

Molecular Characterization of Fowl Adenovirus Serotype 4 (FAV-4) Isolate Associated with Fowl Hydropericardium-Hepatitis Syndrome in Pakistan

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Abstract.- A study was conducted to characterize local fowl adenovirus serotype 4 (FAV-4) associated with hydropericardium syndrome (HPS) at molecular level. For this purpose, chicken livers naturally infected with HPS virus were aseptically collected from a field outbreak in commercial broilers from Faisalabad, Pakistan. Virus purification was done using a 15-45% (w/v) sucrose gradient. Virus was confirmed by agar gel precipitation test. Viral DNA was extracted and variable region hexon gene of 0.7 kb in size was amplified by PCR and sequenced. Nucleotide sequence (730 bp) and deduced amino acid sequence (243) of isolate was compared with already published sequences of FAV4. Sequence analysis of the variable region hexon gene confirmed that the virus belonged to avian adenovirus serotype 4. The nucleotide sequence of the Pakistani isolate had a 94% to 98% homology with Indian isolates, 97% homology with Belgium and Russian isolates and 96% homology with Canadian isolate. The amino acid sequences of the Pakistani isolate had an almost 87% to 98% homology with Indian, 92% and 95% homology with Belgian, 96% homology with Russian and 95% homology with Canadian isolate.

Key words: amino acid sequencing, fowl adenovirus 4, hexon gene, hydropericardium-hepatitis syndrome, Pakistani isolate

INTRODUCTION

Hydropericardium syndrome (HPS) since its first epidemic in 1987 reported from Angara Goth near Karachi, Pakistan caused huge economic losses to poultry industry in many parts of world (Jaffery, 1988; Abdul-Aziz and Al-Attar, 1991; Gowda and Satyanarayana, 1994). The disease was characterized by abrupt onset, with high mortality ranging from 12% to 75%, accumulation of a jelly-like fluid in the pericardial sac with deformed and flabby heart; pulmonary edema, nephritis, enlarged mottled and friable livers, with intranuclear inclusion bodies (INIBs) in the hepatocytes (Ahmad *et al.*, 1989; Cheema *et al.*, 1989). The causative organism of HPS is fowl adenovirus (FAV) serotype 4 belonging to group 1 FAV of Adenoviridae family (Mazaheri *et al.*, 1998; Balamurugan *et al.*, 2002). As far as structure of fowl adenoviruses are

concerned, they are non-enveloped with icosahedral structure, composed of 252 capsomers. Out of these 252 capsomers, there are 12 vertex capsomers (penton bases) and 240 non-vertex capsomers (hexons). There are seven polypeptide present in virion capsid. Hexon as major protein of the adenovirus capsid, known to have region related to virus neutralizing and serotype specificity (Norby, 1969; Norby and Wadell, 1969; Toogood *et al.*, 1989).

The traditional diagnosis of HPSV in Pakistan is based on the clinical signs and pathological alteration (Anjum *et al.*, 1989; Muneer *et al.*, 1989). The detection of viral antigen from the clinical samples by conventional methods like cell culture, electron microscopy, as well as different serological tests *e.g.*, virus neutralization test, fluorescent antibody test and enzyme linked immunosorbent assay (ELISA) (Hassan *et al.*, 1993; Kumar *et al.*, 1997; Balamurugan *et al.*, 2001) are available, which are cumbersome and time consuming conventional techniques. For proper diagnosis of HPS virus, need of the time is to standardize a rapid and accurate diagnostic test in

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our local conditions (Khan *et al.*, 2005). PCR, a technique for amplifying a specific DNA fragment *in vitro* is an exquisitely sensitive and rapid molecular diagnostic technique, being used widely for the diagnosis of a variety of avian pathogens (Cavanagh, 2001). This manuscript for the first time describes PCR based detection and sequence analysis of variable region hexon gene of HPSV from Pakistan, which will further enable to characterize the FAV4 at molecular level.

