ASSOCIATION OF FOWL ADENOVIRUS SEROTYPE 12 WITH HYDROPERICARDIUM SYNDROME OF POULTRY IN INDIA

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Summary. – Eight fowl adenovirus (FAdV) isolates obtained from different geographical regions of India were typed by a virus-neutralization test (VNT) using rabbit antisera against all the 12 serotypes of FAdV and by PCR for the hyper-variable region of hexon gene combined with restriction fragment length polymorphism (RFLP) analysis using *Alu*I and *Mbo*I restriction enzymes. It was found that six isolates belonged to FAdV-4, one to FAdV-12 and one to both of them. This study revealed the involvement of FAdV-12 alone or in association with FAdV-4 in precipitating inclusion body hepatitis - hydropericardium syndrome (IBH-HPS) among poultry flocks in the country.

Key words: fowl adenovirus; inclusion body hepatitis; hydropericardium syndrome; typing; PCR; restriction analysis; restriction fragment length polymorphism

Introduction

Inclusion body hepatitis (IBH) associated with hydropericardium syndrome (IBH-HPS) has been first reported from Angara Goth, Karachi, Pakistan in three to seven week-old broilers (Jaffery, 1988; Hassan, 1989) and later from Jammu-Kashmir and Punjab, India which subsequently spread to other parts of the country (Sreenivas Gowda and Satyanarayan, 1994; Kataria *et al.*, 1995; Oberoi *et al.*, 1996; Chandra *et al.*, 1997). The disease manifestation includes a characteristic hydropericardium with the heart simulating a de-shelled litchi fruit (*Litchi chinensis*; hence the name "litchi disease") and hepatitis with basophilic intranuclear inclusion bodies in hepatocytes and a mortality rate of 10–70%.

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Abbreviataions: CEL = chick embryo liver; CPE = cytopathic effect; FAdV(s) = fowl adenovirus(es); FAdV-4 = FAdV serotype 4; FAdV-12 = FAdV serotype 12; IBH = inclusion body hepatitis; HPS = hydropericardium syndrome; RFLP = restriction fragment length polymorphism; VNT = virus neutralization test

Fowl adenoviruses (FAdVs) belonging to 5 species (*Fowl adenovirus A-E)* and 12 serotypes (FAdV-1 to FAdV-12) are extensively distributed among commercial chickens worldwide and all except FAdV-11 are associated with inclusion body hepatitis (McFerran, 1997). In India, sporadic IBH outbreaks have been reported also earlier, but recently, the occurrence of IBH-HPS accounting for severe economic losses to the poultry industry have been described (Kataria *et al.,* 1996; Chandra *et al.*, 1997). FAdV isolates from cases of IBH-HPS in the country have been identified as serotype 4 using standard antisera to the 12 different serotypes by VNT (Jadhao *et al*., 1997). All the serotypes of FAdV share a common group-specific antigen, thus giving positive reaction with specific serum in all serological tests other than serum neutralization.

PCR for a target gene sequence has been applied as a rapid diagnostic tool for the detection of FAdVs in recent years (Raue and Hess, 1998; Barua, 2002). Restriction analysis can detect differences in the genome, which are of relevance for serology. Restriction analysis can further be used to differentiate individual FAdV serotypes and strains.

In spite of an intensive vaccination program the disease incidence has recently increased, which necessitated an investigation of the involvement of certain FAdV serotypes, if any, in the causation of IBH-HPS. Cases of IBH-HPS have

Table 1. The FAdV isolates examined in the study

No.	Isolate	Place of origin	Species
1	383/AD/97	Haryana	Chicken (*Gallus domesticus*)
2	421/AD/98	Uttar Pradesh	
3	488/AD/98	Lucknow, Uttar Pradesh	
4	507/AD/01	Coimbatore, Tamil Nadu	Japanese quail *(Coturnix coturnix japonica)*
5	528/AD/01	Izatnagar, Uttar Pradesh	Turkey *(Maleagris gallopava)*
6	608/AD/02	Amaria, Uttar Pradesh	Chicken *(Gallus domesticus)*
7	617/AD/02	Khatima, Uttaranchal	
8	387/AD/97	Bangalore, Karnataka	

also been encountered in quail and turkey, which are not considered natural hosts of FAdV-4. FAdV isolates have been obtained from such cases in our laboratory.

