

In vitro* Studies of Chicken Egg Yolk Antibodies Generated against *Salmonella pullorum

¹T. Diraviyam, ¹S. Elakkiya, ¹T. Subbraj, ¹S. Avanthi, ²S. Meenatchisundaram and ¹A. Michael

¹Department of Microbiology, PSG College of Arts and Science, Coimbatore, India

²Department of Biosciences, Nehru Arts and Science College, Coimbatore, India

Abstract: The present investigation is focused to generate chicken Egg yolk antibodies against *Salmonella pullorum* and their *in-vitro* characterization. *Pullorum* disease is leading cause of morbidity and mortality in poultry and highly responsible for significant economic loss. Mortality in such outbreaks may approach 90% if untreated. Treatment primarily is a salvage operation and does not prevent from becoming a carrier. Therefore, the prevention of this disease in breeder level through vaccination is more convenient for the control of vertical transmission. One of the recent researches has revealed that the combination of IBDV vaccine and chicken IgY generated against IBDV was superior in preventing IBDV infection in Broiler chickens rather than using them alone. Based on this recent finding, the chicken egg yolk antibody (IgY) raised against *Salmonella pullorum*. IgY antibodies were purified by (Polson *et al.*, 1980) method and Water dilution method followed by DEAE cellulose ion exchange column chromatography. The total IgY concentration was relatively constant, average IgY concentration was 6.62 mg/mL during the immunization period. Titre of IgY antibodies was 1:10000 on 120th day after first immunization determined by ELISA. The agglutination was observed in both Rapid Slide Agglutination and Micro-titre plate (up to 1:2048 dilutions). It indicated the presence of IgY against *S. pullorum*. Present study concluded that the generated IgY was specific against *S. pullorum* whole cell antigen and it could effectively bind with that. The raised antibodies could be used for the passive immunotherapy to protect the young chicks from horizontal transmission of *Pullorum* disease by improving the immunological strength against infectious disease.

Key words: ELISA, IgY, microagglutination, *Pullorum* disease

INTRODUCTION

Poultry meat is an important food product and the broiler chicken related industry is an economically important component of the agro industry. The poultry meat industry all over the world is growing fastest among all the meat categories (Chalghoumi *et al.*, 2009). The industry however is under pressure and faces many challenges because of infectious diseases. *Salmonella enterica* causes a number of significant poultry diseases and is also a major human pathogen. Younger birds are more susceptible to infection by *Salmonella* spp. In particular *Pullorum* disease affects only young birds, which is a leading cause of morbidity and mortality in poultry and highly responsible for significant economic loss. Most acute outbreaks occur in birds are under three weeks of age. Mortality in such outbreaks may approach 90% if untreated. Treatment primarily is a salvage operation and does not prevent from becoming a carrier. Consequently, do not keep recovered flocks; the usage of antibiotics is limited.

Therefore, the prevention of this disease in breeder level through vaccination is more convenient for the control of transmission. Because, the serum immunoglobulins are readily transferred from hen serum to the yolk of egg, then the chick embryos absorbs maternal antibodies while development. These maternal antibodies protect chicks from disease until they disappear. If alteration in such temporary immunological protection through maternal immunoglobulins such as IgG, IgM and IgA accompanies with the infection in chicks, results in immune suppression (Sander *et al.*, 1998) and also the vaccination strategy cannot be applied in broilers due to the short life span of the bird, so there is a need for an alternative way to combat with the pathogens. One of the recent study revealed that the combination of IBDV vaccine and chicken IgY generated against IBDV was superior in preventing IBDV infection in Broiler chickens rather than using them alone (Wafaa and El-ghany, 2011). Hence, the oral administration of chicken IgY has drawn attention of researchers. The present investigation is focused to generate and

characterize the chicken antibodies against *Salmonella pullorum*. This could be used for therapeutic and diagnostic purpose to protect the broiler chicks from the *Pullorum* disease.

MATERIALS AND METHODS

The present research study was carried out during the period of November 2010 to May 2011 in the Department of Microbiology, PSG College of Arts and Science, Coimbatore, Tamil Nadu, India.

Bacterial antigen: *Salmonella pullorum* (killed) plain antigen as a suspension in phenol saline and *Salmonella pullorum* coloured antigen (in solution containing 1% formalin, 1% KH₂PO₄ and 0.8% NaCl) were obtained from the Division of Biological Products, Indian Veterinary Research Institute (IVRI), Izatnagr, India. The killed *Salmonella pullorum* plain antigen in Phenol saline was washed with saline by centrifugation at 10,000 rpm for 15 min and the concentration was adjusted to OD equivalent to McFarland's Standard No.2 (which is corresponding to 3×10⁶ cells/mL). Then the cell suspension was aliquoted and stored at -70°C for further use.

