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Fiber gene based molecular and biological characterization of hydropericardium-hepatitis syndrome associated avian adenoviruses

Yasmeen, S.¹; Siddique, N.^{2*}; Athar Abbas, M.²; Ali, A.²; Rafique, S.³; Rashid, F.²; Shah, A. U.⁴; Mehmood, F.⁵; Begum, I.⁶; Javaid, T.⁷; Jaffery, S. M. H.⁸; Ali, R.⁹ and Naeem, Kh.²

¹Mphil in Animal Genomics and Biotechnology, National Reference Lab for Poultry Diseases, Animal Sciences Institute, National Agricultural Research Centre, Islamabad-45500, Pakistan; ²National Reference Lab for Poultry Diseases, Animal Sciences Institute, National Agricultural Research Centre, Islamabad-45500, Pakistan; ³Ph.D. Scholar, National Reference Lab for Poultry Diseases, Animal Sciences Institute, National Agricultural Research Centre, Islamabad-45500, Pakistan; ⁴MSc in Biotechnology, National Reference Lab for Poultry Diseases, Animal Sciences Institute, National Agricultural Research Centre, Islamabad-45500, Pakistan; ⁵Mphil in Microbiology, National Reference Lab for Poultry Diseases, Animal Sciences Institute, National Agricultural Research Centre, Islamabad-45500, Pakistan; ⁶Mphil in Biology, National Reference Lab for Poultry Diseases, Animal Sciences Institute, National Agricultural Research Centre, Islamabad-45500, Pakistan; ⁷Al-Ferooz Poultry Clinic, Rawalpindi-46000, Pakistan; ⁸Jaffmac Consultant Services, Lahore, Pakistan; ⁹Remount Veterinary and Farms Directorate, GHQ, Rawalpindi, Pakistan

*Correspondence: N. Siddique, National Reference Lab for Poultry Diseases, Animal Sciences Institute, National Agricultural Research Centre, Islamabad-45500, Pakistan. E-mail: naila.nrlpd@gmail.com

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Summary

This study was designed to perform biological and molecular characterization of avian adenoviruses (AAVs) recovered from suspected cases of hydropericardium-hepatitis syndrome (HHS) in commercial poultry. Initially the samples were screened by Agar Gel Precipitation Test (AGPT) for the presence of AAVs followed by its confirmation and typing through polymerase chain reaction (PCR) focusing on already reported serotypes AAV-4, AAV-8 and AAV-10 elsewhere. These PCR-positive samples were further subjected to amplification of fiber gene, followed by conducting restriction fragment length polymorphism (RFLP) using restriction enzyme *Alu*. The selected isolates were further propagated through cell culture and pathogenic potential of selected isolates was determined by infecting chickens. In this study, out of a total 190 samples, 57.8% of suspected cases were found positive for AAV presence through AGPT while sub-type identification using PCR revealed 46.3% for these viruses belonging to AAV-4, 41.8% to AAV-8 and 11.8% showed co-infection of AAV-4 and AAV-8. AAV-10 was not detected in any of the tested samples. On the basis of RFLP pattern, AAV-4 isolates were further divided into four sub-groups (A-D) while AAV-8 isolates had identical RFLP pattern. To further evaluate the pathogenic potential of these sub-groups of AAV-4 isolates, specific pathogen free (SPF) chicks were challenged with selected isolates belonging to each of the sub-groups, resulting in variable pattern of pathogenicity. It is concluded that any variation in the fiber gene of AAV-4 isolates may affect its pathogenicity and eventually specificity of the vaccines used against such infections. Therefore, regular monitoring of the circulating AAV serotypes may be helpful in understanding the pathogenic potential of emerging AAVs, which may lead to development of more effective response strategies accordingly.

