BRUNA LETÍCIA DOMINGUES MOLINARI

DIAGNÓSTICO E ESTUDO MOLECULAR DE CEPAS BRASILEIRAS DE ROTAVÍRUS SUÍNO ESPÉCIES B E H

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Tese apresentada ao Programa de Pós-graduação em Ciência Animal – área de concentração: Sanidade Animal – da Universidade Estadual de Londrina como requisito parcial para a obtenção do título de Doutora em Ciência Animal.

Orientador: Prof. Dr. Amauri Alcindo Alfieri

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Londrina, 30 de agosto de 2018.

O presente trabalho foi realizado no Laboratório de Virologia Animal (LabViral) e no Laboratório de Saúde Animal, Unidade de Biologia Molecular (LAMSA / BioMol) Departamento de Medicina Veterinária Preventiva, Centro de Ciências Agrárias, Universidade Estadual de Londrina, como requisito para a obtenção do título de Doutora em Ciência Animal pelo Programa de Pós-Graduação em Ciência Animal (Área de Concentração: Sanidade Animal), sob a orientação do Prof. Dr. Amauri Alcindo Alfieri.

Os recursos financeiros para o desenvolvimento do projeto foram obtidos junto às agências e órgãos de fomento à pesquisa, abaixo relacionados:

1. CNPq: Conselho Nacional de Desenvolvimento Científico e Tecnológico / MCT

2. CAPES: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior / MEC

3. FAP/PR: Fundação Araucária de Apoio ao Desenvolvimento Científico e Tecnológico do Paraná / SETI

4. FINEP: Financiadora de Estudos e Projetos / MCT

DEDICATÓRIA

A minha famíla!

AGRADEDIMENTOS

A Deus, por tudo.

Aos professores Amauri e Alice Alfieri, exemplos de dedicação, por todo seu carinho e paciência, pelos conhecimentos compartilhados e por terem aceitado me orientar durante essa jornada.

As professoras e amigas que constituíram a banca de qualificação, Raquel e Elis. A ajuda de vocês, técnica ou não, foi excencial durante toda essa jornada "doida" que é a pós- graduação.

À Universidade Estadual de Londrina e aos professores do Programa de Pós-graduação em Ciência Animal, pela formação acadêmica e científica.

Aos amigos de longa data, pelo companheirismo, carinho, preocupação, pelas conversas, festas e risadas. Por tornarem minha vida mais alegre.

Aos meus pais e irmão, por sempre acreditarem em mim e me apoiarem em todas minhas decisões. Por sua dedicação e amor incondicional. Por serem exemplos a se seguir. Aos dois, minha admiração, respeito e amor eternos.

Ao meu esposo por sua enorme paciência, generosidade, carinho e dedicação. Com certeza não teria conseguido encarar os desafios que foram surgindo durante esta jornada sem a sua ajuda!

À toda minha família, por seu amor e pela constante preocupação e

torcida. A todos que contribuíram para a realização deste trabalho.

Muito obrigada!

"Ter mais disposição para fracassar e colocar-se em posições de vulnerabilidade pode, muitas vezes, ser o caminho mais rápido para o sucesso."

(Anjali Sud – CEO da Vimeo)

MOLINARI, Bruna Letícia Domingues. **Diagnóstico e estudo molecula de cepas brasileiras de rotavírus suíno espécies B e H**. 2018. 81 f. Tese (Doutorado em Ciência Animal) – Universidade Estadual de Londrina, Londrina, 2018.

RESUMO

A gastroenterite em leitões lactentes e recém-desmamados representa a principal causa de morbidade e mortalidade durante o período neonatal. A síndrome é caracterizada por infecção entérica multifatorial e multietiológica. Entre as causas infecciosas, o rotavírus (RV) é o principal agente etiológico viral. Por serem mais frequentes, as espécies de rotavírus A (RVA) e C (RVC) são as mais estudadas. No entanto, surtos de diarreia causados por espécies de rotavírus B (RVB) em leitões lactentes estão sendo relatados em rebanhos de suínos das regiões sul e centro-oeste do Brasil. Por sua vez, a espécie de rotavírus H (RVH) foi descrita apenas recentemente na suinocultura brasileira. Os objetivos deste estudo foram avaliar a presença de cepas de RV em um surto de diarreia em leitões lactentes e recém-desmamos que ocorreu em uma granja com programa de vacinação contra patógenos entéricos, incluindo RVA, e realizar a caracterização molecular das cepas de RVB e RVH identificadas. Para isso, três estudos independentes foram realizados. O objetivo do primeiro estudo foi determinar as sequências de nucleotídeos (nt) e de aminoácidos (aa) dos genes que codificam as proteínas VP6, VP7, VP4 e NSP4 de seis cepas de campo identificadas como RVH (BR59, BR60, BR61, BR62, BR63 e BR64) no surto de diarreia. A partir da cepa de RVH suína, SKA-1, *primers* específicos foram selecionados para amplicação dos genes citados acima. Com base nas altas identidades encontradas entre as sequências de nt (~99%) dos genes VP6, VP4, VP7 e NSP4 entre cinco das cepas estudadas (BR59 a BR63), é possível considerá-las pertencentes a mesma linhagem de RV, denominada RVH / BRA-1. Em contraste, uma vez que a amostra fecal BR64 apresentou uma diferença relativamente alta (81,6% e 83,4% de identidade para nt e aa, respectivamente) na sequência referente à proteína VP7, quando comparada com as outras cinco amostras, a mesma foi denominada cepa RVH / BRA-2. No segundo estudo, com o objetivo de triar todas as amostras diarreicas (n = 50) obtidas no surto para RVA, B, C e H, realizou-se RT-PCR com *primers* específicos para cada espécie. De acordo com os testes, RVC (78%) foi mais prevalente nas infecções singulares (34%) e mistas (44%), seguido pelo RVA (46%), RVB (32%) e RVH (18%). A análise filogenética de três cepas de RVA permitiu a caracterização de dois genotipos G / P distintos, representados por G5P[13] e G9P[23], diferentemente do G5P[7] presente em vacinas comerciais. Independentemente da espécie de RV, as infecções mistas (54%) foram mais prevalentes do que as infecções por um único agente. RVB e RVH foram detectados apenas em associação com outros grupos de RV, sugerindo uma ação secundária dessas espécies no surto relatado. Finalmente, no terceiro estudo, com base na qualidade do produto amplificado por RT-PCR, a amostra fecal de RVB identificada como BR62, foi selecionada para análises moleculares adicionais. As sequências de nt e aa do gene VP7 foram determinadas e a análise comparativa desta cepa com as cepas dos outros 21 genotipos de VP7 previamente identificados mostraram que a cepa suína brasileira pertence a um novo genotipo, G22, e parece estar circulando em diferentes partes do mundo. Os estudos reforçam o papel dos RV como importantes agentes causadores de diarreia demonstram a variabilidade genética entre as espécies. Além disso, esta é a primeira detecção do genotipo de RVB, G22, em rebanhos suinícolas brasileiros.

Palavras-chave: Suínos. Diarreia. Rotavirus. RT-PCR. Infecção mista. Genotipos.

MOLINARI, Bruna Letícia Domingues. **Diagnostic and molecular study of Brazilian porcine rotavirus strains species B and H.** 2018. 81 p. Thesis (Doctorated degree in Animal Science) – Universidade Estadual de Londrina, Londrina, 2018.

ABSTRACT

Gastroenterits in suckling and newly weaned piglets represents the major cause of morbidity and mortality during the neonatal period. The syndrome is a multifactorial and multi etiologic enteric infection. Among the infectious causes, rotavirus (RV) is the main viral etiologic agent. To be more frequent rotavirus species A (RVA) and C (RVC) are most studied. However, outbreaks of diarrhea caused by rotavirus species B (RVB) in suckling piglets are being reported from pig herds of the southern and midwest regions of Brazil. The rotavirus species H (RVH) was only recently described in Brazilian pig farming. The aims of this study was to evaluate the presence of RV strains in suckling and newly post-weaning diarrhea outbreak that occurred in a pig farm with vaccination program against enteric pathogens, including RVA, and perform the molecular characterization of RVB and RVH field strains identified in this diarrhea outbreak. For this, three independent studies were carried out. The objective of the first study was to determine the VP6, VP7, VP4, and NSP4 nucleotide (nt) and amino acid (aa) sequences of six (BR59, BR60, BR61, BR62, BR63, and BR64) RVH field strains identified in the diarrhea outbreak. Specific primers were designed based on the porcine RVH strain SKA-1. Based on the high nt sequence identities (~99%) of the VP6, VP4, VP7, and NSP4 genes among five of the studied specimens (BR59 to BR63), they are considered the same local RV strain denominated RVH/BRA-1. In contrast, once that the fecal sample BR64 showed a relatively high difference (81.6% nt identity and 83.4% aa identity) in the VP7 sequence when compared to the other five specimens it was named RVH/BRA-2 strain. On the second study, with the objective of screening all the diarrheic samples (n = 50) obtained from the outbreak for RVA, B, C, and H, RT-PCR with specific primers were performed. 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] present in commercial 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. Finally, in the third study, based on the quality of the RT-PCR amplified product, the RVB-positive fecal sample identified as BR62 was selected for further molecular analyses. The VP7 nt and deduced aa sequences were determined and comparative analysis of this strain with the strains of the other 21 previously identified VP7 genotypes showed that the Brazilian porcine RVB strain belongs to a novel G22 VP7 genotype, and seems to be circulating in different parts of the world. The studies reinforce the rule of RV as important diarrhea agents and contribute to show the genetic variability among the species. Additionally, this is the first detection of RVB G22 genotype in Brazilian pig herds.

Key Words: Swine. Diarrhea. Rotavirus. RT-PCR. Mixed infection. Genotypes.

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Topics on rotavirus species B and H

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Keywords: Piglets, diarrhea, rotaviruses, RVB, RVH.

Abstract

Diarrhea in suckling and recently weaned piglets represents the major cause of morbidity and mortality during the neonatal period. The syndrome is a multifactorial and multi etiologic enteric infection. Among the infectious causes, rotavirus is the main viral etiologic agent. Pigs are susceptible to enteric infections by several rotavirus species highlighting the rotavirus A (RVA), RVB, RVC, and RVH. Since RVA and RVC are more frequent, they are most studied. However, outbreaks of diarrhea caused by RVB in suckling piglets are being reported from pig herds of the southern and midwest regions of Brazil. The RVH was only recently described in Brazilian pig farming. Considering the few reports available on the frequency of occurrence of RVB and RVH in Brazilian pig herds, this review aims to present some characteristics related to the classical and molecular virology of these viruses as well as epidemiological aspects of these infections.

Introduction

Rotaviruses (RVs) represent a global public health problem once it is considered one of the most common causative agents of acute gastroenteritis in children and many mammalian and avian species [21, 37].

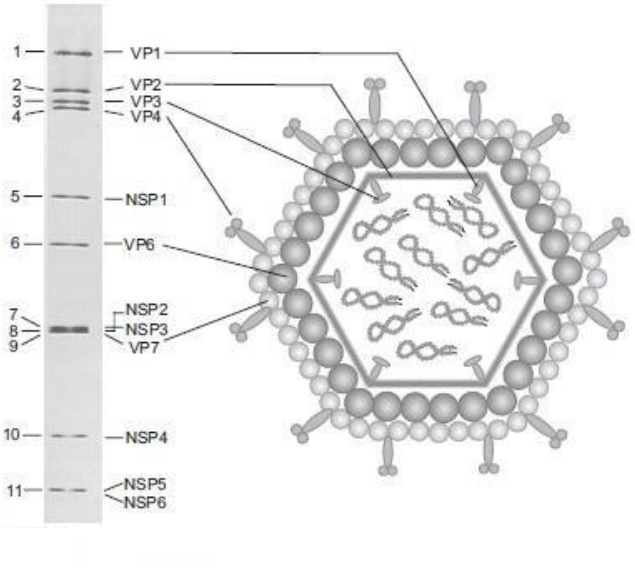
The first descriptions of RVs date back to the 1970s, when viral particles originally designated as reovirus-like/orbivirus-like were detected by electron microscopy infecting the cells of the upper portions of the duodenal epithelium of children with acute gastroenteritis [8, 23]. Based on the electron microscope analysis of the viral particles and its morphological similarity with a wheel (from the Latin *rota*), Flewett and colleagues [24] proposed the Rotavirus nomenclature for the described viruses. After their identification, human rotavirus was then associated with previous descriptions of identical viral particles related to epidemic diarrhea in infant mice (EDIM) virus [1], calves (NCDV – Nebraska calf diarrhea virus) [53], and with the SA11 (simian agent) virus strain detected in a healthy monkey [42].

In the past 44 years, with the advances of molecular diagnostic techniques, it was possible to explore many aspects of RVs, including classification, epidemiology, structure, and pathogenesis. Currently, the rotaviruses are grouped into 9 species (RVA to RVI) [31]. Once that RVA is the most common RV species detected in both human and animals, a great number of studies is available, including review articles [21]. On the other hand, there is a lack of information about the other RV species. Considering that the first RVH detections are recent, and that this RV species seems to be genetically connected with RVB [3, 34], the aim of this review is to gather information about these two RV species, give an up-to-date review of the most important research achievements related to them and formulate some perspectives about remaining open questions.

Structure of *Rotavirus* genus

Rotavirus virion, unprovided of a glycoprotein envelope, presents approximately 75-100 nm in diameter and a capsid composed of three concentric protein layers of icosahedral symmetry [21, 32]. The viral genome consists of 11 segments of double strand RNA (dsRNA) which encode 6 structural (VP – Viral Protein) and 5 or 6 non-structural (NSP) proteins. The genes are monocistronic, meaning that each one encodes only one protein, except for genome segment 11, which can encode two proteins depending on the RV species [21] (Figure 1).

The inner layer of the capsid, also called core-shell, is formed by the viral protein 2 (VP2) associated with the enzyme complexes, consisting of VP1 and VP3, which are located inside the core-shell and in intense contact with the segments of the viral genome [51]. The capsid middle layer is constituted by VP6, which is in contact with the inner core protein VP2 and with VP7 and VP4 proteins that form the outer layer of viral capsid [21] (Figure 1). The structural protein VP6 is the most abundant virion protein, representing about 50 to 60% of the viral mass [21]. For being considered one of the most immunogenic and antigenic proteins, it is frequently detected in immunological assay diagnostic systems [50].



Source: Alfieri and colleagues **[5]**. (Adapted from Estes, 2001 **[20]**). With permission of the authors and the publisher.

Figure 1. Aspects of rotavirus structure. Polyacrylamide gel electrophoresis (PAGE) showing the 11 genomic segments of dsRNA from rotavirus A (RVA) strain SA11 (left). The proteins encoded by each segment (middle) - the nomenclature of the VPs (VP1 to VP4, VP6, and VP7) and the NSPs (NSP1 to NSP6) present in mature viral particles are followed by numbers

in decreasing order of molecular mass, according to the migration order in PAGE. A simplified illustration of the viral particle and its components (right).

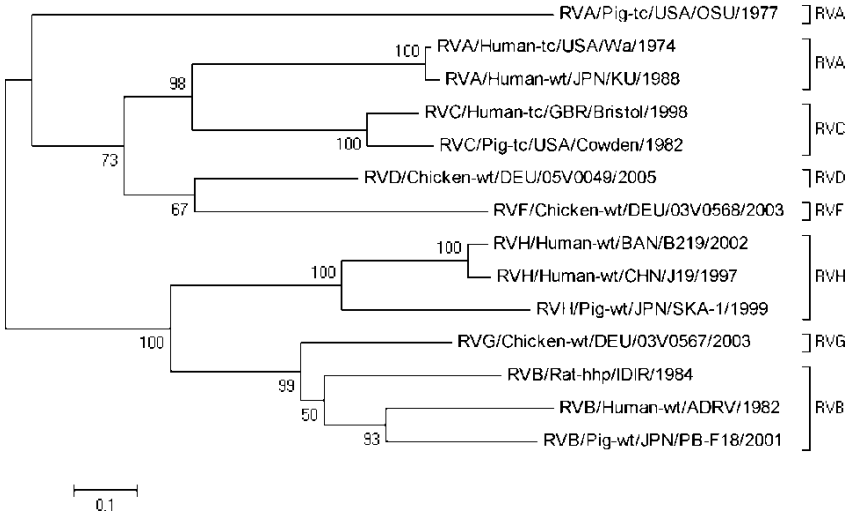
The NSPs are synthesized in infected cells and are involved in the viral replicative cycle stages and interactions with host proteins that influence the pathogenesis and immune response to the infection [26].

Rotavirus classification

Rotaviruses are members of the genus *Rotavirus*, one of the 15 genera that belong to *Reoviridae* family [31]. Based on antigenic and molecular characteristics of the VP6 protein, RVs are classified into 9 groups (RVA to RVI, Figure 2), also termed species [31]. Additionally, after the detection of RV in bats in Serbia, the creation of group J is being proposed [7].

Once that RVA is the most common RV species isolated from humans and animals, initially, a binary classification system, based on the antigenic and molecular variability of the VP7 (G types) and VP4 (P types) proteins, was implanted for them [21]. To date, 36 G types and 51 P types of RVA have been detected in humans and several animal species, including mammals and avian. All these RVA genotypes were recognized by the Rotavirus Classification Working Group (RCWG) demonstrating the wide antigenic and molecular diversity of this RV species [61]. Later, similar genotype differentiation for RVB and RVC species has been established [18, 43-45].

Due to the great genetic diversity found in RVA strains and the possibility of reassortment occurring in all segments of the genome, a new classification system based on the eleven genes was proposed and the genotypes adopted for the proteins VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 were Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, respectively. This classification system is also called whole genome or complete genotype constellation and the appropriate cut-off values were established for each RV gene [47-49]. Although this system has been used for classification of other RVs, it is not completely established for non-RVA species.



Source: Elaborated by the authors.

Figure 2. Phylogenetic tree showing the different species of rotaviruses (RV).

Rotavirus B

Species or group B rotavirus, designated as Adult Diarrhea Rotavirus (ADRV), was first detected in a large epidemic of diarrhea affecting more than one million people in China during 1982-1983 [15, 30]. Since then, human RVB has been reported in sporadic outbreaks of adult and childhood diarrhea in China [22, 74], India [38, 40], Bangladesh [62, 65], Myanmar [6], and Nepal [4].

In addition to humans, RVB strains have also been identified in cattle [10]; pigs [9], rats [19], and lambs [67]. The first descriptions of RVB in cattle occurred in the United States of America (USA) and in the United Kingdom [12-14, 52]. Although RVB has been detected both in calves and in adult cows with diarrhea, most of the studies have suggested that RVB might be more associated with episodes of cow diarrhea [12, 27, 64]. In addition to the geographical regions mentioned above, bovine RVB has also been described in Japan and Germany [59, 70].

