Preparation of Chicken (IgY) Antibodies Consortium for the Prevention of Enteric Infections in Poultry

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ABSTRACT

Enteric diseases are an important concern to the poultry industry because loss of productivity, increased mortality, and the associated contamination of poultry products for human consumption (human food safety). With concerns about antibiotic resistance, there is increasing interests in finding alternatives to antibiotics for poultry production. Among the candidate replacements for antibiotics through nutritional strategies, passive oral administration of hen egg yolk antibody (IgY) is an emerging and promising nutritional strategy to control infections in broiler chicken industry. Hence, the present study was focused to develop chicken egg yolk antibodies against E. coli, and Salmonella enterica and prepare antibody consortium. White leghorn chickens were immunized with E. coli and S. enterica whole cell antigens, then the egg yolk antibodies were purified from immunized egg by Polson et al., (1980) method and DEAE cellulose ion exchange chromatography. The molecular weight of the purified antibodies was confirmed through SDS PAGE. The titre of egg yolk antibodies was determined by ELISA to confirm the presence of specific antibodies. Titre of IgY antibodies was 1:10000 after third booster dose. The binding activity of antibodies was determined by Growth Inhibition Assay; the growth rate in the presence of IgY fraction was decreased, the binding of specific antibodies with the bacterial cells may be a reason for less motility and opportunity to take nutrients and to proliferate than a free-motile single bacterial cell. The purified chicken antibodies can be used to prepare consortium for passive immunization to protect the young chicks from enteric infections. Further studies have to be conducted to evaluate the potency of these antibodies in chickens. The outcome of this research work would be an alternative to the current antibiotic treatments.

Key words: IgY consortium, Enteric infection, Poultry, growth inhibition assay.

INTRODUCTION

Poultry is one of the fastest growing segments of the agriculture sector. Poultry meat is an important food product. Most importantly, there is a demand for meat of high nutritional value and free of microbiological and chemical hazards. Poultry meat production by major producing
countries is forecast to increase nearly 4% in 2006 to 65.6 million tons. With significant growth in Brazil, China, India and the United States as well as a rebound in production in Asia, poultry production continues to expand worldwide.

India is the fifth largest producer of eggs and ninth largest producer of poultry meat in the world, producing over 34 billion eggs and about 600,000 tons of poultry meat in 2004. In overall market for poultry products, India was pointed 17 in poultry production. And Analysts estimated that the poultry sector in India has been growing at a much faster rate, along with other industries such as BPO and Securities market. Over the past decade the poultry industry in India has contributed approximately US $ 229 million, to the Gross National Product. But during the last few years there is also 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 *E.coli*, *Salmonella* and *Campylobacter* have 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. They are not only unsafe for human consumption but also cause huge economic loss to the farmers. About 30% of chicken products carry live *Salmonella*, and 60-80% of chickens carry *Campylobacter*.

Since the development of antibiotic resistance strains limited the therapeutic antibiotic usage. The use of feed additives is more and more accepted as a valuable way to combat these bacterial infections in broilers production. With concerns about antibiotic resistance, there is increasing interest in finding alternatives to antibiotics for poultry production [1]. Among the candidate replacements for antibiotics through nutritional strategies, passive immunization by oral administration of hen egg yolk antibody (IgY) is an emerging and promising nutritional strategy to control infections in broiler chicken industry [2]. Some studies reported that *Salmonella*-specific IgY can prevent fatal Salmonellosis in mice or calves by oral administration [3, 4, 5]. On the basis of these previous reports and advantage of IgY over the mammalian antibodies, the present study focused to develop chicken egg yolk antibodies consortium against *E. coli* and *Salmonella enterica* for passive immunization, particularly in poultry to establish protective immunity against two different bacterial pathogens in single attempt. This will help the poultry industry to provide safe and healthy poultry products.

**MATERIALS AND METHODS**

**Experimental animal**

Twenty one weeks old egg laying white leghorn chickens in good health was obtained from Amman poultry farm, Palani, Tamil Nadu, India. The birds were maintained and fed with layer mesh. The birds were injected with different antigen.

