

## Monitoring and molecular characterization of group D rotavirus in Brazilian poultry farms<sup>1</sup>

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**ABSTRACT.-** Beserra L.A.R., Bernardes N.T.C.G., Brandão P.E. & Gregori F. 2015. **Monitoring and molecular characterization of group D rotavirus in Brazilian poultry farms.** *Pesquisa Veterinária Brasileira* 35(6):536-540. Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Avenida Professor Dr. Orlando Marques de Paiva 87, São Paulo, SP 05508-270, Brazil. E-mail: [laila\\_andreia@hotmail.com](mailto:laila_andreia@hotmail.com)

Rotaviruses are etiological agents of diarrhea both in humans and in several animal species. Data on avian Group D rotaviruses (RVD) are scarce, especially in Brazil. We detected RVD in 4 pools of intestinal contents of broilers, layer and broiler breeders out of a total of 111 pools from 8 Brazilian states, representing an occurrence of 3.6%, by a specific RVD RT-PCR targeting the VP6 gene. Phylogenetic tree confirmed that the Brazilian strains belong to group D and 3 of the sequences were identical in terms of amino acid whereas one showed 99.5% identity with the others. The sequences described in this study are similar to other sequences previously detected in Brazil, confirming the conserved nature of the VP6 protein.

INDEX TERMS: VP6, rotavirus, group D, avian, broilers.

**RESUMO.- [Monitoramento e caracterização molecular de rotavírus do grupo D em criações comerciais brasileiras.]** Rotavírus são agentes etiológicos de diarreia tanto em humanos como em várias espécies animais. Dados sobre rotavírus do grupo D (RVD) em aves são escassos, especialmente no Brasil. Nós detectamos RVD em 4 pools de conteúdo intestinal de frango de corte, poedeiras e matrizes de um total de 111 pools originários de 8 estados brasileiros, representando uma ocorrência de 3,6% a partir de uma RT-PCR específica para RVD, tendo como alvo o gene VP6. A árvore filogenética confirmou que as amostras brasileiras pertencem ao grupo D e três das sequências obtidas foram idênticas em termos de aminoácidos enquanto uma apresentou 99,5% de identidade com as demais. As sequências aqui definidas são semelhantes a outras sequências previamente definidas no Brasil, confirmando a natureza conservada da proteína VP6.

TERMOS DE INDEXAÇÃO: VP6, rotavírus, grupo D, aves, frangos.

## INTRODUCTION

Rotaviruses are members of the *Reoviridae* family, and they are a major cause of acute gastroenteritis in young children and several mammalian and avian species (Brussow et al. 1992, Estes & Kapikian 2007). They are non-enveloped icosahedral particles and its genome comprises 11 segments of double-stranded RNA, which encodes six structural proteins (VP1 to VP4, VP6 and VP7) and six nonstructural proteins (NSP1 to NSP6). Based on antibody reactivity or genetic sequencing of structural protein VP6 of the internal capsid, rotaviruses have been classified into the groups A to G (Raming et al. 2005, Estes & Kapikian 2007). Recently, a new rotavirus group, originally designated as ADRV-N (New Adult Diarrhea Rotavirus), has been proposed as group H rotavirus (Alam et al. 2007, Matthijnssens et al. 2011).

In avian species, rotavirus groups A, D, F and G have been detected so far (McNulty et al. 1980, McNulty 2008, Trojnar et al. 2010, Johne et al. 2011). The group A rotaviruses (RVA) are mainly associated with diarrhea in humans, various domesticated and captive mammals, and poultry (Matthijnssens et al. 2008, Matthijnssens & Van Ranst 2012). Rotaviruses of groups D (RVD), F (RVF) and G (RVG) have been seen exclusively in poultry (McNulty 2008, Villarreal et al. 2006). Recent studies have demonstrated that RVD is frequently detected in avian species (Otto et al.

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2006, Savita et al. 2008, Islam et al. 2009). However, a few molecular studies are available revealing its genetic information (Dhama et al. 2015).

