in epidemiologic surveys using only the ss-PAGE technique.

Infection rates for rotavirus group C in piglet fecal samples using the ss-PAGE analysis

Table 2: Distribution of porcine rotaviruses detected by RT-PCR assays in 144 diarrheic fecal samples by infection type (single or mixed) and pig age*

Infection	Rotavirus group detected	No. of positive samples				Total (%)
		1 week old	2 weeks old	3 weeks old	4 weeks old	
Single	A	9	5	9	11	34 (23.6)
	B	8	6	2	3	19 (13.2)
	C	3	0	2	0	5 (3.5)
Mixed	A + B	7	4	2	4	17 (11.8)
	A + C	5	1	2	4	12 (8.3)
	B + C	3	2	0	0	5 (3.5)
	A + B + C	2	1	3	5	11 (7.6)
Subtotal	NA	37	19	20	27	103 (71.5)
Negative†	NA	13	4	13	11	41 (28.5)
Total	NA	50	23	33	38	144 (100.0)

* Source of sample collection described in Table 1. Pooled fecal samples represented either one litter in the farrowing unit (≤ 3 weeks old) or one pen in the nursery (4 weeks old). Samples were previously tested for porcine rotaviruses by ss-PAGE with inconclusive results.

† No rotavirus detected.

RT-PCR = reverse transcriptase-polymerase chain reaction; ss-PAGE = silver-stained polyacrylamide gel electrophoresis; NA = not applicable

are frequently reported at approximately 0.35%.[12] However, in the present study, 3.5% of diarrheic samples were positive for group C rotavirus in single infections, and group C was also identified in association with groups A and B in 19.4% of samples. The overall rate of group-C-positive samples (22.9%) shows that the rotavirus group C infection rate is also higher than previously described.

In the group of 144 samples with inconclusive ss-PAGE results, it was possible to demonstrate that rotavirus groups B and C occurred more often in mixed than in single infections. In Italy, Martella et al[16] also demonstrated that in piglets, group C infections associated with other viruses, such as rotavirus group A and porcine enteric calicivirus, were more frequent (26.1%) than were single infections (2.7%). Jeong et al[30] in South Korea, using an RT-PCR assay, found rotavirus group C in 26.3% of fecal samples analyzed, with 12.4% in single and 13.9% in mixed infections. In Brazil, mixed infections with rotavirus groups, rotaviruses, and porcine sapovirus, and rotaviruses and other enteropathogens, have also already been described.[28,31-33] The synergistic effects of viruses causing diarrhea in young animals and even of viruses and other enteropathogens contributing to more severe lesions and diarrhea have already been reported.[16,30]

Using a smaller number of piglet fecal samples (n = 34) initially analyzed by ss-PAGE for rotavirus, Alfieri et al[28] subsequently tested these samples using an RT-PCR assay associated with a digoxigenin-labeled oligonucleotide probe and reported a 50% greater rate of detection of group C in single and mixed infections. These results support the evidence that using more sensitive diagnostic techniques improves the accuracy of estimating real infection rates of atypical rotaviruses in pig herds.

In several countries, including Brazil, the importance of rotavirus group A in the etiology of diarrhea in suckling and recently weaned pigs is well characterized.[9-11,34,35] Due to the high viral load excreted during infection, low-cost techniques that are relatively simple to perform, such as ELISA, latex agglutination, and ss-PAGE, are routinely used to diagnose rotavirus group A infections in humans, animals, and avian species.[20,21,36] In this study, the frequency of diagnosis of rotavirus group A in single and mixed infections increased considerably with use of an RT-PCR assay with consensual primers for genes VP7 and VP4.

Classic genotypes of porcine rotavirus group A have been described as causing infection in children.[37] Some evidence also supports the hypothesis that groups B and C rotavirus of animal origin have zoonotic potential.[18,38,39] In humans and animals, diagnostic tests for atypical rotavirus are rarely included in laboratory routines. In this study, use of highly sensitive molecular techniques revealed a high frequency of infection by rotavirus groups B and C in diarrheic pigs 1 to 4 weeks old. Future studies examining use of these molecular techniques to detect heterologous infections by viral strains of animal and human origin may characterize the zoonotic potential of these atypical rotavirus groups.

The present study is the first large-scale epidemiological survey on the prevalence of porcine rotavirus groups B and C infections in diarrheic pigs from Brazil. The results demonstrate the high frequency of infections caused by group B and C rotaviruses. The high rate of fecal samples positive for group A rotavirus and atypical rotavirus found in a representative number of pig herds from four Brazilian states allow us to conclude that rotavirus groups A, B, and C infections are endemic and widespread in Brazilian pig herds.

Implications

- Use of the ss-PAGE technique alone for diagnosis of rotavirus infection in diarrheic pigs may generate many false-negative results.
- Under the conditions of this study, the rates of detection of rotavirus groups B and C by RT-PCR assay in fecal samples from 1- to 4-week-old pigs are much higher than previously described.
- The importance of rotavirus groups B and C in the porcine neonatal diarrhea complex needs to be clarified.

- Single and mixed infections with rotavirus groups A, B, and C are common in suckling and recently weaned pigs.
- Rotavirus groups A, B, and C infections are endemic and widespread in Brazilian pig herds.

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