Generation and Passive protection of Chicken Egg Yolk antibodies (IgY) against *Campylobacter jejuni*

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Abstract

Indian Poultry industry contributes a major role in the economy through the food processing sector. Pathogens' causing the diseases in poultry leads to significant losses in poultry industry. Campylobacteriosis is a zoonotic infection caused by *Campylobacter jejuni* colonizes the gastrointestinal tracts of a wide range of wild and domestic animals. Chickens are widely recognized as the main source of human exposure to *Campylobacter jejuni* infections. *Campylobacter* associated diarrhoea in patients have been suggested to be linked to the consumption of meat which leads to Guillain Barre´syndrome, diarrhoea, and other intestinal infections. The use of antibiotics to eliminate or to prevent *Campylobacter jejuni* infection is not recommended for various reasons. Chicken egg yolk antibody (IgY) was raised against *Campylobacter jejuni* by immunizing the whole cell antigen prepared from the isolates obtained from the chicken intestine. The specific activity of the purified IgY shows highest titre, determined by ELISA. IgY activity while treated with different sugars like sucrose, lactose and dextran and its stability was evaluated under different conditions. IgY was stable at 60-70°C in the presence of sugars at pH 5-7. Thus the specific IgY has the ability to survive in the gastrointestinal tract and its binding activity to the bacterial cells helps to decrease the growth rate that provides passive protection against *Campylobacter jejuni* infections in Chickens.

Keywords: Poultry, Intestinal infections, IgY, ELISA, Stability.

Introduction

Campylobacter jejuni ("Camp-e-low-back-ter-j-june-eye") is a frequent cause of human gastrointestinal diseases in worldwide (Butzler, 2004). The common source of this bacterium is the gastrointestinal tract of various animals, mainly in the chickens and turkeys. It is mainly present in their faeces (Doyle, 1984; Smitherman *et al*., 1984; and Yoshida *et al*., 1987). Consumption of such contaminated poultry meat is recognized as the main sources of campylobacteriosis in humans (Bryan and Doyle, 1995; Kinde *et al*., 1983; Park *et al*., 1981). After two to five days of consuming contaminated food, gastrointestinal symptoms including abdominal cramps, nausea, vomiting, fever and diarrhoea will appear (Allos, 2001). In addition to the gastrointestinal infection, may cause a neurological disease which is frequently associated with GBS (Guillan Barre Syndrome) (Nachamkin and Allos, 1998; Scott *et al*., 1997).

The pathogenic process of *C. jejuni* is poorly understood as compared to other enteric pathogens. Pathogenic mechanisms like production of CDT (Cytolethal Distending Toxin) (Konkel *et al*., 2001) that hinders the cells from dividing and activating the immune system; cholera like enterotoxin (Polson *et al*., 1980) and cytotoxin (Polson *et al*., 1980; Yokoyama *et al*., 1998): develops the ability to adhere and invade epithelial cells (Konkel and Joens, 1989; Oosterom, 1994) plays an important role in mucosal inflammatory response.

C. jejuni is a normal commensal in the chicken gut but when an infection occurs, the population of the organisms will get increased. During slaughter this can be able to transfer from the intestine to their skin surface and also gets penetrated into their muscles (Russell *et al*., 1993). The moist condition on its surface helps this pathogen for survival, during storage (Fricker and Park, 1989). The infection rates during slaughtering, marketing and other processing range from 22-95% (Newell and Wagenaar, 2000; Rollins, 1991).

Contamination can be reduced by improving the bio security levels in the hatchery, a competitive exclusion technology or using chlorinated water. But in most cases it shows unsatisfactory results (Michino and Otsuki, 2000; Newell and Wagenaar, 2000). Routine use of antibiotic prophylaxis to prevent campylobacter infection is not recommended. No effective immunological interventions are currently available for the control of campylobacter associated infections and it may also disturb the normal bacterial population in the intestine.

Chicken eggs act an ideal alternative antibody source to mammals, because the IgY within the chickens' blood is transported to the egg and accumulates within the ingredient in giant quantities. Hens usually lay about 280 eggs in a year. Egg yolk contains a considerable amount of IgY, around 100-150 m/egg (Rose *et al*., 1974.). Therefore, an immunized hen yields more than 40 g of IgY a year through eggs, equivalent to that from 40 rabbits. In the sense of animal welfare, the use of laying hens for antibody production represents a refinement and a reduction in animal use. It is a refinement in that the painful and invasive blood sampling or scarifying are replaced by collecting eggs.

Chicken egg yolk was recognized as an inexpensive alternative antibody source (IgY) as its immune therapeutic application has proved successful for the treatment of various gastrointestinal infections. It also has advantageous characteristics over mammalian IgG, including stability under various physic-chemical conditions and its suitability as an immunological tool (Losch *et al*., 1986; Marquardt *et al*., 1999; Shimizu *et al*., 1994; Shimizu *et al*., 1992 and Shimizu *et al*., 1988). Egg yolk antibody as a food grade has been substantiating the effective use for bacterial passive immunization. IgY demonstrated an immune function in preventing bacterial shedding or infection in vivo (Ikemori *et al*., 1992; Imberechts *et al*., 1997; Peralta *et al*., 1994; Yolken *et al*., 1988 and Yoshida et al., 1987).

