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## Investigation of Enteric Viruses in the Feces of Neotropical Migratory Birds Captured on the Coast of the State of Pará, Brazil

### ABSTRACT

Migratory birds can become long-distance vectors for a wide range of microorganisms and can cause human disease, being the Brazilian coast a gateway for northern migratory birds. These animals are considered natural reservoirs of different viruses that cause important diseases, being relevant research of viral pathogens in migratory birds to epidemiology surveillance. The objective of the study was to investigate the presence of avian rotavirus (AvRV), avian reovirus (ARV) and picobirnavirus (PBV) in Neotropical migratory birds captured on the coast of Brazil. A total of 23 individual fecal samples of the migratory birds species *Calidris pusilla* (20 birds), *Numenius phaeopus* (1 bird) and *Charadrius semipalmatus* (2 birds) were collected. Fecal suspensions were prepared from the collected samples for subsequent extraction of double-stranded RNA (dsRNA), which was subjected to polyacrylamide gel electrophoresis (PAGE) and reverse transcription polymerase chain reaction (RT-PCR). The electrophoretic profiles were not detected by PAGE, and the amplification for the studied viruses PBV, ARV and AvRV (specie D, gene VP6 and NSP4) were negative. Positivity for AvRVD, VP7 gene was of 4.35% (1/23) for the migratory bird *Calidris pusilla*. After sequencing and building the tree of phylogenetic relationships avian Rotavirus Group D identified in this study was phylogenetically related and grouped into one branch, together to previously reported AvRVD from Brazil in chicken flocks with 99.8% nucleotide and 100% amino acid similarities.

### INTRODUCTION

There are huge numbers of wild animals distributed throughout the world and the diversity of wildlife species is immense. Infectious pathogens that originate in wild animals have become important throughout the world, as they have had substantial impacts on human health, agricultural production, wildlife-based economies and wildlife conservation (Bengis *et al.*, 2004). Wild birds and especially migratory species can become long-distance vectors for a wide range of microorganisms and can cause human disease (Tsiodras *et al.*, 2008). The Brazilian coast is a gateway for northern migratory birds, and there are records of migratory birds extending from the coastal Amazon to the northeastern region of the country. These birds generally arrive in the country from August to October and return to their breeding areas from March to May (Nunes *et al.*, 2006).

The areas where northern migratory birds concentrate are important in the context of epidemiological surveillance of the countries that are included in migratory routes because these animals are considered natural reservoirs of different viruses that cause important diseases, such as avian influenza, Newcastle disease and West Nile virus. These



diseases are of major concern to public authorities and populations (Bengis *et al.*, 2004; Estes & Greenberg, 2013).

The avian rotaviruses (AvRV) belong to the family *Reoviridae*, genus *Rotavirus*, and have a genome consisting of 11 segments of non-enveloped, icosahedral, double-stranded RNA with viral genome that encodes six structural proteins (VP1-VP4, VP6 and VP7) and six non-structural proteins (NSP1-NSP6) (Estes & Greenberg, 2013). As based on the serological reactivity and genetic variability of VP6 gene, there are at least nine different groups/species, named RVA-RVI and more recently, rotavirus J was proposed as tenth specie (Matthijnssens *et al.*, 2012; Mihalov-Kovács *et al.*, 2015). Birds can be infected by species A, D, F and G (Johns *et al.*, 2011; Otto *et al.*, 2012).

The first report of rotavirus infection in birds was described by Bergeland *et al.* (1977) in the United States from the intestinal contents of some 2 to 3 week old turkeys with watery diarrhea and high mortality. In Brazil, AvRV was first reported by Alfieri *et al.* (1989) from diarrheic broiler chickens. Bezerra *et al.* (2012) analyzed pools of fecal samples from farms distributed among municipalities of the Metropolitan Mesoregion of Belém and detected rotavirus group D by polyacrylamide gel electrophoresis (PAGE) and reverse transcription PCR (RT-PCR). However, there are no data of the occurrence of AvRV in literature on the occurrence of these viruses in migratory birds.

