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Full Length Research Paper

Molecular characterization of fowl adenoviruses associated with hydro-pericardium syndrome in broilers

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Hydropericardium syndrome (HPS) is a viral disease of poultry, which is common in hot and humid season during summer in Pakistan. Fowl adenovirus serotype 4 (FAdV-4) classified into the species Fowl adenovirus C and the genus Aviadenovirus, which were identified as the causative agent of the disease during field outbreaks. The virus was obtained from suspected broiler birds showing typical signs and postmortem lesions that is ascites, hydropericardium, multifocal hepatic necrosis and hepatitis. The virions were partially purified from the livers of infected birds, by sucrose density gradient ultracentrifugation. A variable part of the hexon gene (L1 and part of P1, 730 bp) was amplified by polymerase chain reaction (PCR) and its nucleotide sequence was determined. During nucleotide sequence analysis, the viruses were identified as FAdV- 4. The presence of FAdV-4 was also supported by scanning electron microscopy. Phylogenetic analysis of the partial hexon gene of the viruses determined their close relation with previously sequenced FAdV-4 isolates from India.

Key words: Fowl adenovirus serotype 4 (FAdV-4), hydro-pericardium syndrome, hexon, electron microscopy, phylogenetic analysis, taxonomy.

INTRODUCTION

Hydro-pericardium syndrome (HPS) is an important disease of poultry particularly effecting 3 to 6 week old broiler chicks. The disease is characterized by sudden onset with 20 to 70% mortality, accumulation of fluid in the pericardial sac, enlarged discolored liver with necrotic foci and intra-nuclear inclusion bodies in hepatocytes (Dahiya et al., 2002; Anjum et al., 1989). The causative agent is fowl adenovirus serotype 4 (FAV-4), which belongs to the genus Aviadenovirus of the family Adenoviridae. Adenoviruses are non-enveloped, medium sized, icosahedral particles with linear, double stranded DNA genome. Adenoviruses are classified into four recognized genera which include Atadenovirus, Siadenovirus, Mastadenovirus and Aviadenovirus while the only confirmed Fish adenovirus felled into fifth clade (Davison et al., 2003). Harrach et al. (2011) suggested to place the Turkey adenovirus species in genus Aviadenovirus and renamed the species in the family Adenoviridae by adding letter designation "*Possum adenovirus* A, *Goose adenovirus* A, *Canine adenovirus* A, *Tree shree adenovirus* A and *Frog adenovirus* A". Transmission of the disease occurs through oral - fecal

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route, both vertically and horizontally. The first outbreak of HPS was reported in 1987 from *Angara Goth* near Karachi, Pakistan, so the disease was locally named as *Angara* disease (Anonymous, 1988; Roy et al., 2004). Later the syndrome was also found in medium weight layers and broiler breeders (Anjum, 1988). Afzal et al. (1991) and Kim et al. (2008) noticed that HPS was common in broilers at 3 to 5 week of age, whereas outbreaks were also reported in layers and breeders. The syndrome can be reproduced in broilers by the inoculation of a liver homogenate from affected birds (Anjum, 1990; Nakamura et al., 1999). Polymerase chain reaction (PCR) amplification of a hyper variable region (L1 and part of P1) of the hexon gene and its phylogenetic analysis has revealed that FAdV-4 isolates from chickens with HPS in Japan were identical but they were differentiated from the isolates of India and Pakistan (Meulemans et al., 2004; Masaji et al., 2009). This work was carried out to characterize the viruses causing HPS in Pakistan and to determine their phylogenetic relationship with other regional isolates.

MATERIALS AND METHODS

Collection of infected samples

Postmortems of suspected birds were conducted and livers were collected from naturally infected broilers showing typical post mortem lesions (enlarged liver, serous fluid in the pericardial sac and abdomen) of HPS. Details of samples are given in Table 1. One gram of liver was homogenized in 10 ml of phosphate buffer saline (PBS) and centrifuged at 3000 rpm for 15 min at 4°C. The supernatant was collected and stored at -20°C after addition of antibiotics that is Penicillin 10, 0000 I.U/ml, Streptomycin 10 mg/ml and Gentamycin 250 µg/ml (Roy et al., 2004).