Key words: Avian adenoviruses, Inclusion body hepatitis, Hydropericardium-hepatitis syndrome, Hydropericardium syndrome

Introduction

Avian adenoviruses (AAVs) in poultry are the etiologic agents of two very important diseases known as inclusion body hepatitis (IBH) and hydropericardium syndrome (HPS). Although in some cases each disease is observed separately, under field circumstances the two conditions have been frequently observed as a single entity; therefore, the name hydropericardium-hepatitis syndrome (HHS) has been widely used to describe the pathologic condition. The syndrome is an acute disease of young chickens associated with anemia, hemorrhagic disorders and hydropericardium. Avian adenovirus infections have always been a threat to the national gross domestic poultry productions ever since one of its

serotypes was reported for the first time in 1987 from Angara Goth near Karachi, Pakistan (Khawaja *et al.*, 1988). The disease was given the name of Angara disease (AD) and its etiological agent was later found to be AAV serotype-4. Also known as HPS, the disease is characterized by accumulation of a clear but straw colored fluid in the pericardial sac of chickens (Jaffery, 1988). Likewise, the infected liver becomes swollen, friable and congested appearing dark to yellow in color. The AD was initially reported to primarily target 3-5 week old broilers, but it was later on reported to infect layers and breeders of variable age (Shukla *et al.*, 1997). Rare outbreaks of the disease have also been recorded in broilers of older age (Asrani *et al.*, 1997). Mortality as high as 80% has also been reported in cases depending

on the immune status of the chicks, pathogenicity of the virus and immuno-suppression caused by secondary infections (Cowen, 1992).

The disease is infectious in nature and is transmitted both horizontally and vertically. Horizontal transmission occurs through faeces and fomite while disease in progeny occurs through vertical transmission (Hafez, 2011). AAV-4 is also known to be persistent and causes immuno-suppression in chickens (Naeem *et al.*, 1995a). Avian adenoviruses belong to the family Adenoviridae, AAVs are further subdivided into three genera along with Mastadenoviruses and Ichtadenoviruses. Fowl adenoviruses are members of group I AAVs currently belonging to the genus Aviadenovirus which includes 5 species and twelve serotypes belonging to chicken, however, the adenoviruses isolated from the geese, turkeys and other birds are kept in other species (Fitzgerald, 2013). Both HPS and IBH were found in chicken infected with FAdV type 4 while those having FAdV types 8b and 11 exhibited IBH lesions only (Choi *et al.*, 2012). Another indicated a new fowl adenovirus genotype, FAdV 12 strain 380 and FAdV11 strain C2B on the basis of phylogenetic analysis (Thakor *et al.*, 2012).

Laboratory diagnosis of AAVs employs both conventional and molecular methods. Conventional methods include a combination of virus isolation in cell culture, histopathology, and electron microscopy. It is concluded that polymerase chain reaction (PCR) genotyping is a reliable method for identification of FAdV. Also, it is more rapid than virus neutralization and direct sequence analysis (Steer *et al.*, 2011).

Fiber gene is reported to play an important role in infectivity and pathogenicity of AAVs. RFLP analysis of AAV isolates of IBH and HPS/IBH revealed marked genomic differences resulting in characterization of these variants in highly virulent, mildly virulent and non-pathogenic strains (Tan *et al.*, 2001). PCR-RFLP analysis of short fiber protein gene may play an important role in differentiation of FAdV-4 strains (Mase *et al.*, 2010).

Gel based vaccine prepared from fresh HPS infected liver homogenate is more effective and economical as compared to that of HPS-infected chicken embryo hepatocyte homogenate (CEHH) vaccine (Mehmood *et al.*, 2011). Subsequently, cell culture based oil-emulsified vaccines have been found to induce greater antibody titers and are used effectively (Naeem *et al.*, 1995b). However, it has been seen in the field that outbreaks of HPS/HHS still occur primarily in broilers and broiler-breeders despite the use of available vaccines. It was initially hypothesized that this may be due to the induction of some mutations in the circulating AAV-4 or co-infection with other AAV serotypes of group I with it, thereby rendering current vaccines ineffective. The study reported here is focused on isolation, biological typing and molecular characterization of the AAVs recovered from HHS cases in the field.

