Characterization of avian adenovirus type-4 causing hydro-pericardium syndrome in broilers in Karachi, Pakistan

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Summary

Avian adenovirus was isolated from naturally infected birds during 1990 to 2003 in the vicinity of Karachi, Pakistan. The virus is known to cause hydro-pericardium syndrome (HPS) in poultry. The mortality was recorded 20-50% in commercial broilers. Fifty field samples were collected; however, six samples were selected for molecular studies. These isolates were grown and characterized by cell culture using chicken embryo liver cell culture, chicken embryo pathogenicity, agar gel precipitation (AGP), serum neutralization test, DNA extraction, polymerase chain reaction (PCR) and restriction enzyme analysis (REA). Reference strain J2-A (ATCC VR-829) was used to confirm that HPS isolates belongs to avian adenovirus type-4. The disease was reproduced in 28-day-old broilers by intramuscular inoculation of cell extracts. Typical hydro-pericardium was observed in experimental chickens 72 h post-inoculation. The 50% neutralization endpoint of pooled sera of these isolates was not recorded more than 1:40. The results showed that there was no difference in PCR product of 1319 bp with H3/H4 primer on agarose gels, which is characteristic of group 1 avian adenovirus (AA). Restriction fragment length polymorphism (RFLP) analysis of PCR products using restriction enzyme *Hpa* II resulted in five bands confirming the presence of AA in the analyzed samples.

Key words: Hydro-pericardium syndrome, Fowl adenovirus, Polymerase chain reaction, RFLP

Introduction

The avian adenovirus infections are believed to cause heavy economic losses by increasing mortality in chicken, diminished weight gain, poor feed conversion, drop in egg production and poor egg quality. It may also be involved in immuno-suppression leading to increase incidence of secondary infection (Sambrook *et al*., 1989; Rabbani and Naeem, 1996). A number of useful methods have been developed for the diagnosis of avian adenovirus infections, including virus isolation in cell culture (Cowen *et al*., 1978), indirect immunofluorescent assay, virus neutralization (Adair *et al*., 1980; Adair *et al*., 1986), enzyme linked immunosorbant assay and the double immuno-diffusion (Adair *et al*., 1980; Adair *et al*., 1986; Cowen, 1987). However, most of them are laborious and time-consuming. The main problem with any serological test for adeno-virus is associated with the interpretation of results, as antibodies against adenoviruses are commonly found in the blood of both healthy and infected birds. Polymerase chain reaction (PCR) (Saiki *et al*., 1985) has been applied as a rapid diagnostic tool for the detection of avian viral and bacterial pathogens (Nguyen *et al*., 1994; Ganesh *et al*., 2002; Swati *et al*., 2002). This method is not only rapid, but also more sensitive and specific than other diagnostic tests. Utilizing most advanced techniques of molecular biology the hydro-pericardium syndrome (HPS) agent can be characterized in order to develop a highly specific vaccine and in turn

providing a quick, reliable and specific diagnosis. This study deals with the molecular characterization of six Pakistani avian adenoviruses isolated during 1990-2003, through a combination of PCR and restriction fragment length polymorphism (RFLP).

Materials and Methods

Viruses and antisera

The viruses enlisted in Table 1 were used in this study. The sera against these isolates were raised at Sindh Poultry Vaccine Centre (SPVC) and the reference serum procured from ATCC-USA. A 10% liver homogenate was prepared using infected liver samples in phosphate buffered saline (PBS), pH = 7.4.

Table 1: Locality of broiler farms in the vicinity of Karachi, Pakistan used for the isolation of virus since 1990-2003

Year	Source	Region
1990	Broiler	Korangi
1994	Broiler	Poultry Estate 2
1995	Broiler	Poultry Estate 1
1996	Broiler	Hawksbay
1997	Broiler	Dhabegi
2000	Broiler	Angara Goth
2003	Broiler	Steel Town

Virus isolation in chicken embryo liver cell culture

The chicken's embryo liver cell culture (CELC) was prepared according to the procedure described by Schat and Purchase (1989). The confluent monolayer was infected with 100 µl of liver homogenate and incubated for half an hour. Then the maintenance media was added, supplemented with 5% bovine serum and incubated at 37°C for 2-3 days to observe the cytopathic effect (CPE). The plates showing positive CPE were frozen and thawed repeatedly for release of antigen. Extracts from CPE positive plates were injected in 28-day-old broiler chicken via intramuscular route to reproduce the infection. Agar gel precipitation (AGP) test of cell extract was also employed with reference antisera (FAV-4).

