Serotype Characterization of Pakistani Aviadenovirus Strains Causing Inclusion Body Hepatitis and Hydropericardium Syndrome

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ABSTRACT— ***Controlling fowl adenoviral (FAdVs) outbreaks in commercial chicken is of immense importance since such outbreaks of disease can cause economic losses by infecting a large number of broiler chicken. The current study was performed for detection and serotype characterization of FAdVs causing inclusion body hepatitis and hydropericardium syndrome (IBH-HPS) in Pakistani commercial broiler chicken in the year 2015. Tissue samples (liver) of infected birds, manifesting signs of IBH-HPS, were used as source for DNA isolation and detection of FAdVs was done using PCR (polymerase chain reaction) to amplify hexon gene region. Serotype identification and characterization was based on Restriction Fragment Length Polymorphism (RFLP) results, using the restriction enzyme BfoI, together with Sanger dideoxy sequencing. From the cases analyzed, 87% were diagnosed with FAdV 11, therefore marked as the main cause of IBH-HPS, serotype 1 was detected in 10% of cases and least cases were detected of serotype 8 (4%) and serotype 4 (2%). BLAST was used to identify close homologues to the sequenced samples, multiple sequence alignment was performed in MEGA 7.0 Clustal w tool and phylogenetic tree was built to infer relationship between sequenced samples and previous FAdV isolates based on the sequence alignment of partial loop1 region of hexon gene. The sequences of FAdV isolates sequenced in the study were subsequently submitted to NCBI database.***

Keywords— Fowl adenovirus, FAdVs, inclusion body hepatitis, hydropericardium syndrome

1. INTRODUCTION

Adenoviruses belonging to the family *Adenoviridae* possess a linear genome of double stranded DNA, are non-enveloped, and have icosahedral symmetry [1, 2]. The genome is linked to various proteins including a terminal protein (TP) at the 5'end, which harbors inverted terminal repeats (ITRs) [3]. The major proteins in their capsid are hexon (II) and fibre (IV) with a penton base (III) non-covalently attached, and numerous minor proteins: VI, VIII, IX, IIIa and IVa2 [4].

Aviadenoviruses have been divided into eight species comprising *Falcon adenovirus A, Fowl adenovirus (FAdV)-A, FAdV-B, FadV-C, FadV-D, FAdV-E, Goose adenovirus A* and *Turkey adenovirus B* [5]. Fowl adenoviruses ubiquitously infect a huge number of birds causing inclusion body hepatitis, quail bronchitis, gizzard erosion, hydro-pericardium syndrome, and pancreatic necrosis [6]. Such infections in poultry chickens lead to heavy economic losses due to high mortality rate, poor weight gain, poor feed conversion and meager egg production. Additionally, adenovirus infection may lead to immuno-suppression, thus increasing the risk of secondary infection [7].

Hydro-pericardium syndrome (HPS) emerged in Angara Goth near Karachi, Pakistan in 1987, which severely affected poultry [8]. HPS has a sudden onset, and generally has mortality rate up to 75%. Gathering of a transparent or straw-colored fluid in the pericardial sac, along with nephritis, hepatic necrosis and intra-nuclear inclusion body formation in eosinophils or basophils within hepatocytes are the main characteristics of this disease [1, 9, 10].

Various methods have been employed for the diagnosis of FAdV infections like examination of histopathological changes, isolation of virus in cell culture and serological tests such as virus neutralization test, fluorescent antibody test and ELISA, albeit such methods are onerous, time consuming and labor intensive [6, 8]. Further, a major predicament in serological tests is that the antibodies against adenoviruses are present in healthy as well

as infected birds, which poses difficulty in result interpretation [7]. In recent years, the applications of molecular diagnostics methods like Polymerase chain reaction and restriction digestion has gathered significance, being sensitive and rapid techniques, allowing detection of viral DNA as well as identification and typing, which is very important to control the disease [8].

