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RESEARCH ARTICLE

Induction of Immunosuppression in Broiler Chicken upon Co-infection of Avian Adenovirus-4 with Low Pathogenic Avian Influenza H9N2

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ABSTRACT

Despite the infectious nature of Angara Disease (AD), the disease has also been clinically observed in association with other infectious diseases of poultry, which alone may not be capable to cause high mortality. The present study was designed to evaluate the pathology of co-infection of Avian Adenovirus-4 (AAV-4) and Avian Influenza virus (AIV) serotype H9N2 on the health of broiler chicken. In this regard, 12-day old chicks were first experimentally infected with AAV-4 followed by re-infection with AIV H9N2. The chickens infected only with AAV-4 showed virus detection from primary and secondary lymphoid organs up to 7 days post-infection. However, the duration of detection was extended up to 28 days post AIV-H9N2 infection when these birds were co-infected with Avian Influenza serotype H9N2. Moreover, a reduced serological response was observed against AIV-H9N2 in the presence of AAV-4 infection in chicken in comparison with the serological response against AIV H9N2 from healthy birds.

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INTRODUCTION

Adenoviruses are known for their infectious nature throughout the world in various species. These viruses are grouped and serotyped on the basis of their pathogenicity, disease pattern and other biological characteristics within poultry. Avian Adenovirus serotype-4 from group-1Avian Adenovirus, is known to cause AD in different poultry breeds in Pakistan and elsewhere (McFerran, 1997; Jabeen *et al.,* 2015).

AAV-4 is known to persist in chickens and causing some immunosuppression (Naeem *et al*., 1995; Balamurugan and Kataria, 2004). It may therefore, be possible that such immunosuppressed chicks become co-infected with some other relatively mild or low pathogenic microorganism resulting increased morbidity and mortality in the affected flocks. H9N2 subtype of AI is endemic in Pakistan (Munir *et al.*, 2013; Siddique *et al.*, 2016) and is reported to contain NS gene similar to highly pathogenic H7N3 and H5N1 (Iqbal *et al.*, 2009), which suggests that H9N2 can undergo extensive genetic reassortement. Most recently in some countries H9N2 strain was found that has the ability to gain basic amino acids in the HA connecting the peptide sequence that can become highly pathogenic (Abdel-Moneim *et al*., 2012; Shanmuganatham *et al.,* 2013).

Recent outbreaks of such conditions in broiler flocks reared under un-controlled environmental conditions in Pakistan have been investigated to reveal the co-infection of AAV-4 and AIV H9N2. The present study was designed to explore the impact of co-infection of AAV-4 & AIV-H9N2 on the clinical picture of disease among broilers.

MATERIALS AND METHODS

Experimental Design

For primary infection: A total of 60, day-old commercial broiler chicks were randomly divided into three treatment groups (A, B and C), each consisting of 20 birds (Table 1). Group A and B were challenged with AAV-4, group C was control group. Tissue samples (Liver, Kidneys, Spleen, Bursa, Thymus, and Caecal Tonsils) were either

collected from dead birds or post slaughtering of one bird from each group on daily basis. The sera collected from daily bleeding were analyzed by Agar Gel Precipitation Test (AGPT) against reference AAV-4 antigen while the tissue samples were analyzed through AGPT and PCR for the presence of AAV-4.

For induction of immunosuppression: A total of 72, day-old commercial broiler chicks were randomly divided into twelve treatment groups (G1-G12), each group consisting of 6 birds, G11 was only given AIV-H9 infection intranasally and G12 is non-infected control. For this purpose, the broiler chicks were subjected to co-infection by AAV-4 and LPAIV H9N2 (Table 2). The birds were sampled for serum on weekly basis, while swabs (oral and cloacal) were taken twice a week post AIV infection. The tissues from the dead birds were taken on the day of mortality and at the termination of experiment at day 30 post AIV infection.