Materials and Methods

Source of samples

The study is based on data of 190 clinical specimens of liver and spleen, collected from HHS suspected chicken flocks during March 2012-September 2012. The sampling was done from 19 districts of different poultry populated regions of Pakistan including Punjab, Khyber Pukhtunkhwa, Azad Kashmir and Islamabad Capital Territory (ICT). Out of a total 190 samples, 98 samples were HPS vaccinated while 92 samples were originated from non-HPS vaccinated flocks.

Sample collection

The samples were collected through the National Avian Diseases Surveillance System (NADSS) established at the National Reference Laboratory for Poultry Diseases (NRLPD), Animal Science Institute (ASI), National Agricultural Research Centre (NARC), Islamabad. Under this system, liver and spleen specimens from HPS/IBH suspected cases were transported to NRLPD. Fresh tissue samples were processed at the earliest or were stored in freezer (-20°C) till further process.

Sample preparation

The morbid tissue samples were thawed and a 20% (w/v) suspension was prepared in phosphate buffered saline. It was further homogenized in stomacher (Biomaster, Seward Ltd., UK) and subjected to centrifugation at $800 \times g$ for 10 min at 4°C. The supernatant collected was stored at -70°C until further use.

AAV antigen detection by AGPT

The standard procedure of Agar Gel Precipitation Test (AGPT) was employed to detect the group specific antigen of AAV from the field samples (Crowle, 1973). For this purpose, first antisera against AAV - was raised in chickens by inoculating 0.3 ml of inactivated adenovirus vaccine (ANGAVAC, Merial) sub-cutaneously to 4 chicks of 10-day age. Blood samples were drawn after 14 days. The sera collected from all the birds was pooled and centrifuged at $800 \times g$. It was subsequently inactivated by placing it in a 56°C water bath for 30 min and then stored at 4°C for immediate use and at -70°C for long term storage.

Molecular analysis: PCR for differential diagnosis

The processed tissue samples declared positive for AAVs by AGPT were then subjected to PCR for sub-type detection using AAV universal as well as type-specific primers for the *Hexone* gene (Ganesh *et al.*, 2002).

Here for extracting viral DNA an Easy DNA Kit (Invitrogen Inc., USA) was used and for PCR reaction the kit from Thermo Scientific was used, following the

manufacturer's protocol. Various serotypes of AAV were confirmed using type specific PCR already standardized at NRLPD (unpublished data). Briefly the target sequences were amplified using a GeneAmp 9700 PCR Thermal Cycler (ABI, Foster, CA) for a 50 μ L reaction mixture comprised of 25 μ L of DreamTaq Green PCR Master Mix (2x) (Thermo Scientific Cat#K1081) with 1 μ L of 1 μ M each forward and reverse primer and 5 μ L of template DNA mixed with 18 μ L of nuclease-free water. The PCR profile for the amplification of type specific PCR at 95°C for 5 min for first denaturation followed by 35 cycles of 94°C for 20 s, annealing at 58°C for 30 s (AAV-4 and AAV-10) and 56°C for 40 s (AAV-8) and extension at 72°C for 40 s with final extension at 72°C for 5 min was used to amplify 317, 201 and 438 bp products for AAV-4, 8 and 10, respectively.

Molecular characterization of selected AAV-4 and AAV-8

PCR amplification of fiber gene

PCR amplification of 30 samples from each serotype (AAV-4 and AAV-8) confirmed by type-specific differential PCR, was conducted by targeting the fiber gene. For this purpose the DNA was extracted using Easy DNA Kit (Invitrogen Inc., USA). PCR was performed using the fiber gene-specific primers as described earlier (Mase *et al.*, 2010).