The objective of the present study was to analyze the variants of adenovirus in Pakistan, affecting the broiler chicken with inclusion body hepatitis-hydropericardium syndrome (IBH-HPS) in 2015. For achieving this objective, liver samples of infected chicken were processed for DNA isolation, and analyzed by PCR amplification, RFLP and Sanger dideoxy sequencing. Phylogenetic relationship was built based on the sequence alignment of loop1 of hexon gene of fowl adenoviruses.

2. MATERIALS AND METHODS

2.1 Sample Collection

Samples were collected as authorized by GP lab over the year 2015. Infected (dead) bird samples that came for analysis were dissected in GP lab post-mortem unit to collect liver samples for DNA isolation. Samples were stored at -20 °C before further use.

2.2 DNA Extraction

DNA was extracted from chicken liver using slightly modified method with QIAamp DNA Min Kit. Briefly, the tissue sample (20mg) was ground and mixed with 180 µL of ATL buffer and 20µL proteinase K added with incubation at 50 °C with shaking for 1-2 hr until all the tissue has been lysed. Then 200µL of AL buffer was added and 10-15 min incubation was given at 72 °C followed by precipitation with 250 µL of absolute ethanol. Precipitated DNA was bound to QIAamp mini spin column by centrifugation at 8000 rpm for 1 min. DNA was washed with AWI (500 µL) and AWII (500 µL) using 13000 rpm for 3 min followed by elution with AE buffer (200 µL). Purified DNA was stored at -20°C until further use.

2.3 PCR Amplification

PCR was performed using H1 (TGGACATGGGGGCGACCTA) and H2 (AAGGGATTGACGTTGTCCA) as reported by Raue and Hess (1998). Reaction mixtures were prepared using 12.5 µL of 2X DreamTaq Green PCR Master Mix (Thermo Fisher Scientific), 1 µL of each H1 and H2 primer, 3 µL DNA template and 7.5 µL of nuclease-free water. Reaction was run in Bio-Rad T100™ Thermal Cycler with the following conditions: initial denaturation of 95°C for 5 min followed by 35 cycles of denaturation (95°C for 45 sec), annealing (55°C for 45 sec), and extension (72°C for 90 sec) and a final extension of 72°C for 5 min. PCR products were analyzed by 1.5% agarose gel electrophoresis, containing 10 µg/µL ethidium bromide, and visualized using benchtop UV transilluminator. 100 bp DNA ladder (SM0323, Thermo Fisher Scientific) was used as marker for size determination.

2.4 Amplicon Purification and RFLP Analysis

GeneAll® Expin™ SV was used for purification of PCR product. In brief, DNA band was excised and weighed and was dissolved in GB buffer (3µl per mg gel slice) at 50°C and 150 µL isopropanol was added to the mixture and vortexed followed by loading on SV column and centrifugation at 8000 rpm. Wash buffer NW (700 µL) was added to column and centrifuged for 30 sec at 12000 rpm and finally the DNA was eluted in 50 µL of elution (EB) buffer. For restriction digestion, mixture was prepared in a volume of 30 µL containing 10 µL of purified amplicon, 2 µL of 10X FastDigest Green Buffer (Thermo Fisher Scientific) and 17 µL nuclease-free water and 1 µL of Fast Digest BfoI FD2184 (Thermo Fisher Scientific). Incubation was given at 37 °C for 5 min and subsequent inactivation at 65 °C for 10 min.

2.5 Sequencing and Phylogenetic Analysis

Sequencing of amplified hexon gene regions was performed from 1st BASE DNA sequencing services (Axil Scientific Pte Ltd., Singapore). Sequences were analyzed by BLAST [12] and mutations were identified in the strains by alignment with the sequences displaying high sequence identity and coverage. After further verification from respective sequence chromatograms, the sequences were truncated and submitted to NCBI, GenBank.

Multiple sequence alignment was performed using Clustal w tool in MEGA 7.0. Phylogenetic analysis based on the sequence alignment of the loop 1 region of hexon gene was carried out using MEGA 7.0 [13] with Neighbor-Joining method to infer the evolutionary history [14] and 1000 bootstrap replicates [15] were implemented. The evolutionary distances were calculated using the Tajima-Nei method [16] and were represented as units of the number of base substitutions per site. Gamma distribution (shape parameter = 2) was used to model the rate variation among sites. All alignment gaps and missing data were eliminated. The analysis involved 32 nucleotide sequences and a total of 487 positions in the final dataset.

