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Whole-genomic analysis of 12 porcine group A rotaviruses isolated from symptomatic piglets in Brazil during the years of 2012–2013

Fernanda D.F. Silva *, Luis R.L. Espinoza, Paloma O. Tonietti, Bruna R.P. Barbosa, Fabio Gregori

Department of Preventive Veterinary Medicine and Animal Health, College of Veterinary Medicine, University of São Paulo, Av. Professor Dr. Orlando Marques de Paiva, 87, 05508-270 São Paulo, SP, Brazil

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ABSTRACT

Group A rotaviruses (RVAs) are leading causes of viral diarrhea in children and in the young of many animal species, particularly swine. In the current study, porcine RVAs were found in fecal specimens from symptomatic piglets on 4 farms in Brazil during the years of 2012–2013. Using RT-PCR, Sanger nucleotide sequencing, and phylogenetic analyses, the whole genomes of 12 Brazilian porcine RVA strains were analyzed. Specifically, the full-length open reading frame (ORF) sequences were determined for the NSP2-, NSP3-, and VP6-coding genes, and partial ORF sequences were determined for the VP1-, VP2-, VP3-, VP4-, VP7-, NSP1-, NSP4-, and NSP5/6-coding genes. The results indicate that all 12 strains had an overall porcine-RVA-like backbone with most segments being designated as genotype 1, with the exception of the VP6- and NSP1-coding genes, which were genotypes I5 and A8, respectively. These results add to our growing understanding of porcine RVA genetic diversity and will provide a platform for monitoring the role of animals as genetic reservoirs of emerging human RVAs strains.

1. Introduction

Neonatal diarrhea is a multifactorial disease and a major health problem for most swine herds worldwide. Group A rotavirus (RVA) infection is one of leading causes of acute viral diarrhea in suckling and weaned piglets (Chang et al., 2012; Estes and Greenberg, 2013). RVAs also cause gastroenteritis in humans, being responsible for an estimated 453,000 deaths among children less than 5 years of age (Tate et al., 2012).

While there is an ever-increasing amount of genetic information for human RVA strains, much less is known about porcine RVA strains. Moreover, the limited information that we do have suggests that porcine RVAs contain gene segments that are genetically-similar to those of RVAs isolated from humans, horses, and cows (Ha et al., 2009; Hwang et al., 2012; Ghosh et al., 2012; Matthijnssens et al., 2008a). This observation indicates that interspecies transmissions may occur between RVAs originating from different animal species and highlights the need for surveillance studies in swine herds.

The non-enveloped RVA virion has a triple-layered capsid surrounding 11 double-stranded RNA genome segments, which together encode 6 structural (VP1-VP4, VP6, and VP7) and 5 or 6 non-structural (NSP1–NSP5/6) proteins (Attoui et al., 2011). Since VP4 and VP7 induce neutralizing antibodies and protective immunity, these proteins play a central role in strain selection for vaccine production and form the basis of a binary classification system, namely the P (Protease-sensitive) and G (Glycosylated) genotypes (Estes and Greenberg, 2013). To date, 27 G- and 37 P-genotypes with different combinations have been described for RVAs found in many host species (Matthijnssens et al., 2011; Trojnar et al., 2013). Epidemiological surveillance studies show that porcine RVA strains exhibit G-genotypes of G3-G5, G9, and G11, and P-genotypes of P[6], P[7], P[13], P[19] and P[23] (Collins et al., 2010; Matthijnssens et al., 2011; Papp et al., 2013). However, RVAs can evolve by several mechanisms, including point mutation, recombination, and reassortment, thereby generating a large diversity of genotypes and in nature (Ghosh and Kobayashi, 2011). Such diversity may allow for the emergence of new strains bearing properties derived from both parental lineages, which might eventually have implications for human infection and vaccine efficacy (Gentsch et al., 2005).