In Brazil, there have been reports on avian rotavirus occurrence since 1989, with frequency ranging from 8.5% (32/378 samples) in broilers (Alfieri et al. 1989) to 45.3% (58/128 samples) from intestinal contents of layers and broilers by PAGE (polyacrylamide gel electrophoresis) (Villarreal et al. 2006). Most of the avian rotaviruses detected in Brazil belong to RVA (Silva et al. 2012, Asano et al. 2011, Beserra et al. 2014). Nevertheless, RVD and RVF have been recently described in the country (Bezerra et al. 2012, Beserra & Gregori 2014, Bezerra et al. 2014). Here we report the occurrence of RVD in feces of broilers, layers and broiler breeders on Brazilian poultry farms representing 8 different states, using a RT-PCR technique based on the amplification of the VP6 gene, followed by nucleotide sequencing reaction of the amplicons in order to better understand the phylogeny of the strains detected.

## MATERIALS AND METHODS

**Samples.** A total of 111 pools of intestinal contents were used in this study. Each pool consisted in enteric content of 3-5 birds within a single batch, collected between the years of 2008 and 2012. They are from 8 different Brazilian states, São Paulo (n=52), Paraná (n=21), Santa Catarina (n=16), Rio Grande do Sul (n=8), Espírito Santo (n=2), Goiás (n=10), Bahia (n=1) and Ceará (n=1), including broilers (58/111, 52.25%), layers (14/11, 12.6%) and broiler breeders (39/111, 35.13%) farms, without symptoms of diarrhea. This study was approved by the Committee on Ethics for Animal Trials of the School of Veterinary Medicine, University of São Paulo, under protocol number 2236/2010.

**RT-PCR.** Samples were prepared as 10% (v/v) suspensions in DEPC-treated water, clarified at 12,000 x g for 15 min at 4°C, and the supernatant was used for RT-PCR. Total RNA was extracted with TRIzol Reagent® (Invitrogen™) and cDNA was synthesized using random primers (Invitrogen™) and M-MLV Reverse Transcriptase (Invitrogen™) as described by the manufacturer. The cDNA was subjected to PCR targeting the VP6 gene, with RVD specific primers and thermal cycling conditions described by Bezerra et al. (2012) generating a 742 bp gel band.

**DNA sequencing.** Amplicons were purified with EXOSAP-it (USB®) reagent, submitted to bidirectional DNA sequencing with BigDye 3.1 (Applied Biosystems®) and resolved in an ABI-3500 Genetic Analyzer (Applied Biosystems®), according to the respective manufacturer's instructions. The VP6 gene sequences from each sample were aligned with homologous sequences representing different rotavirus groups retrieved from GenBank with CLUSTAL/W 2.1 (Larkin et al. 2007), and a phylogenetic tree was generated with the neighbor-joining distance algorithm and the maximum composite likelihood model with 1,000 bootstrap replicates using MEGA 5.1 (Tamura et al. 2011). Deduced amino acid identities of the generated sequences were calculated with Bioedit 7.1.3.0 (Hall 1999) software.

## RESULTS

### Detection of RVD

Out of 111 pools of intestinal contents tested by RT-PCR, four (3.6%) showed positive results for RVD. Two samples were detected in broilers from São Paulo in 2012, one was detected in broiler breeder from Paraná in 2009, and one in layer from Goiás State in 2010. The results are presented in Table 1.

**Table 1. RVD positive samples characterized in this study, according to sample ID, Brazilian State, type of farm, collection date and VP6 Genbank Accession number**

Sample ID	State	Farm	Collection date	Accession number
AVRVBR1	Paraná	Broiler breeder	2009	KC689306
AVRVBR2	Goiás	Layer	2010	KC689307
AVRVBR3	São Paulo	Poultry	2012	KC689308
AVRVBR4	São Paulo	Poultry	2012	KC689309

### Sequencing analysis of the VP6 gene

Amplicons from all positive samples were successfully sequenced and partial nucleotide sequences obtained in this study ranged from 669 nt to 698 nt (residues 25 to 693/722 nt of VP6 gene using as reference strain 10V0133 - accession number JN034682). The phylogenetic tree of VP6 nucleotide sequences depicted that the four Brazilian rotavirus strains segregated with the rotavirus D representatives, while the topology maintained the remaining groups (A, B, C, E, F, G, and H) apart from each other and supported by high bootstrap values, as shown in Figure 1.

