DAVID ISAIAS DE LA TORRE DUQUE

**Detection and molecular characterization of enteric viruses in
commercial birds**

São Paulo

2019

DAVID ISAIAS DE LA TORRE DUQUE

**Detection and molecular characterization of enteric viruses in
commercial birds**

Tese apresentada ao Programa de Pós-Graduação em Patologia Experimental e Comparada da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para obtenção do título de Doutor em Ciências.

Departamento:

Patologia - VPT

Área de concentração:

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Orientador:

Prof. Dr. Antonio José Piantino Ferreira

São Paulo

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CERTIFICADO

Certificamos que a proposta intitulada "Detecção e Caracterização Molecular de Vírus Entéricos em Aves Comerciais", protocolada sob o CEUA nº 8582030517, sob a responsabilidade de **Antonio José Piantino Ferreira e equipe; David Isaias de La Torre Duque** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 10/05/2017.

We certify that the proposal "Detection and Molecular Characterization of Enteric Viruses in Commercial Birds", utilizing 300 Birds (males and females), protocol number CEUA 8582030517, under the responsibility of **Antonio José Piantino Ferreira and team; David Isaias de La Torre Duque** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 05/10/2017.

Finalidade da Proposta: **Pesquisa**

Vigência da Proposta: de **12/2015 a 11/2018**

Área: **Ornitopatologia**

Origem: **Amostras biológicas estocadas**

Espécie: **Aves**

sexo: **Machos e Fêmeas**

Idade: **1 a 365 dias**

N: **300**

Linhagem: **Leghorn**

Peso: **50 a 3000 g**

Resumo: As doenças entéricas que atingem as aves comerciais, são um fator importante a considerar devido à importância econômica que a indústria avícola representa na economia de muitos países, e em especial aqueles da América Latina. Dentre os principais agentes etiológicos das doenças entéricas nas aves comerciais, além das bactérias, parasitas e fungos, podemos mencionar alguns tipos de vírus como o adenovírus aviário do grupo I (FAdV-I), parvovírus aviário (ChPV), vírus da bronquite infecciosa das galinhas (BIG), astrovírus das galinhas (CAstV), vírus da nefrite aviária (ANV), reovírus aviário (ARV), rotavírus A (RV-A) e o tremovírus, que antigamente foi chamado como vírus da encefalomielite aviária (AE), e que embora não produza alterações digestivas nas aves, a principal forma de transmissão é horizontal a través da rota fecal-oral. O estudo epidemiológico dos vírus entéricos nas aves, além de ajudar na compreensão da distribuição geográfica das diferentes cepas virais, também contribui no entendimento da dinâmica das infecções entéricas, em uma tentativa de achar a relação dos diferentes vírus com as principais síndromes multifatoriais como o Runtling Stunting Syndrome (RSS). O objetivo do presente trabalho, é detectar e caracterizar os principais vírus entéricos com importância epidemiológica na avicultura dentro e fora do território brasileiro, usando o material biológico armazenado na coleção do Laboratório de Ornitopatologia da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo e a través de técnicas moleculares como RT-PCR, PCR e sequenciamento de primeira geração.

Local do experimento: Laboratório de Ornitopatologia da Faculdade de Medicina Veterinária e Zootecnia

São Paulo, 02 de junho de 2017

Prof. Dra. Denise Tabacchi Fantoni

Presidente da Comissão de Ética no Uso de Animais

Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Roseli da Costa Gomes

Secretaria Executiva da Comissão de Ética no Uso de Animais

Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

FOLHA DE AVALIAÇÃO

Autor: DE LA TORRE DUQUE, David Isaías

Título: **Detection and molecular characterization of enteric viruses in commercial birds**

Tese apresentada ao Programa de Pós-Graduação em Patologia Experimental e Comparada da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para obtenção do título de Doutor em Ciências

Data: ____/____/____

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DEDICATORIA

Ao meu querido filho Emilio José, que com seu espírito me motiva todos os dias para superar os desafios que a vida me reserva, procurando sempre o sucesso para compartilhá-lo com ele.

A todos que eu amo, minha esposa Cristina, meus pais Wilson e Lidia, meus irmãos Consuelo e Wilmer, meus sobrinhos e sobrinhas Katrin, Brenda, Jéssica, Aaron, Jaidé, Michu e Samy, para quem compartilho meu esforço e aliás mostro que a perseverança pode fazer com que todos os objetivos sejam alcançados, independentemente dos obstáculos que temos em nossas vidas.

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A Deus que sempre me guiou no melhor caminho em busca da felicidade.

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Aos meus pais que sempre se esforçaram para me dar o melhor em todas as fases da minha vida, por seus sacrifícios, compreensão, paciência e amor, especialmente nos momentos mais difíceis da minha vida.

A minha esposa Cristina, que esteve do meu lado incondicionalmente em todos os momentos da nossa aventura fora do país.

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Ao meu amigo e professor Luis que confiou em mim desde o início dos meus estudos e que abriu as portas de sua casa quando eu precisei.

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À Faculdade de Medicina Veterinária e Zootecnia, ao Departamento de Patologia e à Universidade de São Paulo, por me permitir realizar meus estudos de doutorado.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 1706271.

As três coisas mais difíceis do mundo são:
guardar um segredo, perdoar uma ofensa e
aproveitar o tempo.

Benjamin Franklin

ABSTRACT

DE LA TORRE DUQUE, D. I. **Detection and molecular characterization of enteric viruses in commercial birds**. [Detecção e Caracterização Molecular de Vírus Entéricos em Aves Comerciais]. 2019. 100 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2019.

Enteric viruses in commercial birds are associated with infections that affect the health and productive rates of animals, causing economic losses for the poultry industry in Brazil and worldwide. The objective of this study is to identify the main enteric viruses that infect commercial poultry flocks in Brazil and in some cases in neighboring countries where the poultry industry is an important factor for economic development. For this study, frozen samples of bird organs from different states of Brazil and Ecuador were used, as well as results previously obtained by the avian disease diagnosis service of the Laboratory of Avian Pathology at the School of Veterinary Medicine and Animal Sciences of the University of São Paulo. Diagnoses were performed through conventional polymerase chain reaction (PCR) and reverse transcription-PCR, and the characterization of different viruses was performed by sequence analysis of specific genes from randomly selected samples from each virus diagnosed. The results provide important information about virus serotypes and their geographic distribution within the Brazilian territory. Three serotypes (8a, 8b, and 11) of fowl adenovirus group I (FAdV-I) were found in digestive, respiratory, and immunological organs, including the heart and kidneys. In addition to FAdV-I, during the period from 2010 to 2017, chicken parvovirus, chicken astrovirus, avian nephritis virus, infectious bronchitis virus, avian reovirus, and avian rotavirus were found in samples from 11 different Brazilian states (Mato Grosso, Goiás, Piauí, Ceará, Paraíba, Pernambuco, Bahia, Minas Gerais, Espírito Santo, São Paulo, and Santa Catarina). Chicken parvovirus was molecularly diagnosed for the first time in samples of commercial birds from Ecuador. Tremovirus A (AEV) was diagnosed in outbreaks of avian encephalomyelitis in broilers during the period from 2006 to 2015, even though vaccination in breeders is included in the regimen to prevent the vertical transmission of this virus. These results demonstrate the diversity of viruses that affect commercial birds individually or in combination with other viruses, while the exact role of each one within enteric diseases is still not well

understood, requiring experimental studies to achieve more knowledge about the pathogenesis of these etiological agents.

Keywords: Molecular detection. Enteric virus. Poultry. PCR. Characterization.

RESUMO

DE LA TORRE DUQUE, D. I. **Deteção e Caracterização Molecular de Vírus Entéricos em Aves Comerciais.** [Detection and molecular characterization of enteric viruses in commercial birds]. 2019. 100 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2019.

Os vírus entéricos em aves comerciais estão associados a infecções que causam alterações na saúde e nos parâmetros produtivos dos animais, gerando perdas econômicas para a indústria avícola no Brasil e no mundo. O objetivo deste estudo foi identificar os principais vírus entéricos que afetam os lotes de aves comerciais no Brasil, e em alguns casos, nos países vizinhos onde a indústria avícola é um fator importante para o desenvolvimento de sua economia. Para o desenvolvimento deste trabalho, foram utilizadas amostras congeladas de órgãos de aves de diferentes Estados do Brasil e do Equador, assim como os resultados obtidos anteriormente pelo serviço de diagnóstico de doenças aviárias do Laboratório de Ornitopatologia da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo. Os diagnósticos foram realizados através da Reação em Cadeia da Polimerase (PCR) convencional e pela técnica da Transcriptase Reversa – PCR. A caracterização de alguns vírus foi realizada pela análise de sequências de nucleotídeos de genes específicos em amostras selecionadas aleatoriamente para cada vírus diagnosticado. Os resultados forneceram informações importantes sobre os sorotipos de vírus e sua distribuição geográfica no território brasileiro. Três sorotipos (8a, 8b e 11) do Adenovírus Aviário do Grupo I (FAdV-I) foram encontrados em órgãos digestivos, respiratórios ou imunológicos, incluindo coração e rins. Além de FAdV-I, durante o período de 2010 a 2017, Parvovírus das galinhas (ChPV), Astrovírus das galinhas (CAstV), Vírus da Nefrite Aviária (ANV), Vírus da Bronquite Infecciosa (IBV), Reovírus Aviário (AReo) e Rotavírus Aviário (ARtV), também foram encontrados em amostras de 11 diferentes Estados do Brasil, ou seja, Mato Grosso, Goiás, Piauí, Ceará, Paraíba, Pernambuco, Bahia, Minas Gerais, Espírito Santo, São Paulo e Santa Catarina. O ChPV foi diagnosticado por métodos moleculares pela primeira vez em amostras de aves comerciais do Equador. O Tremovírus A (AEV) foi diagnosticado em surtos de encefalomielite aviária em frangos de corte durante o período de 2006 a 2015, embora a vacinação seja uma rotina para prevenir a transmissão vertical deste vírus. Estes resultados demonstraram a diversidade de vírus que afetam as aves

comerciais, sendo que estas infecções podem ser causadas por um único vírus ou por associação entre vírus. O exato papel de cada um destes vírus nas doenças entéricas ainda não foi bem compreendido, exigindo estudos experimentais para se obter mais informações e conhecimento sobre a patogênese desses agentes etiológicos.

Palavras-chave: Detecção molecular. Vírus entéricos. Avicultura. PCR. Caracterização.

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1 INTRODUCTION

The poultry industry is an important economic activity in Brazil and throughout the world. The high diversification of businesses related to poultry farming and the intensification of growing methods requires a continuous development of biological technologies that improve the productive parameters of animals, focused on a sustainable economy and adequate maintenance of animal welfare. Some data that show the importance of the poultry industry are summarized below. The global consumption of meat per capita at the end of 2018 is estimated at 43.7 kg/year, and to achieve this demand, meat production worldwide is estimated at 335 million tons, of which 121 million tons corresponds to chicken meat. Brazil is the fourth largest producer of poultry meat worldwide, with 13.2 million tons produced per year. Approximately 30% of its production is intended for export, turning Brazil into the largest poultry exporter in the world with 4.3 million tons exported mainly to Asian countries and the Middle East and generating an approximate income of USD \$ 7.236 million for the Brazilian economy (Food and Agriculture Organization of the United Nations – FAO, 2018; Associação Brasileira de Proteína Animal – BPA, 2018). In parallel, the poultry industry in Brazil reached a production of 50,400 million eggs in 2017, constituting a population of 242.8 million permanently housed laying hens (Instituto Brasileiro de Geografia e Estatística – IBGE, 2017).

These data demonstrate the importance of the prevention and control of diseases in poultry farming, from a sanitary and economic point of view. In this study, we focus on enteric diseases that affect commercial birds, especially those caused by viruses. When enteric diseases occur, one or more agents may be involved in the development of infections, and in some cases, an agent may trigger the action of others causing multiple infections or showing similar symptoms among several pathogens. This hinders effective diagnosis in the field and requires the use of laboratory techniques for the identification of the causative agent.

There are many viral pathogens associated with enteric diseases that cause common symptoms collectively known as runting-stunting syndrome (RSS). Studies conducted with next-generation sequencing in birds with RSS demonstrated the coinfection of viral families such as Adenoviridae, Picornaviridae, Astroviridae,

Caliciviridae, Parvoviridae, Reoviridae, and Herpesviridae, among others (DEVANEY, 2016; LIMA et al., 2019). Fowl adenovirus (FAdV), chicken parvovirus (ChPV), chicken astroviruses (CAstV and ANV), avian reovirus (ARV) and avian rotavirus (ARtV) are some of the viruses with the highest incidence in enteric diseases (KOO et al., 2013; KAITHAL et al., 2016; NUÑEZ et al., 2016), which have been studied individually to determine their impact on bird health and to demonstrate an association between viral infection and symptoms of dwarfism, malabsorption, diarrhea and weight loss, especially in young birds (OTTO et al., 2006; BANYAI et al., 2011; ZSAK; CHA; DAY, 2013; FINKLER et al., 2016; NUÑEZ et al., 2018). Despite the virulence and pathogenicity described in experimental studies and field findings, these viruses have also been found in healthy birds (DAY et al., 2010; SHAH, JD et al., 2016; KAPGATE, et al. 2018), which has aroused interest in the analysis of the conditions in which the viruses affect the health of the birds and in the study of the genetic variability of the viruses that renders some strains more pathogenic than others.

Many laboratory techniques have been used for the diagnosis of viral diseases: hemagglutination assay, enzyme-linked immunosorbent assay (ELISA), fluorescent antibody (FA) staining, virus isolation, electron microscopy, and others that are laborious and time-consuming, but since the development of the polymerase chain reaction (PCR) (SAIKI et al., 1985), the presence of pathogens has been rapidly and more sensitively diagnosed. Similarly, through DNA sequencing, a more specific characterization of the different viruses infecting animal species has been possible.

The aim of this study is to apply molecular techniques for the diagnosis, characterization and epidemiological analysis of the main enteric viruses inside and outside the Brazilian territory using biological material kindly provided by poultry companies through the diagnostic service of the Avian Pathology Laboratory at the Faculty of Veterinary Medicine of the University of São Paulo, Brazil. The results show the geographical distribution of these pathogens, their association with production problems and the need to develop and implement vaccination programs for the prevention and control of enteric diseases.

2 MOLECULAR CHARACTERIZATION OF FOWL ADENOVIRUS GROUP I (FADV-I) IN COMMERCIAL CHICKEN BROILERS IN BRAZIL

DE LA TORRE, D.; NUÑEZ, L.; SANTANDER, S.; ASTOLFI-FERREIRA, C.S.; FERREIRA, A.J.P.

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ABSTRACT. - Avian adenovirus has been reported in many countries and is an infectious agent related with inclusion body hepatitis, hepatitis-hydropericardium syndrome (HHS), and respiratory and enteric conditions in chickens worldwide. The objective of this study was to detect and establish the molecular sequences of the hexon gene from the avian adenovirus strains of group I (FAdV-I) isolated from birds with hepatitis-hydropericardium syndrome (HHS), malabsorption syndrome and runting-stunting syndrome, to characterize the serotype of virus affecting commercial flocks in Brazil. Molecular characterization was performed by polymerase chain reaction (PCR), using specific primers to amplify the Loop 1 (L1) variable region of the hexon gene in the FAdV-I genome and subsequent sequencing of the PCR product for each positive sample. The results have revealed the presence of the FAdV-8a, FAdV-8b, and FAdV-11 serotypes circulating in Brazilian chicken flocks. Phylogenetic analysis grouped these sequences into three (3) distinct groups, 14 samples were aligned with the FAdV-11 group, three (3) samples in the FAdV-8b group and one (1) sample in the FAdV-8a group. The serotypes FAdV-8a, FAdV-8b, and FAdV-11 are circulating in Brazilian chicken flocks. Therefore, these results are very important for improvement biosecurity measurements and vaccine production.

2.1 INTRODUCTION

Avian adenovirus group I (FAdV-I) mainly infects birds and is classified within the Aviadenovirus genus in the Adenoviridae family (KING et al., 2012). Avian adenovirus is composed by 3 groups (I-III) (HESS, 2000) and was characterized into 5 species (A-E) and 12 serotypes (FAdV-1, FAdV-2, FAdV-3, FAdV-4, FAdV-5, FAdV-6, FAdV-7, FAdV-8a, FAdV-8b, FAdV-9, FAdV-10, FAdV-11) (McCONNELL; FITZGERALD, 2008).

The viral particles are non-enveloped and icosahedral in geometry. They are 70-90 nm in diameter, with a linear double-stranded DNA genome between 35 and 45 kb in size (JIANG et al. 1999). The capsid has 252 capsomeres, of which 240 are formed by the Hexon protein and 12 by the penton protein, which forms the vertices from which fibres that have antigenic properties become detached (McCONNELL; FITZGERALD, 2008).

Inclusion body hepatitis (IBH) associated with FAdV-I occurs worldwide (ALEMNESH et al., 2012). It is an acute disease that mainly affects young chickens between 3-7 weeks of age (STEER et al., 2015) and is caused by several avian adenovirus serotypes (McCONNELL; FITZGERALD, 2008). FAdV-I can be transmitted vertically and horizontally through progeny and faeces, respectively (ALEMNESH et al., 2012). There are studies demonstrating infections caused by this virus in Brazil, which is associated with enteric diseases, liver problems, and hydropericardium (METTIFOGO et al., 2014b). Furthermore, there are reports of adenovirus infections resulting in increased feed conversion rate and increased mortality (TORO et al., 1999; Hess, 2000; SENTÍES-CUÉ et al., 2010). Therefore, the effects of avian adenovirus infections are of great economic importance in poultry farming (MITTAL et al., 2014) and associated with enteric virus, such as ANV (GOWTHAMAN et al., 2015). In addition to the liver, the virus can be found in the upper respiratory tract, digestive tract, pancreas, kidneys, spleen and heart (NUÑEZ; PIANTINO, 2013). Studies with experimentally inoculated birds have demonstrated that avian adenovirus infection can result in weight loss, apathy, and mortality (ALVARADO et al., 2007).

2.2 MATERIALS AND METHODS

2.2.1 Virus samples

The samples were stored during the period of 2011 to 2015, where 18 strains identified as avian adenovirus group I obtained from chickens at eight farms in different Brazilian states were identified by PCR. The birds presented clinical signs related to enteric problems, hydropericardium, and low zootechnical performance. Viral strains were identified from the following organs: liver, bursa, heart, intestine, pancreas, trachea, and kidneys (METTIFOGO et al., 2014a) (Table 1).

2.2.2 DNA extraction

For isolation of DNA, macerated tissue of each organ and Phosphate Buffered Saline (PBS) 0.1M, pH 7.4 was added to 1.5 ml tubes at a 1:1 ratio. The mixture was vortexed for 10 s, and subjected to three freeze-thaw cycles. Extraction was performed using the phenol/chloroform method, as described by Chomczynski (1993).

2.2.3 Polymerase chain reaction (PCR) for detection of FAdV-I

The PCR reaction was carried out as described by Meulemans et al. (2001), with modifications. The reaction mix contained 0.5 μ M each of the sense and antisense primers, 1X PCR buffer, 200 μ M of each deoxynucleotide triphosphate (dNTP), 1.5 mM MgCl₂, 1 U of Platinum™ Taq DNA polymerase (Invitrogen™ by Life Technologies, Carlsbad, CA, USA), 2.5 μ l of extracted DNA and ultrapure water to bring the total volume to 25 μ l. The amplification reaction occurred under the following conditions: a thermal cycle of 94°C for five min, followed by 35 cycles of denaturation at 94°C for 60 s, annealing at 52°C for 45 s, and extension at 72°C for 60 s. The final extension cycle was 72°C for 10 min. The reaction was stored at -20°C. The primers used in the PCR reaction correspond to the conserved segments of the Pedestal 1 (P1) regions adjacent to the Loop 1 (L1) variable segment of the hexon gene (hexon A: CAARTTCAGRCAGACGGT and hexon B: TAGTGATGMCGSGACATCAT). This resulted in amplification of an 897 bp segment (MEULEMANS et al., 2001).

