Mini Review

# Co-circulation of Astrovirus, Rotavirus and Coronavirus in Brazilian Avian Farms

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**Abstract** | Several viruses are associated with enteric diseases in avian species, causing decreased growth performance and mortality. In this study, 59 pools of intestinal content of layers, broilers, and breeders were investigated from different Brazilian regions for the presence of rotavirus (RVA, RVD, and RVF), avian coronavirus (ACoV), and avian astrovirus (AAstV), using PCR reactions followed by nucleotide sequencing and analysis. Twenty-nine pools (29/59; 49%) appeared positive for AAstV, 6 pools (6/59; 10%) were positive for RVD, forty-three pools (43/59; 72.8%) were positive for ACoV, and twenty-four pools (24/59; 40.67%) presented concomitant viral infections of two or three investigated agents. The relative high frequency of occurrence of the studied viruses both in single or concomitant infection emphasizes the need for continued monitoring of poultry flocks to better understand its epidemiology and understanding the impact of one virus on the pathobiology of second virus.

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## Introduction

Enteric diseases in avian species, especially in turkeys and poultry, have an important economic impact due to decreased growth performance and mortality (Saif, 2008). Different factors under field conditions such as diversity of etiologic agents, single or concomitant infections, broad range of disease signs, immunological status, and management practices, makes diarrhea prevention a constant challenge (Day and Zsak, 2013). Among viral species associated with the enteric disease syndrome, Avian Astroviruses (AAstV), Rotaviruses (ARV) and Coronaviruses (ACoV) are frequently described worldwide (Pantin-Jackwood et al., 2007; Jindal et al., 2009; Spackman et al., 2010; Moura-Alvarez et al., 2014).

Astroviruses, single-stranded, positive-sense RNA virus (Matthew et al., 2002) belongs to the *Astroviridae* family and comprises two genera, *Mamastrovirus* and *Avastrovirus,* that infected mammals and avian species, respectively (Bosch et al., 2012). The *Avastrovirus* genera is further subdivided into three species: *Avastrovirus 1*, including Turkey Astrovirus type 1 (TAstV-1), *Avastrovirus 2*, including Avian Nephritis virus (ANV-1 and ANV-2) and *Avastrovirus 3*, including Turkey Astrovirus type 2 (TAstV-2) and Duck Astrovirus (DAstV) (Bosch et al., 2012).

Rotaviruses, non-enveloped double-stranded RNA (family *Reoviridae*, subfamily *Sedoreovirinae*), are classified based on the reactivity or genetic sequencing of the nonstructural protein VP6 into groups A to H (Attoui et al., 2012; Matthijnssens et al., 2012); being groups A (RVA), D (RVD), F (RVF) and G (RVG) detected in avian species (Trojnar et al., 2010; Johne et al., 2011; Ogden et al., 2012; Kindler et al., 2013). Using the metagenomic approach and the VP6-based molecular classification scheme, Mihalov-Kóvacs et al. (2015) found evidence for a novel rotavirus species that they tentatively called Rotavirus I.

Regarding coronaviruses, positive-sense single-stranded RNA viruses (order *Nidovirales,* family *Coronaviridae*, subfamily *Coronavirinae*), are classified in four genera, *Alphacoronavirus* (α-CoV), *Betacoronavirus* (*β-CoV), Gammacoronavirus* (*γ-CoV*) and *Deltacoronavirus* (*δ-CoV*) (de Groot et al., 2012; Woo et al., 2012; Fehr and Perlman, 2015). Avian Coronavirus (ACoV), is a *γ-CoV* that infects domesticated (Infectious Bronchitis Virus in chickens and Turkey Coronavirus) and wild birds and can cause poultry diseases related to respiratory, reproductive and renal systems and enteritis (Cavanagh, 2003; Cavanagh, 2005; Villarreal et al., 2007).

According to some studies, co-circulation of these viruses may cause a negative impact on intestinal absorptive functions (Ismail et al., 2003). Moreover, AAstV could be associated with ARV and ACoV, likely causing runting-stunting syndrome (RSS), mainly, in broilers and PEMS (poult enteritis mortality syndrome) in other species of birds, respectively (Barnes et al., 2000).

