Preparation and Evaluation of Immune Complexes as Vaccine Candidate for Fowl Adenovirus Infection associated with Hydropericardium Syndrome in Domestic Fowl

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ABSTRACT

Immune complex (Icx) vaccine is an antigen-antibody complex which will engage all immunocytes with Fc binding capabilities, enhance early maturation and B memory cell formation. *In vitro* preformed Icx have been shown to be 100 times more efficient in inducing humoral immune response *in vivo* than the native protein antigen. In present study, immune complexes were prepared by mixing varying quantities of HPS virus with different concentrations of egg derived immunoglobulins (IgY). The efficacy of immune complex formulations as vaccine was checked in broiler birds. The Icx vaccinated birds exhibited protection in the range of 53.4% to 66.7% following challenge with virulent HPS virus. Birds vaccinated with 40 and 80 units of IgY with 100 $TCID_{50}$ HPS virus did not shed virus in feces following challenge and induced better antibody response. No significant change in body weight gain was observed between survived birds from vaccinated and control groups. The study showed that immune complex vaccines significantly reduced the mortality and stopped the excretion of the virus in feces thus afforded protection and prevented the horizontal transmission of virus by oral-fecal route.

Keywords: Immune complex vaccine, Poultry, Hydropericardium syndrome, Fowl adenovirus

Hydropericardium syndrome (HPS), primarily a disease of broiler birds of 3-6 weeks of age, is caused by Fowl Adenovirus-4 (Mazaheri *et al.*, 1998; Kataria *et al.*, 2013). Disease has also been reported in 7-day-old broiler birds. The disease is characterized by sudden onset of death, with mortality up to 80% (Shane, 1996; Asthana *et al.*, 2013; Kataria *et al.*, 2013). The accumulation of straw/amber coloured jelly–like fluid in the pericardial sac mainly characterizes the disease. Other predominant lesions include enlarged and discoloured liver with foci of haemorrhage and/or necrosis and enlarged kidneys with distended tubules (Abe *et al.*, 1998; Roy *et al.*, 2004). Transmission of the virus occurs horizontally by oral-fecal route (Akhtar, 1995) and vertically to the offspring (Nagy *et al.*, 2006).

Inactivated vaccines produced in primary cell culture systems have shown promising results against HPS in broilers (Gupta *et al.*, 2005; Asthana *et al.*, 2013, Shah *et al.*, 2017). However, inactivated vaccines have disadvantages like low level of immunity, requirement of additional immunizations, increased dependence on adjuvant and expensive production. Maternal antibodies neutralize the vaccine virus and thus play major role in vaccination failure. Therefore, there is a need for development of safe live virus vaccines which can boost humoral immune response at early period of chick life and at the same time shield the vaccine virus from maternal antibodies. The capacity of immune complexes to augment antibody (Ab) responses is well established. The enhancing effects of immune complexes have been attributed mainly to Fc-mediated adjuvant activity (Hioe

et al., 2009). Immune complex vaccine improve the safety due to very low antigen involved which is complexed with antibody in immune complex vaccine, protective even in the face of maternal antibodies, offer long lasting immunity, lower effective dosing schedule and eliminate use of adjuvants (Haddad *et al.*, 1994; Schat *et al.*, 2011). Immune complex vaccine can be administered to day old chicks or *in ovo*; in absence of maternal antibodies it starts produce immune response by the age chicks susceptible to disease. In presence of maternal antibodies immune complex antibody is able to shield the vaccine virus and release slowly when maternal antibodies decline (Coletti *et al.*, 2001; Chansiripornchai *et al.*, 20119; Mazengia, 2012).

Present research work describes formulation of an immune complex vaccine, using chicken egg derived antibodies, against HPS virus infection in domestic fowl and level of protection it afforded in broiler chicks.

MATERIALS AND METHODS

Virus isolates

A field isolate of FAV4 (HPS-G) (Asthana *et al.*, 2011) was used to hyper immunize layer birds for production of egg yolk antibodies (IgY). Another field isolate of FAV4 (HPS-K) (Asthana *et al.*, 2011) was used for challenge studies. Both isolates were propagated and titrated in chicken embryo liver cell culture and maintained in Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, G.B. Pant University of Agriculture & Technology, Pantnagar.

Egg yolk antibodies (IgY)

IgY were raised against HPS virus in layer birds as per the method described by Rani *et al.* (2012). IgY were purified from eggs laid between days 35-40 post immunization using chicken IgY purification kit (Bangalore Genei, India) as per manufactures recommendations.

