POST INFECTION DISSEMINATION PATTERN OF AVIAN ADENO-VIRUS INVOLVED IN HYDROPERICARDIUM SYNDROME

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ABSTRACT

To determine the post-infection tissue dissemination pattern of Avian Adenovirus-4 (AAV-4), the virus was inoculated in broilers via subcut or oral routes. The birds were sacrificed at 12 hour interval and their organs were examined by indirect fluorescent antibody (IFA) assay for the presence of viral antigen. A distinct variation in the virus retention pattern in different organs of the inoculated birds was observed. Both the kidneys and liver in the inoculated birds showed the persistence of AAV-4 beyond 60 hours post inoculation. Histopathological studies revealed basophilic intranuclear inclusions in liver and kidneys, and also showed a low level of inflammatory cell infiltration in these organs.

Keywords: Avian Adeno-virus, hydropericardium syndrome, post infection, dessemination pattern

INTRODUCTION

Since 1987, Poultry industry in Pakistan has been confronted with hydropericardium syndrome (HPS) affecting broiler chicks in particular, with colossal economic losses (Ahmad *et al.*, 1990). Disease has been mostly seen anytime during 2-6 weeks of age in broiler chicks, however, it is also reported quite oftenly in broiler breeders but less commonly in layers (Ashraf *et al.*, 2000). In broilers, the disease suddenly appears and the course of disease is usually 10-15 days and mortality rate is 30-60%. The syndrome is characterized by accumulation of clear straw coloured fluid in pericardium; swollen discoloured and friable liver and pale enlarged kidneys with distended tubules (Ahmad *et al.*, 1990).

Histopathological investigations earlier reported intranuclear inclusion bodies in liver and kidney cells of the birds died of HPS (Cheema *et al.*, 1989; Anjum *et al.*, 1989). No information, however, is available regarding the dissemination and organ predilection of the purified avian adenovirus-4 (AAV-4) upon infection in experimental birds. To elaborate the pathogenesis of AAV-4 it is necessary to determine the pattern of disease spread after infection, and to further know the major target organs of the virus during infection. This would lead to better understanding of viral pathogenesis on cellular level. An earlier attempt in this direction revealed viral predilection for lymphoid organs (Naeem *et al.*, 1995). However, its dissemination pattern in other vital organs was not studied in detail, which has now been investigated here.

MATERIALS AND METHODS

Chickens

Day old broiler chickens were obtained from a local hatchery and reared in isolated rooms at the Experimental Animal Facility, Animal Sciences Institute, National Agricultural Research Centre, Islamabad, under strict biosecurity measures. The chicken were provided feed and water *ad libidum*.

Virus

An avian adenovirus type-4 was purified from the field and propagated in chicken embryo liver cells (CEL) using standard procedures. The virus was inoculated in the experimental birds at the rate of $10^{6.4}$ $TCID_{50}$/bird.

Hyperimmune serum

The purified AAV-4 propagated in CEL was used to prepare hyperimune serum in chicken, following standard protocol. Briefly, three selected chickens were tested negative of AAV-4 antibodies. They were injected intramuscularly with 1 ml of killed avian adenovirus-4 which was earlier emulsified with equal quantity of Freund's complete adjuvant. Three weeks later a similar virus preparation was emulsified with incomplete Freund's adjuvant and 1 ml was injected subcutaneously into each chickens. After three more weeks serum samples were collected and tested by AGPT to detect adenovirus-4 antibodies.

Agar Gel Precipitation Test (AGPT)

AGPT was conducted on hyperimmune serum samples and liver homogenate prepared at different stages to detect HPS-associated adenovireus antibodies and viral presence respectively following the standard protocol (Beard, 1970).

Indirect Fluorescent Antibody Assay (IFA)

For the detection of viral antigens from various organs of the inoculated birds, IFA was standardized and proceeded as follows:

A 4 mm3 AAV-4 infected liver sections were made using cryomicrotom (Miles, USA). For this purpose, the tissue was first embedded in cryofoam (O.T.C. compound Miles Inc., USA) and frozen sectioned accordingly. The sectioned tissue was placed on Poly-L-Lysine coated slides (Sigma, USA) and fixed in cold acetone for 10 minutes at –20°C. After drying the slides in air, area covered with tissue were circled with grease pencil and the slides were stored at –20°C. For selecting working dilutions of the conjugate and antisera, different dilutions (1/20 - 1/640) of antibodies to AAV-4 and two dilutions of FITC conjugate (1/50 - 1/100) were prepared using the above slides from the infected liver. The dilutions showing appropriate fluorescence with minimum background were selected for further testing of unknown tissue samples.

To perform the detection of AAV-4 antigen in experimental tissues, these tissues were processed for cryosectioning as described above. The tissues within the encircled area of slides were first flooded with 1:20 dilution of hyperimmune sera of AAV-4 and incubated at 37°C in humid chamber for 30 minutes. After rinsing 3 times, 5 minutes each, with PBS(pH 7.2) and air drying, the slides were flooded with 1:100 dilution of anti-chicken FITC conjugate (Sigma, USA) and incubated as described earlier. The slides were rinsed with PBS (pH 8.5) as described above, and counter-stained by dipping in Evan's blue for 20 seconds and washed with distilled water. Slides were air dried and mounted with FA-mounting media (50% glyceral + 50% PBS, pH 7.2) and were examined under fluorescent microscope.