Restriction fragment length polymorphism (RFLP)

After obtaining the amplified region of target gene (fiber gene) of the selected AAV-4 and AAV-8 isolates, DNA digestions were carried out using *AluI* restriction endonucleases (Thermo Scientific, USA) according to the manufacturer's recommendations. The product was electrophoresed and photographed for record.

Biological characterization

Tissue culture propagation of RFLP-based sub-group

The RFLP variants of AAV-4 and AAV-8 were subjected to tissue culture propagation. For this purpose, chicken embryo liver (CEL) cells were prepared according to the procedure described by (Schat and Sellers, 2008). Here, 0.2 ml of each sub-group specific isolate was inoculated onto 60-70% confluent monolayer of CEL cells in a culture flask. The flask was incubated at 37°C for 25-30 min, followed by the addition of 5 ml of EMEM maintenance culture media. The flask was incubated at 37°C and observed for the production of cytopathic effects (CPE) for the next 72 h. The flasks showing specific CPE were freeze-thawed thrice. The material was centrifuged at 800 \times g for 10 min. The pellet was discarded and 1 ml of the supernatant was used for virus titration using method of (Reed and Muench, 1938) whereas the remaining quantity was stored at -70°C for further studies.

Pathogenicity testing of RFLP-based sub-groups

Here one representative sample from each sub-group was further tested to see their biological activities. For

this purpose, 1 ml of infectious culture fluids ($10^{4.5}$ TCID₅₀) representing each of the five RFLP-based sub-groups was subcutaneously inoculated into each of the five 10-day-old specific pathogen free (SPF) chicks per group. For this purpose sub-groups A-D were inoculated with AAV-4 and group E was inoculated with AAV-8. Group-F served as negative control, which was inoculated with phosphate buffered saline via subcutaneous route. Each group was kept separately in chicken isolators. All the chicks were observed daily for 14 days after which they were slaughtered humanely. Post mortem observation of any dead bird was also recorded. The data of pathogenicity was subsequently evaluated by comparison with RFLP results to determine the impact of genetic variation on the pathogenic potential of different field isolates of AAVs circulating in poultry.

Analysis of co-infection of AAV-4 and AAV-8

Twenty SPF chicks ten-days-old were randomly allocated into four groups (group A-D). Group A and B were subcutaneously inoculated with 1 ml (7.4 TCID₅₀) of AAV-4 and AAV-8, respectively. Group C was inoculated with 0.5 ml each of AAV-4 and AAV-8. Group D was kept as control and was inoculated subcutaneously with 0.1 ml of phosphate buffered saline.

Results

Area-wise distribution of avian adenoviruses

During AAV surveillance study, out of 190 samples from AD/IBH suspected poultry flocks, 110 samples were found positive for AAV through AGPT. Of the total 110 positive samples, 75 isolates were recovered from Punjab province, 15 from KPK province, 12 from AJK and 8 isolates were recovered from ICT (Table 1).

Table 1: Area-wise distribution of avian adenovirus presence in infected tissues

Area	Samples tested	AGPT ^a positive	AGPT ^a negative	Percentage positive
Islamabad	15	8	7	53.3%
Punjab	135	75	60	55.5%
KPK ^b	23	15	8	65.2%
AJK ^c	17	12	5	70.5%
Total	190	110	77	59.4%

^a AGPT: Agar Gel Precipitation Test, ^b KPK: Khyber Pukhtunkhwa, and ^c AJK: Azad Jamu Kashmir

Serotype identification using PCR

The PCR based evaluation of the AGPT positive samples revealed that out of 110 AAV positive samples, 51 were positive for serotype AAV-4, 46 for AAV-8 and the remaining 13 samples were positive for both AAV-4 and AAV-8. None of the samples was positive for the presence of AAV-10 (Fig. 1).

