RESEARCH ARTICLE

Molecular Detection, Phylogenetic Analysis and Differentiation of Fowl Adenovirus from Chickens with Inclusion Body Hepatitis in Haryana

Aman Kumar[1]*, Koushlesh Ranjan, [2] Minakshi Prasad[1], Nand Kumar Mahajan[3], Sushila Maan[1]

Abstract

Inclusion Body Hepatitis (IBH) is responsible for huge economic losses to the poultry industry and is caused by Fowl adenoviruses (FAdV). A total of thirty pooled poultry liver homogenates from thirty different poultry farms suspected of IBH have been processed for the PCR-based detection and chicken embryo liver cell culture-based virus isolation. Out of 30 pooled samples, 18 were found positive in PCR; however, only 9 chicken embryo liver cell culture samples having CPE found positive in PCR. The nucleic acid obtained from FAdV specific Polymerase Chain Reaction (PCR) were further genotyped by *in-silico* restriction enzyme analysis (REA) using enzymes *BsiW*I, *Kpn*I, and *Hpa*I. Upon phylogenetic analysis, all the nine positive samples of FAdVs clustered in distinct clads of FAdV serotypes 8a, 8b, and 11, which is in correlation with the REA pattern. Thus, nucleic acid sequencing followed by phylogenetic analysis revealed the presence of FAdV-D (serotype 11) and FAdV-E (serotype 8a and 8b) in broiler chickens.

Keywords: Fowl Adenovirus, Inclusion Body Hepatitis, Polymerase Chain Reaction, Restriction enzyme, Serotype.

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Introduction

FAdVs are a non-enveloped virus with a diameter of 70-100 nm. The genome of FAdVs consists of double-stranded DNA of 43-45kb. They belong to the genus *Aviadenovirus* under the family *Adenoviridae*. Based on restriction enzyme digestion patterns, FAdV can be categorized into five different species (FAdV A to FAdV E) (Harrach *et al*., 2011). Furthermore, based on cross-neutralization assays they can also be categorized into 12 serotypes, i.e., FAdV1 to FAdV8a, FAdV8b, FAdV9, FAdV10, and FAdV11 (Hess, 2000). However, one more serotype, i.e., FAdV-12, has also been reported from different regions of the world (Mittal *et al*., 2014). FAdV consists of major surface hexon protein, which acts as subtype-specific antigenic determinants and forms major surface exposed capsid structure (Russell, 2009). The molecular characterization of hexon gene is an important tool for establishing the epidemiological status and genetic relations of FAdV field strains with previously known viruses (Niczyporuk, 2016).

The FAdV serotypes causing serious illness to poultry are also associated with IBH in chickens, leading up to10-30% of mortality (Lim *et al*., 2011). The Adenoviruses belonging to FAdV-D and FAdV-E species have been reported from several IBH cases globally (Nakamura *et al*., 2011). Moreover, FAdV-1 and FAdV-8 cause primarily GEU, and FAdV-4 is associated mainly with HHS leading to mortality up to 30-80% in poultry (Kim *et al*., 2008). A study showed that FAdVs are opportunistic pathogens in immuno-compromised or previously ill birds having simultaneous infections of other pathogens such as Infectious Bursal Disease (IBD), Chicken Infectious Anemia (CIA), etc. (Adair and Fitzgerald, 2008). FAdVs have also been identified as a causative agent for respiratory illness in poultry birds (Mittal *et al*., 2014). Although FAdV is prevalent in India, its molecular diversity is still not fully characterized. Therefore, the current study was designed to identify and characterize the FAdV as a causative agent of IBH using molecular and cell culture techniques.

[1]Department of Animal Biotechnology, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana, India

[2]Department of Veterinary Physiology and Biochemistry, SVP University of Agriculture and Technology, Meerut, Uttar Pradesh, India

[3]Department of Veterinary Public health and Epidemiology, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana, India

Corresponding Author: Aman Kumar, Department of Animal Biotechnology, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana, India, e-mail: aman.abt@luvas.edu.in

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Materials and Methods

Collection of Samples

A total of thirty (n = 30) commercial broiler poultry farms suspected for inclusion body hepatitis (IBH) from different districts of Haryana state and its adjoining districts of Rajasthan were selected for the study from January 2009 to April 2010. The samples were collected after the necropsy of dead birds with a history of respiratory diseases. The liver tissue samples from 2 to 4 affected birds in a flock were collected in 50% buffered glycerol and were pooled to make a single pooled sample for virus isolation and PCR-based confirmation of etiological agents. The collected samples were given specific laboratory identification numbers in serial from 1 AD to 30 AD.