To provide a better understanding of the overall genetic relatedness among human and animal RVA strains, a whole genome classification system was developed (Matthijnssens et al., 2008b). In this system, a genotype is given to each of the 11 genome segments. Therefore, the acronym Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx describes the genotype constellation of the

* Corresponding author. Tel.: +55 11 3091 1386; fax: +55 11 3091 7928.
E-mail addresses: vete_fer@yahoo.com.br (F.D.F. Silva), lunaluis@usp.br (L.R.L. Espinoza), palomatonietti@gmail.com (P.O. Tonietti), bruna888@hotmail.com (B.R.P. Barbosa), acme@usp.br (F. Gregori).

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VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6-coding genes, respectively. Two main genotype constellations have been described so far for human RVAs: (i) constellation 1 (prototype strain: human Wa) of Gx-P[x]-I1-R1-C1-M1-A1-N1-T1-E1-H1, and (ii) constellation 2 (prototype strain: human DS-1) of Gx-P[x]-I2-R2-C2-M2-A2-N2-T2-E2-H2 (Nakagomi et al., 1989; Matthijnssens et al., 2010a). Culture-adapted porcine RVAs were found to contain several genotype 1 genes, suggesting that they may share an evolutionary ancestor with human Wa-like strains.

Despite the importance of swine as reservoirs for RVA genetic diversity, there is a paucity of phylogenetic studies including all 11 genome segments of strains prevalent in swine farms. In this study, RT-PCR, Sanger nucleotide sequencing, and phylogenetic analysis was performed for each gene of 12 porcine RVAs from Brazil. Our data indicate that all strains tested had an overall porcine-RVA-like backbone, although some segments appeared genetically similar to RVAs isolated from other animal species. To our knowledge, this is the first study to whole-genome analysis of porcine RVAs from Brazil.

2. Materials and methods

2.1. Specimen collection, RNA extraction, and RVA diagnostics

Between the years of 2012–2013, 126 fecal samples from nursing and suckling piglets suffering from diarrhea were collected from 4 swine herds in São Paulo and Mato Grosso states in Brazil. All samples were transported to the laboratory chilled on ice and stored at −18 °C. Fecal samples were prepared as 50% (v/v) suspensions in DEPC-treated ultrapure water and centrifuged at 5000×*g* for 10 min at 4 °C. Total RNA was extracted from 250 μL of the supernatant using TRIzol Reagent™ (Invitrogen, Carlsbad, CA, USA). Samples were screened as positive for RVA by nested RT-PCR as previously described (Ben Salem et al., 2010).

2.2. RT-PCR and Sanger nucleotide sequencing

To amplify the viral genome for nucleotide sequencing, RT-PCR was performed. First, cDNA was synthesized using Random Primers (Invitrogen, Carlsbad, CA, USA) and Super Script III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) as per manufacturer's protocol. Different PCR runs were performed using a panel of previously designed and new primer pairs (Supplementary Table 1) and thermocycling conditions (Supplementary Table 2), targeting different viral genes and fragments. Subsequently, 2.5 μL cDNA was added into the reaction mix comprising 1× PCR Buffer™ (Invitrogen, Carlsbad, CA, USA), 0.2 mM of each dNTP, 0.5 μM of each positive and negative primer, 1.5 mM $MgCl_2$, and 3 U Platinum Taq Polymerase™ (Invitrogen, Carlsbad, CA, USA) and nuclease-free water to obtain a final volume of 25 μL. After completion of thermocycles, PCR products were separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and visualized by UV transillumination. The amplicons were purified using ExoSAP-IT® PCR Product Cleanup (USB Products Affymetrix, Cleveland, USA) and nucleotide sequences of the RT-PCR amplicons for the 11 genome segments were sequenced in both directions using BigDye Terminator 3.1™ cycle sequencing kit (Applied Biosystems, Foster City, USA), according to the manufacturer's protocol and a 3500™ Genetic Analyzer system (Applied Biosystems, Foster City, USA).