Transmission of infection

Ten healthy broiler birds of 3 weeks old from a commercial poultry farm were experimentally infected with 1 ml of 10 % liver homogenate and observed for signs and symptoms of the disease up to 10 days post inoculation, as shown in Table 2. Five broiler birds of same age were inoculated with 1 ml of sterile PBS and kept as control (Kumar et al., 1997). Birds were observed daily for clinical signs and mortality. At post mortem examinations, the livers were collected for re-isolation of the virus as described previously.

Semi-purification of the virus

Partial purification of the virus was performed by ultra centrifugation of the supernatant, described in previous (Kumar and Chandra 2004). The supernatants were layered on preformed, 15 to 35% sucrose gradients prepared in 0.1 M PBS and centrifuged at 80,000 rpm for 2 h at 4°C. An opaque band was found between 25 and 35% sucrose gradient. This band was collected, diluted with PBS and spun again in a centrifuge at 80,000 rpm for 2 h at 4°C. The pellet was resuspended in 0.5 ml of PBS and stored at -20°C. Purity of the viral suspension was determined using spectrophotometer, by measuring the absorbance ratio at 260 and 280 nm as described by Kumar and Chandra (2004). This virus suspension was used for DNA extraction.

Viral DNA extraction

DNA was extracted as described by Ganesh et al. (2002) with some modifications. The semi purified viral suspension was digested by adding 200 µl lysis buffer (Tris-HCl, NaCl, SDS, and Proteinase K to final conc. of 25 , 100 mM, 0.5% and 25 µg/ml respectively) in 200 µl viral sample. Subsequently, 100 µl of phenol:chloroform (a:a) was added and well mixed. After centrifugation, the supernatant was moved into a new tube, and the concentration of NaCl was adjusted at 0.2 M. DNA was precipitated by chilled (-20ºC) ethanol. The DNA was pelleted by centrifugation. The DNA pellet was dried, re-dissolved in 20 µl of double-distilled water and stored at –20ºC.

Detection of viral DNA by polymerase chain reaction (PCR)

Amplification of a 730 bp variable part of the hexon gene was done by PCR to confirm the presence of viral DNA. The primers (FAVHL 5'- GACATGGGGTCGACCTATTTCGACAT-3' and reverse primer FAVHR 5'- AGTGATGACGGGACATCAT-3') as well as the conditions of the PCR (35 cycles consisting of 94°C for 60 s, 57°C for 60 s and 72°C for 120 s) as described by Ganesh et al. (2001). The PCR products were analyzed along with 1 Kb DNA ladder on 1.5% agarose gels containing 0.1% ethidium bromide. The electrophoresis was performed at 100 Volts for 30 to 40 min in 1 X TAE buffer. After electrophoresis, PCR products were visualized by placing the gel on ultra violet light transluminator.

DNA sequencing of the polymerase chain reaction (PCR) product

Nucleotide sequence of the PCR products were determined from both sides using forward and reverse primers, on an ABI 3100 capillary sequencer. The sequence data were searched in public access databases (GenBank, EMBL) with BLAST (Basic Local Alignment Search Tool) as described by Altschul et al. (1990).

Scanning electron microscopy (SEM)

Surface structure of the virus was analyzed under field emission scanning electron microscope (FESEM) as described by Hong et al. (2005) with some modifications. Virus particles were adsorbed on the surface of carbon coated grids by placing a drop of the semi purified viral suspension on the grid and drained out extra fluid. Specimens were negatively stained by adding a drop of 2% uranyl acetate stain for few seconds and extra stain was drained. Specimens were air dried and observed under Scanning Electron Microscope (Model No: JEOL JFM 7500 F). Micrograph was taken under low-dose conditions at 3.0 KV and at magnification of 30,000 ×.

Phylogenetic analysis

Multiple sequence alignment and phylogenetic analysis were done on observed divergence basis by Multiple Sequence Alignment program of "DNAMAN, Lynnon Biosoft" (Lynnon Corporation, Quebec, Canada). Pair-wise comparison of nucleotide sequences of the variable part of the hexon gene was done. A rooted genetic dendrogram was constructed by neighbor-joining method and phylogenetic relationships of our isolate with other reported isolates were determined. All sequences used for multiple alignments were obtained from public access databases GenBank and EMBL,

Table 1. Case history of flocks with natural outbreaks used for virus isolation.

