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Molecular characterization of fowl adenovirus in commercial chicken flocks in Ecuador

Caracterización molecular del adenovirus aviar en pollos comerciales del Ecuador

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Abstract

Fowl Adenovirus Group I (FAdV-I) is considered an important pathogen in the poultry industry in Ecuador and worldwide. Inclusion Body Hepatitis (IBH) and Hydropericardium Syndrome (HPS) are the main diseases associated with this virus. Despite being an etiological agent that is usually related to immunosuppressive diseases such as Chicken Anemia Virus or Gumboro disease, recent studies have demonstrated the pathological activity of FAdV-I as the primary responsible for certain diseases. Since the 1990s, several outbreaks of IBH and HPS have been reported in many Latin American countries including Ecuador, identifying the serotype 4 of FADV-I as the causal agent of these outbreaks. The aim of this study was to characterize by the Polymerase Chain Reaction (PCR) and first-generation sequencing, the FAdV-I serotypes circulating in commercial poultry farms in Ecuador. Several organs of broilers with enteric problems were collected from 13 farms in the northern area of Ecuador, and subsequently impregnated in FTA cards for transport and processing. 4/13 (30.8%) samples were positive for FAdV-I, and by sequencing and bioinformatic analysis of DNA amplified by PCR, serotypes 6 and 11 of FAdV-I were characterized. These data show us the variety of serotypes circulating in poultry farms in Ecuador that could be influencing the health status of the country's poultry industry.
Keywords: Fowl Adenovirus, Inclusion body hepatitis, Hidropericardium syndrome

Resumen

El Adenovirus Aviar del grupo I (FAdV-I), es considerado un patógeno de mucha importancia en la industria avícola en el Ecuador y a nivel mundial. La Hepatitis por Cuerpos de Inclusión (IBH) y el Síndrome del Hidropericardio (HPS), son las principales enfermedades asociadas a este virus. A pesar de ser un agente etiológico que por lo general se encuentra relacionado a enfermedades inmunodepresoras como la Anemia Infecciosa de las Aves o la enfermedad de Gumboro, recientes estudios han demostrado la actividad patológica del FAdV-I como responsable primario de estas enfermedades. Desde la década de 1990, se reportaron varios brotes importantes de IBH y HPS en muchos países de América Latina incluyendo el Ecuador, logrando identificar principalmente al serotipo 4 del FAdV-I como agente causal de estos brotes. El objetivo de este estudio fue caracterizar por medio de la Reacción en Cadena de la Polimerasa (PCR) y secuenciamiento de primera generación, los serotipos del FAdV-I que se encuentran circulando en granjas de aves comerciales en el Ecuador. Para esto fueron colectados órganos de pollos de engorde con problemas entéricos de 13 diferentes granjas a lo largo de la zona norte del Ecuador, y posteriormente fueron impregnados en tarjetas FTA para su transporte y procesamiento. 4/13 (30,8%) muestras fueron positivas a FAdV-I y mediante el secuenciamiento y análisis bioinformático del ADN amplificado por la PCR, se caracterizaron los serotipos 6 y 11 del FAdV-I. Estos datos demuestran la variedad de serotipos del FAdV-I presentes actualmente en granjas avícolas del Ecuador que podrían estar influenciando en el estado sanitario de la industria avícola del país.
Palabras clave: Adenovirus Aviar, Hepatitis por Cuerpos de Inclusión, Síndrome del Hidropericardio

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1 Introduction

Fowl adenovirus (FAdV) is a virus classified within the adenovirus genus in the Adenoviridae family (King *et al*., 2012). This genus has three groups classified as: FAdV-I (Cause of HPS: Hydropericardium syndrome and IBH: Hepatitis per inclusion bodies), FAdV-II (causal agent of HEV: Turkey hemorrhagic Enteritis) and FAdV-III (causal agent of EDS: Low posture Syndrome) (Hess, 2000). FAdV-I has been classified into 5 species (A-E) and 12 serotypes (1–8a, 8b–11) (Adams *et al*., 2017). The viral particle has icosahedral form that lacks of capsule and has a diameter of 70 to 90 nm. The viral genome consists of a double DNA strand with a size of approximately 45 KB (Jiang *et al*., 1999). The design of the viral capsule consists of the union of 252 capsomers, being 240 formed by the protein Hexon and the remaining 12 by the protein Penton. Two fibers of different size are released from each vertex in which is located the Penton protein, which is formed by proteins (fiber proteins) that give the antigenic capacity to the virus (Adair y Fitzgerald, 2008).