2.2.4 Purification and sequencing

The product amplified by the FAdV-I hexon gene PCR was purified using the GPX™ PCR DNA and Gel Band Purification kit (GE Healthcare, Piscataway, New Jersey, USA) according to the manufacturer's instructions. Each purified product was sequenced in sense and antisense directions using the BigDye® Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems by Life Technologies, Carlsbad, California, USA). Sequencing reactions were performed on an ABI 3730 DNA Analyzer (Applied Biosystems by Life Technologies, Carlsbad, CA, USA).

Table 1 – Sample identification for Brazilian FAdV-I study. Description of samples' characteristics obtained from poultry enterprises with poor development and malabsorption syndrome

Sample designation	Type of bird (origin of samples) and organs	Case history	Serotype identified	GenBank accession number
USP-BR-418.1	Broiler - Liver	Hydropericardium – hepatitis	FAdV-8b	KY229168
USP-BR-418.14	Broiler - Bursa	Hydropericardium – hepatitis	FAdV-11	KY229169
USP-BR-420.12	Broiler - Heart	Malabsorption – undigested feed	FAdV-8b	KY229185
USP-BR-420.16	Broiler - bursa	Malabsorption – undigested feed	FAdV-8b	KY229170
USP-BR-420.17	Broiler - intestine	Malabsorption – undigested feed	FAdV-11	KY229171
USP-BR-420.18	Broiler - pancreas	Malabsorption – undigested feed	FAdV-11	KY229172
USP-BR-420.26	Broiler - bursa	Malabsorption – undigested feed	FAdV-11	KY229173
USP-BR-420.27	Broiler - intestine	Malabsorption – undigested feed	FAdV-11	KY229174
USP-BR-420.28	Broiler - pancreas	Malabsorption – undigested feed	FAdV-11	KY229175
USP-BR-424.4	Broiler - pancreas	Malabsorption – undigested feed	FAdV-11	KY229176
USP-BR-453.2	Broiler - trachea	NI	FAdV-8a	KY229177
USP-BR-471.14	Broiler - trachea	NI	FAdV-11	KY229184
USP-BR-475.1	Broiler - kidney	Runting-Stunting Syndrome	FAdV-11	KY229178
USP-BR-475.3	Broiler - pancreas	Runting-Stunting Syndrome	FAdV-11	KY229179
USP-BR-475.4	Broiler - pancreas	Runting-Stunting Syndrome	FAdV-11	KY229180
USP-BR-475.11	Broiler - kidney	Runting-Stunting Syndrome	FAdV-11	KY229182
USP-BR-102.8D	NI - liver	NI	FAdV-11	KY229181
USP-BR-G21.B	NI- liver	NI	FAdV-11	KY229183

Source: (DE LA TORRE, D. 2018).

Legend: FAdV= Fowl Adenovirus group I; USP= University of São Paulo; BR= Brazil; NI: not Informed.

2.2.5 Phylogenetic analysis

The NT sequences were assembled and edited using the CLC Main Work Bench 7.7.1 software (CLC Bio-Qiagen, Aarhus, Denmark) and aligned with reference sequences representative of the FAdV-I serotypes (KING et al., 2012) present in GenBank with the following accession numbers: FAdV-1 Strain CELO (AC_000014) from Germany; FAdV-2 strain P7-A (AF339915) and FAdV-3 strain 75 (AF508949), both from Belgium; FAdV-4 strain ON1 (NC_015323) from Canada; FAdV-5 strain 340 (AF508952), FAdV-6 strain CR119 (AF508954) and FAdV-7 strain YR36 (AF508955),

all from Belgium; FAdV-8a strain TR59 (KT862810) from Japan; FAdV-8b strain 764 (KT862811) from the United Kingdom; FAdV-9 strain A-2A (AF083975.2) from Canada; FAdV-10 strain C-2B (KT717889) from the USA and FAdV-11 strain 380 (AF339925) from Belgium. Alignment was performed using the CLUSTAL W method available in Clustal X 2.0 software. Phylogenetic analysis of NT was inferred using the maximum likelihood model (TAMURA et al., 2011) integrated into MEGA software 7.0.18. The nucleotide (NT) and amino acid (AA) sequence similarity matrix was generated in BioEdit Sequence Alignment Editor v. 7.2.5.

2.3 RESULTS

2.3.1 Analysis of sequencing results

Sequencing and alignment of sequences in GenBank showed that 14/18 viral strains (77.7%) corresponded to the FAdV-11 serotype, 3/18 strains (16.6%) corresponded to the FAdV-8b serotype, and 1/18 strains (5.5%) corresponded to the FAdV-8a serotype (Table 2). The sequences were deposited in GenBank under the accession numbers described below: USP-BR-418.1 (KY229168), USP-BR-418.14 (KY229169), USP-BR-420.12 (KY229185), USP-BR-420.16 (KY229170), USP-BR-420.17 (KY229171), USP-BR-420.18 (KY229172), USP-BR-420.26 (KY229173), USP-BR-420.27 (KY229174), USP-BR-420.28 (KY229175), USP-BR-424.4 (KY229176), USP-BR-453.2 (KY229177), USP-BR-471.14 (KY229184), USP-BR-475.1 (KY229178), USP-BR-475.3 (KY229179), USP-BR-475.4 (KY229180), USP-BR-475.11 (KY229182), USP-BR-102.8D (KY229181), and USP-BR-G21.B (KY229183). The phylogenetic tree was inferred using phylogenetic reconstruction analysis with the Maximum Likelihood statistical model with 1000 replicates (Bootstrap), the Tamura-Nei substitution model and a Gamma distribution with Invariant sites (TN93 + G + I). This model was suggested by previous analysis with the Maximum Likelihood statistical method using MEGA 7 Software. Phylogenetic analysis revealed that of the 18 viral strains, fourteen (14/18) belonged to serotype 11 of the D species, three (3/18) belonged to serotype 8b and one (1/18) to serotype 8a, both of species E (Figure 1).

Figure 1 - Phylogenetic tree for Brazilian FAdV-I study

Source: (DE LA TORRE, D. 2018).

Legend: Phylogenetic relationships of Brazilian FAdV-I sequences from chickens presented enteric problems, impairment, malabsorption diseases, hydropericardium and hepatitis in comparison to isolates from different countries. The tree was inferred based on alignments of partial hexon gene segment L1 and adjacent P1, using the Maximum Likelihood statistical method included in the MEGA 5 software. Numbers along the branches refer to bootstrap value for 1,000 replicates. The scale bar represents the number of substitutions per site. Highlighted names represent the sequences used in this study, and letters A to E represent fowl adenovirus groups. DAdV-2 Duck adenovirus 2 sequence was employed as the out-group.

The similarity matrix of the USP-BR-418.1 sample with serotype 8b showed NT and AA similarities of 99.3% and 99.6% respectively. The USP-BR-418.14 sample had 97.5% (NT) and 96.8% (AA) similarity to serotype 11. Both samples USP-BR-420.12 and USP-BR-420.16 had 99.0% (NT) and 99.6% (AA) similarity to serotype 8b. The sequences USP-BR-420.17, USP-BR-420.18, USP-BR-420.26, USP-BR-420.27, USP-BR-420.28 and USP-BR-424.4 showed 97.6% (NT) and 96.8% (AA) similarity to serotype 11, and the USP-BR-453.2 sample showed 99.2% (NT) and 99.6% (AA) similarity to serotype 8a. The samples USP-BR-471.14, USP-BR-475.1, USP-BR-475.3, USP-BR-475.4, USP-BR-475.11 and USP-BR-102.8D showed 97.5% (n) and 96.8% (AA) similarity to serotype 11, while the USP-BR-G21.B sample had similarities of 97.2% (NT) and 96.0% (AA) for serotype 11 (Table 2).

Table 2 – Similarity matrix of Brazilian FAdV-I study. Molecular relationship among the FAdV-I Brazilian isolates and the main reference strains for determining the serotypes based on deduced AA and NT identities

Seq->	AMINO ACID SEQUENCES																													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1 KY229168 USP-BR-418.1	-	81.7%	100.0%	100.0%	81.7%	81.7%	81.7%	81.7%	81.7%	81.7%	88.3%	81.7%	81.7%	81.7%	81.7%	81.7%	81.7%	80.9%	66.6%	81.7%	81.7%	60.4%	72.9%	87.5%	91.8%	87.9%	99.6%	81.3%	60.4%	81.3%
2 KY229169 USP-BR-418.14	70.7%	-	81.7%	81.7%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	83.6%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	99.2%	66.2%	96.4%	82.8%	61.0%	73.3%	82.4%	82.4%	84.0%	81.3%	82.8%	59.9%	96.8%
3 KY229185 USP-BR-420.12	99.4%	70.2%	-	100.0%	81.7%	81.7%	81.7%	81.7%	81.7%	81.7%	88.3%	81.7%	81.7%	81.7%	81.7%	81.7%	81.7%	80.9%	66.6%	81.7%	81.7%	60.4%	72.9%	87.5%	91.8%	87.9%	99.6%	81.3%	60.4%	81.3%
4 KY229170 USP-BR-420.16	99.4%	70.2%	100.0%	-	81.7%	81.7%	81.7%	81.7%	81.7%	81.7%	88.3%	81.7%	81.7%	81.7%	81.7%	81.7%	81.7%	80.9%	66.6%	81.7%	81.7%	60.4%	72.9%	87.5%	91.8%	87.9%	99.6%	81.3%	60.4%	81.3%
5 KY229171 USP-BR-420.17	71.1%	99.0%	70.5%	70.5%	-	100.0%	100.0%	100.0%	100.0%	100.0%	83.6%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	99.2%	66.2%	96.4%	82.8%	61.0%	73.3%	82.4%	82.4%	84.0%	81.3%	82.8%	59.9%	96.8%
6 KY229172 USP-BR-420.18	71.1%	99.0%	70.5%	70.5%	100.0%	-	100.0%	100.0%	100.0%	100.0%	83.6%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	99.2%	66.2%	96.4%	82.8%	61.0%	73.3%	82.4%	82.4%	84.0%	81.3%	82.8%	59.9%	96.8%
7 KY229173 USP-BR-420.26	71.1%	99.0%	70.5%	70.5%	100.0%	100.0%	-	100.0%	100.0%	100.0%	83.6%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	99.2%	66.2%	96.4%	82.8%	61.0%	73.3%	82.4%	82.4%	84.0%	81.3%	82.8%	59.9%	96.8%
8 KY229174 USP-BR-420.27	71.1%	99.0%	70.5%	70.5%	100.0%	100.0%	100.0%	-	100.0%	100.0%	83.6%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	99.2%	66.2%	96.4%	82.8%	61.0%	73.3%	82.4%	82.4%	84.0%	81.3%	82.8%	59.9%	96.8%
9 KY229175 USP-BR-420.28	71.1%	99.0%	70.5%	70.5%	100.0%	100.0%	100.0%	100.0%	-	100.0%	83.6%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	99.2%	66.2%	96.4%	82.8%	61.0%	73.3%	82.4%	82.4%	84.0%	81.3%	82.8%	59.9%	96.8%
10 KY229176 USP-BR-424.4	71.1%	99.0%	70.5%	70.5%	100.0%	100.0%	100.0%	100.0%	100.0%	-	83.6%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	99.2%	66.2%	96.4%	82.8%	61.0%	73.3%	82.4%	82.4%	84.0%	81.3%	82.8%	59.9%	96.8%
11 KY229177 USP-BR-453.2	84.1%	72.2%	84.4%	84.4%	72.7%	72.7%	72.7%	72.7%	72.7%	72.7%	-	83.6%	83.6%	83.6%	83.6%	83.6%	83.6%	82.8%	67.0%	83.6%	82.8%	63.1%	73.7%	89.1%	88.7%	99.6%	88.7%	82.1%	63.1%	83.2%
12 KY229184 USP-BR-471.14	70.7%	100.0%	70.2%	70.2%	99.0%	99.0%	99.0%	99.0%	99.0%	99.0%	72.2%	-	100.0%	100.0%	100.0%	100.0%	100.0%	99.2%	66.2%	96.4%	82.8%	61.0%	73.3%	82.4%	82.4%	84.0%	81.3%	82.8%	59.9%	96.8%
13 KY229178 USP-BR-475.1	70.8%	98.9%	70.3%	70.3%	99.6%	99.6%	99.6%	99.6%	99.6%	99.6%	72.6%	98.9%	-	100.0%	100.0%	100.0%	100.0%	99.2%	66.2%	96.4%	82.8%	61.0%	73.3%	82.4%	82.4%	84.0%	81.3%	82.8%	59.9%	96.8%
14 KY229179 USP-BR-475.3	70.8%	98.9%	70.3%	70.3%	99.6%	99.6%	99.6%	99.6%	99.6%	99.6%	72.6%	98.9%	100.0%	-	100.0%	100.0%	100.0%	99.2%	66.2%	96.4%	82.8%	61.0%	73.3%	82.4%	82.4%	84.0%	81.3%	82.8%	59.9%	96.8%
15 KY229180 USP-BR-475.4	70.8%	98.9%	70.3%	70.3%	99.6%	99.6%	99.6%	99.6%	99.6%	99.6%	72.6%	98.9%	100.0%	100.0%	-	100.0%	100.0%	99.2%	66.2%	96.4%	82.8%	61.0%	73.3%	82.4%	82.4%	84.0%	81.3%	82.8%	59.9%	96.8%
16 KY229182 USP-BR-475.11	70.8%	98.9%	70.3%	70.3%	99.6%	99.6%	99.6%	99.6%	99.6%	99.6%	72.6%	98.9%	100.0%	100.0%	100.0%	-	100.0%	99.2%	66.2%	96.4%	82.8%	61.0%	73.3%	82.4%	82.4%	84.0%	81.3%	82.8%	59.9%	96.8%
17 KY229181 USP-BR-102.8D	71.1%	98.9%	70.5%	70.5%	99.8%	99.8%	99.8%	99.8%	99.8%	99.8%	72.7%	98.9%	99.4%	99.4%	99.4%	99.4%	-	99.2%	66.2%	96.4%	82.8%	61.0%	73.3%	82.4%	82.4%	84.0%	81.3%	82.8%	59.9%	96.8%
18 KY229183 USP-BR-G21.B	70.8%	98.6%	70.3%	70.3%	99.6%	99.6%	99.6%	99.6%	99.6%	99.6%	72.5%	98.6%	99.2%	99.2%	99.2%	99.2%	99.7%	-	65.5%	95.7%	82.0%	60.7%	72.9%	81.7%	81.7%	83.2%	80.5%	82.0%	59.5%	96.0%
19 AC_000014-DE-Serotype 1	65.2%	64.0%	65.4%	65.4%	64.2%	64.2%	64.2%	64.2%	64.2%	64.2%	66.5%	64.0%	64.2%	64.2%	64.2%	64.2%	64.1%	64.0%	-	66.6%	68.6%	68.7%	68.7%	66.2%	68.0%	67.0%	67.0%	68.2%	69.5%	66.2%
20 AF339915-Serotype 2	71.3%	95.9%	70.8%	70.8%	95.5%	95.5%	95.5%	95.5%	95.5%	95.5%	72.9%	95.9%	95.7%	95.7%	95.7%	95.7%	95.4%	95.1%	63.2%	-	83.9%	61.4%	72.9%	82.4%	82.4%	84.0%	81.3%	83.9%	61.4%	96.8%
21 AF508949-Serotype 3	70.8%	77.6%	70.3%	70.3%	77.8%	77.8%	77.8%	77.8%	77.8%	77.8%	70.9%	77.6%	78.0%	78.0%	78.0%	78.0%	77.7%	77.5%	65.1%	76.7%	-	61.8%	73.7%	82.8%	82.4%	82.8%	81.7%	92.9%	62.2%	83.9%
22 NC_015323-Serotype 4	61.4%	61.2%	61.2%	61.2%	61.5%	61.5%	61.5%	61.5%	61.5%	61.5%	63.3%	61.2%	61.4%	61.4%	61.4%	61.4%	61.5%	61.4%	68.2%	62.3%	60.3%	-	61.0%	59.3%	60.8%	62.7%	60.8%	61.0%	95.2%	61.0%
23 AF508952-Serotype 5	68.8%	67.8%	68.3%	68.3%	68.2%	68.2%	68.2%	68.2%	68.2%	68.2%	70.8%	67.8%	68.1%	68.1%	68.1%	68.1%	68.2%	68.2%	66.5%	68.2%	67.3%	62.9%	-	73.3%	73.7%	73.7%	72.9%	72.9%	61.3%	72.9%
24 AF508954-Serotype 6	83.2%	73.1%	83.0%	83.0%	73.4%	73.4%	73.4%	73.4%	73.4%	73.4%	85.3%	73.1%	73.5%	73.5%	73.5%	73.5%	73.4%	73.1%	66.5%	73.5%	72.4%	62.3%	71.3%	-	92.2%	89.1%	87.9%	82.4%	60.0%	81.7%
25 AF508955-Serotype 7	89.6%	71.1%	89.8%	89.8%	71.6%	71.6%	71.6%	71.6%	71.6%	71.6%	86.9%	71.1%	71.5%	71.5%	71.5%	71.5%	71.6%	71.3%	67.5%	71.7%	70.8%	63.2%	70.5%	87.0%	-	88.3%	92.2%	81.7%	60.0%	81.7%
26 KT862810-Serotype 8a	83.9%	72.5%	83.9%	83.9%	73.0%	73.0%	73.0%	73.0%	73.0%	73.0%	99.2%	72.5%	72.9%	72.9%	72.9%	72.9%	73.0%	72.7%	66.4%	73.4%	70.5%	63.4%	70.8%	84.7%	86.2%	-	88.3%	82.1%	63.1%	83.6%
27 KT862811-Serotype 8b	99.3%	70.3%	99.0%	99.0%	70.8%	70.8%	70.8%	70.8%	70.8%	70.8%	84.5%	70.3%	70.5%	70.5%	70.5%	70.5%	70.8%	70.5%	65.4%	70.9%	70.8%	61.4%	69.1%	83.6%	90.0%	84.0%	-	81.3%	60.8%	80.9%
28 AF083975.2-Serotype 9	71.1%	78.6%	70.5%	70.5%	78.6%	78.6%	78.6%	78.6%	78.6%	78.6%	70.3%	78.6%	78.8%	78.8%	78.8%	78.8%	78.8%	78.5%	65.2%	77.5%	95.0%	60.9%	67.0%	72.1%	71.3%	70.2%	71.1%	-	62.2%	83.5%
29 KT717889-Serotype 10	61.4%	61.1%	61.2%	61.2%	61.4%	61.4%	61.4%	61.4%	61.4%	61.4%	63.6%	61.1%	61.2%	61.2%	61.2%	61.2%	61.4%	61.2%	68.4%	62.3%	60.3%	96.6%	62.9%	62.7%	63.0%	63.8%	61.4%	61.0%	-	60.7%
30 AF339925-Serotype 11	70.9%	97.5%	70.4%	70.4%	97.6%	97.6%	97.6%	97.6%	97.6%	97.6%	72.7%	97.5%	97.5%	97.5%	97.5%	97.5%	97.5%	97.2%	64.1%	96.3%	78.4%	61.1%	67.8%	73.5%	71.3%	73.0%	70.5%	79.1%	61.1%	-
NUCLEOTIDE SEQUENCES																														

Source: (DE LA TORRE, D. 2018).