Source of viruses: The AAV-4 and Low Pathogenic AIV (LPAIV) serotype H9N2 were obtained from the repository of National Reference Laboratory for Poultry Diseases (NRLPD), Animal Science Institute (ASI), National Agriculture Research Center **(**NARC), Islamabad. The selection of above viruses was made on the basis of their involvement in clinically overt infection of AD and Avian Influenza. The following isolates were used in this study:

1. AAV/Chicken/Murree/NARC-26946/2011 (AAV-4)
2. A/Chicken/Abbottabad/NARC-25717/2011(AIV-H9N2)

Virus isolation and identification: The swabs and tissue samples were subjected to virological evaluation through AGPT, Polymerase Chain Reaction (PCR), Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and virus isolation and identification.

For detection of AAV4/AIV in tissue samples, a 20% w/v tissue homogenate was prepared using biomaster stomacher-80 (Seward Ltd., UK) for 2 minutes at high speed. The homogenate was then centrifuged at 400g refrigeration at 4^0C for 10 minutes using 5804R centrifuge (Eppendorf, Germany). The supernatant was collected as virus source and stored at -20^0C until further use. While 0.5 ml of normal saline was added to each swab sample and mixed gently in sterile sampling tubes and centrifuged at 2000 rpm at 4^0C for 10 minutes using 5804R centrifuge (Eppendorf, Germany). The supernatant was collected as virus source and stored at -20^0C until further use. In case of AAV, AGPT was performed for detection of viral antigen using standard protocol as described earlier (Crowle, 1973).

For further identification of the AAV-4 from the tissues and swab samples collected during the study; DNA extraction was done by easy DNA Kit (Invitrogen Inc. USA) following the manufacturer's protocols while PCR was performed using Dream Taq Green PCR Master Mix (Thermo Fisher Scientific Inc.; Fermentas Cat # K1081) using the earlier developed protocol (Jiang *et al*., 1999) with certain modifications.

For isolation and identification of AIV-H9N2, 0.1 ml of sterile inoculum was injected in 9 days old embryonated chicken eggs through allantoic route. The allantoic fluid was collected after 48 hrs and Hemagglutination Assay (HA) was performed for confirmation of presence of influenza viruses (Senne, 1998). The HI protocols were used to identify virus subtypes in allantoic fluid (Pedersen, 2008). Further confirmation of virus was carried out by RT-PCR. For this purpose, RNA extraction was done with QIAmp Viral RNA mini kit according to the manufactures instructions (QIAmp Viral RNA mini kit, QIAGEN, USA, CAT # 52906). RT-PCR was performed using Superscript-II One Step RT-PCR Kit with Platinum Taq (Invitrogen Inc. USA) by following standard protocols (Siddique *et al*., 2008).

Serological analysis: The sera were analyzed against AAV-4 using AGPT following the standard protocols (McFerran, 1997). While the serological response against AIV-H9N2 was evaluated by hemagglutination inhibition (HI) test using the standard protocols (Pedersen, 2008).

RESULTS

Virological analyses: In immunosuppression experiment, higher morbidity and mortality was observed in the groups infected with AAV subcutaneously as compared to the groups orally inoculated. Group 1-5 which were orally infected with AAV-4, a total of 6 birds died among all birds of these groups, while tissues of only 5 birds were found positive for AAV detected by PCR. However, Groups 6 to 10 which were subcutaneously infected with AAV-4, morbidity was observed in almost all birds of each group except for control group. Moreover, a total of 8 birds died in different groups subcutaneously infected with AAV-4 which were found positive for AAV through PCR. While no swabs were found positive for AAV in both the categories of AAV infected birds either with AGPT or PCR (Table 3).

Although most of the birds of each group infected with AIV were found positive for AIV through PCR for viral shedding in the swabs, no shedding was observed in AIV control groups while tissues of 7 birds were found positive for AIV-H9N2 through PCR from all the groups co-infected with AIV and AAV (Table 3). Moreover, tissues of only 3 out of 6 dead birds were found positive for AIV through PCR in group 11 which was infected with AIV only (AIV positive control group). In addition, there was no detection either from tissues or swabs from the group 12 which was kept as negative control for both AIV and AAV (Table 3).