Prevalence of AAVs in vaccinated and non-vaccinated flocks

The data showed that 63 AAV positive samples

originated from the flocks already vaccinated with AAV-4 and 47 from the flocks not vaccinated against AD. Out of these 63 AAV samples, 32 were typed as AAV-4 while 20 were identified as AAV-8. Co-infection of both AAV-4 and AAV-8 was observed in 11 samples. On the other hand, from 47 samples originated from non-AD-vaccinated flocks, 19 AAV-4 and 26 AAV-8 were detected. Here, 2 of the samples were found to have co-infection of AAV-4 and AAV-8 (Table 2).

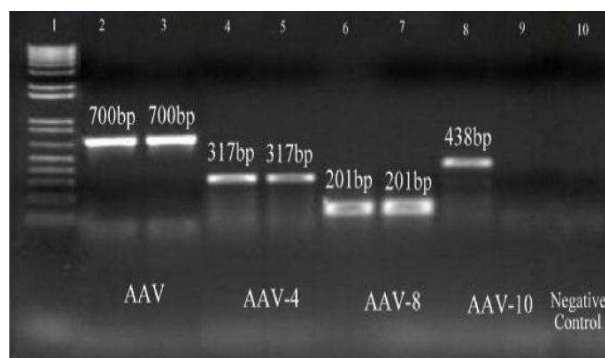


Fig. 1: Agarose gel electrophoresis of PCR amplified *hexon* gene of AAV, AAV-4, AAV-8 and AAV-10. Lane 1: Marker, 1 Kb plus step ladder DNA, Lane 2: AAV, positive control for AAV (700 bp), Lane 3: AAV, positive sample for AAV (700 bp), Lane 4: AAV-4, positive control for AAV-4 (317 bp), Lane 5: AAV-4, positive sample for AAV-4 (317 bp), Lane 6: AAV-8, positive control for AAV-8 (201 bp), Lane 7: AAV-8, positive sample for AAV-8 (201 bp), Lane 8: AAV-10, positive control for AAV-10 (438 bp), Lane 9: AAV-10, negative sample for AAV-10, and Lane 10: PBS, negative control

Molecular characterization: RFLP of fiber gene

Around 65 isolates were subjected to RFLP analysis of fiber gene, AAVs were characterized into five groups. Groups A, B, C and D were identified as AAV-4 using type-specific PCR. The isolate in group A has four restriction sites for *AluI*. In group B, six isolates of AAV-4 has identical pattern having three restriction sites. The variants included in sub-group C and D revealed two and one restriction sites, respectively. However, all the tested AAV-8 isolates in group E showed identical pattern of RFLP, having two *AluI*

restriction sites (Mase *et al.*, 2010) (Fig. 2).

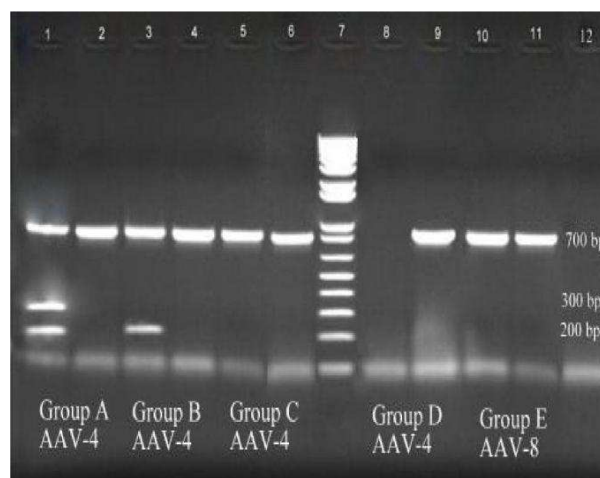


Fig. 2: Agarose gel electrophoresis of PCR amplified RFLP of fiber gene of various variants of AAV