The avian picobirnavirus isolate (PBV) belongs to the family *Picobirnaviridae*, genus *Picobirnavirus* with two species: *Human picobirnavirus* (type species) and *Rabbit picobirnavirus* (designated species) (Malik *et al.*, 2016). In Brazil, this agent was first described in the intestinal contents of wild black-footed pigmy rice rats (*Oryzomys nigripes*) and later identified during an outbreak of gastroenteritis involving humans in Brazil (Pereira *et al.*, 1988). In birds, the first report of infection was described in the same year by Alfieri *et al.* (1988) in broilers. Worldwide the PBV has a wide host range and has been detected in both domestic and captive animals, however, in migratory birds there are no reports on the circulation of this virus.

The avian reovirus (ARV) belongs to the genus *Orthoreovirus*, subfamily *Spinareovirinae*, family *Reoviridae*, and it is a non-enveloped, icosahedral, dsRNA virus. The first isolate in birds was described by Fahey & Crawley (Goldenberg *et al.*, 2010) and was initially associated with chronic respiratory disease in broilers. In Brazil, evidence of avian reovirus circulation was first reported after the detection of neutralizing

antibodies against the virus in free ranged chicken (Dos Santos *et al.*, 2008).

Due to the limited knowledge of the migratory routes of birds in the coastal region of Brazil and noting the importance of these viral agents in the epidemiology of *Gallus gallus domesticus*, the present study aimed to determine the presence of AvRV, ARV and PBV in Neotropical migratory since there are no data in literature on the occurrence of these viruses in this birds.

## MATERIALS AND METHODS

### Clinical Specimens

Twenty-three fecal samples were collected from migratory birds captured on Corvina Beach (S 00°36.648' / W 047°22.860'), Pará, Brazil. Twenty-one samples belonged to the order Charadriiformes, family Scolopacidae, with 20 samples from the species *Calidris pusilla*, one sample from *Numenius phaeopus* and two samples belonged to the order Ciconiiformes, family Charadriidae from the species *Charadrius semipalmatus*.

*Calidris pusilla* is distributed from the west to northern Alaska to northern Canada. During the winter, it is present on the Pacific coast of Mexico to the south of Peru; on the Atlantic coast of the Yucatan Peninsula and the West Indies and also in the coast of Argentina. These birds live in high and low Arctic and subarctic tundra in the wet, often near natural pools, rivers, lakes, river deltas and also dry areas and sandy scrubland along the rivers.

The birds were captured using mist nets with the aid of aluminum rods after ebb tides in locations with food availability. Feces were collected of the cloacae with the aid of a plastic spoon or individually in cardboard boxes lined with aluminum foil and stored in 1.5 mL Eppendorf tubes and stored in a freezer. After feces collection, the birds were properly identified by ornithologists and biologists showing any clinical signs of diarrhea and released back into the wild.

### Sample preparation

A 1 mL volume of 10% phosphate buffered saline (PBS pH 7.0) and 160 µL of chloroform were added to each fecal sample. The samples were then stirred for 20 minutes and centrifuged at 3,000 rpm for 10 minutes at 4°C. The supernatant was carefully collected and stored in a plastic vial at -20°C. The viral genome was extracted from the fecal suspensions using guanidine isothiocyanate/silica powder glass according to the



method described by Boom *et al.* (1990) and subjected to PAGE according to Pereira *et al.* (1993).

### RT-PCR

The AvRVD (Avian Rotavirus D) was screened by RT-PCR using the RD6F/RD6R primers for amplification of VP6 gene according to the method of Bezerra *et al.* (2012). Additionally, a pair of primers were designed to AvRVD VP7 gene RDVP7-F (5' GGA GTG ACG CAC CTG TAT G 3') and RDVP7-R (5' TAG CGT TTG TTG ATT CAA TG 3').

For AvRV belonging to group A the primers Seg10-c-s and Seg10-c-as were used according to Trojnar *et al.* (2009) and the primers Rotvp6-f and Rotvp6-r were used according to Ursu *et al.* (2011) for the NSP4 and VP6 genes, respectively.

For detection of PBVs (genogroup I), the primers PicoB25(+) and PicoB43(-) were used according to Rosen *et al.* (2000) to amplify the RdRp gene. For amplification of the ARV S2 gene, the primers PAF and PAR were used according to Zhang *et al.* (2006).

