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Persistence and Transmission Pattern of Avian Adenovirus-4 in Broiler Breeders

S. Ashraf, S. A. Malik and K. Naeem*
Quaid-e-Azam Univeristy, Islamabad, Pakistan
*Animal Health Laboratories, Animal Sciences Institute,
National Agricultural Research Centre, Islamabad, 45500, Pakistan

Abstract: This study was undertaken to investigate the persistence of Avian Adenovirus-4 (AAV-4) in broiler breeders after the infection of Angara Disease (AD) and also to study its transmission pattern in the progeny. In the affected breeders the presence of AAV-4 was detected in organs of embryonated eggs and egg albumen derived from breeders at 30, 37 and 44 weeks of age, using Dot- ELISA. Shedding of the virus was found to be at its peak at the start of the laying period, decreasing successively thereafter. It has been proposed to vaccinate the breeders against AD to protect the progeny and eliminating the carriers so as to prevent the virus from circulating in the population.

Key Words: Angara Disease, Hydropericardium syndrome, Avian adenovirus, Persistence

Introduction

Angara Disease (AD), also called hydropericardium syndrome (HPS) is an acute disease of poultry affecting both the broilers and broiler-breeder chickens. It was first reported from ''Angara Goth'' near Karachi, Pakistan in 1987, quickly spreading to the other parts of the country (Jaffery, 1988). The disease has now been reported from different other countries also (Shane, 1995).

The course of disease is 10 to 15 days during which the mortality rate of 12 percent to 75 percent has been reported in broilers whereas mortality upto 10 percent has been observed in breeders. The disease is marked by hydropericardium, hepatitis, pulmonary oedema and inclusion body hepatitis. The typical gross lesion is the accumulation of clear, straw colored fluid in the pericardium. Other lesions include discolored and friable liver, pale kidneys with distended tubules, and heart appears flaccid. Histologically large round basophilic inclusion bodies in hepatocytes are also present (Anjum *et al*., 1989).

Initially the syndrome was attributed to a nutritional disorder but its transmission by subcutaneous inoculation of liver homogenate from infected birds confirmed its infectious nature (Chishti *et al*., 1989). The subsequent studies proposed role of adenovirus alongwith some other agents in producing AD (Afzal *et al*., 1991). However, later on avian adenovirus belonging to serotype-4 of group 1 adenovirus named as PARC-1 isolate, was identified as the sole pathogen for AD (Naeem *et al*., 1995b). The virus has been propagated in cell culture and embryonated eggs and used for preparation of vaccines (Naeem *et al.,* 1995a). The isolates of AAV serotype-4 recovered from AD outbreaks from Pakistan and Ecuador have also been found to reproduce disease by oral inoculation in chicks up to 1 week of age. Furthermore, these isolates have also been grouped under serotype-4 on the basis of restriction enzyme analysis (Mazaheri *et al*., 1998).

AAV-4 has been found to have a predilection for lymphoid organs, which results in immunoppression in the affected birds (Naeem *et al*., 1995b) resulting in subsequent bacterial infections. Apart from broilers, the disease has now oftenly been seen in broiler breeder flocks. Here the disease occurs between 10-14 weeks of age and typical signs of angara disease appear in only 10-20% of the flock, and the mortality may go upto 20 percent (personnal communication). An over all disease incidence of 10% is estimated among the broiler-breeder population of 3.5 million per annum in Pakistan. It has also been observed that progeny from the previously infected breeders suffer more from AD even if they are vaccinated against AD at 10-15 days of age.

Adenoviruses are known to transmit horizontally and vertically from the viremic parent flocks but no such information is available in the case of AD associated AAV-4. It is, therefore, still unclear if breeder stocks are the major reservoirs of infection, and whether they have any role in the transmission of this infection in their progeny. This paper describes the persistence and vertical transmission pattern of AAV-4 in the broiler-breeders infected with AD.

Materials and Methods

Viruses and antisera: The avian adenovirus serotype–4 was isolated from field cases of HPS and was purified by limiting dilution method and grown in chicken embryo liver (CEL) cells from 10-day old embryos. Eagle's minimum essential medium (EMEM) containing 10 percent fetal calf serum was used for cell propagation. The antiserum against the purified AAV-4 earlier produced in SPF chickens using standard procedures was used here (Naeem *et al.,* 1995b).