Hence, in this present study, eight FAdV isolates originating from different geographical locations and different avian host species were characterized by serotyping and PCR-RFLP to detect possible differences among them.

Materials and Methods

Viruses and cells. The FAdV isolates analyzed in this study were obtained from Division of Avian Diseases, Indian Veterinary Research Institute, Izatnagar, India (Table 1). Primary chick embryo liver (CEL) cell cultures were prepared according to Adair *et al*. (1979). The FAdV isolates underwent two passages in CEL cell cultures. All the isolates produced characteristic cytopathic effect (CPE) with cell degeneration and rounding within 48 to 96 hrs.

Antisera. Standard rabbit antisera against 12 different FAdV serotypes were kindly supplied by Dr. J.B. McFerran, Department of Agriculture, Veterinary Research Laboratory, Stormount, Belfast, Northern Ireland, UK.

VNT. A micro-VNT was performed according to Erny *et al.* (1995). The reciprocal value of the highest dilution of serum causing complete inhibition of cytopathic effect (CPE) in 50% of the wells was taken for calculation of the titer (Jadhao *et al.*, 1997).

PCR. Viral DNAs were extracted from cells infected with the isolates showing 70–80% CPE according to Shinagawa *et al.* (1983). The DNA pellets thus obtained were suspended in 20 µl of TE buffer (10 mmol/l Tris and 1 mmol/l EDTA pH 8.0) and stored at -20°C until used. The purity and concentration of the extracted DNAs was checked by UV spectrophotometry. PCR amplification of the hypervariable region of the hexon gene of FAdV was performed according to Ganesh *et al.* (2002) with some modifications. The reaction mixture (25 µl) contained 30 ng of viral DNA, 2.5 µmol/l dNTPs, 5 pmoles each of forward and reverse primers (forward: 5'-GACATGGGGTCGACCTATTTCGA CAT-3', reverse: 5'-AGTGATGACGGGACATCAT-3'), 1 U of *Taq* DNA polymerase, 10 mmol/l Tris-HCl pH 8.3, 1.5 mmol/l $MgCl_2$, 50 mmol/l KCl and 0.001% gelatin. After initial denaturation at 95°C for 7 mins it was subjected to 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 57°C for 1 min and extension at 72°C for 2 mins and final extension at 72°C for 5 mins. The PCR products were separated by electrophoresis in 1.5% agarose gels.

Restriction analysis. The PCR products generated from FAdV isolates were purified using Wizard™ PCR Preps DNA Purification System (Promega). Purified PCR products were subjected to digestion with *Alu*I and *Mbo*I according to the manufacturer's instructions. The digested products along with a 50 bp DNA ladder (Sigma) were electrophoresed in 2% agarose gel (Sambrook *et al.,* 1989).

Table 2. Serotyping of the FAdV isolates by VNT

Isolate	Virus neutralization titers with FAdV serotype-specific antisera											
	1	2	3	4	5	6	7	8	9	10	11	12
383/AD/97	–	–	–	400	–	–	–	–	–	–	–	–
387/AD/97	–	–	–	400	–	–	–	–	–	–	–	–
421/AD/98[a]	–	–	–	200	–	–	–	–	–	–	–	200
488/AD/98	–	–	–	400	–	–	–	–	–	–	–	–
507/AD/01	–	–	–	400	–	–	–	–	–	–	–	–
528/AD/01	–	–	–	400	–	–	–	–	–	–	–	–
608/AD/02	–	–	–	400	–	–	–	–	–	–	–	–
617/AD/02	–	–	–	–	–	–	–	–	–	–	–	800

(–) = < 50.

[a]Neutralized by 1:200 dilution of a 1:1 mixture (vol/vol) of FAdV-4 and FAdV-12 antisera.

