Indian Journal of Animal Sciences **82** (6): 552–556, June 2012

Therapeutic efficacy of chicken egg yolk immunoglobulins against hydropericardium syndrome in broiler chickens

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Received: 1 September 2010; Accepted: 10 November 2011

ABSTRACT

The aim of the present study was to assess the role of passive immunization using immunoglobulins derived from chicken egg yolk in protection against Hydropericardium syndrome (HPS) in broilers. Immunoglobulins against HPS virus were produced by egg yolk technology. Three methods viz., Dextran sulphate, Polyethylene glycol and Ammonium sulphate method were compared to purify IgY from egg yolk. AGPT and RID were used for confirmation and quantitation of IgY against HPS virus, respectively. ELISA was used to compare IgY titers in serum and yolk samples at different time intervals. IgY titre in yolk at 14, 21, 28 and 56 days PIm was more than that in serum, however peak titer in egg yolk was at 49 days PIm. Administration of IgY to experimentally infected broiler chickens afforded 86.66% protection. These findings demonstrate that IgY obtained from hens immunized by HPS virus could provide an alternative approach for treatment of Hydropericardium syndrome in broilers.

Key words: Egg yolk antibodies, FAV-4, Hydropericardium syndrome, Immunoglobulin therapy

Hydropericardium syndrome (HPS), primarily a disease of broiler chicken was first reported in Angara, Goth, Karachi, Pakistan in 1987 (Khawaja *et al*. 1988). Since then disease established itself in Pakistan and then spread to other parts of world including India (Chandra *et al*. 2000). The sudden onset of the disease, mortality rate as high as 75%, accumulation of clear/amber or straw colored watery or jelly like fluid in the pericardial sac, a condition called hydropericardium in clinically healthy 3–5 week old broiler chickens are some of the salient features of the disease (Kumar *et al*.1997). Although, cell culture and autogenous vaccines are available but either they are not always successful or carry the risk of introduction of avian viruses (Balamurugan and Kataria 2004).

Furthermore, no treatment is available after the disease sets in or broiler flock is exposed to HPS virus. Consequently, there is a need to develop a therapy in the face of outbreak or outbreak is imminent. IgY can be used for prevention and treatment of several infections in poultry like *Salmonella Enterica* serovar Typhimurium, enterotoxigenic *Escherichia coli*, rotavirus (Chalghoumi *et al*. 2009), Newcastle disease virus (Yegani and Krover 2007), avian influenza virus (Rahimi *et al*. 2007) and birnavirus (Malik *et al*. 2006). However, despite being a convenient source of antibodies, there has been no report so far on the use of IgY in the prevention and treatment of hydropericardium syndrome in broilers. Present study describes production of IgY against HPS virus, purification from egg yolk and evaluation of its efficacy as therapeutic agent against HPS virus infection in domestic fowl.

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MATERIALS AND METHODS

Hydropericardium syndrome virus: A field isolate of HPS virus (FAV-4) adapted in chicken embryo liver (CEL) cell culture (Kumar *et al*. 1997) having a titre of 10^8 $TCID_{50}$/ml was used for immunization as well as experimental induction of HPS in broilers.

Experimental birds: Layers (5) and broilers (45) for this study were procured from Poultry farm of Pantnagar university. The layer birds were used for production of IgY against HPS virus while broilers were used to determine the therapeutic efficacy of IgY against HPS infection in birds. These birds were reared in deep litter system during the course of the experiment and provided feed and water *ad lib*.

Table 1. Schedule for immunization of birds for raising IgY against HPS virus (Route of administration-subcutaneous)

Day of inoculation	Immunogen	Dose/bird (ml)
0	Heat inactivated virus	0.1
2	Live HPS virus + FCA	0.2
3	Live HPS virus + FIA	0.3
4	Live HPS virus + FIA	0.4
5	Live HPS virus + FIA	0.5
6	Live HPS virus + FIA	0.6
7	Live HPS virus + FIA	0.7
8	Live HPS virus + FIA	0.8
9	Live HPS virus + FIA	0.9
10	Live HPS virus + FIA	1.0
17	Live HPS virus + FIA	0.5
24	Live HPS virus + FIA	0.5
51	Live HPS virus + FIA	0.5

FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant.