Histopathology

For histopathology, the tissues were fixed in 10% formalin. Tissues were sectioned at 8-10 um and were processed in ascending grades of alcohol starting from 5 to 100% for 1.5 hours in each concentration. Tissues were then dipped in xylene for three hours and paraffin embedding was done by automatic tissue processor (Karl Kolb, W.Germany). Sections were floated on warm water at 50°C. Albumin (5% w/v) coated slides were used for placing the sectioned tissues. The slides were incubated at 37°C overnight so that the tissues get attached with the surface of slides. Then the slides were stained with hematoxylin and eosin and examined under the light microscope as per standard procedures (Anjum *et al.*, 1989; Cheema *et al.*, 1989).

Infectivity Test for Major infected Organs

Heart, lungs, liver and kidneys collected from infected chicks were minced individually and their 20% homogenates were prepared separately using normal saline solution. Each homogenate was centrifuged at 3000 rpm for 15 minutes. The supernatant was used as inoculum at the rate of 1 ml per organ for subcutaneous inoculation in 15-day old broiler birds and their mortality due to HPS was recorded.

Experimental Design

The experimental birds were bled at 15 day of age and tested negative for the presence of antibodies against AAV-4. For first experiment (#I) thirty birds were divided into a test group (20 birds) and an uninfected control (10 birds). All the groups except control were inoculated with 1ml of the virus inoculum ($10^{6.4}TCID_{50}$). The control group was injected with normal saline solution. Every time four birds from each test group and 2 from control were sacrificed at 12, 24, 36, 48 and 60 hours post-inoculation. The liver, lungs, kidneys and heart from them were saved each time for conducting the following tests; a) for indirect fluorescent antibody assay, the organs were stored at -20°C, and b) for histopathology the organs were placed in 10% formalin. For disease reproduction experiment (#II) another group of 90 birds were split into test (80 birds) and control groups (10 birds). Among test birds, each time 4 birds were injected with either of liver, kidneys, lungs or heart homogenates obtained at different time interval in experiment #I. The control group acted as uninfected control. Mortality showing HPS related lesions was recorded in each sub-group. For Experiment #III, another 40 test birds were grouped into sub-groups of 4 birds each and given AAV-4 on 3,6,9,12 and 15 days of age either by oral route ($10^{6.4}$ $TCID_{50}$/bird) or by subcut inoculation ($10^{6.4}$ $TCID_{50}$/bird). Mortality due to HPS was recorded in this group.

RESULTS

Detection of the viral antigen(s) in various organs was conducted with indirect fluorescent antibody assay

(IFA). Here the standardized dilution for conjugate and hyperimmune sera used were, 1:20 and 1:100, respectively. Initially no antigen was detected during first 12 hour post infection from any organ of the bird. In the lungs, viral antigens were detected at 12 hours, 24 hours and 36 hours post inoculation only. The heart showed the presence of viral antigens at 24, 36, 48 and 60 hour post inoculation (Table 1). No AAV-4 antigen was detected in the negative control group.

Gross and histopathological changes observed in various organs at different time interval post inoculation using HPS associated adenovirus are shown in Table 2.

When the organs from experimentally inoculated birds were used to determine the presence of AAV-4 in experiment #I, it revealed that inoculation of kidney homogenate from 12 hours, 24 hours and 36 hours post-inoculation group produced HPS lesions. Inoculation of heart homogenate of 12 hours and 24 hours resulted in the production of HPS with typical disease lesions. Lungs homogenate of only 36 hours and 48 hours reproduced the Hydropericardium Syndrome (Table 3).

In disease transmission experiment (Exp. #III) 60-80% mortality was seen in group injected the virus subcutaneously among the inoculated groups. However 50% mortality was observed in birds orally administered with the virus at the age of 3 & 6 days . No mortality was observed thereafter in this group (Table 4).

DISCUSSION

In the past, it has been proposed repeatedly that adenovirus serotype-4 has the major role in the development of HPS in birds (Ashraf *et al.*, 2000). However, sufficient information regarding viral pathogenicity and its cellular dissemination pattern has not been investigated.

The experiment reported here regarding the route of disease transmission indicated that the most successful route of infection is subcutaneous inoculation irrespective of age of the bird, however, the oral route administration was only successfully upto 7 day of age. Khawaja *et al.* (1988) reported 100% mortality through intrapericardial route while 80 and 40% mortality was reported by sub-cut and oral routes, respectively. They also reported 40% mortality in un-inoculated birds kept in close contact with the diseased birds. In another study it was found that AAV-4 was capable to reproduce the disease in birds during first week of age via oral administration (Mazaheri, 1998). These findings indicate that the disease can be reproduced by various parentral routes out of which the subcutaneous route was considered more effective for experimental purposes. However, the oral route appears to be the natural route of infection whereby the virus is taken up by the host at very early age of life which may later on results in overt disease.