Legend: USP= University of São Paulo BR= Brazil.

2.4 DISCUSSION

Avian adenovirus has been reported in many countries and is a virus associated with diseases such as inclusion body hepatitis (IBH), hydropericardium syndrome (HS), and respiratory and enteric conditions (CHRISTENSEN; SAIFUDDIN, 1989; GOODWIN, 1993a; ALVARADO et al., 2007; OJKIC et al., 2008; MITTAL et al., 2014; LI et al., 2016). FAdV-I has also been described in South American countries like Ecuador, Chile and Peru (MAZAHARI et al., 1998; TORO et al., 1999; RODRIGUEZ et al., 2014), including Brazil (NUÑEZ et al., 2016). In this study, FAdV-I was detected in its target tissues where replication occurs (GOODWIN et al., 1993b; STEER et al., 2015), based on PCR amplification of the 897 bp fragment of the hexon gene (MEULEMANS et al., 2001). In addition, phylogenetic analysis was carried out based on the NT sequence and its resulting AA sequence. Sequencing of this fragment and its subsequent analysis allowed characterization of different serotypes among the samples. The results obtained in this study corroborate those found by other authors and highlight the importance of this method for the detection and characterization of FAdV-I serotypes (MEULEMANS et al., 2001; MITTAL et al., 2014; NICZYPORUK, 2016). Although infections caused by FAdV-I are commonly related to primary immunosuppressive infections such as Infectious Chicken Anemia (CAV) or Infection Bursa Disease (IBD) (GOMIS et al., 2006), there are also reports of infection by FAdV-I without immunological changes (CHRISTENSEN; SAIFUDDIN, 1989). In addition, the FAdV-4 serotype has been associated with hydropericardium syndrome (LIM et al., 2011). Besides, all the FAdV-I serotypes are associated with inclusion body hepatitis corpuscles (OJKIC et al., 2008), whereas the FAdV-1 serotype is associated with gizzard erosions (SEUBERLICH et al., 2010). This study showed the presence of group I adenoviruses and their serotypes with the associated enteric disorders described in Table 1. The molecular characterization classified the samples as serotypes 8a, 8b and 11, making this the first report of the presence of these serotypes as possible causes of enteric infections in Brazilian poultry. These samples belonged to birds with signs of enteric disease and feed malabsorption, so these clinical signs could be related to these serotypes. The samples were from eight different chicken farms, and in seven of them (87.5%), the presence of serotype 11 occurred in different organs such as the liver, intestine, bursa, kidneys, pancreas, and trachea. This result suggests that serotype 11 could be predominant in Brazil. The trachea is an important

viral replication organ as well as the pharynx, heart, and kidneys (NUÑEZ; PIANTINO, 2013). In this study, serotypes 11 and 8a were found in trachea, corroborating the results of Goodwin (1993b). The bursa is also an important organ where FAdV-I is found in natural infections (STEER et al., 2015), which is confirmed in this report, which shows that serotypes 11 and 8b were present in the bursa. In the samples obtained from farms 418 and 420, serotypes 8b and 11 were found, demonstrating the presence of mixed infections that have also been described by Meulemans et al. (2001). However, it is unknown whether mixed FAdV-I infections influence the severity of the disease or whether there is synergism between the serotypes (MITTAL et al. 2014).

Phylogenetic analysis based on the NT sequence of the variable region L1 of the hexon gene classified the NT sequences into 3 distinct groups and confirmed the presence of the serotype 11 of the D species and the serotypes 8a and 8b of the E species in Brazilian poultry flocks. These results emphasize the worldwide distribution of FAdV-I, since serotype 8a aligned with a reference strain from Japan, serotype 8b with a United Kingdom (UK) reference strain, and serotype 11 with a reference strain from Belgium. Other studies will be necessary to associate the pathogenicity of these serotypes with the clinical changes described. It is necessary to provide more epidemiological data to establish the prevalence of these viruses and justify the development of vaccine programs in breeders, broiler and laying hens to prevent vertical and horizontal virus infections and prevent the spread of this virus (GOMIS et al., 2006; GRGIC et al., 2006).

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3 MOLECULAR DIAGNOSTIC OF CHICKEN PARVOVIRUS (ChPV) AFFECTING COMMERCIAL FLOCKS IN ECUADOR

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ABSTRACT. - Enteric diseases affect poultry and cause important economic losses in many countries worldwide. Avian parvovirus has been linked to enteric conditions, such as malabsorption and runting-stunting syndrome (RSS), characterized by diarrhoea, and reduced weight gain and growth retardation. In 2013 and 2016, 79 samples were collected from different organs of chickens in Ecuador that exhibited signs of diarrhea and stunting syndrome, and analysed for the presence of chicken parvovirus (ChPV). The detection method of ChPV applied was Polymerase Chain Reaction (PCR), using primers designed from the conserved region of the viral genome that encodes the non-structural protein NS1. Out of the 79 samples, 50.6% (40/79) were positive for ChPV, and their nucleotide and amino acid sequences were analysed to determine their phylogenetic relationship with the sequences reported in the United States, Canada, China, South Korea, Croatia, Poland, Hungary, and Brazil. Strong similarity of nucleotide and amino acid sequences among all analyzed sequences and between the analysed and reference sequences was demonstrated, and the phylogenetic analysis clustered all the sequences within the same group, demonstrating a strong relation between the studied strains and the reference chicken parvovirus strains.

3.1 INTRODUCTION

The intestinal health of birds is related to animal welfare and the productive capacity of animals. Enteric problems cause economic losses around the world, especially in young chickens, due to the costs of therapeutic treatments, decreased productivity and even increased morbidity and mortality. Viral diseases are characterized by the presence of diarrhoea, decreased weight gain, and increased feed conversion (GOODWIN et al., 1993; OTTO et al., 2006; PANTIN-JACKWOOD et

al., 2008; KANG et al., 2012). Several viruses are associated with enteric problems in chickens, such as avian coronavirus (IBV), avian reovirus (AReo), avian astrovirus (CAstV), avian rotavirus-A (ARtV-A), fowl aviadenovirus (FAdV-I), and chicken parvovirus (ChPV) (GUY, 1998; ZSAK et al., 2008; NUÑEZ; FERREIRA, 2013), but the specific activity of each virus and their interactions, in gut health changes, are not widely known (PANTIN-JACKWOOD et al., 2008; DOMANSKA-BLICHARZ et al., 2012; METTIFOGO et al., 2014).

Avian parvovirus was first reported by Kisary et al. (1984) who found parvovirus-like virus particles that cause Derzsy's disease in geese, using electron microscopy with gut samples from chickens with runting-stunting syndrome (RSS). The family Parvoviridae contains two subfamilies: Parvovirinae that infect vertebrates and Densovirinae that infect invertebrates (NUÑEZ; FERREIRA, 2013).

The ChPV belongs to the genus Aveparvovirus, which also includes the turkey parvovirus (COTMORE et al., 2014). Viral particles of ChPV are small, 19-24 nm in diameter, non-enveloped, and have icosahedral symmetry. The linear genome is single-stranded DNA and is ~5 kilobases (Kb) long (KISARY et al., 1984; COTMORE; TATTERSALL, 1995; DOMANSKA-BLICHARZ et al., 2012). The genome contains 3 open reading frames (ORFs), including ORF 5', which is 2085 NT long, ORF 3', which is 2028 NT long, and a small ORF that is 306 NT long located between the 5' and 3' ORFs. The 5' ORF encodes a non-structural protein, NS1, whereas the 3' ORF appears to encode the capsid proteins VP1, VP2 and VP3, and the small ORF function is not defined (DAY; ZSAK, 2010).

ChPV is associated to enteric diseases that cause diarrhoea, growth retardation and lower than average weight gain, specially in 2- to 7-year-old chicks, and it is considered to be one of the aetiological agents for RSS (ZSAK et al., 2013), also called malabsorption syndrome (MAS), helicopter disease, infectious stunting syndrome and brittle bone disease (FINKLER et al., 2016). Viral replication and pathogenic effects mainly occur in cells with high proliferative rates (HUEFFER; PARRISH, 2003).

The aim of this study is to determine the presence of ChPV in organs obtained from broilers in Ecuador with signs of enteric disease, using Polymerase Chain Reaction (PCR) and NT sequencing procedures.

3.2 MATERIAL AND METHODS

3.2.1 Samples

During the years 2013 and 2016, 79 samples were received at the Laboratory of Avian Diseases from University of São Paulo, corresponding to imprints of different organs such as thymus, spleen, trachea, lung, air sac, gut, cecal tonsil, bursa, kidney and bone marrow from broilers raised in Ecuador and between 1 to 4 weeks of age. The samples were used for molecular analysis of enteric viruses that could be affecting commercial flocks, whose clinical history included enteric problems such as diarrhea, malabsorption, and delayed growth. These birds belonged to different commercial lots distributed in the northern region of Ecuador, and after the necropsy of the birds, several imprints were collected on FTA cards (GE Healthcare, Buckinghamshire, UK) for shipment to Brazil.

3.2.2 DNA isolation

The material impregnated on the FTA cards was cut and suspended in PBS 0.1M, pH 7.4, in a 1:1 proportion, then macerated into 2 ml microtubes using the Tissue Lyser LT Bead Mill (Qiagen, Hilden, Germany) instrument for 5 min. The material was finally centrifuged for 30 min at 12,000 x g and at 4°C. An aliquot of the supernatant was then collected for the extraction of DNA by the Phenol/Chloroform technique described by Chomczynski (1993). The extracted DNA was stored at -20°C.

3.2.3 Polymerase chain reaction for the detection of chicken parvovirus

The primers used in this reaction were those described by Zsak et al. (2009), PVF1 5'-TTCTAATAACGATATCACT-3' and PVR1 5'-TTTGCCTTGCGGTGAAGTCTGGCTCG-3' corresponding to the conserved region of the non-structural NS gene, which amplify a fragment of 561 bp. The PCR reaction conditions for ChPV amplification were performed as reported by Zsak et al. (2009), with some variations. PCR components were mixed in a DNA-free microtube that included 1X

reaction buffer, 200 µM of each dNTP, 0.5 µM of each primer, 1.25 U of Platinum™ Taq DNA polymerase (Invitrogen™), and 2 µl of extracted DNA. Thermocycling parameters included one cycle of DNA denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for one min, followed by a final extension at 72°C for 10 min. PCR products from all samples were run on 1.5% Agarose gel using SyBR® Safe DNA gel stain (Invitrogen™) and a 100 bp DNA Ladder (Invitrogen™) to determinate the size of bands.

3.2.4 DNA sequencing and nucleotide sequence analysis

The amplified product was purified using the GPX™ PCR DNA and Gel Band Purification kit (GE Healthcare) according to the manufacturer's instructions. Each purified product was sequenced in the forward and reverse direction using the BigDye® Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems by Life Technologies, Carlsbad, CA, USA). Sequencing reactions were done on an ABI 3730 DNA Analyzer (Applied Biosystems). The sequences obtained were assembled and edited with the CLC Main Workbench 7.7.3 software and aligned together with previous reported sequences obtained from the GenBank database belonging to Brazil, Canada, Croatia, China, Hungary, South Korea, Poland, and the United States, using the CLUSTAL W method available in the ClustalX 2.1 software. Accession numbers of the reference sequences are detailed in the phylogenetic tree (Figure 2). The phylogenetic tree was inferred using the neighbour-joining method, with 1,000 bootstrap replicates integrated in the MEGA 7.0.18 software. The NT and AA sequence similarity matrix was generated in the BioEdit Sequence Alignment Editor v. 7.2.5.

3.3 RESULTS

3.3.1 PCR

PCR products were run on 1.5% agarose gel, and the location of the DNA band of each positive sample confirmed the amplification of the 561 bp segment from 40/79 samples, of which 17/42 corresponded to the samples received in 2013 and 23/37 to the samples received in 2016. Details of the positive samples are described in Table 3.

Table 3 – Sample identification for Ecuadorian Chicken Parvovirus study. Sample identification and origin, type of bird, clinical signs, year of collection and accession number from the NCBI GenBank database

Sample ID	Type of sample	Bird	Clinical Signs		Year	Accession number
			Diarrhea	Stunting		
EC 513-14	spleen	Broiler	YES	YES	2013	KY649239
EC 513-16	lung	Broiler	YES	YES	2013	KY649240
EC 513-17	trachea	Broiler	YES	YES	2013	KY649241
EC 513-18	kidney	Broiler	YES	YES	2013	KY649242
EC 513-19	timus	Broiler	YES	YES	2013	KY649243
EC 513-22	air sac	Broiler	YES	YES	2013	KY649244
EC 513-23	trachea	Broiler	YES	YES	2013	KY649245
EC 513-24	timus	Broiler	YES	YES	2013	KY649246
EC 513-25	bone marrow	Broiler	YES	YES	2013	KY649247
EC 513-26	spleen	Broiler	YES	YES	2013	KY649248
EC 513-29	trachea	Broiler	YES	YES	2013	KY649249
EC 513-30	trachea	Broiler	YES	YES	2013	KY649250
EC 513-32	trachea	Broiler	YES	YES	2013	KY649251
EC 513-33	cecal tonsils	Broiler	YES	YES	2013	KY649252
EC 513-34	gut	Broiler	YES	YES	2013	KY649253
EC 513-37	cecal tonsils	Broiler	YES	YES	2013	KY649254
EC 513-38	cecal tonsils	Broiler	YES	YES	2013	KY649255
EC 722-3	trachea	Broiler	YES	YES	2016	KY649256
EC 722-15	trachea	Broiler	YES	YES	2016	KY649257
EC 722-17	kidney	Broiler	YES	YES	2016	KY64925S
EC 722-18	bursa	Broiler	YES	YES	2016	KY649259
EC 722-19	bursa	Broiler	YES	YES	2016	KY649260
EC 722-20	bursa	Broiler	YES	YES	2016	KY649261
EC 722-21	bursa	Broiler	YES	YES	2016	KY649262
EC 722-22	bursa	Broiler	YES	YES	2016	KY649263
EC 722-23	bursa	Broiler	YES	YES	2016	KY649264
EC 722-24	bursa	Broiler	YES	YES	2016	KY649265
EC 722-25	bursa	Broiler	YES	YES	2016	KY649266
EC 722-26	bursa	Broiler	YES	YES	2016	KY649267
EC 722-27	bursa	Broiler	YES	YES	2016	KY649268
EC 722-28	bursa	Broiler	YES	YES	2016	KY649269
EC 722-29	bursa	Broiler	YES	YES	2016	KY649270
EC 722-30	bursa	Broiler	YES	YES	2016	KY649271
EC 722-31	bursa	Broiler	YES	YES	2016	KY649272
EC 722-32	bursa	Broiler	YES	YES	2016	KY649273
EC 722-33	bursa	Broiler	YES	YES	2016	KY649274
EC 722-34	bursa	Broiler	YES	YES	2016	KY649275
EC 722-35	bursa	Broiler	YES	YES	2016	KY649276
EC 722-36	bursa	Broiler	YES	YES	2016	KY649277
EC 722-37	bursa	Broiler	YES	YES	2016	KY649278

Source: (DE LA TORRE, D. 2018).

Legend: EC=Ecuador.

Figure 2 – Phylogenetic tree for Ecuadorian Chicken Parvovirus study

Source: (DE LA TORRE, D. 2018).

Legend: Phylogenetic analysis of the NT sequences of ChPV from Ecuador. The sequence NC_001701.1 in red (goose parvovirus) was placed as a control outside the group. Numbers along the back refer to bootstrap values for 1,000 replicates. The scale bar represents the number of substitutions per site. The sequences obtained in the present work are shown in blue. EC=Ecuador, BR=Brazil, CA=Canada, HR=Croatia, HU=Hungary, PL=Poland, CH=China, US=United States, KR=South Korea.

3.3.2 DNA sequencing and phylogenetic analysis

It was possible to sequence all the positive results, obtaining a total of 40 sequences of different organs: 20 bursae, seven in tracheas, three in caecal tonsils, two in spleens and kidneys, and thymuses, one in each in following organs, as air sac, bone marrow, intestine, and lung. The details of all positive samples including GenBank accession numbers are given in Table 3. The 40 sequenced fragments were

analysed with a size of 398 bp, showing a high percentage of similarity between NT (89.6% - 100%) and AA (90.1% - 100%). Furthermore, there was a high percentage of similarity between sequences in Brazil (91.9% - 99.2% NT and 91.6% - 100% AA), Canada (87.9% - 94.2% NT and 87.8% - 96.2% AA), the United States (90.4% - 97.4% NT and 91.6% - 100% AA), Croatia (91.7% - 99.4% NT and 91.6% - 100% AA), Poland (92.2% - 98.2% NT and 91.6% - 100% AA), China (90.7% - 98.2% NT and 92.4% - 99.2% AA), South Korea (88.4% - 96.2% NT and 87.8% - 96.2% AA) and Hungary (91.9% - 98.7% NT and 92.4% - 99.2% AA). The similarity matrix is detailed in Table 4. In the phylogenetic analysis, all the sequences were clustered in the same group, demonstrating that the sequences obtained in this study are related to the reference sequences originating in North America, Brazil, Europe and Asia, as shown in Figure 2.

