Longitudinal study of rotavirus C VP6 genotype I6 in diarrheic piglets up to 1 week old

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Abstract

The reports of rotavirus C (RVC) involvement in diarrhea outbreaks in newborn piglets have been increasing in recent years. This longitudinal study, conducted over a 37-day period, aimed to evaluate the frequency of RVC infection in piglets aged up to 7 days obtained from a pig herd with a previous diagnosis of RVC infection in this age group. Piglets from 50 different litters were monitored daily for the occurrence of diarrhea, and all litters were classified into the following categories: sow parity order (PO) 1 to 5; litter size (LS) ≤ 10 piglets and > 10 piglets; and piglet birth weight (BW) 1.2 to 1.3 kg and > 1.3 to 1.4 kg. Two hundred six diarrheic fecal samples were collected and classified according to the fecal consistency score (pasty, semiliquid, liquid). Ten fecal samples were collected from asymptomatic piglets (control group). Fecal samples were screened for rotavirus (RV) by silver stained-polyacrylamide gel electrophoresis (ss-PAGE), and samples with inconclusive and negative-ss-PAGE results were submitted to RVC VP6 gene amplification by RT-PCR. RVC was identified in 71 (34.5%) samples, in 1 (10%) sample of the control group, and in piglets from 33 (66%) litters. The electrophoretic profile of RV species A was identified in only two samples. Of the 72 RVC-positive samples, 51 (70.8%) presented semiliquid or liquid consistency. There was no significant difference in either group regarding the production parameters (PO, LS, BW) evaluated. An analysis of the whole VP6 gene of three RVC field strains collected on the first, fifteenth, and last day of the experiment enabled us to identify genotype I6. This report describes the first longitudinal study examining epidemiological aspects of RVC infection in newborn piglets.

Keywords Pig · Newborn · Diarrhea · RVC · Epidemiology

Introduction

Neonatal diarrhea is a major sanitary problem in piglet production units and leads to substantial economic losses for all pork production chains. Although enteric infections can occur

throughout the nursing period, when the diarrhea episodes affect very young animals, that is, no more than 1 week old, they are more troubling due to the severe dehydration that results [1, 2]. Diarrhea is a syndrome triggered by several factors, such as host immunity, management procedures, and different classes of microorganisms, such as bacteria, viruses, and protozoa. Regarding viruses, rotavirus (RV) is recognized worldwide as the major etiological agent of diarrhea in suckling and recently weaned piglets [3–7].

Rotavirus A (RVA) diarrhea outbreaks in piglets during the first week of life have been described throughout the world, and RVA outbreaks are more frequent than episodes of diarrhea caused by other RV species [8, 9]. However, recently, reports of rotavirus C (RVC) involvement in outbreaks of enteric infection in newborn piglets have increased in pig herds from several countries, including Brazil [6, 10–12]. Due to the high morbidity rates and very young age of piglets, these outbreaks often increase the mortality rates in this period of life [5].

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Commercial diagnostic techniques for rapid RV identification, such as ELISA assay or latex agglutination, are specific for detecting RVA. Due to the difficulty of replication and isolation of nongroup-A RV in cell culture, there are no rapid diagnostic tests available commercially for detection of other RV species, such as RVC [13]. The lack of robust diagnostic systems that allow the simultaneous analysis of a large number of fecal samples causes the RVC outbreaks not to be identified or to be underreported in pig herds worldwide [8].

Most reports of diarrhea outbreaks in newborn piglets aged up to 7 days describe cross-sectional studies with retrospective or prospective characteristics [5, 10, 14]. Considering the increased frequency of RVC diarrhea outbreaks reported in very young piglets, this study, conducted over a 37-day period, aimed to evaluate the frequency of RVC infection in piglets from a herd with a previous diagnosis of RVC infection in this age group.

Materials and methods

Herd and sampling

The piglet fecal samples included in this study were collected during the winter season (July to August) in a complete-cycle pig farm with a previous RVC diagnosis infection in piglets up to 7 days old. The pig farm is located in Guarapuava City (25° 23' 42" S and 51° 27' 28" W), Paraná State, South Region of Brazil. The pig herd consists of four different sites. Site 1 houses the boars, pregnant sows, and gilts. One week before parturition, the pregnant sows are transferred to site 2, where they stay until their litters complete 3 weeks. The postweaned piglets are housed in site 3 for up to 9 weeks, and in site 4, the growing-finishing pigs are housed until slaughter.

The units are separated by a minimum distance of 15 m of grass and protected by a fence. Except for the passage used to transport the animals, there is no contact between the units. To maintaining the animal health, external and internal biosecurity measures and strict management are adopted, especially: traffic control of vehicles, trucks, agricultural machinery; control of the presence of pets near the units; visit control; exclusive staff for each unit; rigorous cleaning and disinfection procedures of barns and equipment, a sanitary break for 3 to 5 days.