MATERIALS AND METHODS

Virus isolation and purification

Liver samples of clinically affected birds with hydropericardium syndrome (HPS) were collected from district Faisalabad, Pakistan. A 20% (w/v) homogenate of infected liver tissue was prepared in phosphate buffered saline (pH 7.2). Antibiotics (Penicillin 1000 I.U. /ml and Streptomycin 100 µg/ml) were added. This homogenized infected liver material was then subjected to sonication (Rapidis 600; ultrasonics Ltd., USA) at 20 kHz, 105 W twice for 3 minutes. Sonicated homogenized infected liver material was centrifuged at 3000 rpm for 20 minutes in a refrigerated centrifuge (4°C) to sediment tissue debris. The supernatant collected was clarified by filtration through 0.45 µm and 0.2 µm membrane filters (Millipore Corporation, USA). The virus was then pelleted using the method of Naeem *et al.* (1995) with few modifications through a 35% (w/v) sucrose cushion in Tri-buffered saline (TBS10m mol/L Tris-HCl and 150 m mol/L NaCl; pH 7.4) at 9000xg for 2 h at 4°C. Viral pellet was again purified by centrifugation using 15-45% (w/v) sucrose gradients in TBS at 9000xg for 4 h at 4°C. Viral band at 25-35% sucrose concentration was collected and again pelleted at 9000x g for 2h at 4°C. The virus pellet was re-suspended in TBS and stored at -70°C until used.

Agar gel precipitation test (AGPT)

Hyperimmune serum was prepared in five, 2-week-old broiler birds as described by Kumar and Chandra (2004). Briefly, three broiler birds were initially inoculated with 0.5 ml of HPS vaccine (Bioangara plus, Sana lab) S/C route followed by weekly injections of 0.75, 1 ml and 1.5 ml of same

preparation mixed with equal volume of Freund's complete adjuvant. Two birds were inoculated with PBS as negative control. The blood samples were collected 14 days post-boosting. Sera were separated, heat inactivated in water bath at 56°C for 30 minutes and stored at -20°C until used. Virus confirmation was done by AGPT (Kumar *et al.*, 1997).

DNA extraction

Viral DNA from purified viral suspension was extracted following the procedure of Ganesh *et al.* (2002) with slight modifications. For viral coat protein digestion 1mL of viral suspension was mixed with 30µL of 10% SDS and vortexed twice for ½ minute. Proteinase K (50 µg/µL) was added in an amount of 10µL and suspension was incubated at 37°C for 1 hour. Equal volume of chloroform-isoamyl alcohol was added and the tubes were inverted vertically 5–10 times, followed by spinning at 11600g for 10 min in a centrifuge (MSB010 CX1.5, MSE, UK). Three molar NaCl (60µL) was added to the supernatant. The DNA was precipitated using equal volume of ice-cold 100% ethanol and kept at -20°C for 60 min. After brief centrifugation, the pellet was washed with 70% ethanol and then allowed to dry. Further more DNA quality was checked by measuring the ratio of absorbance (A_{260}/A_{280}). Finally, after checking the purity, DNA was re-suspended using 100 µL of sterile water (d₃H₂O). The homogenate from uninfected liver was used as control.

Amplification of variable region of hexon gene

The extracted DNA was subjected to PCR using the method of Ganesh *et al.* (2002) with few modifications. Briefly the amplification reaction contained 5µL of target DNA, 0.5µL of (1.0 U) of Taq DNA polymerase (MBI, Fermentas), 50 mM KCl, 3 mM MgCl₂, 1 µL of (100µM) of each dNTP, 18 pmol of each upstream FAVL (5'GACATGGGGTCGACCTATTTTCGACAT3'OH) and downstream FAVR (5'AGTGATGACGGGACATCA T3'OH) primers (MWG-Biotech, Germany) and 0.01% gelatin. PCR was then performed for 40 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min and polymerization at 72°C for 2 min. The initial denaturation step was conducted at

94°C for 3 min, and the final polymerization step was at 72°C for 10 min. All amplification products were analyzed on a 1.2% agarose gels and stained with ethidium bromide.