Agar gel precipitation test: Agar gel precipitation test (AGPT) was conducted following the standard protocol (Crowle, 1973). AGPT was performed both for the detection of AAV-4 antigen and AAV-4 antibodies.

Dot-Enzyme linked immunosorbent assay: Dot-Enzyme Linked Immunosorbent Assay (DOT-ELISA) was used for the detection of viral antigen in the test material. It was slightly modified from earlier standardized technique (Rabbani *et al.,* 1998a). Briefly, nitrocellulose sheets of 0.45 µm pore size were cut into strips of 4.0 x 0.5 cm and marked with lead pencil for the orientation of the antigen dots. Two µl sample of the tissue culture propagated AAV-4 dilutions ranging from 10^{-1} - 10^{-6} were placed on each strip at 1 cm apart and allowed to dry at room temperature. For negative controls organ homogenate of 18 day SPF embryo was processed and placed similarly on the same strip to determine the specificity of the test. The strips were treated with 3% gelatin (Sigma Chemicals Co., St Louis, MO) in Tris buffer saline (TBS; 50 mM Tris, 150 mM NaCl, pH = 7.5) for 90 minutes for blocking the unreacted protein binding sites. The strips were then placed in antiserum against AAV-4 diluted separately from 1:20 - 1:160 in TBS for 90 minutes. A negative control strip was also included using SPF antisera of uninfected chicks. After 4 washings in TBS, upto 5 minutes each time, the strips were separately submerged in rabbit anti-chicken IgG conjugated with horseradish peroxidase (Sigma Chemicals, Co.) diluted to 1:500, 1:1000 and 1:2000 in TBS. After 90 minutes of incubation at room temperature the strips were washed again in the manner described above and were developed in 50 mM TBS containing 0.15 percent (w/v) diaminobenzidine (DAB), 0.04 percent (w/v) nickel chloride (Ni Cl_2), and 0.02 percent (w/v) hydrogen peroxide (H_2O_2). The reagents were allowed to react for 25 seconds. The strips were washed in tap water and air dried. In positive samples the site of the antigen-antibody complex turned slate black. The highest dilution of viral antigen with maximum antibody titer at the highest conjugate dilutions giving the positive results with no background signal was considered to be the ideal dilutions of each conjugate, antigen and antibodies for future testing. To determine the specificity of the test, antigens from AAV-serotypes 3, 6, 8, 9 and

11 was also run as control. These preparations were obtained from NVSL, Ames, Iowa and used as 1/10 dilutions. The serum dilutions of AAV-4 giving negative results against above AAV-serotypes were selected for use in future testing. After the selection ofoptimum concentration of antigen, antisera and conjugate, the test was run with unknown samples.
In routine testing, a negative antigen control, using triturated organs from SPF-embryos after 18 days of incubation were used. Furthermore, SPF originated negative serum was also employed as a control.

Experimental Design:
The test material was collected from the following groups:
Organs from broiler-breeders naturally infected with AD: Four dead chickens, from 10 week-old broiler-breeder flock of 10,000 birds showing typical signs and lesions of AD were obtained. Out of them the organs collected were liver, lungs, kidneys, spleen, cecal tonsils, thymus, heart and bursa. A 20 percent (w/v) suspension of each organ was made in PBS and freeze-thawed thrice before centrifuging to get rid of debris. Antibiotics solution was added to it using penicillin 1000 IU/ml and streptomycin 1000 ug/ml. This material was tested by Dot-ELISA and AGPT for the presence of viral antigen. After filtration from 0.20 um syringe filter the samples were also inoculated onto monolayers of chicken embryo liver (CEL) cells for virus isolation.

Eggs from the iinfected flock: Eggs from the parent flock, which earlier suffered from AD outbreak were obtained at the age of 30, 37 and 44 weeks of age. The albumin from these eggs was collected and stored at -20°C till used. This material was also used in AGPT Dot-ELISA and cell culture propagation for the detection of AAV-4 antigen.