Table 4 – Similarity matrix of similarity for Ecuadorian Chicken Parvovirus study. Similarity matrix for NT and AA sequences from ChPV of this study and GenBank sequences

Ecuadorian isolates compared with GenBank sequences of ChPV		Ecuadorian isolates																				GenBank sequences						
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1	EC_513-14	-	1,000	1,000	0,969	1,000	1,000	1,000	0,992	1,000	1,000	1,000	0,984	1,000	1,000	1,000	1,000	0,992	0,992	0,931	0,931	0,992	0,946	1,000	0,992	0,962	0,984	0,992
2	EC_513-16	0,987	-	1,000	0,969	1,000	1,000	1,000	0,992	1,000	1,000	1,000	0,984	1,000	1,000	1,000	1,000	0,992	0,992	0,931	0,931	0,992	0,946	1,000	0,992	0,962	0,984	0,992
3	EC_513-17	1,000	0,987	-	0,969	1,000	1,000	1,000	0,992	1,000	1,000	1,000	0,984	1,000	1,000	1,000	1,000	0,992	0,992	0,931	0,931	0,992	0,946	1,000	0,992	0,962	0,984	0,992
4	EC_513-18	0,954	0,957	0,954	-	0,969	0,969	0,969	0,962	0,969	0,969	0,969	0,984	0,969	0,969	0,969	0,969	0,977	0,962	0,924	0,924	0,962	0,946	0,969	0,977	0,931	0,984	0,962
5	EC_513-19	0,979	0,992	0,979	0,959	-	1,000	1,000	0,992	1,000	1,000	1,000	0,984	1,000	1,000	1,000	1,000	0,992	0,992	0,931	0,931	0,992	0,946	1,000	0,992	0,962	0,984	0,992
6	EC_513-22	1,000	0,987	1,000	0,954	0,979	-	1,000	0,992	1,000	1,000	1,000	0,984	1,000	1,000	1,000	1,000	0,992	0,992	0,931	0,931	0,992	0,946	1,000	0,992	0,962	0,984	0,992
7	EC_513-23	0,997	0,984	0,997	0,957	0,977	0,997	-	0,992	1,000	1,000	1,000	0,984	1,000	1,000	1,000	1,000	0,992	0,992	0,931	0,931	0,992	0,946	1,000	0,992	0,962	0,984	0,992
8	EC_513-24	0,989	0,977	0,989	0,954	0,969	0,989	0,992	-	0,992	0,992	0,992	0,977	0,992	0,992	0,992	0,992	0,984	1,000	0,931	0,931	1,000	0,939	0,992	0,984	0,954	0,977	0,984
9	EC_513-25	0,997	0,984	0,997	0,957	0,977	0,997	1,000	0,992	-	1,000	1,000	0,984	1,000	1,000	1,000	1,000	0,992	0,992	0,931	0,931	0,992	0,946	1,000	0,992	0,962	0,984	0,992
10	EC_513-26	0,997	0,984	0,997	0,957	0,977	0,997	1,000	0,992	1,000	-	1,000	0,984	1,000	1,000	1,000	1,000	0,992	0,992	0,931	0,931	0,992	0,946	1,000	0,992	0,962	0,984	0,992
11	EC_513-29	0,974	0,977	0,974	0,969	0,979	0,974	0,977	0,974	0,977	0,977	-	0,984	1,000	1,000	1,000	1,000	0,992	0,992	0,931	0,931	0,992	0,946	1,000	0,992	0,962	0,984	0,992
12	EC_513-30	0,964	0,977	0,964	0,979	0,979	0,964	0,967	0,964	0,967	0,967	0,974	-	0,984	0,984	0,984	0,984	0,992	0,977	0,931	0,931	0,977	0,962	0,984	0,992	0,946	1,000	0,977
13	EC_513-32	0,962	0,974	0,962	0,959	0,982	0,962	0,964	0,962	0,964	0,964	0,979	0,977	-	1,000	1,000	1,000	0,992	0,992	0,931	0,931	0,992	0,946	1,000	0,992	0,962	0,984	0,992
14	EC_513-33	0,992	0,979	0,992	0,952	0,972	0,992	0,989	0,987	0,989	0,989	0,982	0,962	0,964	-	1,000	1,000	0,992	0,992	0,931	0,931	0,992	0,946	1,000	0,992	0,962	0,984	0,992
15	EC_513-34	0,992	0,979	0,992	0,952	0,972	0,992	0,989	0,987	0,989	0,989	0,982	0,962	0,964	1,000	-	1,000	0,992	0,992	0,931	0,931	0,992	0,946	1,000	0,992	0,962	0,984	0,992
16	EC_513-37	0,977	0,989	0,977	0,957	0,992	0,977	0,974	0,967	0,974	0,974	0,987	0,977	0,979	0,979	0,979	-	0,992	0,992	0,931	0,931	0,992	0,946	1,000	0,992	0,962	0,984	0,992
17	EC_513-38	0,974	0,987	0,974	0,969	0,989	0,974	0,977	0,969	0,977	0,977	0,979	0,989	0,977	0,967	0,967	0,987	-	0,984	0,924	0,924	0,984	0,954	0,992	0,984	0,954	0,992	0,984
18	EC_722-3	0,989	0,977	0,989	0,954	0,969	0,989	0,992	0,994	0,992	0,992	0,979	0,964	0,967	0,992	0,992	0,972	0,969	-	0,931	0,931	1,000	0,939	0,992	0,984	0,954	0,977	0,984
19	EC_722-15	0,904	0,917	0,904	0,896	0,917	0,904	0,902	0,904	0,902	0,902	0,912	0,914	0,924	0,907	0,907	0,917	0,909	0,909	-	1,000	0,931	0,893	0,931	0,939	0,893	0,931	0,931
20	EC_722-17	0,904	0,917	0,904	0,896	0,917	0,904	0,902	0,904	0,902	0,902	0,912	0,914	0,924	0,907	0,907	0,917	0,909	0,909	1,000	-	0,931	0,893	0,931	0,939	0,893	0,931	0,931
21	BR_JX861894	0,954	0,967	0,954	0,952	0,974	0,954	0,957	0,959	0,957	0,957	0,972	0,969	0,992	0,957	0,957	0,972	0,969	0,964	0,922	0,922	-	0,939	0,992	0,984	0,954	0,977	0,984
22	CA_JF267316	0,912	0,924	0,912	0,932	0,929	0,912	0,914	0,912	0,914	0,914	0,927	0,942	0,937	0,914	0,914	0,929	0,932	0,917	0,879	0,879	0,929	-	0,946	0,954	0,984	0,962	0,939
23	HR_JF428870	0,962	0,974	0,962	0,959	0,982	0,962	0,964	0,962	0,964	0,964	0,979	0,977	0,994	0,964	0,964	0,979	0,977	0,967	0,919	0,919	0,992	0,932	-	0,992	0,962	0,984	0,992
24	HU_GQ281296	0,954	0,967	0,954	0,962	0,974	0,954	0,957	0,954	0,957	0,957	0,972	0,979	0,987	0,957	0,957	0,972	0,969	0,959	0,919	0,919	0,984	0,939	0,987	-	0,954	0,992	0,984
25	KR_KC593420	0,924	0,937	0,924	0,922	0,944	0,924	0,927	0,924	0,927	0,927	0,942	0,939	0,962	0,927	0,927	0,942	0,939	0,929	0,886	0,886	0,959	0,968	0,962	0,954	-	0,946	0,954
26	PL_JQ178301	0,952	0,964	0,952	0,964	0,969	0,952	0,954	0,952	0,954	0,954	0,969	0,982	0,982	0,954	0,954	0,969	0,972	0,957	0,922	0,922	0,974	0,949	0,977	0,984	0,944	-	0,977
27	PL_JQ178303	0,952	0,964	0,952	0,944	0,972	0,952	0,954	0,947	0,954	0,954	0,964	0,962	0,979	0,949	0,949	0,969	0,967	0,952	0,922	0,922	0,972	0,927	0,974	0,972	0,942	0,972	-
28	US_GQ260159	0,944	0,957	0,944	0,967	0,962	0,944	0,947	0,944	0,947	0,947	0,967	0,974	0,969	0,952	0,952	0,967	0,964	0,949	0,904	0,904	0,962	0,942	0,969	0,977	0,932	0,977	0,954
29	CN_KU523900	0,957	0,969	0,957	0,962	0,977	0,957	0,959	0,957	0,959	0,959	0,967	0,982	0,974	0,954	0,954	0,969	0,977	0,957	0,907	0,907	0,972	0,927	0,979	0,977	0,942	0,967	0,959

Ecuadorian isolates compared with GenBank sequences of ChPV		Ecuadorian isolates																				GenBank sequences							
		30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	
30	EC_722-18	-	1,000	0,984	0,984	0,984	0,977	0,977	0,969	0,969	0,962	1,000	1,000	0,984	1,000	1,000	0,984	0,984	0,946	0,916	0,916	0,992	0,946	1,000	0,992	0,962	0,984	0,992	
31	EC_722-19	1,000	-	0,984	0,984	0,984	0,977	0,977	0,969	0,969	0,962	1,000	1,000	0,984	1,000	1,000	0,984	0,984	0,946	0,916	0,916	0,992	0,946	1,000	0,992	0,962	0,984	0,992	
32	EC_722-20	0,987	0,987	-	1,000	0,984	0,962	0,992	0,984	0,984	0,977	0,984	0,984	0,984	0,984	1,000	1,000	0,946	0,916	0,916	0,977	0,962	0,984	0,992	0,946	1,000	0,977		
33	EC_722-21	0,984	0,984	0,997	-	0,984	0,962	0,992	0,984	0,984	0,977	0,984	0,984	0,984	0,984	1,000	1,000	0,946	0,916	0,916	0,977	0,962	0,984	0,992	0,946	1,000	0,977		
34	EC_722-22	0,979	0,979	0,972	0,969	-	0,977	0,977	0,977	0,977	0,962	0,984	0,984	1,000	0,984	0,984	0,984	0,984	0,946	0,916	0,916	0,977	0,946	0,984	0,977	0,946	0,984	0,977	
35	EC_722-23	0,972	0,972	0,959	0,957	0,969	-	0,969	0,954	0,954	0,954	0,977	0,977	0,977	0,977	0,977	0,962	0,962	0,962	0,931	0,931	0,969	0,924	0,977	0,969	0,939	0,962	0,984	
36	EC_722-24	0,974	0,974	0,982	0,979	0,969	0,957	-	0,977	0,977	0,984	0,977	0,977	0,977	0,977	0,977	0,992	0,992	0,946	0,916	0,916	0,969	0,954	0,977	0,984	0,939	0,992	0,984	
37	EC_722-25	0,964	0,964	0,972	0,969	0,959	0,944	0,964	-	1,000	0,962	0,969	0,969	0,977	0,969	0,969	0,984	0,984	0,939	0,909	0,909	0,962	0,946	0,969	0,977	0,931	0,984	0,962	
38	EC_722-26	0,964	0,964	0,972	0,969	0,959	0,944	0,964	1,000	-	0,962	0,969	0,969	0,977	0,969	0,969	0,984	0,984	0,939	0,909	0,909	0,962	0,946	0,969	0,977	0,931	0,984	0,962	
39	EC_722-27	0,952	0,952	0,959	0,957	0,952	0,949	0,977	0,957	0,957	-	0,962	0,962	0,962	0,962	0,962	0,977	0,977	0,931	0,901	0,901	0,954	0,939	0,962	0,969	0,924	0,977	0,969	
40	EC_722-28	1,000	1,000	0,987	0,984	0,979	0,972	0,974	0,964	0,964	0,952	-	1,000	0,984	1,000	1,000	0,984	0,984	0,946	0,916	0,916	0,992	0,946	1,000	0,992	0,962	0,984	0,992	
41	EC_722-29	0,982	0,982	0,969	0,967	0,962	0,959	0,957	0,957	0,957	0,939	0,982	-	0,984	1,000	1,000	0,984	0,984	0,946	0,916	0,916	0,992	0,946	1,000	0,992	0,962	0,984	0,992	
42	EC_722-30	0,987	0,987	0,984	0,982	0,987	0,969	0,977	0,969	0,969	0,957	0,987	0,969	-	0,984	0,984	0,984	0,984	0,946	0,916	0,916	0,977	0,946	0,984	0,977	0,946	0,984	0,977	
43	EC_722-31	1,000	1,000	0,987	0,984	0,979	0,972	0,974	0,964	0,964	0,952	1,000	0,982	0,987	-	1,000	0,984	0,984	0,946	0,916	0,916	0,992	0,946	1,000	0,992	0,962	0,984	0,992	
44	EC_722-32	0,989	0,989	0,977	0,974	0,969	0,967	0,964	0,959	0,959	0,947	0,989	0,992	0,977	0,989	-	0,984	0,984	0,946	0,916	0,916	0,992	0,946	1,000	0,992	0,962	0,984	0,992	
45	EC_722-33	0,984	0,984	0,992	0,994	0,969	0,957	0,979	0,964	0,964	0,957	0,984	0,967	0,977	0,984	0,974	-	1,000	0,946	0,916	0,916	0,977	0,962	0,984	0,992	0,946	1,000	0,977	
46	EC_722-34	0,987	0,987	0,994	0,992	0,977	0,959	0,987	0,977	0,977	0,964	0,987	0,969	0,989	0,987	0,977	0,987	-	0,946	0,916	0,916	0,977	0,962	0,984	0,992	0,946	1,000	0,977	
47	EC_722-35	0,929	0,929	0,924	0,922	0,929	0,957	0,922	0,912	0,912	0,914	0,929	0,917	0,929	0,929	0,924	0,919	0,924	-	0,962	0,962	0,946	0,909	0,946	0,954	0,909	0,946	0,946	
48	EC_722-36	0,919	0,919	0,914	0,912	0,917	0,947	0,912	0,899	0,899	0,904	0,919	0,907	0,919	0,919	0,914	0,909	0,914	0,979	-	1,000	0,916	0,878	0,916	0,924	0,878	0,916	0,916	
49	EC_722-37	0,919	0,919	0,914	0,912	0,917	0,947	0,912	0,899	0,899	0,904	0,919	0,907	0,919	0,919	0,914	0,909	0,914	0,979	1,000	-	0,916	0,878	0,916	0,924	0,878	0,916	0,916	
50	BR_JX861894	0,972	0,972	0,959	0,957	0,977	0,967	0,957	0,954	0,954	0,947	0,972	0,954	0,972	0,972	0,962	0,957	0,964	0,927	0,919	0,919	-	0,939	0,992	0,984	0,954	0,977	0,984	
51	CA_JF267316	0,929	0,929	0,932	0,929	0,934	0,927	0,929	0,929	0,929	0,927	0,929	0,912	0,939	0,929	0,919	0,924	0,937	0,891	0,881	0,881	0,929	-	0,946	0,954	0,984	0,962	0,939	
52	HR_JF428870	0,979	0,979	0,967	0,964	0,979	0,969	0,959	0,962	0,962	0,949	0,979	0,962	0,979	0,979	0,969	0,964	0,972	0,929	0,917	0,917	0,992	0,932	-	0,992	0,962	0,984	0,992	
53	HU_GQ281296	0,972	0,972	0,969	0,972	0,977	0,967	0,967	0,964	0,964	0,957	0,972	0,954	0,977	0,972	0,962	0,967	0,974	0,934	0,922	0,922	0,984	0,939	0,987	-	0,954	0,992	0,984	
54	KR_KC593420	0,942	0,942	0,929	0,927	0,947	0,937	0,927	0,924	0,924	0,917	0,942	0,924	0,942	0,942	0,932	0,927	0,934	0,896	0,884	0,884	0,959	0,968	0,962	0,954	-	0,946	0,954	
55	PL_JQ178301	0,969	0,969	0,972	0,969	0,979	0,967	0,969	0,967	0,967	0,962	0,969	0,952	0,979	0,969	0,959	0,964	0,977	0,934	0,922	0,922	0,974	0,949	0,977	0,984	0,944	-	0,977	
56	PL_JQ178303	0,969	0,969	0,957	0,954	0,974	0,974	0,964	0,947	0,947	0,952	0,969	0,952	0,969	0,969	0,959	0,954	0,962	0,934	0,922	0,922	0,972	0,927	0,974	0,972	0,942	0,972	-	
57	US_GQ260159	0,962	0,962	0,964	0,967	0,967	0,949	0,967	0,964	0,964	0,964	0,962	0,944	0,972	0,962	0,952	0,967	0,969	0,917	0,904	0,904	0,962	0,942	0,969	0,977	0,932	0,977	0,954	
58	CN_KU523900	0,974	0,974	0,972	0,969	0,964	0,959	0,969	0,964	0,964	0,959	0,974	0,957	0,969	0,974	0,964	0,974	0,977	0,922	0,912	0,912	0,972	0,927	0,979	0,977	0,942	0,967	0,959	

Source: (DE LA TORRE, D. 2018)

Legend: To the left, NT sequences, and to the top, AA sequences obtained in the study, compared with the reference sequences obtained from GenBank. EC=Ecuador, BR=Brazil, CA=Canada, HR=Croatia, HU=Hungary, PL=Poland, CH=China, US=United States, KR=South Korea.

3.4 DISCUSSION

The primary etiology of RSS or MAS in chickens is still unknown, although several viruses have been identified in animals with RSS, with ChPV being found in many of these disorders (GOODWIN et al., 1993; PANTIN-JACKWOOD et al., 2008; DOMANSKA-BLICHARZ et al., 2012, DEVANEY et al., 2016). ChPV has a worldwide distribution, and it has been associated with enteric diseases in many other countries (KISARY et al., 1984; DECAESSTECKER et al., 1986; GOODWIN et al., 1990; ZSAK et al., 2008, 2009; BIDIN et al., 2011; DOMANSKA-BLICHARZ et al., 2012; TARASIUK et al., 2012; NUÑEZ et al., 2016). Experimentally, ChPV produces intestinal alterations such as diarrhea, decrease in the weight gain and retardation of growth (ZSAK et al., 2013). In the present study, we looked for the presence of ChPV in different imprints of organs fixed in FTA cards collected from birds with enteric problems such as diarrhea and stunting. The results showed the presence of ChPV in 50.6% of the collected samples, demonstrating that the virus is not only related to enteric organs but also to organs of other systems such as the respiratory organs (trachea, lungs, and air sacs), immune organs (thymus, bursa, bone marrow and spleen), and urinary (kidney) systems, as was also demonstrated in the experimental studies of Zsak et al. (2013) and Domanska-Blicharz et al. (2012). Parvovirus infections found in this study corresponded to young chickens, confirming previously published data on the occurrence of the virus in young animals (PALADE et al., 2011; DOMANSKA-BLICHARZ et al., 2012), and this may indicate that vertical infections are occurring in poultry farms in Ecuador.

In this study, we confirmed that the PCR protocol used for the amplification of a genome segment encoding the non-structural protein (NS1) in the 5' ORF region (ZSAK et al., 2009) allowed for the identification of ChPV by the amplification of a DNA fragment of 561 bp. Furthermore, we found a high percentage of similarity between the obtained NT and AA sequences, with others described and submitted to GenBank from North America, Brazil, Europe and Asia. All of the samples used in this study came from broilers affected by enteric disease, so it was not possible to determine the presence of ChPV in animals with no signs of enteric disease to corroborate the prevalence found by Zsak et al. (2008) of natural infections of ChPV in healthy American broiler flocks.

In conclusion, we confirmed the circulation of ChPV in poultry farms located in the northern region of Ecuador, providing the first molecular report of the virus in this country, which is possibly related to the enteric diseases described above. However, the exact role of the virus in enteropathies is not fully understood, and thus, more pathological and epidemiological studies should be done to determine the real pathogenicity and prevalence of these pathogens in the country, to develop vaccines in the future to prevent the vertical and horizontal transmission of ChPV.

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4 DETECTION AND MOLECULAR CHARACTERIZATION OF TREMOVIRUS A OBTAINED IN CASES OF AVIAN ENCEPHALOMYELITIS (AE) OUTBREAKS IN BRAZIL

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ABSTRACT. - This study determined the presence of Tremovirus A as the possible agent related to Avian Encephalomyelitis in broiler chicks from the states of São Paulo (SP) Paraná (PR), Goiás (GO), Santa Catarina (SC) and Rio Grande do Sul (RS), between the years 2006 and 2015. Samples of the nervous, digestive, respiratory, immune, and renal systems, plus muscular organs from broiler chicks with neurological problems such as ataxia and muscle tremors, and four (4) commercial vaccines as positive control, were tested by reverse-transcriptase (RT-PCR) amplification and DNA sequencing. A highly conserved region (P1) of the viral genome, was used to amplify a segment which encodes a structural protein VP4. Out of 112 samples, 46 were positive (42%) for Tremovirus A, that was identified in the nervous, digestive, respiratory, renal and immune systems. The phylogenetic analysis clustered together the nucleotide sequences of the 46 samples, the four commercial vaccine strains and the reference sequence of Calnek strain obtained from the GenBank. According to these results, we conclude that the presence of Tremovirus A in these Brazilian chicken flocks distributed in all states was due to flaws in the biosecurity measurements.

