

Case Report

First Molecular Detection and Characterization of Fowl Aviadenovirus Serotype 11 from Broiler Chickens in Chile

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Abstract: Fowl aviadenovirus (FAdV) is a member of the Aviadenovirus genus within the *Adenoviridae* family. FAdVs are divided into five species based on genomic differences: Fowl aviadenovirus A to Fowl aviadenovirus E (FAdV-A to FAdV-E). They are classified into twelve serotypes (FAdV-1 to FAdV-8a and FAdV-8b to FAdV-11) through cross-neutralization tests. FAdVs are mainly associated with hepatitis hydropericardium syndrome (HHS), adenoviral gizzard erosion (AGE), and inclusion body hepatitis (IBH). The serotypes commonly involved in IBH are FAdV-2, FAdV-11, FAdV-8a, and FAdV-8b. IBH causes significant economic losses in the poultry industry, mainly due to high mortality, reduced productivity, and immunosuppression. This is the first case report on IBH in Chile caused—according to post-mortem findings, molecular analysis, sequencing, and phylogenetic analysis—by FAdV-11. Since the serotype had not previously been reported in Chile, continued monitoring of IBH cases is required to determine the serotype of the circulating FAdVs and adapt preventative vaccination programs.

Keywords: fowl aviadenovirus; hexon gene; inclusion body hepatitis; serotype 11; poultry



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1. Introduction

The *Adenoviridae* family includes non-enveloped viruses with linear dsDNA genomes of 25–48 kb and icosahedral capsids. The capsid comprises 240 non-vertex (hexon) and 12 vertex capsomers (penton). The *Adenoviridae* family is currently divided into the following six genera: mastadenovirus, testadenovirus, atadenovirus, siadenovirus, ichtadenovirus, and aviadenovirus. Belonging to the aviadenovirus genus [1], Fowl aviadenovirus is divided into five species based on genomic differences (FAdV-A to FAdV-E) and classified into twelve serotypes (FAdV-1 to FAdV-8a and FAdV-8b to FAdV-11) through cross-neutralization tests [2].

The hexon gene consists of the variable loop regions, L1, L2, and L4, and the conserved pedestal regions, P1 and P2. The sequencing variable regions (right and left flank) of the hexon gene identify adenoviruses at the species level and classifies them into genotypes, which mostly correspond to serotypes [3]. Phylogenetic and high-resolution melt analyses of the L1 region of the hexon gene can classify fowl aviadenovirus into twelve genotypes that mostly correspond to well-established serotypes [4]. The hexon protein is involved in producing neutralizing antibodies, hemagglutination, pathogenicity, and viral infectivity, making it a virulence factor [3].

Aviadenovirus is distributed worldwide and transmitted both vertically and horizontally. It infects a wide variety of hosts, including ducks, chickens, falcons, birds of prey, ostriches, and parrots. In chickens, aviadenoviruses are associated with avian adenoviral

splenomegaly (AAS), egg drop syndrome (EDS), adenoviral gizzard erosion (AGE), hepatitis hydropericardium syndrome (HHS), and inclusion body hepatitis (IBH) [5]. IBH causes substantial economic losses to the poultry industry, given the high mortality, reduced productivity, and immunosuppression [6]. While it usually affects birds between 3 and 5 weeks old [5], IBH has been reported in chicks less than 7 days old. IBH outbreaks commonly result in 10% of the overall mortality and, on rare occasions, up to 30% [7]. IBH was first detected in broiler chicken flocks with high mortalities in the USA in 1963 [8] and was known to occur as a secondary disease, particularly due to the presence of immunosuppressive agents such as the chicken anemia virus (CAV) [9] or infectious bursal disease virus (IBDV) [10]. Consequently, FAdVs were considered secondary agents. However, IBH outbreaks without predisposing agents have also been reported [11]. Though all FAdV serotypes cause the disease, the most identified are FAdV-D serotypes 2 and 11 and FAdV-E serotypes 8a and 8b [5].