Immunization of hens: 21 weeks old white Leghorn Hens were immunized to obtain *Salmonella pullorum* specific IgY antibody from eggs. 1 mL of antigen (0.5 mL of whole cells suspension (3×10⁶ cells/mL) with an equal volume of Freund's complete adjuvant) was injected into the pectoral muscle at multiples sites. Booster doses were given at 2wk and 4th wk (Sunwoo *et al.*, 1996). The presence of specific IgY against *S. pullorum* in chicken serum was assessed by slide agglutination method at specified time interval. Eggs were collected and stored at 4°C for further use.

Purification of IgY:

Purification by Polson *et al.* (1980) method: The antibodies were extracted from egg yolk by the method of Polson *et al.* (1980) using Polyethylene and Ammonium sulphate precipitate method. Then the content was desalted by dialysis process. The crude fraction of IgY thus obtained was further purified by DEAE cellulose ion exchange column chromatography. The protein content of the purified IgY fraction was determined by the method described by Lowry *et al.* (1951). The purity of Chicken egg yolk antibodies was checked by SDS-PAGE.

Purification by water dilution method (Akita and Nakai, 1992): The egg yolk was separated from immunized egg and mixed with 8 volumes of cold distilled water (acidified with 0.1 N HCl to a pH of 4.0) and then final dilution was made up to 1: 10 using cold,

acidified, distilled water (pH2.0). After mixing well, the mixture was adjusted to pH 5.0 to 5.2 and incubated at 4°C for 12 h. The water soluble fraction) WSF was obtained by centrifugation at 4,500 rpm at 4°C for 20 min and lyophilized. The IgY powder was stored under - 20°C and used for further studies. The protein content and purity of the WSF was determined as mentioned above.

Determination of antibody titer by ELISA: *Salmonella pullorum*-IgY titre was determined by indirect ELISA described by Voller *et al.* (1978). Whole cell antigen suspension of *S. pullorum* was coated in the wells using 0.05 M carbonatebicarbonate buffer (pH 9.6) and incubated at 4°C for overnight. After incubation the plate was washed with PBST then the empty sites were blocked by 1% BSA (200 µL/well) and incubated at 37°C for 1 hour. Plates were subsequently washed and incubated with IgY solution (100 µL/well). Finally 100 µL of diluted (1:1000) rabbit anti-chicken immunoglobulin conjugated with HRP (Genei Pvt. Ltd., Bangalore) was added and incubated. Then the plates were washed and 100 µL of fresh substrate was added and the plates were allowed to stand at room temperature in dark for 20 min. The reaction was stopped by adding 50 µL of 4N H₂SO₄ and plates were read at 490 nm in an ELISA reader. All samples were tested in triplicates (Sunwoo *et al.*, 2000; Lee *et al.*, 2002).

IgY specificity determination: The specificity of *S. pullorum*-IgY was determined by Rapid Slide Agglutination (RSA) and micro-titre plate agglutination method. Rapid slide agglutination test was done on a plastic strip, 20 µL of coloured antigen and 20 µL of *S. pullorum*-IgY were placed and mixed thoroughly by stirring with the help of applicator stick. Then the slide was observed for the appearance of agglutination within 2minutes (Muktaruzzaman *et al.*, 2010).

For microagglutination, the bacteria cell suspension in Phenol saline (IVRI, Izatnagar) was adjusted to a desired optical density 0.42 at 620 nm. 10% antibody solutions (specific and non-specific) were prepared by reconstituting 0.1 g of freeze-dried antibody powder in 0.9ml of sterile saline. *Salmonella pullorum* positive serum (IVRI, Izatnagar) was used as positive control. In a micro-titre plate three rows were assigned for the test, 0.08ml of sterile saline was added in the first well of each row and 0.05 mL was added in the remaining wells of each rows. Then 0.02 mL of *S. pullorum* positive serum was added in the first well of the first row (positive control) and doubling dilution was done by transferring 0.05 mL from the first well to second well, second to third and so on. The same procedures was repeated in the second row (test) and third row (positive control) but except positive serum, 0.02ml of 10% *S. pullorum*-antibody solution and non specific antibody solution were used respectively. Finally 0.05 mL of bacterial cell

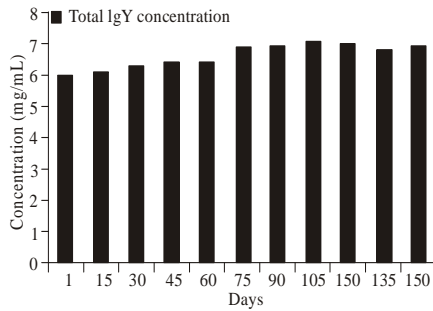


Fig. 1: Total IgY concentration in immunized chicken egg yolk. it was relatively constant during the immunisation period