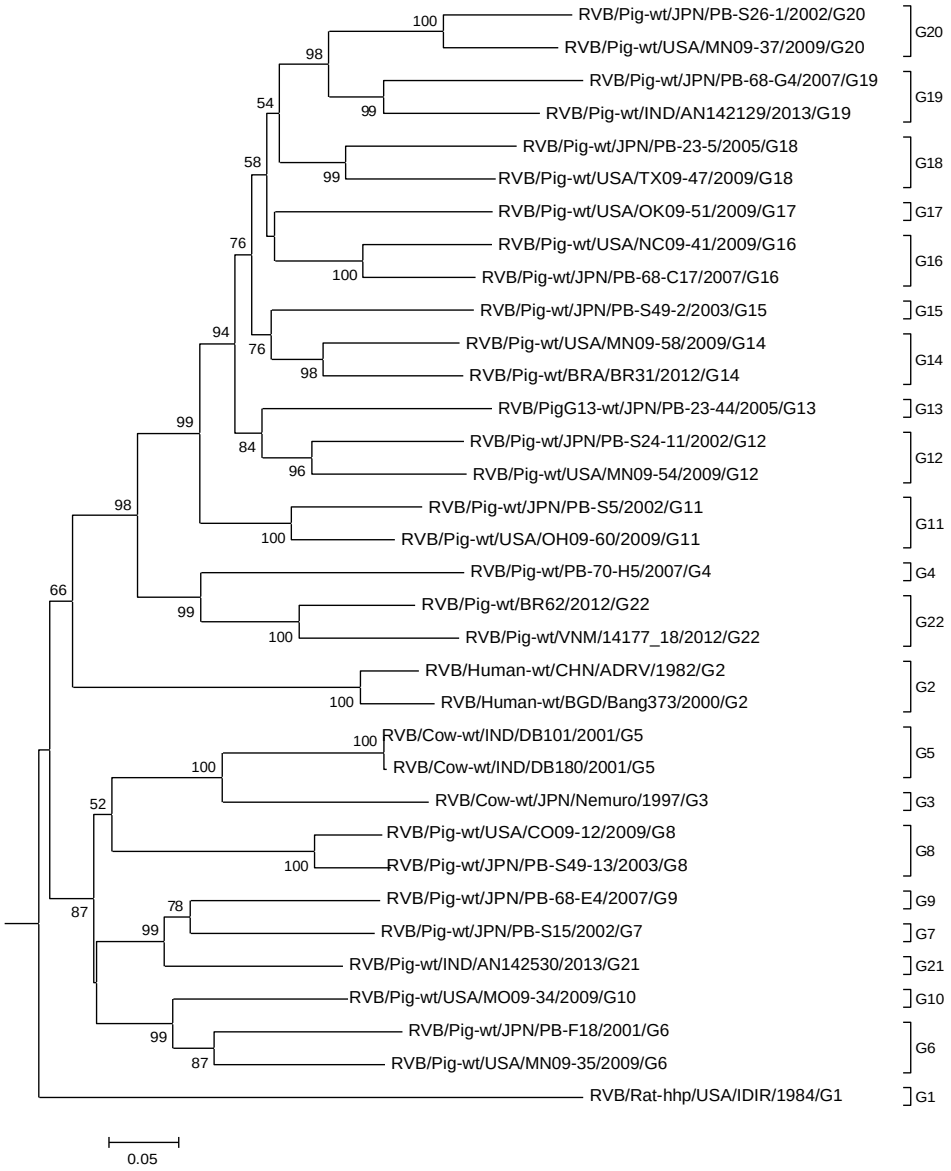
Porcine RVB first detection occurred in a suckling pig with diarrhea from an Ohio pig herd in the 1980s [63]. After that, RVB infections have been sporadically described in pre- and post-weaning porcine diarrhea outbreaks of several locations around the world [25, 28, 39, 46, 56, 68]. The role of porcine RVB as a primary enteric pathogen remains unclear. Marthaler and colleagues [45] detected the presence of the virus in almost half of the stool samples tested, suggesting that RVB was the causative agent of the reported symptoms, including diarrhea. On the other hand, Molinari and colleagues [56] have found RVB in diarrheic fecal samples mostly associated with RVA, RVC, and RVH contributing to the hypothesis that the virus probably acts as a secondary diarrhea agent. However, outbreaks of diarrhea in suckling piglets caused only by RVB have also been described in Brazilian pig herds (Laboratory of Animal Virology-UEL, unpublished data). Complete genome sequences have been determined for several human RVB strains [2, 36, 72, 74]. However, only partial genome sequencing was established for bovine, porcine, and rat strains. Although high prevalences of anti-RVB antibodies have been demonstrated in animal serum, antigenic and molecular features, as well as the prevalence of the infection by this species of RV are still not clear, mainly due to the difficulty of viral isolation in cell cultures and the fact that they are excreted in small amounts (titer) in the feces of infected animals [11, 69].

Despite porcine RVB has been found in several countries, genetic variation among RVB strains remains poorly characterized. Knowledge regarding the genome of porcine RVB remains restricted to information obtained via analyses of the VP6, VP7, NSP1, NSP2, and NSP5 genes [39, 45, 50, 68]. Based on the analyses of the VP7 gene of 50 RVB strains, Kuga and colleagues [39] proposed the classification of RVB strains into G genotypes using cut-off values of 67% and 76% at nucleotide (nt) level and, 66% and 79% at amino acid (aa) level. Thus, they proposed the creation of five genotypes that were further divided into 12 clusters. Three years later, based on the VP7 analyses of 125 RVB strains, Marthaler and colleagues [45] proposed a modification in the cut-off values established by Kuga and colleagues [39]. Using an nt cut-off of 80%, RVB strains were divided into 20 G genotypes. With exception of G1 (murine RVB strains), G2 (human RVB strains), G3 and G5 (cow RVB strains), porcine RVB strains were clustered in all the other genotypes. This fact demonstrates the high genetic variation among the porcine RVB strains and might be a possible explanation for the difficulty in developing a diagnostic platform for the virus.

In addition to the 20 genotypes described by Marthaler and colleagues [45], Suzuki and colleagues [68] detected a new RVB genotype, G21, in pigs from India. More recently, the creation of the G22 genotype including the Vietnamese 14177_18 [60] and the Brazilian BR62 strains was proposed by Molinari and colleagues (unpublished data). Representative strains of the 22 RVB genotypes are demonstrated in Figure 3.

Phylogenetic analysis shows that human RVB strains present remarkable differences when compared with animal RVB strains. This fact may suggest that RVB infections are species-specific. However, a recent study suggested the possible inter-species transmission between caprine and bovine RVB strains [16]. Therefore, future studies about the viral biology are needed to exclude the possibility of interspecies and/or zoonotic transmission.

When considering the geographical regions where the porcine RVB genotypes were described, it is possible to note a viral regionalism. Genotypes G4, G7, G9, G13, G15, and G19 were only detected in Japanese pigs. Additionally, genotypes G21 and G10/G17 were only detected in pigs from India and USA, respectively [45, 68]. Knowledge about viral distribution is extremely important for the development of viral prevention programs. However, it must be taken into account the possibility of diagnostic errors, once that genotype G14, for instance, has already been reported in Japan, the USA, and Brazil [39, 45, 56]. Therefore, the search for RVB infections should always be included in the diagnostic tests for enteric diseases in suckling and recently weaned pigs.

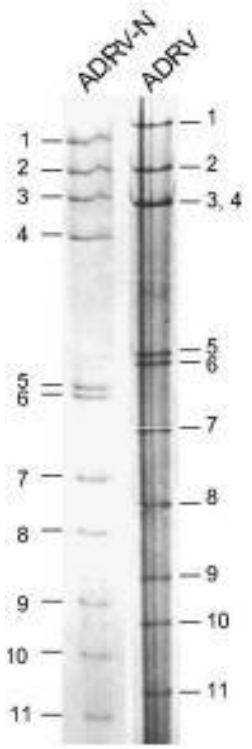


Source: Molinari and colleagues (unpublished data). Adapted. With permission of the authors.

Figure 3. Phylogenetic tree constructed based on the VP7 nucleotide sequences of representative strains for the 22 rotavirus B (RVB) genotypes.

Rotavirus H

Species or group H rotavirus was first described as the cause of an outbreak of adult gastroenteritis in China in 1997 leading to 1,053 hospitalizations [17, 73]. The clinical signs caused by the viral infection were similar to those observed in another outbreak of diarrhea also described in China in 1983, caused by the ADRV, classified as RVB [15, 30]. The electron microscopic analysis indicated that the etiological agent of the outbreak that occurred in 1997 had morphology compatible with the genus *Rotavirus*. However, the PAGE migration profile of the genomic segments was different from the other known dsRNA profiles (Figure 4) [17, 73]. Subsequent analyzes demonstrated that antisera induced by experimental inoculations with RVA, RVB, and RVC did not react with the new RV strain. In addition, specific primers selected for RT-PCR amplification of the VP6 and VP7 genes of the RVB ADRV strain was not able to amplify the genomic fragments of this new RV strain. However, the new RV was successfully isolated in cultures of human embryonic kidney cells and MA-104 cells [17, 33, 73].



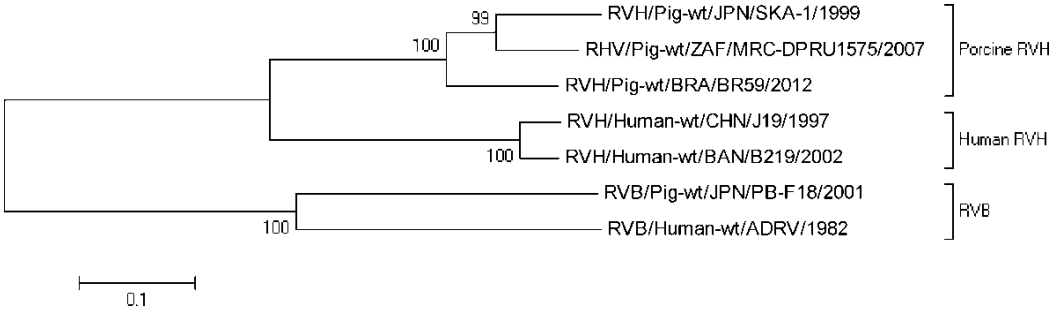
Source: Yang and colleagues **[74]**.

Figure 4. Comparative analysis of the electrophoretic profile of the new rotavirus (NADRV) strain of RVH with the RVB ADRV (Adult Diarrhea Rotavirus) strain.

Based on the electrophoretic profile of the viral genome, serological analyzes, and similarity of the clinical signs caused by the viral infection with those described in the outbreak in China in 1983 caused by the ADRV strain, the new RV received the nomenclature of Novel Adult Diarrhea Rotavirus - NADRV [33, 73]. Only in 2012, Matthijnssens and colleagues [50] proposed the creation of a novel RV species named RVH.

In addition to the NADRV strain, it was also included as RVH species other strains such as the J19 strain, from the outbreak of diarrhea that occurred in China in 1997 [33, 34]; the human RV B219 strain, detected in a fecal sample of a 65-year-old male patient who presented a sporadic case of diarrhea in Bangladesh in 2002 [3, 57]; and SKA-1 porcine RV strain isolated from a piglet with diarrhea in Japan [66, 71] (Figure 5). Recently, Molinari and

colleagues [55], Marthaler and colleagues [44], and Nyaga and colleagues [58] reported the detection of porcine RVH in pig herds from Brazil, the USA, and South Africa, respectively. Additionally, an RVH-like strain was also detected in feces of Korean bats by Kim and colleagues [35].



Source: Elaborated by the authors.

Figure 5. Phylogenetic analysis showing the rotavirus H (RVH) representative strains from porcine and human host species. Two strains of rotavirus B (RVB) of the same host species were included as outgroup.

The epidemiology, antigenic, and molecular characterization of RVH, both of human and animal origin, remains unclear. The development of a classification system based on the viral genome (genotype constellation) has not yet been established. All the three human RVH strains described until now belong to the Asian continent, are related to adult and older people infection and seem to cause clinical signs of diarrhea [3, 33, 34]. The complete genomes of strains J19 and B219 have already been described, and molecular analyses between them showed that the nt and deduced aa sequence similarities of RNA segments ranged between 87.7–94.3% and 88.7–98.7%, respectively [3, 34, 57]. Additionally, once that the aa sequences of VP4 and VP7 presented 95.0% and 96.5% identities, respectively, Nagashima and colleagues [57] suggested that B219 and J19 may have an identical antigenic specificity of VP4 and VP7.

Porcine RVH strain was first detected in a diarrheic fecal sample from a piglet aged < 30 days old in Japan [71]. In the subsequent years, Marthaler and colleagues [44] described the presence of RVH in 30 diarrheic fecal samples from pigs of different ages; however, most of them were from 21-55-day-old pigs from the USA herds of several locations. Additionally, Molinari and colleagues [56] detected nine RVH-positive diarrheic fecal samples in 35-day-old piglets in a Brazilian pig herd, and Nyaga and colleagues [58] described the virus in a 10-week old pig with signs of diarrhea in South Africa. In all the cases, the presence of RVH was more prevalent in post-weaned piglets. This information may contribute to a better understanding of the viral biology.

Although in Japan [71] and South Africa [58] RVH seems to be the cause of the porcine diarrhea episodes, such as the human RVH described until now [3, 33], Marthaler and colleagues [44] and Molinari and colleagues [56] reported the presence of porcine RVH only in fecal samples of piglets co-infected with RV groups A, B, and/or C, implying that porcine RVH likely behaves as an opportunistic agent. Thus, the role of the agent in the development of episodes of diarrhea remains unclear and further studies are needed to elucidate this information. Since the virus does not act as a causative agent for clinical signs, the development of vaccines and prevention measures should be focused on the RV species that may lead to diarrhea and favor infection with RVH, which may worsen the clinical condition.

With respect to the genomic analysis of porcine RVH, the complete genomes of strains from the USA and South Africa (MRC-DPRU1575) have already been established [29, 58]. For the Brazilian and Japanese strains only the VP4, VP6, VP7, and NSP4 genes were determined [54, 71]. Molecular analyses between porcine RVH VP6 genes suggest the USA strains are evolutionarily distinct from those found in Brazil, Japan, and South Africa [44, 58]. Comparisons between the available RVH genomic segments showed that porcine RVH strains from Japan, Brazil, USA, and South Africa are evolutionarily distinct from human RVH strains from China and Bangladesh [3, 34, 44, 54, 57, 58, 71, 74]. Once that human and porcine RVH presented low nt and deduced aa identities and clustered into different branches in the phylogenetic tree there is no evidence suggesting interspecies transmission [58]. This information suggests that pigs do not act as reservoirs for human RVH, and so, do not represent a public health problem in this case. However, once that there is very little information regarding this RV species, further studies are necessary to elucidate the zoonotic potential of RVH transmission.

When compared with other RV species, Marthaler and colleagues [44] and Molinari and colleagues [54] described that porcine RVH strains shared low molecular identities with representative strains from RVA, RVC, RVD, and RVF. However, when compared with RVB, they showed a moderate level of genetic relationship, suggesting that RVB and RVH might have a common ancestral virus. The possibility of a common ancestral between the two RV species has also been proposed for human RVH and RVB strains [3, 34]. This possible genetic relation between both RV species may be a reason for the similar clinical signs reported in the first outbreak of adult gastroenteritis in China in 1997 to those observed in the outbreak caused by RVB in the same country in 1983. Furthermore, the similarity between the RVH and RVB PAGE dsRNA profile may be related to this hypothesis, which may justify why the first Brazilian porcine RVH detection occurred in a diarrheic fecal sample that was first described as RVB-positive by PAGE and RT-PCR [55]. Additionally, this hypothesis may also be related with the fact that the strain SKA-1, detected from a piglet with diarrhea in Japan and isolated in MA-104 cells in 1996 [66] was also suggested to be RVB, even that subsequent molecular analysis showed that the porcine SKA-1 strain belonged to RVH [71]. Once that there is a lack of genetic information about RVH, further studies are necessary to confirm the relation between RVB and RVH. Nevertheless, the lack of appropriate diagnostic tools suggests that RVH may be circulating in pig herds without being noticed.

Recently, Kim and colleagues [35] described the detection of RVH in fecal samples from Korean bats. According to the authors, comparisons of sequences from VP1, VP3, and VP4 genes between the bat strain and the other RVH strains revealed 69.4–72.7%, 71.1–72.9%, and 67.3–72.2% nt identities, respectively. However, the analyses of VP6 sequences at the aa level showed only 62% identity between the bat and other RVH strains [58]. Therefore, complete genome analyses of this RVH-like strain should be performed before it can be definitely considered as belonging to RV species H [58].

Concluding remarks

In a similar way to that described by Ma and colleagues [41] in their paper entitled “The pig as a mixing vessel for influenza viruses: human and veterinary implications” we can propose the same for porcine rotavirus. The pig is undoubtedly the animal species where we have identified the greatest diversity of rotavirus species and genotypes. Due to the genomic feature (segmented dsRNA) and in view of the great diversity of rotavirus types that can infect pigs the possibility of reassortant strains increases considerably. Current studies have revealed a wide diversity of rotavirus species and genotypes in single and mixed infections

with endemic or epidemic presentation in pig herds worldwide. The epidemiology of rotaviruses has shown to be complex, dynamic, and with a very varied spatial and temporal prevalence. Additionally, more frequent genotypes of porcine rotavirus strains have been identified in virus strains isolated from humans and vice versa, thus characterizing the zoonotic potential of rotaviruses. This reinforces the importance of constant monitoring of viral strains in the most varied animal species, particularly in swine, at different geographical regions around the world. Clinical, epidemiological, antigenic, and molecular characteristics of the most prevalent porcine rotavirus strains at any given time are valuable information for monitoring infections and, especially, for understanding the complex relationships of viral evolution.

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2 OBJETIVOS

2 OBJETIVOS

2.1 OBJETIVO GERAL

- Avaliar a presença de rotavírus em um surto de diarreia em suínos recém-desmamados provenientes de um rebanho com programa de vacinação para controle de diarreia neonatal incluindo rotavírus A e, caracterizar molecularmente as cepas de rotavírus B e H detectadas.

2.2 OBJETIVOS ESPECÍFICOS

- Desenvolver *primers* específicos para a amplificação dos genes que codificam as proteínas VP4, VP7 e NSP4 de rotavírus suíno espécie H.

- Utilizar RT-PCR específica (A, B, C, H) para determinar as espécies de rotavírus identificadas no surto;

- Determinar a especificidade das reações de RT-PCR por meio do sequenciamento de nucleotídeos dos *amplicons* obtidos;

- Determinar a frequência de infecções singulares e mistas com relação às possíveis espécies de rotavírus identificadas no surto;

- Realizar análises filogenéticas comparativas incluindo as diferentes cepas de rotavírus identificadas no estudo e cepas virais disponíveis em bases públicas de dados *(GenBank)*.