Virus Isolation

Chicken embryo liver (CEL) cell culture was prepared from 11-day old chicken embryos as per the standard procedure (Mohamed Sohaimi *et al*., 2019). The suspected liver tissue samples were inoculated to CEL culture, incubated at 37°C and 5% CO2 and observed for cytopathic effect (CPE) daily for 7 days. After the appearance of CPE, cultures were passaged for three cycles of freezing and thawing the flasks followed by centrifugation at 1200 x g for 10 min. 500 µL of supernatant was inoculated onto freshly prepared chicken embryo liver cell monolayers.

PCR Amplification

The genomic DNA from cell culture grown virus as well as from tissue homogenate was extracted using the Chelax method as described previously (Walsh *et al*., 1991). The extracted DNA was used in PCR for molecular identification of the virus. The PCR reaction was carried out using Hexon gene-based primer of FAdV H1 (296-314): 5′ TGGACATGGGGGCGACCTA 3′ and FAdV H2 (1514-1496): 5′ AAGGGATTGACGTTGTCCA3′ (Raue and Hess, 1998) to get an amplicon of 1219 bp. The cyclic conditions for amplification were used as initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, extension at extension 72°C for 1.5 min, and final extension at 72°C for 10 min. The PCR amplicons were allowed for 1% agarose gel electrophoresis and visualized with ethidium bromide (0.5 µg/mL) in a gel documentation system (Raue and Hess, 1998).

Nucleic Acid Sequencing

The amplified PCR products were purified using QIAquick® PCR Purification Kit (Qiagen, USA) as per the manufacturer's protocol, the purified PCR products obtained were allowed for direct nucleic acid sequencing from both sides using Hexon gene-specific primers and Big Dye™ Terminator v3.1 Cycle Sequencing Kit (ABI, USA) as per the manufacturer's protocol. The nucleotide sequencing was performed on capillary sequencing machine 'Applied Biosystems® 3130xl Genetic Analyzers' (ABI, USA) in the department of Animal Biotechnology, LUVAS, Hisar.

Nucleotide Sequence Analysis

The nucleic acid sequences generated from the capillary sequencer were allowed for Blastn analysis of NCBI to confirm the virus identity. The forward and reverse sequences of a sample were joined together, and contig was prepared using BioEdit v7.2.5 (Hall, 1999). The web-based ORF Finder tool generated the deduced amino acid sequences from contig sequences (https://www.ncbi.nlm.nih.gov/orffinder/). The percent nucleotides as well as deduced amino acid sequence identity matrix of contig sequences generated from samples and similar sequences of FAdVs from GenBank were calculated using BioEdit v7.2.5 (Hall, 1999). For phylogenetic analysis, the neighbor-joining (NJ) tree was constructed using the p-distance matrix algorithm of sequences in the study along with other sequences of FAdVs from GenBank using MegaX software with 1000 bootstrap value (Kumar *et al*., 2018). *In-silico* restriction enzyme analysis (REA) using online tool restriction mapper v3.0 was also performed for genotyping.

Results and Discussion

Out of 30 samples, 18 were found to be positive for FAdV by PCR; however, only nine-cell culture adopted samples were found positive by PCR. After the second successive passages in cell culture, the cytopathic effect (CPE) was observed in nine samples. At 48 hours of inoculation, detachment and clumping of cells were observed, whereas no such changes were observed in the remaining samples. The hexon gene of FAdV was amplified from extracted nucleic acid, and a specific PCR amplicon of 1219 bp was obtained (Fig. 1). All cell culture adopted PCR positive samples were successfully sequenced and obtained sequences were analysed and submitted to the GenBank database under the following accession numbers: MT708590 (1AD), MT708591 (2AD), MT708592 (4AD), MT708593 (5AD), MT708594 (6AD), MT708595 (7AD), MT708596 (9AD), MT708597 (10AD) and MT708598 (11AD).