Nucleotide sequence analysis

PCR product (0.7 kb) amplified from hexon gene using FAVL/FAVR primer pair was purified in low melting point agarose following the method described by Sambrook and Russel (2001). A gel of low melting point agarose at concentration of 1.0% was prepared in 1x TAE buffer. Approximately, 20µL of PCR products were loaded into the slots and electrophoresis was carried out along with 100 bp DNA molecular weight marker (GeneRuler, MBI Fermentas) at constant 80 V for 60 min.

The DNA band of interest was visualized on a UV Trans-illuminator. Using a sharp razor blade, slices of agarose containing the bands of interest were cut out and transferred into a clean microfuge tube, and were incubated in five volumes of LMT elution buffer at 72°C for ten min. The solution was cooled to room temperature, equal volume of equilibrated phenol was added, vortexed and then aqueous phase was recovered by centrifugation at 4000x g for 10 min at 20°C. The aqueous phase was extracted by using phenol: chloroform (1:1) and once with chloroform. The aqueous phase was transferred to a fresh tube, and then 0.2 volume of 10M ammonium acetate and two volumes of chilled absolute ethanol was added. The mixture was stored at room temperature for 10 min, and then DNA was pelleted by centrifugation at 5000xg for 20 min at 4°C. The DNA pellet was washed twice with 70% ethanol. The pellet was air dried for 30 min, and then dissolved in 20 µL of TE buffer (pH 8.0).

The purified product along with primers were submitted for DNA sequencing to confirm the specificity of amplicon using a commercial service (MWG-Biotech, Germany). The sequence homology of purified HPSV based on variable region hexon were analyzed and compared with already published sequences using BLASTn program (National Center for Biotechnology Information, USA). Phylogenetic analysis of sequences was performed for the determination of relationships among Pakistani and other strains. A neighbour-joining distance tree with Tamura-Nei genetic model was built with Geneious

pro 3.8 with 1000 Bootstrap replicates, using the sequences obtained in this study and aligned by the Clustal/W method. The nucleotide sequence was submitted to the GenBank nucleotide database and assigned the accession number DQ264728.

RESULTS

The AGP test was used for initial confirmation of purified virus. One sharp band of single identity was observed with known hyperimmune serum.

Figure 1 shows 0.7 kb PCR product (lanes 1, 2, 3) which was absent from healthy liver extract (lane 4).

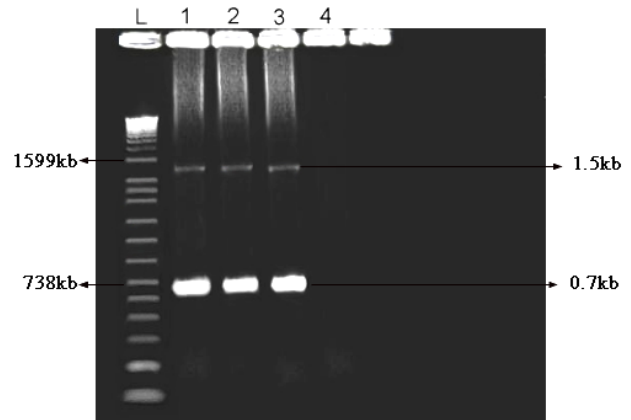


Fig. 1. Visualization of 730-bp PCR product of variable region of hexon gene of HPS virus by agarose gel electrophoresis (1.2%) after staining with ethidium bromide. Lane L, DNA ladder; Lanes 1-3, field isolates; Lane 4, healthy liver tissue extract.

Sequence analysis confirmed the presence of variable region hexon gene of FAV4. Comparative nucleotide analysis of Pakistani isolate revealed a 94% to 98% identity with Indian, almost 97% identity with Belgium and Russian isolates and 96% identity with Canadian isolate (Table I). There are five splice acceptor sites CAGG in the 730 bp sequences. The G + C content of the 730 bp sequences are 59.32 % (Fig. 2).

The deduced amino acid sequence of 730 bp product of Pakistani FAV4 encodes for a 243 amino acid protein. The protein is basic in nature having

Table I.- Homology in nucleotide and its derived amino acid sequences of variable region of hexon gene of Pakistani isolate with that of published data.