3 ARTIGOS PUBLICADOS

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3.1 UNUSUAL OUTBREAK OF POST-WEANING PORCINE DIARRHEA CAUSED BY SINGLE AND MIXED INFECTIONS OF ROTAVIRUS GROUPS A, B, C, AND H

(http://dx.doi.org/10.1016/j.vetmic.2016.08.014)

Veterinary Microbiology 193 (2016) 125–132

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

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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ARTICLE INFO

Article history:
Received 29 July 2016
Received in revised form 13 August 2016
Accepted 16 August 2016

Keywords:
Pigs
Diarrhea
Outbreak
Enteric viruses
Rotavirus mix infection
RVH

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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http://dx.doi.org/10.1016/j.vetmic.2016.08.014
0378-1135/

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).

RVC, and RVH were detected in 23 (46%), 16 (32%), 39 (78%), and 9 (18%) samples, respectively (Table 2).

A single RV group was detected in 23 (46%) of the 50 analyzed diarrheic weaned pigs. RVC was the most prevalent group found in single infections ($n = 17$; 34%), followed by RVA ($n = 5$; 10%) and RVB ($n = 1$; 2%) (Table 2).

In the 27 (54%) diarrheic fecal samples we identified simultaneous infection with more than one RV group, characterizing a post-weaning diarrhea outbreak by mixed infections. Two and three RV groups were identified in 17 (34%) and 10 (20%) diarrheic fecal samples, respectively (Table 3). None of the fecal samples were positive for all RV groups simultaneously. The highest frequency of co-infection was found between RVC, RVB, and RVA (14%). The distribution of RV groups detected in single and mixed infections is presented in Table 3.

On the basis of quality of the amplification products, three (BR43, BR54, and BR55) RT-PCR-positive RVA samples were selected for further molecular analyses. Likewise, one positive sample of each group – RVB (BR31) and RVC (UEL33) – was also selected. The RotaC[2.0] automated genotyping tool for group A RVs and the BLAST searches revealed that RVA strain BR43 has the G5P [13] genotype, whereas BR54 and BR55 have genotype G9P[23].

Comparative sequence analysis was performed on the VP7 gene of the Brazilian strains and the 27 known G genotypes. Brazilian wild-type porcine RVA strain BR43 showed the highest nt identity (91.9%) with the G5 genotype (porcine OSU strain). When compared with the other Brazilian strains, nt identity was 75.6%. The lowest nt identity (61%) was observed with a chicken G19 genotype strain (02V0002G3). The other two remaining Brazilian wild-type porcine RVA strains, BR54 and BR55, presented 100% nt sequence identity with each other. Comparisons between BR54 and BR55 and the other G genotypes strains revealed that the highest nt identity percentages were 93.4 and 91.3 in relation to the human B3458 strain and the porcine JP29-6 strain, respectively, both belonging to G9 genotype.

In the phylogenetic tree, the porcine field strain BR43 described in this study clustered with the G5 genotype strain, while BR54 and BR55 clustered with G9 genotype strains (Fig. 1).

Comparative sequence analysis of the VP4 gene of Brazilian strains and the 37 known P genotypes was also performed. Brazilian wild-type porcine RVA strain BR43 showed the highest (88.9% and 81.4%) nt identity with the porcine P[13] genotype strains HP140 and A46, respectively. When compared with the other Brazilian strains, nt identity was 63.5%. The lowest nt identity (47.1%) was observed with a chicken P[31] genotype strain (06V0661). Brazilian wild-type porcine RVA strains BR54 and BR55 showed 100% nt sequence identity between each other, and when compared to the other strains of P genotypes, the highest nt identity percentage was 91.6 in relation to the A34 porcine strain, which has the P[23] genotype. When compared with the other strains of P genotypes, nt identity was between 48.4% and 72.6%.

In the phylogenetic tree, the porcine field strain BR43 clustered with the P[13] genotype strains. BR54 and BR55 clustered with the P[23] genotype strain (Fig. 2).

Table 2
Rotavirus groups identified in 50 fecal samples collected during a post-weaning diarrhea outbreak in piglets.

Rotavirus groups	Infection type/N° of positive diarrheic fecal samples		
	Single (%)	Mixed (%)	Total (%)
A	5 (10.0)	18 (36.0)	23 (46.0)
B	1 (2.0)	15 (30.0)	16 (32.0)
C	17 (34.0)	22 (44.0)	39 (78.0)
H	–	9 (18.0)	9 (18.0)

Table 3
Mixed types of rotavirus infections found in the post-weaning diarrhea outbreak.

Infection type	Fecal samples ($n = 50$)	
	Positive	%*
RVA + B	3	6.0
RVA + C	7	14.0
RVB + C	1	2.0
RVB + H	1	2.0
RVC + H	5	10.0
Sub-total	**17**	**34.0**
RVA + B + C	7	14.0
RVA + B + H	1	2.0
RVB + C + H	2	4.0
Sub-total	**10**	**20.0**
Total	**27**	**54.0**

* Percent analysis was performed based on the total number ($n = 50$) of diarrheic fecal samples collected in this study.

Phylogenetic analysis of the RVB strain BR31 and RVC strain UEL33 was also performed. A comparison of VP7 gene sequence between BR31 Brazilian wild-type strain and the 21 known RVB G genotypes indicated clustering with other porcine G14 strains (Fig. 3). Nucleotide sequence identity was found to be 84.2%, and 84.4% between BR31 and G14 strains SD09-45 and MN09-58, respectively. The BR31 strain shared 56.5–77.8% nt identity with other RVB G genotypes. The analysis performed in relation to the RVC UEL33 VP6 gene sequence revealed the highest nt identity with BRA1034-10 (92.9%) and Cowden (89.7%) strains belonging to I1 genotype (Fig. 4). Nucleotide identity with other RVC strains of the I genotype was 79.8–85.3%.

As described by Molinari et al. (2015), the amplification and phylogenetic analyses of porcine RVH VP6, VP7, VP4, and NSP4 genes from six diarrheic fecal samples revealed remarkable differences when compared with those of other RV groups (A–G). Comparisons of the six Brazilian RVH samples with representative strains from RV groups confirmed that the VP6, VP7, VP4, and NSP4 genes belonged to group H RV. These genes shared higher identity with RVH strains SKA-1, J19, and B219.

4. Discussion

Porcine diarrhea episodes are a multifactorial and multi-etiological health problem. RVA infections are the most frequent cause of diarrhea outbreaks in pig herds (Estes and Kapikian, 2007). Prevalence of RV infections among pigs, primarily of groups B, C, and H, has been difficult to estimate because of the genetic diversity of porcine RV groups and due to the lack of proper diagnostic tools.

Few studies have assessed the prevalence of simultaneous infections of RV groups A, B, and C in pigs. The presence of single and mixed RV infections in pre- and post-weaning pigs has already been described for fecal samples from pig farms in Japan, the United States (US), Canada, Mexico, and Brazil (Kuga et al., 2009; Médici et al., 2011; Marthaler et al., 2012, 2014a). Regarding RVH, this group was detected only from pig herds in Japan, US, and Brazil. Marthaler et al. (2014b) recently detected the association of RVH with RV groups A, B, and C in fecal samples from pigs of different ages from US herds at several locations. In the present study, RV groups A, B, C, and H were detected in diarrheic fecal samples from a pig herd vaccinated against RV group A. To the best of our knowledge, this is the first detection of simultaneous infection with several RVs from different groups in nursery pigs in a single diarrhea outbreak in Brazil.

Regardless of the RV group, in our study, mixed infections were more prevalent than single infections. In Brazil, piglets are

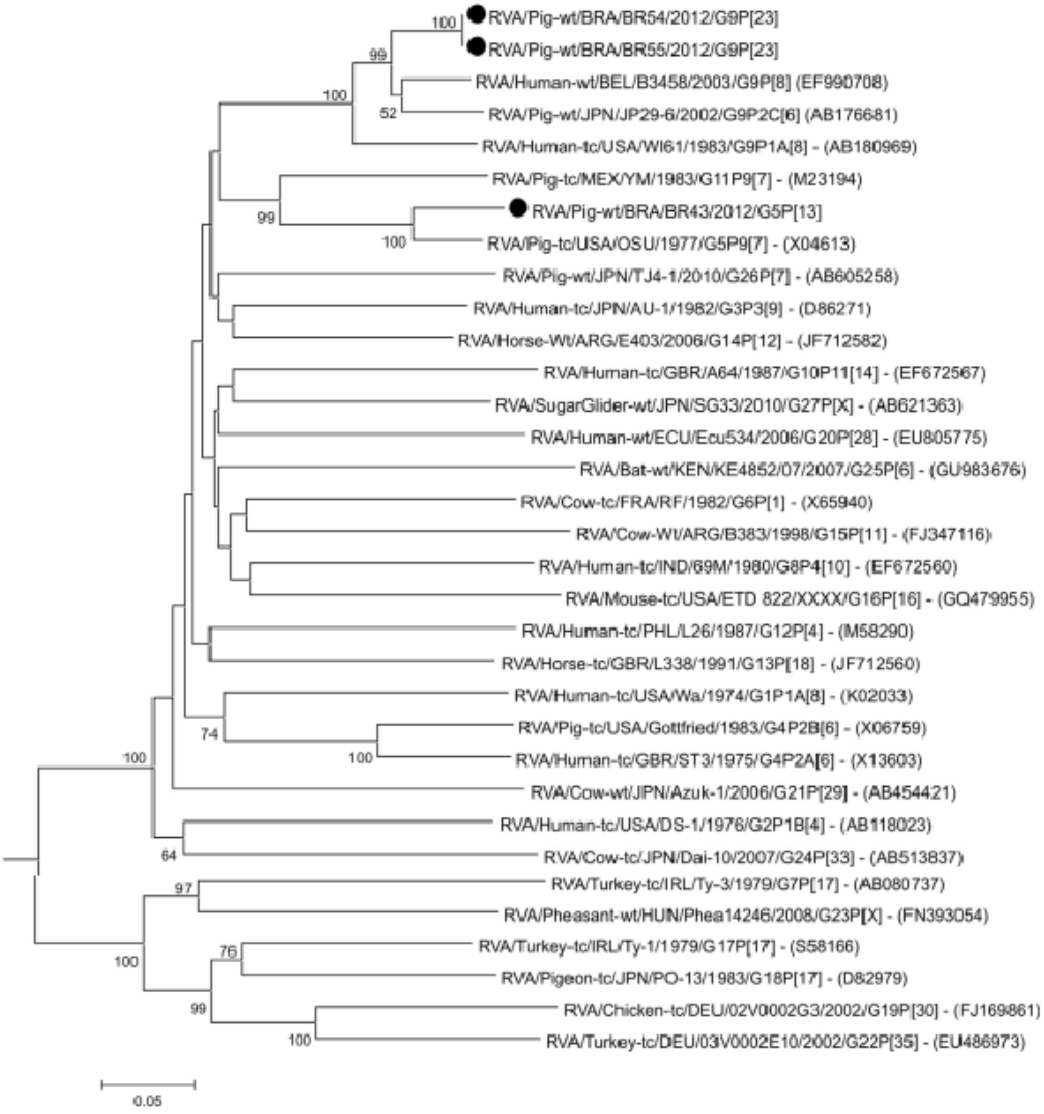

Fig. 1. Phylogenetic tree based on the partial nucleotide sequences of the VP7 gene from the porcine RVA strains described in this study and representative strains of the 27 genotypes recognized thus far. The tree was constructed using neighbor-joining and the Kimura two-parameter model for nucleotide substitution. The bootstrap values are shown at the branch nodes (values <50% not shown). GenBank accession numbers of the reference strains are indicated in parentheses. The Brazilian porcine RVA strains are marked with filled circles.

frequently weaned at 21 days of age. The consequences of the stress caused by environmental, social, and feeding changes can deteriorate the health condition of these animals; this notion may explain the higher rate of RV co-infections. Another possible explanation is contact among piglets from different litters after weaning (Marthaler et al., 2012, 2013).

Although in most studies RVA infections predominate (Martella et al., 2007; Médici et al., 2011; Marthaler et al., 2014a), our analysis here showed that RVC infections occur more frequently (78%), followed by RVA (46%), RVB (32%), and RVH (18%). Despite this result, it must be taken into account that the diarrhea episodes occurred in a pig herd regularly vaccinated against the RVA OSU strain (G5P[7] genotype).

The phylogenetic analysis performed on three field RVA strains allowed the characterization of two distinct G/P genotypes represented by G5P[13] (strain BR43), and G9P[23] (strains BR54 and BR55). The most prevalent genotypes of RVA found in pigs are G3, G4, G5, G9, and G11 in association with P[6] or P[7]. VP4 genotypes P[13], and P[23] have been detected sporadically in pigs (Matthijnssens et al., 2011). Porcine RVA genotypes G5P[13], and G9P[23] have been reported in several Asian countries such as Thailand, China, Japan, and South Korea, as well as in Brazil and Belgium (Okitsu et al., 2013; Silva et al., 2015; Theuns et al., 2016). Our data reinforces the idea that a combination of these G and P genotypes can be found in RVA strains circulating in Brazilian pig herds.

There are reports of diarrhea caused by RVA in children and animals who are regularly vaccinated (Lorenzetti et al., 2011; Gurgel et al., 2014). Although vaccine failures should be considered because of some genotypes similarity, the possibility that diarrhea episodes can be produced by immune pressure developed by mass vaccination causing the emergence of new RVA genotypes cannot be ruled out in this study (Matthijnssens et al., 2009).

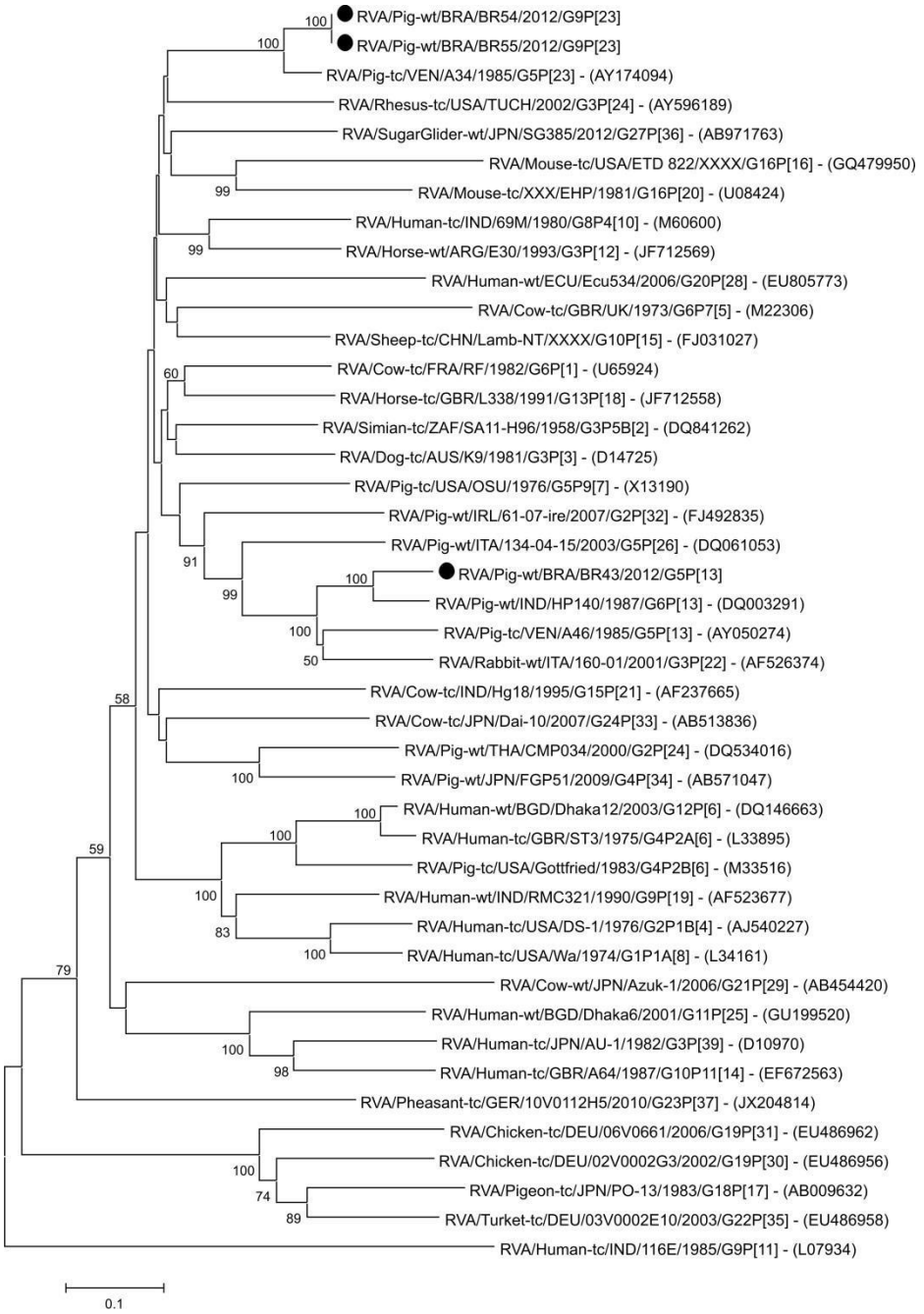

Fig. 2. Phylogenetic tree based on the partial nucleotide sequences of the VP4 gene from the porcine RVA strains described in this study and representative strains of the 37 genotypes recognized thus far. The tree was constructed using neighbor-joining and the Kimura two-parameter model for nucleotide substitution. The bootstrap values are shown at the branch nodes (values <50% not shown). GenBank accession numbers of the reference strains are indicated in parentheses. The Brazilian porcine RVA strain is marked with a filled circle.

In addition, sequencing analyses allowed the confirmation of the RVB and RVC amplicons specificity. To our knowledge, porcine G14 RVB strains were detected only in Japan and US until now (Kuga et al., 2009; Marthaler et al., 2012). The detection of the Brazilian G14 RVB strain may indicate that RVs of this genotype can be circulating in Brazilian pig herds. RVC strains of genotype I1 have already been described in several countries, including Brazil (Médici et al., 2010; Suzuki et al., 2014; Jeong et al., 2015; Stipp et al., 2015). Recently, Stipp and colleagues (2015) demonstrated high heterogeneity of Brazilian porcine RVC strains from several states, including Mato Grosso do Sul. Our results may reinforce the idea that RVC strains of genotype I1 are circulating in Brazilian pig herds.