In primary infection experiment, AAV was found detectable up to 7 days post infection in the birds challenged by either oral or sub cut route of infection. However, a prolonged detection of AAV was observed up to 38 days post AAV infection when the birds were co-infected with AIV-H9N2 at 10 days post AAV infection. A total of 5 birds were found positive for AAV in different groups infected with AAV orally while 8 birds from the groups infected subcutaneously were found positive for AAV by PCR (Table 4).

Table 1: Experimental design of primary infection experiment in chickens infected at 12 days of age with AAV-4 at 10^3 TCID50. Treatment group A and B infected via oral and Sub cut routes respectively, Group C Negative Control.

Treatment groups	No of birds	Route of infection	Virus Load	Amount (ml)
A	20	Oral	10^{-3}	0.1
B	20	Sub-Cut	10^{-3}	0.1
C	20	NIL	NIL	NIL

Table 2: Experimental design of induction of immunosuppression. Treatment groups (G1-G5) were infected with a dose of 10^4 LD_{50}/ml of AAV4 orally and those in treatment groups (G6-G10) were inoculated with a dose of 10^4 LD_{50}/ml of AAV4 subcutaneously

Treatment groups	No. of birds	HPS infection at age 12 days	Challenge with AIV-H9N2 Days Post HPS Infection	Sampling Post AIV-H9N2 Infection		
				Bleeding (weekly)	Swabing (Twice a week)	Organs: collection (after mortality)
G-1	6	Oral	7	-Do-	-Do-	-Do-
G-2	6	Oral	10	-Do-	-Do-	-Do-
G-3	6	Oral	13	-Do-	-Do-	-Do-
G-4	6	Oral	16	-Do-	-Do-	-Do-
G-5	6	Oral	-	-Do-	-Do-	-Do-
G-6	6	S/C	7	-Do-	-Do-	-Do-
G-7	6	S/C	10	-Do-	-Do-	-Do-
G-8	6	S/C	13	-Do-	-Do-	-Do-
G-9	6	S/C	16	-Do-	-Do-	-Do-
G-10	6	S/C	-	-Do-	-Do-	-Do-
G-11	6	-	At age of 19 days	-Do-	-Do-	-Do-
G-12	6	-	-	-Do-	-Do-	-Do-

S/C=subcutaneously.

Table 3: Morbidity, mortality and viral detection in swab/tissues in AAV and AIV co-infected birds

Groups	AAV Infection (route, age)	AIV Infection (days)	Morbidity Pos/Total	Mortality Pos/Total	AAV Detection		AIV Detection	
					Tissues Pos/Total	Swabs Pos/Total	Tissues Pos/Total	Swabs Pos/Total
G1	Oral, 12 D	7 DPA	5/6	2/6	2/6	0/6	2/6	5/6
G2		10 DPA	5/6	1/6	1/6	0/6	0/6	5/6
G3		13 DPA	5/6	0/6	0/6	0/6	0/6	4/6
G4		16 DPA	4/6	2/6	1/6	0/6	2/6	4/6
G5		NI	3/6	1/6	1/6	0/6	0/6	0/6
G6	S/C, 12 D	7 DPA	6/6	2/6	2/6	0/6	1/6	5/6
G7		10 DPA	6/6	2/6	2/6	0/6	1/6	6/6
G8		13 DPA	5/6	0/6	0/6	0/6	0/6	5/6
G9		16 DPA	5/6	2/6	2/6	0/6	1/6	4/6
G10		NI	4/6	2/6	2/6	0/6	0/6	0/6
G11	NI	19 D	5/6	3/6	0/6	0/6	3/6	5/6
G12	NI	NI	0/6	0/6	0/6	0/6	0/6	0/6

D=Days of age, DPA=Days post AAV infection, pos/total=No of positive birds/No of total birds in the group.NI=Not infected.