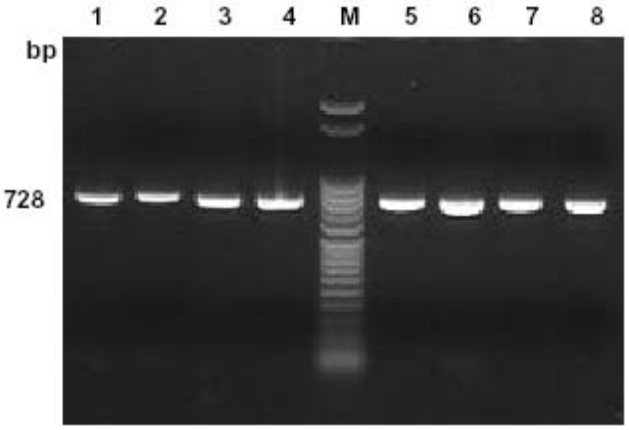

Fig. 1

Agarose gel electrophoresis of PCR products of FAdV isolates

The isolates 383/AD/97 (lane 1), 421/AD/98 (lane 2), 488/AD/98 (lane 3), 507/AD/01 (lane 4), 528/AD/01 (lane 5), 608/AD/02 (lane 6), 617/AD/02 (lane 7), and 387/AD/97 (lane 8). The DNA size marker, a 50 bp DNA ladder (lane M).

Results

Serotyping of FAdV isolates

The results of serotyping of all the eight FAdV isolates by VNT are presented in Table 2. The isolates 383/AD/97; 387/AD/97, 488/AD/98, 507/AD/01, 528/AD/01 and 608/AD/02 were neutralized by the 1:400 dilution of standard FAdV-4 antiserum. The isolate 617/AD/02 was not neutralized by the FAdV-4 antiserum, but it was neutralized by the 1: 800 dilution of FAdV-12 antiserum.

Thus the isolate 617/AD/02 belongs to FAdV-12, a serotype not reported to be associated with IBH-HPS in the Indian subcontinent to date. The isolate 421/AD/98 was not neutralized even by the 1: 50 dilution of any of the antisera against individual FAdV serotypes, but it was neutralized by the 1:200 dilution of a 1:1 mixture of FAdV-4 and FAdV-12 antisera. These results indicated that the isolate 421/AD/98 was a mixture of the both FAdV serotypes.

PCR-RFLP analysis of FAdV isolates

The viral DNAs extracted from the FAdV isolates were found to be free of protein contamination and with A_{260}/A_{280} ratios ≥1.2, indicating adequate purity. PCR amplification of the hypervariable region of hexon gene of FAdVs using DNAs extracted from the FAdV isolates revealed an expected amplicon of 0.7 kb (Fig. 1). Restriction analysis of purified PCR products generated from the FAdV isolates showed following results.

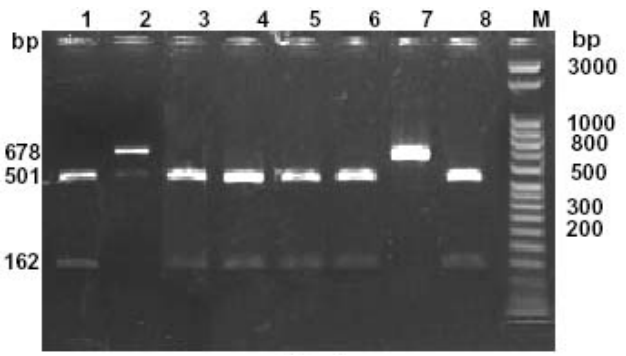

Fig. 2

Restriction profiles of PCR products of FAdV isolates with *Alu*I

The isolates 383/AD/97 (lane 1), 421/AD/98 (lane 2), 488/AD/98 (lane 3), 507/AD/01 (lane 4), 528/AD/01 (lane 5), 608/AD/02 (lane 6), 617/AD/02 (lane 7), and 387/AD/97 (lane 8). The DNA size marker, a 50 bp DNA ladder (lane M).