Marthaler and colleagues (2014a) stated that RVB is detected more often in older pigs, while RVC is more frequently detected in very young piglets. Additionally, there are several studies that show higher prevalence of RVC infection in suckling piglets (Martella et al., 2007; Lorenzetti et al., 2014; Theuns et al., 2016). Despite the relatively high prevalence of RVB (32%) in this study, rates of RVC infection (78%) were much higher, revealing that this

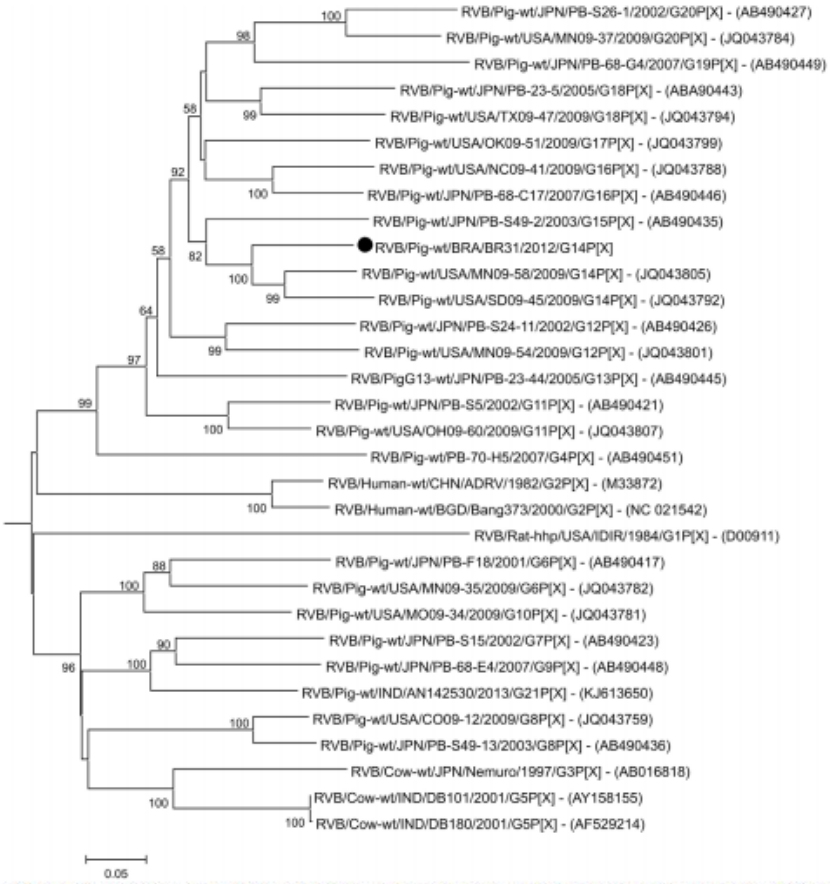

Fig. 3. Phylogenetic tree based on the partial nucleotide sequences of the VP7 gene from the porcine RVB strain described in this study and representative strains of the 21 genotypes recognized thus far. The tree was constructed using neighbor-joining and the Kimura two-parameter model for nucleotide substitution. The bootstrap values are shown at the branch nodes (values <50% not shown). GenBank accession numbers of the reference strains are indicated in parentheses. The Brazilian porcine RVB strain is marked with a filled circle.

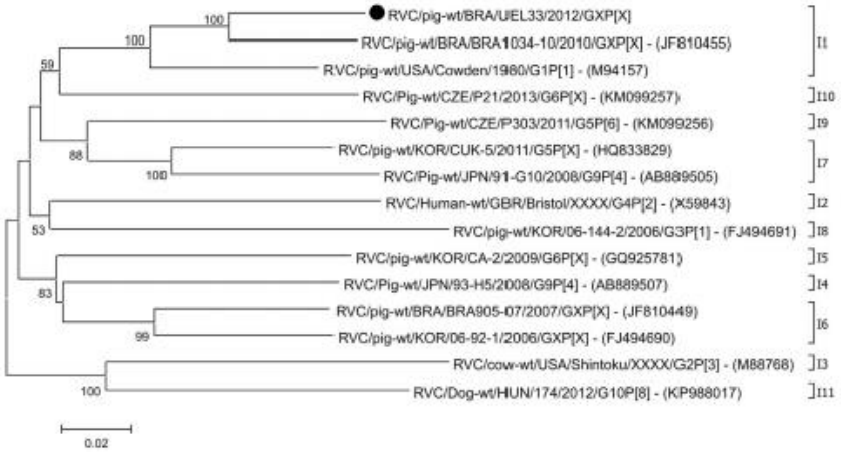

Fig. 4. Phylogenetic tree based on the partial nucleotide sequences of the VP6 gene from the porcine RVC strain described in this study and representative strains of the 11 genotypes recognized thus far. The tree was constructed using neighbor-joining and the Kimura two-parameter model for nucleotide substitution. The bootstrap values are shown at the branch nodes (values <50% not shown). GenBank accession numbers of the reference strains are indicated in parentheses. The Brazilian porcine RVC strain is marked with a filled circle.

RV group prevailed among both single (34%) and mixed (44%) infections during this post-weaning diarrhea outbreak. Because both RV groups showed high detection rates, epidemiological studies should be performed exclusively on RV groups B and C for better clarification of their infection profile.

In one study, researchers detected RVB in almost a half of the stool samples tested (46.8%), suggesting that RVB can be a primary enteric pathogen in pigs (Marthaler et al., 2012). In our results, besides the higher prevalence of RVC and RVA than that of RVB, as described previously (Collins et al., 2008; Kuga et al., 2009), another interesting finding was that only in one sample it was possible to detect single infection with RVB. In the other 15 RVB-positive diarrheic fecal samples analyzed, RV groups A, C, and/or H were also detected. These data suggest that RVB probably acted as a secondary diarrhea agent in this outbreak. Nonetheless, because the RVB detection rate was relatively high, screening for RVB should be actively incorporated into porcine RV studies.

Although RVH was found in nine diarrheic fecal samples, its detection was always associated with the presence of other RV groups. Recently, Marthaler and colleagues (2014b) also described the presence of porcine RVH only in samples co-infected with RV groups A, B, and/or C. Both studies provide information implying that porcine RVH behaves as an opportunistic agent, unlike the human RVH, which was described as a cause of diarrhea episodes (Ji et al., 2002; Alam et al., 2007). In addition, Marthaler and colleagues (2014b) described the presence of RVH in pigs from 4 to more than 55 days old. Nevertheless, of all fecal samples from age groups in which they detected positive results, most (18%) were from 21 to 55-day-old pigs. Our results may contribute to a better understanding of RVH infection, since all Brazilian positive fecal samples belong to pigs with 35 days old, in accordance with the previous study. Information on the epidemiology and pathogenesis of the virus remain scarce. However, the studies on molecular classification of RVH porcine strains are only beginning in Brazil and around the world. Future molecular epidemiologic studies are needed for further information.

In summary, this study suggests that Brazilian pig herds may be constantly challenged by different RV groups. Despite the good nutritional and sanitary management in the pig herd under study, a diarrhea outbreak occurred on the farm and was responsible for important productive and economic losses. The mass vaccination of the pig population in the herd may have contributed to the selection pressure and emergence of infections with the RVA genotypes reported herein. Furthermore, the detection of RV groups B, C, and H in this outbreak suggests that these RVs play a role as causative agents of diarrhea. Therefore, infections with RV groups B, C, and H should not be underestimated and must be included in the diagnostic tests for gastrointestinal diseases in pigs. The constant monitoring of RV infections is necessary for the detection of new genotype emergence within the various RV groups.

In conclusion, this study provides novel information that ratify pre-established concepts regarding certain characteristics of infections with RV; however, new epidemiological information was presented and needs to be addressed in future studies on RVs for a better understanding and prevention of RV infections. Additionally, because diarrhea etiology is complex, the real importance of enteropathogens can be evaluated only by means of a diagnostic platform that should include analyses of several enteric viruses.

Acknowledgments

This work was supported by the Brazilian Institutes CNPq, CAPES, Finep, and Fundação Araucária (FAP/PR). Lorenzetti, E., Alfieri, A.F., and Alfieri, A.A. are recipients of CNPq fellowships.

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3.2 GENETIC VARIABILITY OF VP6, VP7, VP4, AND NSP4 GENES OF PORCINE ROTAVIRUS GROUP H DETECTED IN BRAZIL

(http://dx.doi.org/10.1016/j.virusres.2014.12.003

Virus Research 197 (2015) 48–53

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Genetic variability of VP6, VP7, VP4, and NSP4 genes of porcine rotavirus group H detected in Brazil

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ARTICLE INFO

Article history:
Received 13 October 2014
Received in revised form 2 December 2014
Accepted 2 December 2014
Available online 10 December 2014

Keywords:
Pigs
Diarrhea
Group H rotavirus
RVH
RT-PCR

ABSTRACT

Rotaviruses (RV) are a common cause of viral gastroenteritis in humans and animals. Despite the seven groups/species of RV (A–G), recently it was proposed the creation of a new RV group/specie H (RVH) based on VP6 sequence analysis. In this study we determined the VP6, VP7, VP4, and NSP4 nucleotide and deduced amino acid sequences of 6 (BR59–BR64) RVH-positive stool specimens obtained from piglets with diarrhea in Mato Grosso do Sul, Central-West region of Brazil in 2012, using RT-PCR assay. Based on the high sequence identities (>99%) of the VP6, VP4, VP7, and NSP4 genes among 5 of the studied fecal specimens (BR59–BR63), they are considered the same local rotavirus strain denominated RVH/BRA-1. In contrast, once that the fecal sample BR64 showed a relatively high difference (81.6% nt identity and 83.4% aa identity) in the VP7 sequence when compared to the other 5 specimens it was named RVH/BRA-2 strain. Comparative phylogenetic analysis showed that the 6 RVH strains do not cluster together with any available sequences of members of the established RV groups (RVA–RVG), however, seem to be related to RVB and RVG. These results confirm the presence of RVH in Brazil, demonstrate their genetic diversity, and provide new data that will assist in understanding the viral phylogeny and epidemiology, as well as the explanation of patterns of viral evolution and biological properties of RVH.

1. Introduction

Rotaviruses (RV), classified in the genus *Rotavirus* of the family Reoviridae, are a common cause of viral gastroenteritis in humans and animals (Estes and Kapikian, 2007; Attoui et al., 2012). The viral particles are composed of a triple-layered capsid with a genome consisting of 11 segments of double-stranded RNA (dsRNA), which encode six structural (VP1–VP4, VP6 and VP7) and six non-structural proteins (NSP1–NSP6) (Estes and Kapikian, 2007).

Based on the antigenicity and genetic characteristics of VP6, rotaviruses have been classified into 5 groups/species, A–E (RVA–RVE), and 2 tentative groups/species, RVF and RVG (Attoui et al., 2012). Additionally, also based on molecular analysis of VP6, Matthijnssens et al. (2012) recently proposed the creation of another RV group/species, named as group H (RVH).

Groups A, B, C, and H rotavirus are known to infect both humans and animals, whereas groups D, E, F, and G have been found to infect only animals (Matthijnssens et al., 2010, 2012). Group A rotavirus (RVA) infections cause severe diarrhea in infants and young children worldwide but can also infect adults, other mammals and birds (Estes and Kapikian, 2007). Group B rotavirus (RVB) infections were first associated with cases of severe diarrhea in adults, but have also been detected in cows, pigs, sheep, and rats (Hung et al., 1984; Eiden et al., 1992; Chang et al., 1997; Kuga et al., 2009).

Group H rotavirus was first described as the cause of an outbreak of adult gastroenteritis in China in 1997 (Yang et al., 1998, 2004). Based on the electrophoretic profile of the viral genome, serologic analysis, and similarity of the clinical signs with those observed in the outbreak caused by the RVB strain ADRV (adult diarrhea rotavirus), the novel RV strain was named novel adult diarrhea rotavirus (NADRV) (Yang et al., 1998, 2004; Ji et al., 2002).

In addition to the NADRV strain, other RV strains were included in the new RV group H such as the J19 strain, identified in the same outbreak in China in 1997 (Jiang et al., 2008), the human rotavirus B219, detected in a sporadic case of diarrhea in Bangladesh during 2002 (Alam et al., 2007; Nagashima et al., 2008), and the porcine RV strain SKA-1 that was isolated from a pig with diarrhea in Japan (Wakuda et al., 2011). The complete nucleotide sequences of the 11 dsRNA segments of the human J19 and B219 strains have been determined (Alam et al., 2007; Jiang et al., 2008; Nagashima et al., 2008). Additionally, 4 dsRNA segments encoding the proteins VP4,

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http://dx.doi.org/10.1016/j.virusres.2014.12.003
0168-1702/

VP6, VP7, and NSP4 of the porcine RVH SKA-1 strain were determined (Wakuda et al., 2011), and comparative sequence analysis showed that these RV strains (ADRV-N, J19, B219, and SKA-1) do not cluster together with any available sequences of members of the established RV groups (RVA–RVG) (Matthijnssens et al., 2012).

Recently, based on the VP6 cutoff value proposed by Matthijnssens et al. (2012), RVH was detected outside the Asian continent in 3 (BR59, BR60, and BR63) diarrheic fecal samples in a molecular study of RVB during a neonatal diarrhea outbreak in a Brazilian pig farm (Molinari et al., 2014). PAGE electrophoretic migration profiles of the genome segments of the wild-type Brazilian RVH specimens were similar to those of RVB; however, comparative analysis of the VP6 genes showed that they were closely related to the novel group H rotavirus. Additionally, Marthaler et al. (2014) described the presence of porcine RVH circulating in US pig herds.

In order to establish a possible comparison with other human and porcine RV strains already described, in this study we determined the nucleotide sequences of the RVH most studied genes, VP6, VP4, VP7, and NSP4 from 6 RVH-positive stool specimens obtained from piglets with diarrhea in Brazil. After the sequence data were obtained, the characteristics of the nucleotide sequences and deduced amino acid products were analyzed and compared with data available from other RV groups.

2. Materials and methods

2.1. Rotavirus specimens

A molecular study of RV infection was performed on a pig farm in Mato Grosso do Sul in the Central-West region of Brazil during an outbreak of neonatal diarrhea in 2012. A total of 59 diarrheic fecal specimens were collected from piglets at 12–35 days of age. Within these specimens, samples BR59, BR60, and BR63 had been previously classified as RVH by molecular analysis of VP6 in a study that described the identification of RVH for the first time in the American continent (Molinari et al., 2014).

2.2. RVH screening and RT-PCRs

The viral dsRNA extraction from all stool specimens was performed 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).

RVH screening was performed by RT-PCR using the primer pair VP6/RVN-1F–VP6/RVN-1R as described by Molinari et al. (2014). The specimens BR59, BR60, and BR63 were not included in this step, as they had been previously tested (Molinari et al., 2014).

Subsequently, the positive specimens were submitted to an additional set of RT-PCRs for the amplification of genes VP6, VP4, VP7, and NSP4 using primer pairs that were designed based on the sequence data of the RV strain SKA-1 (Wakuda et al., 2011) (Table 1). Synthesis of cDNA was achieved following RNA denaturation by heating to 94 °C for 5 min and cooling rapidly on ice for 5 min. Reverse transcription was performed in a 20 μl reaction mixture containing 12 μl of the denaturation reaction product annealed to forward and reverse primers, 0.5 mM dNTP mix, 1× RT buffer (50 mM Tris–HCl, pH 8.3, 3 mM $MgCl_2$, 75 mM KCl), 10 mM DTT, and 100 U SuperScript™ II Reverse Transcriptase. The mixture was incubated at 42 °C for 30 min. The reaction was stopped at 95 °C for 5 min. PCR amplification was performed in 50 μl of total reaction mixture by adding 8 μl of cDNA into a reaction cocktail containing 1× PCR buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 1 mM dNTP mix, 20 pmol of each primer, and 2.5 U Platinun®*Taq* DNA Polymerase. The steps of thermal cycling were setup as follows: initial denaturation for 3 min at 94 °C, followed by 35 cycles of 94 °C for 1 min, 48 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 10 min.

The amplified products were analyzed by electrophoresis on a 2% agarose gel in TBE buffer, pH 8.4 (89 mM Tris, 89 mM boric acid, 2 mM EDTA), with 0.5 μg/ml EtBr and visualized under UV light.

2.3. Sequencing and phylogenetic analysis

The amplified cDNA products from the VP6, VP4, VP7, and NSP4 genes of the RVH in diarrheic fecal samples were purified using the GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, UK), quantified in 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). Sequence quality analyses were performed using Phred and CAP3 software (http://aspargin.cenargen.embrapa.br/phph/). Similarity searches were performed with sequences deposited in GenBank using the basic local alignment search tool (BLAST) software (http://blast.ncbi.nlm.nih.gov/Blast.egi). Phylogenetic tree building based on nucleotide (nt) sequences was obtained using the neighbor-joining method from the Kimura two-parameter model, which provided statistical support via bootstrapping with 1000 replicates using the MEGA software package (version 6). The sequence identity matrix was performed using BioEdit software version 7.08.0.

Table 1
Primer sequences designed based on the sequence data of the rotavirus strain SKA-1 (Wakuda et al., 2011), and their position used for RT-PCR amplification of RVH genes.

Viral protein gene	Primer	Sequence (5′–3′)	Position	PCR product (bp)
VP6	VP6/RVN-1F	TGCTACAAGTGACCCACAAGG	11–31	590
	VP6/RVN-1R	GCCATCTTTCCAGTGGCTCT	581–600	
	VP6/RVN-2F	ACCAGGTGGAGCAACAAACA	529–548	716
	VP6/RVN-2R	CAGTGCGTGACCAGATCTCA	1225–1244	
VP7	VP7/RVH-fw	GGAACTTTAAAGCCATGTTGTTC	1–23	817
	VP7/RVH-rv	GGGTATATTTGCCTGACATAACG	795–817	
VP4	VP4/RVH-1F	AGAACCCAGGTGAAGGGTCT	47–66	961
	VP4/RVH-1R	GGTGTAGTGACTGTCGTTGC	988–1007	
	VP4/RVH-2F	TCATGGGTGGAACGTTTGGA	893–912	914
	VP4/RVH-2R	CGCTACTCCGTTCACCCTAC	1787–1806	
	VP4/RVH-3F	ACATCAGGTATAATGTCTTTTGCAT	1678–1702	813
	VP4/RVH-3R	AACGTCATGTACTAATGCCACT	2469–2490	
NSP4	NSP4/RVH-fw	TTCATCAAAGTCACGATGGA	10–29	720
	NSP4/RVH-rv	CAAGGGTGAACACTACCAAG	710–729	

3. Results

Including the diarrheic fecal samples BR59, BR60, and BR63, porcine RVH was detected in 9 of the 59 (15.2%) fecal specimens using the RT-PCR with the primer pair VP6/RVN-1F–VP6/RVN-1R. Six (BR59–BR64) of the 9 positive specimens were selected for further molecular assay analyses based on the quality of the amplified products.

3.1. VP6 gene

The pair-wise comparisons of the VP6 nt sequences and the inferred amino acid (aa) sequences of 5 positive specimens (BR59–BR63) revealed 100% nt and aa identities among themselves. The specimen BR64 showed 99.8% nt identity when compared to the other 5 specimens, but the aa identity was 100%. The 6 sequences were compared with those of other RV representative strains and showed less than 35% nt identity (less than 13% aa identity) with groups A, C, D, and F RV. Similarities of 50.6–51.8% nt (35.8–37.9% aa) and 51.4% nt (38.2% aa) were found for RVB and RVG, respectively.

The specimens shared the highest identity with group H RV. The highest identity was shared with the VP6 gene of the porcine RVH SKA-1 strain (Table 2). Based on phylogenetic analysis, the tree (Fig. 1) inferred from the VP6 aa sequences could be subdivided into 2 major clusters, one containing RVA, RVC, RVD, and RVF, and the other containing RVB, RVG, and RVH. The BR59–BR64 samples grouped closest to the RVH strains, independent of the other RV groups, but were segregated into a different branch, closer to the SKA-1 porcine RVH strain.