4.1 INTRODUCTION

Tremovirus A, also known as Avian Encephalomyelitis Virus (AEV) until 2014 (KNOWLES et al., 2012), was described for the first time in the United States in 1932 (JONES, 1932) and it has been associated to the Avian Encephalomyelitis (AE) disease (TANNOCK; SHAFREN, 1994). This disease mainly affects young birds like

broilers, turkeys, pheasants and quails between 1 or 2 weeks of age (SUAREZ, 2013). However adult birds do not show clinical signs of disease (TANNOCK; SHAFREN, 1994), it may decrease egg production and hatchability (TAYLOR et al., 1955), affecting economically the poultry industry around the world (CALNEK, 1998; BAKHSHESH et al., 2008). Young birds infected with Tremovirus A have shown signs of ataxia, weakness legs, reduced mobility and tremors mainly in head and neck (OLITSKY, 1939). Although the Tremovirus A has a protein similarity with the genus Hepatovirus (MARVIL et al., 1999), it is classified within the genus Tremovirus, of the family Picornaviridae (KNOWLES et al., 2012), and until now this is the only specie in this genus. The viral particle has a diameter of 24-32 nm, non-enveloped, icosahedral and with an external structure composed by 32-42 capsomeres (GOSTING et al., 1980). The virus genome contains 7032 bp encoding a polyprotein P1, P2 and P3. The P1 region codes 4 structural proteins: VP4, VP2 (VP0), VP3 e VP1. The P2 and P3 regions encode the non-structural proteins 2A, 2B e 2C e 3A, 3B, 3C e 3D (MARVIL et al., 1999). There are two types of strains of Tremovirus A, a natural enterotropic strain and a highly neurotropic embryo-adapted strain (SUAREZ, 2013). The enterotropic strain is transmitted horizontally by the oral route, and it is disseminated by feces (BOROS et al., 2014). This strain causes neurological problems in susceptible chicks, and it is practically asymptomatic in birds older than 3-4 weeks of age. However, this virus can cause drop in egg production in breeders and laying hens, in addition to vertical transmission of the virus through the eggs (CALNEK, 1998). The embryo-adapted strain is produced by repeated passages by intracerebral inoculation in chicks or inoculation in embryonated eggs (TANNOCK; SHAFREN, 1994). These strains cause severe neurological changes in birds of any age after intracerebral inoculation and even by parenteral inoculation (CALNEK, 1998), but after oral ingestion, the ability to be transmitted by feces is lost due the incapacity of multiply in digestive cells of birds (IKEDA et al., 1976). The transmission of enteric strains occurs horizontally through the feces and vertically through infected embryonated eggs, both under natural conditions (YU et al., 2015). The time of virus excretion depends on the age of the infected birds, so, birds with less than two weeks of age usually excrete the virus for two weeks, and birds older than 3 weeks of age, excrete the virus for about 5 days (WESTBURY; SINKOVIC, 1978). Vertical transmission occurs when breeders do not show immunological activation against the virus and are infected during the laying period, transmitting the virus to the progeny (FREITAS; BACK, 2015). The aims of this

work were to describe the several cases of neurological disturbs, associated with Tremovirus A, in chicks in the first week of age and establish a molecular characterizes in comparison with vaccine strains used in Brazil.

4.2 MATERIALS AND METHODS

4.2.1 Field samples

During the period of 2006 to 2015, the Laboratory of Avian Diseases – School of Veterinary Medicine of the University of São Paulo, received 112 samples comprised of different organs such as nervous, digestive, respiratory, renal and immune systems (cecal tonsils, bursa and thymus) of broilers from 21 poultry farms distributed in five Brazilian states, with history of neurological problems and suspected of Avian Encephalomyelitis, which are in the states of SP (n=14), PR (23), RS (n=45), GO (n=11), SC (n=19). These samples belonged to broilers between six and 49 days old, as described following: whole brain (n=24), intestine (n=20), gizzard (n=12), proventriculus (n=7), sciatic nerve (n=7), liver (n=7), ceca (n=6), lung (n=5), kidney (n=5), bursa (n=4), thymus (n=3), cecal tonsil (n=2), cloacal swab (n=2), trachea (n=2), breast muscle (n=2), pancreas (n=1), spleen (n=1), heart (n=1) and bone marrow (n=1). Each sample was received, macerated and conserved at -20°C. Approximately 750 µg of the macerated samples were used for RNA extraction. A sample was suspended in PBS 0.1M, pH 7.4, in a 1:1 proportion. The suspensions were vortexed and subjected to three freeze and thaw cycles to release the genetic material from cells. The suspensions were finally centrifugated at 12,000 x g for 30 min at 4°C, and then an aliquot was collected for RNA extraction. Commercial vaccines from Zoetis, Laboratory Bio-Vet, CEVA and Merial were used as positive controls and for comparison of phylogenetic analysis.

4.2.2 Reverse-transcriptase PCR and DNA sequencing

Isolation of RNA was performed by the Phenol-Chloroform method as described by Chomczynski (1987), and the final product was stored at -20°C. RNA concentrations were measured on the Nano Drop 2000 Spectrophotometer (ThermoFisher Scientific Inc., Waltham, MA, USA). The final RNA concentration for the Reverse Transcriptase

(RT) was approximately 1.500 ng/μl. The reaction mix for the RT was developed according to the Moloney Murine Leukemia Virus Reverse Transcriptase (ThermoFisher Scientific Inc., Waltham, MA, USA) kit instructions, using Random Primers synthesized by ThermoFisher Scientific Inc., Waltham, MA, USA. The primers used for the PCR were those described by Xie et al. (2005), MK AE 1 (CTTATGCTGGCCCTGATCGT) e MK AE 2 (TCCCAAATCCACAAACCTAGCC) which were designed to amplify a 619 bp specific segment of the VP2 gene in the P1 region of the viral genome published by Marvil et al. (1999). The PCR reaction mix contained 1X PCR buffer, 200 μM of each dNTP, 0.5 μM of primers MK AE1 and MK AE2, 0.75 μl of 50 mM MgCl, 1 U Platinum™ Taq DNA Polymerase (Invitrogen™), 2,5 μl of cDNA, and ultrapure water to reach the volume of 25 μl. The amplification reaction occurred under the following conditions: a thermal cycle of 95°C for five min, followed by 34 cycles of denaturation at 94°C for one min, annealing at 50°C for 30 s, extension at 68°C for one min and a final extension at 72°C for 10 min. The final reaction was stored at -20°C. The PCR product was purified using the GPX™ PCR DNA and Gel Band Purification kit (GE Healthcare), according to the manufacturer's instruction. Each purified sample was sequenced by the Sanger method using the two primers MK AE1 and MK AE2 for the forward and reserve reactions, using the BigDye® Terminator Cycle Sequencing Kit v3.1 (ThermoFisher Scientific Inc., Waltham, MA, USA). The reactions were analyzed by the ABI 3730 DNA Analyzer (Applied Biosystems).

4.2.3 Sequence analysis

The NT sequences were assembled and edited by using the CLC Main Work Bench 7.7.1, software and aligned with reference sequences: enterotropic strain Calnek (NC_003990), embryo-adapted strain Van Roekel (AY517471), Chinese strain L2Z (AY275539) and strain HM-175 from Hepatovirus genus (NC_001489). The alignment was performed using the CLUSTAL W method, available in the Clustal X 2.0 software (EMBL-EBI, Wellcome Genome Campus, Hinxton, Cambridgeshire, UK). The phylogenetic tree was inferred using Neighbor-Joining statistical method with integrated in the MEGA 7.0.18 software (TAMURA et al., 2011). The similarity matrix for NT and AA, were generated in the BioEdit Sequence Alignment Editor v. 7.2.5 software (BioEdit Sequence Alignment Editor v. 7.2.5 software).

4.3 RESULTS

4.3.1 Reverse-transcriptase - PCR

From 112 samples tested with RT-PCR, the 619 bp specific segment was amplified in 46 samples (42%) (Table 5). The positive results for each organ were: whole brain 10/24 (41.66%), intestine 9/20 (45%), gizzard 5/12 (41.7%), proventriculus 5/7 (71.4%), sciatic nerve 5/7 (71,4%), liver 4/7 (57.1%), ceca 3/6 (50%), lung 2/5 (40%), kidney 2/5 (40%), Bursa 2/4 (50%), thymus 1/3 (33.3%), cecal tonsil 1/2 (50%) in the Table 5. Tremovirus A was not detected in samples of cloaca swabs, pool of enteric and nerve tissues, trachea, breast muscle, pancreas, spleen, heart, and bone marrow.

Table 5 – Results for Tremovirus study. Percentage of positive and negative results for Tremovirus A, for each group of organs from chickens clinically suspected of Avian Encephalomyelitis

Sample organs/number	Number of organ samples that were considered positive or negative in RT-PCR	
	Number of positive/percentage	Number of negative/percentage
Whole brain n= 24	10 (41.66%)	14 (58.33%)
Intestine n= 20	9 (45%)	11 (55%)
Gizzard n= 12	5 (41.7%)	7 (58.3%)
Proventriculus n= 7	5 (71.4%)	2 (28.6%)
Sciatic nerve n= 7	5 (71.4%)	2 (28.6%)
Liver n= 7	4 (57.1%)	3 (42.9%)
Ceca n= 6	3 (50%)	3 (50%)
Lung n= 5	2 (40%)	3 (60%)
Kidney n= 5	2 (40%)	3 (60%)
Bursa n= 4	2 (50%)	2 (50%)
Thymus n= 3	1 (33.3%)	2 (66.7%)
Cecal tonsils n= 2	1 (50%)	1 (50%)
Cloaca (swab) n= 2	0 (0%)	2 (100%)
Trachea n= 2	0 (0%)	2 (100%)
Breast muscle n= 2	0 (0%)	2 (100%)
Pancreas n= 1	0 (0%)	1 (100%)
Spleen n= 1	0 (0%)	1 (100%)
Heart n= 1	0 (0%)	1 (100%)
Bone marrow n= 1	0 (0%)	1 (100%)
Total n= 112	46 (41.1%)	66(58.9%)

Source: (DE LA TORRE, D. 2018).

4.3.2 Sequence analysis

From 46 samples positive for Tremovirus A, were sequenced by Sanger method a total of 38 samples due the optimal concentration of DNA, besides the 4 vaccines used as positive control. The similarity matrix showed that the NT sequences of samples 529-1, 529-3, 529-4, 529-5 and 529-6 had 99.4% similarity with the NT sequences of the four vaccines and the Calnek strain, 94.7% with the Van Roekel and

L2Z strains, and finally 60.2% with the HM 175 strain (Table 6). The NT sequences of the remaining 33 samples had 100% similarity to the NT sequences of the four vaccines and the Calnek strain, 95.2% with the Van Roekel and L2Z strains, and finally 60.1% with the HM 175 strain. Regarding the AA sequences, the sequences of the 38 samples had 100% similarity with the four vaccine sequences and the Calnek strain, 98.9% with the Van Roekel strain, 97.4% with the L2K strain and 55.8% with the HM 175 strain. The sequences obtained from this study were submitted to the GenBank with the following accession codes: USP-BR-463-3 (KY292311), USP-BR-463-4(KY292312), USP-BR-463-5(KY292313), USP-BR-463-6(KY292314), USP-BR-463-7(KY292315), USP-BR-463-8(KY292316), USP-BR-463-9 (KY292317), USP-BR-463-10 (KY292318), USP-BR-463-11 (KY292319), USP-BR-463-12 (KY292320), USP-BR-463-13 (KY292321), USP-BR-463-14 (KY292322), USP-BR-463-15 (KY292323), USP-BR-463-16 (KY292324), USP-BR-463-17 (KY292325), 463-18 (KY292326), USP-BR-463-19 (KY292327), USP-BR-465-1 (KY292328), USP-BR-465-3 (KY292329), USP-BR-465-5 (KY292330), USP-BR-465-6 (KY292331), USP-BR-465-7 (KY292332), USP-BR-465-8 (KY292333), USP-BR-465-9 (KY292334), USP-BR-465-11 (KY292335), USP-BR-465-13 (KY292336), USP-BR-465-16 (KY292337), USP-BR-465-17 (KY292338), USP-BR-465-18 (KY292339), USP-BR-465-19 (KY292340), USP-BR-529-1 (KY292341), USP-BR-529-3 (KY292342), USP-BR-529-4 (KY292343), USP-BR-529-5 (KY292344), USP-BR-529-6 (KY292345), USP-BR-604-7 (KY292346), USP-BR-604-8 (KY292347) e USP-BR-604-14 (KY292348). The phylogenetic analysis (Figure 3) clustered all the samples together with the four vaccines and the Calnek, Van Roekel and L2K strains, and in a different group, the HM 175 (Hepatovirus A) to show the difference between these two genera of the family Picornaviridae.

Table 6 – Similarity matrix of for Tremovirus study. Results of similarity between AA and NT sequences of the 38 positive samples, vaccines sequences and the reference sequences of Calnek, Van Roekel, L2K and HM 175 strains

Brazilian isolates compared with vaccines and GenBank sequences		Brazilian Isolates																			Vaccine Isolates				GenBank sequences			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1	USP-BR-463-3	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
2	USP-BR-463-4	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
3	USP-BR-463-5	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
4	USP-BR-463-6	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
5	USP-BR-463-7	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
6	USP-BR-463-8	1.000	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
7	USP-BR-463-9	1.000	1.000	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
8	USP-BR-463-1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
9	USP-BR-463-1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
10	USP-BR-463-1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
11	USP-BR-463-1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
12	USP-BR-463-1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
13	USP-BR-463-1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
14	USP-BR-463-1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
15	USP-BR-463-1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
16	USP-BR-463-1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
17	USP-BR-463-1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
18	USP-BR-465-1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
19	USP-BR-465-3	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
20	Vaccine-1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	0.989	0.974	0.558	
21	Vaccine-2	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	1.000	1.000	0.989	0.974	0.558	
22	Vaccine-3	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	1.000	0.989	0.974	0.558	
23	Vaccine-4	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	0.989	0.974	0.558	
24	Calnek	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	0.989	0.974	0.558
25	Van Roekel	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	-	0.984	0.558
26	L2Z	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.988	-	0.543
27	HM175	0.601	0.601	0.601	0.601	0.601	0.601	0.601	0.601	0.601	0.601	0.601	0.601	0.601	0.601	0.601	0.601	0.601	0.601	0.601	0.601	0.601	0.601	0.601	0.587	0.585	-	

Brazilian isolates compared with vaccines and GenBank sequences		Brazilian Isolates																		Vaccine Isolates				GenBank sequences				
		28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54
28	USP-BR-465-5	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
29	USP-BR-465-6	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
30	USP-BR-465-7	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
31	USP-BR-465-8	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
32	USP-BR-465-9	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
33	USP-BR-465-1	1.000	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
34	USP-BR-465-1	1.000	1.000	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
35	USP-BR-465-1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
36	USP-BR-465-1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
37	USP-BR-465-1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
38	USP-BR-465-1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
39	USP-BR-529-1	0.994	0.994	0.994	0.994	0.994	0.994	0.994	0.994	0.994	0.994	0.994	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
40	USP-BR-529-3	0.994	0.994	0.994	0.994	0.994	0.994	0.994	0.994	0.994	0.994	0.994	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
41	USP-BR-529-4	0.994	0.994	0.994	0.994	0.994	0.994	0.994	0.994	0.994	0.994	0.994	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
42	USP-BR-529-5	0.994	0.994	0.994	0.994	0.994	0.994	0.994	0.994	0.994	0.994	0.994	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
43	USP-BR-529-6	0.994	0.994	0.994	0.994	0.994	0.994	0.994	0.994	0.994	0.994	0.994	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
44	USP-BR-604-7	0.998	0.998	0.998	0.998	0.998	0.998	0.998	0.998	0.998	0.998	0.998	0.996	0.996	0.996	0.996	0.996	-	1.000	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
45	USP-BR-604-8	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.994	0.994	0.994	0.994	0.994	0.998	-	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558	
46	USP-BR-604-1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.994	0.994	0.994	0.994	0.994	0.998	1.000	-	1.000	1.000	1.000	1.000	1.000	0.989	0.974	0.558
47	Vaccine-1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.994	0.994	0.994	0.994	0.994	0.998	1.000	1.000	-	1.000	1.000	1.000	1.000	0.989	0.974	0.558
48	Vaccine-2	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.994	0.994	0.994	0.994	0.994	0.998	1.000	1.000	1.000	-	1.000	1.000	1.000	0.989	0.974	0.558
49	Vaccine-3	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.994	0.994	0.994	0.994	0.994	0.998	1.000	1.000	1.000	1.000	-	1.000	1.000	0.989	0.974	0.558
50	Vaccine-4	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.994	0.994	0.994	0.994	0.994	0.998	1.000	1.000	1.000	1.000	1.000	-	1.000	0.989	0.974	0.558
51	Calnek	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.994	0.994	0.994	0.994	0.994	0.998	1.000	1.000	1.000	1.000	1.000	-	0.989	0.974	0.558	
52	Van Roekel	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.947	0.947	0.947	0.947	0.947	0.951	0.952	0.952	0.952	0.952	0.952	0.952	-	0.984	0.558	
53	L2Z	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.952	0.947	0.947	0.947	0.947	0.947	0.951	0.952	0.952	0.952	0.952	0.952	0.952	0.988	-	0.543	
54	HM175	0.601	0.601	0.601	0.601	0.601	0.601	0.601	0.601	0.601	0.601	0.601	0.602	0.602	0.602	0.602	0.602	0.602	0.601	0.601	0.601	0.601	0.601	0.601	0.601	0.587	0.585	-

Source: (DE LA TORRE, D. 2018).

Legend: To the left, NT sequences, and to the top, AA sequences.

Figure 3 – Phylogenetic tree for Tremovirus study

Source: (DE LA TORRE, D. 2018).

Legend: Phylogenetic tree inferred by using the Neighbor-Joining statistical method with 1,000 bootstrap replications. All sequences were clustered with the four vaccines used as control and the reference sequences obtained from the GenBank (Calnek, Van Roekel and L2K). The MH 175 strain was placed as an external group. BR=Brazil, USP=University of São Paulo.

4.4 DISCUSSION

Avian encephalomyelitis is a disease distributed worldwide (TANNOCK; SHAFREN, 1994; BOROS et al., 2014; HARYANTO et al., 2016; WELCHMAN et al., 2009) and it has been described in Brazil in the state of Rio de Janeiro in 1962 (DA SILVA; SOUTO; PEREIRA, 1972) and in the state of São Paulo in 1964 (BUENO, 1964). In 2015, it was reported an increase in cases of Avian Encephalomyelitis diagnosed through histopathology and serological analysis in the southern region of Brazil during 2012 and 2013 (FREITAS; BACK, 2015). The results obtained in this study, confirm those of Freitas and Back (FREITAS; BACK, 2015), finding the Tremovirus A in Avian Encephalomyelitis outbreaks, but using molecular techniques as diagnostic tools. The Avian Encephalomyelitis associated to Tremovirus A is a disease that affects the nervous system in Young birds causing ataxia, weakness, tremors in the head and neck (TANNOCK; SHAFREN, 1994), resulting in economic loss for poultry farmers due to a drop-in food consumption and consequent decline in meat conversion (BOROS et al., 2014). The highest susceptibility to this virus, where the clinical signs occurs, are the two first weeks of life (SUAREZ, 2013; TANNOCK; SHAFREN, 1994), which support our findings due the age of birds tested with positive results that were not more than 15 days old for 41 samples, and 5 with not revealed age. The main symptom of Avian Encephalomyelitis is tremor in head and neck caused by a disorder in the nervous system (BOROS et al., 2014), so, these organs are routinely tested for diagnosis of this disease. In this study, in addition to finding the virus in 39.1% of brains and 71.4% of sciatic nerves, the virus was also found in digestive, respiratory, immune and renal systems. Thus, in addition to the organs of the nervous system, the digestive organs were also important for the diagnostic of Tremovirus A confirming the reports described by Tannock and Shafren (1994), because in this study, Tremovirus A was detected in 36.8% of intestines, 41,7% of gizzards, 71.4% of proventriculus, 57.1% of livers and 50% of cecum. The virus was also found in 40% of lungs, 40% of kidneys, 50% of bursas and 33.3% of thymus, demonstrating the possibility of finding this virus in these organs during outbreaks of AE. Although the infections by Tremovirus A do not show clinical signs that affect the health of adult animals, they represent an economic risk for poultry farmers because there is a decrease in egg production (YU et al., 2015), decrease in the number of

births and increase of embryonic mortality in the case of breeder infections (TAYLOR et al., 1955). Another factor to be considered is that the progeny derived from these infected breeders, will have neurological signs associated with Avian Encephalomyelitis in the first weeks of life (CALNEK, 1998). The results obtained in this study suggest a possible failure in the vaccination procedures in breeders, either due to errors in the management of the vaccine, poor storage conditions, changes in the vaccination schedule or delay in vaccine application with possible viral inactivation. In other hand, it is important to emphasize that the thanks to the vaccination of the breeders, occurs the transfer of antibodies to the progeny (WESTBURY; SINKOVIC, 1978), and that failures in vaccination procedures will decrease antibodies concentration in the progeny, and if they are challenged with a field virus, the infection will affect the chicks. Another hypothesis to be considered is that breeders were not vaccinated, or they got the field virus during the laying stage due to biosafety failures. Through analysis of the AA sequences, 100% of the samples had similarity to the vaccines and the reference strain (Calnek). The phylogenetic tree confirmed the data of the similarity matrix, clustering all the sequences within the same group of reference strains of Tremovirus A. The strain MH 175 was placed in a different group because it corresponds to a distinct genus called Hepatovirus (KNOWLES et al., 2012), which would explain one of the reasons for Tremovirus A to be withdrawn from the genus Hepatovirus for the new genus Tremovirus A (KNOWLES et al., 2012). Finally, all the viruses found in the samples correspond to the same molecular pattern of Tremovirus A. Probably, occurred a fail in the biosecurity procedure for preventing the outbreaks of AE in progeny, due to practice of vaccination nearby laying phase.