Accession	Description	Nucleotide	Protein
AY581275	Fowl adenovirus 528/AD/01/turkey/India hexon gene	98%	98%
AY581274	Fowl adenovirus 507/AD/01/quail/India hexon gene	98%	98%
AY581298	Fowl adenovirus 4 isolate Nepal hexon gene	98%	98%
AY581297	Fowl adenovirus 4 isolate Bangalore hexon gene	98%	98%
AY581296	Fowl adenovirus 4 isolate 608 hexon gene	98%	98%
AY581295	Fowl adenovirus 4 isolate 488 hexon gene	98%	98%
EU177546	Fowl adenovirus 4 isolate RC hexon protein gene	98%	98%
EU177544	Fowl adenovirus 4 isolate GC hexon protein gene	98%	98%
AY581299	Fowl adenovirus 4 isolate Std hexon gene	98%	98%
EU177545	Fowl adenovirus 4 isolate KC hexon protein gene	98%	97%
AJ459805	Fowl adenovirus 4 hexon gene for hexon capsid protein, isolate Indian	98%	97%
AF508951	Fowl adenovirus 4 strain KR5 hexon gene	97%	95%
AF339917	Fowl adenovirus 4 strain ATCC VR-829 hexon gene	97%	92%
AJ431719	Fowl adenovirus 4 hexon gene	97%	96%
EF685395	Fowl adenovirus C isolate 04-50388 hexon protein gene	96%	95%
AJ554049	Fowl adenovirus 4 hexon gene for hexon capsid protein	94%	92%
AF154246	Fowl adenovirus 4 hexon protein gene	94%	87%

ten arginine, three histidine and seven lysine residues. The amino acid sequences of the Pakistani isolate had an almost 87% to 98% identity with

Indian, 92% and 95% identity with Belgian, 96% and 95% identity with Russian and Canadian isolate respectively (Fig. 3).

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1 GACATGGGGT CGACCTATT CGACATCAAG GGAATCCTAG ACCGAGGGCC GTCCTTCAAG 60
61 CCCTACTGCG GCACGGCTTA CAACCCGCTG GCTCCCAAGG AGTCATGTT TAACCACTGG 120
121 TCGGAGACGG CACCCGGGCA GAACGTGTC GCCTCCGGTC AGCTGTCCAA TGTCTATACC 180
181 AACACGAGCA CCTCCAAAGA CACGACGGCG GCGCAGGTGA CGAAGATTTC CGGCCTCTTC 240
241 CCCAATCCCA ACCAGGGACC CCGAAGAAAT CCTCTCCGAC AGGTAGAAAA CGCCAACACC 300
301 GCGTGTCTCG GTCGCTTCGC CAGGTCTCAG TACAATTACG CTTACGGTGC CTACGTCAAG 360
361 CCGGTGCGCC CCGACGGTTC CCAGTCCCTC ACGCAGACCC CACTACTGGCA AGACGATAAC 420
421 ACGGGCGTAT CGTACCTGGG AGCGGTGGCC GTCGAGGACT ACACCAACAG CCTCTCGTAC 480
481 CCAGATACCA TAGTGTGTC GCCTCCGAG GACTACGAG ATTATAACAT AGGCACACAG 540
541 CGTGCCTCA GGCCTCACTA CATCGGGTTC AGGGATAACT TCATTAACT GCTGTATCAC 600
601 GACTCCGGCG TGTGCTCGG CACCCTCAAC TCGGAGCGTT CGGGCATGAA CGTGGTGGTC 660
661 GAGCTGCCC ACCGGAATAC CGAGCTCAGC TACCAGTACA TGCTGGCCGA CATGATGTCC 720
721 CGTCATCACT

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Fig. 2. Nucleotide sequence of 730 bp variable region of hexon gene (underlining showing CAGG sites).

Phylogenetic tree was made with already published sequences (Fig. 4), which revealed that Pakistani strain clustered most closely with Indian strains with high bootstrap confidence levels, and differ phylogenetically from Belgium, Russian and Canadian subtypes.