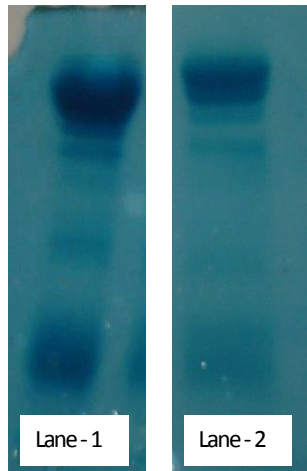


Fig. 2: SDS-PAGE profile for IgY, A single protein band of high molecular weight (180 KDa) was observed. Lane -1: IgY fraction purified by Water Dilution; Lane -2: IgY fraction purified by PEG method

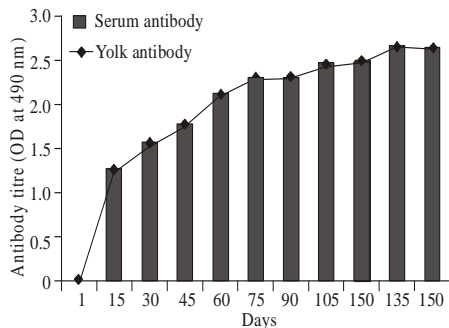


Fig. 3: Kinetics of specific IgY against *S. pullorum* in immunized chicken egg yolk estimated by ELISA. There was a gradual increase in antibody titre with subsequent booster dose administration

suspension was added to all the wells of each rows and incubated at 25°C for overnight. After incubation, plate was observed the titre was defined as the highest dilution

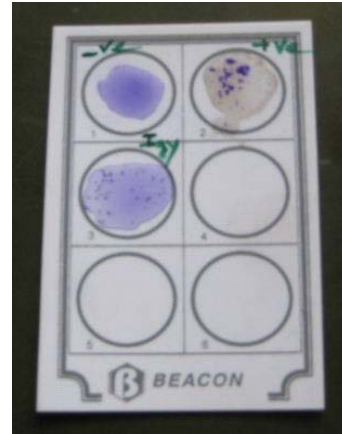


Fig. 4: Rapid slide agglutination test, clumping was appeared for IgY corresponding to positive control

of *S. pullorum*-antibody powder showing agglutination by comparing with positive and negative controls.

RESULTS

IgY from immunized eggs were purified by both PEG method (Polson *et al.*, 1980) and Water dilution method. The water soluble fraction obtained by PEG method was further purified by Iron Exchange Chromatography and then concentrated by lyophilization. IgY fractions of each method were subjected to protein estimation to detect antibody concentration. The concentration of antibodies increased in the egg yolk was relatively constant during immunization period, and the average yield of IgY was 6.62 mg/mL (Fig. 1).

The quantity of antibody yield was higher in the water dilution method when compare to PEG method. The molecular weight of the purified IgY's were confirmed as 180KDa through SDS PAGE (Laemmli, 1970). The electrophoretic band pattern obtained in this study was revealed that the purity of IgY was higher in IgY fraction purified by (Polson *et al.*, 1980) method when compare to water dilution method (Fig. 2). The titre of antibodies in the immunized chicken egg yolk was determined by ELISA, thus indicated that the specific IgY concentration in the egg yolk was significantly increased when the chickens received booster doses at regular intervals. High peak titre of 1:10000 was observed during 120th day of observation (Fig. 3).

The specificity of Anti-*S. pullorum* IgY was evaluated by Rapid slide agglutination and micro-titre plate agglutination method. The presence of clumping in the test sample is similar to that of positive control indicated the agglutination reaction (Fig. 4). It indicated the presence of specific IgY antibody against *S. pullorum*. The agglutination was observed in micro-titre plate up to 1:2048 dilution by comparing with positive and negative controls.