Biological characterization

Pathogenicity testing of RFLP-based sub-group

The isolates selected on RFLP-based evaluation showed variable intensity of pathogenicity upon inoculation in susceptible chickens. The main lesions observed in all the sub-groups included pulmonary edema, swollen kidneys, lesions on liver and hydropericarditis (Figs. 3A-B). Enlarged liver of chicken were found when infected with AAV-4 (Fig. 4). In addition to these lesions, sub-group E, infected with AAV-8, had pale liver, jaundice like lesions, very small quantity of fluid produced in pericardial sac, diffuse hemorrhagic and necrotizing foci in swollen liver (Fig. 5 and 6). Maximum pathognomic lesions were observed in sub-group D, which is a variant of AAV-4, as shown in (Table 3).

Analysis of co-infection of AAV-4 and AAV-8

Group III, which represented co-infection of AAV-4 and AAV-8, showed nephritis and lesions on liver in addition to hydropericarditis, pale liver, hepatitis and pulmonary edema recorded in group I.

Table 2: Distribution of AAV-4 and AAV-8 isolates recovered from AD-vaccinated and non-vaccinated groups

Status of AD-vaccination	Distribution of AGPT positive samples for AAV	AAV-4 positive (I)	AAV-8 positive (II)	AAV-4 and AAV-8 positive (III)
Non-vaccinated group	47	19	26	2
Vaccinated group	63	32	20	11
Total	110	51	46	13

Table 3: Comparison of lesions produced by various RFLP-based sub-groups of AAV within serotype-4 and serotype-8

Sub-group labelling	AAV serotype	HP ^c	Pale liver	Hepatitis	Jaundice	Pulmonary edema
A	AAV-4 ^a	++	+	++		+
B	AAV-4 ^a	++	+	+		+
C	AAV-4 ^a	++	+	++		
D	AAV-4 ^a	+++	+	+++		
E	AAV-8 ^b	+	++	+	+	
F (control)	No infection					

^a AAV-4: Avian adenovirus serotype-4, ^b AAV-8: Avian adenovirus serotype-8, and ^c HP: Hydropericarditis