**Bacterial strain**

Bacterial strains used for the present study were *Escherichia coli* (E) and *Salmonella enterica* (S). The bacterial cultures were obtained from Suguna poultry farms, Udumalpet, Tamil Nadu, India. They were cultured on nutrient agar plates, Macconkey agar plates and preserved in nutrient agar slants. Sub culturing was done (once a month) as desired and used for further studies.

**Preparation of whole cell antigen**

The whole cell antigen was prepared under standard indigenous conditions. Pure isolated colonies of S and E were incubated at 37°C overnight. They were then transferred to 250 ml of
broth and incubated overnight on a rotator platform at 200 rpm. Cells were harvested by centrifugation at 3000 rpm for 15 to 20 minutes at 4°C. Supernatant was discarded and the pellet was washed three times with 0.04 M PBS (pH 7.2, 3500 rpm, 15 min, 4°C). The pellet was resuspended in phosphate buffer saline. Subsequently, bacteria were killed with 3% formalin (V/V), final concentration for 2 hrs at 37°C (continuous shaking at 2000 rpm) followed by vigorous vortexing at 1600 rpm for 25 hrs at 4°C. Then bacterial suspension was centrifuged at 10,000 rpm for 10 minutes. Then supernatant was discarded. The pellets were resuspended in saline and stored under refrigeration. Complete killing of the bacteria was tested by resuspending an aliquot of the cell pellet in PBS saline and plating 199 ul of this suspension into nutrient agar and Macconkey agar medium. The plates were incubated overnight at 37°C and examined for the presence of bacterial growth.

**Immunization of chickens**

For first immunization, the five month old white leghorn chickens were intra muscularly injected at multiple sites of the breast muscles with prepared bacterial antigens. Booster doses were given with two weeks intervals. Blood was sampled at intervals of two weeks of immunization and checked for the presence of antibodies. Further, eggs laid by the chicken under the test were collected regularly and stored at 4°C.

**Purification and concentration of egg yolk antibodies**

The egg yolk was separated from white, washed with distilled water to remove as much albumin as possible and rolled on a paper towel to remove adhering egg white. The membrane was punctured and the yolk without the membrane was allowed to flow into a graduated cylinder.

The egg yolk antibodies were purified by the method of Polson et al. (1980) [6]. To the 20ml of egg yolk, an equal amount of buffer “S” (10mM phosphate, 100mM NaCl, pH 7.4 containing 0.01% sodium azide) was added to the yolk and stirred. To this mixture 10.5% PEG 6000 in buffer “S” was added to a final concentration of 3.5%. The mixture was stirred for 30 minutes at room temperature. The stirred mixture was centrifuged at 11,000 rpm for 20 minutes. The supernatant was filtered through double-layered cheese cloth. The 42% PEG in buffer “S” was added to make final concentration of 12.5% PEG. The mixture was stirred thoroughly for 30 minutes at room temperature and centrifuged at 11,000 rpm for 20 minutes. The pellet was redissolved in buffer “S” to the original yolk volume and equal volume of 4M Ammonium sulphate (pH 7) was added and incubated at 0°C for 30 minutes. The solution was centrifuged at 11,000 rpm for 20 minutes. The precipitate was re-dissolved in buffer “S” (without NaCl) and was desalted by dialysis to remove ammonium sulphate.

**Purification of IgY by DEAE Cellulose Ion Exchange Column Chromatography**

The chicken egg yolk antibodies were purified by DEAE cellulose Ion Exchange Column Chromatography. DEAE cellulose column size 2x30 cm was used. Once the column was set, it was equilibrated with 25mM phosphate buffer, pH 8.0 (till the out flow of buffer showed pH 8.0). Then, the level of buffer in column was allowed to run down to the matrix and the outlet was closed. The immunoglobulin IgY sample layered on the top of the column and was allowed to run till all the sample had entered the bed. Then a continuous constant flow of 25mM phosphate buffer was maintained until all the un-retained protein came out. IgY was eluted with 250mM phosphate buffer pH 8.0. The protein content of the eluted fractions was estimated by Lowry et al. (1951) procedure.
Titration of antibodies by ELISA
The antibody titer was assayed by an ELISA procedure as described by Sunwoo et al. (2000) [7] with modifications. The specific binding activity of IgY against whole bacterial cells was tested as follows. A microtiter plate was coated with 100 µL of S. paratyphi A (1.11 mg of cells/mL; 10 µg of protein/mL) and E. coli (10^7 cells per well) whole cells in carbonate-bicarbonate buffer (0.05 M, pH 9.6). The (diluted 1:1,000 in PBS, 100 µL per well) specific egg yolk antibodies (IgY) was reacted with coated antigens. The same volume (100 µL) of rabbit anti-chicken IgG conjugated with horseradish peroxidase3 (diluted 1:1,000 in PBS) and freshly prepared substrate solution, 2-2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)3 in 0.05 M phosphate citrate buffer (pH 5.0) containing 30% hydrogen peroxide were used for secondary antibody and substrate, respectively. Absorbance of the mixture was read at 490nm by a kinetic micro plate reader.