Serum neutralization test

Serum neutralization test (SNT) was carried out by the micro neutralization method (Erny *et al*., 1995) using β method. The 50% tissue culture infective does ($TCID_{50}$) and the neutralization endpoint was calculated (Table 2).

DNA extraction

Viral DNA from HPS infected tissue was extracted using TRI reagent (Product No. T9424 Sigma, USA), according to the manufacturer's protocol. Briefly, 50-100 mg of tissue (liver) was homogenized in 1 ml TRI reagent followed by centrifugation (12000 × g) at 4°C for 15 min. Aqueous phase was transferred to a fresh tube, 0.2 ml chloroform was added to it and then centrifuged at 12000 × g for 15 min at 4°C. The upper aqueous phase was carefully discarded and 0.3 ml of 100% ethanol was

Table 2: Data for the calculation of the 50% endpoint of a neutralization test by a method of Reed and Muench

Dilution of serum		Infectivity	Response		Accumulated values		Ratio	Percent infected
Numerical	Log_{10}	Ratio [a]	Infected	Non-Infected	Infected	Non-Infected		
1:2	$10^{-0.3}$	0/8	0	8	0	39	0/39	0
1:4	$10^{-0.6}$	0/8	0	8	0	31	0/31	0
1:8	$10^{-0.9}$	0/8	0	8	0	23	0/23	0
1:16	$10^{-1.2}$	1/8	1	7	1	15	1/16	6.25
1:32	$10^{-1.5}$	3/8	3	5	4	8	4/12	33
1:64	$10^{-1.8}$	6/8	6	2	10	3	10/13	77
1:128	$10^{-2.1}$	7/8	7	1	17	1	17/18	94.4
1:256	$10^{-2.4}$	8/8	8	0	25	0	25/25	100

[a] Number of infected over number of inoculated (experimental number). Proportionate distance (P.D.) = (Percent infected at dilution next above 50% – 50) / (Percent infected at dilution next above 50% – Percent infected at next dilution below 50%)

added to the remaining suspension followed by incubation at room temperature for 2-3 min. The tubes were centrifuged at 2000 × g for 5 min at 4°C, the supernatant was discarded and DNA pellet was washed twice with 1 ml of 0.1 M sodium citrate prepared in 10% ethanol. Then, the pellet was dried under vacuum for 5-10 min and reconstituted in 600 µl of 8 mM NaOH (pH = 7.5 adjusted by 159 µl of 1 M HEPES).

Polymerase chain reaction and restriction fragment length polymorphisim (RFLP)

The H3 (forward) (AACGTCAACCCC TTCAACCACC) and H4 (reverse) (TTGCC TGTGGCGAAAGGCG) primers as reported earlier were used in this study (Raue and Hess, 1998). PCR was carried out according to manufacturer's protocol (Promega, Madison, WI. USA) using Gene Amp PCR System 9700. The internal control in PCR was FAV serotype 4 reference strain J2-A (ATCC VR-829). Initial denaturation was carried out at 94°C for 2 min followed by 35 cycles of 1 min denaturation at 94°C, 1 min at 55°C for annealing, 1 min 30 sec at 72°C for extension and final extension of 7 min at 72°C. The products were electrophoresed on 1% agarose gel (0.5 µg/ml ethidium bromide) and visualized using gel documentation system (Gel Doc. Bio-Rad-2000). RFLP analysis was performed on positive samples in a reaction volume of 20 µl (5 µl PCR product and 10 units of *Hpa* II) at 37°C for 3 h followed by gel electrophoresis (2% agarose gel, 0.5 µg/ml ethidium bromide).