2.3. Contig assembly, nucleotide alignments, and phylogenetic analysis

The nucleotide sequences obtained from the selected strains were assembled using Bioedit 7.0.5 software (Hall, 1999), and analyzed using the RotaC 2.0 automated genotyping tool to confirm the RVA identity of all sequences (Maes et al., 2009). Nucleotide alignments were constructed using a multiple sequence alignment program, Clustal W v.2.1 (Larkin et al., 2007) and the sequence identity calculated using Bioedit 7.0.5 software (Hall, 1999). Phylogenetic trees were constructed and molecular analyses were performed with MEGA® 6.06 software by using the maximum likelihood criterion for partial or complete ORF (Tamura et al., 2013). The nucleotide substitution models were determined for each gene separately according to the lowest Bayesian Information Criterion (BIC), provided by the same software (Tamura et al., 2013), indicated as follows (gene/model): VP7/TN93; VP4/GTR, VP6/T92; VP1/TN93; VP2/TN93; VP3/T92; NSP1/GTR; NSP2/T92; NSP3/T92; NSP4/HKW and NSP5/T92. Statistical support was obtained by 100 bootstrap replicates.

2.4. Nucleotide sequence accession numbers

Sequences from this study were deposited into the GenBank database under the following accession numbers: VP7 (KJ482509, KJ482520–KJ482523, KJ482525, KJ482526, KJ482528, KJ482531); VP4 (KJ482460, KJ482465–KJ482471, KJ482473, KJ482474, KJ482476); VP6 (KC855060, KJ482487–KJ482493, KJ482494–KJ482496, KJ482498); VP1 (KJ482382, KJ482387–KJ482393, KJ482395–KJ482397, KJ482402); VP2 (KJ482409, KJ482414–KJ482420, KJ482422, KJ482423, KJ482425, KJ482429); VP3 (KJ482436, KJ482441–KJ482447, KJ482448, KJ482449–KJ482450, KJ482455); NSP1 (KC855054, KJ482249–KJ482255, KJ482257, KJ482258, KJ482260, KJ482267); NSP2 (KJ482274, KJ482279–KJ482285, KJ482287, KJ482288, KJ482289, KJ482291); NSP3 (KC855057, KJ482302–KJ482308, KJ482310, KJ482311, KJ482313, KJ482320); NSP4 (KJ482328, KJ482332–KJ482338, KJ482340, KJ482341, KJ482343, KJ482348); NSP5 (KJ482353, KJ482358–KJ482362, KJ482364–KJ482366, KJ482371).

3. Results

3.1. Genotype constellation of porcine RVAs from Brazil

As shown in Table 1, 6 different genotype constellations were found for the 12 Brazilian porcine RVAs sequenced in this study. Whereas a wide variety of G- and P-genotypes were detected for the VP7- and VP4- coding genes (i.e., G3, G5, G9, G11, P[6], P[13], and P[23]), the following constellation was found for the other nine genes: I5-R1-C1-M1-A8-N1-T1/T7-E1-H1. Although the T7 genotype for the NSP3-coding gene was found in the majority (n = 7/12) of Brazilian strains, the T1 genotype was also encountered (n = 5/12).

3.2. Analysis of the VP7- and VP4-coding genes

The partial ORF sequence of the VP7-coding gene were deduced (nucleotides 117–839) for all 12 Brazilian porcine RVAs and compared with those of RVA strains from swine, humans, and bovine (Matthijnssens et al., 2011). The VP7-coding gene of the Brazilian porcine strains clustered in the phylogenetic trees with those of G3, G5, G9, and G11 strains (Fig. 1A). The finding of G3, G5, and G11 genotypes for the Brazilian porcine strains was not surprising, as these genotypes are common or exclusively found in porcine RVAs worldwide. In contrast, the finding of G9 strains was more surprising, as this genotype is often found in humans RVAs.

Intra-genotypic heterogeneity was highest for the VP7-coding genes belonging to the G3 and G5 genotypes, but it was less for the G9 genotype. As an example, the G3 VP7-coding gene of strain RVA/Pig-wt/BRA/ROTA09/2013/G3P[13] clustered distantly from