In Brazil, single or concomitant detection of these viruses in poultry and turkeys, have been reported by several research groups (Villarreal, et al., 2006a,b; Bunger et al., 2009; Moura-Alvarez, et al., 2013; Nuñez et al., 2013; Mettifogo et al., 2014; Moura-Alvarez, et al., 2014). A rapid, comprehensive and continuous monitoring of these prevalent viruses related to diarrhea is a key to better understanding of viral dynamics and to provide helpful data on critical analysis of preventive measures on farms. Therefore, this study aimed to investigate the frequency of occurrence and molecular diversity of AAstV, ARV and ACoV from broilers, layers and broiler breeders from Brazilian poultry farms.

## Materials and Methods

### Samples

In 2013, a total of 59 flocks from 6 different Brazilian avian production states were evaluated: Rio Grande do Sul (RS); Paraná (PR); São Paulo (SP); Minas Gerais (MG); Mato Grosso do Sul (MS); Pernambuco (PE). Samples were collected as pools of complete intestinal content from five birds (broilers, layers and broiler breeders), aged between 1 to 70 weeks, with or without clinical signs, that included moderate increase or high mortality, drop in performance, lack of uniformity and disorders of respiratory, reproductive and enteric tracts. 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 3070/2013.

### Reverse Transcription

Samples for all screening PCR's were prepared as 50% (vol/vol) suspensions in DEPC treated water, clarified at 12,000 x g for 15 min at 4ºC, and the supernatant was used for RNA extraction. Total RNA was extracted with TRIzol Reagent™ (Life Technologies, Carlsbad, USA) and cDNA was synthesized using 50 ng of random primers and 200 U of SuperScript III Reverse Transcriptase™ (Life Technologies, Carlsbad, USA) as described by the manufacturer.

**Astrovirus Nested-PCR:** It was adopted a heminested PCR protocol targeting the *RdRp* gene amplification of all astroviruses, using primers and thermocycling conditions described by Chu et al. (2008). The expected product size was 422 base pairs (bp).

**Rotavirus PCR**: The cDNA was subjected to three different PCR's targeting the NSP5/VP6 gene, with RVA, RVD and RVF primers and thermal cycling conditions (Salem et al., 2010; Bezerra et al., 2012; Beserra et al., 2014) generating a 317 bp, 742 bp and 881 bp gel band, respectively.

**Avian Coronavirus Nested-PCR:** Each sample was screened for ACoV as described by Cavanagh et al. (2002), targeting the 3'-UTR with amplicons of 179 bp. Positive 3'-UTR samples were then tested by a genotype S-gene targeted multiplex RT-PCR for Massachusetts, D274 and 4/91 genotypes (Cavanagh et al., 1999) and a pan-IBV S1 gene (ACoV) RT-PCR described by Torres et al. (2014) (450 bp amplicon). All amplification steps for screening and

**Table 1:** *Frequency of occurrence of single and mixed virus detection from avian fecal material (n=59) according to farming type (Layer n=24; Broiler n=28; Breeder n=7).*

| Virus(es) | Frequency % (n) | Farming Type Frequency % (n) | | |
|---|---|---|---|---|
| | | Layer | Broiler | Breeder |
| AstV[A] | 8.47% (5) | 40% (2) | 60% (3) | - |
| RVD[B] | 0% (0) | - | - | - |
| ACoV[C] | 32.2% (19) | 31.57%(6) | 57,89%(11) | 10,52%(2) |
| AstV+ ACoV | 30.51% (18) | 38.9%(7) | 33.3%(6) | 27.8%(5) |
| AstV+ ACoV +RVD | 10.17% (6) | - | 100% (6) | - |
| Negative for all viruses | 18.64% (11) | 81.9% (9) | 18.1%(2) | - |