Production of IgY against HPS virus: IgY was raised against HPS virus as per the Malik *et al*. (2006) with slight modifications. Before injecting the birds with HPS virus antigen, the blood was collected from all the 5 birds for collection of serum. Initially, birds were first injected with 0.1 ml of heat inactivated virus via subcutaneous route and then hyper-immunized by injecting antigen emulsified in equal volume of Freund's incomplete adjuvant and complete adjuvant by subcutaneous route as per the schedule given in Table 1.

Collection of blood: The blood was collected at 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, and 70 day post-immunization (pIm), serum was separated and stored at –20°C until used.

Collection of eggs and purification of IgY: Eggs were collected daily from day 1 to day 70 and stored at 4°C till used for further processing. For purification of IgY from chicken egg yolk 3 methods, viz. polyethylene glycol method (PEG) (Rahimi *et al*. 2007), dextran sulphate method and ammonium sulphate (Ams) method (Malik *et al*. 2006) were used and on the basis of purity and yield; best method was selected for further purification of IgY from pooled yolks to be used in passive immunotherapy in this study.

Protein concentration of purified IgY suspensions was estimated by spectrophotometric method (Warberg and Christian 1942).

Purity of purified IgY samples was determined by SDS-PAGE as per Ko and Ahn (2007).

Agar gel precipitation test (AGPT): Agar gel precipitation test was performed according to Malik *et al*. (2006) to detect the presence of precipitating antibodies in serum, egg yolk and purified IgY samples.

Radial immuno-diffusion (RID) test: Radial immuno diffusion test was performed according to Akita and Nakai (1992) to quantify the IgY in purified IgY suspensions obtained from 3 purification procedures. A standard curve was obtained by plotting the diameter square (d^2) of the precipitation rings against known concentrations (0.2 mg/ml to 25.6 mg/ml) of pure IgY. Samples obtained from 3 purification methods were diluted to 1:4 for RID analysis and IgY concentrations were determined by reference to standard curve using the regression equation.

Detection of antibodies in serum and yolk: Antibody titres in egg yolk and serum collected at day 7, 14, 21, 28, 35, 42, 49, 56, 63 and day 70 were determined by indirect ELISA. Known negative controls were also included with test sample. All samples were tested in triplicates.

Indirect ELISA was done as per Choi *et al*. (2010) with modifications. The plate was first coated with 100 ml of semi-purified antigen diluted with 1:100 in coating buffer and incubated at 4°C overnight. coated plates were washed 3 times each with washing buffer for 5 min, wells were blocked with 100 µl blocking buffer (2% BSA in PBS- T) per well and incubated at 37°C for 1 h and then washed 3 times. After washing, test samples diluted with 1:100 were added in 100 µl volumes in triplicates. The plates were then incubated at 37°C for 2 h. Following incubation the plate was washed 3 times with PBS-T. The rabbit anti chicken HRPO conjugate (1:5000) was added @100 µl/well and incubated for 120 min at 37°C. After 3 times washing 100 µl OPD (orthophenylene diamine) substrate was added and incubated for 30 min at room temperature. The reaction was stopped by $5NH_2SO_4$ solution to each well and optical density of substrate reaction was measured spectrophotometrically at 492 nm wave length in an ELISA reader. The absorbance values were determined for serum and yolk. The mean absorbance values were calculated.

Therapeutic efficacy of IgY against hydro pericardium syndrome (HPS): Broilers (45) 3-wk-old without detectable anti-HPS antibodies were allocated into 3 groups. Each bird in group 1 was administered orally with HPS virus infected cell culture supernatant (10^8 $TCID_{50}$/ml) @2.0 ml HPS antigen/bird. After 12 h passive immunotherapy (PI) each bird in group 1 injected subcutaneously with 15 ml of IgY suspension purified by dextran suphate method (5ml/bird in 2 divided doses at 12 h interval on 3 consecutive days). The second group of 15 birds (group 2) was inoculated similarly with HPS virus (2.0 ml HPS Ag/bird) but received no IgY. The third (untreated control) group of 15 birds (group 3) received neither IgY nor HPS virus. Following inoculation of HPS virus, 3 birds were sacrificed and sampled from each group (feacal sample, liver tissue, caecal tonsils) at 3, 7, 14, 21 and 28 days post infection. Each bird was examined for body weight, clinical signs, gross and post-mortem lesions. Data were analyzed by ANOVA.