3.2. VP4 gene

The VP4 gene sequences of specimens BR61, BR62, and BR64 showed a very small difference (99.9% nt and 100% aa identities) when compared to BR59, BR60, and BR63. Based on the analysis with representative RV strains, all 6 samples shared the greatest similarity with RV strains belonging to group H. When compared restricted to the SKA-1 porcine RVH strain the 6 samples showed the highest identities, that were on average 88.66% at the nt level and 93.23% at the aa level. The sequence analyses results from the other RV groups are summarized in Table 2. The phylogenetic tree including the 6 specimens (BR59–BR64) of porcine RVH and those of representative RV strains showed the same pattern as that of the VP6 gene (Fig. 1).

3.3. VP7 gene

The identity analysis of the VP7 sequences of the 6 Brazilian specimens revealed that the RVH present in the diarrheic fecal samples BR59–BR63 shared 100% similarity at the nt and aa levels among themselves. In contrast, sample BR64 showed only 81.6% nt identity and 83.4% aa identity when compared with the 5 specimens above mentioned, and it shared the highest identity with the porcine RVH strain SKA-1. In general, the 6 samples showed the highest similarity to the strains belonging to group H RV (63–86.9% at nt and 57.4–89.9% at aa levels). Comparisons with representative strains from RV groups revealed low identities with RVA, RVC, RVD, and RVF and relatively high identities with groups B and G (Table 2). Samples BR59–BR63 formed a new branch grouped into the same cluster as the other RVH strains during phylogenetic analysis of the deduced VP7 aa sequences. Closer to the 5 samples, the Brazilian specimen BR64 belonged to the branch containing the Japanese SKA-1 porcine RVH strain (Fig. 1).

Table 2
Sequence identities of the 6 Brazilian porcine RVH genes to representative rotavirus strains from different groups.

Protein/RVH strains	Percentage of nucleotide (amino acid) sequence identity										
	Group A	Group B			Group C	Group H			Group D	Group F	Group G
	OSU	CAL-1	ADRV	IDIR	Cowden	SKA-1	J19	B219	05V0049	03V0568	03V0567
VP6											
BR59–BR63	33.4 (11.9)	51 (37.1)	51.7 (35.8)	50.7 (37.9)	33.3 (9.7)	85.6 (96.9)	71 (75.7)	71 (75.4)	34.9 (12.4)	34.6 (8.7)	51.3 (38.2)
BR64	33.3 (11.9)	51.2 (37.1)	51.8 (35.8)	50.6 (37.9)	33.4 (9.7)	85.7 (96.9)	71.2 (75.7)	71 (75.4)	34.9 (12.4)	34.7 (8.7)	51.4 (38.2)
VP7											
BR59–BR63	32.4 (14.3)	42.5 (20.8)	43.1 (20.8)	42.5 (22)	32.3 (10.4)	81.1 (81.8)	63.8 (57)	63.8 (57.8)	32.9 (9.7)	29.5 (11.7)	44.2 (19.1)
BR64	31.6 (14)	42.6 (20.4)	42.2 (20.4)	40.8 (20.8)	32.6 (11.1)	86.9 (89.9)	63 (57.4)	63.4 (58.2)	34 (10.4)	28.8 (12.8)	44 (20.7)
VP4											
BR59, BR60, BR63	30.1 (11)	41 (22.3)	40.9 (21.6)	42 (23.5)	31 (9.8)	88.7 (93.3)	58.4 (50.8)	58.5 (50.9)	30.8 (9.8)	30.7 (12.9)	42.8 (22.2)
BR 61, BR62, BR64	30.1 (11)	41 (22.3)	40.9 (21.6)	42 (23.5)	31 (9.8)	88.6 (93.3)	58.4 (50.8)	58.4 (50.9)	30.8 (9.8)	30.7 (12.9)	42.8 (22.2)
NSP4											
BR59–BR63	31.2 (6.8)	33.4 (16.3)	33.2 (13.7)	30.6 (-)	36.9 (6.8)	81.6 (80.6)	59.1 (32.4)	60 (33.7)	35.4 (6.2)	31 (6.8)	37 (8.7)
BR64	31.2 (6.8)	33.4 (16.3)	33.4 (13.7)	30.9 (-)	37.2 (6.8)	81.4 (80.6)	59.5 (32.4)	60.3 (33.7)	35.4 (6.2)	31.1 (6.8)	37 (8.7)

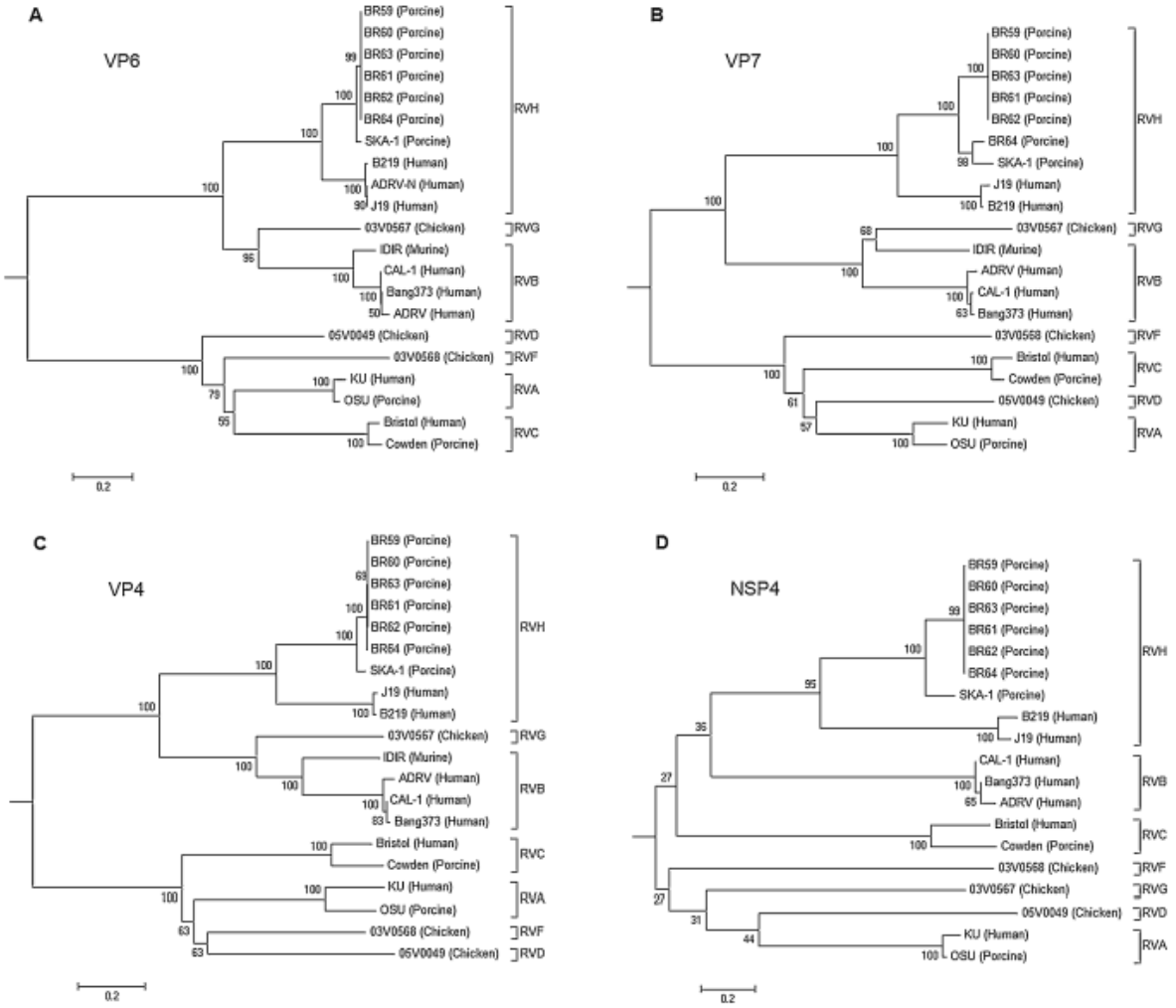

Fig. 1. Phylogenetic trees constructed from the deduced aa sequences of (A) VP6, (B) VP7, (C) VP4, and (D) NSP4 genes of the 6 Brazilian specimens BR59, BR60, BR61, BR62, BR63, and BR64, and representative rotavirus strains belonging to groups A–D and F–H. The 4 trees were constructed using the neighbor-joining method and the Poisson correction method. Bootstrapping was statistically supported with 1000 replicates. Scale bars indicate aa substitutions per site. Sequences used in the alignment present the following GenBank accession numbers: VP6 genes: BR59 (KF021619), BR60 (KF021620), BR61 (KM359479), BR62 (KM359480), BR63 (KF021621), BR64 (KM359481), SKA-1 (AB576626), J19 (DQ113902), B219 (DQ168033), IDIR (M84456), ADRV (M55982), CAL-1 (AB037931), Bang373 (AY238389), KU (AB022768), OSU (AF317123), Bristol (X59843), Cowden (M94157), 05V0049 (GU733448), 03V0568 (HQ403603), 03V0567 (HQ403604); VP7 genes: BR59 (KM359482), BR60 (KM359483), BR61 (KM359485), BR62 (KM359486), BR63 (KM359484), BR64 (KM359487), SKA-1 (AB576627), J19 (DQ113905), B219 (DQ168034), IDIR (D00911), ADRV (M33872), CAL-1 (AF184083), Bang373 (AY238385), KU (D16343), OSU (X04613), Bristol (X77257), Cowden (M61101), 05V0049 (GU733451), 03V0568 (JQ919998), 03V0567 (JQ920007); VP4 genes: BR59 (KM359488), BR60 (KM359489), BR61 (KM359491), BR62 (KM359492), BR64 (KM359493), SKA-1 (AB576625), J19 (DQ113899), B219 (EF453358), IDIR (X16949), ADRV (M55982), CAL-1 (AF184084), Bang373 (AY238388), KU (AB222784), OSU (X13190), Bristol (X79442), Cowden (M74218), 05V0049 (GU733445), 03V0568 (JQ919997), 03V0567 (JQ920006); NSP4 genes: BR59 (KM359494), BR60 (KM359495), BR61 (KM359497), BR62 (KM359498), BR63 (KM359496), BR64 (KM359499), SKA-1 (AB576628), J19 (DQ113906), B219 (EF453359), IDIR (U03557), ADRV (AY548957), CAL-1 (AY238387), Bang373 (AY238384), KU (AB022772), OSU (D88831), Bristol (X83967), Cowden (AF093202), 05V0049 (GU733452), 03V0568 (JQ920002), 03V0567 (JQ920011).

3.4. *NSP4 gene*

The sequences of the 6 Brazilian porcine RVH strains were compared among themselves and among the RV representative strains. In the analysis among themselves, only sample BR64 showed some differences (99.3% nt identity), which were restricted to nt sequences. The level of aa identity was 100% among each other. In the analysis with the other RV strains, the highest identity was with the RVH representative strains (59.5–81.4% at nt and 32.4–80.6% at aa levels), and the aa similarity among the 6 specimens and the RVH J19 and B219 human strains was on average only 34%. The sequence analyses results of RVA, RVB, RVC, RVD, RVF, and RVG are summarized in Table 2. The phylogenetic tree inferred from the deduced NSP4 aa sequences showed the same pattern as those from VP6 and VP4 genes (Fig. 1).

4. Discussion

In the present study, an RT-PCR assay using specific primers for amplification of nearly the full length of porcine RVH VP6,

VP7, VP4, and NSP4 genes was developed. Then, the sequences of these 4 genes were determined for porcine RVH strains presented in 6 (BR59–BR64) diarrheic fecal samples. The nt and deduced aa sequences of the 6 samples studied were remarkably distinct from those of other (A–G) RV groups. To our knowledge, this is the first report to describe the nucleotide and deduced amino acid sequences of Brazilian porcine RVH strains VP6, VP7, VP4, and NSP4 genes.

Based on the extremely high sequence identities (>99%) of the VP6, VP4, VP7, and NSP4 genes among 5 of the studied fecal specimens (BR59–BR63), they are considered as virtually identical, suggesting that these specimens represented the same local rotavirus strain denominated RVH/BRA-1. In contrast, although the 6 specimens were collected on the same farm, fecal sample BR64 showed a relatively high difference (81.6% nt identity and 83.4% aa identity) in the VP7 sequence when compared to the other 5 specimens and was named the RVH/BRA-2 strain.

Regardless of the differences found in the VP7 sequences, comparisons of the 6 Brazilian RVH samples with representative strains from RV groups confirmed that the VP6, VP7, VP4, and NSP4 genes belonged to group H RV. These genes had higher identities with RVH strains SKA-1, J19, and B219, and in the phylogenetic trees they were grouped with these RVH strains in a cluster clearly distinguished from those of RVA, RVB, RVC, RVD, RVF, and RVG.

The VP6, VP4, VP7, and NSP4 nt and aa sequences of the 6 Brazilian samples shared low identities with representative strain sequences from RV groups A, C, D, and F. However, when compared with RV groups B and G, they showed a moderate level of relatedness (30.6–51.8% nt identity and 8.7–38.2% aa identity). This similarity is also evident in the phylogenetic trees, in which the 6 samples cluster closer to groups B and G than to the other RV groups. In previous reports, based on sequence identities and structural similarities, a genetic relationship between human RVH and RVB strains was suggested, as well as the possibility that these 2 rotavirus groups might have originated from a common ancestral virus and have an animal as their reservoir host (Alam et al., 2007; Jiang et al., 2008). Although in our study RVH was detected from pigs, our molecular findings reinforce the idea of a common ancestor between RVH and RVB. As this virus has been described in humans and swine, the possibility of pigs acting as an RVH reservoir cannot be ignored. However, further studies are necessary to clarify the zoonotic potential of RVH transmission.

The genetic diversity of the VP7 gene sequences has already been studied for RVA–RVC. The Rotavirus Classification Working Group established a sequence identity cutoff value of 89% in aa for the definition of RVA G genotypes (Matthijnssens et al., 2008a,b). In addition, a cutoff value of 66% in aa identity was proposed for the classification of RVB VP7 genotypes (Kuga et al., 2009). According to Kuga et al. (2009), this difference between the RVA and RVB VP7 sequence cutoff value may reflect differences in the evolutionary period or rate between the rotavirus groups. Taking into account the considerable difference found between the identities of the VP7 aa sequences from the RVH/BRA-1 and RVH/BRA-2 strains and the fact that it shared the highest identity with the porcine RVH strain SKA-1 (86.9% at nt and 89.9% at aa levels), we suggest that the RVH/BRA-2 strain belongs to the same genotype as the Japanese strain SKA-1, which would be different from the other Brazilian strain RVH/BRA-1. However, considering the genetic relationship proposed for RVH and RVB, and based on the cutoff value proposed for RVB VP7 aa sequences, the cluster formed by human and porcine RVH strains should be divided into just 2 genotypes, one containing the human J19 and B219 RVH strains and, despite their differences, the other containing all the porcine RVH strains (SKA-1, RVH/BRA-1, and RVH/BRA-2). Based on VP7 genotype characterization, a specific classification system for RVH should be established.

In conclusion, we determined the nt sequences of porcine RVH VP6, VP4, VP7, and NSP4 genes and demonstrated their genetic diversity. Very little information is available regarding this new rotavirus group, which infects both humans and animals. To date, these viruses have only been detected in small areas of Asia, USA, and Brazil, which suggests that they are widely spread. The lack of information on these viruses may be associated with misdiagnoses. These findings provide valuable information for future studies regarding rotavirus evolution and infection, and its impact on diarrheal diseases.

Acknowledgements

This work was supported by the Brazilian Institutes CNPq (grant no. 309.137/2013-6), CAPES (grant no. 05740790921), Finep (grant no. 0102022508), and Fundação Araucária (FAP/PR) (grant no. 192/2014). A.F. Alfieri and A.A. Alfieri are recipients of CNPq fellowships.

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3.3 MOLECULAR CHARACTERIZATION OF A NEW G (VP7) GENOTYPE IN GROUP B PORCINE ROTAVIRUS

(INTERVIROLOGY. 2018 JUL 16:1-7. doi: 10.1159/000490388)

Short Communication

Intervirology

Intervirology
DOI: 10.1159/000490388

Received: November 13, 2017
Accepted: May 24, 2018
Published online: July 16, 2018

Molecular Characterization of a New G (VP7) Genotype in Group B Porcine Rotavirus

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Keywords
Diarrhea outbreak · Novel G genotype · Pigs · Reverse transcription PCR · Rotavirus group B

Abstract
Rotaviruses (RVs), a common cause of viral gastroenteritis in humans and animals, are classified into 9 established groups/species (RVA–RVI). Although RVB has been found in several countries, genetic variation among RVB field strains remains poorly characterized. RVB strains can be classified into G genotypes based on a nucleotide (nt) homology that exceeds a cutoff value of 80% for the gene that encodes the structural protein VP7. In this study, we determined the VP7 nt and deduced amino acid sequences of one RVB strain (RB62) identified in a diarrheic fecal sample obtained from a piglet in Brazil in 2012. Comparative analysis of this strain and the strains of the other 21 previously identified VP7 genotypes showed that the highest nt identity (71.2%) was found with the porcine PB-70-H5 strain within the G4 genotype. However, when compared with the nonclassified Vietnamese RVB G genotype 14177_18 strain, the nt sequence identity was of 82.9%. These results led us to conclude that the Brazilian strain BR62 and the Vietnamese strain 14177_18 belong to a novel G genotype (G22).

Rotaviruses (RVs), which are members of the *Reoviridae* family, are a common cause of viral gastroenteritis in humans and animals. RVs consist of nonenveloped particles and possess a genome composed of 11 segments of double-stranded RNA that encode 6 structural (VP1-VP4, VP6, and VP7) and 6 nonstructural (NSP1–NSP5/6) proteins [1].

RVs are classified into 9 established groups/species (RVA–RVI) based on genetic and antigenic differences in the structural VP6 protein [2–4]. RV strains from groups A, B, C, and H are known to infect humans and various animal species, whereas strains from the other RV groups have been found to infect only animals [3–5].

Among animals, RVB has been found in pigs, cattle, goats, and rats [1]. However, in contrast to RVA, porcine RVB infections have been sporadically reported in pig

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herds in the UK [6], Australia [7], New Zealand [8], Japan [9], the Czech Republic [10], Brazil [11], the USA [12], and Vietnam [13]. The relatively few worldwide reports of RVB infection in animals may be attributed to the fact that small quantities of this virus are excreted in the feces of infected animals, which affects the diagnosis of RVB infections [9, 12, 14].