The farm adopted good nutritional and sanitary practices, including “all-in-all-out” management and vaccination of the sows with a commercial vaccine for neonatal diarrhea control (first dose at 70 days and second dose at 90 days gestation) that included *Escherichia coli* (K88, K99, F41, and 987P), *Clostridium perfringens* type C, and RVA (genotypes G4 and G5), according to the manufacturer’s instructions.

For this study, over 37 days, piglets up to 7 days old from 50 litters were monitored daily (morning, noon, and late afternoon) for the occurrence of diarrhea and fecal samples were

collected direct from each piglet with diarrhea during monitoring. According to the fecal consistency, a score of diarrhea was attributed at each sample. In total, 206 diarrheic fecal samples were collected, of which 74 (35.9%) samples were classified as pasty, 61 (29.6%) as semiliquid, and 71 (34.5%) as liquid. Additionally, 10 fecal samples with normal consistency were randomly collected from asymptomatic piglets from the same litters and used as negative controls.

Some production parameters for the litters or piglets were also evaluated in this study. All litters were classified according to the sow parity order (PO) in five categories (PO1 to 5). Regarding the litter size (LS), the litters were further divided into two groups: litters with up to 10 piglets ($LS \leq 10$) and litters with more than 10 piglets ($LS > 10$). According to the birth weight (BW) the piglets were classified in BW1.2 to 1.3 kg and BW > 1.3 to 1.4 kg.

Nucleic acid extraction

The nucleic acid extraction was performed from aliquots of 500 µL of fecal suspension prepared at 10–20% (w/v) in Tris/Ca⁺⁺ buffer (50 mM Tris-HCl; 10 mM NaCl; 1.5 mM 2-mercaptoethanol; 3 mM CaCl₂; pH 7.2) using the combination of phenol/chloroform/isoamyl alcohol (25:24:1) and silica/guanidinium isothiocyanate [15] nucleic acid extraction methods with modifications [16]. The nucleic acid was eluted in ultrapure diethylpyrocarbonate (DEPC)-treated water and stored at –80 °C until use. An aliquot of Tris/Ca⁺⁺ buffer was included in each nucleic acid extraction and RT-PCR procedure as a negative control.

ss-PAGE

All fecal samples were screened for dsRNA of RV by a silver stained-polyacrylamide gel electrophoresis (ss-PAGE) technique as described previously [17, 18].

RT-PCR

Fecal samples with both ss-PAGE-negative and ss-PAGE-inconclusive results were submitted to RVC-specific partial VP6 gene amplification, which was performed using a primer pair previously described [19]. The RT-PCR products were analyzed by electrophoresis in 2% agarose gel in Tris-borate-EDTA buffer (89 mM Tris; 89 mM boric acid; 2 mM EDTA; pH 8.4), stained with ethidium bromide (0.5 µg/mL), and visualized under UV light. Three RVC-positive fecal samples in partial VP6 gene amplification collected on the first, fifteenth, and last day of the experiment were selected for molecular analysis of the complete VP6 gene, and RT-PCR amplification was performed as previously described [20].

Sequencing and phylogenetic analysis

The amplicons were purified using PureLink Quick Gel Extraction Kit (Invitrogen, Carlsbad, CA, USA) and quantified using a Qubit Fluorometer with a Quant-iT dsDNA BR Assay Kit (Invitrogen, Eugene, OR, USA). An ABI3500 Genetic Analyzer sequencer and the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) were used for sequencing, which was performed with forward and reverse primers. The obtained sequences were analyzed by Phred (<http://asparagin.cenargen.embrapa.br/phph/>), and the sequences were accepted if base quality was ≥ 20 . The consensus sequences were determined by CAP3 software (<http://asparagin.cenargen.embrapa.br/phph/>), and similarity searches were verified with all sequences deposited in GenBank using the Basic Local Alignment Search Tool (BLAST) software (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Multiple and pairwise alignments were accomplished using ClustalW in MEGA software (v. 7) , and the sequence identity matrix was performed using BioEdit software (v. 7.2.5). The phylogenetic tree was obtained by the neighbor-joining method with the Kimura two-parameter model using MEGA software (v. 7). Bootstrap values were determined for 1000 replicates.

Statistical analysis

The ss-PAGE and RT-PCR-positive fecal samples were grouped according to their litters. When the litter had one or more RVC-positive fecal samples, it was considered to be a positive litter. Statistical analysis was performed using the chi-square (χ^2) test with confidence limits of 95%. The results were considered to be statistically significant when two-tailed P values were ≤ 0.05 .

Results

According to the fecal consistency, the RVC dsRNA was mostly detected in the liquid fecal samples (42.2%) but without a significant difference ($P \leq 0.05$) compared with other fecal consistency scores (Table 1).