IgY thus obtained were titrated by virus neutralization test (beta procedure) as described by Haddad *et al.* (1994). Briefly, serially diluted purified HPS IgY suspension was tested against a fixed dose of HPS virus (100 $TCID_{50}$) which was incubated and residual virus producing cytopathic effects was assayed using Chicken embryo liver cell (CEL) culture. The mean neutralization dose (ND_{50}) of IgY suspension was calculated according to the method of Reed and Muench (1938). One unit of IgY activity was taken as the reciprocal of dilution that protects 50% of the CEL cells from the cytopathic effect of HPS virus.

Preparation of HPS immune complex (HPS-Icx) vaccine

The HPS-Icx vaccine was prepared as per the method of Whitfill *et al.* (1995) with some modifications. HPS-G isolate having a titre of 6.5 $\log_{10}$ $TCID_{50}$/ml was used to prepare HPS-Icx vaccine. The neutralizing antibody titre of purified IgY was 2500 units/50μl, which was suitably diluted in serum free medium to get 80 and 40 units of IgY per 50μl.

In experiment 1, two formulations of each dose (100μl) of the complex vaccine was prepared by mixing 50μl each of 100 $TCID_{50}$/50μl of HPS virus antigen with 40 and 80 units of HPS IgY/50μl.

In experiment 2, two formulations of each dose (100μl) of the complex vaccine was prepared by mixing 50 μl each of 200 $TCID_{50}$/50μl of HPS virus antigen with 40 and 80 units of HPS IgY/50μl.

Vaccine formulations were incubated at room temperature for one hour with gentle shaking every 15 minutes. These formulations were administered to one week old broiler chickens.

Experimental evaluation of HPS-Icx Vaccine

Ninety-day-old broiler chicks were procured from local commercial hatchery and randomly assigned to 6 groups of 15 chicks each. Chicks in Group I and II were vaccinated at one week age with 100 $TCID_{50}$ HPSV with 40 and 80 units of IgY, respectively. Group III and IV chicks were vaccinated at one week age with 200 $TCID_{50}$ HPSV mixed with 40 and 80 units of IgY. Chicks of Group V were unvaccinated, challenged at 21st day of age and served as positive control and Group VI chicks were unvaccinated and unchallenged and served as negative control group.

All 15 birds in group I-V were challenged intramuscularly with 1.0 ml of HPS-K virus isolate (7.5 $\log_{10}$ $TCID_{50}$/ml) at 14 day post vaccination.

All the vaccinated groups were observed for mortality, body weight changes, faecal shedding of virus and level of antibody titers post vaccination at weekly interval. Mortality patterns of different groups were obtained by calculating total no. of birds died in each group post challenge. Body weight changes of birds in different groups following post challenge were obtained by taking body weight of all the birds at weekly interval till the end of experiment. The antibody levels were assessed by indirect ELISA (2003). Fecal samples were collected, with the help of sterilized swabs and then diluted in HBSS (pH 7.2), and stored at -20ºC until tested by sandwich ELISA for the presence of viral antigens.

Detection of viral antigens in fecal samples

HPS virus antigens in fecal samples were detected by sandwich ELISA (Saifuddin and Wilks, 1991). Briefly, Wells of ELISA plate were coated with 100µl of rabbit anti-HPSV sera diluted to 1:400 in coating buffer (carbonate buffer, pH 9.6) and incubated at room temperature for 90 minutes. Coated wells were washed 5 times each with washing buffer for 5 minutes and tapped thoroughly. The unreacted sites of the wells were blocked with 100 µl of 5% skimmed milk powder in PBS-T for 90 minutes at room temperature and washed as mentioned above.

Then test fecal samples, known negative fecal and tissue samples were added in 100µl volume in triplicate. Following incubation at room temperature for 90 minutes the plate was washed and tapped thoroughly. Then chicken anti-HPSV sera was diluted (1:200) and dispensed at 100 µl per well followed by incubation at room temperature for 90 minutes. The plate was washed and anti-chicken HRPO conjugates 1:500 dilution was dispensed @ 100 µl per well and incubated at room temperature for 90 minutes. After washing and tapping, 100µl of freshly prepared substrate solution ortho-phenylenediamine dihydrochloride (OPD) in citrate buffer (pH 4.6) was added and incubated at room temperature for 30 min in dark. The enzyme substrate reaction was stopped by adding 100µl of 1N H_2SO_4 to each well and optical density of substrate reaction was measured spectrophotometrically at 492 nm wavelength in ELISA reader. The results of the ELISA were interpreted by comparing 6 negative controls fecal samples with that of test fecal samples collected from birds survived in the Group I and II. The mean absorbance value and standard deviation (SD) were calculated for negative fecal samples. A test sample was considered positive if the absorbance value was greater than the mean value of the negative fecal samples plus 3 x standard deviation (Saifudin and Wilks, 1991).

