



Investigation of avian rotavirus infections in broiler chicks from commercial flocks with different performance efficiency indexes

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Abstract

The aim of this study was to investigate and compare the frequency of occurrence of avian rotavirus (AvRV) in poultry flocks according to its Performance Efficiency Index (PEI) scores. A total of 256 individual intestinal content samples of small sized-chicks (runts) with clinical signs of Runting Stunting Syndrome (RSS) and 24 clinically healthy chicks (control) were collected from twelve flocks in southern Brazil with different PEI scores: good ($n=4$, PEI mean = 365); moderate ($n=4$, PEI mean = 342) or poor ($n=4$, PEI mean = 319). Silver-stained polyacrylamide gel electrophoresis (ss-PAGE) was used to detect and identify the AvRV species followed by RT-PCR and sequencing of the partial VP6 gene for species confirmation. AvRV was detected in 83% (10/12) of the flocks and 23.4% (60/256) of the chicks. The electrophoretic migration patterns of viral dsRNA segments were compatible with AvRV species A (AvRV-A), D (AvRV-D) and F (AvRV-F) in 9 (15%), 18 (30%), and 33 (55%) of the positive chicks fecal samples, respectively. The AvRV species identified by ss-PAGE were confirmed by RT-PCR and partial sequence analysis of the VP6 gene. The AvRV detection rate was statistically higher ($p=0.007$) in chicks from flocks with poor PEI when compared to those with good PEI. The occurrence of AvRV-D and AvRV-F was statistically higher in 7 to 9 days old chicks, while AvRV-A was detected only in 13 to 14 days old animals.

Keywords Rotavirus A · Rotavirus D · Rotavirus F · Ss-PAGE · Poultry flocks · Runting stunting syndrome

Introduction

Enteric diseases are one of the most important health problems that affect the poultry industry worldwide. Several pathogens have been identified as causes of intestinal tract infections of young chickens including those with signs of Runting Stunting Syndrome (RSS), also known as malabsorption syndrome. RSS is characterized by enteritis, anorexia, poor feathering, and severe weight loss, mostly within the first two weeks of age (Rebel et al. 2006). Commercial

poultry flocks with RSS can determine significant economic losses mainly due to poor feed conversion and high discard rate which directly affect the flock's Performance Efficiency Index (PEI). The PEI is used by integrated poultry companies to evaluate flock performance and is equivalent to the European Broiler Index (EBI) that considers average daily gain (ADG), viability, and feed conversion ratio (FCR) (Marcu et al. 2013). Concerning enteric viruses, different agents have been identified in association with RSS including avian rotavirus (AvRV) (Devaney et al. 2016; Kang et al. 2012; Otto et al. 2006).

Rotaviruses are recognized causes of enteric diseases in a wide variety of avian and mammalian species. These viruses belong to the *Reoviridae* family and are classified into nine distinct species or groups (A to D and F to J) based on the antigenicity and genetic characteristics of capsid protein VP6 (ICTV 2020; Matthijnssens et al. 2012). The rotavirus genome comprises 11 segments of double-stranded RNA (dsRNA) that migrate differently on silver-stained polyacrylamide gel electrophoresis (ss-PAGE), according to the

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rotavirus species. Birds can be infected by species of rotavirus species A (AvRV-A), D (AvRV-D), F (AvRV-F), and G (AvRV-G) (Todd and McNulty 1986).

Due to the multifactorial nature of RSS, is extremely difficult to establish cause-and-effect relationships conclusively on a field situation. However, to our best knowledge, epidemiological surveys in broiler chickens with signs of RSS in association with AvRV infections and PEI have never been conducted before and can be an interesting approach to evaluate the role of this virus on the flock's performance.

The aim of this study was to investigate and compare the frequency of occurrence of AvRV in intestinal content samples from broiler chicks with signs of RSS from commercial broiler flocks of a highly productive region of southern Brazil with different PEI scores.