FAdV-I has been associated with two major diseases present in commercial birds, Inclusion Bodies Hepatitis (IBH) which was first described in 1963 in the United States (Helmboldt y Frazier, 1963), and currently associated with 12 Serotypes of FAdV-I, and Hydropericardium syndrome (HPS), which is a disease that mainly affects young broilers from 3 to 6 weeks old, and has been associated with FAdV-I serotype 4 (Balamurugan y Kataria, 2004). Currently, the virus is distributed around the world (Alemnesh *et al*., 2012), being responsible for producing a decrease in the food consumption, and increase in food conversion and mortality, and although there is the association of IBH and HPS with immunosuppressive diseases such as Bursa Infectious Disease (IBV) and Infectious Bird Anemia (CAV), FAdV-1 has been detected as a primary agent in the development of IBH and HPS without the presence of other concomitant diseases (Sentíes-Cué *et al*., 2010). Its contagion form is mainly by mouth, although vertical transmission is an important contagion form due to a poorly developed immune system of the progeny (Toro *et al*., 2001; Nakamura *et al*., 2011).

Hepatitis outbreaks by inclusion bodies have been reported in several Latin American countries such as Brazil, Chile, Peru, Ecuador and Mexico (Hess, 2000; Toro *et al*., 2001). Mazaheri *et al*. (1998) reported the presence of FAdV-I serotype 4 in Ecuador, being isolated from hepatic tissues in birds with HPS and characterized by neutralization tests and restriction enzyme analysis in viral DNA (Toro *et al*., 2000)). Given the global distribution of all serotypes of this virus it is important to know the geographic distribution of each serotype in Ecuador; therefore, the objective of this study focuses on the molecular characterization of the FAdV-I currently circulating in the commercial poultry farms of Ecuador in order to have the scientific knowledge that allows to make decisions and to apply sanitary measures for the benefit of the poultry industry of the region.

2 Methodology

2.1 Viral samples

In 2016 biological samples were collected from different farms in the northern area of the Ecuadorian Highlands, whose birds presented symptoms related to hepatitis, malabsorption, increased conversion and increased mortality. The collected samples were taken by imprint on 13 Whatman FTA cards (GE Healthcare Company, Little Chalfont, Buckinghamshire, UK) of organs such as kidneys, Tracheae, Bursa of Fabricio, lungs and tonsils of birds from 4 to 7 weeks old, and were then transported to the Ornitho-pathology laboratory of the Faculty of Veterinary Medicine and Animal Science of Universidad do São Paulo in Brazil, where the corresponding molecular analyses were carried out. Each FTA card was impregnated with 2 to 4 samples of different organs and different birds, which corresponded to 13 different farms.

2.2 Maceration and viral extraction

The material was processed into a laminar flow, disinfected with alcohol at 70° and direct exposure to ultraviolet light (UVC) for 15 minutes. All of the material impregnated on each FTA card was cut off for the final obtaining of 13 different samples that were placed in 2 ml microtubes with PBS (Phosphate Bufered Saline), 0.1 M; PH 7.4 in 1:1 proportion. Maceration was carried out using the Tissuelyser LT Bead Mill (Qiagen, Hilden, Germany) at 50 oscillations per second for 5 minutes.

The macerated product was centrifuged for 30 minutes at 12000 x g and at 4 °C (Koo *et al*., 2013).

Finally, 200 µL of the supernatant was collected for isolating the genetic material.

2.3 DNA extraction and polymerase chain reaction (PCR)

For the extraction of the genetic material with phenol and chloroform, the methodology described by Chomczynski (1993) was used. For the PCR reaction initiators designed by Meulemans *et al*. (2001) were used which cover part of the preserved regions called Pedestal 1 (P1) adjacent to the variable region called Loop 1 (L1) in the nucleotides sequence of the Hexon gene, allowing to amplify the 12 different serotypes of FAdV-I.