Specificity of antibodies by Growth Inhibition Assay
This assay was conducted to investigate whether the binding activity of anti-Salmonella enterica IgY anti-E.coli IgY could inhibit Salmonella and E.coli growth in a liquid medium. The same strain of S. paratyphi A and E. coli were used as an antigen for immunizing chickens and the same strains were sub cultured on MacConkey agar plates and suspended in Nutrient broth. The suspensions were adjusted to an optical density of 0.05 at 600 nm, corresponding to a cell density of approximately 2.7 X 10^7 CFU/mL. Two milliliters of prepared bacterial culture were mixed with 2 mL of Nutrient broth and incubated at 37°C with shaking. The turbidity of the culture (optical density at 600 nm) was measured by a spectrophotometer at 1-h intervals and simultaneously plate count was also performed. The growth curve was plotted until the stationary phase was reached.

The IgY solutions were sterilized by using a 0.22-µm membrane filter. Two milliliters of specific or nonspecific IgY solution were then added to the same volume of prepared S. enterica and E.coil cultures. The bacteria and IgY mixtures were incubated at 37°C with shaking. Aliquots of samples (100 µL) were taken at 0, 2, 4, and 6 h of incubation. Plate counts were performed by the spread plate method on nutrient agar plates and simultaneously OD values were also taken at 600nm. The inoculated plates were incubated at 37°C overnight. The number of colony forming units per plate was counted to determine the total number of bacteria colony forming units per ml of sample.

RESULTS

Generation of Antibodies in Hen
The 21 week old white leghorn Hens were immunized intramuscularly with prepared bacterial antigens to generate anti-Salmonella enterica and anti-E.coli antibodies with two week intervals. Eggs were collected from fourth week of immunization and stored at 4°C. Then the antibodies were separated from chicken egg yolk.

Isolation and Purification of Antibodies
The method used for purification of chicken egg yolk antibodies were PEG and ammonium sulphate precipitation described by Polson et al (1980) .The precipitate was desalted by dialysis to remove ammonium sulphate. The egg yolk antibodies were further purified by ion exchange chromatography using Anion exchanger like DEAE – Cellulose and immunoglobulin fractions were recovered. The recovered antibodies were detected by Protein estimation and ELISA.
Protein Estimation
The total protein concentration of purified IgY was estimated by UV – Visible Spectrophotometer. The concentration of antibodies increased in the egg yolk with subsequent booster doses with an average yield of 36mg per egg yolk after third booster doses (45 days after first immunization).

Protein profile by SDS – PAGE
The chicken egg yolk antibodies and its molecular weight was determined by Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gel at 100 V and 10 mA according to the method of Laemmli(1970). The SDS- PAGE (Fig1) shows a single band with a molecular weight of 180 KDa in both lane 1 and 2.

Fig1. SDS-PAGE Profile of IgY

Lane 1 – Salmonella enteric – IgY
Lane 2 – E. coli - IgY

Fig2. Titre of IgY specific to E.coli antigen was found to be increased after booster doses
Estimation of antibody titer by ELISA
The antibody titer potency of each IgY fractions obtained above was determined by the following modified ELISA as described by Lee et al (2002) [8]. The antibody titer increases at the time of booster injections, even a minute increase in antibody titer can be traced by this assay. The comparative results show that the antibody titer potencies changes in the courses of immunization (Fig2 and Fig3). The rate of dilution of antibodies given an OD_{490} value in 1:10000 dilution.