RESULTS AND DISCUSSION

Purification of IgY from egg yolk: IgY purified by 3 methods showed precipitin lines against the HPS virus

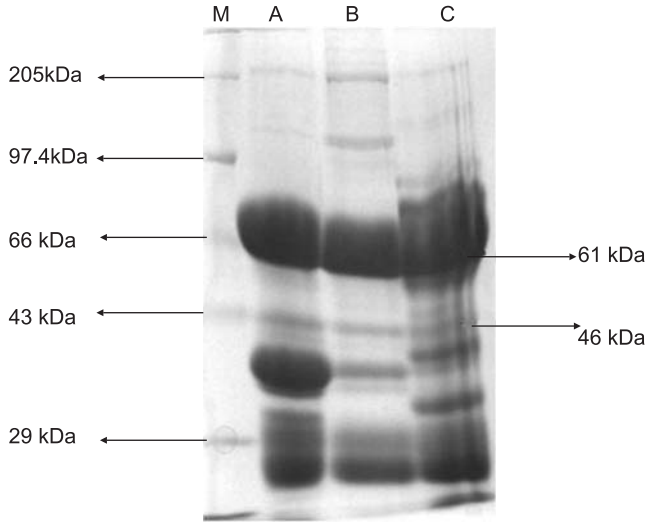

Fig. 1. SDS-PAGE analysis of purified IgY samples showing bands heavy (61 kDa) and light chain (46 kDa) M, Marker; A, Dextran sulphate; B, Ammonium sulphate; C, polyethylene glycol.

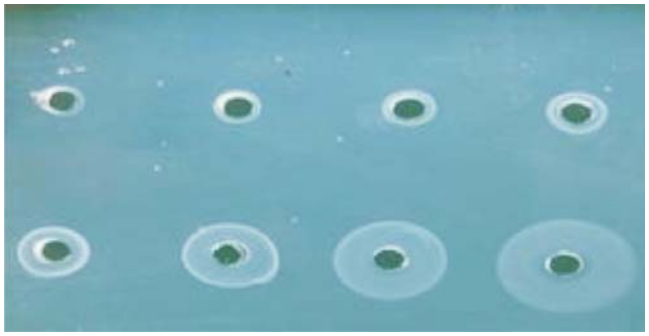

Fig. 2. Different concentrations of standard IgY showing concentric rings in radial immunodiffusion.

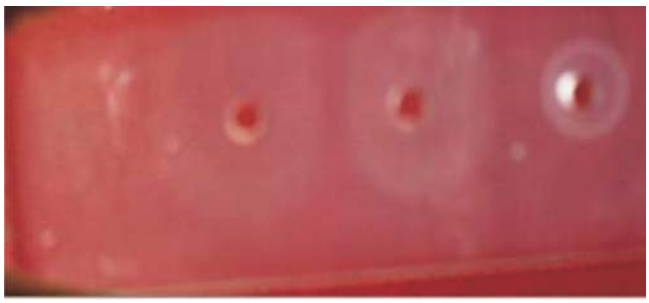

Fig. 3. Purified IgY samples showing concentric rings in radial immunodiffusion (From left to right dextran sulphate, Ammonium sulphate, polyethylene glycol).