3. RESULTS

DNA was extracted from the infected liver samples using QIAamp DNA Min Kit and PCR was done using optimized conditions with H1 and H2 primers, originally described by Raue and Hess (1998). These primers hybridize to

conserved (pedestal) regions of hexon gene and amplify sequence (1219 bp product) including loop1 and loop2 regions. Figure 1 shows amplified products of hexon gene from DNA isolated from infected samples.

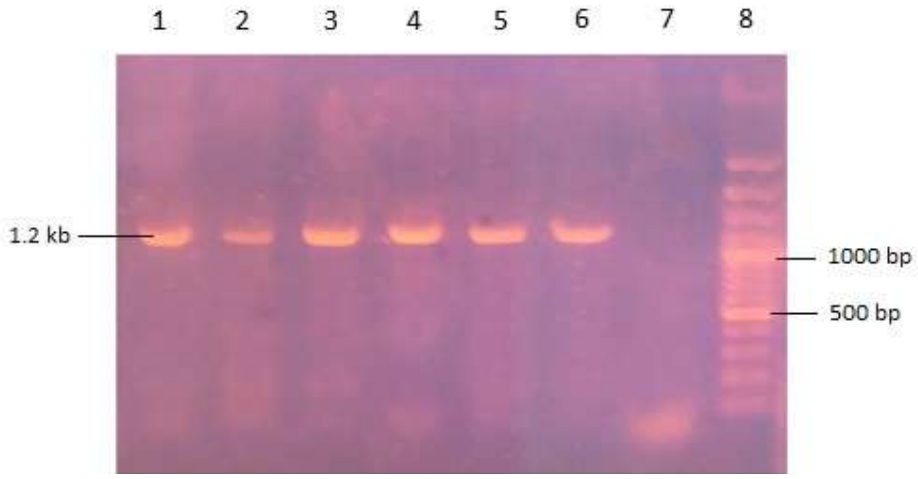

Figure 1: Agarose gel electrophoresis of amplified product (hexon gene) from adenoviral DNA using H1 and H2 primers. Lane 1-6: Amplified product, Lane 7: Negative control and Lane 8: 100 bp DNA marker (SM0323)

Restriction analysis was performed using *Bfo*I restriction enzyme for identification of serotypes based on fragments produced with *Hae*II (isoschizomer of *Bfo*I) as described by Raue and Hess (1998). Figure 2 indicates the restricted products of amplified hexon gene region for identification of serotypes.

Amplified products were sequenced by Sanger dideoxy DNA sequencing and the sequences were identified by BLAST and aligned with clustal omega to compare the sequences with respective homologues and screened for mutations. After analyses, the adenoviral hexon gene sequences were finally submitted to NCBI, GenBank with the accession numbers: KX179503, KX179504, KX179505, KX247371, KX247372, KX247373 and KX247374 and KX247375. From a total of about 100 samples analyzed, adenovirus serotype 11 was identified the major causative agent accounting for 87% of the infected cases followed by serotype 1, causing 10% of cases. Serotype 8 and 4 were identified in 4% and 2% of cases, respectively.

Phylogenetic analysis based on the sequence alignment of the loop 1 region of hexon gene was carried out using the sequences of avian adenoviruses obtained from this study with the selected closest homologues, exhibiting strong identity (≥96%) as identified from BLAST. The sequences along with the homologues were downloaded in FASTA format and imported in MEGA 7.0 for alignment using Clustal w tool and subsequently analyzed for constructing phylogenetic tree using Neighbor-Joining method and 1000 bootstrap replicates. Phylogenetic relationship between the sequences have been depicted in Figure 3. Adenovirus strains, based on their hexon gene loop L1 sequences, have been clustered into their corresponding groups in the phylogenetic tree.

