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Identification and differentiation of pathogenic and non–pathogenic serotypes of fowl adenoviruses using PCR-RFLP and sequencing

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ABSTRACT

Two cell culture adapted non-pathogenic isolates and 5 cell culture adapted pathogenic isolates of fowl adenoviruses were amplified using hexon gene H1/H2 and H3/H4 primers. The H1/H2 and H3/H4 PCR products were digested with HaeII and HpaII respectively. The species and serotype of our FAV isolates were arrived by comparing the polymerase chain reaction – restriction fragment length polymorphism pattern of FAV 1–12 reference strains. Out of 7 isolates, RFLP pattern of 2 isolates matched exactly either with RFLP pattern of both HaeII and HpaII enzymes or any one enzyme of FAV reference strains. Four of our pathogenic isolates and 1 non-pathogenic were not matching with RFLP pattern of reference strains. The 5 isolates which gave different restriction pattern later confirmed as species D (serotype 2) and species C (serotype 4) based on nucleotide sequence data on blast analysis. Hence, BLAST analysis using nucleotide sequence data may be used as effective tool for serotype identification of FAV field isolates. More interestingly, the prevalence of 2 non-pathogenic serotypes in 1 poultry farm in the same period was recorded. Earlier hydropericardium syndrome/inclusion body hepatitis was associated with serotype 4 in India. However, in recent outbreaks of inclusion body hepatitis in Tamil Nadu, prevalence of pathogenic serotype 2 in vaccinated flock was recorded, and serotype 4 was found to be non-pathogenic**.** Based on polymerase chain reaction-restriction fragment length polymorphism pattern, pathogenic and non-pathogenic field isolates of serotype 2 and serotype 4 could be differentiated.

Key words: Fowl adenovirus, Hexon gene, Inclusion body hepatitis, Restriction fragment length polymorphism, Serotype, Sequencing

Fowl adenoviruses belonging to the genus adenovirus are classified into 5 species (A, B, C, D and E) and 11 serotypes (Ojkic *et al*. 1984). Fowl adenovirus comprises species A (serotype1), species B (serotype 5), species C (serotype 4 and 10), species D (2,3, 9 and 11) and species E (serotypes 6,7,8a and 8b) (Benko *et al.* 2005). The diagnostic methods such as virus isolation and immunological assays could not be used as suitable method for serotype identification of fowl adenovirus isolates (Hess 2000). The virus neutralization assay in cell culture is used as a gold standard method for serotype identification (Monreal *et al*. 1980). However, the availability of serum against all serotypes is the major limitation for this assay. To circumvent this problem, a more rapid and precise polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) method for serotype identification of fowl adenovirus was developed (Raue and Hess 1998).

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In the field condition, only some of FAV isolates can cause clinical diseases such as inclusion body hepatitis (IBH), hydropericardium syndrome, respiratory disease and tenosynovitis (Mcconell Adair *et al*. 2008). Most of adenoviruses isolates do not produce clinical disease as a primary pathogen and however their role as opportunistic factors is indicated (Fitzgerald 2008). Hence in this study, serotype identification of FAV from both pathogenic isolates of inclusion body hepatitis (IBH)-suspected broiler birds and non-pathogenic isolates of apparently healthy broiler birds was attempted using PCR-RFLP and sequence analysis.

MATERIALS AND METHODS

Sample collection: Tracheal and cloacal swabs (50) were collected from apparently healthy broiler birds from Institute of Poultry Research and Management, Madhavaram, Tamil Nadu, India during 2011 and placed in a tube containing sterile phosphate buffered saline (pH 7.2) (PBS) and in the flock no mortality was reported. The contents were centrifuged at 10 000 rpm for 15 min to remove debris. The supernatant was collected and stored at –20°C until further use.

Liver samples (4) were collected from dead broiler birds during 2011 showing characteristic lesions of inclusion body hepatitis. Liver tissue (1g) was ground using a sterile pestle and mortar and mixed with 10 ml of sterile PBS. The homogenate was centrifuged at 10 000 rpm for 15 min to pellet the suspended particles. The supernatant was collected and stored at –20°C until further use. One liver sample collected from broiler birds with hydropericardium syndrome during the year 2004 available in our department was used in this study.

Isolation of DNA: DNA was isolated from 50 pooled tracheal and cloacal swabs (healthy birds) and 5 liver samples (dead birds) using commercially available kit according to the manufacturer's instructions.

Polymerase chain reaction: For initial screening of samples, the crude DNA was subjected to PCR using the partial hexon gene already published primers namely FAV HL (5'-GAC-ATG-GGG-TCG-ACC-TAT-TTC-GAC-AT-3'OH) and FAV HR (5'-AGT-GAT-GAC-GGG-ACA-TCA-T-3'OH) (Ganesh *et al.* 2002). The expected amplicon size of 700bp was checked in 1.5% agarose gel using UV transilluminator after ethidium bromide staining. These PCR positive samples were further used for virus isolation studies.