Results

Hydro-pericardium syndrome isolates of different years produced cytopathogenic effect (CPE) characterized by appearance of round cells in monolayer of liver cells at fourth passage. The presence of fowl adenovirus producing experimental disease in 28-day-old broiler chickens (Fig. 2) and the presence of specific antibodies against adenovirus infection confirmed by the AGP test (Fig. 1). All isolates were effectively neutralized by the pooled antisera. The neutralization endpoint of these pooled sera was not more than 1:40 as calculated in Table 2. An alternative method to the conventional serological tests for sero-typing is based on PCR which is a simple method for amplifying a specific DNA fragment and in recent years has become widely accepted as a tool for the diagnosis of avian virus infections (Alexander and Nagy, 1997; Smith *et al*., 1998). The method showed high specificity and sensitivity for the detection of fowl adenoviruses, especially for FAV-8 and the CELO virus (Clavijo *et al*., 1996; Jiang *et al*., 1999).

The PCR results for all HPS viruses along with reference strain J2-A using the primer pair H3/H4 are shown in Fig. 3. For optimisation, different concentration of the reagents used for PCR protocol and cycling parameters were tested. The results showed that 25 µl of reaction mix containing 10 pmol of H3 and H4 primer, 1.2 mM $MgCl_2$, 0.2 mM dNTPs, 0.75 U Taq DNA polymerase and 1 × PCR buffer was the best condition for conducting PCR of six isolates of HPS which were belong to different years. The best cycling profile was initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1 min and the final cycle followed by a long extension at 72°C for 5 min. The length of the PCR product (1319 bp) and the restriction profiles of the isolates used in this study and the reference strain J2-A were identical in accordance to earlier reports (Raue and Hess, 1998; Toro *et al*., 1999; Xie *et al*., 1999; Swati *et al*., 2002). The number of bands identified after digestion was 5, 150, 200, 250, 300 and 500 bp (Fig. 4).

Discussion

There has been an increasing demand for broiler meat in Pakistan during the past two decades, which mushroomed the broiler farming throughout the country. Variety of factors have been identified affecting the performance of broilers and inadequate management that eventually leads to huge economic loss. Hydro-pericardium syndrome primarily affects 3- to 6-week-old broiler flocks with mortality up to 80% (Kumar *et al*., 2003). Previously, it has been

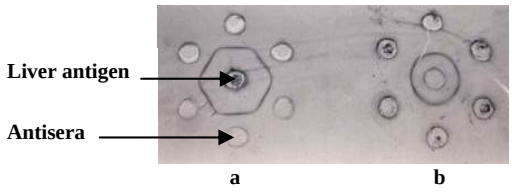

Fig. 1: Agar gel precipitation (AGP) test between antigen and antisera of six isolates. (a) the antisera is in peripheral wells and liver antigen in the central well (b) vice versa

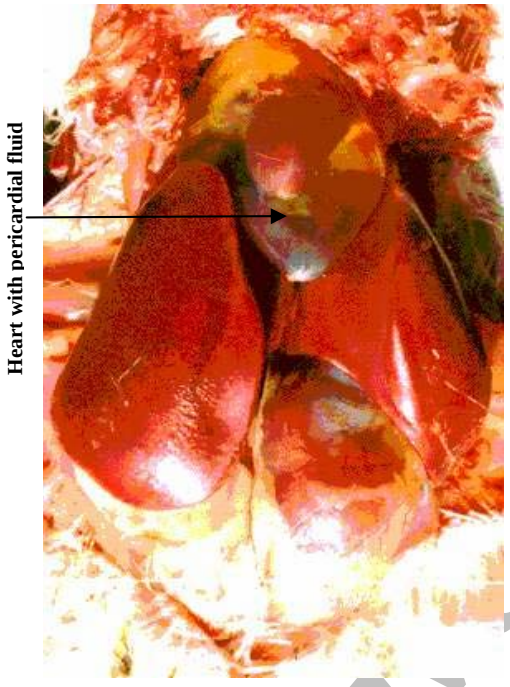

Fig. 2: An infected enlarged liver and heart with yellowish viscous pericardial fluid