Although RVB has been found in several countries, genetic variation among RVB strains remains poorly characterized. Knowledge regarding the genome of porcine RVB remains restricted to information obtained via analyses of the VP6, VP7, NSP1, NSP2, and NSP5 proteins [3, 9, 11, 12, 14–16]. Similar to RVA strains, RVB strains are categorized using a sequence-based classification system [12, 16, 17]. RVB strains can be classified into G genotypes based on a nucleotide (nt) cutoff value of 80% for the gene that encodes the structural protein VP7 [17]. To our knowledge, 21 RVB G genotypes have previously been described [13, 17].

In this study, we determined the VP7 nt and deduced amino acid (aa) sequences of one RVB strain identified in a diarrheic fecal sample obtained from a piglet in Brazil in 2012. Comparative analysis of this strain and the strains of the other 21 previously identified VP7 genotypes showed that the Brazilian porcine RVB strain belongs to a novel VP7 genotype, G22, and seems to be circulating in different parts of the world.

A total of 50 diarrheic fecal samples, collected from pigs 28 and 35 days of age, from a post-weaning diarrhea outbreak that occurred in a pig herd located in Mato Grosso do Sul, Brazil, in 2012, were subjected to reverse transcription PCR to investigate the presence of RV groups A, B, C, and H. According to Molinari et al. [18], all samples were tested for RVB using the primer pair described by Marthaler et al. [12], which was designed to amplify a fragment (778 bp) of the VP7 gene of RVB. Sixteen diarrheic fecal samples were positive for RVB [19]. Based on the quality of the amplified product, the RVB-positive fecal sample identified as BR62 was selected for further molecular analyses.

To confirm the specificity of the RVB amplicon, the amplified product was purified using a 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 a BigDye Terminator v3.1 Cycle Sequencing Reaction Kit (Applied Biosystems, Foster City, CA, USA) on an automated sequencer (ABI3500; Applied Biosystems). Sequence quality analyses were performed using the Phred and CAP3 software packages (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 nt sequences were constructed using the neighbor joining method from the Kimura two-parameter model, which provided statistical support via bootstrapping with 1,000 replicates in the MEGA software package (version 6). Nt and aa sequence identity matrices were constructed using BioEdit software version 7.08.0. The nt sequence for the VP7 gene of the BR62 RVB strain described in this study was deposited in the GenBank database under accession No. MF072691.

A comparative sequence analysis involving the VP7 genes of the Brazilian RVB strain BR62 and strains belonging to the 21 known RVB G genotypes was performed. Additionally, the VP7 gene of the unclassified porcine RVB strain 14177_18, recently described in Vietnam, was included in this analysis [20].

Compared with the strains belonging to the 21 established RVB G genotypes, the Brazilian wild-type porcine RVB strain BR62 showed the highest nt identity (71.2%) with the porcine PB-70-H5 strain within the G4 genotype. The lowest nt identity (54.6%) was observed with the IDIR strain within the G1 genotype. Pairwise comparisons with the other genotypes showed similarities ranging from 61.8 to 70.9% at the nt level (Table 1). In contrast, a comparison of the BR62 strain and the Vietnamese 14177_18 strain revealed an nt sequence identity of 82.9%.

A phylogenetic analysis based on deduced aa sequences for RVB VP7 was also performed. This analysis indicated that the BR62 strain showed the highest aa identity (88.7%) with the Vietnamese 14177_18 strain. The BR62 strain's aa identity with other RVB strains ranged from 48.6 to 75.9% (Table 1).

In phylogenetic trees based on VP7 nt and aa sequences for VP7, the porcine field strain BR62 described in this study constituted a branch separate from the strains belonging to the 21 previously identified RVB genotypes. Additionally, the unclassified Vietnamese 14177_18 strain was clustered with the BR62 strain (Fig. 1).

Although less prevalent than RVA infections, RVB infections in humans and pigs have been reported in different regions throughout the world [19, 20–22]. However, small quantities of this virus are excreted in the feces of infected animals, and it is difficult to adapt RVB strains to cell culture [9, 12, 14, 23]; as a result, the serological and molecular characterization of RVB strains remains limited.

Table 1. Nucleotide and deduced amino acid sequence identity of the VP7 gene of the Brazilian strain BR62 and representatives of the different RVB G genotypes

Strain	Identity with the BR62 strain, %	
	nucleotide	amino acid
RVB/Rat-hhp/USA/IDIR/1984/G1P[X]	54.6	48.6
RVB/Human-wt/BGD/Bang373/2000/G2P[X]	64.3	64.1
RVB/Cow-wt/JPN/Nemuro/1997/G3P[X]	61.8	62.0
RVB/Pig-wt/PB-70-H5/2007/G4P[X]	71.2	75.9
RVB/Cow-wt/IND/DB101/2001/G5P[X]	63.8	62.0
RVB/Pig-wt/USA/MN09-35/2009/G6P[X]	64.7	61.4
RVB/Pig-wt/JPN/PB-S15/2002/G7P[X]	64.3	63.6
RVB/Pig-wt/USA/CO09-12/2009/G8P[X]	64.7	60.9
RVB/Pig-wt/JPN/PB-68-E4/2007/G9P[X]	62.2	60.9
RVB/Pig-wt/USA/MO09-34/2009/G10P[X]	64.8	64.1
RVB/Pig-wt/JPN/PB-S5/2002/G11P[X]	70.9	70.0
RVB/Pig-wt/JPN/PB-S24-11/2002/G12P[X]	69.1	68.9
RVB/PigG13-wt/JPN/PB-23-44/2005/G13P[X]	64.1	65.2
RVB/Pig-wt/BRA/BR31/2012/G14P[X]	67.9	67.3
RVB/Pig-wt/JPN/PB-S49-2/2003/G15P[X]	66.3	67.3
RVB/Pig-wt/JPN/PB-68-C17/2007/G16P[X]	66.1	64.1
RVB/Pig-wt/USA/OK09-51/2009/G17P[X]	66.6	62.5
RVB/Pig-wt/USA/TX09-47/2009/G18P[X]	66.6	64.1
RVB/Pig-wt/IND/AN142129/2013/G19P[X]	64.3	61.4
RVB/Pig-wt/JPN/PB-S26-1/2002/G20P[X]	64.8	60.4
RVB/Pig-wt/IND/AN142530/2013/G21P[X]	64.8	63.1
RVB/Pig-wt/VNM/14177_18/2012/GXP[X]	82.9	88.7

In 2009, Kuga et al. [9] proposed a classification system of G genotypes for RVB strains based on analyses of the VP7 genes of 38 porcine RVB strains. Using nt cutoffs of 67 and 76% (66 and 79%, respectively, at the aa level), they proposed the creation of 5 genotypes divided into 12 clusters. Several years later, after phylogenetic analyses of 68 new RVB VP7 sequences had been performed, Marthaler et al. [12] proposed a modification to the cutoff values established by Kuga et al. [9]. Using an nt cutoff value of 80%, they identified 20 RVB G genotypes. Additionally, using this nt cutoff value proposed by Marthaler et al. [12], Suzuki et al. [14] described a new RVB G genotype (G21) isolated from pigs in India.

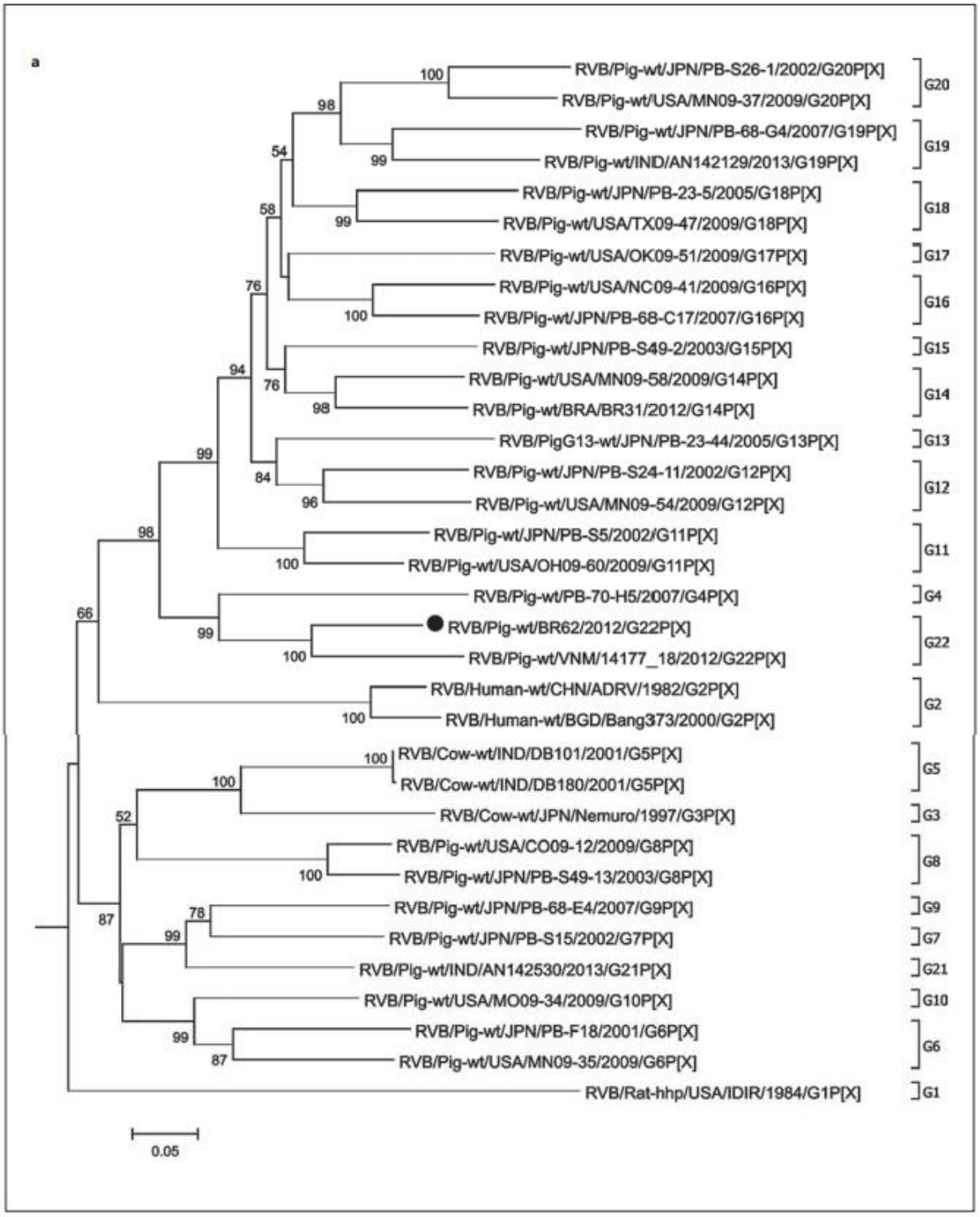
In our study, the VP7 sequence of the BR62 RVB strain was compared with the VP7 sequences of representative strains for all 21 previously described G genotypes. The BR62 RVB strain had the highest nt identity (71.2%) with the porcine RVB strain PB-70-H5, which belongs to the G4 genotype. However, given that the VP7 gene sequence of the Brazilian strain BR62 did not share ≥80% nt identity with any of the VP7 gene sequences of RVB strains with previously identified genotypes, we propose the creation of G22, a new RVB G genotype.

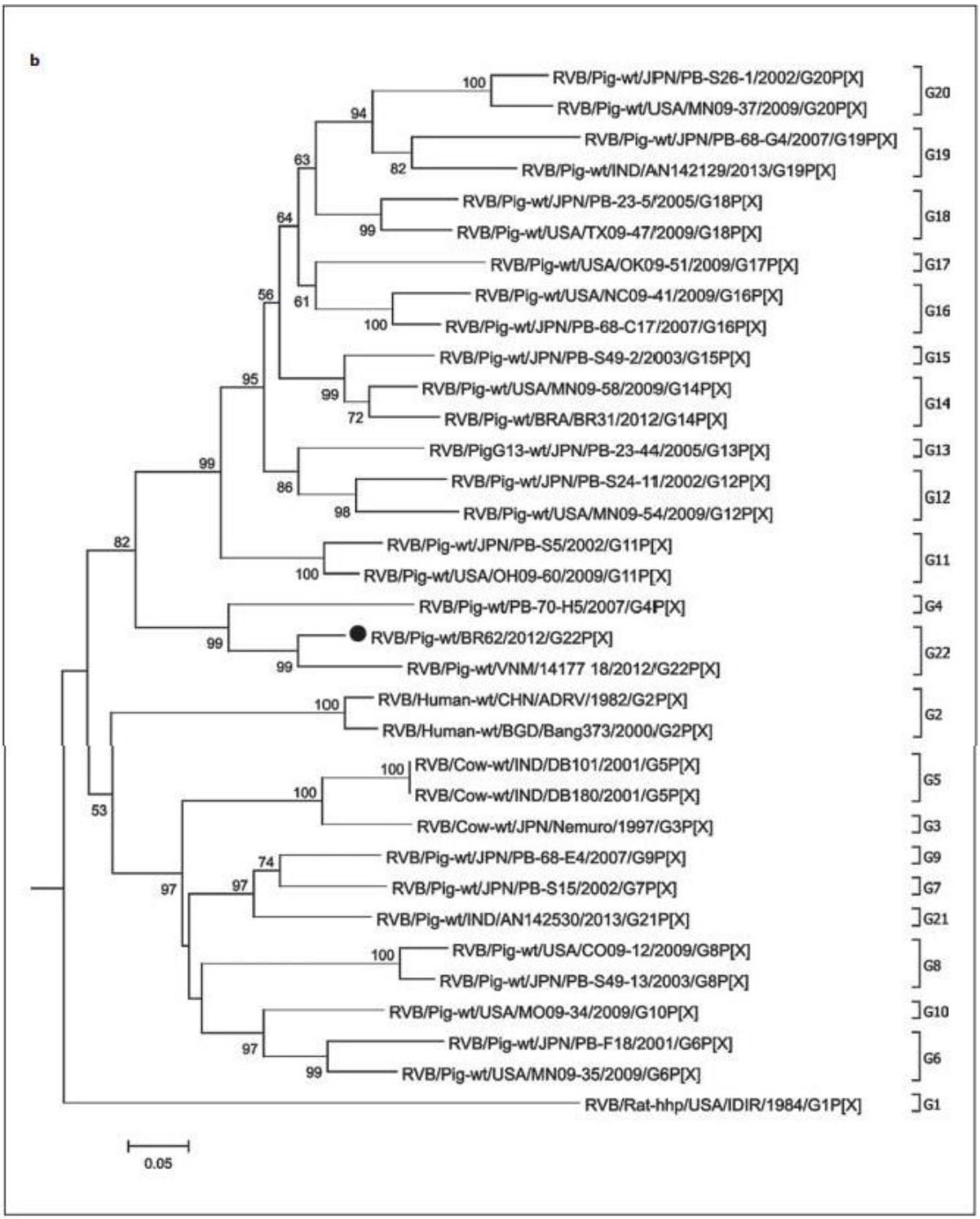
In contrast, an analysis comparing the Brazilian BR62 strain and the unclassified Vietnamese RVB 14177_18 strain revealed an nt sequence identity of 82.9%. This result led us to believe that strain 14177_18 and the Brazilian strain BR62 belong to the same genotype (G22). This assumption is supported by our nt-based phylogenetic tree, in which strains BR62 and 14177_18 are clustered together but placed in a different branch than strains belonging to the other 21 RVB G genotypes. Moreover, this reasoning suggests that porcine RVB strains with genotypes distinct from previously described genotypes might be circulating in several countries, which may interfere with the control and prophylaxis of RV infections, as well as the zoonotic role of the virus. Therefore, screening for RVB should be actively incorporated into porcine RV studies, and RVB should receive increased research attention.

Regarding analyses of the deduced aa sequences for VP7 for the BR62 strain and other RVB strains, the BR62

Fig. 1. Phylogenetic trees constructed based on the nucleotide (**a**) and deduced amino acid (**b**) sequences of the VP7 gene from the porcine RVB strain BR62 described in this study, as well as representative strains for the 21 previously identified RVB genotypes. The scale bars indicate nt/aa substitutions per site. The bootstrap values are shown at branch nodes (values <50% are not shown). The Brazilian porcine RVB strain is marked with a filled circle.

(For figure see next pages.)


a
RVB/Pig-wt/JPN/PB-S26-1/2002/G20P[X]
RVB/Pig-wt/USA/MN09-37/2009/G20P[X]
G20
RVB/Pig-wt/JPN/PB-68-G4/2007/G19P[X]
RVB/Pig-wt/IND/AN142129/2013/G19P[X]
G19
RVB/Pig-wt/JPN/PB-23-5/2005/G18P[X]
RVB/Pig-wt/USA/TX09-47/2009/G18P[X]
G18
RVB/Pig-wt/USA/OK09-51/2009/G17P[X]
G17
RVB/Pig-wt/USA/NC09-41/2009/G16P[X]
RVB/Pig-wt/JPN/PB-68-C17/2007/G16P[X]
G16
RVB/Pig-wt/JPN/PB-S49-2/2003/G15P[X]
G15
RVB/Pig-wt/USA/MN09-58/2009/G14P[X]
RVB/Pig-wt/BRA/BR31/2012/G14P[X]
G14
RVB/PigG13-wt/JPN/PB-23-44/2005/G13P[X]
G13
RVB/Pig-wt/JPN/PB-S24-11/2002/G12P[X]
RVB/Pig-wt/USA/MN09-54/2009/G12P[X]
G12
RVB/Pig-wt/JPN/PB-S5/2002/G11P[X]
RVB/Pig-wt/USA/OH09-60/2009/G11P[X]
G11
RVB/Pig-wt/PB-70-H5/2007/G4P[X]
G4
RVB/Pig-wt/BR62/2012/G22P[X]
RVB/Pig-wt/VNM/14177_18/2012/G22P[X]
G22
RVB/Human-wt/CHN/ADRV/1982/G2P[X]
RVB/Human-wt/BGD/Bang373/2000/G2P[X]
G2
RVB/Cow-wt/IND/DB101/2001/G5P[X]
RVB/Cow-wt/IND/DB180/2001/G5P[X]
G5
RVB/Cow-wt/JPN/Nemuro/1997/G3P[X]
G3
RVB/Pig-wt/USA/CO09-12/2009/G8P[X]
RVB/Pig-wt/JPN/PB-S49-13/2003/G8P[X]
G8
RVB/Pig-wt/JPN/PB-68-E4/2007/G9P[X]
G9
RVB/Pig-wt/JPN/PB-S15/2002/G7P[X]
G7
RVB/Pig-wt/IND/AN142530/2013/G21P[X]
G21
RVB/Pig-wt/USA/MO09-34/2009/G10P[X]
G10
RVB/Pig-wt/JPN/PB-F18/2001/G6P[X]
RVB/Pig-wt/USA/MN09-35/2009/G6P[X]
G6
RVB/Rat-hhp/USA/IDIR/1984/G1P[X]
G1
0.05