Virus isolation in cell culture: The chicken embryo liver culture was prepared from 12- to 13-day-old chicken embryonated egg for the propagation of both pathogenic and non pathogenic strains of fowl adenoviruses (Schat *et al*. 1998).The chick embryo liver cells were grown in Dulbecco's Modified Eagles medium supplemented with 10% foetal bovine serum incubated at 37°C with 5% CO_2.Ten passages were done for each positive sample.

PCR –RFLP analysis: The infected culture fluid of each isolate was subjected to PCR using already published 2 sets of primers namely H1/ H2 (H1 – 5'-TGG-ACA-TGG-GGG-CGA-CCT-A-3',H2- 3'AAG-GGA-TTG-ACG-TTG-TCC-A-5') and H3/H4 (H3-5'- AAC-GTC-AAC-CCC-TTC-AAC-CAC-C-3', H4- 3' TTG-CCT-GTG-GCG-AAA-GGC-G- 5') (Raue and Hess 1998).The H1/H2 and H3/H4 primer amplified products were cleaved further with HaeII and HpaII enzymes respectively. The restriction profile pattern of pathogenic and non pathogenic FAV isolates was analysed in 2% agarose gel and compared with FAV1 –12 reference serotype strains for identification of serotype of our field isolates (Raue and Hess 1998).

Sequencing of PCR products: The PCR products of each isolate were purified using a silica membrane based column purification kit. The purified products were checked in 1% agarose gel and subjected for sequencing in both directions. These obtained sequences were assembled and aligned using Gene tool software. The assembled hexon gene sequences of both pathogenic and non-pathogenic serotype of our isolates were compared with respective FAV reference serotype hexon gene sequences.

Experimental infection of chicken with non pathogenic isolates: Unvaccinated broiler chicks (12), 3- to 4- week-old were received from Institute of Poultry Research and Management, Madhavaram, Tamil Nadu, India to check the pathogenicity of isolates obtained from apparently healthy flock namely NPFAVTN1 and 2 isolates. Four chicks were infected with each isolate of about 0.1 ml inoculum subcutaneously (Parthiban *et al*. 2005) and the remaining birds were kept as controls.

RESULTS AND DISCUSSION

On initial screening of 50 samples from healthy birds, 2 samples were found positive for the presence of FAV by PCR. Similarly, out of 5 liver samples screened from infected birds, all the 5 samples were positive by PCR. These initial PCR positive samples were further isolated in primary chicken embryo liver cells and the characteristic CPE like rounding of cells and detachment of cell sheet were observed (Rehmani *et al*. 2009).

On PCR amplification of cell culture adapted 2 non-pathogenic isolates and 5 pathogenic isolates using H1/H2 and H3/H4 primers yielded 1219 bp and 1319bp amplicons respectively. The HaeII digestion of H1/H2 PCR product for all isolates is presented in Fig.1. Similarly, HpaII digestion of H3/ H4 PCR product for all isolates is shown in Fig. 2.

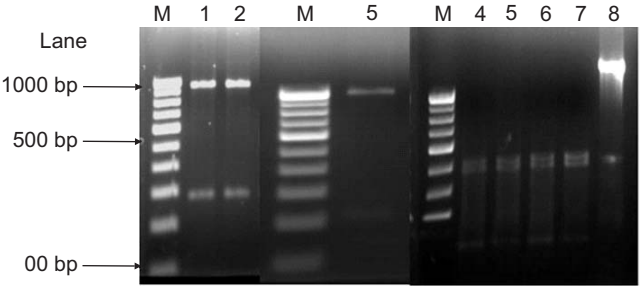

Fig. 1. Restriction profile for H1/H2 PCR products digested with Hae II enzyme. Lane M, 100bp DNA marker; lane 1, NPFAVTN1; lane 2, PFAVTN5; lane 3, NPFAVTN2; lane 4, PFAVTN1; lane 5, PFAVTN2; lane 6, PFAVTN3; lane 7, PFAVTN4; lane 8: Undigested PCR product (1219bp).

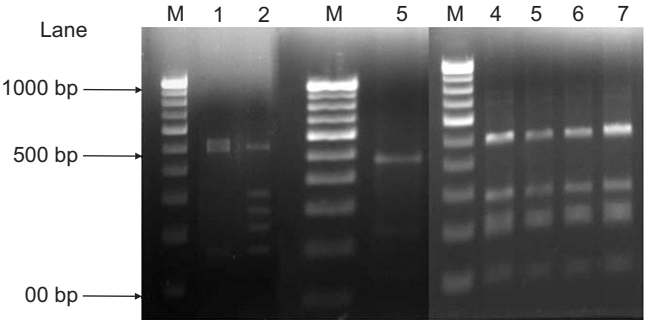

Fig. 2. Restriction profile for H3/H4 PCR products digested with Hpa II enzyme. Lane M, 100bp DNA marker; lane 1, NPFAVTN1; lane 2, PFAVTN5; lane 3, NPFAVTN2; lane 4, PFAVTN1; lane 5, PFAVTN2; lane 6, PFAVTN3; lane 7, PFAVTN4.