The maximum nucleotide identity among the strains of this study was 99.7% (KC689309 x KC689308), and the minimum identity was 94.1% (KC689309 x KC689306). With regard to amino acid identities, three of the sequences were identical (KC689307, KC689308, KC689309) whereas one (KC689306) showed 99.5% identity with the others, presenting a substitution I102V, using as reference rotavirus D strain JN034682.

With respect identities between prototype strain 10V0133 (JN034682) and the sequences generated in this study, high identities were also found (91-92%, in terms of nucleotide and 99.5-100% for amino acids).

The nucleotide comparison of the strains from this study with RVD previously described in Brazil revealed a high identity (99% nucleotide identity) among KJ101589 (Bezerra et al. 2012, Bezerra et al. 2014) and KC689307. Likewise, the putative amino acids sequences all samples showed 100% identity, except the strain KC689306 (99.5-99.9% amino acid identity) (Bezerra et al. 2012, Bezerra et al. 2014).

The phylogenetic analysis of the sequences, depicted two major clades formed by rotavirus A/C/D/F and rotavirus B/G/H (Fig.1), supported by high bootstrap values, despite being a partial fragment of the VP6 gene. Taken together, the use of a specific RVD primer, the high amino acid sequence identity (99.5%) and the segregation of the Brazilian Samples in the group D clade, show that the samples detected belong to the group D.

### Accession numbers

The sequences were deposited in GenBank under the accessions numbers KC689306 to KC689309.

## DISCUSSION

Viral enteric diseases in poultry, especially in turkeys and chickens, have an important economic impact because of production losses due to poor weight gain (Saif 2008).

Avian rotaviruses, their genetic variability and ability

for interspecies transmission is very limited (Trojnar et al. 2013), and the first complete genome sequence of a group D rotavirus has been determined in order to characterize the group D rotaviruses in more detail (Trojnar et al. 2010).

Were detected a low number of positive samples in this study likewise previous reports, *e.g.*, from example India where there are some reports about the occurrence of the RVD. Savita et al. (2008) detected 7 positive samples for RVD from 77 (fecal and environmental samples) by PAGE (polyacrylamide gel electrophoresis), and Nitire et al. (2010) detected 4 samples (7.4% or 4/54) with a migration pattern characteristic of the RVD by PAGE and all of these samples were positive for PCR targeting VP6 gene. Katoor et al. (2013) report the first two sequence-confirmed RVD (4.7% or 2/42) infections in 1–2 week-old broiler chicks from northern India with an initial test of RVD by PAGE and VP6 gene-based PCR assay for confirmation.

Alam et al. (2011) described the occurrence of the 0.38% (1/267) RVD in fecal samples from chickens by PAGE-SS (polyacrylamide gel electrophoresis and silver staining) in Bangladesh.

In contrast, Otto et al. (2012) showed a high prevalence of the RVD in chicken and turkey from commercial flocks in Europe and Bangladesh. They tested 199 samples from five different European countries and detected 56 (28.2% or 56/199) positive samples by Page and PCR. They also tested 393 fecal samples from three different European countries and Bangladesh and detected 259 positive samples (65.9% or 259/393) by RT-PCR.

In Brazil, RVD was previously detected in Northern Brazilian Region by a specific RT-PCR assay, with a high occurrence ranging from 53% (16/30 samples) to 35.3% (30/85) (Bezerra et al. 2012, Bezerra et al. 2014).

The strains characterized in this study are very similar to those found by Bezerra et al. (2012) and Bezerra et al. (2014), but were collected in different Brazilian states. Considering the analysis of group-specific VP6 protein showed amino acid changes at only one position, *i.e.*, 102, when compared to the reference RVD used in this study (GenBank accession number JN034682) and to other sequences described in Brazil, confirming the conserved nature of this protein. In fact, the comparative sequence analysis based on partial VP6 gene of Indian RVD strains revealed higher homology with the VP6 genes of the avian RVD from Brazil, Germany, Netherlands, Bangladesh and UK (Kattoor et al. 2013).