Materials and methods

Broiler flocks

Twelve commercial broiler chicken flocks with stocking densities between 12 and 15.6 birds/m² were evaluated in 2015. All the selected poultry farms were located in Paraná State, southern Brazil. This region is highly productive, which accounted for 24.8% of the national poultry production in 2015 (BRASIL 2016). Based on the PEI score (Marcu et al. 2013) the flocks were classified into three categories: good ($n=4$; mean PEI = 365), moderate ($n=4$; mean PEI = 342), or poor ($n=4$; mean PEI = 319).

Animals and samples

Twenty to twenty-two small-sized broiler chicks (runts) with clinical signs suggestive of RSS (lethargy, retarded growth, sticky droppings adhering to the cloaca and retarded feather development) and two normal-sized asymptomatic broiler chicks (control) were selected from each flock, performing a total of 280 mixed-sex broiler chicks of Cobb 500 strain. All chicks were between 7 and 14 days old and had feed and water ad libitum. The chicks were humanely euthanized by cervical dislocation and immediately submitted for necropsy to collect the enteric contents that were stored at -20 °C until analysis.

Nucleic acid extraction and ss-PAGE

The nucleic acid was extracted from 10% intestinal contents in Tris-calcium buffer (10 mM Tris-HCl; 1.5 mM CaCl₂; pH 7.3) using a combination of phenol/chloroform/isoamyl alcohol (25:24:1) and silica/guanidine thiocyanate according to Alfieri et al. (2006). DPEC water was used as negative control and a positive fecal sample for bovine rotavirus

species A was included as the positive control. The extracted nucleic acid was submitted to 7.5% PAGE followed by silver staining (Herring et al. 1982).

RT-PCR and sequencing

The AvRV ss-PAGE-positive fecal samples were submitted to RT-PCR with specific primers targeting the VP6 protein gene of the AvRV-A (Schumann et al. 2009), AvRV-D (Bezerra et al. 2012) and AvRV-F (Mascarenhas et al. 2016) for the species confirmation. The amplicons were purified by PureLink[®] Quick Gel Extraction Kit (Invitrogen, Carlsbad, CA, USA), quantified in a Qubit[™] Fluorometer, using QuantiT[™] dsDNA BR Assay Kit (Invitrogen, Eugene, OR, USA). The sequencing reactions were performed in both directions with forward and reverse primers using an ABI3500 Genetic Analyzer sequencer and the BigDye[®] Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequence quality analysis was performed using Phred software and the consensus sequences were assembled using the CAP3 software (<http://asparagin.cenargen.embrapa.br/phph/>). Sequence similarity searches were performed using the Basic Local Alignment Search Tool (BLAST) software (<http://blast.ncbi.nlm.nih.gov/>). A nucleotide sequence of each rotavirus species was deposited in GenBank under accession numbers MZ935731 (AvRV-D), MZ964147 (AvRV-A), and MZ964148 (AvRV-F).

Statistical analysis

The frequency data of AvRV and the different species detected in the broilers chicks were submitted to analysis of variance (ANOVA) concerning the flock PEI and the age groups (7 to 9 days and 13 to 14 days). Means for PEI groups were compared using the Tukey test, whereas the means for age groups were compared based on the F-test from ANOVA. A significance level of 0.05 was adopted for all analyses, which were performed in the Statistical Analysis System (SAS), v9.0.

Results

AvRV detection by ss-PAGE and confirmation by RT-PCR and sequencing

AvRV was detected in 83% (10/12) of evaluated broiler flocks and the identification of rotavirus species was obtained in 23.4% (60/256) of the intestinal content samples analyzed by ss-PAGE. The electrophoretic migration profiles were characteristics of AvRV-A, AvRV-D, and AvRV-F in 9 (15%), 18 (30%), and 33 (55%) of the positive fecal samples, respectively. The distribution of AvRV in the evaluated

Table 1 Distribution of avian rotavirus (AvRV) species (A, D, and F) detected in the enteric contents of broiler chickens according to the Performance Efficiency Index (PEI) of commercial broiler flocks from Paraná state, southern Brazil (February to October 2015)