Of the 206 piglet diarrheic fecal samples analyzed by the ss-PAGE technique, 45 (21.85%) were RVC positive, 130 (63.1%) were RVC negative, and 31 (15.05%) showed inconclusive results. Out of the 161 ss-PAGE-negative and ss-PAGE-inconclusive samples, 26 (16.15%) were positive in the partial RVC VP6 gene amplification. Thus, 34.5% (71/206) of diarrheic fecal samples evaluated in this study were positive for RVC. In the control group ($n = 10$), only 1 (10%) fecal sample was RVC-positive. This fecal sample with normal consistency was collected in a litter that was subsequently considered RVC-positive once diarrheic fecal samples

Table 1 Fecal samples of piglets aged up to 1 week and positive for porcine rotavirus C, distributed according to fecal consistency

Fecal consistency	Samples evaluated ($n = 216$)		Total
	Positive (%)	Negative (%)	
Normal-control group	1 (10.0)	9 (90.0)	10
Pasty	20 (27.0)	54 (73.0)	74
Semiliquid	21 (34.4)	40 (65.6)	61
Liquid	30 (42.2)	41 (57.8)	71

$P = 0.0958$

collected in this litter were RVC-positive. Additionally, dsRNA with the electropherotype of RVA was identified in only two diarrheic fecal samples by the ss-PAGE technique.

When the results of RVC dsRNA detection were distributed by the 50 litters evaluated in this study, 33 (66%) litters had positive fecal samples and were considered positive for RVC infection. Thereafter, the other characteristics concerning the litters were analyzed. According to the sow PO, the frequency of RVC detection varied from 33.3 to 87.5%; the LS > 10 and the BW > 1.3 to 1.4 kg showed higher detection (73.5% and 71.0%, respectively) of RVC compared with other evaluated groups. However, in any of the evaluated production parameters (PO1 to 5; LS ≤ 10 and LS > 10 ; and BW 1.2 to 1.3 kg and BW > 1.3 to 1.4 kg) significant differences ($P \leq 0.05$) were observed between the groups (Table 2).

In the molecular analysis of the whole VP6 gene, the three porcine RVC strains exhibited 100% nucleotide (nt) identity to each other and 86.2 to 96.4% nt identity with RVC strains belonging to the I6 genotype. As the three sequences are identical, only one RVC strain (GenBank accession number: MN248749) was used in the phylogenetic tree that grouped with strains that belong to the I6 genotype (Fig. 1).

Discussion

Porcine RVC has been considered an important cause of enteritis in suckling piglets and weaned pigs around the world [6, 10, 12, 21, 22]. In this study, 34.5% of the evaluated diarrheic fecal samples of piglets up to 1 week old were positive for RVC. This age is considered a crucial stage of the piglet lifetime due to the higher occurrence of diarrhea and, consequently, reduced daily weight gain and increased mortality rates [2]. Several cross-sectional studies also demonstrated that RVC infection in Brazilian pig herds occurs primarily in newborn and suckling piglets [5, 6, 8, 12, 20, 23].

Although RVC dsRNA has been detected in asymptomatic pigs [24, 25], RVC was more frequently detected in younger animals, such as newborn piglets and recently weaned pigs with clinical signs of diarrhea [5, 6, 21]. Thus, the score of

Table 2 Distribution of positive and negative litters up to 7 days old for porcine rotavirus C infection according to some production parameters, such as sow parity order, litter size, and birth weight

Parameter	Groups	Litters (<i>n</i> = 50)		
		Positive (%)	Negative (%)	Total
Parity order (PO) (<i>P</i> = 0.3060)	PO1	12 (70.6)	5 (29.4)	17
	PO2	6 (66.6)	3 (33.4)	9
	PO3	6 (60.0)	4 (40.0)	10
	PO4	7 (87.5)	1 (12.5)	8
	PO5	2 (33.3)	4 (66.7)	6
Litter size (LS) <i>P</i> = 0.1013	LS ≤ 10	8 (50.0)	8 (50.0)	16
	LS > 10	25 (73.5)	9 (26.5)	34
Birth weight (BW) <i>P</i> = 0.3435	BW 1.2 to 1.3 kg	11 (57.9)	8 (42.1)	19
	BW > 1.3 to 1.4 kg	22 (71.0)	9 (29.0)	31

diarrhea may interfere with the frequency of detection of RV, as previously shown [26]. In our study, in addition to the frequency of detection of RVC increasing with decreasing fecal consistency, there was no significant difference ($P \leq 0.05$) between the diarrhea scores analyzed.