antigen in agar gel precipitation test within 24 h of incubation at 37°C. Similarly, Rabbit antichicken IgY gave single bands with standard IgY and IgY purified by 3 different methods. Further, Radial Immunodiffusion (RID) was done to quantify IgY in preparations obtained from three purification methods (Figs 2, 3). IgY yield, total protein content and % purity of IgY from all the 3 methods are depicted in Table 2. SDS-PAGE analysis of purified IgY suspensions 2 major protein bands with molecular weights of 46 kDa (light chain) and 61 kDa (heavy chain), and few less intense bands (Fig. 1). These results indicated that the dextran sulphate method gave highest total protein content, less number of contaminating proteins and highest IgY concentration, therefore, this method was used for further purification of IgY from pooled egg yolk samples for use in passive immunotherapy against infectious hydropericardium in broilers. Garcia *et al*. (2005) reported that the greatest concentrations of immunoglobulins were recovered by dextran sulphate method, producing 4.6 mg of IgY/ml of initial egg yolk. Malik *et al.* (2006) compared eight extraction methods of IgY and demonstrated that dextran sulphate method was most effective, quick and simple for antibody purification. We estimated that 1 ml of the egg yolk (15 ml per egg) contains about 5 mg of IgY. A hen lays about 250 eggs (about 4 000 ml of egg yolk) in a year; thus, the eggs laid by an immunized hen in a year would yield 20 g of IgY.

Table 2. Characteristics of IgY suspensions obtained from different purification methods

Purification method	Protein (mg/ml of egg yolk)	IgY (mg/ml of egg yolk)	% purity
Dextran sulphate	6.07	5	82.37
Ammonium sulphate	5.12	4.38	85.54
Polyethylene glycol	2.21	1.44	65.01

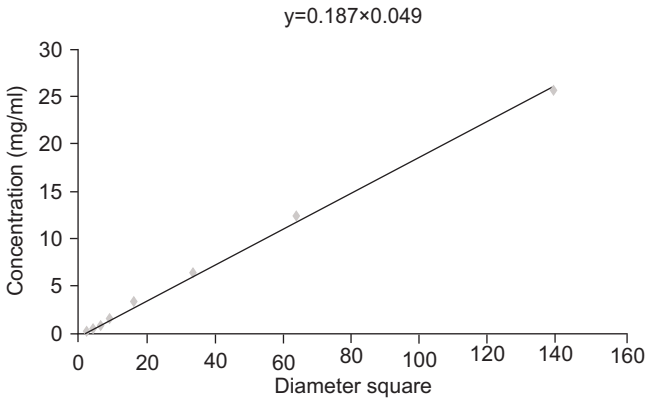

Fig. 4. Graph showing standard IgY concentrations and square of diameter of rings

Comparison of IgY titres in Serum and yolk by ELISA: The IgY titres in serum and egg yolk at different time intervals were determined by enzyme linked immunosorbant assay (ELISA) (Fig. 5). The concentration in yolk was maximum on day 49 PIm but exceeded to that in serum on 14, 21, 28 and 56 day PIm. However, the antibody concentration in serum was maximum on day 63 PIm. Similarly, Malik *et al*. (2006) reported higher concentration of IgY in yolk compared to serum on day 7 and 28 day PIm. However, lower titres have been reported in eggs of chickens immunized with the

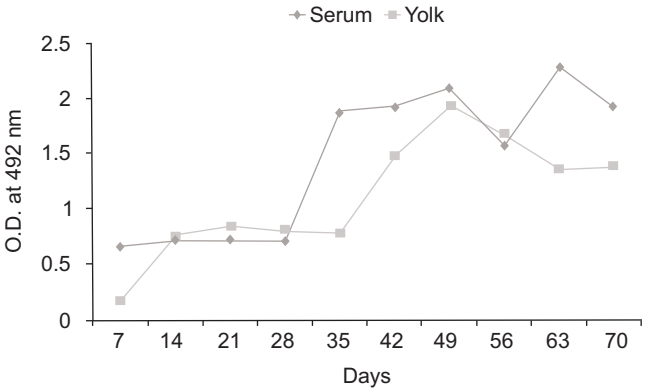

Fig 5. IgY titers at different time intervals in serum and yolk determined by ELISA

Newcastle disease virus (Piela *et al*. 1984). These differences may be due to immunological differences between the viruses as well as to the use of different immunization schemes, different breeds of hens and different assays employed (Bizhanov *et al*. 2000). Other reports have noted high specific immune response over 3–15 months in the egg yolk from hens inoculated with various antigens (Akita and Nakai 1993).