b
RVB/Pig-wt/JPN/PB-S26-1/2002/G20P[X]
RVB/Pig-wt/USA/MN09-37/2009/G20P[X]
RVB/Pig-wt/JPN/PB-68-G4/2007/G19P[X]
RVB/Pig-wt/IND/AN142129/2013/G19P[X]
RVB/Pig-wt/JPN/PB-23-5/2005/G18P[X]
RVB/Pig-wt/USA/TX09-47/2009/G18P[X]
RVB/Pig-wt/USA/OK09-51/2009/G17P[X]
RVB/Pig-wt/USA/NC09-41/2009/G16P[X]
RVB/Pig-wt/JPN/PB-68-C17/2007/G16P[X]
RVB/Pig-wt/JPN/PB-S49-2/2003/G15P[X]
RVB/Pig-wt/USA/MN09-58/2009/G14P[X]
RVB/Pig-wt/BRA/BR31/2012/G14P[X]
RVB/PigG13-wt/JPN/PB-23-44/2005/G13P[X]
RVB/Pig-wt/JPN/PB-S24-11/2002/G12P[X]
RVB/Pig-wt/USA/MN09-54/2009/G12P[X]
RVB/Pig-wt/JPN/PB-S5/2002/G11P[X]
RVB/Pig-wt/USA/OH09-60/2009/G11P[X]
RVB/Pig-wt/PB-70-H5/2007/G4P[X]
RVB/Pig-wt/BR62/2012/G22P[X]
RVB/Pig-wt/VNM/14177 18/2012/G22P[X]
RVB/Human-wt/CHN/ADRV/1982/G2P[X]
RVB/Human-wt/BGD/Bang373/2000/G2P[X]
RVB/Cow-wt/IND/DB101/2001/G5P[X]
RVB/Cow-wt/IND/DB180/2001/G5P[X]
RVB/Cow-wt/JPN/Nemuro/1997/G3P[X]
RVB/Pig-wt/JPN/PB-68-E4/2007/G9P[X]
RVB/Pig-wt/JPN/PB-S15/2002/G7P[X]
RVB/Pig-wt/IND/AN142530/2013/G21P[X]
RVB/Pig-wt/USA/CO09-12/2009/G8P[X]
RVB/Pig-wt/JPN/PB-S49-13/2003/G8P[X]
RVB/Pig-wt/USA/MO09-34/2009/G10P[X]
RVB/Pig-wt/JPN/PB-F18/2001/G6P[X]
RVB/Pig-wt/USA/MN09-35/2009/G6P[X]
RVB/Rat-hhp/USA/IDIR/1984/G1P[X]
G20
G19
G18
G17
G16
G15
G14
G13
G12
G11
G4
G22
G2
G5
G3
G9
G7
G21
G8
G10
G6
G1
0.05

strain exhibited the highest (88.7%) aa identity with the Vietnamese 14177_18 strain. According to Kuga et al. [9], aa cutoff values of 66 and 79% produced good correlations between G serotypes and G genotypes. Given this assumption, the Brazilian BR62 and Vietnamese 14177_18 strains could be classified as belonging to the same serotype. In contrast, Marthaler et al. [12] indicated that an aa cutoff value >89% is appropriate for most RVB strains belonging to the same genotype, with certain exceptions (strains belonging to genotypes G6, G7, G12, and G16). Thus, two hypotheses seem reasonable: either the BR62 strain does not belong to the same serotype as the 14177_18 strain, or the new proposed G22 genotype is in the group of RVB genotypes with sufficient aa diversity such that the applicable cutoff threshold is lower than the suggested value of 89%. If the second hypothesis is correct, the BR62 and 14177_18 strains would belong to the same serotype. The aa phylogenetic tree shows evidence that might support this conclusion; once again, the BR62 and 14177_18 strains are clustered together, but placed in a different branch than the other 21 RVB G genotypes. However, future serological assays should be performed to confirm these assumptions and provide more information about the viral immunology.

In conclusion, the genetic diversity observed among porcine RVB strains may be underestimated. In this study, two strains from different countries that belong to G22, a new RVB G genotype, could be identified. Although RVA infections are more prevalent and pathogenic than RVB infections, constant monitoring of RV infections is necessary to detect the emergence of new genotypes within various RV groups. Future studies of the epidemiology and molecular evolution of RVB genes should be developed to better understand and prevent RV infections. In addition, the idea that certain RV genotypes may exhibit a zoonotic potential and that animals can serve as reservoirs of such viruses reinforces the need for further studies.

Acknowledgements

This work was supported by the Brazilian Institutes CNPq, CAPES, Finep, and Fundação Araucária (FAP/PR). A.F.A. and A.A.A. are recipients of CNPq fellowships.

Statement of Ethics

The study was submitted to the Ethics Committee on Animal Experiments of Universidade Estadual de Londrina and approved under the identification No. 11363.2015.16. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Disclosure Statement

All authors declare that they have no conflicts of interest.

Funding Sources

This study was funded by CNPq (grant No. 309.137/2013-6).

Author Contributions

B.L.D.M. developed this study as part of her postgraduate program under the guidance of A.A.A. and A.F.A. A.F.A. is the graduate student's co-advisor. She contributed to the design of the research as well as to corrections referring to the scientific manuscript. A.A.A. is the graduate student's advisor. He contributed to the design of the research as well as to corrections referring to the scientific manuscript.

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4 CONCLUSÕES

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- A RT-PCR com os *primers* selecionados para os genes VP4, VP7 e NSP4 possibilitou a amplificação dos produtos esperados para as amostras de RVH incluídas neste estudo.

- A análise filogenética das sequências obtidas a partir dos genes VP4, VP7 e NSP4 confirma a classificação das cepas estudadas como pertencentes à espécie RVH.

- A detecção de cepas de RVA em um rebanho vacinado (Cepa OSU – G5P[7]) demonstra que a imunidade cruzada existente entre genotipos diferentes de uma mesma espécie não é suficiente para proteção integral.

- A grande variedade genética existente entre as cepas de rotavírus pode comprometer a implantação de programas de controle vacinal efetivos.

- O RVH participa dos quadros clínicos de diarreia quando em associação com outras espécies de rotavírus.

- A análise filogenética da sequência de nucleotídeos do gene VP7 da cepa de RVB BR62 confirmou que a mesma pertence a um novo genotipo, G22.

- A elevada similaridade entre o gene VP7 da cepa brasileira BR62 e da cepa vietnamita 14177_18 sugere que o genotipo G22 pode estar circulando silenciosamente em diferentes países.

- O presente estudo reforça a necessidade de pesquisa de forma rotineira dos rotavírus espécies B, C e H em surtos de diarreia.

5 PERSPECTIVAS

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Considerando a importância da saúde intestinal no sistema atual de produção de suínos, o monitoramento de micro-organismos entéricos com potencial enteropatogênico é de grande importância para a manutenção das taxas de produção e de produtividade da cadeira produtiva de suínos. O uso de programas de vacinação para controle e profilaxia de diarreia neonatal em leitões lactentes é uma das ferramentas mais importantes e tem-se mostrado eficiente na redução das taxas de morbidade e de mortalidade de leitões na maternidade. Com relação ao RV, as vacinas comerciais brasileiras contemplam apenas duas cepas de RVA (G4P[6] e G5P[7]. No campo, provavelmente devido à pressão de seleção exercida pelo programa de vacinação, observa-se a recorrência de focos de diarreia por RV em leitões. Devido à diversidade de genotipos e espécies de RV passíveis de infectar suínos, o presente estudo, caracterizado pela multiplicidade de espécies virais presentes em um mesmo surto de diarreia, abre a perspectiva da implantação de sistemas de monitoramento das espécies de RV circulantes em rebanhos suinícolas. Para isso, além de RVA é fundamental que o diagnóstico contemple outras espécies virais, como RVB, RVC e RVH. Adicionalmente, além do diagnóstico etiológico de focos e/ou surtos de diarreia neonatal em leitões, a definição tanto dos genotipos G e P quanto, principalmente, da constelação de genes que constituem os RV é fundamental para a compreensão da epidemiologia clássica e molecular dessa virose. Sem dúvida, estudos nessa direção impactam na saúde intestinal e, consequentemente, na saúde animal. Por fim, considerando a possibilidade da ocorrência de infecções heterólogas e a caracterização do aspecto zoonótico, estudos nessa direção também podem apresentar importantes reflexos em saúde pública.

6 APÊNDICE

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6.1 Número de acesso em base pública de dados (GenBank) das sequências genômicas das cepas de Rotavírus descritas no estudo

Porcine rotavirus H strains BR 59 VP6 gene, partial cds
1197 bp linear RNA
Accession: **KF021619.1**

Porcine rotavirus H strain BR60 VP6 gene, partial cds
1197 bp linear RNA
Accession: **KF021620.1**

Porcine rotavirus H strain BR61 VP6 gene, partial cds
1199 bp linear RNA
Accession: **KM359479.1**

Porcine rotavirus H strain BR62 VP6 gene, partial cds
1199 bp linear RNA
Accession: **KM359480.1**

Porcine rotavirus H strain BR63 VP6 gene, partial cds
1197 bp linear RNA
Accession: **KF021621.1**

Porcine rotavirus H strain BR64 VP6 gene, partial cds
1199 bp linear RNA
Accession: **KM359481.1**

Porcine rotavirus H strain BR59 VP7 gene, partial cds
760 bp linear RNA
Accession: **KM359482.1**

Porcine rotavirus H strain BR60 VP7 gene, partial cds
760 bp linear RNA
Accession: **KM359483.1**

Porcine rotavirus H strain BR61 VP7 gene, partial cds
801 bp linear RNA
Accession: **KM359485.1**

Porcine rotavirus H strain BR62 VP7 gene, partial cds
778 bp linear RNA
Accession: **KM359486.1**

Porcine rotavirus H strain BR63 VP7 gene, partial cds
760 bp linear RNA
Accession: **KM359484.1**

Porcine rotavirus H strain BR64 VP7 gene, partial cds
787 bp linear RNA
Accession: **KM359487.1**

Porcine rotavirus H strain BR59 VP4 gene, partial cds
2398 bp linear RNA
Accession: **KM359488.1**

Porcine rotavirus H strain BR60 VP4 gene, partial cds
2398 bp linear RNA
Accession: **KM359489.1**

Porcine rotavirus H strain BR61 VP4 gene, partial cds
2423 bp linear RNA
Accession: **KM359491.1**

Porcine rotavirus H strain BR62 VP4 gene, partial cds
2423 bp linear RNA
Accession: **KM359492.1**

Porcine rotavirus H strain BR64 VP4 gene, partial cds
2415 bp linear RNA
Accession: **KM359493.1**

Porcine rotavirus H strain BR59 NSP4 gene, complete cds
696 bp linear RNA
Accession: **KM359494.1**

Porcine rotavirus H strain BR60 NSP4 gene, complete cds
696 bp linear RNA
Accession: **KM359495.1**

Porcine rotavirus H strain BR61 NSP4 gene, complete cds
691 bp linear RNA
Accession: **KM359497.1**

Porcine rotavirus H strain BR62 NSP4 gene, complete cds
715 bp linear RNA
Accession: **KM359498.1**

Porcine rotavirus H strain BR63 NSP4 gene, complete cds
696 bp linear RNA
Accession: **KM359496.1**

Porcine rotavirus H strain BR64 NSP4 gene, complete cds
704 bp linear RNA
Accession: **KM359499.1**

Porcine rotavirus A strain RVA/Pig-wt/BRA/BR43/2012/G5P[13] VP7 gene, partial cds
937 bp linear RNA
Accession: **KX376970.1**

Porcine rotavirus A strain RVA/Pig-wt/BRA/BR54/2012/G9P[23] VP7 gene, partial cds
928 bp linear RNA
Accession: **KX376971.1**

Porcine rotavirus A strain RVA/Pig-wt/BRA/BR55/2012/G9P[23] VP7 gene, partial cds
946 bp linear RNA
Accession: **KX376972.1**

Porcine rotavirus A strain RVA/Pig-wt/BRA/BR43/2012/G5P[13] VP4 gene, partial cds
830 bp linear RNA
Accession: **KX376973.1**

Porcine rotavirus A strain RVA/Pig-wt/BRA/BR54/2012/G9P[23] VP4 gene, partial cds
808 bp linear RNA
Accession: **KX376974.1**

Porcine rotavirus A strain RVA/Pig-wt/BRA/BR55/2012/G9P[23] VP4 gene, partial cds
810 bp linear RNA
Accession: **KX376975.1**

Porcine rotavirus B strain RVB/Pig-wt/BRA/BR31/2012/G14P[X] VP7 gene, complete cds
771 bp linear RNA
Accession: **KX376976.1**

Porcine rotavirus C strain RVC/Pig-wt/BRA/BR33/2012/GXP[X] VP6 gene, partial cds
1239 bp linear RNA
Accession: **KX376977.1**

Porcine rotavirus B isolate BR62 VP7 gene, complete cds
777 bp linear RNA
Accession: **MF072691.1**

7 ANEXOS

7 ANEXOS

ANEXO A: Lista de Reagentes

1. Acetona, P.A. (CH_3COCH_3) P.M. 58,08 (Dinâmica®)
2. Ácido acético glacial, P.A. (CH_3COOH) P.M. 60,05 (Nuclear®)
3. Ácido bórico (H_3BO_3) P.M. 61,83 (Sicalab®)
4. Ácido clorídrico (HCl) P.M. 36,46 (Reagen®)
5. Ácido etilenodiaminotetraácido sal di-sódico – EDTA, P.A. ($C_{10}H_{14}N_2O_8Na_22H_2O$) P.M. 372,24 (Reagen®)
6. Acrilamida P.M. 71,08 (Gibco BRL®)
7. Ágar Noble (Difco®)
8. Agarose (Invitrogen™ Life Technologies)
9. Água DEPC (Dietil pirocarbonato) (Invitrogen Life Technologies®)
10. Álcool etílico absoluto (C_2H_2OH) P.M. 46,07 (Nuclear®)
11. Álcool isoamílico ($(CH_3)_2CHCH_2CH_2OH$) P.M. 88,15 (Synth®)
12. Azul de bromofenol (Sigma®)
13. Bicarbonato de sódio P.A. ($NaHCO_3$) P.M. 84,01 (Biotec®)
14. *BigDye Terminator v3.1 Cycle Sequencing kit* (Applied Biosystems®)
15. Bis-acrilamida P.M. 154,2 (Sigma®)
16. Borohidreto de sódio P.M. 37,83 (Sigma®)
17. Brometo de etídeo ($C_{21}H_{20}N_3Br$) P.M. 394,3 (Sigma®)
18. Cloreto de cálcio puro ($CaCl_2$) P.M. 110,94 (Invitrogen™ Life Technologies)
19. Cloreto de magnésio 50 mM ($MgCl_2$) (Invitrogen Life Technologies®)
20. Cloreto de potássio, P.A. (KCl) P.M. 74,56 (Reagen®)
21. Cloreto de sódio, P.A. (NaCl) P.M. 58,45 (Reagen®)
22. Clorofórmio, P.A. ($CHCl_3$) P.M. 119,38 (Dinâmica®)
23. Dióxido de sílica (SiO_2) P.M. 60,08 (Sigma®)
24. Dithiothreitol (DTT-10 mM) (Invitrogen Life Technologies™)
25. DNA Ladder (123 bp) (Invitrogen Life Technologies™)
26. dNTP Set (100 mM), 4 x 250 µL; 25 µmol each (100 mM dATP Solution, 100 mM dCTP Solution, 100 mM dGTP Solution, 100 mM dTTP Solution) (Invitrogen Life Technologies™)

27. Dodecil sulfato de sódio – Lauril Sulfato de Sódio – SDS ($C_{12}H_{25}NaO_4S$) P.M. 288,38 (Synth®)
28. Fenol (C_6H_5OH) P.M. 94,11 (Invitrogen Life Technologies™)
29. Fosfato de sódio dibásico anidro (Na_2HPO_4) P.M. 141,96 (Synth®)
30. Fosfato de sódio dihidratado ($Na_2HPO_4.2H_2O$) P.M 177,99 (Merck®)
31. Fosfato de sódio monobásico ($NaH_2PO_4 \cdot 2H_2O$) P.M. 155,99 (Reagen®)
32. GFX PCR DNA and *Gel Band Purification Kit* (GE Healthcare, UK)
33. Glicina, P.A. (Nuclear®)
34. Glicose ($C_6H_{12}O_6$) P.M. 180,16 (Reagen®)
35. Hidróxido de sódio, P.A. (NaOH) P.M. 40,00 (Dinâmica®)
36. Hidroximetil amino metano – TRIS 99% P.M. 121,14 (Inlab®)
37. Isotiocianato de guanidina P.M. 118,16 (Gibco BRL®)
38. Metanol P.A. (CH_3OH) P.M. 32,04 (Allkimia®)
39. Nitrato de Prata (Sinth®)
40. Oligonucleotídeo iniciador (*primer*) VP6/RVN-1F (VP6/RVH) (foward; 5´-TGCTACAAGTGACCCACAAGG -3´) – MOLINARI *et al*. (2014) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)
41. Oligonucleotídeo iniciador (*primer*) VP6/RVN-1R (VP6/RVH) (reverse; 5´-GCCATCTTTCCAGTGGCTCT -3´) – MOLINARI *et al*. (2014) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)
42. Oligonucleotídeo iniciador (*primer*) VP6/RVN-2F (VP6/RVH) (foward; 5´-ACCAGGTGGAGCAACAAACA -3´) – MOLINARI *et al*. (2014) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)
43. Oligonucleotídeo iniciador (*primer*) VP6/RVN-2R (VP6/RVH) (reverse; 5´-CAGTGCGTGACCAGATCTCA -3´) – MOLINARI *et al*. (2014) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)
44. Oligonucleotídeo iniciador (*primer*) VP7/RVH-fw (VP7/RVH) (foward; 5´-GGAACTTTAAAGCCATGTTGTTC -3´) – MOLINARI *et al*. (2015) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)
45. Oligonucleotídeo iniciador (*primer*) VP7/RVH-rv (VP7/RVH) (reverse; 5´-GGGTATATTTGCCTGACATAACG -3´) – MOLINARI *et al*. (2015) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)