The nucleotide sequences obtained were allowed for BLASTn analysis which showed maximum similarity with

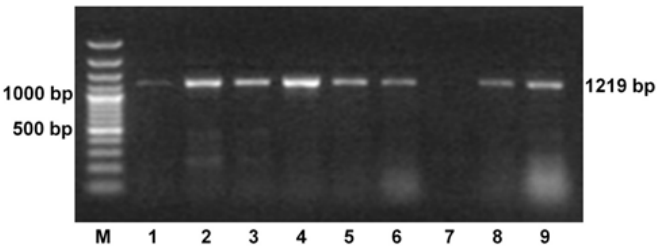

Fig. 1: Agarose gel electrophoresis of Hexon gene of Fowl Adenovirus samples. Lane M: Marker 1kb; Lane 1:1AD; Lane 2:2AD; Lane 3: 4AD; Lane 4: 5AD; Lane 5: 11AD; Lane 6: 6AD; Lane 7: Negative control; Lane 8: 7AD and Lane 9: 9AD

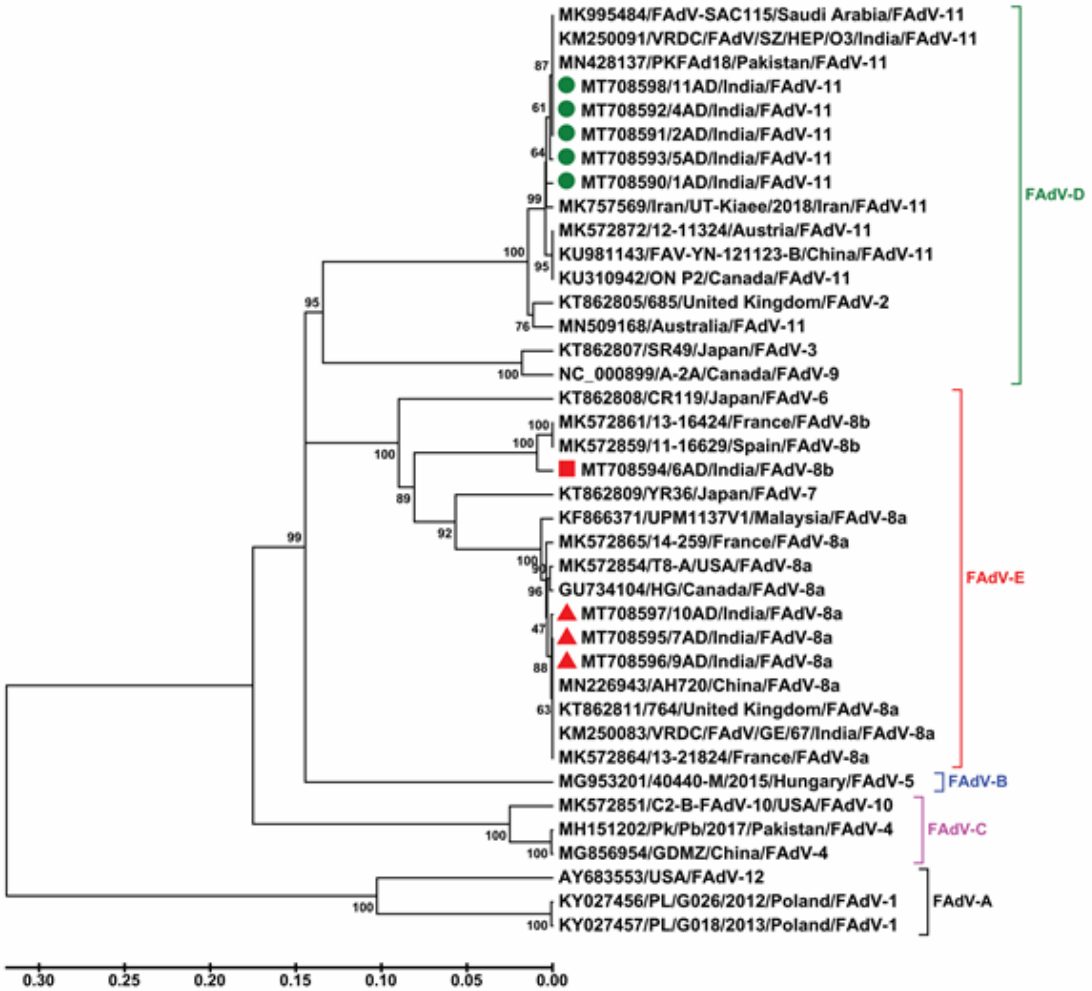

Fig. 2: Phylogenetic analysis of partial Hexon gene sequence of Fowl adenovirus with similar sequences from Genbank. A neighbor-joining tree was constructed using p distance matrix algorithm of Mega X program (Kumar *et al*., 2018) with 1000 bootstrap values.