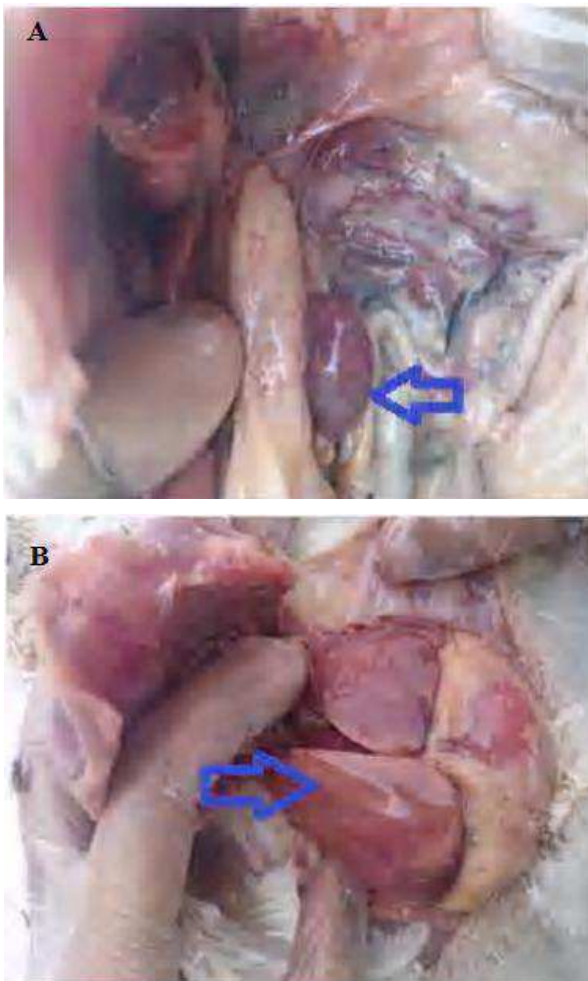


Fig. 3: Chicken infected with AAV. A) Swollen kidneys, and B) lesions on liver

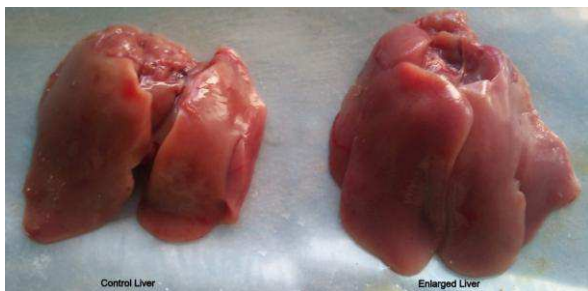


Fig. 4: Enlarged liver of chicken infected with AAV-4 (right), and normal liver (left)



Fig. 5: Pale liver of chicken infected with AAV-8 (right), and normal liver (left)

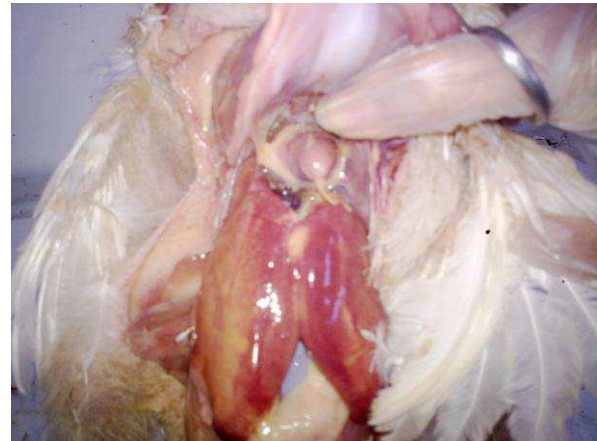


Fig. 6: AAV-8 infected chicken showing diffuse hemorrhagic and necrotizing foci in swollen liver

Geographical origin of RFLP variants of AAV

Sub-group A of RFLP variants of AAV belonged to a vaccinated flock from Islamabad. Sub-group B, comprising the maximum number of isolates was traced back to Rawalpindi, Chakwal and Lahore districts of Punjab. Sub-group C was found in AAV-4 isolate belonging to Poonch district, Azad Jamu Kashmir. Sub-group D, containing only one restriction site for *AluI* belonged to a broiler vaccinated flock from Rawalpindi district. Sub-group E, containing the AAV-8 isolates belonged to Rawalpindi and Attock districts of Punjab province.

Discussion

Angara disease, also known as HPS is one of the highly fatal viral diseases causing heavy losses to the poultry industry of Pakistan. It was reported for the first time in the autumn of 1987 from Angara Goth, located near Karachi, Pakistan (Khawaja *et al.*, 1988). Since then, the disease has become prevalent worldwide. It has been reported in India (Gowda and Satyanarayana, 1994), Iraq (Abdul-Aziz and Al-Attar, 1991), Japan (Abe *et al.*, 1998), Russia (Borisov *et al.*, 1997) and America (Shane, 1996).

AD has been under control at most of the places in Pakistan since the regular use of homologous inactivated vaccines locally prepared from AD infected liver, especially in broilers (Kumar *et al.*, 1997), or later on by using the imported killed oil based vaccines. However the disease has been reoccurring occasionally during the past few years along with the condition named as HHS. It has been recorded primarily in both vaccinated and unvaccinated broiler breeder flocks. Based on the prevalent clinical picture among infected flocks, it was hypothesized that this disease could be spreading either due to vaccine failure on account of some mutation in the circulating AAV-4 or co-infection of more than one AAV serotype, modifying the clinical picture of the disease under field conditions.

