



Molecular and pathotypic characterization of fowl adenovirus associated with inclusion body hepatitis in Indian chickens

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ABSTRACT

Inclusion body hepatitis (IBH) is a disease of chickens characterized by acute mortality with severe anemia caused by fowl adenoviruses (FAdV). IBH is one of the most important re-emerging diseases worldwide. In India earlier IBH/ Hydropericardium Syndrome (HPS) outbreaks were mainly due to (FAdV) type 4; however, in recent outbreaks, other serotypes are involved, despite regular vaccination in breeders and young chicks. The aim of the present study was isolation and molecular characterization of FAdV associated with IBH in India. A total of 193 liver samples of IBH suspected broiler chickens were collected from different regions of India. Liver samples were initially screened by histopathology and further, a total of 127 samples which microscopically showed basophilic, intra-nuclear inclusion bodies were processed for FAdV detection. A total of 69 samples were found to be positive for Hexon gene in PCR, of which 28 were sequenced. These samples showed more than 94% sequence homology with FAdV2 and 97% with FAdV11, which was confirmed by Restriction Enzyme Analysis (REA). Tissue Culture Infective Dose₅₀ (TCID₅₀) and Egg Infective Dose₅₀ (EID₅₀) titer of isolated FAdV was found to be 10^{6.5}/ml. Pathogenicity study in SPF chicks showed 100% mortality up to Post Infection Day (PID)-6. These findings conclude that the prevalence of FAdV-11 is currently causing IBH outbreaks India. However, further genetic and pathogenic analysis of FAdV is required, which would provide useful information for the development of the efficient commercial IBH vaccine.

Keywords: Fowl Adenovirus (FAdV), Hexon gene, Histopathology, Inclusion Body Hepatitis (IBH), PCR, Serotype-11, SPF chicks

Inclusion Body Hepatitis (IBH) is a disease of chicken characterized by acute mortality, often with severe anemia, caused by an adenovirus. In northern parts of India, IBH was reported in broilers with causing mortality 10–60% (Sreenivas Gowda and Satyanarayana 1994). Fowl adenoviruses (FAdVs) are main causative agents of IBH worldwide. Adenoviruses are non-enveloped icosahedral in shape, size in between 70 to 100 nm in diameter and containing a linear dsDNA of approximately 45 kb in size of its genome (Horne *et al.* 1959). The major structural proteins are hexon and fiber proteins, which are non-covalently linked to the penton base, a structure known as penton (Parthiban *et al.* 2014). Isolation of FAdV is an important procedure for subsequent identification and classification of FAdVs and its serotypes. Isolation can be done using primary chick embryo liver cell culture (CEL)

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or primary chicken embryo kidney (CEK) cells and SPF eggs (Singh *et al.* 2004). In India many outbreaks of IBH being reported, despite regular vaccination in breeders and young chicks.

The present study was designed to isolate and characterize the FAdVs in the current IBH outbreaks in Indian broiler chickens by PCR as well as using various *in vitro* and *in vivo* studies.

MATERIALS AND METHODS

Materials and reagents: The materials and reagents used in the present study were procured from HiMedia: Dulbecco's modified eagle's medium (DMEM), Trypsin phosphate verses glucose (TPVG), Trypsin-EDTA, Antibiotic solution (Penicillin, Streptomycin and Gentamicin), Fetal bovine serum (FBS), M-199 medium, Hanks balanced salt solution (HBSS), Phosphate buffer saline (PBS) and Viral transport medium (VTM). Agilent: Taq DNA polymerase and Taq Buffer. Bangalore Genei: *AspI* restriction enzyme, dNTPs, 10×Buffer, 100 bp DNA ladder, 6× loading dye. QIAGEN: DNA Extraction Kit. Tarsons: Tissue culture flasks and tissue culture plates.

Sample collection: Total of 193 liver tissue samples of IBH suspected broiler chickens were collected from different parts of India (Maharashtra, Telangana, Karnataka, and West Bengal). Liver samples from same flocks were pooled in VTM and transferred to the laboratory and were stored at -80°C for further use.