FAdV- D and E. The contig sequences were prepared from forward and reverse sequences using BioEdit v7.2.5 program (Hall, 1999). Similar sequences for both the nucleotide and deduced amino acid sequences were selected from GenBank, and the percent nucleotide and amino acid identity matrix were analyzed.

The phylogenetic analysis of hexon gene sequences of all the samples in the study and similar sequences from different world regions (Fig. 2) was done using the Mega X program. Further, the nucleotide sequences of all the nine samples and representative isolates of serotype FAdV-8a, FAdV-8b, and FAdV-11 were subjected to *in-silico* restriction enzyme analysis (REA) using online tool restriction mapper v3.0 (http://www.restrictionmapper.org/) (Table 1).

The percent nucleotide and amino acid identity matrix revealed that samples 1AD, 2AD, 4AD, 5AD, and 11AD showed a maximum identity of 95.8-100 % nucleotide (nt) and 94.7-100 % amino acid (aa) with serotype 11 of FAdV-D from Pakistan (MN428137), Saudi Arabia (MK995484), India (KM250091), Iran (MK757569), Austria (MK572872) , China (KU981143), Canada (KU310942) and Australia (MN509168). Moreover, these samples also showed 98.7–100% nt and 96.8–100% aa identity among themselves, indicating the distinctness of viruses from each other. Similarly, samples 7AD, 9AD, and 10 AD showed a maximum of 98.2–100% nt and 96.8–100% aa identity with serotype 8a of FAdV-E from China (MN226943), United Kingdom (KT862811), India (KM250083), France (MK572864 and MK572865) , USA (MK572854) Canada (GU734104) and Malaysia (KF866371). Nucleic acid and deduced amino acid sequences of 7AD, 9AD and 10 AD showed 98.3–100% nt and 97.5–100% aa identity among themselves, indicating that these viruses are distinct from each other. However, sample 6AD showed a maximum of 98.2% nt and 98.5% aa identity with FAdV-E serotype-8b from Spain (MK572859) and France (MK572861). Some of the studies suggested that only specific types of FAdV-D and FAdV-E, such as FAdV serotype 2, 8a, 8b, or 11 could be associated with IBH cases in poultry (Schachner *et al*., 2016).

The hexon gene-based phylogenetic analysis revealed that samples 1AD, 2AD, 4AD, 5AD, and 11AD have maximum closeness with serotype 11 of FAdV-D and form a close cluster with serotype 11 viruses from different regions of the world,

Table 1: *In-silico* restriction enzyme analysis of Hexon gene sequences (296 bp - 1514bp) of Fowl Adenovirus (FAdV)

S.N.	*Sequence*	*Species*	*Serotype*	*BsiWI (CGTACG)*	*HpaI (GTTAAC)*	*KpnI (GGTACC)*
1	MT708590/1AD/India	FAdV D	11	-	-	259
2	MT708591/2AD/India	FAdV D	11	-	-	259
3	MT708592/4AD/India	FAdV D	11	-	-	259
4	MT708593/5AD/India	FAdV D	11	-	-	259
5	MT708598/11AD/India	FAdV D	11	-	-	259
6	MN428137/PKFAd18/Pakistan	FAdV D	11	-	-	259
7	MK995484/FAdV-SAC115/Saudi_Arabia	FAdV D	11	-	-	259
8	KM250091/VRDC/FAdV/SZ/HEP/O3/India	FAdV D	11	-	-	259
9	MK757569/Iran/UT-Kiaee/2018/Iran	FAdV D	11	-	-	259
10	MK572872/12-11324/Austria	FAdV D	11	-	-	259
11	KU981143/FAV-YN-121123-B/China	FAdV D	11	-	-	259
12	KU310942/ON_P2/Canada	FAdV D	11	-	-	259
13	MN509168/Australia	FAdV D	11	-	-	259
14	MT708595/7AD/India	FAdV E	8a	838	545	-
15	MT708596/9AD/India	FAdV E	8a	838	545	-
16	MT708597/10AD/India	FAdV E	8a	838	545	-
17	MN226943/AH720/China	FAdV E	8a	838	545	-
18	KM250083/VRDC/FAdV/GE/67/India	FAdV E	8a	838	545	-
19	MK572854/T8-A/USA	FAdV E	8a	838	545	-
20	GU734104/HG/Canada	FAdV E	8a	838	545	-
21	KF866371/UPM1137V1/Malaysia	FAdV E	8a	838	545	-
22	MT708594/6AD/India	FAdV E	8b	838	920	-
23	MK572861/13-16424/France	FAdV E	8b	370, 838	920	-
24	MK572859/11-16629/Spain	FAdV E	8b	370, 838	920	-