The specimens from experimentally inoculated birds when examined by IFA indicated that viral presence can be easily detected from the organs through this technique especially in liver and kidneys. The fluorescent nuclei indicated the presence of the intranuclear inclusion bodies in the cells of these organs. Only a few positive foci of viral presence were seen in tissues taken from lungs of infected birds. This means that the virus may have variable distribution pattern in cells on the other hand. It may have persisted in the cells but did not replicate in the tissues. So in this case it got cleared from the lungs at a faster rate whereas it persisted in the kidneys and liver for a longer duration. The presence of intranuclear inclusion bodies seen by hematoxylin and eosin staining in liver also supported IFA results.

Inclusion body hepatitis (IBH) can be confused with HPS on the basis of intranuclear inclusion bodies in liver cells and necrosis of liver. However, high mortality rate, inflamed liver distinct and hydropericarditis and severe nephritis are major symptoms of HPS. On the basis of similarity in disease symptoms with IBH, the severity of these lesions in case of HPS in liver and kidneys, it was earlier considered that some distinct serotype of adeno-virus group may be the causal agent of HPS. Some further studies suggested in the past that adeno virus and some other agent(s) act synergistically to produce the syndrome (Afzal *et al.*, 1991). However, later on AAV-4 alone was proved to reproduce the disease in the field (Naeem *et al.*, 1995).

The major histopathological lesions persistently observed in liver were intranuclear inclusion bodies in hepatocytes. Similar results have also been reported by Anjum (1988) and Khan *et al.* (1988). Kidneys showed cellular infiltration and congestion. Despite the fact that the heart reflected the major signs of the syndrome, no changes were seen histologically in this organ. This reflects that perhaps the changes produced in the pericardial sac were due to some yet unidentified physiological phenomenon, developed due to the presence of the infectious agent of HPS.

1. **Table 1: Detection of AAV-4 antigen in different organs after experimental inoculation using IFA**

Organs	Detection of AAV-4 antigen at hours post-inoculation*				
	12	24	36	48	60
Liver	0/4	3/4	3/4	4/4	3/4
Lungs	0/4	2/4	3/4	3/4	0/4
Kidneys	0/4	3/4	3/4	3/4	2/4
Heart	0/4	0/4	2/4	3/4	0/4

* All the organs from negative control group were clear from viral presence.

Table 2: Histopathological changes observed in various organs at different time interval post-inoculation using HPS associated adenovirus

S.No.	Organs examined	Gross lesions	Microscopic lesion
1.	Liver	Pale, swollen and friable at 36 hours, 48 hours and 60 hours PI. Necrotic focci were also seen.	Intranuclear basophilic inclusion bodies observed at 24, 36, 48 and 60 hours post infection.
2.	Lungs	Oedematous and congested at 36 hour and 48 hours PI.	No lesions.
3.	Kidneys	Pale, inflamed, swollen and enlarged at 48 hours and 60 hours. Pinpoint haemorrhages were also observed.	Cellular infiltration and congestion observed at 48 hours and 60 hours PI.
4.	Heart	Flabby, accumulation of watery fluid in pericardial sac observed at 48 hours and 60 hours.	No specific microscopic lesions.

Table 3: Reproduction of Hydropericardium syndrome using organs collected from the infected birds at different time interval post-inoculation

Organs	Mortality recorded using different organ homogenate at variable time interval post-inoculation				
	12 hours	24 hours	36 hours	48 hours	60 hours
Liver	1/4	1/4	3/4	1/4	1/4
Lungs	0/4	0/4	1/4	1/4	0/4
Kidneys	1/4	1/4	1/4	0/4	0/4
Heart	1/4	1/4	0/4	0/4	0/4

The numerator is showing the birds affected with Hydropericarditis out of the four inoculated birds (denominator), In all the control birds no HPS was detected.

Table 4: HPS production upon inoculation of AAV-4 via different routes

Route of inoculation	Rate of mortality on days in inoculated groups				
	3	6	7	12	15
Oral	2/4	2/4	1/4	0/4	0/4
Sub-cut	3/4	3/4	4/4	3/4	3/4

The numerator is showing the birds affected with Hydropericarditis out of the four inoculated birds (denominator); In all the control birds no HPS was detected.

Although a number of informative findings have been recorded in this study, numerous questions yet remain to be answered. To know more about the viral pathogenesis, it would be important to study the virus replication at molecular level. This would help to identify the type of changes occurring at the cellular level that lead to the development of hydropericarditis and eventually the death of the infected birds. More information would also be required to understand the nature of viral persistence from the date of infection till the occurrence of the disease. The findings of this study might also be helpful in understanding the reasons for disease re-occurrence in the vaccinated flocks 3-5 weeks post-vaccination. More studies in this regard would, therefore, be required to resolve various aspects of Hydropericardium syndrome in chicken.

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