Commercial flocks		ss-PAGE positive results				
PEI	Flocks (n)	Age of chicks (days)	chicks (n)	AvRV-A	AvRV-D	AvRV-F
Good	4	8–14	87	1	2	9
Regular	4	9–14	84	3	nd ^a	17
Poor	4	7–14	85	5	16	7
TOTAL	12		256	9	18	33

^and not detected

flocks is shown in Table 1. Figure 1 illustrates the electrophoretic profiles of AvRV dsRNA detected in this study. The RT-PCR and sequencing analysis confirmed the AvRV species previously identified from analysis of the electrophoretic profile by ss-PAGE.

A higher frequency of AvRV detection was observed in chicks from broiler flocks with poor PEI ($P=0.007$) when compared to flocks with good PEI (Table 2). Regarding the distribution of AvRV species and the age group of affected chicks, the AvRV-A occurred only in broiler chicks at 13 to 14 days old ($P<0.001$). In contrast, species D and F were observed significantly ($P=0.033$ and $P=0.050$, respectively) in chicks at 7 to 9 days of age.

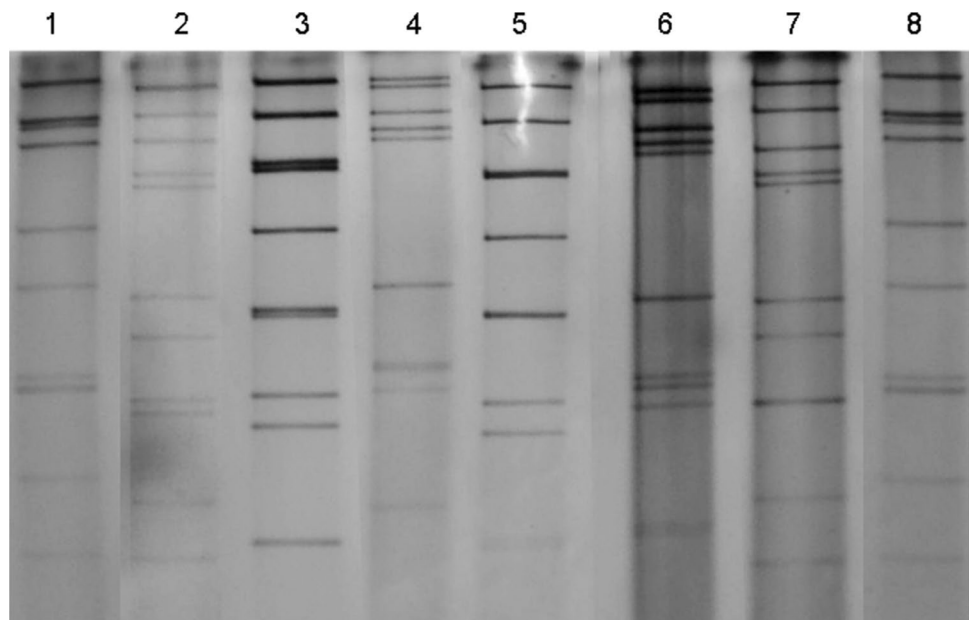
AvRV was detected in only 2 (8.3%) out of 24 asymptomatic control broiler chicks evaluated, with one sample positive for AvRV-A and one for AvRV-D, both from flocks with poor PEI scores.