The AAV prevalence data in this study indicated that in AJK province where mostly broiler and layer flocks

are raised under poor biosecurity conditions, highest number of positive cases (70.5%) of AAV infection was recorded. On the other hand 53% AAV positive samples were recorded as the lowest incidence recorded in this study from ICT. More specifically, the highest percentage of AAV-4 was recovered from Punjab province whereas the highest percentage of AAV-8 was found in KPK. In addition, the highest number of co-infection of AAV-4 and AAV-8 was recorded in KPK. Interestingly no detection of AAV-10 was recorded from any of the samples tested here.

It was also observed that the AD-vaccinated flocks had higher percentage (65.3%) of positive samples for AAV-4 and AAV-8 infection, alone or as co-infection as compared to non-vaccinated flocks (57.9%). This may be due to the fact that most of the AD-vaccines available in the market are made from AAV serotype-4 only and thus may not be effective in protecting the flocks against other serotypes of AAVs, including AAV-8.

Fiber gene was amplified from 10 isolates of AAV-4 and from four isolates of AAV-8, using the specific primers designed for this purpose. Fiber gene is previously reported to affect infectivity and pathogenicity traits of AAVs (Tan *et al.*, 2001). On the basis of PCR-RFLP analysis of fiber gene using restriction enzyme *AluI*, AAV-4 was characterized into four sub-groups. In only one of these sub-groups, the pattern of restriction sites for *AluI* is in accordance with the earlier reported AAV-4 sequence data available at NCBI Gene-Bank. On the other hand, all the four isolates of AAV-8 had identical RFLP pattern for *AluI*.

The PCR-RFLP-based sub-groups of fiber gene were observed to have variable pathogenic potential. Previously, route and quantity of challenge-dose along with type of strain were known to be deciding factors for organisms to be highly pathogenic, low pathogenic or non-pathogenic. In addition to these signs, AAV-8 inoculated chicks had earlier been reported to induce jaundice like lesions all over the body. Also, the fluid in pericardial sac was lesser than that found in AAV-4 infected chickens (Mazaheri *et al.*, 1998). In case of co-infection of AAV-4 and AAV-8 in this study, nephritis and necrotic lesions on liver were observed in addition to the typical clinical signs of AD or IBH. Nephritis and necrotic lesions were not observed in groups inoculated with AAV-4 or AAV-8 only. This particular study suggests that variation in fiber gene of AAVs circulating in the field along with the presence of co-infection of AAV-4 and AAV-8 may be responsible for the failure or reduced efficacy of the available AD-vaccines. As most of the AD-vaccines available in the market are based on AAV-4 serotype only, they may not be providing sufficient protection against the infection from AAV-8. The variation observed in fiber gene of AAV-4 along with co-infection of AAV-4 and AAV-8 may therefore have an effect on the pathogenesis of adenoviruses, resulting in occurrence of HHS/AD/IBH despite using the available vaccines.

Based on the above investigation it appears that use of inactivated vaccines containing both AAV-4 and

AAV-8 may help to play an effective role in the control of HHS in poultry. Furthermore, use of the prevalent sub-groups of AAV-4 and AAV-8 in the existing vaccines may lead to more effective control of HHS condition. In addition to this, there is a strong need to continuously monitor the prevalent strains of different avian pathogens, including Avian Adeno Viruses, in Pakistan for developing proper disease control strategy, especially in the face of non-judicial usage of vaccines prepared from a variety of non-matching imported strains of various avian pathogens, irrespective of their presence or absence in this country.

It is concluded that there is involvement of more than one serotype (AAV-4 and AAV-8) in the existing condition of HHP. Available vaccines can only provide immunity against AAV-4, and on the basis of RFLP of fiber gene, AAV-4 is classified into five sub-groups but AAV-8 isolates form only one group. Variation in fiber gene of the circulating AAV-4 along with the evidence of co-infection of AAV-4 and AAV-8 may have a major impact on the pattern of disease, resulting in the occurrence of HHS despite the use of monovalent AAV-4 based vaccines.

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Conflict of interest

The authors of the paper affirm that there is no conflict of interest concerning the publication of the current paper.

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