i.e., India (KM250091), Iran (MK757569), Austria (MK572872), Pakistan (MN428137), Saudi Arabia (MK995484), China (KU981143), Canada (KU310942) and Australia (MN509168). The FAdV-11 was previously also isolated from outbreaks of IBH in poultry flocks in India (Thakor *et al*., 2012; Gulhane *et al*., 2016). Moreover, samples 7AD, 9AD, and 10AD form a close cluster with serotype 8a viruses of FAdV-E from France (MK572864), India (KM250083), China (MN226943), United Kingdom (KT862811), Canada (GU734104), USA (MK572854), France (MK572865) and Malaysia (KF866371). The remaining sample 6AD was placed closer to FAdV E serotype 8b from France (MK572861) and Spain (MK572859) and formed a close cluster.

A previous study suggested that FAdV-11 and other serotypes belonging to FAdV-D species were isolated from poultry in Poland (Niczyporuk, 2016). Similarly, viruses belonging to FAdV-D and FAdV-E were isolated in Belgium from several poultry samples (De Herdt *et al*., 2013). Serotypes FAdV-8b and FAdV-11 have predominantly been reported from Australia (Steer *et al*., 2011) and Turkey (Sahindokuyucu *et al*., 2020). Similarly, in other studies, FAdV serotypes 4, 8b, and 11 from Korea (Choi *et al*., 2012; Lim *et al*., 2012) and FAdV serotypes 8a, 8b, and 11 from China have been reported (Wang *et al*., 2018).

Furthermore, nucleic acid sequences of hexon gene of FAdV were allowed for *in-silico* REA using web-based available restriction mapper v3.0 (http://www.restrictionmapper.org/) tool. Upon REA with restriction enzyme *BsiW*I (CGTACG), all the serotype 11 viruses from FAdV-D (1AD, 2AD, 4AD, 5AD, 11AD, and representative isolates from GenBank) did not show any restriction site, but serotype 8a FAdV-E virus (7AD, 9AD, 10AD and representative isolates from GenBank) showed a single restriction site at 838bp. Similarly, the Indian FAdV-8b virus (6AD) showed a single restriction site at 838 bp. However, another serotype 8 b viruses from France (MK572861) and Spain (MK572859) showed additional restriction sites with *BsiW*I restriction enzyme at positions 370 and 838 bp. The absence of restriction site at position 370 in 6 AD sample is attributed to a single transition mutation from Guanine (G) to Adenine (A) in the recognition site of the restriction enzyme.

Similarly, REA with *Kpn*I (GGTACC) revealed the single restriction site at nucleotide position 259 in all the serotype 11 of FAdV-D viruses. However, enzyme *Kpn*I did not show any restriction site in serotype 8a and 8b of FAdV-E viruses.

Interestingly, REA with *Hpa*I (GTTAAC) did not show any restriction site in serotype 11 viruses but, showed a single restriction site at nucleotide position 545 in serotype 8a and at position 920 in serotype 8 b viruses. Thus, restriction enzymes *BsiW*I and *Hpa*I may be used for *in-silco* REA-based differentiation of serotype 11, serotype 8 a, and 8 b. The REA patterns were also in correlation with the phylogenetic analysis result. The REA patterns observed with *BSiWI* enzyme correlated with the previously obtained RFLP analysis pattern (Gulhane *et al*., 2016). A previous study showed that REA using *Sty*I, *BsiW*I and *Mlu*I enzyme may be used to identify and differentiate FAdV-8 from other FAdV serotypes in India (Mittal *et al*., 2014). The PCR products showing specific restriction patterns may also be correlated with nucleic acid sequencing of fowl adenovirus types.

The current study revealed that different FAdV serotypes such as FAdV-8a, 8b, and 11 were involved as the causative agent of IBH in Indian poultry birds. Prevalence of serotypes 8a and 8b were uncommon and not reported earlier. Therefore, further investigation is required to decipher their exact pathological role as the causative agent in IBH. Moreover, the involvement of different FAdV serotypes in the production of IBH imposes extensive studies on virulence and pathogenicity of different FAdV serotypes. The study also preaches about the necessity and possibility of the development of specific vaccines which may confer protection against more than one FAdV serotypes simultaneously.

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