Organs from embryonated eggs: Fertile eggs after eighteen days incubation, originated from the parent flock that had earlier suffered from an outbreak of AD, were obtained at the flock age of 30, 37 and 44 weeks. The organs namely liver, lungs, kidneys, and spleen were aseptically dissected out and 20 percent (w/v) suspension of each was made in PBS (pH = 7.2) and processed as above. The samples were tested by AGPT, Dot-ELISA and cell culture inoculation.

Results

Standardization of Dot-ELISA: On the basis of Dot-ELISA standardization results, a 1:1000 dilution of the conjugate, 1:40 of the antibody and 1:10 of the antigen dilution were used for all the subsequent testing of the material through Dot-ELISA.

Detection and isolation of AAV-4 from AD infected birds: All the samples appeared negative by AGPT where as all the tissue samples appeared positive by Dot-ELISA. The samples of liver, spleen and bursa of fabricius appeared positive for AAV-4 when propagated in CEL cells, while lungs, kidneys, thymus, cecal tonsils and heart appeared negative (Table 1).

Detection and isolation of AAV-4 from the egg albumin of infected flock: Fifty four samples of the eggs were tested from different age groups. All these samples were tested negative by AGPT for AAV-4. However, by Dot-ELISA out of 17 samples in group A, 14 were positive, whereas by cell culture 15 were found positive. In group B, 5 out of 7 samples were found positive both by Dot-ELISA and cell culture inoculation. In group C, only 2 out of 30 were positive by Dot-ELISA, whereas 6 were positive by cell culture inoculation (Table 2).

Detection and isolation of AAV-4 from embryonated eggs: AGPT again did not detect any viral antigen in these cases. In the group A, out of 30 samples examined by Dot-ELISA, 20 livers, 30 spleens, 10 lungs, and 30 kidneys were tested positive. In this group, 20/30 samples were found positive by cell culture inoculation. In the group B, 5 out of 17 livers, 1 out of 17 spleens, 1 out of 17 lungs and none out of 17 kidneys were tested positive. Whereas from this group, 3 out

Table 1: Presence of AAV-4 antigen in the organs of 10-week old broiler breeders

Test Performed	AAV-4 positive / tested ratio from:							
	Liver	Lungs	Kidneys	Spleen	Thymus	Cecal tonsils	Heart	Bursa of Fabricius
AGPT	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Dot-ELISA	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
Tissue Culture	4/4	0/4	0/4	4/4	0/4	0/4	0/4	4/4

Table 2: Presence of AAV-4 antigen in albumin of eggs collected from infected birds

Group (Age of birds)	Tests employed	No. of Samples Examined	No. of Positive Samples	Percentage (%)
A	AGPT	17	0	0.00
(30 Weeks)	DOT-ELISA	17	14	82.35
	Tissue Culture	17	15	88.24
B	AGPT	7	0	0.00
(37 Weeks)	DOT-ELISA	7	5	71.43
	Tissue Culture	7	5	71.43
C	AGPT	30	0	0.00
(44 Weeks)	DOT-ELISA	30	2	6.60
	Tissue Culture	30	6	20.00

Table 3: Detection of AAV-4 in embryos from laying birds at different age

Group	No. of samples	Tests Employed	Type of Test Material (Positive/Tested)			
			Liver	Spleen	Lungs	Kidneys
A	30	AGPT	0/30	0/30	0/30	0/30
		DOT-ELISA	20/30	30/30	10/30	30/30
		Tissue Culture	20/30	0/30	ND	ND
B	17	AGPT	0/17	0/17	0/17	0/17
		DOT-ELISA	5/17	1/17	0/17	0/17
		Tissue Culture	3/17	1/17	ND	ND
C	10	AGPT	0/10	0/10	0/10	0/10
		DOT-ELISA	0/10	0/10	0/10	0/10
		Tissue Culture	0/10	0/10	ND	ND

AGPT = Agar Gel Precipitation Test
Dot-ELISA = Dot-Enzyme Linked Immunosorbent Assay
ND = Not Done
AAV-4 = Avian Adenovirus-4

of 17 liver samples and 1 out of 17 spleen samples were positive for the presence of AAV-4 upon propagation in CEL cells. In group C, none of 10 organ samples were found positive for AAV-4 by Dot-ELISA or cell culture inoculation (Table 3).