[A]: *Astroviruses;* [B]: *Rotavirus D;* [C]: *Avian coronavirus*

**Table 2:** *Frequency of occurrence of single and mixed virus detection from avian fecal material (n=59) according to farming type and geographical origin (Brazilian States): MS, MG, PR, PE, RS, SP.*

| Virus(es) | Frequency % (n) | Farming Type (n) | | |
|---|---|---|---|---|
| | | Layer | Broiler | Breeder |
| AstV[A] | 8.47% (5) | MS(1) RS(1) | PR(1) RS(2) | - |
| RVD[B] | 0% (0) | - | - | - |
| ACoV[C] | 32.2% (19) | PE(1) RS(5) | PR(3) RS(8) | RS(2) |
| AstV+ ACoV | 30.51%(18) | MG(1) MS(1) PE(1) RS(3) PR(1) | PR(3) RS(3) | RS(5) |
| AstV+ ACoV +RVD | 10.17% (6) | - | PR(2) SP(4) | - |
| Negative for all viruses | 18.64% (11) | MS(2) PE(2) RS(4) SP(1) | PR(1) RS(1) | - |

[A]: *Astroviruses;* [B]: *Rotavirus D;* [C]: *Avian coronavirus*

genotyping were carried out with GoTaq™ Green Master Mix (Promega, Madison, USA) as per manufacturer's instructions.

**RT-PCR controls:** The rotavirus strain NCDV (Nebraska calf diarrhea virus), strain AstV Brazil 1 and ACoV Massachusetts strain vaccine (NOBILIS™ IB Ma5; MSD Animal Health, Boxmeer, The Netherlands) were used as positive controls for reverse transcription-polymerase chain reaction (RT-PCR). As negative controls, ultrapure water treated with 0.1% DEPC was included.

### Nucleotide sequencing and phylogenetic analysis

Nucleotide sequences from AstV, ARV and ACoV were submitted to phylogenetic analysis in order to confirm the virus genera. PCR products were purified with EXOSAP-it (USB®) reagent, submitted to bidirectional DNA sequencing with BigDye 3.1 (Cycle Sequencing Kit, Applied Biosystems™, Carlsbad, USA) and resolved in an ABI-3500 Genetic Analyser (Applied Biosystems™, Carlsbad, USA), according to manufacturer's instructions. The nucleotide sequences of each sample were aligned with homologous representatives from different Astrovirus, Rotavirus or Coronavirus strains retrieved from GenBank, with CLUSTAL/W 2.1 (Larkin et al., 2007) software, and a phylogenetic tree was generated with the neighbor-joining distance method and the maximum composite likelihood substitution model with 1,000 bootstrap replicates using MEGA 6.0 (Tamura et al., 2013) (data not shown).

## Results and Discussion

In the present study, 59 pools of complete intestinal content were used; being 81.45% (48/59) positive samples for at least one the screened viruses. The results per single or concomitant detection of virus (es), bird type, and state of origin, are summarized in Table 1 and Table 2.

### Astrovirus

Twenty-nine pools (29/59; 49%) were considered positive for astrovirus (*Avastrovirus*). Regarding type species, ANV was detected in twenty-one (21/29; 72.4%) samples, TAstV-1 in six (6/29; 20.6%) samples and CAstV in two (2/29; 6.89%) samples.

### Rotavirus

Out of 59 pools of intestinal contents tested by RT-

PCR, 6 showed positive results (6/59; 10%) for RVD, whereas none of the samples were positive for RVA and RVF. The phylogenetic tree of VP6 nucleotide sequences depicted that the six Brazilian rotavirus strains segregated with the typical rotavirus D representatives, while the topology maintained the remaining groups (A, B, C, F, G, H and I) apart from each other and supported by high bootstrap values (>90%) (not shown).

### Avian Coronavirus

Viral RNA was detected in forty-three sampled pools (43/59; 72.8%). The multiplex typing PCR revealed that all strains of ACoV detected do not belong to the Massachusetts, D274 and 4/91 genotypes but were classified by the phylogenetic analysis of the pan-IBV S1 gene as Brazilian type (not shown). All sequences were related to GI-11 genotype according to Valastro et al. (2013).

## Concomitant viral infections

A total of 24 (24/59; 40.67%) pools presented concomitant viral infections. Of these, 18 (18/24; 75%) presented AstV+ACoV and 6 (6/24; 25%) AstV+RVD+ACoV and the higher frequencies of occurrence were found in layers and broilers (Table 1).