4.5 REFERENCES

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5 ENTERIC VIRUS DIVERSITY DETECTED BY MOLECULAR METHODS IN BRAZILIAN POULTRY FLOCKS

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ABSTRACT. - Enteric viruses play an important role in the Brazilian poultry industry due to the economic impact of resulting low yields of broilers, layers, and breeders. The most common enteric viruses affecting commercial flocks in Brazil include Fowl Adenovirus of group I (FAdV-I), Chicken Parvovirus (ChPV), Chicken Astrovirus (CAstV), Avian Nephritis Virus (ANV), Infectious Bronchitis Virus (IBV), Avian Reovirus (AReo), and Avian Rotavirus (ARtV). The aim of this study was to identify single and multiple infections using data obtained from 270 samples from eleven Brazilian states, corresponding to the period between 2010 and 2017. This was accompanied by an analysis of the relationship between the age of birds, clinical signs, and geographical distribution, using Polymerase Chain Reaction (PCR) and Reverse Transcription-PCR (RT-PCR) techniques. Twenty-five profiles of virus combinations were detected. Single infections were encountered in 86.3% of samples, and multiple infections were present in the remaining 13.7%. Both single and multiple infections affected all kinds of commercial chickens with digestive problems, stunting syndrome, decreases in egg and meat production, increased mortality, and respiratory signs. FAdV-I, ChPV, CAstV, ANV, and ARtV were mostly detected in young broilers, in contrast with IBV, which was detected in hens from one to greater than 51 weeks of age. These results exhibit the complexity of enteric diseases and the still poorly understood role of each pathogen as a unique etiological agent.

5.1 INTRODUCTION

Enteric viruses are the etiological agents for a series of health disturbances for commercial chickens around the world. They cause severe economic losses for the poultry industry because they negatively affect productive parameters, causing growth retardation, low feed consumption, high mortality, poor egg and meat production, and

runting-stunting syndrome (RSS) (OTTO et al., 2006; KANG et al., 2012; DEVANEY et al., 2016; NUÑEZ et al., 2016). These kinds of infections affect mostly young birds, but it is common to find viral infections in birds of all ages, including broilers, layers, and breeders (JINDAL et al., 2009). The main enteric viruses reported to cause enteric diseases are found in single and multiple infections and include the Fowl Adenovirus of group I (FAdV-I); Chicken Parvovirus (ChPV); two viruses from the Astroviridae family: Chicken Astrovirus (CAstV) and Avian Nephritis Virus (ANV); two viruses from the Reoviridae family: Avian Reovirus (AReo) and Avian Rotavirus (ARtV); and a member of the Coronaviridae family, Infectious Bronchitis Virus (IBV) (DECAESSTECKER et al., 1988; ROUSSAN et al., 2012; MOURA-ALVAREZ et al., 2013; METTIFOGO et al., 2014; NUÑEZ et al., 2016;). Several laboratory analytical methods have been used to detect enteric viruses in organic tissues from sick and healthy birds. Conventional polymerase chain reaction (PCR) and reverse transcription-polymerase chain reaction (RT-PCR) are two of the most commonly used methods for diagnosis and characterization of viruses in the poultry industry (MEULEMANS et al., 2001; CAVANAGH et al., 2002; DAY et al., 2007; PANTIN-JACKWOOD et al., 2008; ZSAK et al., 2008). The objective of this study was to determine the prevalence of enteric viruses affecting commercial chicken flocks in Brazil, encompassing an analysis of the relationships between single and multiple infections, the age of birds, clinical signs, and geographical distribution in the Brazilian states of Mato Grosso, Goiás, Piauí, Ceará, Paraíba, Pernambuco, Bahia, Minas Gerais, Espírito Santo, São Paulo, and Santa Catarina.

5.2 MATERIALS AND METHODS

5.2.1 Sample collection

For this study, we used 270 positive samples of single and multiple enteric viral infections from broilers, layers and breeders of different ages, collected from eleven Brazilian states: Mato Grosso, Goiás, Piauí, Ceará, Paraíba, Pernambuco, Bahia, Minas Gerais, Espírito Santo, São Paulo, and Santa Catarina. The samples were collected between 2010 and 2017. Each sample was composed of a pool of maximum five organs of the same type, and each sample came from different farms across the mentioned states. The main reported symptoms of the birds were enteritis, diarrhea,

decreased feed absorption, a decrease in production, mortality, signs of respiratory disease, and stunting syndrome. Samples were processed and analyzed by conventional PCR and RT-PCR for detection of seven main enteric viruses reported in Brazil: FAdV-I, ChPV, CAstV, ANV, IBV, AReo, and ARtV. The organs used for viral detection were the intestines, liver, pancreas, and caecal tonsils, and also cloacal swabs were used for fecal samples. The results were categorized according to the viral infection in each sample, the type of birds (broilers, layers, or breeders), the age of birds (days for broilers and weeks for layers and breeders), clinical signs, and origin of the samples based on their geographical distribution in Brazil. Known positive samples for FAdV-1, ChPV, CAstV, ANV, and ARtV, validated through Sanger sequencing, and commercial vaccines for IBV and AReo were used as positive controls.

5.2.2 DNA and RNA extraction

The tissues were mechanically macerated and suspended in PBS 0.1M, pH 7.4, in a 1:1 proportion in 1500 µL microtubes. The mixture was vortexed, subjected to three freeze-thaw cycles, and subsequently clarified at 12,000 x g for 20 min at 4°C. An aliquot of each supernatant was used for DNA and RNA extraction, using the organic phenol-chloroform method, according to procedures described by Green et al., (2017) and Chomczynski and Sacchi (1987). The concentration and quality of nucleic acids were evaluated in a NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA). Final DNA and RNA preparations were stored at -20°C.

5.2.3 Reverse-transcription reaction

The samples screened for viruses with RNA genomes (CAstV, ANV, IBV, AReo, and ARtV), were submitted to a reverse transcriptase (RT) reaction to produce complementary DNA (cDNA), which was then used in the corresponding PCR. RT was conducted with the extracted RNA at a concentration of 3–5 µg/µL, which was denatured at 95°C for 5 min and added to a mix containing 250 ng of Random primers, 500 µg/ml of anchored oligo (dT)₁₂ primer, 500 µM of each dNTP, 4 µL of 5X First-Strand Buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM Magnesium Chloride) (Invitrogen™, Carlsbad, CA, USA), 2 µL of 100 mM Dithiothreitol (DTT) (Invitrogen™), 200 units of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT)

(Invitrogen™), and sterile distilled water to reach 20 µL in final volume. The reaction was performed with the following temperature conditions: an initial incubation at 25°C for 10 min, a second incubation at 37°C for 50 min, and inactivation of the reaction at 70°C for 15 min. RT products were stored at –20°C.

5.2.4 PCR for fowl adenovirus

The primers used for detection of FAdV-I were those described in (MEULEMANS et al., 2001), as shown in Table 7, and amplify an 897 bp segment corresponding to the variable region Loop 1 of the gene that encodes the structural protein, Hexon. Through analysis of the NT and AA sequences, it is possible to characterize the 12 different serotypes of FAdV-I (FAdV-1–FAdV-8a, FAdV-8b–FAdV-11) (MEULEMANS et al., 2001). DNA amplification was carried out in 200 µL microtubes by adding 2.5 µL of extracted DNA with a concentration of 1 µg/µL, to a mix containing 1X PCR magnesium free (–Mg) Buffer (Invitrogen™), 200 µM of each dNTP, 0.5 µM of each primer, (Hexon A and Hexon B), 1 U of Platinum™ Taq DNA Polymerase, 1.5 mM of MgCl₂, and sterile distilled water to reach 22.5 µL. The following temperature conditions were used for the PCR reactions: a cycle of 94°C for 5 min, 35 cycles at 94°C for 60 s, 52°C for 45 s, and 72°C for 1 min, followed by a final extension at 72°C for 10 min.

5.2.5 PCR for chicken parvovirus

The primers used for detection of ChPV were those described in Table 7 (ZSAK et al., 2009) and amplify a 561 bp segment corresponding to a well-conserved region of the non-structural gene (NS) usually used for detection of chicken and turkey parvovirus (ZSAK et al., 2009). DNA amplification was carried out in 200 µL microtubes by adding 2 µL of extracted DNA with a concentration of 1 µg/µL into a mix containing 1X PCR magnesium free (–Mg) Buffer, 200 µM of each dNTP, 0.5 µM of each primer (PVF1 and PVR1), 1.25 U of Platinum™ Taq DNA Polymerase (Invitrogen™), 2 mM of MgCl₂, and enough sterile distilled water to reach 23 µL. The following temperature conditions were used for the PCR reaction: a cycle of 94°C for 3 min, 35 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, followed by a final extension at 72°C for 10 min.

5.2.6 RT-PCR for chicken astrovirus and avian nephritis virus

PCR was conducted with the cDNA produced by the RT reaction, using the primers described in Table 7 (DAY et al., 2007). This was used for the diagnosis of CAstV and ANV, which results in the amplification of 362 bp and 473 bp segments, respectively, both of which are located in the RNA polymerase gene (ORF 1B) (DAY et al., 2007). DNA amplification was done in 200 μ L microtubes by adding 2.5 μ L of cDNA to a mix containing 1X PCR magnesium free (–Mg) Buffer, 200 μ M of each dNTP, 0.5 μ M of each primer (Cas pol 1F and Cas pol 1R for CAstV; ANV pol 1F and ANV pol 1R for ANV), 1 U of Platinum™ Taq DNA Polymerase (Invitrogen™), 1.5 mM of MgCl₂, and enough sterile distilled water to reach 22.5 μ L. The following temperature conditions were used for the PCR reaction: a cycle at 95°C for 5 min, 35 cycles at 94°C for 30 s, 50°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min.

5.2.7 RT-PCR for coronavirus

PCR was conducted with the cDNA produced by the RT reaction, using the primers for detection of all known coronaviruses, described in Table 7 (CAVANAGH et al., 2002), which amplifies a 266 bp segment located in a highly conserved region of the 3'-untranslated region (3'-UTR) in the first PCR reaction (UTR-11 and UTR-41) and a 179 bp segment in a second, hemi-nested reaction (UTR-31 and UTR-41) (CAVANAGH et al., 2002). Amplification of the first and second PCR reactions was performed in 200 μ L microtubes by adding 2.5 μ L of cDNA in the first PCR reaction and 1 μ L of the PCR product for the second reaction. The mix for the two PCR reactions contained 1X PCR magnesium free (–Mg) Buffer, 200 μ M of each dNTP, 0.5 μ M of each primer (UTR-11, UTR-31 and UTR-41), 1.25 U of Platinum™ Taq DNA Polymerase (Invitrogen™), 2 mM of MgCl₂, and enough sterile distilled water to reach 22.5 μ L for the first PCR reaction and 24 μ L for the second PCR hemi-nested reaction. The following temperature conditions were used for both PCR reactions: a cycle at 94°C for 3 min, 35 cycles at 94°C for 1 min, 48°C for 90 s, 72°C for 90 s, and a final extension at 72°C for 10 min.

5.2.8 RT-PCR for avian rotavirus

PCR was conducted with the cDNA produced by the RT reaction, using the primers described in Table 7 (DAY et al., 2007), which amplify a 630 bp segment located in Segment 10 (S10) of the viral genome, encoding the non-structural protein, NSP4 (DAY et al., 2007). DNA amplifications were conducted in 200 μ L microtubes by adding 2.5 μ L of cDNA to a mix containing 1X PCR magnesium free (–Mg) Buffer, 200 μ M of each dNTP, 0.5 μ M of each primer (NSP4-F30 and NSP4-R660), 1 U of Platinum™ Taq DNA Polymerase (Invitrogen™), 1.5 mM of MgCl₂, and enough sterile distilled water to reach 22.5 μ L. The following temperature conditions were used for the PCR reaction: a cycle at 95°C for 5 min, 35 cycles at 94°C for 30 s, 50°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min.

5.2.9 RT-PCR for avian reovirus

PCR was conducted with the cDNA produced by the RT reaction, using the primers described in Table 7 (PANTIN-JACKWOOD et al., 2008), which amplify a 1120 bp fragment located in Segment 4 (S4) of the viral genome, encoding the structural protein 4 (VP4) (PANTIN-JACKWOOD et al., 2008). DNA amplifications were conducted in 200 μ L microtubes by adding 5 μ L of cDNA to a mix containing 1X PCR magnesium free (–Mg) Buffer, 200 μ M of each dNTP, 0.5 μ M of each primer (S4-F13 and S4-R1133), 1.25 U of Platinum™ Taq DNA Polymerase (Invitrogen™), 2 mM de MgCl₂, and enough sterile distilled water to reach 20 μ L. The following temperature conditions were used for the PCR reaction: a cycle at 94°C for 5 min, 35 cycles of 94°C for 30 s, 50°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min.

5.2.10 Evaluation of RT-PCR and PCR products

RT-PCR and PCR products were analyzed by electrophoretic diffusion in 1.5% Ultrapure™ Agarose (Invitrogen™) gels submerged in 0.5X Tris-borate-Ethylenediamine Tetraacetic acid (EDTA). The size of DNA fragments was estimated by comparison with the 100 bp DNA Ladder (Invitrogen™). A UV trans-illumination camera was used to visualize the DNA bands.

5.2.11 Sequencing and phylogenetic analysis

Some samples were chosen randomly for DNA sequencing to validate the RT-PCR and PCR specific reactions: FAdV-I (n = 3), ChPV (n = 3), CAstV (n = 3), ANV (n = 4), AReo (n = 1), and ARtV (n = 3). The NT sequences for each sample were submitted to GenBank with the exception of the FAdV-I and ChPV sequences, which had already been submitted in previous studies. IBV sequences were not analyzed phylogenetically due to the high diversity of coronaviruses in different animal species. PCR products from each sample were purified using the GPX™ PCR DNA and Gel Band Purification kit (GE Healthcare), following the manufacturer's instructions. Purified products were sequenced in the forward and reverse directions using the BigDye® Terminator Cycle Sequencing Kit v3.1 (Invitrogen™) based on the manufacturer's instructions. Sequenced products were analyzed in an ABI 3730 DNA Analyzer (Applied Biosystems). Electropherograms obtained from the sequencing process were assembled and edited in the CLC Main WorkBench 7.7.3 (CLC Bio-Qiagen) software and aligned with the Clustal W method available in the ClustalX 2.1 software (Des Higgins Conway Institute, UCD, Dublin, Ireland), using reference sequences from the NCBI GenBank for each enteric virus with the following access codes: FAdV-I: FAdV-1 (NC_001720), FAdV-2 (AF339915), FAdV-3 (AF508949), FAdV-4 (NC_015323), FAdV-5 (NC_021221), FAdV-6 (AF508954), FAdV-7 (AF508955), FAdV-8a (KT862810), FAdV-8b (JN112373, KT862811, KX258422, KU981150, and KU981150), FAdV-9 (NC_000899), FAdV-10 (KT717889), and FAdV-11 (AF339925); ChPV: (KU569462, GQ281296, KJ486491, JQ178304, KT347548, KX133426, HQ680340, NC_024452, JF267323, KM598417, and KY649278); CAstV: (KC633180, JX945871, KT386328, KX397575, JF414802, and DQ324839); ANV: (DQ324833, KM254166, KM985702, HM029238, and NC_003790); ARtV: (KX185128, JX474761, GQ353331, KT347547, EU400310, JQ085408, LC088136, FJ169862, and LK932176); AReo: (KX398291, KC865795, JQ954693, EU400285, KP173692, JN641885, JN641884, KF741745, and DQ198858). Phylogenetic trees were inferred using the neighbor-joining statistical method, with 1000 bootstraps replications, integrated in the MEGA 7 software (Pennsylvania State University, PA, USA).

5.2.12 Statistical analysis

Descriptive statistics were used to represent the variability of the positive samples, using the single and multiple viral infections, type of birds (broilers, layers and breeders), age of birds (days for broilers and weeks for layers and breeders), clinical signs (respiratory signs, digestive signs, stunting-mortality-drop production, and no clinical signs), and geographic distribution of the samples. Minitab 18 (Minitab® Statistical Software, v18.1, Minitab Inc., State College, PA, USA) was used to develop the variance analysis of frequencies of single and multiple infections for each virus, and clinical signs pertaining to each type of bird. The alfa value for significance level was $\alpha = 0.05$.

Table 7 – Primers used for Brazilian enteric virus study

*Virus	**Gene Target	Primer Name	Primer Sequence	bp	Reference
FAdV-I	Hexon	Hexon A	5'-CAARTTCAGRCAGACGGT-3'	897	Meulemans, 2001
		Hexon B	5'-TAGTGATGMC GSGACATCAT-3'		
ChPV	NS	PVF1	5'-TTCTAATAACGATATCACTCAAGTTTC-3'	561	Zsak, 2009
		PVR1	5'-TTTGCGCTTGCGGTGAAGTCT GGCTCG-3'		
IBV	UTR	UTR 11	5'- GCTCTAACTCTATACTAGCCTA-3'	179	Cavanagh, 2002
		UTR 31	5'- GGGCGTCCAAGTGCTGTACCC-3'		
		UTR 41	5'- ATGTCTATCGCCAGGGAATGTC-3'		
CAstV	ORF 1b	CAS pol 1F	5'-GAYCARCGAATGCGRAGRITG-3'	362	Day, 2007
		CAS pol 1R	5'-TCAGTGGAAGTGGGKARTCTAC-3'		
ANV	ORF 1b	ANV pol 1F	5'-GYTGCGCGCYTCYTTTGAYACCRT-3'	473	Day, 2007
		ANV pol 1R	5'-CRTTGGCCCKRTARTCTTTRTGAY-3'		
AReo	S4	S4-F13	5'-GTGCGTGTGGAGTTTCCCG-3'	1120	Pantin-J. 2008
		S4-R1133	5'-TACGCCATCCTAGCTGGA-3'		
ARtV	NSP4	NSP4-F30	5'-GTGCGGAAAGATGGAGAAC-3'	630	Day, 2007
		NSP4-R660	5'-GTTGGGGTACCAGGGATTAA-3'		

Source: (DE LA TORRE, D. 2018).

Legend: *Primer sequences and amplicon sizes are expressed in base pairs (bp)

**Genes abbreviations are based on the nomenclature suggested by authors cited in the reference column.

NS=nonstructural; UTR=untranslated region; ORF=open reading frame; S4=segment 4; NSP4=nonstructural protein 4.