Table 1. Identification of species and serotype of local FAV isolates by comparing the already published PCR-RFLP pattern of reference FAV 1– 12 serotypes

Sl no	Isolates	RFLP pattern		Species	Serotype
		Hae II	Hpa II		
1	NPFAVTN 1*	Not matching	Matching	D	2
2	NPFAVTN2	Not Matching	Not matching	C	4
3	PFAVTN1*	Not matching	Not matching	D	2
	PFAVTN2				
	PFAVTN3				
	PFAVTN4				
4	PFAVTN5	Matching	Matching	C	4

NPFAV*, Non pathogenic fowl adenovirus; PFAV*, pathogenic fowl adenovirus.

The species and serotype identification of our isolates was done by comparing the PCR-RFLP pattern of FAV 1–12 reference strains (Raue and Hess 1998) and the results are presented in Table 1. The nucleotide homology percentage of each pathogenic and non- pathogenic isolates against the respective reference strain are compared and presented in Table 2. The 2 isolates namely NPFAVTN1 and NPFAVTN2 obtained from healthy flock, on experimental infection also did not produce any clinical signs and mortality for a period of 4 weeks.

On PCR amplification of H1/H2 and H3/H4 primer resulting in fragment size of 1219bp and 1319bp, respectively, for all the isolates which indicated they are of FAV origin (Singh *et al.* 2002). In case of early detection of fowl adenoviral genome, PCR assay was more sensitive than conventional assays (Parthiban *et al.* 2005). Hence in this study, for initial screening of field samples sensitive PCR assay was used. Hexon gene was used for serotype identification because the major viral surface protein adenoviruses are the hexon protein on which type, group and subgroup antigenic determinant were located (Norby 1969). The HaeII digestion of H1/H2 PCR product produced different RFLP patterns except for FAV4 and FAV5. Similarly, Hpa II digestion of H3/H4 PCR product produced very specific restriction profile for most of FAV serotypes except for FAV3,6, 7,8, 9 and 10 (Raue and Hess 1998). Using only one region of hexon gene, it is difficult to differentiate some of the strains of FAV. Hence in our study, both regions of hexon gene were used for serotype identification. Out of our local 7 isolates, RFLP pattern of 2 isolates matched exactly either with RFLP pattern of both HaeII and HpaII enzymes or any one enzyme of FAV reference strains. Four of our pathogenic isolates and one non-pathogenic strain were not matching with RFLP pattern of reference strains, even though RFLP can be very efficiently used as a tool for serotype identification of FAV isolates (Singh *et al*. 2002, Meulmans *et al*. 2001). However, minute variation in nucleotide changes between our field strain and reference strain may result in different RFLP pattern. Hence, nucleotide sequencing data were used for further confirmation of FAV serotype identification (Meulmans *et al.* 2004). The 4 pathogenic isolates and 1 non-pathogenic isolate gave different restriction pattern and later confirmed as species D (serotype 2) showing 93–95% homology and species C (serotype 4) showing 96% homology, respectively, based on nucleotide sequence data on blast analysis. Hence, BLAST analysis using nucleotide sequence data may be used as effective tool for serotype identification of FAV field isolates.

Table 2. Percentage homology of local FAV field isolate by comparing the respective reference serotype Hexon gene nucleotide sequence

Field isolates	Reference strain serotype and accession No	Homology percentage
NPFAVTN1*	2 & AF339915 (P7-A)	95%
NPFAVTN2	4 & AJ431719 (KR95)	95%
PFAVTN1*	2 &AY683543	93%
PFAVTN2	2 &AY683543	93%
PFAVTN3	2 &AY683543	95%
PFAVTN4	2 &AY683543	94%
PFAVTN5	4 &AJ431719(KR95)	96%

NPFAV*, Non pathogenic fowl adenovirus; PFAV*, pathogenic fowl adenovirus.

Regarding pathogenicity the mild to hyper virulent nature of viruses were recorded in the same serotype (Pallister and Sheppard 1996). Even though our isolates were collected from apparently healthy flock with no mortality, the pathogenicity of these isolates were further confirmed by mean of experimental infection and they were found to be non-pathogenic in nature. More interestingly, the 2 non-pathogenic serotypes were prevalent in one poultry farm in the same period. In this study, the restriction patterns of both pathogenic and non-pathogenic stains of FAV serotype 2 and 4 are same. Similarly, Pallister and Sheppard (1996) reported that mild and hypervirulent exhibited similar restriction pattern for serotype 8. Most of the authors reported serotypes 2 and 4 associated with inclusion body hepatitis outbreaks

(McFerran 1997, Ojkic *et al.* 2008). In contrast in this study, both pathogenic and non-pathogenic strains belonging to same serotype 2 and 4 were observed because mutation in the hexon gene may affect the amount of virus escaping from the endosomes into the cell and thereby alter the virulence of virus as reported by Palliser *et al*. (1994). Even though non-pathogenic virus is not acting as primary pathogen for any disease condition, it was used as vector to express number of complex viral antigen (Sheppard *et al.* 1998, Francosis *et al*. 2004). However, our future attempts on full length hexon gene sequencing of both pathogenic and non-pathogenic serotypes will give more information about the homology percentage and nucleotide variation between the pathogenic and non-pathogenic strains.

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