Table 4: Prolonged persistence of AAV in birds co-infected with AAV and AIV.

Groups	AAV Infection (route, age)	AIV Infection(days)	AAV Detection (days post AAV infection)						
			10	13	14	16	18	19	38
G1		7 DPA	-	-	1	-	-	1	-
G2	Oral, 12D	10 DPA	-	-	-	-	-	-	1
G3		13 DPA	-	-	-	-	-	-	-
G4		16 DPA	-	-	-	-	-	1	-
G5		-	-	1	-	-	-	-	-
G6	S/C, 12 D	7 DPA	1	-	-	1	-	-	-
G7		10 DPA	-	1	-	-	-	1	-
G8		13 DPA	-	-	-	-	-	-	-
G9		16 DPA	-	-	-	-	1	1	-
G10		-	1	1	-	-	-	-	-
G11	-	19 D	-	-	-	-	-	-	-
G12	-	-	-	-	-	-	-	-	-

D=Days of age, DPA=Days post AAV infection, Pos/total=No of positive birds/No of total birds in the group.

Moreover, the dissemination of AAV was observed with variation when the birds were co-infected with AIV and AAV. The data showed that dissemination of AAV in the tissues increased up to day 19 and then again reduced to be localized in liver only and was detectable up to day 38 post AAV infection (Fig. 1).

Serological analyses: The serum samples were analyzed by AGPT to detect seroconversion against AAV. It was observed that seroconversion against AAV in broiler chicks was detectable up to 18 days post infection (dpi) with AAV when no other stress was subjected to the chicks (data not shown). However, in case of co-infection with AIV at various intervals after AAV infection, a prolonged and higher seroconversion against AAV was observed in both the groups infected with AAV either orally or subcutaneously (Fig. 2). No seroconversion was observed in groups kept as negative controls for AAV.

The seroconversion against AIV-H9N2 was determined through HI test. Increase in the HI titers up to 21dpi and then a slight decline in the titer against AIV was observed in co-infected groups. However, the positive control group for AIV (G-11) showed a consistent increase in antibody titers against AIV up to day 28 post AIV infection. While no seroconversion against AIV in the negative control groups (G-5, G-10 & G-12) was observed (Fig. 3).

DISCUSSION

The members of genus Avian Adenovirus-I are emerging as a cause of disease problems in poultry especially in the broiler chickens. They are known to cause infectious diseases like IBH and HPS in the broiler chicken (Cowen, 1992). Quite insufficient information on the prevalence, persistence, post infection dissemination pattern and immunosuppressive role of these agents is available in literature.

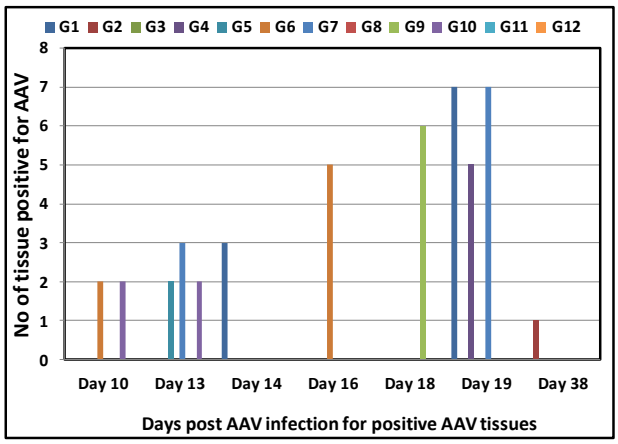

Fig. 1: Post Infection Dissemination Pattern of AAV in terms of number of tissue positive for presence of AAV through PCR. Different days when AAV positive mortalities observed in various treatment groups are shown on x-axis. Whereas y-axis indicates the maximum number AAV-4 positive tissues from each bird at that specific day post AAV-4 infection. Different treatments administered to groups G1-G12 are; G1-G4 were infected through orally and AIV intranasally, G5 was positive AAV control infected orally, G6-G9 were infected AAV via sub cut route and AIV intranasally, G10 was positive AAV control infected subcutaneously, G11 was only given AIV H9 infection intranasally and G12 was non-infected control.