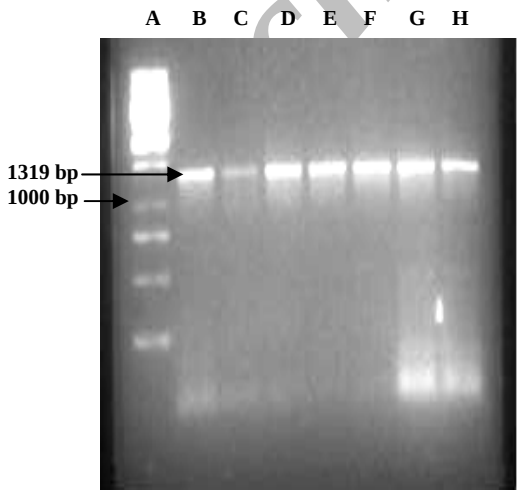

Fig. 3: PCR amplified products of HPS isolates after agarose gel electrophoresis. Lane A = molecular marker 100 bps, Lane B = FAV-4 (J2-A), Lane C = HPS/SPVC/1990, Lane D = HPS/SPVC/1994, Lane E = HPS/SPVC/1996, Lane F = HPS/SPVC/1997, Lane G = HPS/SPVC/2000, Lane H = HPS/SPVC/2003

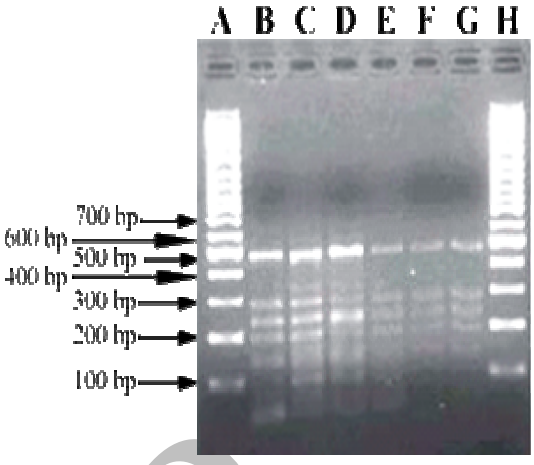

Fig. 4: RFLP profile of PCR amplified products of HPS isolates after digestion with *Hpa* II enzyme. Lane A = molecular marker 100 bps, Lane B = FAV-4 (J2-A), Lane C = HPS/SPVC/1990, Lane D = HPS/SPVC/1994, Lane E = HPS/SPVC/1996, Lane F = HPS/SPVC/1997, Lane G = HPS/SPVC/2000, Lane H = molecular marker 100 bps

suggested that HPS/FAV-4 could be grown in chicken liver and kidney monolayers (Rabbani and Naeem, 1996). These observations were confirmed in general in the present study.

The avian adenovirus group-specific PCR has been reported and suggested to be a rapid, sensitive and specific test to demonstrate all different serotypes of avian adenovirus infection and might be used for avian adenovirus surveillance of specific pathogen-free flocks (Xie *et al*., 1999; Guys *et al*., 2004). Such an assay coupled with restriction endonuclease analysis has been attempted for differentiation of all the 12 FAV serotypes (Raue and Hess, 1998). In the present investigation, PCR with the primers H3/H4 and subsequent *Hpa* II digestion was used for serotype identification of six isolates as it has proven for the identification of FAV serotypes (Raue and Hess, 1998). These profiles are identical to those of FAV isolates on the basis of molecular weight on agarose gel and confirm the identification of Pakistani isolate of hydro-pericardium syndrome as FAV-4. Similar results with the same length of PCR product have been reported for isolate code 341 and 215 during the outbreaks in 1996-97 in Chile and the reference strain KR5 (FAV-4), confirming the classification of all three isolates as

FAV-4 (Toro *et al*., 1999). During the course of the present investigation, a report on isolates from Haryana, India was published confirming the isolates as FAV serotype 4 employing PCR assay coupled with restriction endonuclease analysis during 1998 (Swati *et al*., 2002).

This study provides genomic information about the serotype causing HPS which may be helpful for researchers dealing with the phylogenetic assay of FAV and may indirectly support an urgent need for re-evaluation and harmonization of the FAVs classification and taxonomy. It may also provide the basis for future investigations to identify the fiber gene and protein components for developing sub-unit vaccine (Fingerut *et al*., 2003).

References

Adair, BM; McFerran, JB and Calvert, VM (1980). Development of a microtitre fluorescent antibody test for serological detection of adenovirus infection in birds. Avian Pathol., 9: 291-300.

Adair, BM; Tood, D; Mcferran, JB and Mckillop, ER (1986). Comparative serological studies with egg drop syndrome virus. Avian Pathol., 15: 677-685.