DISCUSSION

Locally isolated HPS virus was initially purified by 15–45% sucrose density gradient. Virus-containing band between 25 and 35% sucrose bands gave one precipitation band of single identity with known hyperimmune serum. Sucrose density gradient centrifugation has been used for the purification of HPS virus from naturally infected birds (Haq *et al.*, 1997; Ganesh *et al.*, 2001). These workers used 10–55% sucrose density gradient and obtained the virus-containing band between 25 and 35% sucrose bands. Caesium chloride step gradient ultracentrifugation has also been used for the purification of type 1 avian adenovirus (Maiti and Sarkar, 1997). DNA of the purified HPS was extracted following the procedure described by Ganesh *et al.* (2002). The results of present studies indicated that this procedure worked well for extraction of DNA from purified HPS virus. Quality of DNA was first checked by running on gel and then by measuring the absorbance ration at A_{260}/A_{280} that was 1.808. Quick assessment of nucleic acid purity could be done by determining the ratios of

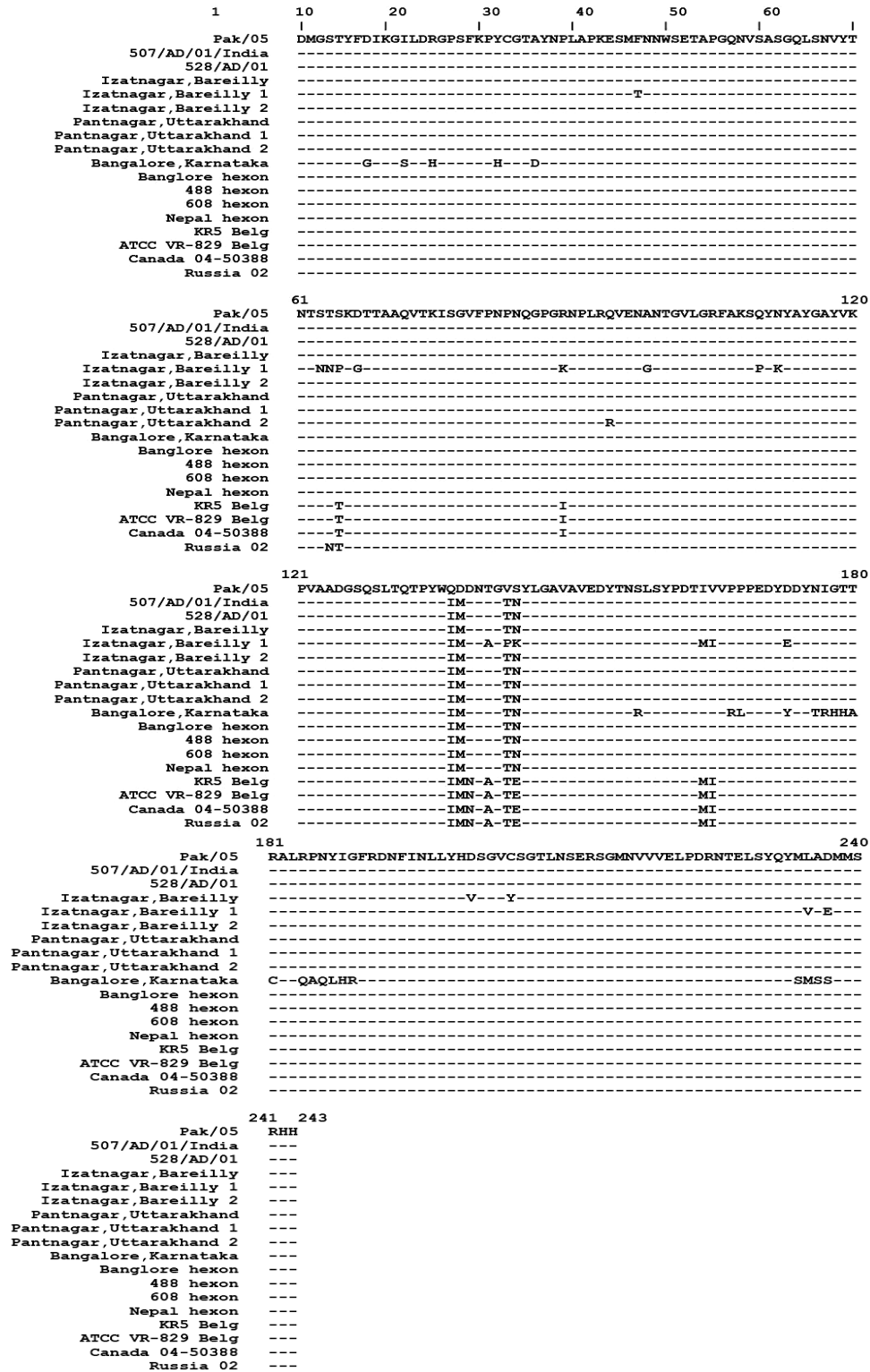


Fig. 3. Comparative amino acid homology of variable region of hexon gene of fowl adenovirus serotype 4 isolate from Pakistan with the publish data.