IBH cases have recently been reported in Morocco [12], Japan [13], Spain [14], India [15], Poland [16], Greece [17], Palestine [18], and Bangladesh [19]. The disease has also been reported in Germany, France, South Africa [20], and the USA [21]. In South America, IBH cases have been reported in Peru [22], Brazil [23], and Ecuador [24]. In Chile, an outbreak of inclusion body hepatitis/hydropericardium syndrome associated with FAdV-C serotype 4 was reported in 1999 using virus neutralization tests (VNTs) and restriction enzyme analysis of a DNA fragment. This case report is the first on inclusion body hepatitis in Chile that was caused—according to post-mortem findings, molecular analysis, sequencing, and phylogenetic analysis—by the FAdV-D serotype 11.

2. Materials and Methods

The Universidad de Chile's Avian Pathology Laboratory received birds between 4 and 6 days old that were suspected of having IBH from four broiler chicken farms in Chile's Metropolitan Region. The farmed flocks had experienced sudden mortality of approximately 10% without previous associated clinical signs. The birds were euthanized by cervical dislocation, and post-mortem examination revealed pale, enlarged, friable livers with whitish necrotic foci and a reticular pattern. No other significant injuries were observed.

Liver samples were aseptically collected and divided up for molecular and microbiological analysis. For molecular analysis, the samples were sliced and homogenized in 10% phosphate-buffered saline (PBS; pH 7.2) containing 200 U/mL of penicillin and 0.2 mg/mL of streptomycin. The homogenates were vortexed for 10 s, subjected to three freeze-thaw cycles, and then centrifuged at $3000 \times g$ for 20 min at 4 °C. The supernatant was filtered through a 0.45 µm filter, transferred to a sterile tube, and preserved at −80 °C. For microbiological analysis, samples were cultured in tryptone soy agar with 5% blood and MacConkey agar, then incubated in 5% CO₂ at 37 °C for 24 h.

Viral DNA was extracted using a PureLink™ Viral RNA/DNA Mini Kit (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. DNA was eluted in 50 µL of nuclease-free water. For viral identification, the DNA samples underwent a polymerase chain reaction following the method described by Meulemans et al. (2001) [25]. For the phylogenetic analysis, the DNA samples underwent a polymerase chain reaction following the method described by Raue and Hess (1998) [26]. The sequences of the primers used are indicated in Table 1. PCR was used to amplify a portion of the hexon gene, covering loops 1 and 2, in a final volume of 25 µL containing 0.5 µmol of each primer, 2.5 µL of 10× buffer, 1.25 mM dNTP Mix, 0.75 µL of 50 mM MgCl₂, 2.5 U of DNA polymerase (Taq Platinum™, Invitrogen, Waltham, MA, USA), 2.5 µL of extracted DNA, and ultrapure water to bring the total volume to 25 µL. The following conditions were used for PCR: one 5 min cycle of initial denaturation at 94 °C, followed by 35 1 min cycles of denaturation at 94 °C, a 1 min annealing at 60 °C, a 1 min 30s extension at 72 °C, and a final 7 min extension at 72 °C. The PCR products were analyzed by 1% agarose gel electrophoresis using GelRed®

Nucleic Acid Stain (Millipore, Burlington, MA, USA) and visualized by Trans Lum SOLO transilluminator (Biotop, Jing'an District, Shanghai, China).

Table 1. Primers that were used in this study.

Primers	Sequence (5' to 3')	Position ¹	PCR Product (bp)	Reference
Hexon A	CAARTTCAGRCAGACGGT	144–161	897	[25]
Hexon B	TAGTGATGMC GSGACATCAT	1041–1021		
H1	TGGGACATGGGGGCGACCTA	296–314	1219	[26]
H2	AAGGGATTGACGTTGTCCA	1514–1496		

¹ Position on the FAdV1 hexon gene.

The amplified PCR products from the liver samples were purified using a Pure-Link™ Quick Gel Extraction Kit (Invitrogen, USA) according to the manufacturer's instructions. Austral Omics (Valdivia, Los Ríos Region, Chile) provided sequencing services. Raw nucleotide sequences of the FAdV hexon genes were edited, aligned, and used for phylogenetic analysis. The sequences from each sample were assembled using Bioedit v7.2.5. Twenty-seven hexon gene sequences belonging to the main FAdV serotypes were retrieved from GenBank and included in the analysis, along with the four sequences from this case. The datasets were aligned using MAFFT v.7 online software (<http://mafft.cbrc.jp/alignment/server/large.html>) (accessed on 3 April 2024). A maximum-likelihood tree was created using PhyML 3.0, and a bootstrap expectation (TBE) process with 1000 replicates supported the robustness of the nodes. The tree was visualized and edited in the FigTree v. 7.4.4 software. The divergence of the Chilean field isolates in relation to the five FAdV species was estimated using MEGA X. analyses with the maximum composite likelihood model.