The mix of 25 µL for the polymerase chain reaction (PCR) consisted of 1X PCR free magnesium buffer, 1.25 mm of each deoxyribonucleotides triphosphate, 0.5 mm of each initiator, 1.25 U of Platinum® Taq polymerase (Invitrogen® by Life Technologies, Carlsbad, CA), and 2.5 µL of DNA extracted at an approximate concentration of 1000 ng/µL. The amplification conditions were as follows: 94 °C for 5 minutes, followed by 35 cycles of 95 °C for 1 minute, 52 °C for 45 seconds and 72 °C for 1 minute, ending with a final extension of 72 °C for 10 minutes. The final product was electrophoresis using agarose gel at a concentration of 1.5% to verify the amplification of DNA fragments with a size of approximately 897 pb.

2.4 Purification and sequencing

The amplified product was purified using the GPXTM PCR DNA and Gel Band purification kit (GE Healthcare, Piscataway, New Jerser, USA) according to the manufacturer's instructions. Each purified product was sequenced in Senso and Antisenso using the BigDye® Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems by Life Technologies, Carlsbad, California, USA). The final reactions were analyzed in an ABI 3730 DNA Analyzer (Applied Biosystems by Life Technologies, Carlsbad, California, USA).

2.5 Phylogenetic analysis

Nucleotide sequences were edited using CLC Main Workbench 7 software, and aligned with the CLUSTAL W method available in CLUSTAL X 2.0 software. The phylogenetic tree was inferred using the maximum likelihood method based on the Tamura 3-parameter model (Tamura, 1992), with 1000 repetitions, available in the software Mega 7. For this, the nucleotide sequences generated in this study were used as well as international sequences of FAdV-I serotypes 6 and 11 taken from the GeneBank (National Center for Biotechnology Information). The reference sequences for the alignment and construction of the phylogenetic tree were taken from Brazil, Mexico, United States, Canada, United Kingdom, Europe, Belgium, China, Japan, South Korea and India, whose access numbers are described in the Figure 1.