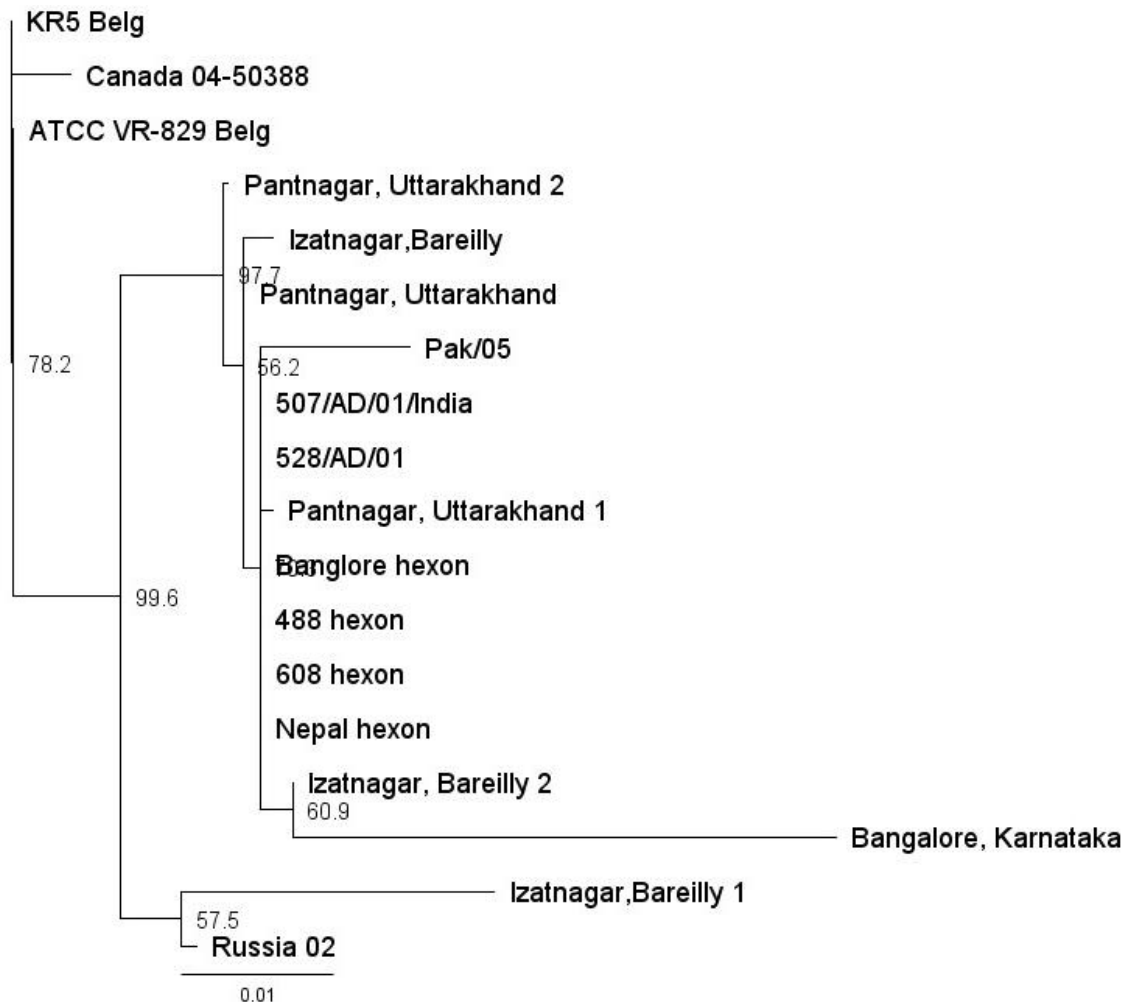


Fig. 4. Phylogenetic relationship among Pakistani, Indian, Belgium, Russian and Canadian isolates, following alignment of sequences from the variable region hexon gene. Bootstrap confidence levels are presented.

spectrophotometric absorbance of sample at A_{260}/A_{280} . This procedure was first described by Warburg and Christian (1942) as a means to measure protein purity in the presence of nucleic acid contamination, it is most commonly used today to assess purity of nucleic acid samples. The results of present study are also in line with the findings of other workers (Manchester, 1995) who used the same technique for quantification and to check the purity of DNA samples.

The hexon gene provides the basis for the PCR based identification of various avian adenoviruses (Hess, 2000). The hexon as a major antigenic protein of adenovirus exposed on the capsid of virion. PCR based amplification and restriction analysis has been used to reveal the

differences between reference strain and field isolates of FAV4 (Toro *et al.*, 1999). Hess *et al.* (1999) also used the restriction analysis to categorize twelve fowl adenoviruses from seven countries including Pakistan. They described that after Hpa II digestion isolates from India and Pakistan may possibly be separated from rest of isolates. Sheppard *et al.* (1995) mapped, cloned and sequenced the hexon gene of FAV10, the first avian adenovirus for which the hexon gene sequence is available. The amplicon defined by the selected primers carries the variable region of hexon gene. Initial heating of DNA at 95°C is very much essential before the amplification of 0.7 kb band that is due to high G + C contents (59.32%). Ganesh *et al.* (2002) found 57.2% G + C contents in their

isolate.