To obtain complementary DNA (cDNA), the total reaction volume was 25  $\mu$ L, and this included 3  $\mu$ L of extracted dsRNA, 1  $\mu$ L of each primer pair 20mM, 16.5  $\mu$ L of RNase- and DNase-free H<sub>2</sub>O, 2.5  $\mu$ L of 10x First-strand buffer (Invitrogen®, Life Technologies, Carlsbad, CA, USA), 1  $\mu$ L of deoxynucleotide triphosphates (dNTPs -10 mM, Invitrogen®), 0.75  $\mu$ L of MgCl<sub>2</sub> (50mM, Invitrogen®) and 0.25  $\mu$ L of reverse transcriptase (Enzyme Super Script II; 20 U, Invitrogen®), for 1 hour at 42°C. The products (cDNA) were then subjected to PCR amplification with final volume of 50  $\mu$ L, with the addition 25  $\mu$ L cDNA, 18.5  $\mu$ L of RNase- and DNase-free H<sub>2</sub>O, 2.5  $\mu$ L of 10x First-strand buffer (Invitrogen®), 3  $\mu$ L of dNTPs (10mM, Invitrogen®), 0.75  $\mu$ L of MgCl<sub>2</sub> (50mM, Invitrogen®) and 0.25  $\mu$ L of Taq DNA Polymerase (2.5 U/ $\mu$ L, Invitrogen®).

The cycling conditions for amplification of the AvRVD VP6 gene were as follows: 93°C for 3 minutes, followed by 35 cycles at 93°C, 55°C and 72°C for 1 minute each, with a final cycle at 68°C for 7 minutes to amplify a 742 bp fragment. For amplification of the AvRVA NSP4 gene, the following cycling conditions were used: 93°C for 3 minutes, followed by 35 cycles at 94°C and 50°C for 1 minute each and 72°C for 2 minutes, ending with one cycle at 68°C for 7 minutes to obtain a 645 bp fragment and AvRVD VP7 were used: 94°C for 2 minutes, followed by 35 cycles at 94°C, 55°C and 72°C for 1 minute each, with a final cycle at 72°C for 5 minutes to amplify a 595 bp fragment. Amplification of the AvRVA VP6 gene was conducted at 95°C for 3 minutes, followed by 40

cycles at 95°C and 50°C for 30 seconds each and 72°C for 45 seconds, ending with one cycle at 68°C for 7 minutes to obtain a 550 bp fragment.

The positive control used in RT-PCR to AvRVD VP6 and VP7 genes was a field isolate RVD/Chicken-wt/27/PA/BRA/2008/ (Access number Genebank: JQ065735; KC669408) and for negative control ultra pure DNase/RNase free water was used.

For amplification of the PBV RdRp gene, the following cycling conditions were used: 94°C for 5 minutes, followed by 35 cycles at 94°C for 45 seconds, 45°C for 30 seconds and 72°C for 1 minute, with a final step at 72°C for 10 minutes to amplify a 201 bp fragment. The cycling conditions for amplification of the ARV S2 gene were as follows: 93°C for 3 minutes, followed by 35 cycles at 93°C, 53°C and 72°C for 1 minute each, with a final step at 68°C for 7 minutes to amplify a 625 bp fragment.

A total of 10  $\mu$ L of the amplified products were separated on a 1.5% agarose gel with 2  $\mu$ L of bromophenol blue and 2  $\mu$ L of molecular weight marker (123 bp, Invitrogen®) diluted in 10  $\mu$ L of 1X Tris/Borate/EDTA (TBE). The gel was then subjected to horizontal electrophoresis for 60 minutes with a mean voltage and amperage of 120 V and 400mA, respectively. The bands were observed with the GEL DOC 1000 instrument (Bio-Rad Laboratories, Hercules, CA, USA).

### Nucleotide sequencing and phylogenetic analysis

The positive samples were sequenced in the ABI Prism 3500 genetic analyzer (Applied Biosystems, Foster city, CA, USA) using ABI PRISM™ Big Dye Terminator Cycle Sequencing (Applied Biosystems, USA) and the same PCR primers with each reaction were repeated for three times to forward and reverse.

