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Unusual outbreak of post-weaning porcine diarrhea caused by single and mixed infections of rotavirus groups A, B, C, and H

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ABSTRACT

Rotaviruses (RVs) are a major cause of severe diarrhea in humans and animals. Five of the nine RV groups (RVA, RVB, RVC, RVE, and RVH) have been previously detected in pigs; however, in pig herds worldwide, most studies highlight diarrhea outbreaks caused by RVA. In the present study, we describe detection and characterization of RV groups A, B, C, and H in fecal samples from pigs with single and mixed infections during a post-weaning diarrhea outbreak. The outbreak occurred in a single pig herd routinely vaccinated with an inactivated commercial vaccine for neonatal diarrhea control that included the RVA OSU (G5P[7]) strain. RVC (78%) was the most prevalent group found in single (34%) and mixed (44%) infections, followed by RVA (46%), RVB (32%), and RVH (18%). Phylogenetic analysis of three RVA strains allowed the characterization of two distinct G/P genotypes represented by G5P[13] and G9P[23], different from G5P [7] found in vaccines. Regardless of the RV group, mixed infections (54%) were more prevalent than single infections. Detection of RVB or RVH was associated with the presence of other RV groups, suggesting a secondary action of these RV groups in the reported outbreak. The detection of RV groups B, C, and H in the same pig herd suggests that these RVs act as causative agents of diarrhea and should be included in the diagnostic tests of porcine enteric diseases. These data provide new epidemiological information on RV diversity that need to be addressed in future studies for a better understanding and prevention of RV infections.

1. Introduction

Rotaviruses (RVs) belong to the *Reoviridae* family and are a major cause of severe diarrhea in humans and animals (Estes and Kapikian, 2007; Attoui et al., 2012). The non-enveloped particles of RVs are composed of a triple-layered capsid and the genome consists of 11 segments of double-stranded (ds) RNA that encode six structural (VP1-VP4, VP6, and VP7) and six non-structural (NSP1-NSP6) proteins (Estes and Kapikian, 2007).

RVs are classified into eight groups/species (RVA-RVH) on the basis of antigenic and genetic characteristics of the inner capsid protein VP6 (Attoui et al., 2012; Matthijnssens et al., 2012). RV strains from groups A, B, C, and H are known to infect humans and various animal species, while strains from groups D, E, F, and G have been found to infect only animals (Matthijnssens et al., 2010, 2012). Recently, Mihalov-Kovács et al. (2015) proposed a new RV group candidate (RVI). RVI was found infecting sheltered dogs.

Five of the nine RV groups (RVA, RVB, RVC, RVE, and RVH) have been detected in pigs (Estes and Kapikian, 2007; Wakuda et al., 2011). Among these groups, RVA is considered the most frequent cause of diarrhea in piglets worldwide (Médici et al., 2011; Marthaler et al., 2014a). In contrast, porcine RVB infections have been reported only sporadically, because RVs from this group are excreted in small amounts in feces of infected animals (Kuga et al., 2009; Suzuki et al., 2012; Marthaler et al., 2012). RVC was first detected in swine diarrhea episodes and is associated with sporadic cases or large outbreaks of gastroenteritis, mainly in suckling piglets around the world (Saif et al., 1980; Médici et al., 2010; Lorenzetti et al., 2014). RVE has only been described as a cause of porcine diarrhea in the 1980s; these data call into question its significant prevalence and importance (Pedley et al., 1986). The

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first detection of porcine RVH occurred in 1999 in Japan (Wakuda et al., 2011). Subsequently, Molinari et al. (2014) detected RVH in piglets from Brazil and Marthaler et al. (2014b) described its presence in US pig herds; however, the role of RVH in the pathogenesis of gastroenteritis is still unknown.

Most studies that report the epidemiology or the occurrence of rotavirus diarrhea outbreaks in pig herds worldwide deal with RVA; however, other RV groups are related to single and mixed infections in pigs with or without signs of diarrhea (Kuga et al., 2009; Médici et al., 2011; Marthaler et al., 2012, 2014a,b; Lorenzetti et al., 2014).

In the present study, we describe detection and characterization of multiple RV groups in single and mixed infections during a post-weaning porcine diarrhea outbreak in a pig herd vaccinated with the RVA OSU strain.

2. Materials and methods

2.1. Herd and fecal samples

The herd, located in the State of Mato Grosso do Sul, Central-West region of Brazil, had a complete cycle of 650 sows in a confinement system (all-in-all-out) with standard nutritional and health management practices. All of the sows were routinely vaccinated with an inactivated commercial vaccine for neonatal diarrhea control that included the RVA OSU (G5P[7]) strain, *Escherichia coli*, and *Clostridium perfringens* types C and D, according to the manufacturer's instructions.