3. Results

The birds presented pale, enlarged, and friable livers with whitish necrotic foci and a reticular pattern (Figure 1). Pathogenic bacteria were not isolated from the liver samples. Agarose gel electrophoresis of the PCR products revealed amplified DNA products of 897 bp in the DNA samples from the livers of the birds from the four affected farms (Figure 2), confirming fowl adenovirus as a causal agent of IBH in broiler chickens in Chile. Genetic analysis based on the hexon gene sequence showed a 7.6% divergence between the strains in this study (CL) and FAdVs from species D. For the rest of the species, a 29.4–39.4% divergence was observed (Table 2). Comparison of the nucleotide sequences from this study with those retrieved from GenBank KX247375/FAdV-11 revealed a difference of four nucleotides (Table 3). The synonymous mutations in positions 273, 450, and 462 resulted in valine, leucine, and proline residues, respectively. Meanwhile, the mutation in position 223 caused an aspartic acid residue to replace the asparagine residue. The combination of nucleotide homology and the topology of the phylogenetic tree for a partial region of the hexon gene compared to the GenBank sequences was sufficient to classify the Chilean viruses as FAdV-D serotype 11 (Figure 3).

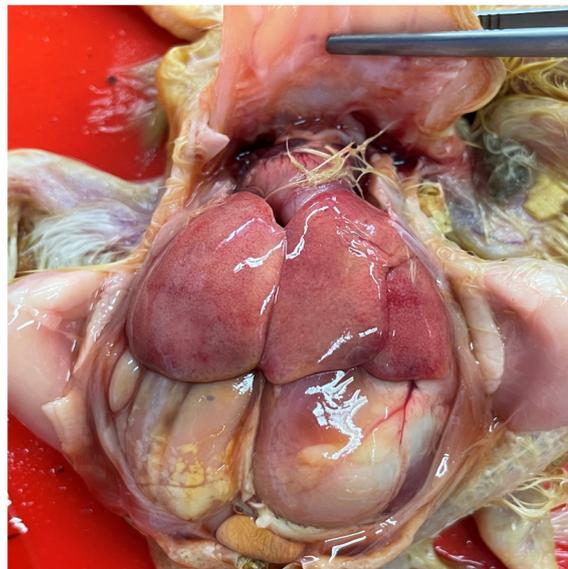


Figure 1. Pale and enlarged liver with whitish necrotic foci with a reticular pattern.

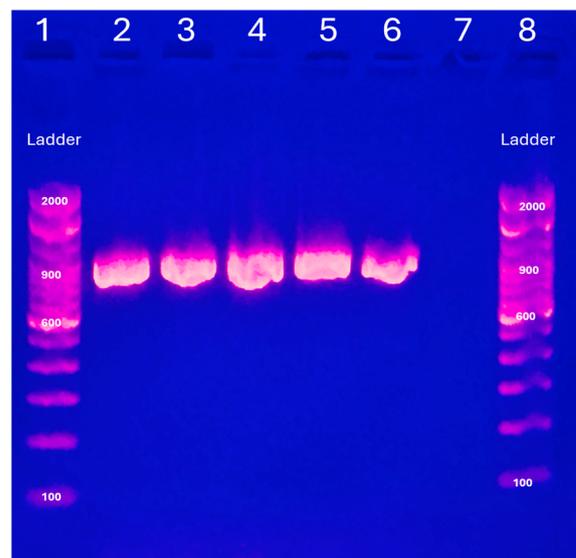


Figure 2. PCR amplicons of the hexon gene of the fowl adenovirus from liver samples in agarose gel. Lane 1: 100 bp DNA Ladder. Lanes 2–5: Fowl Adenovirus DNA. Lane 6: Positive control. Lane 7: Amplification of negative control. Lane 8: 100 bp DNA Ladder.

Table 2. Estimates of evolutionary divergence over sequence pairs between species.