Due to the epitheliochorial nature of the sow placenta, the piglet born dependent of the passive immune transferred by sow via colostrum [27]. Several studies demonstrated that the health condition and performance of progeny improve with increasing sow PO [28, 29]. Therefore, the transfer of maternal

Fig. 1 Phylogenetic tree with 1128 bp amplicon (92–1219 nt) of the VP6 gene from Brazilian and other representative RVC strains of the genotypes. The tree was constructed using the neighbor-joining method and the Kimura 2-parameter model as a nucleotide substitution model. The percentages of bootstrap support are shown at each node. Bootstrap values lower than 45% are not shown. The sequence from this study is indicated by a filled circle

antibodies by colostrum intake is crucial for piglet protection against RV infection in the first one or second weeks of life [30]. In this study, except for the sows PO4, which showed a higher percentage of RVC, the frequency of RVC detection decreased with the increase in PO. However, no significant difference ($P \leq 0.05$) between the PO groups was found.

RVC detection was more frequent in litters with LS > 10 than in LS ≤ 10 ; however, there was no significant difference ($P \leq 0.05$) between the groups. A previous study confirmed that LS, in addition to increasing the variability in mean BW, did not demonstrate a significant effect in the acquisition of passive immunity [31], suggesting that in our study, the piglets from larger litters received passive immunization similar to the smaller litters.

Several studies have demonstrated that BW less than 1 kg affects the growth and survival of pigs, since lighter piglets have disadvantages when competing for teats with their littermates, leading to poor acquisition of passive immunity [27, 32, 33] and, consequently, more susceptibility to enteric infections. In the present study, the two BW groups evaluated did not exhibit a significant difference ($P \leq 0.05$) between them, probably because there are no animals less than 1.2 kg included in our analysis.

In contrast to neonatal diarrhea caused by RVA, for which there are commercial vaccines for control and prophylaxis, for the infections caused by other RV species, including RVC, this prophylactic tool is not available. Therefore, in neonatal diarrhea outbreaks caused by non-A RV species, to mitigate the risk of diarrhea, it is required to monitor and increase the general measures of health management to improve pork productivity.

In the pig farm of this study, with the goal of reducing the number and intensity of diarrhea episodes in piglets up to 7 days old, some general management measures were implemented, such as the following: (i) rigorously cleaning and disinfecting facilities, utensils, and equipment; (ii) exchanging the active ingredient of disinfectant that had been used for more than 1 year; (iii) applying shock dose and subsequent maintenance dose of glutaraldehyde; (iv) “all-in-all-out” management in the maternity pens by at least 1 week; (v) changing management flow of maternity always starting with the pens with asymptomatic piglets and only then going to the pens with diarrheic animals; (vi) intensifying farrowing assistance actions; and (vii) continuing the program of vaccinating all prepartum sows against neonatal diarrhea with multivalent vaccines, including *Escherichia coli*, *Clostridium perfringens* type C, and RVA. These management practices have reduced the number and intensity of diarrhea episodes in piglets up to 1 week old, thereby decreasing the mortality rate. However, RVC was not eliminated and sporadic cases continued to occur (data not shown).

The significance of the RVC involvement in the diarrhea episodes observed in this study is highlighted when we found

that of 206 diarrheic fecal samples evaluated by ss-PAGE, only two were positive for RVA (data not shown). Additionally, the electrophoretic profile compatible with rotavirus B and rotavirus H was not identified in any of the porcine fecal samples included in the study.

The detection of RVC in diarrheic fecal samples throughout all evaluated periods increases the viral load in the environment, contributing to the spread of the virus to other litters and other age groups present in the herd. To the best of our knowledge, only cross-sectional studies investigating RVC have been conducted to date in Brazil [5, 6, 20, 23] and other countries [21, 22, 34]. Therefore, this report describes the first longitudinal study regarding the epidemiological aspects of RVC infection, since the litters born each week in the same pig herd were monitored over a period of 37 days.

Although the samples were collected at three different time points (beginning, middle, and end) over the experiment, in the molecular analysis, the VP6 gene of three selected RVC field strains showed 100% nt identity with each other and high nt identity with I6 genotype strains. In the phylogenetic tree, the RVC/Pig-wt/BRA/383/2012 strain grouped in the cluster of the I6 genotype of porcine RVC strains available in GenBank. Two previous studies also demonstrated the circulation of this RVC genotype in Brazilian pig herds [6, 20].

Conclusion

The results of this study highlight the importance of RVC in the etiology of porcine neonatal diarrhea, particularly in newborn piglets. As there are no commercial vaccines available for immunoprophylaxis of RVC infection, special attention should be given to the health management measures for control and prevention of diarrhea to reduce the viral load in the environment and thus alleviate the challenges facing newborn piglets.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study was submitted to the Ethics Committee on Animal Experiments of the Universidade Estadual de Londrina and approved under the identification number 11363.2015.16. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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