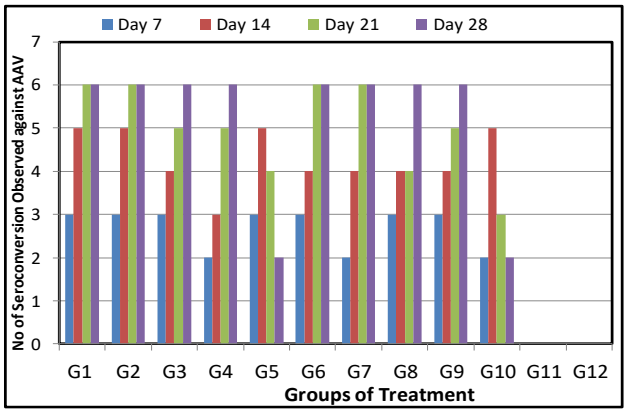

Fig. 2: Seroconversion against AAV-4 detected through AGPT at different time intervals post infection. Different treatments administered to groups G1-G12 are shown on x-axis. Whereas y-axis indicates the number of AAV-4 antibodies positive birds at different days post AAV-4 infection. Different treatment groups are; G1-G4 were infected orally and AIV intranasally, G5 was positive AAV control infected orally, G6-G9 were infected AAV via sub cut route and AIV intranasally, G10 was positive AAV control infected subcutaneously, G11 was only given AIV H9 infection intranasally and G12 was non-infected control.

H9N2, A low pathogenic avian influenza virus (LPAIV) causes only transient mild clinical signs and seldom causes mortality in experimentally infected SPF chickens and poses a potential health risk to humans (Farzin *et al*, 2016; Lee *et al*., 2007). Previously it was believed that high mortalities in the field were due to environmental stress and concurrent secondary infection (Bano *et al*., 2003; Iqbal *et al*., 2013). Later on, it was found that the clinical signs were more severe in SPF chickens receiving cyclosporine (immunosuppressant agent) and infected with H9N2 than those observed in chickens which was only infected with H9N2 (Kwon *et al*., 2008). More severe results were obtained if ORT infection is followed by H9N2 AIV infection (Pan *et al*, 2012) In Pakistan, in recent years, a sudden rise in the HPS incidence along with co-infection of AIV-H9N2 virus in commercial poultry has been observed. This combined infection of poultry resulted in heavy economic losses due to high mortality during the age of day 5-35 especially in case of broiler chicks. The situation was also observed with strong immunosuppression in the birds co-infected with AIV and AAV and various disease problems in elderly age later on in the affected flocks. Moreover, the isolation of AAV agent after 4th week of age was also surprising in the affected flocks. The situation was also confirmed by successful isolation of these co-infecting viruses at National Reference Lab for Poultry Diseases (NRLPD), Islamabad, Pakistan. During this study, quite interesting findings regarding HPS were observed. A 38 days persistence period of the HPS agent was observed in case of co-infection in the broiler birds. The current observations are in agreement with the previous observations where a persistence up to 44th week of age has been reported in broiler breeders naturally infected with AAV (Ashraf *et al*., 2000). However, a persistence of only up to 7dpi was observed when the birds were challenged with AAV only and when there was no other stress subjected. These observations indicated that if any stress is subjected to the commercial poultry birds infected with AAV, the persistence could be prolonged.