Discussion

In the present survey, 23.4% of intestinal contents of broiler chicks evaluated were positive for AvRV by ss-PAGE. In studies that also used ss-PAGE as a diagnostic tool, the frequencies of occurrence of AvRV ranged from 0.86 to 64% in European countries and India (Karim et al. 2007; Otto et al. 2006, 2012). In Brazil, the frequencies ranged from 8.5 to 18% in the Minas Gerais and Paraná states (Alferi et al. 1989; Tamehiro et al. 2003). AvRV dsRNA was detected in two control samples, however, the frequency found in asymptomatic broiler chicks was much lower than that in RSS broiler chicks, as already reported by other studies (Bezerra et al. 2012, 2014; McNulty and Reynolds 2008; Otto et al. 2006, 2012; Tamehiro et al. 2003). The wide variation in detection rates can be influenced by various factors including host susceptibility (age, lineage, and immunological condition), sample representativeness (number of samples and collection method) as well as laboratory analytical method (Karim et al. 2007; Otto et al. 2012; Pauly et al. 2017; Silva et al. 2013). The studies with the highest frequency rates sampled mostly symptomatic animals from flocks with RSS, individual samples, age group up to two weeks of age (considered the most susceptible) and RNA extraction protocols based on silica adsorption (Otto et al. 2006, 2012).

The highest detection rate of AvRV was observed in broilers chicks housed in poor PEI flocks with a statistical difference in comparison to the good PEI flocks. Several infectious risk factors have already been identified to negatively affect the flock’s PEI in conventional broilers production systems, including coccidiosis and clostridiosis (Jones et al. 2018; Van Limbergen et al. 2020). However, to our knowledge, this is the first study in the world that evaluated the

Fig. 1 Silver-stained polyacrylamide gel electrophoresis of the extracted nucleic acid of the enteric contents of runt chicks. The keys represent the number of genomic segments according to the electrophoretic migration profile of the AvRV species. Lanes 1 and 8: bovine RVA positive control (4:2:3:2 pattern); lanes 4 and 6: AvRV-A electropherogroup (5:1:3:2 pattern); lanes 2 and 7: AvRV-D electropherogroup (5:2:2:2 pattern); lanes 3 and 5: AvRV-F electropherogroup (4:1:2:2:2 pattern)



relationship between AvRV frequency and flock productive indexes. Silva et al. (2013) and Pauly et al. (2017) reported a higher occurrence of AvRV in flocks with high bird density, a non-infectious risk factor that magnifies poultry stress and facilitates the dissemination of AvRV.

AvRV-F was the most frequent (55%), followed by AvRV-D (30%) and AvRV-A (15%). In other studies, that also used the ss-PAGE as a diagnostic method, the detection rates for AvRV-A and AvRV-D ranged between 1.43 and 36% (Alfieri et al. 1989; Bezerra et al. 2012, 2014; Karim et al. 2007; Otto et al. 2006; Tamehiro et al. 2003). With the use of more sensitive techniques such as RT-PCR, the detection rates varied from 16.1% to 58.8% and 39.2% to 53% for AvRV-A and AvRV-D, respectively (Bezerra et al. 2012; Otto et al. 2006, 2012).

The lowest concentration of rotavirus detected in human fecal samples by ss-PAGE was 10^8 virions/mL (Kohnno et al. 2000). Due to the low sensitivity of the ss-PAGE technique

(Otto et al. 2006), positive results obtained in this assay indicate a high viral excretion at the time of sampling.

In contrast to the observation that the AvRV-F species occurs less frequently in broilers (Beserra and Gregori 2014; Bezerra et al. 2012; Otto et al. 2006) and is excreted in lower titers (Mascarenhas et al. 2016), in our study, the AvRV-F species was the most detected. Besides, in Brazil, AvRV-F had only been detected by RT-PCR assay with rates ranging from 9.4% to 18.5% (Beserra and Gregori 2014; Mascarenhas et al. 2016). This is the first report to identify AvRV-F by electrophoretic profile on ss-PAGE in Brazil. The individual collection of intestinal contents may be one of the explanations for the higher frequency of AvRV-F reported in our study. Beserra and Gregori (2014) and Mascarenhas et al. (2016) evaluated pools of fecal samples from different individuals. In this type of sampling, a positive fecal sample may become undetectable by the ss-PAGE technique after reducing the viral concentration by mixing with negative fecal samples. Meanwhile, AvRV-D was not detected in flocks with regular PEI score. Among the possible explanations for this are the absence of virus circulation in the flocks or the sampled birds would be with low viral load at the time of collection.