5.3 RESULTS

5.3.1 Single and multiple viral infections

A total of 333 viruses were found in 270 samples, and the most common virus detected corresponded to IBV, showing a 58.9% (196/333) occurrence, followed by ANV with 12.6% (42/333), FAdV-I with 8.4% (28/333), CAstV with 8.1% (24/333), ChPV with 6.6% (22/333), ARtV with 5.1% (17/333), and AReo with 0.3% (1/333). Single infections were found in 86.3% (233/270) of the samples, with IBV being the most predominant virus diagnosed as a unique agent. ANV, CAstV, IBV, ChPV, and ARtV were found in simple and combined (2 to 5 viruses) infections. FAdV-I was found in unique and combined (2 to 3 viruses) infections. Multiple infection analysis showed that 2 viral infections were found in 6.3% (17/270) of samples, followed by 3 viral infections in 5.6% (15/270), 4 viral infections in 1.5% (4/270), and 5 viral infections in 0.4% (1/270). Single and multiple viral infections are described in Table 8. Multiple infections occurred with 25 profile combinations of viruses (Table 9). ANV, CAstV, IBV, ChPV, and ARtV were present in combination with each other, resulting in between two and five virus combinations. FAdV-I was present in combination with ChPV, ANV, and IBV. AReo showed a single infection. The variance analysis showed no significant difference between the frequencies of single and multiple viruses ($p = 0.36$).

Table 8 – Results of frequencies for Brazilian enteric virus study. Frequencies of single and multiple viral infections diagnosed in 270 samples

Number of Viruses	FAdV-I	ANV	CAstV	IBV	ChPV	AReo	ARtV	Single and Multiple Positive Samples
1 virus	22	19	15	164	8	1	4	233/270 (86.3%)
2 viruses	3	6	7	14	1	0	3	17/270 (6.3%)
3 viruses	3	12	1	14	10	0	5	15/270 (5.6%)
4 viruses	0	4	3	3	2	0	4	4/270 (1.5%)
5 viruses	0	1	1	1	1	0	1	1/270 (0.4%)
Number of positive samples for each virus	28	42	27	196	22	1	17	-
% of samples positive for each virus ($n = 333$)	8.4%	12.6%	8.1%	58.9%	6.6%	0.3%	5.1%	-

Source: (DE LA TORRE, D. 2018).

Table 9 – Results of Brazilian enteric virus detected. Enteric virus detection patterns from 270 samples in different types of organs from chicken commercial flocks

Patterns	Virus Examined in 270 Digestive Organs						Number of Samples in Each Pattern (%)	
	FAdV-I	ChPV	CAstV	ANV	IBV	AReo		
1	+	-	-	-	-	-	22	8.1%
2	+	-	-	+	-	-	1	0.4%
3	+	-	-	-	+	-	2	0.7%
4	+	+	-	-	+	-	2	0.7%
5	+	-	-	+	+	-	1	0.4%
6	-	-	-	+	-	-	19	7%
7	-	-	-	+	+	-	3	1.1%
8	-	-	+	+	-	-	1	0.4%
9	-	-	-	+	-	-	1	0.4%
10	-	+	-	+	+	-	7	2.6%
11	-	-	-	+	+	-	3	1.1%
12	-	+	-	+	-	-	1	0.4%
13	-	-	+	+	+	-	2	0.7%
14	-	+	+	+	-	-	1	0.4%
15	-	+	-	+	+	-	1	0.4%
16	-	+	+	+	+	-	1	0.4%
17	-	-	+	-	-	-	15	5.6%
18	-	-	+	-	+	-	6	2.2%
19	-	-	+	-	+	-	1	0.4%
20	-	-	-	-	+	-	164	60.7%
21	-	+	-	-	+	-	1	0.4%
22	-	-	-	-	+	-	2	0.7%
23	-	+	-	-	-	-	8	3%
24	-	-	-	-	-	-	4	1.5%
25	-	-	-	-	-	+	1	0.4%

Source: (DE LA TORRE, D. 2018).

5.3.2 Age and type of birds

The samples received for viral diagnostics were from broilers, layers, and breeders of all ages. According to the age of birds, the samples corresponding to layer and breeder hens were divided into 5 groups, with intervals of 10 weeks of age, from week 1 to week 50, and two more groups, one for birds older than 51 weeks, and a group for which the ages were not reported (N/R; Table 10).

Table 10 – Results of number of positive samples according to the age of layer and breeder flocks

Age of Flocks	Layers		Breeders	
	Number of Positive Samples	% of Samples Positive for Each Week of Age	Number of Positive Samples	% of Samples Positive for Each Week of Age
1-10 weeks	8	16,3%	7	18,9%
11-20 weeks	9	18,4%	0	0%
21-30 weeks	2	4,1%	0	0%
31-40 weeks	8	16,3%	5	13,5%
41-50 weeks	0	0%	7	18,9%
>51 weeks	11	22,4%	14	37,8%
Not reported	11	22,4%	4	10,8%

Source: (DE LA TORRE, D. 2018).

The samples corresponding to broilers were divided into 6 groups with intervals of 7 days of age, from day 1 to day 42, and two more groups, one for birds older than 43 days, and an N/R age group (Table 11).

Table 11 – Number of positive samples according to the age of broilers

Age of Flocks	Number of Positive Samples	% of Samples Positive for Each Day of Age
1–7 days	12	7,4%
8–14 days	25	15,3%
15–21 days	24	14,7%
22–28 days	11	6,7%
29–35 days	8	4,9%
36–42 days	53	32,5%
>43 days	19	11,7%
Not reported	11	6,7%

Source: (DE LA TORRE, D. 2018).

Positive samples from layer and breeder hens older than 51 weeks of age showed the highest frequency of viral infections: 22.4% for layers and 37.8% for breeders. The viral infection frequencies of the other layer groups were 18.4% for the 11–20-week group, 16.3% for the 1–10-week and 31–40-week groups, 4.1% for the 21–30-week group, and 22.4% for the N/R age group. In the case of breeders, the frequency of viral infections was 18.9% for the 1–10-week and 41–50-week groups, 13.5% for the 31–40-week group, and 10.8% for the N/R age group (Table 10). Broilers showed the highest frequency of viral infections, at 37.8% for the 36–42-day group. Viral infection rates in the other groups were 15.3% for the 8–14-day group, 14.7% for the 15–21-day group, 11.7% for the 43-day group, 7.4% for the 1–7-day group, 6.7% for the 22–28-day group, 4.9% for the 29–35-day group, and 6.7% for the N/R age group (Table 11). Single viral infections were found mostly in broilers, with 129/233 (55.4%), followed by 49/233 (21%) in layers and 34/233 (14.6%) in breeders. Multiple viral infections were found with more frequency in broilers, with 14/17 (82.4%) with two viruses, 15/15 (100%) with three viruses, and 4/4 (100%) with four viruses. The only sample with five viral infections also belonged to the broilers group. A total of 3/17 (17.6%) samples with multiple viral infections (3 viruses) were found in samples from breeders (Table 12).

The organs corresponding to positive results for FAdV, ChPV, CAstV, and ARtV included the liver, intestines, and pancreas. AReo was present in one sample of intestines and IBV was present in all the five organs used for this study (Table 13).

FAdV-I was found in samples from breeders (12/28) and broilers (14/28). ChPV was found in samples from broilers (20/22). CAstV was found in samples from breeders (10/27) and broilers (17/27). ANV was found in samples from broilers (41/42). IBV was found in samples from layers (49/196), breeders (16/196), and broilers (116/196). AReo was found in one sample, but the origin was not reported. ARtV was found in samples from breeders (2/17) and broilers (15/17). All data describing the positive samples according to the age of layers, breeders, and broilers affected by the enteric viruses in this study are shown in Table 14.

Table 12 – Frequency of single and multiple virus infections and clinical signs according to the type of birds

Item	Positive Samples with Single and Multiple Viral Infections					Clinical Signs				
	1 Virus	2 Viruses	3 Viruses	4 Viruses	5 Viruses	Respiratory Problems	Digestive Problems	Poor Production *	No Clinical Signs	Not Reported
Broilers (<i>n</i> = 163)	129 (55.4%)	14 (82.4%)	15 (100%)	4 (100%)	1 (100%)	12 (30.8%)	9 (100%)	55 (79.7%)	1 (20%)	86 (58%)
Layers (<i>n</i> = 49)	49 (21%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	23 (59%)	0 (0%)	2 (2.9%)	0 (0%)	24 (16.2%)
Breeders (<i>n</i> = 37)	34 (14.6%)	3 (17.6%)	0 (0%)	0 (0%)	0 (0%)	3 (7.7%)	0 (0%)	12 (17.4%)	4 (80%)	18 (12.2%)
Not reported (<i>n</i> = 21)	21 (9%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (2.6%)	0 (0%)	0 (0%)	0 (0%)	20 (13.5%)
Number of positive samples (<i>n</i> = 270)	233	17	15	4	1	39	9	69	5	148
% of positive samples independently of chicken line	86,3%	6,3%	5,6%	1,5%	0,4%	14,4%	3,3%	25,6%	1,9%	54,8%

Source: (DE LA TORRE, D. 2018).

Legend: *Poor production was defined as culling, stunting, and mortality.

Table 13 – Frequency of viruses found in different digestive organs

Sample	FAdV-I	ChPV	CAstV	ANV	IBV	AReo	ARtV
Liver (<i>n</i> = 39)	19	3	1	1	14	0	2
Intestines (<i>n</i> = 89)	4	14	19	27	52	1	4
Pancreas (<i>n</i> = 25)	5	5	7	14	13	0	11
Caecal tonsils (<i>n</i> = 20)	0	0	0	0	20	0	0
Cloacal swabs (<i>n</i> = 97)	0	0	0	0	97	0	0
Total (viruses = 333)	28	22	27	42	196	1	17

Source: (DE LA TORRE, D. 2018).

Table 14 – Frequency of viruses affecting birds according to the age of birds

Chicken Lines	Age of Birds	FAdV-I	ChPV	CAstV	ANV	IBV	AReo	ARtV
Layers	1–10 weeks	0	0	0	0	8	0	0
	11–20 weeks	0	0	0	0	9	0	0
	21–30 weeks	0	0	0	0	2	0	0
	31–40 weeks	0	0	0	0	8	0	0
	41–50 weeks	0	0	0	0	0	0	0
	>51 weeks	0	0	0	0	11	0	0
	N/R	0	0	0	0	11	0	0
Breeders	1–10 weeks	7	0	0	0	0	0	0
	11–20 weeks	0	0	0	0	0	0	0
	21–30 weeks	0	0	0	0	0	0	0
	31–40 weeks	0	0	0	0	5	0	0
	41–50 weeks	4	0	0	0	3	0	0
	>51 weeks	1	0	9	0	7	0	0
	N/R	0	0	1	0	1	0	2
Broilers	1–7 days	0	2	0	10	5	0	0
	8–14 days	0	8	4	16	13	0	5
	15–21 days	6	5	6	6	21	0	5
	22–28 days	1	3	0	4	3	0	3
	29–35 days	2	0	0	0	6	0	0
	36–42 days	4	0	0	1	48	0	1
	>43 days	1	0	0	0	18	0	0
	N/R	0	2	7	4	2	0	1
Not reported neither age nor line		2	2	0	1	15	1	0
Total viruses = 333 *		28	22	27	42	196	1	17

Source: (DE LA TORRE, D. 2018).

Legend: *values based on 333 viruses detected. N/R= Not Reported age of birds .

5.3.3 Clinical signs

Positive samples were divided into five groups according to clinical signs and type of birds. Respiratory signs were reported in 39/270 samples, digestive signs in 9/270, stunting-mortality-decreased production signs in 69/270, no clinical signs in 5/270, and the remaining 148/270 samples did not include reports regarding clinical signs. The most common disease symptoms reported were stunting syndrome, mortality, and a decrease in egg and meat production, which occurred in 69/270 (25.6%) of the positive samples. Broilers showed the highest values of stunting-mortality-decrease in production, with 55/69 (79.7%), followed by breeders with 12/69 (17.4%) and layers with 2/69 (2.9%). An important factor to be considered is that respiratory pathogens causing respiratory problems were found in digestive organs, for example IBV, with 12/39 (30.8%) positive samples in broilers, 23/39 (59%) in layers, and 3/39 (7.7%) in breeders. Digestive diseases were not commonly reported as having unique clinical signs, and only 9 positive samples were obtained from broilers. All frequencies of clinical signs are described in Table 12. The variance analysis showed no significant difference between clinical signs for each type of bird ($p = 0.45$).

5.3.4 Geographic origin

Samples originated from 12 Brazilian states from the north-east, east, and south-east regions. Most of the samples positive for enteric viruses were from the state of São Paulo 82/270 (30.4%). IBV was the main virus found in 11 states, accounting for 133/333 of the total viruses detected, excluding the state of Paraíba. FAdV-I was detected in samples originating from Pernambuco, Santa Catarina, and São Paulo, with a total of 26/333 virus detected. ChPV and ANV were detected in samples from Bahia, Ceara, Minas Gerais, Paraíba, Pernambuco, and São Paulo, with a total of 15/333 and 37/333 detections, respectively. CAstV and ARtV were detected in the state of Minas Gerais with 20/333 and 15/333 virus detected, respectively, though ARtV was also present in 1 detection of Pernambuco. The origin of the remaining viruses detected, i.e. FAdV-I (2/333), ChPV (7/333), CAstV (7/333), ANV (5/333), IBV (63/333), AReo (1/333), and ARtV (2/333), were not reported. Data describing the geographical distribution of enteric viruses are shown in Table 15.

Table 15 – Frequencies of viruses detected in 12 Brazilian states

Brazilian States— Samples Origin	Total Positive Samples *	% of Samples Positive for Each State	Virus Strains Examined in This Study **						
			FAdV-I	ChPV	CAstV	ANV	IBV	AReo	ARtV
Bahia	6	2.2%		3 (13.6) ^A		6 (14.3)	5 (2.6)		
Ceara	5	1.9%		2 (9.1)		2 (4.8)	1 (0.5)		
Espirito Santo	3	1.1%					3 (1.5)		
Goias	13	4.8%					13 (6.6)		
Mato Grosso	11	4.1%					11 (5.6)		
Minas Gerais	50	18.5%		4 (18.2)	20 (74.1)	14 (33.3)	33 (16.8)		11 (64.7)
Paraiba	4	1.5%		1 (4.5)		3 (7.1)			
Pernambuco	9	3.3%	1 (3.6)	2 (9.1)		5 (11.9)	1 (0.5)		1 (5.9)
Piaui	1	0.4%					1 (0.5)		
Santa Catarina	7	2.6%	7 (25)						
São Paulo	82	30.4%	18 (64.3)	3 (13.6)		7 (16.7)	65 (33.2)		3 (17.6)
Not informed	79	29.3%	2 (7.1)	7 (31.8)	7 (25.9)	5 (11.9)	63 (32.1)	1 (100)	2 (11.8)
Total	270	100%	28 (100)	22 (100)	27 (100)	42 (100)	196 (100)	1 (100)	17 (100)

Source: (DE LA TORRE, D. 2018).

Legend: A=The numbers in parentheses represent the percentage values of each sample. The blanks in the table indicate that the sample showed negative results in the PCR assay.

*Values calculated based on 270 samples received in the Avian Pathology Laboratory.

**Values calculated based on 333 viruses detected in the 270 samples.

5.3.5 Phylogenetic analysis

The following accession numbers correspond to the sequences used in the phylogenetic analysis: FAdV-I: isolate 420-12 (KY229185), isolate 424-4 (KY229176), and isolate 471-14 (KY229184); ChPV: isolate 691-1 (MF784849), isolate 752-3 (MF784850), and isolate 786-1 (MF784851); ANV: isolate 475-5 (MF683400), isolate 752-3 (MF683401), isolate 656-3 (MF683402), and isolate 691-6 (MF683403); CAstV: isolate 541-2 (KR013275), isolate 541-12 (KR013255), and isolate 541-16 (KR013252); ARtV: isolate 480-1 (MF683404), isolate 480-7 (MF683405), and isolate 480-5 (MF683406); AReo: isolate 806-1 (MF693911). FAdV-I and CAstV sequences were already submitted to the GenBank database in previous studies (Nuñez, et al., 2016). Fowl adenovirus sequences were characterized according to their position in the phylogenetic tree (Figure 4).

Figure 4 – Phylogenetic tree for FAdV-I strains

Source: (DE LA TORRE, D. 2018).

Legend: The tree was inferred with the neighbor-joining statistical method and based on the partial sequence of the Hexon gene. Numbers along the branches refer to bootstrap values for 1000 replicates. The five species of FAdV-I are grouped and identified with letters A–E.

Isolate 424-4 was clustered with the reference sequence of strain TR59 with a bootstrap value of 100 in the common ancestral line, suggesting that this isolate should be classified into the FAdV-8a serotype. Isolate 420-12 was clustered with reference sequences of strain 764, and some isolates originated from China and Peru, with a bootstrap value of 100 in the common ancestor of the 6 sequences, suggesting that this isolate should be classified as FAdV-8b serotype. Isolate 471-14 clustered with the reference sequence of strain ATCC, suggesting that this isolate should be classified as FAdV-11 serotype. Isolates 475-5, 656-3, 691-6, and 752-3, clustered with reference sequences of Avian Nephritis Virus from the United States, Korea, Australia, China, and Japan, and a second group of isolates (541-2, 541-12, and 541-16) were clustered with reference sequences of chicken astrovirus from India and the United States, showing high bootstrap values for each group of both types of astroviruses detected in this study (Figure 5).

Figure 5 – Phylogenetic tree for ANV and CAstV strains

Source: (DE LA TORRE, D. 2018).

Legend: The tree was inferred with the neighbor-joining statistical method and based on the partial sequence of the ORF-1b gene in both of them. Numbers along the branches refer to bootstrap values for 1000 replicates.

The three sequences that were positive for ARtV were analyzed with reference sequences from the United States, Peru, South Korea, Ireland, Germany, and Nigeria, all of them clustering with a high bootstrap value with the common ancestor. Reference sequences for groups D, F, and G rotavirus were used as external groups to show the differentiation of the positive samples from Avian Rotavirus A (Figure 6).

Figure 6 – Phylogenetic tree for ARtV strains

Source: (DE LA TORRE, D. 2018).

Legend: The tree was inferred with the neighbor-joining statistical method and based on the partial sequence of the NSP4 gene. Numbers along the branches refer to bootstrap values for 1000 replicates. Sequences of ARtV G, D, and F were used as out-group controls.

Isolate 806-1 clustered with reference sequences of AReo from Hungary. The branch lengths and low bootstrap values from the common ancestor for reference sequences from Hungary, the United States, and China, all show the magnitude of the genetic change among different isolates from these countries (Figure 7).

Figure 7 – Phylogenetic tree for AReo strains

Source: (DE LA TORRE, D. 2018).

Legend: The tree was inferred with the neighbor-joining statistical method and based on the partial sequence of segment S4. Numbers along the branches refer to bootstrap values for 1000 replicates. Sequence of Duck Reovirus was used as out-group control.

The three isolates corresponding to ChPV were analyzed phylogenetically with strains from different countries from America, Europe, and Asia (Figure 8).

Figure 8 – Phylogenetic tree for ChPV strains

Source: (DE LA TORRE, D. 2018).

Legend: The tree was inferred with the neighbor-joining statistical method and based on the partial sequence of NS gene. Numbers along the branches refer to bootstrap values for 1000 replicates. Sequence of Goose parvovirus was used as out-group control.

Isolate 786-1 clustered with an isolate of ChPV from Peru, whereas isolates 752-3 and 691-1 clustered in a different group, together with Brazilian isolates, but remained in the main group with all isolates of chicken parvovirus. Goose parvovirus was used in this tree as an external group. The phylogenetic trees were inferred using the neighbor-joining method (SAITOU; NEI, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are joined to the branches (FELSENTEIN, 1985). The tree was drawn to scale, with branch lengths in the same units as the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura-Nei method (TAMURA; NEI, 1993) and are in units of the number of base substitutions per site.