RESULTS AND DISCUSSION

Production of IgY and preparation of Immune complexes

In present investigation four white leghorn layer birds were used for production of IgY against a local isolate HPS virus. Use of hens represents both refinement and reduction in animal use for large scale production of polyclonal antibody (Schade *et al.*, 2011) which can be employed for therapeutic and diagnostic purposes (Lemamy *et al.*, 1999). Rani *et al.* (2012) used purified HPS IgY for treatment of experimentally induced HPS in broilers. Purification of IgY from yolk is convenient and simple using different purification methods. IgY purification kits are also available commercially for obtaining high yield and purity.

Virus Neutralization Test was carried out using CEL cells as indicator system to calculate neutralizing antibody titre of IgY and it was observed that IgY was efficient to neutralize the virus *in vitro* and form immune complex which prevent the CEL cells from cytopathic effects of HPS virus. An Immune complex vaccine against HPS virus prepared by mixing cell culture HPS virus antigen with IgY against HPS virus at different formulations. Vaccination was carried out in chicks of 1 week age, so that it can produce immunity by the time birds become susceptible to HPS virus infection at the age of 3-5 weeks. Whitfill *et al.* (1995) in their studies administered IBD immune complex vaccine subcutaneously into nape of day old chicks and also *in ovo* into 18 day old embryos. Gupta *et al.* (2005) reported vaccination with inactivated cell culture vaccine to 10 day old chicks, so as to protect birds from Hydropericardium syndrome when they reached market age.

Experimental evaluation of HPS-Icx Vaccine

Single dose of 80 units of HPS IgY complexed with 100 $TCID_{50}/50\mu l$ *of* HPS virus demonstrated protective

immunity against challenge virus at 14 day post vaccination. There was 66.7% protection (Group II) compared to 53.4% of protection offered by 40 units of IgY/50μl (group I birds). Our findings were in correlation with Whitfill *et al.* (1995) studies on Immune complex vaccine against IBD reported that IBDV immune complex vaccine prepared by 100 EID_{50}/50μl mixed 20-80 units of bursal disease antibody (BDA) showed increase antibody response 3 days post challenge with IBDV (31st day post vaccination). In our study peak antibody response at 1 week post challenge in groups I and II, demonstrated protective immunity against the challenge virus. Kaur *et al.* (1997) showed that birds vaccinated with 1000 $TCID_{50}$/0.2 ml live adenovirus inoculated at one week of age at dose rate of 1ml per chick showed elevated neutralizing antibody titre 1 week post challenge (4th week after vaccination). These findings show the immunogenic response of live virus as a candidate for vaccine development against IBH-HPS.

Clinical signs and mortality

All the birds in vaccinated and unvaccinated groups were active and healthy up to 14 days post vaccination, showing no signs of illness. Following challenge, birds did not show any apparent clinical signs till 24 hours. After that few birds in vaccinated group and most of the birds in unvaccinated challenge group developed greenish-yellow diarrhea, by 36 hours some of the birds in both vaccinated group and unvaccinated challenge group were dull, depressed, debilitated. Some birds exhibited ruffled feathers, pale skin and attained a crouching posture by resting their chest and beak on ground with closed eyelids. Some birds struggled vigorously and died. Clinical signs were more prominent and extensive in birds died on 3rd and 4th day post challenge (d.p.c.). Hydropericardium was not observed in the dead group I and II birds on 3rd d.p.c.