Moreover, the increased AAV dissemination in various organs also suggested that the dissemination pattern of AAV varies according to the replication period of virus in the birds (Figure 1). More prolonged stress subjected to the poultry in the field, the more organs will be involved in the replication of AAV. Although the virus was detected in various organs during the study, however 100% virus detection in liver of positive samples suggested that liver was the primary site of the virus replication followed by the spleen and kidneys which had 63.64% and 36.36% detection rate, respectively (data not shown). Therefore, the predilection organs of AAV are only liver and spleen (secondary lymphoid) where the agent could survive and replicate more readily than the other lymphoid organs, either primary or secondary. (Naeem *et al*., 2001; Balamurugan and Kataria, 2006; Schonewille *et al*., 2008).

These observations lead to hypothesis that the Avian Adenovirus might be present in the flocks in some latent phase, but the replication of the virus gets triggered to produce the clinical signs whenever there is a stress either by some infectious agent or due to variable extreme environmental conditions as in the developing countries like Pakistan where environmentally controlled sheds are not available readily for the rearing of commercial poultry.

The AAV virus was found to be an immunosuppressive agent as there was marked decrease in the development of HI antibody titers against AIV H9N2 in the groups co-infected with HPS followed by AIV-H9N2 during these experiments. However, a continuous increase in HI antibody titers against AIV, in the groups infected with AIV-H9N2 only, reflects that reactivation of AAV after external stress to the infection carrier birds can cause immunosuppression and increased mortality (Naeem *et al*., 1995; Toro *et al*., 2000). Similar pattern of HI antibody titers was also found by (Miniawy *et al.*, 2014).

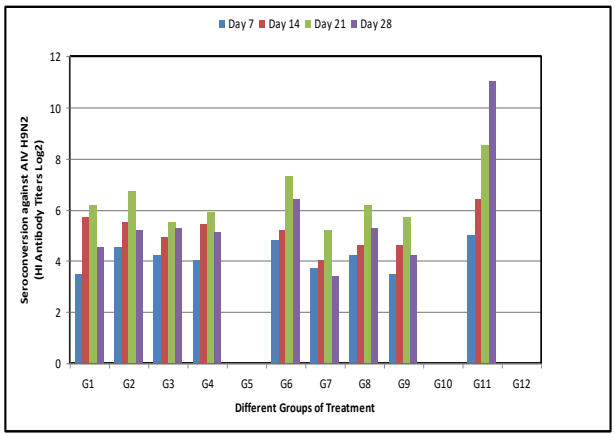

Fig. 3: Seroconversion against AIV-H9N2 detected through HI at different time intervals post infection. Different treatments administered to groups G1-G12 are shown on x-axis. Whereas y-axis indicates HI value of log2 based titer for seroconversion against AIV-H9N2 at different days post AIV infection. Different treatment groups are; G1-G4 were infected through orally and AIV intranasally, G5 was positive AAV control infected orally, G6-G9 were infected AAV via sub cut route and AIV intranasally, G10 was positive AAV control infected subcutaneously, G11 was only given AIV H9 infection intranasally and G12 was non-infected control.

The present study suggested that not only AAV-4 has the capability of infecting lymphoid organs but also the carrier birds of this particular virus show a decreased ability of seroconversion against H9N2 AIV. Immunosuppression was confirmed by the reduced serologic response of infected birds against H9N2.

Conclusions: The present study provided an insight about the role AAV-4 as immunosuppressive agent in broiler chicks. In the absence of secondary infection, AAV is detectable up to 7 days post infection while in the presence of coinfection, AAV could be detectable up to 38 days. This indicates that replication of virus gets triggered whenever it finds some secondary infection like H9N2 AIV. Moreover, the data also described a decrease HI antibody titers against H9N2 AIV in case of co-infection which can cause immunosuppression and high mortality.

Authors contribution: FR, MAA, NS and KN intended and conceived the study. FR and MAA wrote the manuscript. FR, AS, MS and SR edited the manuscript and finalized the data. FR and SR performed the molecular part. FR, SA and FM performed the serological part. NS and SR provided consultation. All authors read and approved the final manuscript.

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