Therapeutic efficacy of IgY against hydropericardium syndrome in broilers: Therapeutic administration of IgY preparation in broilers infected with HPS virus was evaluated. All birds of group 2 were dull, depressed, off-fed, having greenish-yellow diarrohea, and huddling at the corner at 3 day of post- infection. Similar signs were observed at day 7. However all clinical symptoms were disappeared at day 14 and no clinical symptoms were observed in group 1. Mortality in group 1 and 2 started at day 3 and continued up to day 4 PI. Neither mortality nor clinical symptoms were observed in control birds of group 3. Mortality in treatment group (group 1) was 13.33% while in infection group (group 2) it was 66.66%, which proves that IgY administration has caused significant decrease in mortality. Malik *et al*. (2006) used IgY for passive immunization against infectious bursal disease (IBD) and reported a maximum recovery of 92%, they further opined that remaining 8% did not exhibit any recovery because of highly decreased level of their own antibody and amount of injected antibodies was unable to cure them. El-Ghany (2011) concluded that both the IBDV vaccine and polyclonal IgY are relatively equally effective in prevention of IBDV infection in broiler chickens.

The development of lesions following exposure to HPS-antigen was stopped/delayed by passive immunization with anti-HPS IgY. Lesions developed rapidly in the birds without passive immunization (group 2). At the third day PI lesions included pale yellow liver with large areas of necrotic patches and petechial haemorrhages. Spleen was congested with necrotic foci and kidneys were severely congested with haemorrhages and necrotic foci. Lungs were severely congested. Accumulation of clear watery/jelly- like/amber colored fluid in the pericardial sac (fluid varying from 1–4 ml) was also recorded. Bursa of fabricius was enlarged and intestine was congested and haemorrhagic. Some birds showed extensive haemorrhages on thigh muscles. Lesions were extensive in 9 birds that died at day third PI. Similar lesions were present at day 7 PI. However, no gross lesions were detectable until the day third PI in treatment group birds but at day 4 PI the lesions were observed. After day 7, PI similar lesions were observed in 3 birds of group 1 but group 2 birds showed no gross lesions. After day 14, PI 1 bird of group 1 showed haemorrhages on skeletal muscle and swollen kidneys. At day 21 and 28 PI no lesions were recorded. The control birds (group 3) remained free of any detectable HPS lesions throughout the experiment. Only 2 birds died in group 1 showed lesions associated with HPS but severity of lesions was less as compared birds died in group 2. Kermani-Arab *et al*. (1976) studied the effects of IgY antibody on the development of Marek's Disease (MD) and concluded that passive immunization with IgY delayed development of viremia and MD lesions. Body weight is an important economic criterion for broilers and significant reduction in body weights of birds of infection group in comparison to control group shows that IgY treatment has prevented loss in body weight in treatment group, however, it was nonsignificant in comparison to infection group (Table 3).

Present study demonstrates that IgY prepared from the egg yolk of hens immunized with hydropericardium syndrome virus (FAV-4) is effective in the treatment of hydropericardium syndrome in broilers. HPS is a disease of broilers causing heavy mortality and occurs in the most countries of world having broiler population. Treatment of hydropericardium syndrome by administration of active antibodies specific to hydropericardium syndrome virus may have merit, due to reduction in mortality during outbreak, prevention of spread of infection leading to reduced economic losses. It is now well established that egg yolk from an immunized hen has an antibody capable of specific recognition in an abundant quantity and is, therefore, economical. To the best of our knowledge this is first study

Table 3. Effect of HPS virus infection and passive-immunization on body weight of broilers

Day	Group 1	Group 2	Group 3
0	200.67±10.44	176.33±8.41	185.33±11.54
3	234.55±16.51	174.00±5.796[a]	265.00±13.685[b]
7	244.55±14.03	188.00±6.811[a]	281.67±17.917[b]
14	285.00±13.964	270.00±12.910	282.22±10.78
21	478.00±33.40	-	498.33±39.07
28	500.00±5.164	-	570.00±39.07

Means bearing different superscripts differs significantly (P< 0.01).

reporting use of IgY for passive-immunization against hydropericardium syndrome in domestic fowl.

ACKNOWLEDGEMENTS

The author wish to thank the Dean, College of Veterinary and Animal Sciences and Director, Experimentation Station, G.B. Pant University of Agriculture and Technology, Pantnagar, India for providing necessary facilities.

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