5.4 DISCUSSION

Many etiological agents, such as bacteria, fungi, parasites, mycotoxins, and viruses, are related to enteric diseases. Enteric viruses have been considered the main causative etiological agents of RSS and acute enteric disturbances that negatively affect the yield of commercial chickens (OTTO et al., 2006; KANG et al., 2012; KOO et al., 2013; ZSAK et al., 2013; NUNEZ et al., 2016). The most common viruses associated with the enteric diseases of chickens and turkeys include FAdV-I, ChPV, CAstV, ANV, IBV, AReo, and ARtV (ROUSSAN et al., 2012; METTIFOGO et al., 2014). Clinical signs reported for each sample used in this study were related to digestive problems (diarrhea and feed intake), respiratory signs, stunting, high mortality, and a decrease in egg and meat production, which is consistent with results for clinical signs of enteric viruses obtained in other studies around the world (JINDAL et al., 2010; PALADE et al., 2011; PANTIN-JACKWOOD et al., 2011; ROUSSAN et al., 2012; DEVANAY et al., 2016; FINKLER et al., 2016; NUÑEZ et al., 2016a). Molecular techniques such as PCR and NT sequencing were employed due to their wide use in the diagnosis of enteric viruses (PANTIN-JACKWOOD et al., 2006; KOO et al., 2013; METTIFOGO et al., 2014). The association of enteric virus with the age of broilers, breeders, and layers (Tables 10 and 11) showed that molecular diagnosis of these viruses can be performed at different stages of production, which can be useful in the control of vertical infections. For example, in our results, ChPV, ANV, and IBV were present in broilers in the first week of age (Table 14), constituting possible vertical transmission. A high number of enteric viruses were found in birds from all ages and in different stages of production, both in broilers and hens (Tables 10 and 11). Enteric viral infections mostly affect young birds, probably due to their immature intestine epithelium, which is the first organ affected during a viral infection. This results in poor feed absorption with the consequent general symptoms (JINDAL et al., 2009). FAdV-I was detected in young and old birds (Tables 10 and 11), corroborating results obtained previously (NIU et al., 2018), where the virus was found in several samples from broilers, layers, and domestic chickens of all ages, even though the clinical signs were more severe in younger birds (TORO et al., 2000). ChPV infections affect young birds in their first four weeks (ZSAK et al., 2013; FINKLER et al., 2016), which is supported by our findings since the virus was detected in 20 infected samples from broilers between 1 and 28 days old (Table 14). AReo was found in 15/333 detections,

corresponding to broilers in the growth phase (Table 14), especially between 8 to 28 days old, showing that rotavirus infections mainly affect young birds, promoting RSS and enteric lesions (OTTO et al., 2006). Both astroviruses, CAstV and ANV, were found in samples from young broilers, despite having nine positive samples from breeders older than 51 weeks old (Table 14). Astrovirus infections usually affect young birds (JINDAL et al., 2010), although these viruses can be found in older birds in single and multiple infections with other enteric viruses (MOURA-ALVAREZ et al., 2013). IBV affects birds of virtually all ages (NUÑEZ et al., 2013), as demonstrated by our results, where samples of broilers, layers, and breeders from one week and greater than 51 weeks (Table 14) presented positive results for coronavirus in all digestive organs tested, despite the fact that some clinical signs reported for these samples were respiratory signs. Enteric viruses such as FAdV-I, ChPV, CAstV, ANV, IBV, and ARtV were present in the liver, intestines, and pancreas. Caecal tonsils and cloacal swabs were used exclusively for diagnosis of IBV (Table 13). Viral particles of FAdV-I were found in the liver, intestines, and pancreas. The organs where this virus can be detected are the liver, intestines, caecal tonsils, proventriculus, bursa of Fabricius, thymus, spleen, lungs and kidneys (GOODWIN et al., 1993; OJKIC et al., 2008; ROMANOVA et al., 2009; ABSALON et al., 2017), especially in the first week of infection. Based on the pancreatic tissue samples that were positive for FAdV-I (5/28) (Table 13), we confirmed the importance of this organ for diagnosis of diseases caused by fowl adenovirus and even in cases of pancreatitis (SCHMIDT; REAVILL, 2014). ChPV was detected in the liver, intestines, and pancreas, which are areas commonly used for ChPV detection due its pathogenicity as an enteric virus, considering the variety of organs where this virus can be found, including the brain, duodenal loop, and even on cloacal swabs (MARUSAK et al., 2010; FINKLER et al., 2016; NUÑEZ et al., 2016b). Astroviruses, such as CAstV and ANV, are commonly detected in the digestive organs and fecal samples of sick and healthy birds (ZHAO et al., 2011; HUNGARICA et al., 2015; CHAMINGS et al., 2015), supporting our findings of astrovirus in the liver, intestines, and pancreas (Table 13). IBV can be detected in the intestinal content, trachea, lung, liver, bursa of Fabricius, pancreas, thymus, kidney, proventriculus, and spleen of affected birds (PANTIN-JACKWOOD, et al., 2008; FAN et al., 2012), according to the dynamic distribution of the virus, resulting in a wide range of useful organs for molecular detection of this virus, which in our case helped to determine the presence of the pathogen in liver, intestine, pancreas, cloacal swabs, and caecal

tonsils (Table 13). In our study, we found three different serotypes of FAdV-I, without ruling out the possibility of multiple FAdV infections in the same sample because multiple strains of different serotypes can affect to the same animal (NIU et al., 2018). Multiple infections between FAdV-I and other enteric viruses were found in our study, especially with ChPV, ANV, and IBV, supporting previous results showing that FAdV-I is often present in co-infections with other enteric viruses including CAstV, AReo, and ARtV (ROUSSAN et al., 2012). ChPV was present in co-infections with one to four viruses, including IBV, ANV, FAdV-I, and CAstV, which has also been reported in metagenomic (DEVANEY et al., 2016), and epidemiological surveys with broilers showing RSS (NUÑEZ et al., 2016b). The most common virus found in this study belongs to the family Coronaviridae, which indicates the presence of Infectious Bronchitis Virus in the processed samples. A high percentage of positive samples (60.7%) (Table 15), indicated the presence of single IBV infections, probably due to the absence of co-infections with other enteric viruses or because only IBV analysis was requested. IBV co-infections with multiple enteric viruses were demonstrated in 32 samples (Table 14), making it difficult to define the exact role of IBV in enteric diseases (DAY et al., 2015), especially in multiple viral infections. All of the enteric viruses detected during 2010 and 2017 are widely distributed around the world. FAdV-I, found in this study, is a common virus, widely distributed in Brazil and well-reported throughout the world (CHRISTENSEN; SAIFUDDIN, 1989; TORO, et al., 1999; PANTIN-JACKWOOD et al., 2008; LIM et al., 2011; MAARTENS et al., 2014; METTIFOGO et al., 2014; NICZYPORUK, 2016), demonstrating its wide distribution among commercial birds. The phylogenetic tree showed that ChPV has a well-conserved genome in non-structural genic regions, as evidenced by the branch sizes in comparison with reference sequences from GenBank, further demonstrating the distribution of ChPV around the world (ZSAK et al., 2008; DOMANSKA-BLICHARZ et al., 2012; KOO et al., 2015). Astroviruses affecting commercial flocks are also widely distributed worldwide (PANTIN-JACKWOOD et al., 2006; HEWSON et al., 2010; ZHAO et al., 2011). Phylogenetic analysis of astroviruses clustered ANV and CAstV in two different groups, and each of them was sub-grouped with the NT sequences of the RNA polymerase gene from isolates originating in North-America and Asia, providing a resource to determine the global distribution of these viruses. The ARtV analyzed in this study showed a close phylogenetic relationship with isolates from the United States, Peru, South Korea, Ireland, Germany, Nigeria, and Brazil, particularly in the

length of the branches in the phylogenetic tree, which may confirm the complete distribution of the virus around the world (JINDAL et al., 2010; KOO et al., 2013; JINDAL et al., 2014; FUJII et al., 2016; PAULY et al., 2017). Coronaviruses such as IBV are some of the most widely distributed enteric viruses in Brazil and have attracted special interest from the poultry industry due the economic losses caused by its impact on the health of birds (CAVANAGH 2007; SEGER et al., 2016; HUTTON et al., 2017). The geographical distribution of the enteric viruses analyzed in this study was reported previously in some regions from the Brazilian territory. According to this study, FAdV-I was disseminated in the states of Paraíba, Pernambuco, São Paulo, and Santa Catarina, in addition to previously reported isolates of FAdV-I and ChPV from the state of Rio Grande do Sul (LIMA et al., 2017). CAstV was found in samples from Minas Gerais, and the other member of the family, Astroviridae (ANV), was found in samples from Ceará, Paraíba, Pernambuco, Bahia, Minas Gerais, and São Paulo, increasing the previously reported distribution of astroviruses in the states of Rio Grande do Sul, Paraná, and Mato Grosso (ESPINOZA et al., 2016a, 2016b). ChPV was distributed in the states of Ceará, Paraíba, Pernambuco, Bahia, Minas Gerais, and São Paulo, supporting the results of (NUÑEZ et al., 2016b), who reported the presence of this virus in an outbreak in São Paulo, and expanding upon the report of (FINKLER et al., 2016), who worked with isolates of ChPV from poultry farms in the state of Rio Grande do Sul. The ARtV results suggest that this virus was disseminated in the states of Pernambuco, Minas Gerais, and São Paulo, consistent with other previous reports that describe the presence of ARtV in the states of Minas Gerais, Paraná, Rio Grande do Sul, and Goiás (RIOS et al., 2012; BESERRA et al., 2014). IBV is the most disseminated virus among Poultry flocks in Brazil (VILLARREAL et al., 2007). This virus requires more specific analysis for the characterization of the different genotypes that affect commercial chickens in Brazilian states and other countries in South America, to determine the epidemiological dynamics and pathogenesis of the virus within the poultry industry.

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6 CONCLUSIONS

Avian adenovirus has been considered a secondary pathogen that affects birds, especially when they suffer from immunosuppressive diseases such as chicken anemia virus infection or Gumboro disease, and it has also been identified in visceral tissues of animals with runting-stunting syndrome and even in healthy animals. For this reason, it is difficult to understand the exact role of this virus in coinfections with multiple pathogens, as in animals with low adenovirus infection levels. Nevertheless, it is important to recognize the existence of circulating adenovirus in poultry farms in Brazil, as well as the serotypes that somehow help precede possible outbreaks of adenovirus-related diseases and have promising use in prevention and control regimens for this pathogen.

Despite the extensive knowledge about molecular techniques and the presence of pathogens associated with enteric diseases in commercial birds, in Ecuador, the importance of the diagnosis, prevention and control of other pathogens outside the conventional ones included in vaccination programs has not been considered. For this reason, it is important to report for the first time the presence of at least one of the viruses associated with malabsorption problems in birds using molecular biology and bioinformatics approaches, which will surely mark the beginning of enteric virus investigations by universities in Ecuador.

The avian encephalomyelitis virus mainly affects the central nervous system of birds; however, it was included in this study due to its important mechanism of transmission through the oral route. In our analysis, no differences were found in one of the structural genes in the virus genome between vaccine viruses and field sample viruses, which this author concludes was the result of improper practice in the routine vaccination of laying hens, which caused transmission of the live vaccine virus through human or environmental vectors to other broiler farms, in turn causing outbreaks of encephalomyelitis in this region of Brazil, with no new cases being reported so far in the Ornithopathology Laboratory.

Based on the collection of samples from different states and regions of Brazil, it can be concluded that the most common enteric viruses reported in commercial birds around the world are also circulating among Brazilian farms throughout the country. This information helps to understand the need to increase the prevention and control

of avian diseases, which includes monitoring the vertical transmission of viral pathogens and passive antibodies, and the development of vaccines to reduce field outbreaks and increase or at least not affect the zootechnical parameters that today's birds are capable of producing. Finally, all the information available in this work, and the data uploaded to GenBank, are of great importance from an epidemiological point of view and will help to understand the dynamics of current viral strains, their geographical distribution, pathogenicity and subsequent genetic evolution, facing the constant challenges in the molecular characterization of enteric viruses around the world.

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APPENDIX 1

LIST OF ABBREVIATIONS

°C	Degrees Celsius
μl	Microliters
μM	Micromolar
10X	Ten times higher in the solute concentration
5X	Five times higher in the solute concentration
AA	Amino acid
AE	Avian encephalomyelitis
AEV	Avian encephalomyelitis virus
ANV	Avian nephritis virus
AReo	Avian reovirus
ARtV	Avian rotavirus
bp	Base pair
BR	Brazil
CA	Canada
cDNA	Complementary deoxyribonucleic acid
CH	China
ChPV	Chicken parvovirus
DAdV	Duck adenovirus
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiotreitol
EC	Ecuador
EDTA	Ethylenediamine Tetraacetic
FAdV	Fowl adenovirus
FTA	Flinders Technology Associates
g	Gravitational acceleration (9.8 m/s ²)

GO	Goias
HR	Croatia
HS	Hydropericardium syndrome
HU	Hungary
IBH	Inclusion body hepatitis
IBV	Infectious bronchitis virus
Kb	Kilobase
KR	South Korea
M	Molar
MAS	Malabsorption syndrome
min	Minutes
ml	Milliliters
mM	Millimolar
NCBI	National center for biotechnology Information
ng	Nanograms
NS	Non-structural
NT	Nucleotide
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Potential of Hydrogen
PL	Poland
PR	Parana
RNA	Ribonucleic acid
RSS	Runting-stunting syndrome
RT	Reverse transcription
RT-PCR	Reverse transcription – polymerase chain reaction
s	Seconds
SC	Santa Catarina
SP	São Paulo

U	Unit of enzyme's catalytic activity
UK	United Kingdom
US	United States
USP	University de São Paulo
UTR	Untranslated region
UV	Ultraviolet

ATTACHMENT 1

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Atenciosamente,

Irenilza de Alencar Nääs
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 A OBTAINED IN CASES OF AVIAN ENCEPHALOMYELITIS (AE)
 OUTBREAKS IN BRAZIL. Brazilian Journal of Poultry
 Science. DOI: 10.1590/1806-9061-2018-0744

On Mon, Sep 16, 2019 at 10:35 AM Antônio José Piantino Ferreira <ajpferr@usp.br> wrote:

Prezada Profa. Irenilza - Editora da revista Brazilian Journal of Poultry Science:
 Vimos por meio deste solicitar a V. Sa. a autorização para utilizar os seguintes trabalhos
 científicos, publicados na revista BJPS sob nossa autoria, como parte da tese de doutorado
 do aluno DAVID DE LA TORRE, como descrito abaixo. Esta solicitação se refere às normas
 vigentes na USP para uso de trabalhos científicos publicados pelos autores para uso em
 teses da Universidade. Esperamos contar a atenção de V. Sa. referente à autorização de uso.
 Atenciosamente,

MOLECULAR DIAGNOSTIC OF CHICKEN PARVOVIRUS (ChPV)
 AFFECTING COMMERCIAL FLOCKS IN ECUADOR. Brazilian Journal of
 Poultry Science. DOI: 10.1590/1806-9061-2018-0730

- DETECTION AND MOLECULAR CHARACTERIZATION OF
 TREMOVIRUS A OBTAINED IN CASES OF AVIAN ENCEPHALOMYELITIS
 (AE) OUTBREAKS IN BRAZIL. Brazilian Journal of Poultry
 Science. DOI: 10.1590/1806-9061-2018-0744

Irenilza A. Nääs

"Imagination is the highest form of research." Albert Einstein

ATTACHMENT 2

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David De la Torre <daviddelatorreduque@gmail.com>

Authorization to use a manuscript

Antônio José Piantino Ferreira <ajpferr@usp.br>

16 de septiembre de 2019, 11:17

Para: pbutaye@rossvet.edu.kn

Cc: DAVID DE LA TORRE <daviddelatorreduque@gmail.com>, lin.li@mdpi.com

Dear Prof. Patrick Butaye (Editor-in-Chief), on behalf of manuscript's authors of manuscript published in the Veterinary Science - ENTERIC VIRUS DIVERSITY DETECTED BY MOLECULAR METHODS IN BRAZILIAN POULTRY FLOCKS, Veterinary Sciences. DOI: 10.3390/vetsci5020038 - we would like to obtain an authorization to use this work as part of a PH.D. thesis at the University of São Paulo. This work will be used as part of requirements for getting PH.D. degree of an author of work - DAVID DE LA TORRE. I hope to obtain of you as soon as possible an authorization for using this work as part of thesis. I appreciate for your attention to our request.

Best regards.

Antonio Piantino - Corresponding author.

David De la Torre <daviddelatorreduque@gmail.com>

Authorization to use a manuscript

Antônio José Piantino Ferreira <ajpferr@usp.br>

16 de septiembre de 2019, 11:59

Para: DAVID DE LA TORRE <daviddelatorreduque@gmail.com>

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From: Butaye, Patrick <pbutaye@rossvet.edu.kn>

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Subject: RE: Authorization to use a manuscript

To: Antônio José Piantino Ferreira <ajpferr@usp.br>

Cc: DAVID DE LA TORRE <daviddelatorreduque@gmail.com>, lin.li@mdpi.com
<lin.li@mdpi.com>

Dear Colleague, Dear Antonio,

It is clear that the manuscript can be used as a part of a PhD Thesis as it is the scientific merit of the team.

Best wishes

Patrick

Dr. Patrick Butaye, DVM, PhD

Professor of Microbiology

veterinary.rossu.edu

Connect with RUSVM:

PO Box 334 | Basseterre, St. Kitts | West Indies

ATTACHMENT 3

Co-authors authorization to use the articles in this these

São Paulo, September 19th, 2019.

We hereby authorize David Isaías De la Torre Duque, to use the article **“Molecular characterization of fowl adenovirus group I in commercial broiler chickens in Brazil”**, DOI: 1007/s13337-018-0430-z, in the doctoral thesis called **“Detection and molecular characterization of enteric viruses in commercial birds”**.

Luis F Nuñez Naranjo

Silvana H Santander Parra

Antonio J Piantino Ferreira

Claudete Astolfi Ferreira

São Paulo, September 19th, 2019.

We hereby authorize David Isaías De la Torre Duque, to use the article **“Enteric Virus Diversity Examined by Molecular Methods in Brazilian Poultry Flocks”**, DOI: 10.3390/vetsci5020038, in the doctoral thesis called **“Detection and molecular characterization of enteric viruses in commercial birds”**.

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Antonio J Piantino Ferreira

Claudete Astolfi Ferreira

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We hereby authorize David Isaías De la Torre Duque, to use the article **“Detection by Rt-PCR and Molecular Characterization of Tremovirus A Obtained from Clinical Cases of Avian Encephalomyelitis (AE) Outbreaks in Brazil”**, DOI: 10.1590/1806-9061-2018-0744, in the doctoral thesis called **“Detection and molecular characterization of enteric viruses in commercial birds”**.

Luis F Nuñez Naranjo

Silvana H Santander Parra

Antonio J Piantino Ferreira

Claudete Astolfi Ferreira

São Paulo, September 19th, 2019.

We hereby authorize David Isaías De la Torre Duque, to use the article **“Molecular Diagnostic of Chicken Parvovirus (ChPV) Affecting Broiler Flocks in Ecuador”**, DOI: 10.1590/1806-9061-2018-0730, in the doctoral thesis called **“Detection and molecular characterization of enteric viruses in commercial birds”**.

Luis F Nuñez Naranjo

Silvana H Santander Parra

Byron H Puga Torres

Antonio J Piantino Ferreira

Claudete Astolfi Ferreira