Initial screening: Initial screening of IBH was carried out based on clinical and necropsy findings and cytological examination by performing histopathology of collected liver samples.

Sample processing: Approximately 1–2 g of IBH positive 127 liver samples detected by histopathology were taken into a sterile mortar-pestle and gradually ground aseptically. 10% liver suspension was prepared in M-199 medium containing antibiotics (Gentamicin-10 $\mu\text{g}/\text{ml}$, Penicillin 100 U/ml and Streptomycin 100 $\mu\text{g}/\text{ml}$) and three freeze-thaw cycles were given to the samples and were stored at -80°C for further experiment.

Isolation of FAdV by egg inoculation method: This experiment was carried out in 5–7 day old embryonated SPF chicken eggs (Cowen 1988). In brief, 10% liver suspension was diluted 1:100 and 1:1000 in sterile PBS (pH-7.4) and 0.2 ml/egg was inoculated by yolk sac route. Eggs were further incubated at 37°C , and daily mortality was observed and recorded.

Preparation of chick embryo liver (CEL) culture: CEL culture was prepared using 12–14 days-old embryonated SPF chicken eggs. Approximately 3×10^5 cells/ml were seeded in tissue culture flasks and incubated at 37°C in a CO_2 incubator until a confluent monolayer was formed. (Soumyalekshmi, Ajith, and Meshram 2014).

Isolation of FAdV in CEL culture: Liver suspension, which was pre-confirmed for the presence of the Hexon gene by PCR, was inoculated on the monolayer CEL culture. Cells were daily observed for CPE till 3–4 days of post-infection. Subsequently, five passages were done in CEL culture, and finally, tissue culture fluid (TCF) were harvested using three freeze-thaw cycles and stored at -80°C for further use.

Egg infectivity dose (EID_{50}): EID_{50} was determined by using different dilutions of liver suspension ranging from 10^{-3} to 10^{-8} in sterile PBS, and 0.2 ml of each dilution were inoculated in SPF eggs. Control eggs were inoculated with 0.2 ml PBS and incubated for 5–6 days post-inoculation at 37°C . After incubation eggs were kept for chilling at 4°C for overnight and harvested aseptically. The liver was collected from all embryos, and presence of lesions was noted. EID_{50} was calculated by Reed and Muench formula (Reed and Muench 1938).

Tissue culture infectivity dose (TCID_{50}): Different concentrations of liver suspension ranging from 10^{-3} to 10^7 were prepared in sterile PBS, and 100 μl of each dilution was inoculated on confluent monolayer of CEL cells for 1 h at 37°C in a CO_2 incubator. After incubation 0.5 ml of DMEM supplemented with 2% FBS was added in each well, and plate was incubated again. The cells were observed daily for CPE, and TCID_{50} titer was calculated by Reed

and Muench formula (Reed and Muench 1938).

Histopathology: For histopathological examination, liver and kidney from the IBH suspected chicks were collected in 10% buffered formalin, embedded in paraffin sectioned at 4 μm , stained with hematoxylin and eosin and examined by light microscopy (Okuda *et al.* 2001).

DNA extraction and Polymerase Chain Reaction (PCR): DNA was extracted from all 127 samples using the QIAGEN DNA extraction kit as per the manufacturer's instructions and PCR was performed as per the method described by Meulemans *et al.* 2001.

Sequencing: Amplified DNA products were gel-purified using Purelink PCR purification kit (Thermo Fisher Scientific) as per the manufacturer's instructions. The purified products were subjected to automated sequencing (Applied Biosystems Big Dye Terminator V3.1) retrieved sequences of hexon gene were then used to construct the phylogenetic tree (Barua *et al.* 2005).

Restriction enzyme analysis (REA): The PCR products generated was purified using WizardTM PCR Preps DNA purification system (Promega, USA), and purified PCR product were subjected to digestion with restriction enzyme *AspI* (Meulemans *et al.* 2001).