Regarding the AstV+ACoV co-circulation, 83.3% (15/18) were positive for ANV+ACoV, 11.1% (2/18) for TAstV-1+ACoV and 5.55% (1/18) for CAstV+ACoV. Finally, 50% (3/6) of the positive samples for the three screened viruses, showed association between ANV+RVD+ACoV, 33.3% (2/6) with TAstV-1+RVD+ACoV and 16.6% (1/6) with CAstV+ RVD+ACoV.

Recent reports suggest that concomitant enteric viral infections may increase the severity of clinical signs in avian species (Spackman et al., 2010). According to previously reports in Brazilian poultry farms (Mettifogo et al., 2014), ACoV had the highest frequency of occurrence (72%) followed by AAstV (48%) and the association of both (18/24; 75%) was mainly detected in layers (8/18; 44%). Increase mortality and decrease production have been shown in the studied farms in which, co-circulation of these two viruses were observed (Mettifogo et al., 2014).

Some lineages of Avian Coronaviruses can cause, for example, gastrointestinal diseases (TCoV - Turkey coronaviruses) (Ambepitiya, et al., 2015) and other ones, as several serotypes of Infectious Bronchitis Virus (IBV) and IBV-like viruses, showed enterotropism even they primarily infect the respiratory tract (Cavanagh, 2007), having an important economic impact due to production decrease and the possibility of secondary infections (Cavanagh, 2003).

Astroviruses appear to be widely disseminated and endemic in poultry in the world, and concomitant infection of flocks with two or more enteric viruses is common (Baxendale and Mebatsion, 2004; Jindal et al., 2010; Pantin-Jackwood et al., 2006, Pantin-Jackwood et al., 2007; Pantin-Jackwood et al., 2008). So far, there is only one report of CAstV in Brazilian poultry (Mettifogo et al., 2014), being the third most frequent virus detected. AAstV have been also identified in turkeys (Silva et al., 2008; Moura-Alvarez et al., 2013) in Brazil, suggesting a possible relationship between astroviruses and enteric diseases in birds (Nunez et al., 2015).

Moreover, in chickens, AAstV and ARV are associated with RSS causing stunted growth, renal injury and death of young chickens (Imada et al., 1979, Shirai et al., 1991). Results showed herein confirm the circulation of group D (RVD) rotavirus in Brazilian poultry farm, though the frequency of occurrence is still low. RVD was previously detected in Brazil by a specific RT-PCR assay, with occurrence ranging from 53% (16/30 samples) and 35.3% (30/85) to 3.6% (4/111) (Bezerra et al., 2012; Bezerra et al., 2014; Beserra et al., 2015).

In the field, rotaviruses are commonly detected via molecular methods in both turkeys and chickens, often in concomitant infections with other viruses such as astroviruses, reoviruses and coronaviruses; 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 (Pantin-Jackwood et al., 2007; Pantin-Jackwood et al., 2008; Jindal et al., 2009; Jindal et al., 2012).

Interestingly, in five broilers farms located in Rio Grande do Sul state, we have detected AAstV (TAstV-1) in single and concomitant infection of AAstV (ANV) and ACoV. These farms were close to two of the biggest Brazilian turkey farms, where Moura et al. (2014) previously reported TAstV-2 and TCoV (besides rotavirus) co-circulation in turkey flocks with severe enteritis and high mortality. One

can speculate interspecies transmition between these species but when comparing sequencing data from different viral genome.

## Conclusions

Three enteric viruses (AAstV, ACoV and RDV) were found in avian farms (layer, broiler, and breeder) from six Brazilian states. This is the first report of the detection of avian astrovirus and coronavirus in association in breeders from Mato Grosso do Sul state, and coronavirus from intestinal contents in breeders Pernambuco state. The relatively high frequency of occurrence of at least one of the studied viruses denotes the importance to performed a multifactorial diagnosis, as well as the need for continued monitoring of poultry flocks to better understand its epidemiology.

## Authors Contribution

Carolina Torres Alejo, Laila Andreia Rodrigues Beserra, Luis Ramiro Luna Espinoza performed the laboratory methodologies and contributed to writing of the manuscript. Paulo Eduardo Brandão provided samples and contributed to writing of the manuscript. Fabio Gregori participated in its design, coordination, and writing of the manuscript. All authors approved the manuscript for submission.

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