No.	Year	Site of collection	Type of birds	No. of birds	Age of birds (days)	Mortality (%)
1	2008	Punjab, Pakistan	Broilers	8000	35	25
2	2009	Punjab, Pakistan	Broilers	6000	26	32

Table 2. Mortality results after experimental infection with 10% liver homogenate of diseased birds.

	Number of birds	Age of broilers	Infection		Mortality (%)
			Dose	Route	
Treatment	10	21 days	1 ml	S/C	80 (8/10)
Control	5	21 days	1 ml (PBS)	S/C	0 (0/5)

Table 3. Details of the sequence data used for multiple sequence alignment.

No.	Accession No.	Virus type	Host animal	Country of origin
1	AF207659.1	BAdV-6	Cattle	Iowa, USA
2	AF339923.1	FAdV-9	Chicken	Belgium
3	AY581275.1	FAdV	Turkey	Izatnagar, India
4	DM380829.1	CELO Virus	Chicken	Germany
5	DQ149638.1	HAdV-45	Human	Vienna, Austria
6	DQ264728.1	FAdV-4	Chicken	Pakistan
7	EF442329.1	Psittacine AdV-1	Parrot	Germany
8	EF685395.1	Fowl adenovirus C	Chicken	Canada
9	EU847626.1	FAdV-4	Chicken	Haryana, India
10	FN394664.1	FAdV-4	Chicken	Hemachal perdesh, India
11	L80007.1	EAdV-2	Horse	Davis, CA, USA

details are shown in Table 3.

RESULTS AND DISCUSSION

During experimental infection, eighty percent mortality was observed in the treatment group while control birds remained healthy. Results are shown in Table 2. Similar results were found by Kumar et al. (1997) who experimentally infected the birds with FAdV-4.

Presence of the virus was confirmed by PCR amplification of a 730 bp fragment from the hexon gene as shown in Figure 1. Nucleotide sequence of the PCR products from two isolates revealed identical sequence that was submitted to the GenBank with accession no. FR686931.1.

The BLAST revealed that the nucleotide sequence of our isolate has 99% identity with several Indian isolates (Haryana, Hemachal Perdesh, Izetnagar; accession no. EU847626.1, FN394664.1, AY581275.1) of FAdV-4, while 98% identity was found with a Pakistani FAdV-4 isolate (accession no. DQ264728.1). The identity values with a German psittacine adenovirus (accession no. EF442329.1) and FAdV-1 (CELO) (accession no. DM380829.1) were found to be 65% and 70% respectively.

Multiple sequence alignment (MSA) of the 730 bp variable region of the hexon gene with the corresponding gene fragment from other adenovirus is presented in Figure 4. MSA revealed that this region of human adenovirus serotype 45 contains 847 bp, and thus is longer than the corresponding part of other mastadenoviruses (equine or bovine). Our results are in line with the findings of Harrach et al. (2011) who found that, adenoviruses which infect the birds are classified into three genera including Atadenovirus, Mastadenovirus and Aviadenovirus. It was also found that the L1 – P1 region of the hexon gene is hypervariable in the middle both; the ends are more conserved thus being suitable for PCR primer design.

The results of phylogenetic analysis showed close relation with the regional (Indian and Pakistani) FAdV-4 isolates as supported by the tree on Figure 3. So it was suggested that isolates of FAdV in India and Pakistan had a common ancestor, which can be differentiated from other FAdV isolates of Canada, Belgium and Germany. Similarly, Meulemans et al. (2004) and Masaji et al. (2009) suggested that FAdV-4 isolates in Japan were