After ordering the sequences multiple alignments, using BioEdit software package version 7.2.5 (Hall, 1999) and MEGA V.6.0 programs (Tamura *et al.*, 2013) were edited to build the tree phylogenetic relationships for comparison with rotavirus sequences of reference viruses to groups A, D, F and G available in the databases, using the neighbor joining method with Kimura 2-parameter correction (Kimura, 1980) and bootstrap analysis (2,000 replicates). The analysis of rotaviruses in Neotropical migratory birds characterized in the present study were all made with rotaviruses detected in chicken flocks of the four species that infect avian (AvRVA, AvRVD, AvRVF and AvRVG) due the lack of data about rotaviruses in Neotropical migratory birds.





## GenBank accession numbers

The sequence data for the VP7 gene from the present study were submitted to the GenBank database with the accession number KX774462.

For the phylogenetic analyzes the following sequences were used:

- RVD/chicken-wt/BRA/94/2010 (KJ101576.1),
- RVD/chicken-wt/BRA/91/PA/2010 (KC669411.1),
- RVD/chicken-wt/BRA/98/2010 (KJ101578.1),
- RVD/chicken-wt/BRA/29/2008 (KJ101568.1),
- RVD/chicken-wt/BRA/40/2009 (KJ101573.1),
- RVD/chicken-wt/BRA/37/PA/2009 (KC669409.1),
- RVD/chicken-wt/BRA/105A/PA/2010 (KC669412.1),
- RVD/chicken-wt/BRA/96/2010 (KJ101577.1),
- RVD/chicken-wt/BRA/43/PA/2009 (KC669414.1),
- RVD/chicken-wt/BRA/69/PA/2010 (KC669410.1),
- RVD/chicken-wt/BRA/36/2009 (KJ101571.1),
- RVD/chicken-wt/BRA/109/PA/2011 (KC669413.1),
- RVD/chicken-wt/DEU/05V0049/2005 (GU733451.1),
- RVD/chicken-wt/KOR/D62/2013 (KM254196.1),
- RVD/chicken/IND/UKD48/2012 (KF142489.1),
- RVA/pheasant-tc/GER/10V0112H5/2010/G23P[37] (JX204816.1),
- RVA/chicken-wt/BRA/USPARV06/2009 (KC962123.1),
- RVA/chicken-wt/KOR/AvRV\_2/2001 (JQ085407.1),
- RVA/chicken-wt/KOR/D62/2013 (KM254183.1),
- RVA/chicken-wt/DEU/Ch\_03V0358F3/2003 (EU486975.1),
- RVF/chicken-wt/KOR/D62/2013 (KM254217.1),
- RVF/chicken-wt/DEU/03V0568/2003 (JQ919998.1),
- RVG/chicken/DEU/3V0567/2003 (JQ920007.1),
- RVG/Pigeon-wt/CHN/HK18/2011 (KC876015.1),
- RVG/chicken/ZAF/MRC\_DPRU1679/2011/GXP[X] (KJ752089.1).

## Ethics

The present study was authorized by the Brazilian Ministry of the Environment under approval number 21312-1 as well as the Chico Mendes Institute for Biodiversity Conservation (ICMbio) and Biodiversity Authorization and Information System (SISBIO). In addition, the study was approved by the Ethics Committee on the Use of Animals of Instituto Evandro Chagas (IEC) under registration number 014/2012.

## RESULTS

### Detection and molecular characterization

Twenty-three fecal samples of the species *Calidris pusilla*, *Numenius phaeopus* and *Charadrius semipalmatus* with no clinical signs of diarrhea were tested by PAGE and RT-PCR to PBV, ARV, AvRVA (Avian Rotavirus A; VP6 and NSP4 genes) and AvRVD (Avian Rotavirus D; VP7 and VP6 genes). The electrophoretic profiles were not detected by PAGE, and the amplification for the studied viruses PBV, ARV, AvRVA and AvRVD (VP6 and NSP4 genes) were negative. Positivity for AvRVD, VP7 gene was of 4.35% (1/23) for the migratory bird *Calidris pusilla*.