	CL	FAdV-A	FAdV-D	FAdV-C	FAdV-E	FAdV-B
CL		0.612	0.071	0.687	0.416	0.563
FAdV-A	0.392		0.611	0.531	0.611	0.529
FAdV-D	0.076	0.384		0.689	0.396	0.532
FAdV-C	0.392	0.328	0.396		0.617	0.647
FAdV-E	0.294	0.354	0.286	0.356		0.446
FAdV-B	0.340	0.348	0.329	0.370	0.299	

The table shows the number of base substitutions per site after averaging over all sequence pairs between groups. Standard error estimate(s) are shown above the diagonal. Evolutionary analyses were conducted in MEGA X using the maximum composite likelihood model and involved 31 nucleotide sequences. Divergence of the isolates was calculated with FAdVs grouped into five species (A–E), and the sequences obtained in this study were grouped as CL.

Table 3. Comparison of the sequences obtained in this study and the sequence of FAdV-D serotype 11.

N°	FAdV Isolate/GenBank Accession No	FAdV Species	FAdV Serotype	Positions of Nucleotide Sequence Hexon Gene			
				223	273	450	462
1	KX247375	D	11	A	A	T	A
2	15927/CHILE/2023	D	11	G	G	C	G
3	15929/CHILE/2023	D	11	G	G	C	G
4	15950/CHILE/2023	D	11	G	G	C	G
5	15996/CHILE/2023	D	11	G	G	C	G

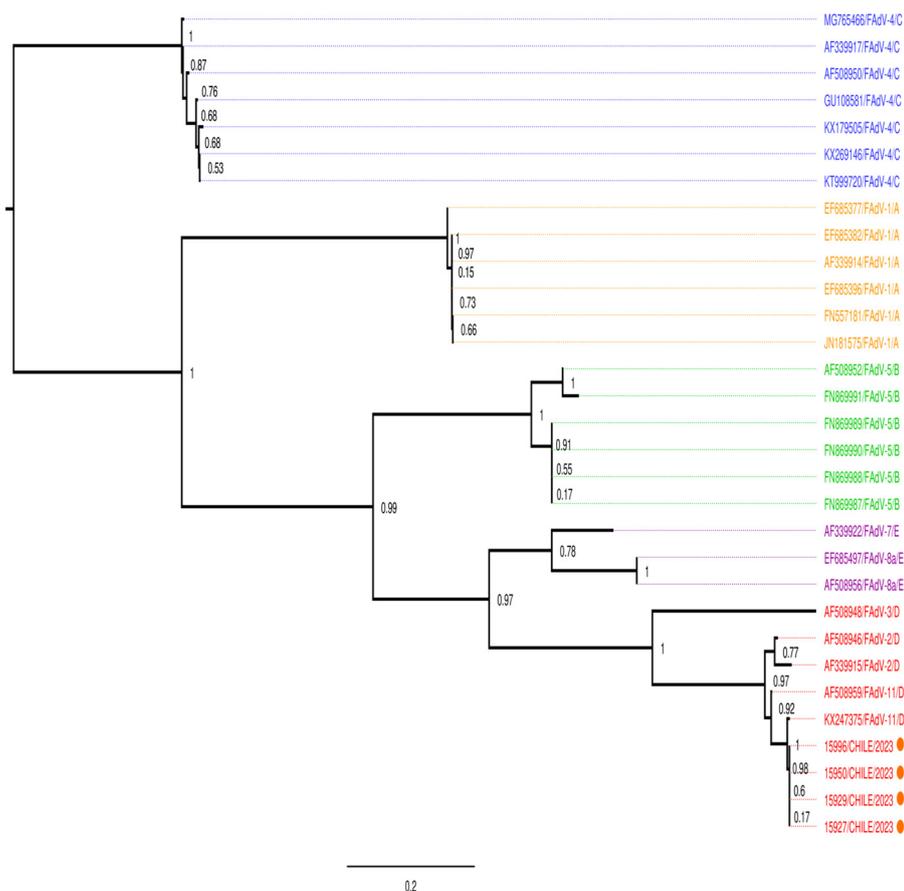


Figure 3. This maximum-likelihood phylogenetic tree is based on the nucleotide sequence of the hexon gene from the FAdVs identified in this study and the GenBank sequences. A bootstrap expectation process with 1000 replicates supported the robustness of the nodes. All bootstrapped values are labeled at major nodes. The sequences from this case are marked with orange circles.