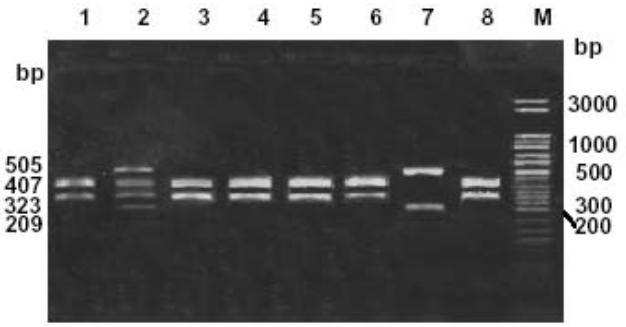

Fig. 3

Restriction profiles of PCR products of FAdV isolates with *Mbo*I

The isolates 383/AD/97 (lane 1), 421/AD/98 (lane 2), 488/AD/98 (lane 3), 507/AD/01 (lane 4), 528/AD/01 (lane 5), 608/AD/02 (lane 6), 617/AD/02 (lane 7), and 387/AD/97 (lane 8). The DNA size marker, a 50 bp DNA ladder (lane M).

On digestion with *Alu*I, all the isolates except 617/AD/02 and 421/AD/98 yielded yielded two fragments of 162 bp and 501 bp. The isolate 617/AD/02 gave only one fragment of about 678 bp. The isolate 421/AD/98 gave three fragments of 162 bp, 501 bp and 678 bp (Fig. 2).

On digestion with *Mbo*I, all the isolates except 421/AD/98 and 617/AD/02 yielded two fragments of 407 bp and 323 bp. The isolate 617/AD/02 gave two fragments of 505 bp and 209 bp and the isolate 421/AD/98 four fragments of 505 bp, 407 bp, 323 bp and 209 bp (Fig. 3).

These results confirmed those of serotyping made by VNT.

Discussion

In the present study, the FAdV isolates were propagated in primary chick embryo liver cell cultures because of a greater sensitivity compared to analogical kidney cultures (Swain *et al.,* 1993). The virus produced cell rounding and degeneration as observed by Adair *et al*. (1979).

FAdV serotypes 1–12 have been reported to be associated with naturally occurring IBH in poultry (McFerran, 1997) and FAdV-4 as the only serotype has been incriminated with IBH-HPS in India (Jadhao *et al*., 1997). The results of the study presented here have revealed the involvement of FAdV-12 in precipitating IBH-HPS among poultry flocks in India.

The PCR used could amplify a 0.7 kb fragment from the loop-1 and part of pedestal-1 of the hexon gene encoding the hypervariable region of hexon protein. The region amplified by the set of primers used in the present study has been reported to vary in serotype comparisons (Barua, 2002). It has been reported that mixed infections with different FAdV serotypes can occur in the same bird (Mockett and Cook, 1983; Erny *et al.,* 1995; Toro *et al.,* 1999). Also we found that the isolate 421/AD/98 was a mixture of FAdV-4 and FAdV-12. Meulemans *et al.* (2001) have analyzed 26 field FAdV isolates by amplification of the L1 coding region of hexon gene and subsequent restriction and RFLP analyses. Based on RFLP, they have found that 8 out of 26 FAdV isolates contained two different serotypes. They concluded that mixed infections with different FAdV serotypes could occur in the same bird.

The present study revealed the prevalence of more than one serotype of FAdV, namely FAdV-4 and FAdV-12 in the country and also their mixed infections causing IBH-HPS in poultry flocks in the subcontinent. Furthermore, it also proved that PCR-RFLP could be effectively used for the detection and differentiation of various FAdV serotypes. The identification of a new FAdV serotype in addition to FAdV-4, associated with IBH-HPS of poultry would complicate further the issue of this disease in India. This would also warrant the use of multivalent vaccines for controlling this disease and also necessitate a thorough improvement of its monitoring and surveillance vis-à-vis adenoviral infections of poultry.

Note of the Editor-in-Chief. The classification of FAdVs into 12 serotypes, namely FAdV-1 to FAdV-12, used in this paper is not compatible with the presently valid classification of FAdVs into 11 main serotypes, namely FAdV-1 to FAdV-11 (van Regenmortel *et al*., 2000).

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