Avian Rotavirus Group D identified in this study was phylogenetically related and grouped into one branch together to previously reported AvRVD from Brazil (KC669409, KC669410) with 95% identity, India (KF142489) 81% identity and South Korea (KM254196), 84% identity, but all recovered from flock chicken (Figure 1).

The data show similarity with rotavirus D in Brazil for the years 2008-2011, with 99.8% nucleotide similarity and 100% amino acid similar to RVD/chicken-wt/BRA/94/2010 (Table 1).

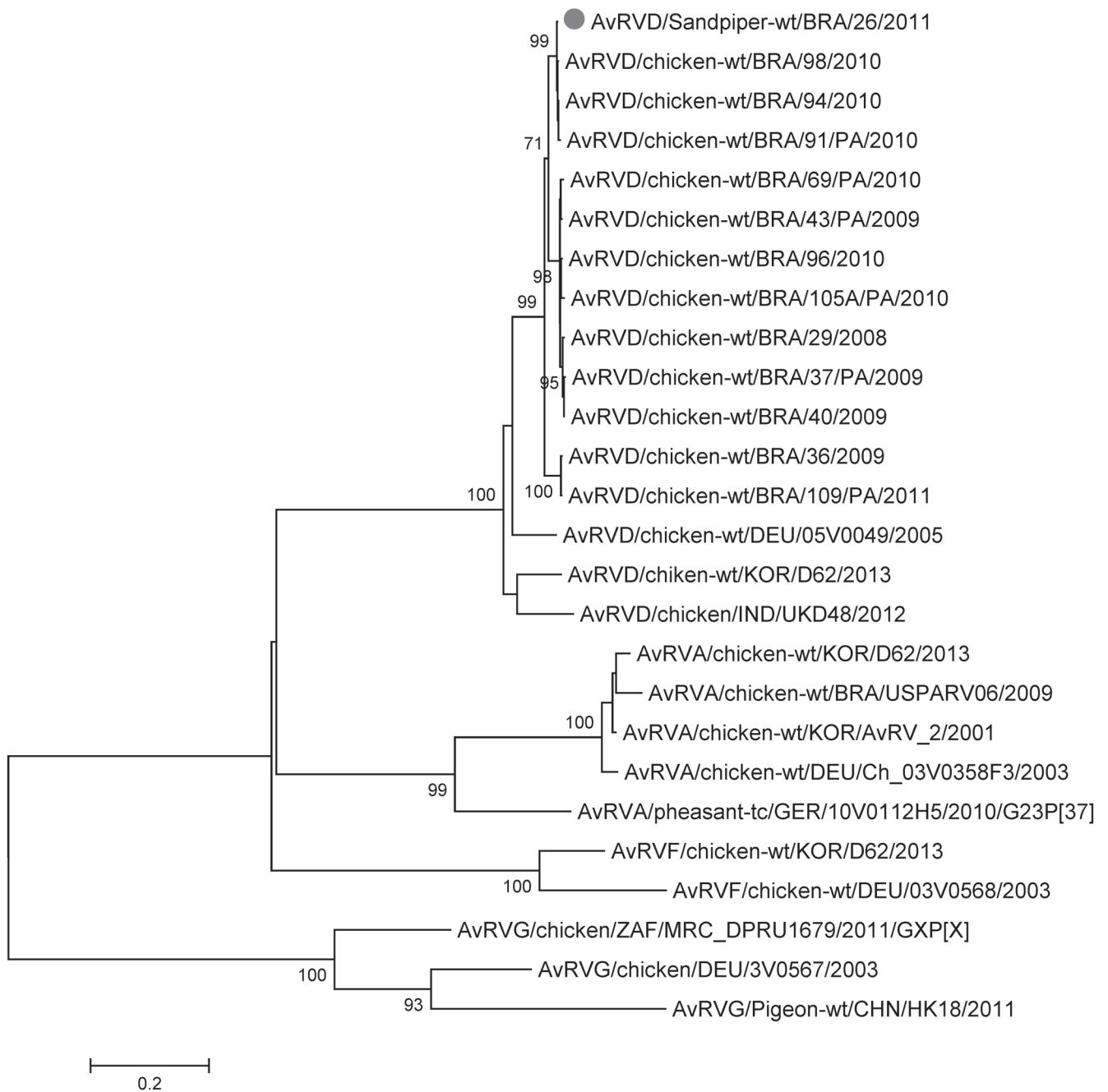
However, when compared with Brazilian prototypes, nucleotide and amino acid similarities were observed, respectively, in the years 2008 (96.03% and 97.59%), 2009 (95.83% and 96.39%), 2010 (95.63% and 97.59%) and 2011 (95.44% and 96.39%) as demonstrated in Table 1.