However, 4 out of 8 dead group III birds showed presence of 3-5 ml straw coloured fluid in pericardial sac. Similarly, 5 out of 7 dead group IV and 6 out of 08 group V dead birds showed presence of 6-8 ml pericardial fluid. Birds died on 4th and 5th d.p.c. in groups I-V showed accumulation of nearly 8-10 ml straw coloured fluid having thick consistency in pericardial sac. It was also observed that amount of pericardial fluid was less in vaccinated groups compared to unvaccinated challenge group. In most of the cases epicardium showed congestion and engorgement

of blood vessels. There were no clinical signs in birds survived in both vaccinated groups. All survived birds in unchallenged control groups did not show clinical signs and were healthy throughout the experiment. Livers of all dead birds were friable, swollen with rounded ends and mottled with multifocal necrotic areas. Some birds revealed severe congestion and haemorrhages, while others exhibited pale livers with large areas of necrotic foci. Kidneys in most of dead birds were swollen, congested and hemorrhagic. Intestine in most of the dead birds were congested and haemorrhagic. Spleen in most of the birds showed congestion, necrotic foci and enlargement. Bursa of fabricius was enlarged in most of the birds died; however, no abnormality could be detected on cut surface as there was no evidence of edema or hemorrhages.

Peak mortality was noticed on 3rd d.p.c. and mortality continued up to day 6 post challenge. Mortality rates in group I, II, III, IV and V were 46.6%, 33.3%, 93%, 100% and 93%, respectively. No mortality was recorded in group VI throughout the experiment (Table 1). Hydropericardium syndrome is highly infectious and devastating disease of broiler birds causing high mortality in the age group of 3-5 weeks, resulting in heavy economic losses to poultry industry (Kataria *et al.*, 2013, Asthana *et al.*, 2013).

Table 1: Mortality patterns in different experimental groups following challenge with virulent HPS virus

Days post challenge	Group I	Group II	Group III	Group IV	Group V	Group VI
Day 2	**0**	**0**	**0**	**0**	**0**	**0**
Day 3	*3*	3	7	8	7	0
Day 4	2	2	5	5	5	0
Day 5	1	0	2	2	1	0
Day 6	1	0	0	0	1	0
Total	**7**	**5**	**14**	**15**	**14**	**0**

High mortality in groups III and IV might be because of high amount of virus leading to improper immune complex formation leaving most of the virus in free State. This free virus may be readily metabolized by body so little exposure of antigen to immune system resulting low antibody formation. Immune complex formation to elicit immune response the amount of virus and antibody should be in proper proportion as showed by Pokric *et al.* (1993) that immune complexes generating effective immune

stimulation were prepared at slight antigen excess or at equivalence. High mortality in groups III, IV and V, may be attributed to the high virulence of challenge virus, absence of protection due to low or no antibody titre. Some strains of broiler birds and route of inoculation also affect the pathogenesis of disease. Roy *et al.* (2004) reported that some B1 white strains of broilers were highly susceptible and recorded mortality rates of 97%, 92.3%, where as in colored B2 broilers, mortality rate was low, ranging from 15.7% to 23.3% in 3rd and 5th week broilers. Route that we used was intramuscular which produced high mortality and reduces Incubation period and duration of disease (Abdul-Aziz *et al.*, 1995). Clinical signs, gross lesions and incubation period were similar to that reported by other investigators (Gowda and Satyanaryana, 1994; Roy *et al.*, 2004, Asthana *et al.*, 2013) in experimental and natural HPSV infection. Incubation period of 48-72 hours was reported by Aleiv *et al.* (1997) and duration of disease of 7 day was observed in our study which was in agreement with findings of Rani *et al.* (2012).

Humoral immune response

Humoral immune response of vaccinated birds was determined by indirect ELISA. In group I, chicks showed an immune response with low antibody titre at 7 days post vaccination (d.p.v.), which increased by day 14 and attained peak by 21 d.p.v., a week following challenge. Later, the titre decreased to half of the peak titre on 28 d.p.v. By 35th d.p.v. it reduced to about 20% of the peak titre. In group II, antibody titre showed similar pattern but was slightly lower than that of group I (Fig. 1).
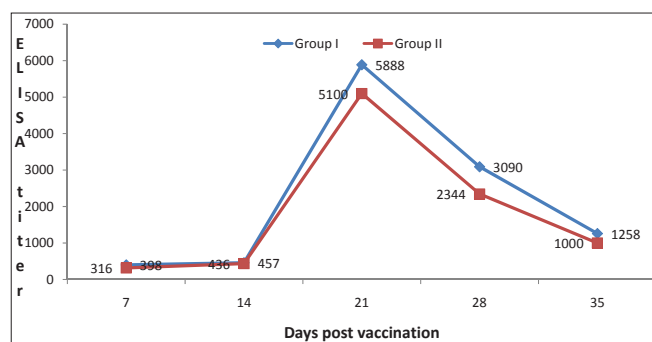

Fig. 1: Antibody titer in Icx vaccinated group I & II birds

Antibody titres in group I chicks were slightly higher throughout the experiment when compared to group II.