DISCUSSION

In India, poultry is the fastest growing segment at over 15% per annum, compared to the overall meat industry growth rate of 5% per annum. The broiler industry is well dominated in southern states in India with nearly 60-70% of total output coming from these states. But during the last few years there is an increase in pathogen loads in poultry farms due to excessive stress on the birds. This stress could be production stress or climatic stress which affects directly or indirectly on immune system of the bird and once immunity goes down birds easily acquire infection. In recent years *Salmonella* has drawn attention of people by emerging as a most serious threat for the poultry due to their wide spreading nature, various sources of contamination and developing resistance against most of the antibiotics. Hence, they are not only unsafe for human consumption but also cause huge economic loss to the farmers. As per EU legislation, from 12/12/2010 if *Salmonella* is detected in fresh poultry meat on the market, the broiler industry needs to take measures to decrease the colonization of the animals and their environment (Chalghoumi *et al.*, 2009). These include pre-harvest, harvest and post-harvest measures. *Salmonella enterica* causes a number of significant poultry diseases and is also a major human pathogen. Particularly younger birds are more susceptible to infection by *Salmonella* spp such as *S. pullorum* and *S. gallinarum*. *Pullorum* disease in young birds is one of the leading cause of morbidity and mortality in poultry and highly responsible for significant economic loss in all the phases of poultry industry from production to marketing. Many research studies are going on globally to control the infection and disease in poultry, in which a recent study by Wafaa and El-ghany (2011) revealed that the combination of IBDV vaccine and chicken IgY generated against IBDV was superior in preventing IBDV infection in Broiler chickens rather than using them alone. Hence, the oral administration of chicken IgY has drawn attention of this present research. Therefore, the present investigation is focused to generate chicken antibodies against *Salmonella pullorum* to inhibit its shedding in broiler chickens by passive immunization using chicken IgY as feed additives.

CONCLUSION

Present study concluded that the generated IgY was specific against *S. pullorum* whole cell antigen and it could effectively bind with that. It was determined by ELISA, Rapid slide agglutination test and micro-agglutination test. Thus, it may suggest that binding activity of IgY with surface molecules of *S. pullorum* could inhibit its growth. In addition, the generation of

antibodies in the chicken is an economic and feasible alternative way to obtain large quantity of polyclonal antibodies than from a similar sized mammal. As a whole, IgY against enteric pathogen could be used for passive immunization in poultry. But, further research is needed for the assessment of stability of IgY in the gastric environment of the birds intestinal tract to emphasis the application of chicken IgY as feed additives in broilers production. It could help the future generation to use chicken IgY as an effective therapeutic agent, by which the poultry industry can provide safe and healthy poultry meat, which will be free from pathogenic microbial contamination.

REFERENCES

- Akita, E.M. and S. Nakai, 1992. Immunoglobulins from Egg Yolk: Isolation and purification. *J. Food Sci.*, 57: 629-634.
- Chalghoumi, R., C. Marcq, A. Thewis, D. Portetelle and Y. Beckers, 2009. Hen egg yolk antibodies (IgY), production and use for passive immunization against bacterial enteric infections in chicken: A review. *Poultry Sci.*, 88: 2081-2092.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680-185.
- Lee, E.N., H.H. Sunwoo, K. Menninen and J.S. Sim, 2002. In Vitro Studies of Chicken Egg Yolk Antibody (IgY) Against *Salmonella enteritidis* and *Salmonella typhimurium*. *Poult. Sci.*, 81: 632-641.
- Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the folin-phenol reagent. *J. Biol. Chem.*, 193: 265-275.
- Muktaruzzaman, M., M.G. Haider, A.K.M. Ahmed, K.J. Alam, M.M. Rahman, M.B. Khatun, M.H. Rahman and M.M. Hossain, 2010. Validation and Refinement of *Salmonella pullorum* (SP) Colored Antigen for Diagnosis of *Salmonella* Infections in the Field. *Int. J. Poultry Sci.*, 9(8): 801-808.
- Polson, A., M.B. Von Wechmar and M.H.V.V. Regenmortel, 1980. Isolation of viral IgY antibodies from yolks of immunized hens. *Immunol. Commun.*, 9: 475-493.
- Sander, J.E., E.M. Willingham, J.L. Wilson and S.G. Thayer, 1998. The effect of inoculating *Enterococcus faecalis* into the yolk sac on chick quality and maternal antibody absorption. *Avian Dis.*, 42: 359-363.
- Sunwoo, H.H., T. Nakano, W.T. Dixon and J.S. Sim, 1996. Immune Responses in Chickens Against Lipopolysaccharide of *Escherichia coli* and *Salmonella typhimurium*. *Poultry Sci.*, 75: 342-345.

- Sunwoo, H.H., X. Li, E.N. Lee, Y.K. Kim and S.J. Sim, 2000. Preparation of Antigen-Specific IgY for Food Application. In: Sim, J.S., S. Nakai and W. Guenter, (Eds.), *Egg Nutrition and Biotechnology*. CAB International, New York, pp: 311-322.
- Voller, B.A. and D.E. Bidwell, 1978. Enzyme immunoassays with special reference to ELISA techniques. *J. Clin. Pathol.*, 31: 507-520.
- Wafaa, A. and A. El-ghany, 2011. Comparison between immunoglobulin IgY and vaccine for prevention of infectious bursal diseases in chickens. *Global Vet.*, 6(1): 6-24.