Nonetheless, a post-weaning diarrhea outbreak unresponsive to wide spectrum antibiotics happened in 2012 and lasted for approximately 2 weeks. The peak of diarrhea episodes occurred between the fifth and ninth day of the 2 weeks. In nursery, the rates of pig morbidity and mortality were around 70% and 11%, respectively. A total of 50 diarrheic fecal samples from weaned pigs of 28 and 35 days of age were selected for virological diagnosis. All fecal samples were stored at −80°C until processing.

2.2. RNA extraction and RT-PCR

Viral dsRNA was extracted from 10 to 20% fecal suspensions in phosphate-buffered saline (PBS) using a combination of the phenol/chloroform/isoamyl alcohol (25:24:1) and the silica/guanidinium isothiocyanate nucleic acid extraction methods described by Alfieri et al. (2006). The presence of RV groups A, B, C, and H was investigated by reverse-transcriptase (RT) PCR assay. The gene target and primers of RT-PCR are described in Table 1.

An aliquot of ultrapure diethylpyrocarbonate (DEPC) treated water was included in each reaction as a negative control. The amplified products were analyzed by electrophoresis in a 2% agarose gel in Tris-borate-EDTA (TBE) buffer, pH 8.4 (89 mM Tris, 89 mM boric acid, 2 mM EDTA), with 0.5 μg/mL ethidium bromide and visualized under ultraviolet (UV) light.

2.3. Sequencing and phylogenetic analysis

To confirm specificity of the RV amplicons the amplified products from groups A, B, and C were purified using the GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, UK), quantified on a Qubit™ Fluorometer (Invitrogen – Life Technologies, Eugene, OR, USA), and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Reaction Kit (Applied Biosystems, Foster City, CA, USA) on an automated sequencer (ABI3500, Applied Biosystems). The resulting sequences were analyzed by means of an automated online rotavirus genotyping tool, RotaC$^{2.0}$ (Maes et al., 2009). Sequence quality analyses were performed using the Phred and CAP3 software pachages (http://aspargin.cenargen.embrapa.br/phph/). Similarity searches were performed against sequences deposited in GenBank using the basic local alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.egi). Phylogenetic trees based on nucleotide (nt) sequences were built using the neighbor-joining method from the Kimura two-parameter model, which provided statistical support via bootstrapping with 1000 replicates in the MEGA software package (version 6). The sequence identity matrix was constructed using the BioEdit software, version 7.08.0.

Analyses of specificity and phylogenetic data of the Brazilian RVH-positive samples have already been described by Molinari et al. (2015).

2.4. Nucleotide sequence accession numbers

The nucleotide sequences described in this study were deposited in the GenBank database under the following accession numbers: RVA VP7 gene: strains BR43 (KX376970), BR54 (KX376971), and BR55 (KX376972); RVA VP4 gene: strains BR43 (KX376973), BR54 (KX376974), and BR55 (KX376975); RVB VP7 gene: strain BR31 (KX376976); RVC VP6 gene: strain UEL33 (KX376977).

3. Results

In all the 50 (100%) diarrheic fecal samples evaluated, it was possible to amplify by RT-PCR fragments of RV dsRNA. RVA, RVB,

Table 1
Characteristics of the primers used for the detection of rotavirus groups A, B, C, and H genes in fecal samples of diarrheic pigs.

RV species	Viral gene	Primer sequence (5'-3')	PCR product (bp)	Ref.
RVA	VP4	F-TGGCTTCGCCATTTLATAGACA R-ATTTCGGACCATTTATAACC	876	Gentsch et al. (1992)
	VP7	F-GGCTTTAAAAGAGAGAATTTCCGTCTGG R-GGTCACATCATACAATTCTAATCTAAG	1062	Gouvea et al. (1990)
RVB	VP7	F-GGAAATAATCAGAGATGGCGT R- TCGCCTAGTCYTCTTTATGC	778	Marthaler et al. (2012); Molinari et al. (2015)*
RVC	VP6	F- GGCTTTAAAAATCTCATTCACAA R- AGCCACATAGTTCACATTTCA	1353	Stipp et al. (2015)
RVH	VP6	F- TGCTACAAGTGACCCACAAGG R- GCCATCTTTCCAGTGGCTCT	590	Molinari et al. (2015)
	VP6	F- ACCAGGTGGAGCAACAAACA R- CAGTGCGTGACCAGATCTCA	716	

* Synthesis of cDNA and PCR amplification were performed as described by Molinari et al. (2015).