46. Oligonucleotídeo iniciador (*primer*) VP4/RVH-1F (VP4/RVH) (foward; 5´-AGAACCCAGGTGAAGGGTCT -3´) – MOLINARI *et al*. (2015) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)
47. Oligonucleotídeo iniciador (*primer*) VP4/RVH-1R (VP4/RVH) (reverse; 5´-GGTGTAGTGACTGTCGTTGC -3´) – MOLINARI *et al*. (2015) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)
48. Oligonucleotídeo iniciador (*primer*) VP4/RVH-2F (VP4/RVH) (foward; 5´-TCATGGGTGGAACGTTTGGA -3´) – MOLINARI *et al*. (2015) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)
49. Oligonucleotídeo iniciador (*primer*) VP4/RVH-2R (VP4/RVH) (reverse; 5´-CGCTACTCCGTTCACCCTAC -3´) – MOLINARI *et al*. (2015) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)
50. Oligonucleotídeo iniciador (*primer*) VP4/RVH-3F (VP4/RVH) (foward; 5´-ACATCAGGTATAATGTCTTTTGCAT -3´) – MOLINARI *et al*. (2015) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)
51. Oligonucleotídeo iniciador (*primer*) VP4/RVH-3R (VP4/RVH) (reverse; 5´-AACGTCATGTACTAATGCCACT -3´) – MOLINARI *et al*. (2015) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)
52. Oligonucleotídeo iniciador (*primer*) NSP4/RVH-fw (NSP4/RVH) (foward; 5´-TTCATCAAAGTCACGATGGA -3´) – MOLINARI *et al*. (2015) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)
53. Oligonucleotídeo iniciador (*primer*) NSP4/RVH-rv (NSP4/RVH) (reverse; 5´-TTCATCAAAGTCACGATGGA -3´) – MOLINARI *et al*. (2015) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)
54. Oligonucleotídeo iniciador (*primer*) VP4/RVAcon3-fw (VP4/RVA) (foward; 5´-TGGCTTCGCCATTTLATAGACA -3´) – GENTSCH *et al*. (1992) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)
55. Oligonucleotídeo iniciador (*primer*) VP4/RVAcon2-rv (VP4/RVA) (reverse; 5´-ATTTCGGACCATTTATAACC -3´) – GENTSCH *et al*. (1992) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)
56. Oligonucleotídeo iniciador (*primer*) VP7/RVABeg9-fw (VP7/RVA) (foward; 5´-GGCTTTAAAAGAGAGAATTTCCGTCTGG -3´) – GOUVEA *et al*. (1990) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)

57. Oligonucleotídeo iniciador (*primer*) VP7/RVAEnd9-rv (VP7/RVA) (reverse; 5´-GGTCACATCATACAATTCTAATCTAAG -3´) – GOUVEA *et al*. (1990) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)
58. Oligonucleotídeo iniciador (*primer*) VP7/RVB-fw (VP7/RVB) (foward; 5´-GGAAATAATCAGAGATGGCGT -3´) – MARTHALER *et al*. (2012) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)
59. Oligonucleotídeo iniciador (*primer*) VP7/RVB-rv (VP7/RVB) (reverse; 5´-TCGCCTAGTCYTCTTTATGC -3´) – MARTHALER *et al*. (2012) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)
60. Oligonucleotídeo iniciador (*primer*) VP6/RVC-fw (VP6/RVC) (foward; 5´-GGCTTTAAAAATCTCATTCACAA -3´) – STIPP *et al*. (2015) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)
61. Oligonucleotídeo iniciador (*primer*) VP6/RVC-rv (VP6/RVC) (reverse; 5´-AGCCACATAGTTCACATTTCA -3´) – STIPP *et al*. (2015) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)
62. PCR-buffer (10x) (200 mM Tris-HCl, pH 8.4, 500 mM KCl) (Invitrogen Life Technologies™)
63. *Platinum* Taq DNA Polymerase recombinant 500 units (Invitrogen Life Technologies™)
64. PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen Life Technologies™)
65. RT-Buffer (5x) (250 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, 375 mM KCl) (Invitrogen Life Technologies™)
66. SuperScript III™ Reverse Transcriptase 200 U/µL (Invitrogen Life Technologies™)
67. QuantIT ™ dsDNA BR assay kit (Invitrogen Life Technologies™)
68. Triton x-100 (Synth®)
69. Tris (Nuclear®)

ANEXO B: Soluções e Tampões

- **Diluição de dNTP**

  - Solução estoque (100 mM) – 100 μL de cada dNTP
  - Solução uso (10 mM) – 10 μL da solução estoque + 90 μL de água ultrapura autoclavada

- **Fenol / clorofórmio – álcool isoamílico (25:24:1)**
  - 25 mL fenol saturado
  - 24 mL clorofórmio
  - 1 mL álcool isoamílico

- **Gel de agarose 2%**
  - 1,0 g de agarose
  - 50 mL de tampão TBE 1x
  - 20 μL de brometo de etídio

- **Gel inferior (7,5%) da PAGE**
  - 5 mL de Lower TRIS
  - 3 mL de acrilamida/bisacrilamida
  - 50 μL de TEMED
  - 560 μL persulfato de amônio 2%
  - 11,44 mL de água bidestilada

- **Gel superior (3,5%) da PAGE**
  - 2,5 mL de Upper TRIS
  - 1 mL de acrilamida/bisacrilamida
  - 100 μL de TEMED
  - 600 μL de persulfato de amônio 2%
  - 6,20 mL de água bidestilada

- **Hidratação da sílica**
  - 6 g de sílica (SIGMA®)

- Adicionar 50 mL de água ultrapura autoclavada
- Agitar lentamente e manter em repouso durante 24 h
- Por sucção, desprezar 44 mL do sobrenadante
- Ressuspender a sílica em 50 mL de água bidestilada
- Manter em repouso durante 5 h para sedimentar
- Desprezar 44 mL do sobrenadante
- Ajustar o pH (pH 2,0)
- Aliquotar

- **Lower TRIS pH 8,8 para PAGE**
  - 36,34 g de TRIS (1,5 M)
  - Água bidestilada q.s.p. 200 mL

- **SDS 10%**
  - 5 g dodecil sulfato de sódio – Lauril sulfato de sódio – SDS ($C_{12}H_{25}NaO_4S$)
  - água bidestilada q.s.p. 50 Ml

- **Solução Acrilamida / Bisacrilamida**
  - 1,3 g de bisacrilamida
  - 50 g de acrilamida
  - Água bidestilada q.s.p 100 mL

- **Solução conservadora para PAGE**
  - 15 mL de álcool etílico P.A.
  - Água bidestilada q.s.p. 300 mL

- **Solução de prata para PAGE**
  - 0,55 g de nitrato de prata
  - Água bidestilada q.s.p. 300 mL

- **Solução fixadora para PAGE**
  - 30 mL de álcool etílico absoluto
  - 1,5 mL de ácido acético

- Água bidestilada q.s.p. 300 mL

- **Solução L6**
  - 120 g de isotiocianato de guanidina (GUSCN)
  - 100 mL de TRIS-HCl 0,1 M pH 6,4
  - 22 mL de EDTA 0,2 M pH 8,0
  - 2,6 g de Triton x-100

- **Solução L2**
  - 120 g de isotiocianato de guanidina (GUSCN)
  - 100 mL de TRIS-HCl 0,1 M pH 6,4

- **Solução reveladora para PAGE**
  - 9 g de hidróxido de sódio
  - 2,5 mL de formaldeído
  - 0,06 g de borohidreto de sódio
  - Água bidestilada q.s.p. 300 mL

- **Solução stop da coloração para PAGE**
  - 15 mL de ácido acético P.A.
  - Água bidestilada q.s.p. 300 mL

- **Tampão de amostra para eletroforese em gel de agarose**
  - 0,25 g de azul de bromofenol 0,25%
  - 45 g de sacarose – sucrose ($C_{12}H_{22}O_{11}$) 45%
  - água bidestilada q.s.p.100 mL

- **Tampão de amostra para eletroforese em gel de poliacrilamida (PAGE)**
  - 0,2 mL de azul de bromofenol 0,25%
  - 6 mL de SDS 10%
  - 1 mL de 2-mercaptoetanol
  - 2,5 mL de TRIS-HCl 0,5 M

- **Tampão de corrida – TBE (Tris – Ácido bórico – EDTA) 10x**
  - 107,78 g de TRIS 0,89 M
  - 55,03 g de ácido bórico 0,89 M
  - 7,45 g de EDTA 0,02 M
  - água bidestilada q.s.p. 1 litro
  - ajustar o pH (pH 8,4)

- **Tampão de corrida para PAGE**
  - 3 g de TRIS 0,24 M
  - 14,4 g de ácido amino acético (glicina) NH_2CH_2COOH 0,19 M
  - água bidestilada q.s.p. 1 litro

- **Tampão de estabilização para o rotavírus (TRIS/Ca^{++}) 10 x – pH 7,2**
  - 12,12 g de TRIS 0,89 M
  - 2,2 g de de cloreto de cálcio 1,5 mM
  - água bidestilada q.s.p. 1 litro

- **Tampão Fosfato Salina – PBS**
  - 137 mM Cloreto de sódio (NaCl)
  - 3 mM Cloreto de potásso (KCl)
  - 8 mM Fosfato de sódio dibásico anidro (Na_2HPO_4)
  - 15 mM Fosfato de potássio monobásico anidro (KH_2PO_4)
  - Água ultrapura autoclavada q.s.p. 500 mL

- **Upper TRIS pH 6,8 para PAGE**
  - 12,12 g de TRIS (0,5 M)
  - Água bidestilada q.s.p 200 mL

ANEXO C: Protocolo de Técnicas

- **Extração do ácido nucléico pela associação das técnicas fenol/clorofórmio/álcool isoamílico e sílica/isotiocianato de guanidina**

1. Suspensão fecal – extração bruta

- 100 μL ou 100 mg de fezes
- 500 μL de PBS
- Vortexar
- Centrifugar a 5000 x *g* / 3 min
- Utilizar 400 μL do sobrenadante para extração

2. Extração do ácido nucléico

Fase I – Fenol

- 400 μL da suspensão fecal
- Adicionar 40 μL de SDS 10%
- Homogeneizar em *vortex*
- Banho-maria 56 °C /20 min
- Centrifugar 10.000 x *g* /30 s
- Adicionar 400 μL de fenol/clorofórmio-álcool isoamílico (25:24:1)
- Homogeneizar em *vortex*
- Banho-maria 56 °C /15 min
- Homogeneizar manualmente por 15 s
- Centrifugar 10.000 x *g* /10 min
- Recolher o sobrenadante em outro microtubo

Fase II – Sílica / isotiocianato de guanidina

- Adicionar 500 μL da solução L6
- Adicionar 25 μL de sílica hidratada
- Homogeneizar em *vortex*
- Agitar em temperatura ambiente /30 min
- Centrifugar 10.000 x *g* /30 s
- Desprezar o sobrenadante em solução contendo NaOH 10 M

- Adicionar 500 μL de solução L2
- Homogeneizar em *vortex*
- Centrifugar 10.000 x *g* /30 s
- Desprezar o sobrenadante em solução contendo NaOH 10 M
- Adicionar 500 μL de solução L2
- Homogeneizar em *vortex*
- Centrifugar 10.000 x *g* /30 s
- Desprezar o sobrenadante em solução contendo NaOH 10 M
- Adicionar 1000 μL de etanol 70% gelado
- Homogeneizar em *vortex*
- Centrifugar 10.000 x *g* /30 s
- Desprezar sobrenadante em descarte comum
- Adicionar 1000 μL de etanol 70% gelado
- Homogeneizar em *vortex*
- Centrifugar 10.000 x *g* /30 s
- Desprezar sobrenadante em descarte comum
- Adicionar 1000 μL de acetona P.A. gelada
- Homogeneizar em *vortex*
- Centrifugar 10.000 x *g* /30 s
- Desprezar sobrenadante
- Secar o *pellet* em termo bloco a 60 ºC (aproximadamente 2 min) ou banho-maria a 56 ºC (15 min)
- Adicionar 50 μL de água DEPC
- Homogeneizar em *vortex*
- Banho-maria 56 ºC/15 min
- Homogeneizar em *vortex*
- Centrifugar 13.000 x *g* /4 min
- Recolher o sobrenadante em microtubo de 500 μL
- Estocar à -20 ºC até a utilização

- **Transcrição reversa (RT)**

  <u>Genes VP6, VP4, VP7, NSP4 RVH e VP7 RVB</u>

Desnaturação (97°C / 5 min)	
Reagentes	**Volume (μL)**
Primer foward (20 pmol)	1
Primer reverse (20 pmol)	1
Água ultrapura	5
RNA	5
Volume final	12

Transcrição reversa (42°C / 30 min; 95°C / 5 min)	
Reagentes	**Volume (μL)**
Buffer 5X	4
DTT	2
dNTP	1
SuperScript™ II (200 U/μL)	0,5
Água	0,5
Produto da desnaturação	8
Volume final	20

- **Reação em cadeia pela polimerase (PCR)**

  Genes VP6, VP4, VP7, NSP4 RVH e VP7 RVB

Reagentes	**Volume (μL)**
Buffer 10 x (pH 8,4)	5
$MgCl_2$	1,5
dNTP (2,5 mM)	2
Platinum®Taq DNA Polymerase (5U/μL)	0,5
Primer foward (20 pmol)	1
Primer reverse (20 pmol)	1
Água ultrapura	34
cDNA	5
Volume final	50

- Ciclos de tempo e temperatura da PCR

Reação	Temperatura (°C)	Tempo (min)	Nº de Ciclos
Desnaturação	94	3	1
Desnaturação	94	1	35
Anelamento	48	1	35
Extensão	72	1	35
Extensão final	72	10	1

- Tamanho esperado dos produtos amplificados

Agente	Gene	Tamanho em pb
	VP6 / RVN - 1	590
	VP6 / RVN - 2	716
	VP7 / RVN	817
RVH	VP4 / RVN - 1	961
	VP4 / RVN - 2	914
	VP4 / RVN - 3	813
	NSP4 / RVN	720
RVB	VP7	778

- **Eletroforese em gel de agarose a 2%**

  - 1,0 g de agarose
  - 50 mL TEB *buffer* (Tris 89 mM; ácido bórico 89 mM; EDTA 2mM) pH 8,4
  - 20 μL de brometo de etídeo (0,5 μg/mL)

  São utilizados 5 μL do amplicon e 1 μL do tampão de amostra. A eletroforese sob voltagem (100V) e amperagem (80A) constantes por aproximadamente 45 min.

- **Purificação de produto de PCR excisado do gel**

  1. Pesar o fragmento excisado do gel em microtubo de 1,5 mL.

2. Adicionar 3 volumes do tampão de solubilização em gel (L3) para cada 1 volume de gel.
3. Incubar o tubo a 50 °C / 15 min, homogeneizando a cada 3 min.
4. Transferir o gel dissolvido com o amplificado de interesse para um tubo coletor com coluna.
5. Centrifugar a 13.000 x *g* / 1 min.
6. Descartar o filtrado e recolocar a coluna no mesmo tubo.
7. Adicionar 500 µL do *Wash buffer 1* (W1) na coluna com tubo coletor.
8. Centrifugar a 13.000 x *g* / 1 min.
9. Descartar o filtrado e recolocar a coluna no mesmo tubo.
10. Centrifugar o tubo novamente à velocidade máxima por 3 min.
11. Descartar o tubo coletor e transferir a coluna para um microtubo de 1,5 mL.
12. Adicionar 30 µL do *Elution buffer 1* (E1) no centro da coluna.
13. Incubar a temperatura ambiente por 1 min.
14. Centrifugar a 13.000 x *g* / 1 min.
15. Estocar o fragmento de DNA purificado a -20°C.

- **Quantificação de produto de PCR**

(Certificar-se de que todos os reagentes estão em temperatura ambiente)

1. Preparar a solução Quant-iT™ *Working Solution* diluindo o reagente Quant-iT™ em *Buffer* Quant-iT™ 1:200. São necessários 200 µL desta solução por amostra e para os padrões 0 e 100.

2. Homogeneizar em *vortex*.

3. No microtubo das amostras adicionar 198 µL da solução Quant-iT™ *Working Solution* a 2 µL do fragmento de DNA purificado.

4. No microtubo do padrão 0 adicionar 190 µL da solução Quant-iT™ *Working Solution* a 10 µL do padrão 0.

5. No microtubo do padrão 100 adicionar 190 µL da solução Quant-iT™ *Working Solution* a 10 µL do padrão 100.

6. Homogeneizar os microtubos em *vortex* por 2-3 s

7. Incubar os microtubos em temperatura ambiente por 2 min

8. Realizar a leitura usando Qubit™ fluorometer (Invitrogen™ Life Technologies, EUA)

9. Multiplicar pelo fator de diluição para determinar a concentração correta da amostra

- **Sequenciamento pelo método Sanger**

Preparo de amostras para o sequenciamento

As amostras e os *primers* devem estar na concentração demonstrada na tabela abaixo:

Tamanho do fragmento (pares de base)	**Concentração da amostra (ng/µL ou µg/mL)**	**Concentração do *primer* (pmol/µL ou µM)**
< 300	2	5
300 – 700	4	5
>700	10	10

Mix do sequenciamento

Reagente*	**Volume**
BigDye Terminator v3.1	2,0 µL
Tampão 5x	1,5 µL
Água ultrapura autoclavada	0,5 µL
Volume final	4,0 µL

**BigDye Terminator v3.1 Cycle Sequencing Kit*

O volume final de mix do sequenciamento é adicionado a 5 µL de amostra purificada + 1 µL de *primer*.

Ciclos de tempo e temperatura da reação de sequenciamento*

Reação	**Temperatura (°C)**	**Tempo**	**Nº de ciclos**
Desnaturação inicial	96	1 min	1
Desnaturação	96	15 seg	35
Anelamento	50	15 seg	35
Extensão	60	4 min	35

*Programa recomendado pela *Applied Biosystems*.

Precipitação com EDTA e Etanol

- Adicionar os 10 µL da reação de sequenciamento em um poço de uma placa *MicroAmp*® *Optical 96-Well Reaction* (0,2 mL) (*Applied Biosystems).*
- Adicionar 2,5 µL de EDTA (ácido etilenodiamino tetra-acético) 125 mM pH 8,0.
- Adicionar 30 µL de etanol 100%.
- Homogeneizar lentamente a placa.
- Incubar a placa por 10 minutos em temperatura ambiente.
- Centrifugar a 2720 x g durante 30 min a 20 °C.
- Desprezar o conteúdo da placa.
- Centrifugar a 2720 x g durante 1 min a 20 °C com a placa invertida sobre papel.
- Adicionar 100 µL de etanol 70%.
- Centrifugar a 2720 x g durante 1 min a 20 °C.
- Desprezar o conteúdo da placa.
- Centrifugar a 2720 x g durante 1 min a 20 °C com a placa invertida sobre papel.
- Cobrir a placa com papel e deixar em temperatura ambiente por 10 min.
- Adicionar 10 µL de formamida (HIDI).
- Adicionar a septa.
- Homogeneizar a placa em vórtex.
- Submeter à placa a um spin no *miniplate spinner.*
- Colocar a placa em termociclador (tampa aberta) por 95 °C por 5 min.
- Colocar a placa em *cooler* ou banho de gelo por 1 min.
- Após a precipitação com EDTA e etanol, a placa é inserida no sequenciador (ABI 3500 *Genetic Analyzer - Applied Biosystems*) para realizar a eletroforese capilar.

ANEXO D: Lista de *Softwares*

- Electropherogram quality analysis - Phred e CAP3

(http://asparagin.cenargen.embrapa.br/phph/)

- BLAST The Basic Local Alignment Search Tool

(http://blast.ncbi.nlm.nih.gov/Blast.cgi)

- MEGA package software version 6

(http://www.megasoftware.net/mega4/mega41.html)

- BioEdit software version 7.1.11

(http://www.mbio.ncsu.edu/bioedit/bioedit.html)