**Table 1** – Analysis of nucleotide and amino acids similarity sample AvRVD/Sandpiper-wt/BRA/2011 compared with the other strains of rotavirus circulating D in Brazil and the world.

	Nucleotide Similarity	Amino Acid Similarity
AvRVD/chicken-wt/BRA/94/2010	99.80%	100.00%
AvRVD/chicken-wt/BRA/29/2008	96.03%	97.59%
AvRVD/chicken-wt/BRA/37/PA/2009	95.83%	96.39%
AvRVD/chicken-wt/BRA/105A/PA/2010	95.63%	97.59%
AvRVD/chicken-wt/BRA/109/PA/2011	95.44%	96.39%
AvRVD/chicken-wt/DEU/05V0049/2005	86.51%	87.95%
AvRVD/chicken-wt/KOR/D62/2013	83.53%	86.14%
AvRVD/chicken/IND/UKD48/2012	81.15%	84.94%

## DISCUSSION

To our knowledge, this is the first report of the detection of rotaviruses in migratory birds in America. AvRVD was detected in 2011 in one sample recovered from *Calidris pusilla* with no clinical signs of diarrhea



**Figure 1** – The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.84996118 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 230 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

captured at Corvina Beach, Salinópolis Municipality, Pará State, Brazil. This result shows the occurrence of AvRVD in Neotropical migratory birds since there is no evidence of rotavirus in migratory birds worldwide.

Ecological associations have been carried out between bird's vectors for a wide range of microorganisms. In some cases, birds may be the main host of the virus (or even single), while in other cases; birds can be virus-amplifying hosts, necessary for the

worsening of epizootic and other epidemic outbreaks. However, regarding wild birds this is the first report (Tsiodras *et al.*, 2008; Dhama *et al.*, 2015). Rotavirus can be identified both in symptomatic and asymptomatic animals. However, detection success increases when diarrheal samples are analyzed due to the possibility of higher viral load. In the present study, the appearance of the feces was observed, and none of the birds showed clinical signs of diarrhea, which may explain



the majority of negative results found. However, cases of AvRV detection in asymptomatic birds were found by Villarreal *et al.* (2006), who concluded that there were no significant differences in the detection of this agent in birds with or without the presence of clinical signs.

The frequency observed in the present study if compared with wild bird is very low. Similar results were found in India by Savita *et al.* (2008) and Islam *et al.* (2009) in fecal samples from farm broilers, which presented a positivity of 11.69% and 13.15%, respectively. Ahmed & Ahmed (2006) conducted a study on 232 fecal samples from broilers between 1 and 2 weeks of age from two districts of Bangladesh and observed 0.86% positivity for AvRV-A. In contrast, Karim *et al.* (2007) observed 13.81% positivity for AvRVD in 630 fecal samples from diarrheal and non-diarrheal stools of poultry at 3 weeks of age from four districts of Bangladesh. Similar results were found in India by Savita *et al.* (2008) and Islam *et al.* (2009) in fecal samples from farm broilers, which presented a positivity of 11.69% and 13.15%, respectively.

Nunes *et al.* (2006) reported that wild and/or migratory birds are carriers of viral pathogens confirming our data that identify the presence of AvRVD. In birds, other viral pathogens were described such as astroviruses, enteroviruses, reoviruses, paramyxovirus and adenovirus, which can cause premature death of the specimens contaminated by other agents in conjunction with AvRV, making it impossible to identify the RV (Rotavirus) in migratory birds (Tamehiro *et al.*, 2003; Nunes *et al.*, 2006; Dhama *et al.*, 2015).