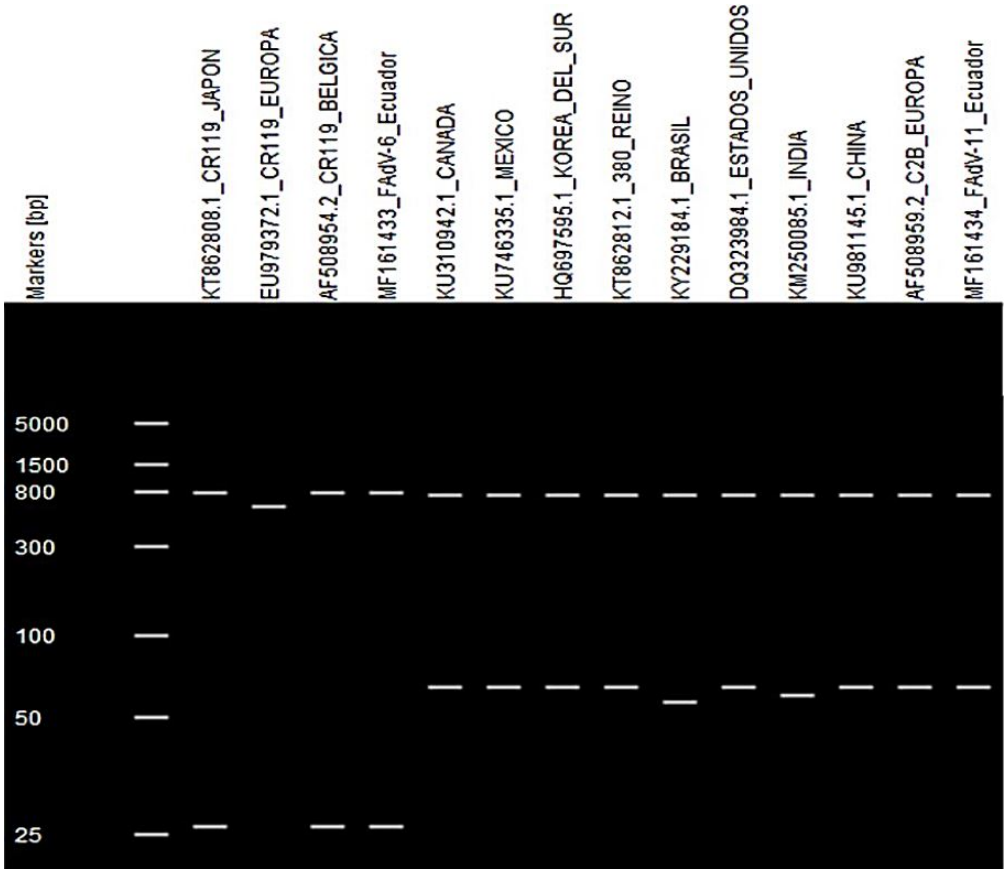

Figure 1. Virtual gel designed by CLC Main Workbench 7.8.1 software, after the action of PstI and EcoRI restriction enzymes on the sequences of FAdV-I Serotypes 6 and 11.

3 Results and discussion

3.1 Polymerase chain reaction (PCR)

The PCR final product for the 13 samples was submitted to agarose gel electrophoresis at 1.5%, with a molecular weight marker of 100 PB (100 bp DNA Ladder, InvitrogenTM), finding 4/13 positive results (30.8%), showing the bands at the approximate height of 897 pb, which were analyzed for the characterization of the respective serotype (Mettifogo *et al*., 2014; Joubert *et al*., 2014; De la Torre *et al*., 2018). According to Meulemans *et al*. (2001), DNA amplified by this reaction may include 12 FAdV-I serotypes, without being able to directly determine the specific serotype, unless restriction enzymes are used to obtain DNA bands in agarose gel with specific sizes for each serotype (Figure 2) (Meulemans *et al*., 2001), or by analyzing nucleotide sequences when compared to sequences stored in the GeneBank database. Through a virtual analysis of the use of restriction enzymes, it was determined that the use of the PstI enzyme caused a DNA cut at the recognition site (CTGCAG) of the 808 pb sequences of the CR119strain of Japan, Belgium and USP-EC-01, resulting in two bands of 786 pb and 26 pb (Figure 2), except CR119 strain of Europe, which did not demonstrate available regions for the action of any restriction enzyme by only having 596 pb. An additional EcoRI enzyme performed DNA cuts at the recognition site (GAATTC) of the other sequences characterized as FAdV-I serotypes 11, including the sequence USP-EC-02, resulting in two bands of 746 bp and 63bp (Figure 2).