It is evident from the sequence analysis that PCR product of Pakistani FAV4 DNA is 730 bp, which show conformity with the observed size in agarose gel and correspond to the specific product, as this was not amplified with DNA from healthy liver tissue. Sequencing reaction reveal that it reads from both the orientations (forward and reverse) and based on overlaps a sequence of 730 bp. On the basis of comparative nucleotide analysis, present study revealed a high level of homology in the variable region of hexon gene within FAV4. Parthiban *et al.* (2005) demonstrated that nucleotide sequences of their isolates had a 98% homology with other Indian isolates and a 96% homology with Belgian and Russian isolates.

According to the findings of Meulemans *et al.* (2001), at amino acid level, FAV4 showed a homology of 51% to FAV1, 91% to FAV9 and 46-48% to other fowl adenoviruses. The 730 bp product of variable region of Pakistani isolate encodes for a 243 amino acid protein that is basic in nature having ten arginine, three histidine and seven lysine residues. Previous studies have shown a protein size of 242 amino acids having eleven arginine, eight histidine and seven lysine residues as demonstrated by Ganesh *et al.* (2001). As Pakistani isolate differ to some extent in amino acid sequence from other FAV4 isolates but its biological properties such as antigenicity and pathogenicity were not altered. Parthiban *et al.* (2005) have found amino acid sequences of their isolate had a more than 98% homology with other Indian isolates and a more than 92% homology with Belgian and Russian isolates.

Hence, it is concluded that amplification of variable region hexon gene is simple and confirmatory for detection of FAV4 and the variable region of hexon gene of Pakistani isolate was highly homologous among all the FAV4 tested both at nucleotide and amino acid level.

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