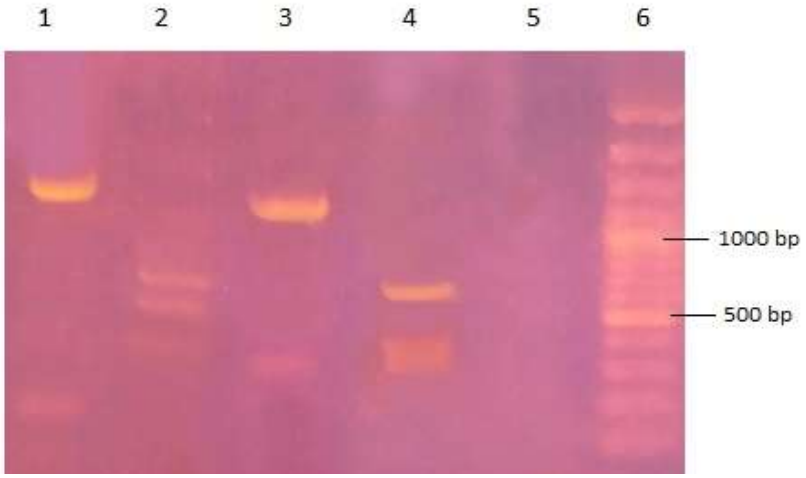

Figure 2: Restriction with the enzyme *BfoI* to determine RFLP, in Agarose gel electrophoresis, of the amplified product for identification of serotypes.
Lane 1: serotype 4, Lane 2: serotype 1, Lane 3: serotype 8, Lane 4: serotype 11, Lane 5: negative control and Lane 6: 100 bp DNA marker (SM0323)

4. DISCUSSION

Fowl adenoviruses (A to E), which belong to group I of aviadenoviruses, are classified into 12 serotypes (FAdV-1 to 8a and 8b to 11), all sharing a communal group antigen [17, 18]. Inclusion body hepatitis and hydropericardium syndrome are among the most significant diseases caused by fowl adenoviruses infections in chickens which results in economic losses both in developing and developed countries of the world [1, 7, 19-21].

Many studies, referring to infection of chicken with fowl adenoviruses and respective analyses using methods based on PCR have been reported. A few studies have been published from Pakistan on characterization of adenoviruses on the basis of PCR and restriction analysis in combination with other techniques. Shamim et al. (2009) characterized avian adenovirus 4 isolates causing hydropericardium syndrome in Karachi. Mansoor et al. (2009) and Shah et al. (2011) have reported molecular characterization of avian adenovirus 4 associated with hydropericardium syndrome in Faisalabad and performed phylogenetic analysis. Jabeen et al. (2015) cloned, sequenced and characterized through bioinformatics, including phylogenetics, an avian adenovirus 4 isolate from 2008 outbreak in Islamabad.

In this study, we characterized avian adenovirus serotypes associated with inclusion body hepatitis and hydropericardium syndrome from outbreak in early summer (April-July) 2015 mainly in Punjab and Northern Areas (KPK) of Pakistan. On studying the IBH-HPS cases from the outbreak in 2015, we found serotype 11 as the main infective agent followed by serotype 1. The adenovirus strains sequenced in this work exhibited significant homology with the isolates of FAdV from Pakistan [8, 22, 23], India [24] and China [1, 25] as well as from other locations including Austria [26, 27], Itlay [28], Russia [29], US [30] and Canada [19, 31]. Some of the highly homologous sequences of published serotypes were used in phylogenetic studies which shows their relationship and possible phylogenetic lineage (Figure 3). Results of sequencing and phylogenetic analysis were in accordance with RFLP results for serotype characterization.