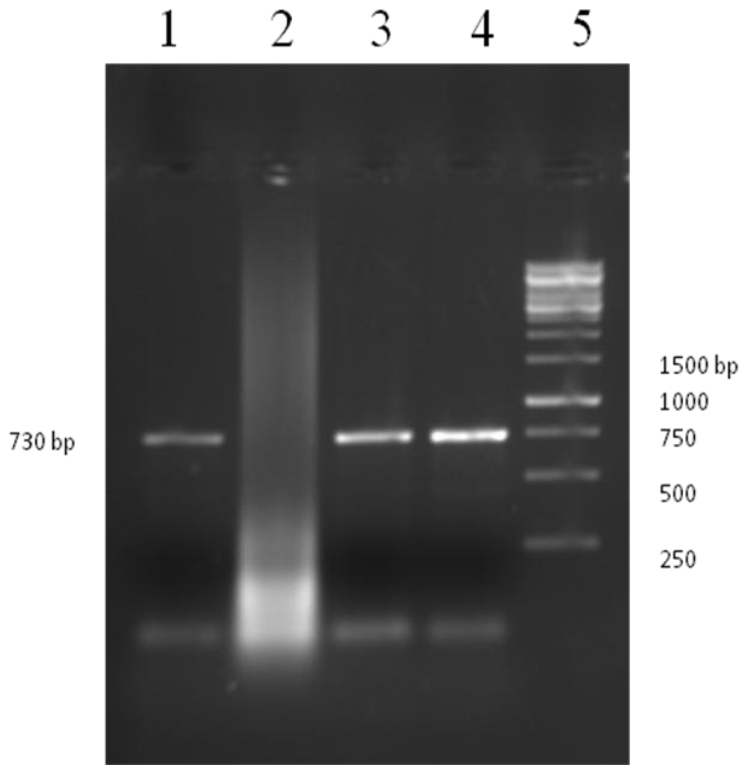

Figure 1. Agarose gel electrophoresis. Lane 1, + ve control; Lane 2, - ve control; Lane 3 and 4, 730 bp PCR product; Lane 5, 1 Kb molecular weight marker.

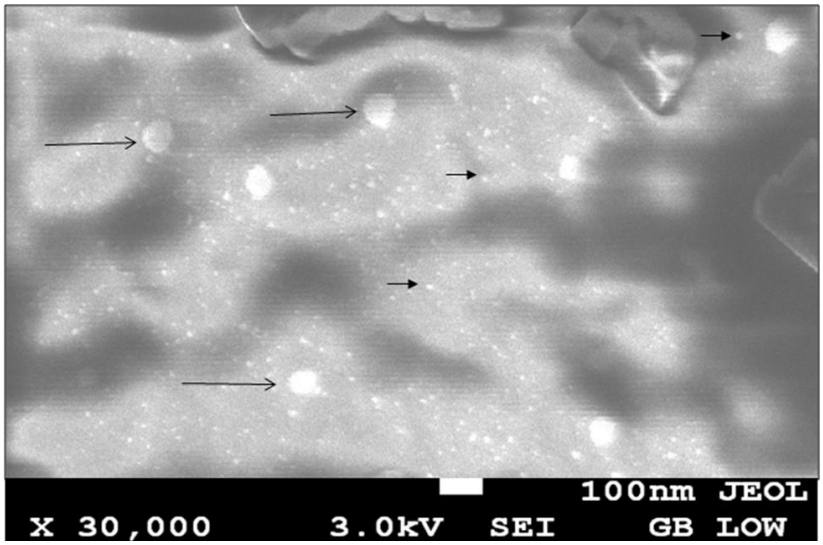

Figure 2. Scanning Electron micrograph of intact FAdV-4 showing icosahedral shape capsids, indicated by long arrows. Hexons found in back ground from disrupted viral particles indicated by small arrows. The scale bar represents 100 nm.

identical but they were different from the isolates of India and Pakistan.

The protein concentration of the semi-purified virus samples was estimated to be 0.85 to 0.90 µg/µl., The SEM photograph of intact virions (Figure 2) revealed typical icosahedral capsids of the virus. The diameter of