Pathogenicity study: The experiment was carried out in day-old SPF chicks as described previously (Junnu *et al.* 2015) with minor modifications. Briefly, the chicks were randomly allocated in six groups. The four groups ($n=8$) were infected intraperitoneally with 0.2 ml of 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} dilution of virus suspension containing $10^{5.5}$ /ml egg infectivity dose (EID_{50}). Fifth group ($n=8$) birds kept as in-contact with infected birds and in control group birds ($n=10$) were administered with 0.2 ml PBS and was kept in a separate isolator with same feed and housing conditions. Birds were clinically examined daily for mortality and any kind of sickness.

RESULTS AND DISCUSSION

Hemboldt and Frazer in 1963, first time reported IBH disease in the USA. Till date, many countries recorded IBH in poultry (El-Tholoth and El-Azm 2019, McFerran and Adair 1977). IBH outbreaks in India, China, South Asia, South Korea, Japan, Mexico, North America, and Eastern Europe caused immense economic losses to the poultry industry worldwide (Niczyporuk 2017, Li *et al.* 2019). In recent years increasing mortality and poor growth rate are the main challenges in poultry industries in India, keeping this in mind, we examined liver samples of 193 IBH suspected commercial broiler chicks from different states of India such as Maharashtra, Karnataka, Telangana, and West Bengal of which histopathologically positive samples were used for further study (Table 1). A similar study was reported from Canada in which 287 liver samples collected and characterized (Ojkic *et al.* 2008).

Liver suspension (10%) were prepared in M-199 medium. DNA was extracted from each sample and further tested using PCR for the presence of the hexon gene (Hess 2010). The hexon protein consists of conserved pedestal

Table 1. IBH suspected chick liver sample collection details from different states of India

State	Total sample collected	Positive in HP screening	PCR positive	Sequencing done for
Maharashtra (MH)	132	98(74%)	61(62%)	23
Karnataka (KN)	10	03(30%)	03(100%)	03
Telangana (TL)	18	08(44%)	05(62%)	02
West Bengal (WB)	33	18(54%)	00(0%)	00
Total	193	127	69	28

Table 2. FAdV hexon gene sequences taken from GenBank with their accession numbers

FAdV serotype (European classification)	GenBank Accession number	Reference
FAdV 1 CELO	AF339914	NCBI
FAdV 2, ATCC VR-827	AF339915	S Barua <i>et al.</i> 2005
FAdV 2	AF508946.1	NCBI
FAdV 2	AF508947.1	NCBI
FAdV 3, ATCC VR-828	AF339916	S Barua <i>et al.</i> 2005
FAdV 4, ATCC VR-829	AF339917	S Barua <i>et al.</i> 2005
FAdV 4b	AF154246.1	NCBI
FAdV 5, ATCC VR-830	AF339919	S Barua <i>et al.</i> 2005
FAdV 6, ATCC VR-831	AF339921	S Barua <i>et al.</i> 2005
FAdV 7, ATCC VR-832	AF339922	S Barua <i>et al.</i> 2005
FAdV 8, ATCC VR-833	AF339918	S Barua <i>et al.</i> 2005
FAdV 9, ATCC VR-834	AF339923	S Barua <i>et al.</i> 2005
FAdV 10, ATCC VR-835	AF339924	S Barua <i>et al.</i> 2005
FAdV 11, strain X11	AF339920	S Barua <i>et al.</i> 2005
FAdV 11	HQ697595	NCBI
FAdV 11	FJ360748 Brazil	NCBI
FAdV 12, strain 380	AF339925	S Barua <i>et al.</i> 2005
FAdV 12	HM748587 HR4	NCBI

regions and variable loops. In PCR, we found that 54.33% infected liver samples are positive for hexon gene (Table 1).