4. Discussion

The incidence of inclusion body hepatitis (IBH) has increased worldwide in recent years [5]. When IBH outbreaks in chickens were reported in Chile in 1999 [27], the diagnosis was based on virus neutralization tests (VNTs) and restriction enzyme analysis of DNA fragments. This case report is the first on IBH in Chile that is caused—according to molecular techniques and phylogenetic analysis—by FAdV-D serotype 11. The molecular technique of PCR is widely used to amplify and detect FAdV-specific genes in liver samples for confirmatory diagnosis of inclusion body hepatitis associated with fowl aviadenovirus. It also enables subsequent purification of the amplified PCR products, sequencing, and phylogenetic analysis to classify FAdVs into different serotypes [28]. The nucleotide

sequence and phylogenetic analysis of loops 1 and 2 of the hexon gene of the four FAdV isolates analyzed herein revealed 92.4% homology, a close evolutionary relationship with FAdV-D serotype 11, and the presence of four point mutations. Given that gene mutations can affect the protein structure and thus, its function, mutations in the region of the hexon gene and the resulting amino acid changes in the hexon protein could be important for pathogenicity or viral infectivity, which must be studied [20].

The results confirmed the presence of FAdV in samples from four poultry farms, all classified as FAdV-D serotype 11. Despite the variety of existing serotypes, only FAdV-C serotype 4 had been reported in Chile. However, other fowl aviadenovirus serotypes, e.g., the FAdV-D serotype 11, are not unexpected in Chile since the serotype has been reported in other South American countries, like Brazil [23] and recently, Ecuador [24]. These FAdVs could have been introduced via infected day-old imported broiler breeder chicks, though confirmation would require analysis of the broiler breeders or the breeders of origin. While fowl aviadenovirus is commonly isolated in birds between 3 and 5 weeks old, our affected birds were between 4 and 6 days old, suggesting there may have been vertical transmission of the virus [5].

IBH is an emerging viral disease that causes significant losses in the poultry industry. In recent years, IBH has increased considerably in different countries worldwide. Most outbreaks are associated with FAdV-2, FAdV-11, FAdV-8a, and FAdV-8b [5]. Despite strict biosafety measures, control and prevention of this disease do not seem to be effective, mainly because FAdV's resistance to commonly used disinfectants makes it difficult to control [29].

Given that the poultry industry's main challenges are increased mortality and poor growth rates, preventing infectious diseases through proper vaccination is essential. To prevent IBH and HHS, Chile vaccinates broiler breeders against FAdV and protects their progeny against the clinical and subclinical forms of the disease, using inactivated FAdV-C serotype 4 and FAdV-E serotype 8 vaccines. While the protection capacity of this vaccination has not been analyzed, tests of the FAdV-E serotypes 8a and 8b strains as possible vaccine candidates suggest they could be effective in protecting against infection by heterologous strains, specifically the FAdV-D serotype 11 [30]. These findings suggest that the vaccine used in Chile (FAdV-E serotype 8) should protect against FAdV-D serotype 11 infections. However, future experiments would need to analyze whether the vaccine strain generates any degree of heterologous protection.

Control of horizontal transmission of FAdV is a key factor in preventing IBH. Two to three days post-infection, birds develop viremia and shed large quantities of the virus in their feces [5]. Furthermore, FAdVs are resistant to commonly used disinfectants and tolerate heat and pH changes well [29]. Therefore, extreme biosafety measures like disinfectants containing iodophor or aldehyde and a minimum of three weeks of inactivity between breeding cycles are advisable to reduce the viral load on the farm [17].

5. Conclusions

To the best of our knowledge, this is the first study to report on the FAdV-D serotype 11 in an IBH outbreak in broiler chickens between 4 and 6 days old in Chile. According to our observations, the IBH cases caused by the FAdV-D serotype 11 (despite breeder vaccination) would indicate a lack of heterologous protection. The emergence of serotypes previously absent in Chile constitutes a latent risk. Continued monitoring of IBH cases is required to determine the serotype of the FAdVs circulating in Chile and gather additional epidemiological data on the prevalence of these viruses in order to adapt preventative vaccination programs and diagnostic tools.

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