It is important to mention that the birds in the present study were all adults, which may have hindered AvRV detection because it is known that young birds are more susceptible to infection due of slow replacement of the apical epithelium of the intestinal villi, which favors the full development of the AvRV replication cycle and the production of viral progeny (Alfieri *et al.*, 2007). This situation does not occur in adult birds due to rapid replacement of the epithelium; thus, the formation of complete and infectious viral particles is hindered, which explains the AvRV detection in the studies by Ahmed & Ahmed (2006), Karim *et al.* (2007) and Islam *et al.* (2009). The present study corroborates the study by Ursu *et al.* (2011), who correlated the low positivity found with the age range (adults) of the wild birds involved in their study, however, these authors detected AvRVA gene VP6.

The limited AvRV studies in Brazil include a study conducted by Tamehiro *et al.* (2003), who was able to detect 8.5% positivity for AvRV by PAGE in 378 fecal samples of 1-7-week-old farm broilers in the

state of Paraná, Brazil. This result was different from that found by Villarreal *et al.* (2006), who observed 45.3% positivity for AvRVA by PAGE in 128 samples of broiler intestinal contents of animals from different states with rotavirus research in Brazil (Alfieri *et al.*, 2007). Although there are no studies on the contact of farm animals and migratory birds, in our study it was observed that the strain identified in migratory birds is closely related to AvRVD circulating in Brazil in the same period, with similar nucleotide the AvRVD/ chicken-wt/BRA/94/2010 of 99.8% and 100% amino acid, showing no change from amino acidic sites, whereas when compared with other samples described in other countries, a lower similarity is observed, suggesting that what was observed on migratory birds reflects the strain circulating in Brazil, indicating that cross-infection of domestic and migratory specimens may occur, but the mechanism of this cross-infection is not known.

Studies conducted on PBV report a wide range of hosts with the detection of these viruses in domestic and captive animals (Alfieri *et al.*, 1988; Pereira *et al.*, 1988; Fregolente *et al.*, 2009). In this study, primers PicoB25 and PicoB43, the same used in the study by Silva *et al.* (2013) were employed, which developed RT-PCR to investigate 85 fecal sample pools from 37 poultry farms distributed among eight municipalities in the state of Pará, Brazil and found 49.4% (42/85) positivity for this virus. However, in this study, no positive samples were obtained, which corroborates the absence of PBV in migratory birds in the literature.

One hypothesis that would explain the negative findings of the present study is that only primers that identify samples belonging to PBV genogroup I were used, thus underestimating the actual prevalence of picobirnaviruses among the migratory birds under, because genogroup II or non-I and non-II samples were not examined in the present study. Therefore, additional studies should be conducted to determine the evolutionary pattern of PBVs circulating among different geographical regions (Ganesh *et al.*, 2011).

As for ARV, there are few studies focused on the molecular epidemiology of this agent in migratory birds, particularly for the S2 gene. However, results that diverged from those found in the present study were observed by Lojkic *et al.* (2010), who identified 13% positivity for ARV in fecal samples from turkeys in Croatia using the same primers as in the present study. In Brazil, Silva *et al.* (2013) used the same primers as in the present study and observed a prevalence of 32.9% (28/85) for ARV by RT-PCR of 85 fecal samples from 37 poultry farms.



In summary, all fecal samples from neotropical migratory birds were negative for PBV, ARV and AvRVA and only AvRVD was detected in *Calidris pusilla* as the only pathogen in these neotropical migratory birds. Nevertheless, it is valid to correlate the importance of the finding in the context of the Brazilian poultry industry, that is configured as an important segment of agribusiness exploring the probable risks of migratory birds as enteric virus reservoir for commercial poultry or vice versa due to previous detections of AvRVD (Avian Rotavirus D), AvRVA (Avian Rotavirus A), AvRVF (Avian Rotavirus F) and AvRVG (Avian Rotavirus G) in broilers in Brazilian territory (Bezerra *et al.*, 2012; Luz *et al.*, 2014; Silva *et al.*, 2013; Mascarenhas *et al.*, 2016).

This result is relevant for the monitoring of migratory birds and highlights the importance of further studies aiming to clarify the actual role of these birds in the epidemiology of rotavirus in the Americas.

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