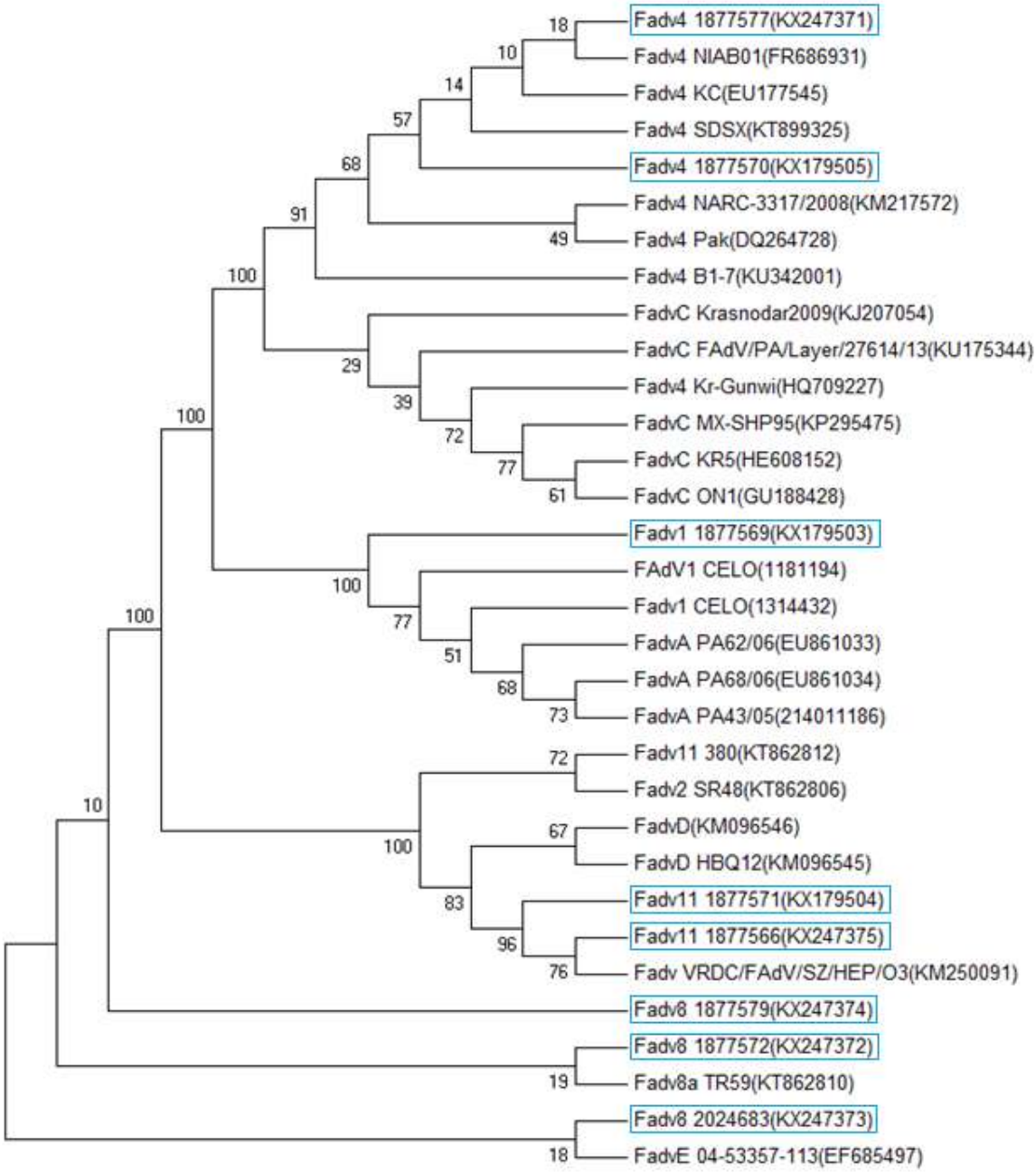

Figure 3: Phylogenetic tree constructed using Neighbor-Joining method based on the sequence alignment of hexon gene loop 1 region. MEGA 7.0 was used for evolutionary analyses, involving 32 nucleotide sequences; those sequenced in this study are highlighted in color with their submitted NCBI accession numbers. Numbers at branches indicate bootstrapping values (%), estimated from 1000 replicates to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

5. CONCLUSION

This strategy based on PCR coupled with RFLP and sequence analysis is an efficacious way for identification and study of fowl adenovirus isolates. Commercial broiler flocks should be routinely tested by PCR for FAdV infection screening and for ensuring proper vaccination prior to infection and disease prognosis. Further, molecular studies can be done to determine the severity of infection caused by Pakistani isolates of FAdVs and to ensure the efficiency of currently available vaccines in controlling any future disease outbreaks in Pakistan.

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