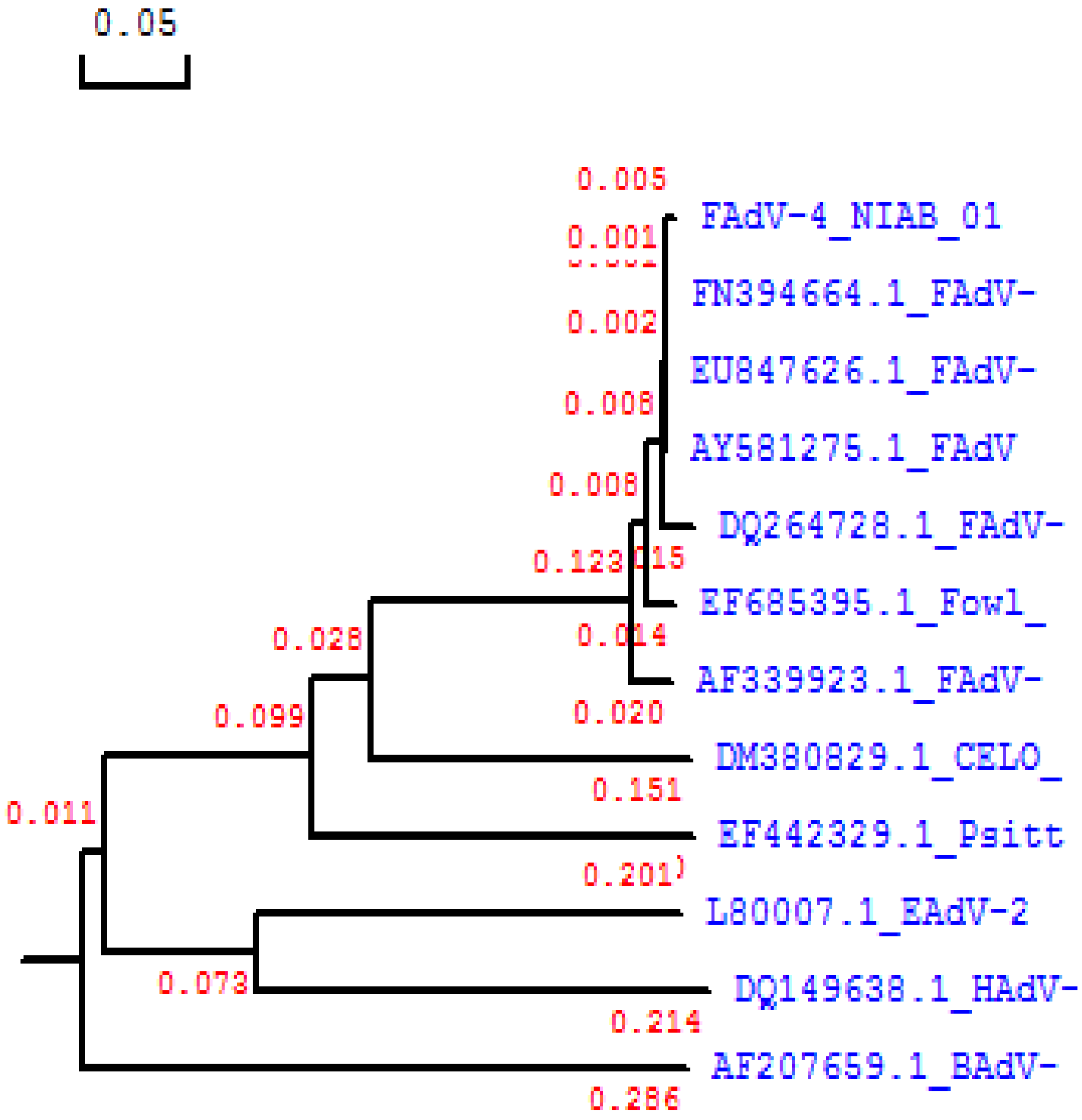

Figure 3. Phylogenetic tree based on a 730 bp hypervariable region of the hexon (FAdV- 4 NIAB 01) a rooted genetic distance dendrogram.

each capsid was estimated to 70 to 80 nm. Laver et al. (1971) also found capsids of same shape and size of an avian adenovirus (CELO). The background of the photograph has small white particles which likely represent the group of hexons from disrupted viral particles as previously found by Hong et al. (2005).

In summary, molecular characterization of two FAdV isolates from Pakistan revealed that these isolates were identical to each other but they were considerably different from isolates of other countries like Belgium and


AF207659.1_BAdV-
L80007.1_EAdV-2
DQ149638.1_HAdV-
EF442329.1_Psitt
DM380829.1_CELO_
EF685395.1_Fowl_
AY581275.1_FAdV
AF339923.1_FAdV-
FN394664.1_FAdV-
EU847626.1_FAdV-
DQ264728.1_FAdV-
FAdV-4_NIAB_01
Consensus

Figure 4. MSA of 730 bp variable region of Hexon gene from various Adenovirus isolates.

Canada. It was also determined that Pakistani isolates had nearest phylogenetic relationship with Indian.

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