Amplified hexon gene DNA products were further sequenced. These were compared with sequences of FAdV-1 to FAdV-11 retrieved from GenBank (Table 2). The sequence similarity was identified by constructing phylogenetic analysis using ClustalW (Fig. 1). Phylogenetic analysis revealed that our sequence showed >94% homology with FAdV-2 and >97% homology with FAdV-11. Sequence obtained in the study have been submitted to GenBank under the accession number MK933773. Similar studies from different countries have reported sequence homology using phylogenetic analysis of the hexon gene sequence of FAdV for e.g., a study conducted in Egypt showed a close genetic relationship with UK (FAdV-2, 3) and Canada (FAdV-4, 5) (El-Tholoth and El-Azm 2019).

REA was performed further to distinguish between the serotypes FAdV2 and FAdV11 using *AspI* (also known as Tth111I) restriction enzyme, as our sequence was showing high homology with both FAdV 2 and FAdV11. *AspI*, which cut the 900 bp product of FAdV11 into 448/452 bp fragments, confirmed the presence of FAdV2 (Meulmans

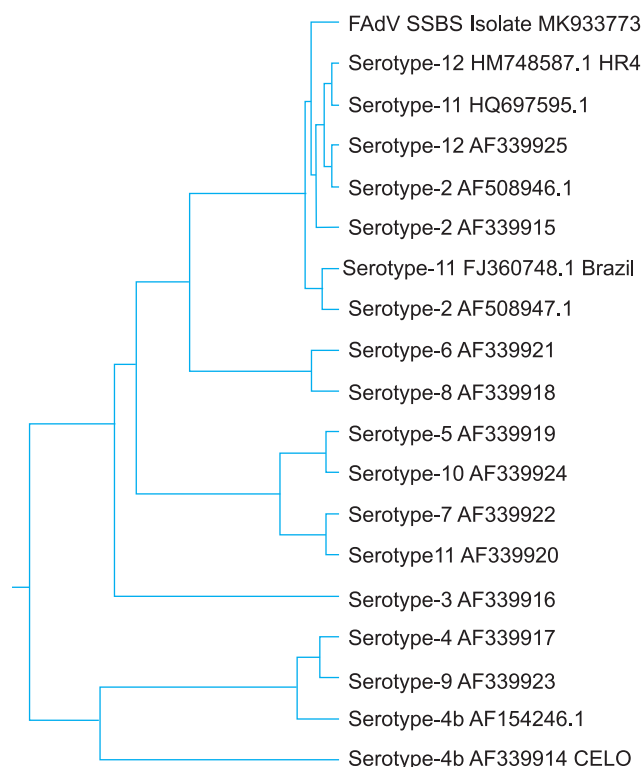


Fig.1. Phylogenetic analysis of Hexon gene sequence of FAdV SSBS isolate showing sequence homology with reported isolates at GenBank database.

et al. 2001). The digested PCR products of hexon gene with restriction enzyme *AspI* which did not show any cut in DNA, hence we confirmed the involvement of FAdV11 in the current Indian IBH outbreaks.

Followed by molecular characterization, isolation of the FAdV was carried out for further confirmation studies. The eggs which were inoculated with FAdV isolates via yolk sac route were harvested on PID-4. The eggs were harvested and hemorrhagic embryo with IBH specific lesions on the liver was considered positive. The IBH specific lesions were more prominent in subsequent passages than the initial. The isolated FAdV has a titer of $10^{6.5}$ /ml EID₅₀ as calculated by Reed and Muench method.

Isolation of FAdV was also carried out in CEL cell culture. FAdV infected cells showed cytopathic effect (CPE)

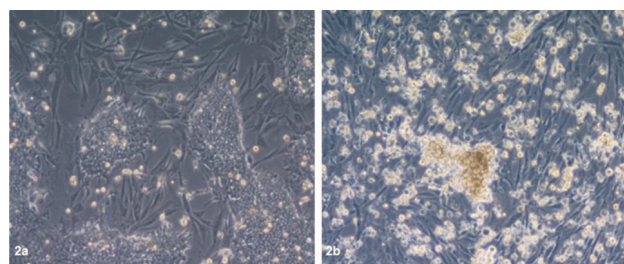


Fig. 2. Cytopathic effect of FAdV on primary chick embryo liver cell culture; a. Primary chick embryo liver cell culture inoculated with PBS as control showing no cytopathic effect. b. Primary chick embryo liver cell culture inoculated with FAdV showing cytopathic effect at PID-3.

rounding, clumping, degeneration, and detachment of cells from monolayer at 72 h post-infection however no cytopathic effects (CPE) were observed in the untreated control CEL cultures (Fig. 2a). Complete detachment of cells monolayer was observed at 96 h post-infection, subsequent passages indicated CPE within 48 to 72 h post-infection (Fig. 2b).