Discussion

Some members of Aviadenoviridae have been known to cause specific infections in poultry in different age groups for the past many years. This includes members of group II and III. However, role of some viruses belonging to group I Aviadenoviridae is now increasingly becoming significant in causing specific infections of inclusion body hepatitis (IBH) and Hydropericardium syndrome in broiler flocks (Cowen, 1992). The avian adenovirus serotype-4 belonging to group-1 aviadenoviridae is now known to cause hydropericardium syndrome in poultry, primarily in broiler flocks (Mazaheri *et al.*, 1998; Naeem *et al.*, 1995b; Rabbani *et al.,* 1998a, b, Voss, 1989). However, little information is available on the prevalence of this disease in older birds and also the information regarding viral transmission and its pathogenesis under field condition is also lacking.

Most of the avian adenoviruses of group-1 are known to excrete through feces, which may lead to infection through oral route, however, airbrone and vertical transmission has also been reported earlier (McFerran and Adair, 1977). Based on the field observations of AD outbreaks in Pakistan high incidence in breeder flocks followed by relatively more outbreak of AD in the progency of those Breeders, The study reported here was planned to determine the persistence of AAV-4 in breeders, which did not show any apparent clinical signs. Furthermore, this study was supposed to present evident for vertical transmission of AAV-4 through eggs.

The results obtained indicate that the probable reason for increase in AD occurrence in the field is the transfer of the virus at the time of egg production to the progeny. The transmitted virus might reactivate when they are exposed to immunosuppression during their life time. The avian adenoviruses are earlier known to persist in chickens in the latent form and are thought to be transferred to the progeny. In this regard, in an earlier study it was reported that after infection, AAV may shed upto 3 months during production (Yates and Fry, 1957).

While testing the egg albumin from the AD infected flock for the presence of AAV-4, it was observed that through Dot-ELISA 82.35 percent of the eggs were tested positive for AAV-4 at 30 weeks of age and later at 37 weeks of age the level increased, but again decreasing to 6.67 percent at 44 weeks of age. This relfects that the virus may be present both at the age of 30 and 37 weeks in the breeder-broiler, but very little virus was detectable at 44 weeks of age. These results were later confirmed by isolation of the virus from infected chickens.

Here Dot-ELISA was used as a method for detecting the presence of viral antigen in comparison with AGPT and cell culture. Dot-ELISA proved to be a more sensitive, simple and less cumbersome test. Especially, the greatest advantage of test lies in its simplicity in screening the breeder flock. On the other hand AGPT was found to be less sensitive and non-specific since it could not detect viral antigen in any of the samples. The AAV-4 virus from Dot-ELISA positive specimens was mostly grown in cell culture also. The presence of AAV-4 in developing embryos in the eggs from the infected flocks was also seen.

At the age of 30 weeks 66% of liver, 33% lungs, 100% kidneys and 100% of spleen were tested positive for AAV-4. The organ distribution of the virus indicated that initially liver, kidneys and spleen were primarily infected. Subsequently at the age of 37 weeks the presence of AAV-4 in various organs decreased except in the liver where rate of positive reactors was 30 percent. However, none of the samples from kidneys, lungs and spleen showed the presence of AAV-4 at 44 weeks of age.

The rate of AAV-4 positive samples both in albumin and other organs appears to be similar, reflecting the reliability of data. The decrease in shedding of the virus with increasing age may be due to the fact that the birds start to overcome the laying stress of the initial stage at later age. This may have contributed towards decreasing the shedding of AAV-4 after 37 weeks of age.

Although most of the samples were found positive using Dot-ELISA and/or cell culture, with the advent of new techniques such as DNA-probes and PCR it may be possible to detect the lowest quantity of the virus in various tissues or egg albumin. It would be, therefore, recommended that some other techniques such as blot hybridization, immuno-histopathology etc., may be employed further to determine the exact cells or the organs where the AAV-4 persists during its latent infection and the state in which the virus is present there. In addition, the cellular changes taking place during its latency and its subsequent reactivation also needs to be investigated further.

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