![Fig 3. Estimation of Antibody titre by ELISA for S. enterica-IgY](image)

**Fig 3.** Titre of IgY specific to *S. enterica* antigen was found to be increased after booster doses

![Fig 4. Growth Inhibition Assay of *E. coli*-IgY](image)

**Fig 4.** Growth inhibition assay showed the reduction in the growth of *E. coli* cells incubated with *E. coli*-specific IgY.
Specificity of antibodies by Growth Inhibition Assay

Growth inhibition assay was performed to check the specific activity of IgY against the bacterial antigens. The growth curves of *E. coli* and *S. enterica* were plotted for the growth of normal bacterial cells and the growth of bacterial cells with IgY fraction separately. Then the plate count was performed in both the plates, which were inoculated with aliquots of samples from cell culture tube and cell culture inoculated with IgY. In which the significant reduction in bacterial growth was observed in the cells incubated with IgY fraction. The results were given in the Fig 4 and Fig 5.

**Fig 5.** Growth Inhibition Assay of *S. enterica* IgY

**DISCUSSION**

There are significant losses in the global poultry industry because of the two major bacterial infections such as Salmonellosis and Colibacillosis including yolk sac infection of chicks, reproductive disorders, and peritonitis of layers, septicemia, and foot abscesses infection of joint membrananes, diarrhea, depression and death in chicks. [12] reported that demographic changes and drug access issues are important reasons in the developed and developing world respectively. “Relentless and dizzying rise of antimicrobial resistance” [13] has contributed in a large measure to the peristana of infections as a major cause of morbidity and mortality.

Recently viable alternatives to the antibiotics, mammalian antibodies IgG and the chicken egg yolk antibodies came into play a major role in the diagnosis of diseases in poultry. Traditionally, the rabbit antibodies (IgG) were used for this purpose. But laboratory production of antibodies involves immunization and bleeding of animals, causing distress to them. However, chicken are known to produce high levels of antibodies then mammalian, thus reducing the number of animals needed. Further the egg yolk receiving the same antibodies from the maternal chicken serum can be suitable alternative to the bleeding of animals. On the basis of the advantages of IgY over the mammalian antibodies entitled in the previous report, the present study focused to
develop egg yolk antibodies to control the morbidity and mortality of the poultry industry from
the infection and diseases caused by the predominant bacterial pathogens such as *E.coli* and
*Salmonella enterica* instead of the treating the infected birds using antibiotics. For this purpose
whole cell antigens were prepared and used to immunize the 21 weeks old white leghorn
chickens to generate IgY. Then eggs were collected, stored and antibodies were purified from
chicken egg yolk and serum by Polson *et al.*, (1980). The molecular weight of the purified IgY’s
were confirmed as 180KDa through SDS PAGE [9]. The electrophoretic band pattern obtained in
this study was similar to that of the bands obtained by Kariyawasam *et al.*, (2003) [10]. The
activity and titer of egg yolk antibodies were determined by ELISA [8] showed that the presence
of antigen specific antibodies for the specific bacterial pathogens. ELISA is a sensitive technique
to find out the antibody titers during different purification processes. Regarding the antibody titer
potency of egg yolk sample a high peak at the dilution of 1:10000 was obtained after third
booster dose, which indicated by the rates of dilution giving an OD490 value of 0.8.

The specific activity of antibodies against specific bacterial pathogens was determined by
Growth Inhibition Assay. The growth curves of *E.Coli* and *S.enterica* were plotted separately
under the same condition with the Growth Inhibitory Assay.Upon addition of IgY fractions in
liquid medium containing bacterial cells, the cells were formed complexes with IgY and
precipitated at the bottom of the culture tubes and the supernatant became clear. As the
incubation was proceeded with shaking, the turbidity of the medium was increased which
indicating that the bacteria were multiplying. The growth rate in the presence of IgY fractions
was decreased due to the binding of specific IgY to the bacterial surface components and
structural alterations of the bacterial surface have been suggested as the mode of action of
specific IgY to inhibit bacterial growth [8].

The present study concluded that chicken antibodies developed against two different pathogens
causing the diseases in poultry and leads to significant losses in poultry industry could be used
for passive immunization using chicken IgY as feed additive to prevent the enteric infections in
poultry. Further studies may be required to evaluate the stability of these antibodies in chicken’s
body system. 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.

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