Alexander, HS and Nagy, E (1997). Polymerase chain reaction to detect infectious laryngotracheitis virus in conjunctival swabs from experimentally infected chickens. Avian Dis., 41: 646-653.

Clavijo, A; Krell, PJ and Nagy, E (1996). Molecular cloning and restriction enzyme mapping of avian adenovirus type 8 DNA. Virus Res., 45: 93-99.

Cowen, BS (1987). A trivalent antigen for the detection of type 1 avian adenovirus precipitin. Avian Dis., 31: 351-354.

Cowen, BS; Mitchell, GB and Calnek, BW (1978). An adenovirus survey of poultry flocks during the growing and laying periods. Avian Dis., 22: 115-121.

Erny, KM; Pallisler, J and Sheppard, M (1995). Immunological and molecular comparison of fowl adenovirus serotype 4 and 10. Arch. Virol., 140: 491-501.

Fingerut, EB; Gutter, G; Gallili, A; Michael, J and Pitcovski, J (2003). A subunit vaccine against the adenovirus egg-drop syndrome using part of its fiber protein. Vaccine. 21: 2761-2766.

Ganesh, K; Suryanarayana, VV and Raghavan, R (2002). Detection of fowl adenovirus associated with hydropericardium hepatitis syndrome by a polymerase chain reaction. Vet. Res. Commun., 26: 73-80.

Guys, M; Couvreur, B; Decaesstecker, M; Boschmans, M and Thierry, P van den Berg (2004). Phylogenetic analysis of fowl adenoviruses. Avian Pathol., 33: 164-167.

Jiang, P; Ojkie, D; Tuboly, T; Huber, P and Nagy, E (1999). Applications of the polymerase chain reaction to detect fowl adenoviruses. Can. J. Vet. Res., 63: 124-128.

Kumar, R; Chandra, R and Shukla, SK (2003). Isolation of etiological agent of hydropericardium syndrome in chicken embryo liver cell culture and its serological characterization. Indian J. Biol., 41: 821-826.

Nguyen, AV; Khan, MI and Liu, Z (1994). Amplification of salmonella chromosomal DNA using the polymerase chain reaction. Avian Dis., 38: 119-126.

Rabbani, M and Naeem, K (1996). *In vitro* and *in vivo* evaluation of avian adenovirus isolates from outbreaks of hydropericardium syndrome. *Proceedings of international symposium on adenovirus and reovirus infections in poultry*. Rauischholzhausen, Germany. P: 24.

Raue, R and Hess, M (1998). Hexon based PCR's combined with restriction enzyme analysis for rapid detection and differentiation of fowl adenoviruses and egg drop syndrome virus. J. Virol. Methods. 73: 211-217.

Saiki, RK; Sckarf, S; Faloona, F; Mullis, KB; Horn, GT; Erlich, HA and Arnheim, N (1985). Enzymatic amplification of beta-globulin genomic sequence and restriction site analysis for diagnosis of sickle cell anemia. Science. 230: 1350-1354.

Sambrook, J; Fritsch, EF and Maniatis, T (1989). *Molecular cloning: a laboratory manual*. 2nd Edn., New York, Cold Spring Harbor Laboratory Press. Section 6.

Schat, KA and Purchase, HG (1998). Cell culture methods. In: *A laboratory manual for the isolation and identification of avian pathogens*. Published by American Association of avian pathologists. PP: 223-234.

Smith, LM; Brown, SR and Howes, K (1998). Development and application of polymerase chain reaction of subgroup J avian leukosis virus. Virus Res., 54: 87-98.

Swati, D; Ravinder, SN; Hess, M and Baldev, RG (2002). Fowl adenovirus serotype 4 associated with outbreaks of infectious hydropericardium in Haryana, India. Avian Dis., 46: 230-233.

Toro, H; Pruses, C; Raue, R; Cerda, I; Geisse, C; Genzalez, C and Hess, M (1999). Characterization of fowl adenoviruses from out-break of inclusion body hepatitis/ hydropericardium syndrome in Chile. Avian Dis., 43: 262-270.

Xie, Z; Fadl, A; Girshick, T and Khan, M (1999). Detection of avian adenovirus by polymerase chain reaction. Avian Dis., 43: 98-105.