There was an increase in antibody titre post vaccination and peak titre was attained a week following challenge infection, which acts as booster to elicit secondary immune response. Gupta *et al.* (2005) reported that peak antibody titre was attained 14 days following post challenge in birds vaccinated with HPS inactivated cell culture vaccine. In comparison with findings of Gupta *et al.* (2005) the immune response in this experiment was attained early and high antibody titre was attained within a week post challenge. These findings suggested assumption that early surge in peak antibody titre following challenge virus may be attributed to presence of B-memory cells and formation of immune complexes by preformed antibodies with challenge virus which help in early selection and maturation of B cells to antibody secreting plasma cells. Marusic *et al.* (1991) suggested that antigen binding into the Immune complex could play an important role in enhancing specific cellular immune responses during the secondary contact with small amount of antigen. Antibodies already present in the circulation of primed animal would rapidly form immune complexes with antigen when it enters the body. This would allow for faster and more efficient binding of antigen to the Antigen presenting cells and consequently stronger stimulation of specific T cells when limiting amounts of antigen enter the body. These findings suggest that there is need for a booster dose required to boost the immune response before challenging with virus. In the same context Klaus (1978) demonstrated the adjuvant effect of antibody in Immune complex is mainly in priming B memory cells and therefore, triggering of memory cells to antibody production would require a further dose of antigen.

Klaus and Humphrey (1986) opined that immune complexes generate memory more rapidly than antigen alone and antibody in the immune complex involves in the generation of B memory cells in germinal centers which are the birth places of B memory cells. Klaus (1978) concluded that *In vitro* preformed Icx have been shown to be 100 times more efficient in inducing humoral immune responses *in vivo* than the native protein antigen. They also demonstrated the adjuvant effect of antibody is manifested priming B memory cells and suggested that triggering of memory cells to antibody production could require a further dose of antigen. From various investigations and this study it is clear that immune complexes induce B memory cells and triggering of which require a booster

dose, which enhances secondary immune response to protect birds from high level of infection.

Detection of viral antigens in fecal samples

All the fecal samples collected from birds of group I and II were screened for presence of viral antigens by sandwich ELISA.

O.D values of group I and II samples ranged between 0.45-0.60 and 0.413-0.609, respectively. O.D values of negative control samples ranged between 0.547-0.658 with mean of 0.614 and standard deviation of 0.03. Test samples were considered positive, if the O.D. value of test sample was more than mean of negative sample plus 3 Standard Deviation. Group I and II yielded O.D values of less than the cut off O.D value for positive sample 0.704. But, one sample collected from the unvaccinated challenge (Group V) on 3rd day post challenge during the peak mortality gave an O.D value of 1.12 which is more than the cut off positive O.D value. These finding indicate that fecal samples of birds from vaccinated Group I and Group II were negative, whereas, unvaccinated birds from group V were positive for presence of HPS virus antigens in feces.

The route of transmission of HPS virus in poultry birds is feco-oral (Akhtar, 1995) and the fecal samples tested from vaccinated Group I & II by sandwich ELISA were negative for presence of HPS virus, which is important from point of view of horizontal transmission of virus. The absence of virus in fecal sample might be due to development of humoral immune response in vaccinated birds of group I and II. Whereas, unvaccinated birds from group V excretes virus in fecal sample from 3rd day post challenge when peak mortality was noticed. Rani *et al.* (2012) noticed similar findings in experimental passive immune therapy using purified IgY against HPS in broilers, detected HPS virus in feces on 3rd day post infection. Kaleta *et al.* (1980) showed that development of neutralizing antibodies coincides with cessation of virus excretion in Egg Drop Syndrome disease. Hwang *et al.* (1980) correlates the cessation of virus excretion with development of humoral and local immunity by appearance of neutralizing antibodies in adenoviral infection.

CONCLUSION

This is the first report that describes preparation of an immune complex vaccine against hydropericardium syndrome in domestic fowl. Present study also showed that egg derived antibodies (IgY) can be used as substitute for serum IgG. Immune complex vaccine significantly reduced the mortality and stopped the excretion of the virus in feces. However, additional studies using maternal antibody-positive chicks in combination with *in-ovo* vaccination along with methods to increase the protection level will be needed to determine if immune complex vaccines will be useful to protect commercial chickens.

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