After passage number five, tissue culture fluids (TCF) was harvested using three freeze-thaw cycles. DNA was extracted from TCF, and PCR was performed. PCR product showed amplification of the hexon gene at 897 bp size (Mariappan *et al.* 2018). The amplified products were sequenced, and homology was observed with FAdV-11 isotype reported sequences on GenBank database. Infectivity titer of isolated virus was also determined in CEL culture. Tenfold serial dilutions of the isolated virus were prepared in sterile PBS ranging from 10^{-3} to 10^{-8} and cells monolayer inoculated with this different dilution of virus and CPE was observed. Plates were observed daily till PID-4. The TCID₅₀ titer was calculated by Reed and Muench formula, and it was found to be $10^{6.5}$ /ml.

Pathogenicity was studied upto a period of seven days. It was found that at PID-7, 100% mortality was observed at 10^{-3} , 10^{-4} and 10^{-5} dilution, whereas in 10^{-6} dilution 50% mortality and in-contact birds 25% mortality was noticed. These results are similar to several other studies (Nakamura *et al.* 1999, Sochonewille *et al.* 2008, Zhao *et al.* 2015). One of the earlier reports showed a maximum of 80% mortality in SPF chicks in 5 days to post-infection with FAdV isolate (Alvarado *et al.* 2007). In yet another reported mortality was 30 to 100% (Ruan *et al.* 2018). The most prominent lesions were observed in the liver. An enlarged dystrophic liver with pale yellow discolouration, crumbly texture, hemorrhages, and visible necrotic foci was observed in both the infected and in-contact chick livers these findings supports the study reported by Dutta *et al.* 2017. These liver samples were further confirmed for the presence of the hexon gene by PCR, which was found to be positive for the hexon gene whereas, in control chicks no amplification for the hexon gene was observed.

During the pathogenicity studies experiment, chicks were sacrificed on PID-3, PID-6, and PID-22 and liver samples were collected in 10% formalin for histopathological evaluation. Histopathological

examination revealed the most prominent lesions in the liver with the presence of intra-nuclear basophilic or eosinophilic inclusion bodies. Inclusion bodies were noticed in many hepatocytes, and hepatocytes degeneration and vacuolation were also observed (Fig. 3b). However, in control chicks IBH specific microscopic changes were not observed (Fig. 3a). These observations are similar to an earlier study (Chitradevi *et al.* 2018; McFerran 1977). Further, the involvement of FAdV11 was confirmed by PCR amplification of hexon gene from these infected chicks.

Our findings conclude that the prevalence of FAdV-11 is currently causing IBH outbreaks in India. However, further genetic and pathogenic analysis of FAdV is required, which would provide useful information for the development of the efficient commercial IBH vaccine.

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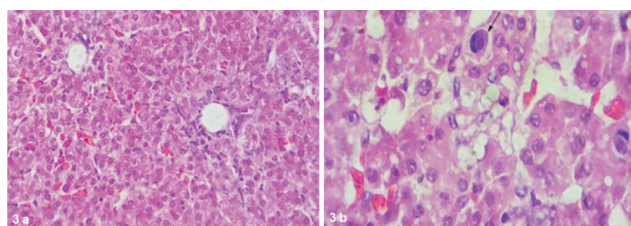


Fig.3. **a.** Control chick liver showing minimal focal hepatocytes degeneration (H&E 40X); **b.** Virus-infected chick liver showing intra-nuclear basophilic inclusion bodies (Arrow) were noticed in many hepatocyte, hepatocytes degeneration and vacuolation also observed (H&E 100X).

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