The four RVD strains detected, two of broiler, one of layer and one of broiler breeder, were from farms with adequate biosafety levels in general, although there are reports of the occurrence of diarrhea on these farms in previous batches. In the field, rotaviruses are commonly detected via molecular methods, however, the situation is complicated by the fact that rotaviruses (and other co-infecting viruses) are often found in healthy flocks, exhibiting no enteric disease signs (Jindal et al. 2009, Jindal et al. 2012, Day et al. 2013). Some reports demonstrating the presence of this virus in healthy flocks of commercial chickens are available (Pantin-Jackwood et al. 2007, Pantin-Jackwood et al. 2008, Jindal et al. 2010). Due to rotavirus features of high particle elimination by infected hosts (Koopmans & Duizer 2004) and resistance to environmental conditions (Harakeh & Butler 1984), coupled with the density of flocks, the conditions for transmission of virus are increased.

However, avian rotaviruses have been isolated from diarrheic chickens, turkey and other avian species in various parts of the world (Nitire et al. 2010). Savita et al. (2008) reported the detection of RVD in diarrheic fecal in central India (17.39% or 8/46), and Ahmed et al. (2006) described the occurrence of the 0.86% (2/232) avian rotavirus in diarrheic fecal samples from broilers in Bangladesh.

In the midst of this, data on the characterization of remaining genes in these strains, including the impact of detected amino acid mutation, can provide more information about the molecular features of the virus and improve the prevention and control of avian rotaviruses.

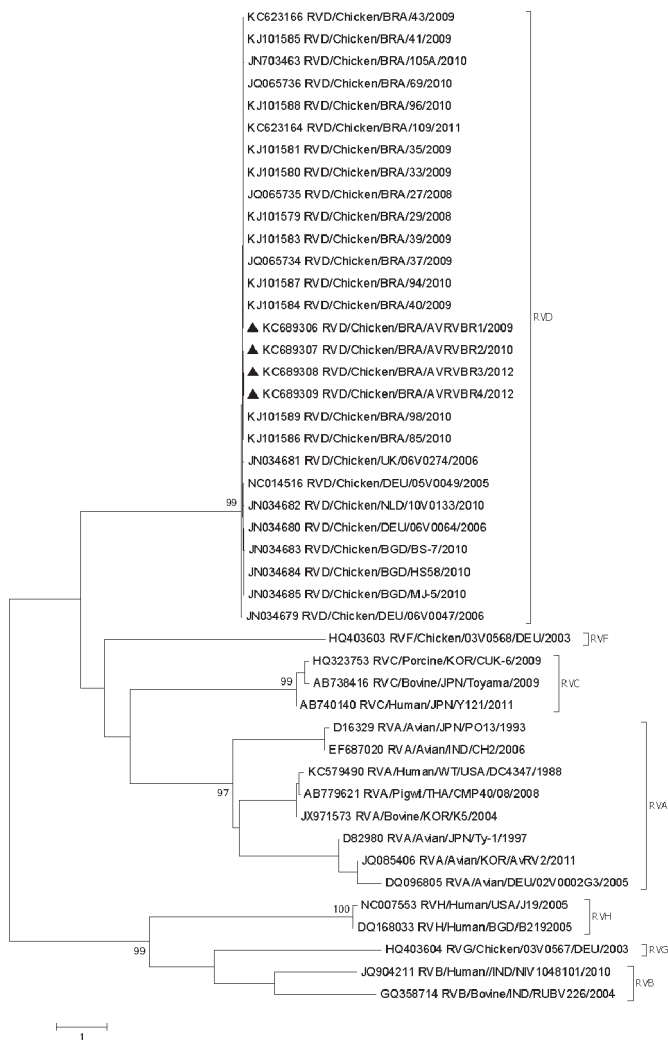


Fig.1. Nucleotide neighbor-joining distance tree (maximum composite likelihood nucleotide substitution model) for the partial VP6 rotavirus gene showing the known groups. Strains detected in the present study are preceded by black triangles. The numbers at each node are bootstrap values greater than 95 from 1,000 replicates. The bar represents the number of substitutions per site.



## CONCLUSION

RVD can be found in all types of poultry farms and it is a possible role might be suggested on the etiology of enteric disease. According to results, samples from RVD in Brazil are similar, but the characterization of remaining genes are necessary to conclude about viral diversity found in the field.

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