Considering the age range of the broilers chicks, several reports have indicated that the highest occurrence of AvRV is in the first two weeks of age (Decaesstecker et al. 1988; Mascarenhas et al. 2016; Tamehiro et al. 2003). Although our sampling represents a narrow range of age (7 to 14 days), the occurrence of AvRV-D and AvRV-F was statistically higher in the 7 to 9 days old age group, while AvRV-A was detected only in chicks between 13 to 14 days old. The non-detection of AvRV-A in broilers from 7 to 9 days of age corroborates

Table 2 Distribution of the avian rotavirus (AvRV) diagnostic frequency by silver-stained polyacrylamide gel electrophoresis (ss-PAGE) in broilers chicks, according to the Performance Efficiency Index (PEI) score of broiler flocks

PEI score	AvRV		
	Positive (%)	Negative (%)	TOTAL
Good	12 (13.8) ^B	75 (86.2)	87
Regular	20 (23.8) ^{AB}	64 (76.2)	84
Poor	28 (32.9) ^A	57 (67.1)	85
TOTAL	60 (23.4)	196 (76.6)	256

Means with no common capital letters, in the same column, differed by Tukey's test ($p < 0.05$)

the finding by McNulty et al. (1983) in a longitudinal survey, where AvRV-A was not detected in broilers younger than 14 days old.

Despite much research have been conducted to elucidate the etiology of RSS, it remains unclear. A large and growing number of viral pathogens and even a combination of multiple viruses have been associated with RSS in broiler chickens, such as reovirus (Songserm et al. 2002), astrovirus (Kang et al. 2012), rotavirus (Otto et al. 2012), calicivirus (Devaney et al. 2016), coronavirus (Hauck et al. 2016), parvovirus (Zsak et al. 2013), birnavirus (Noiva et al. 2015), and gallivirus (Oliveira et al. 2021). Because we did not investigate other pathogens, our findings are not sufficient to establish a causal relationship between AvRV and RSS. Due to the wide spectrum of possible etiologies, the differential diagnosis becomes quite difficult and complex, especially in field situations. In addition to being a time-consuming and very costly process, it is necessary to have well standardized protocols for the accurate detection of different pathogens. Furthermore, the interpretation of results may be challenging since many of these viruses were also detected in clinically healthy animals (Lima et al. 2019). In conclusion, the rate of AvRV detection in intestinal content samples of broiler chicks was high, mainly in flocks with poor PEI. The significant detection of AvRV in chicks with RSS does not constitute an independent indicator for the occurrence of RSS and poor performance of flocks. However, our results suggest that future studies with multivariable modelling should include the AvRV in order to determine whether rotaviruses are potential contributors to the RSS pathogenesis as well as whether it constitutes an important risk factor that threatens the flock's performance under natural field conditions.

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Authors' contributions This study was conceptualized by Jessica Cristhine Gallego and Elisabete Takiuchi; Jessica Cristhine Gallego, Daniela Lorencena, Janaína Lustosa de Mello, Ruana Renostro Delai, Mônica Regina de Matos, Aline de Marco Viott and Sergio Rodrigo Fernandes conducted experimental study; Jessica Cristhine Gallego, Elis Lorenzetti, Amauri Alcindo Alfieri and Elisabete Takiuchi performed sequence analysis; Jessica Cristhine Gallego and Elisabete Takiuchi written original draft preparation; Jessica Cristhine Gallego, Elisabete Takiuchi, Aline de Marco Viott, Sergio Rodrigo Fernandes, Elis Lorenzetti and Amauri Alcindo Alfieri written review and editing. All authors read and approved the final manuscript.

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Availability of data and material The raw data that support the findings of this study are available on request from the corresponding author.

Code availability Not applicable.

Declarations

Ethics approval The entire experimental procedure is following the ethical principles adopted by the local Ethics Commission from the Universidade Federal do Paraná under protocol number 15/2014.

Consent to participate All the authors consent to participate in publication.

Consent for publication All the authors consent to publish the manuscript.

Conflicts of interest The authors declare that they have no conflict of interest.

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