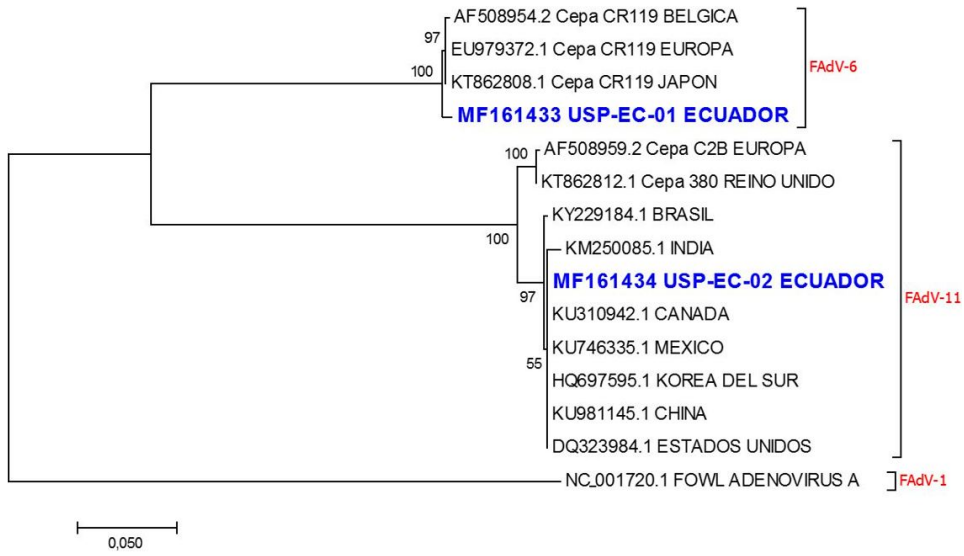

Figure 2. Phylogenetic tree inferred using the Maximum Likelihood method based on the model Tamura 3-parameter (Tamura, 1992). The sample sequences from Ecuador are represented in blue and contain their corresponding access code in the GeneBank database. (FAdV = Fowl Adenovirus). The NC_001720.1 sequence was placed as an external group.

3.2 Sequencing

Sequencing of the 4 positive products of the PCR resulted in the obtaining of 2 different sequences, and since three samples had exactly the same sequences, a consensus was proposed to name it USP-EC-02, and the sequence of the fourth sample was called USP-EC-01. The sequences obtained were entered into the GenBank database with the MF161433 access codes for the USP-EC-01 sequence, and MF161434 for the USP-EC-02 sequence. According to the identity matrix, the nucleotide sequences (NT) and amino acids (AA) of USP-EC-01 have a high identity percentage with the sequences CR119 strains of FAdV-I serotype 6 of Belgium (99.1% of NT and AA), Europe and Japan (99.3% of NT and AA). The NT and AA sequences of USP-EC-02 have a high identity percentage with the sequences of FAdV-I serotype 11 of Mexico, United States, Canada, South Korea and China (100% NT and AA), Brazil (99% NT and AA), India (98.3% NT and AA) , UK (97.5% NT and AA), and Europe (97.3% NT and AA).

3.3 Phylogenetic analysis

The results of the phylogenetic analysis are shown in Figure 2. All the sequences analyzed were grouped into two major groups corresponding to serotypes 6 and 11 of FAdV-I. The sequence USP-EC-01 was grouped to the strain CR119 belonging to FAdV-I serotype 6, registered as originated in Belgium, Europe and Japan, demonstrating the presence of this viral strain in at least 3 continents (Europe, Asia and America). The USP-EC-02 sequence was grouped to sequences from Europe, UK, Brazil, India, Canada, Mexico, South Korea, China and the United States, which have been characterized by FAdV-I serotype 11.

FAdV-1 is responsible for causing two major diseases, including Inclusion Bodies Hepatitis (IBH) and Hydropericardium syndrome (HPS) (Zhao *et al.*, 2015). There are some cases in which the virus has been isolated from birds without signs of disease, so it requires more development of techniques that allow differentiating pathogenic and non-pathogenic strains within the same virus serotype (Absalón *et al.*, 2017). In Ecuador, the presence of FAdV-I serotype 4 has already been reported as a causal agent of Hydropericardium syndrome (Mazaheri *et al.*, 1998), but it is also important to emphasize that all serotypes are related to IBH (Adair y Fitzgerald, 2008); therefore, and epidemiological control of all strains and serotypes circulating in the region must be maintained.

FAdV-I serotype 6 belongs to species E along with serotypes 7, 8a and 8b, being able to have so-

me cross-protection degree with vaccines against FAdV-I existing in Ecuador, which use inactivated serotype 8 viruses, although this has not been yet demonstrated in any experimental study. Serotypes 8b and 11 caused liver lesions at a macro and microscopic level, after being experimentally inoculated visually, and without the presence of immunosuppressive viruses (Steer *et al*., 2015), noting that there are no specific vaccines developed for serotype 11 that are marketed in Ecuador. With these results, it is important to consider the need of the isolation and experimental study of serotypes found in Ecuador with the aim of understanding the epidemiological dynamics and pathogenicity of all circulating FAdV-I strains in the country.

4 Conclusions

This study showed the presence of Serotypes 6 and 11 of Fowl Adenovirus in commercial poultry lots of Ecuador. This provides a contribution to the scientific community for the development of new studies that allow knowing the pathophysiology of circulating strains and if necessary the creation of vaccines to help control the propagation and/or negative effect of these viruses on poultry production in different regions of the country.

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