In this study we use chicken egg yolk antibodies against *Campylobacter jejuni* to inhibit these bacterial growth in chickens which helps to provide a safe meat product for human consumption.

Material and Methods

Samples from Slaughter house

The chicken intestine was purchased from the neighbouring slaughter house. All samples were stored on ice in the laboratory until it processed, and it is processed within 6hrs after collection.

Primary isolation and identification

All intestinal samples from the slaughter house were inoculated directly onto the selective medium; Campylobacter base agar medium supplemented with 10% defribrinated sheep. The plates were incubated under microaerobic conditions (5%O2, 10%CO2) in candle jars at 42ºC for up to 48 hours.

After 48 hours, suspected colonies of *Campylobacter* were examined by oxidase test (Dryslide, BBL), catalase test (3% H2O2) and Gram staining technique. *Campylobacter* spp was identified by the gram negative spiral rods with both positive oxidase and catalase test results and confirmed by the positive result of hippurate hydrolysis tests. The isolates were frozen and stored in 30%glycerol with Brucella broth at -70ºC.

Preparation of Whole cell antigen

The bacteria were cultured under microaerobic conditions in lysed sheep blood agar plates for 24 to 48 h, cells get harvested, and washed two times in phosphate buffered saline (PBS, pH 7.4).The preparations were centrifuge at 7,000*g* for 10 min, and the pellet was stored at 22◦C. The pellets were resuspended in PBS (pH 7.4 (4). The concentration of the culture was adjusted to 0.5 McFarland (10^8 cfu/ml) in PBS by using a colorimeter.

Production and purification of IgY

21 weeks old White Leghorn hens was immunized with 0.5 ml (1:1) of Freund's complete adjuvant and 0.5 ml of the prepared antigen was given intramuscularly to chickens. Two booster doses of 0.5ml, one with Freund's incomplete adjuvant, one without adjuvant was given on 14th day and 21st day respectively by the constant route. Antibodies were detected by slide agglutination method using corresponding antigen on 14th day after second booster and the eggs were collected daily and stored at 4°C until further use. The antibody was purified from the egg yolk by using polson method.

SDS-PAGE analysis

The protein samples were analyzed by SDS-PAGE by the system of (Laemmli, 1970) with a 4% stacking gel and a 12.5% running gel. Proteins were solubilized in SDS solubilizing solution containing 2-mercaptoethanol, boiled for 5 min, and applied to the gel at ca. 25ug per lane. Proteins were electrophoresed by using a Bio-Rad slab gel apparatus. At a constant current of 15 mA per gel until the tracking dye entered

the resolving gel and at 20 mA per gel through the running gel. Proteins were visualized with Coomassie blue stain. The result was compared with standard protein marker.

Protein assay

The total protein concentration of the IgY solution was determined by Lowry *et al*., (1951) method using folin-Ciocalteau reagent. BSA was used as the standard and the results were compared with the standard graph.

IgY powder preparation

For wide application of IgY, IgY powder as a dried concentrated and stable form for long term storage, may be suitable for these intensions. The specific IgY solution was lyophilized by a freeze drier to obtain IgY powder.

Specific Binding Activity of IgY

The ELISA procedure adapted was a modified method of the original ELISA procedure described by (Gupta *et al*., 1992). Polyvinyl micro titration plates were coated with 100µl of the *C. jejuni* antigen that was diluted with carbonate buffer $_{P}^{H}$ 9.0 and incubated at 37°C overnight. After coating the plates were washed with PBS containing 0.05% tween20 (PBST) and nonspecific binding sites blocked by adding 200µl per well of 1% bovine serum albumin in PBS and incubating the plates at 37°C for 1 hour. Plates were subsequently washed with PBST and incubated with 100µl of either polyclonal chicken antibodies or egg yolk antibodies at appropriate dilutions. Control wells had PBST and pre immune sera served as respective controls. Plates were incubated for one hour at 37°C and subsequently washed with PBST. For the chicken antibodies 100µl of rabbit anti chicken immunoglobulin coupled to horseradish peroxides was added at appropriate dilutions and plates incubated for 1 hour at 37°C. After incubation the plates were washed with PBST and enzyme activity determined by adding 100µl of freshly prepared substrate solution (4mg of O-phenylene diamine dissolved in 10ml of 50mM citrate buffer, pH 5.0 containing 10µl hydrogen peroxide). And the plates were allowed to stand at room temperature (dark condition) for 15 minutes. The reaction was stopped by adding 50µl of 4N H_2SO_4 and plates were read at 490nm in an ELISA reader.

IgY determination

The crude IgY concentration was determined by measuring the absorbance at 280 nm, based on 13.6 as extinction coefficient at 280 nm of a 1% IgY solution. The specific IgY concentration in crude IgY was determined by immunoprecipitation. Then 0.5 ml of the crude IgY solution and 1 ml of a 0.01% antigen solution in PBS were added to the same tube and incubated overnight at 37oC. The supernatant was separated by centrifugation (3,000 × g, 30 min) and the absorbance was measured at 280 nm.

The specific IgY concentration was calculated as follows:

$$\text{Specific IgY}(\%) = \frac{\text{Absorbance without antigen - absorbance with antigen}}{\text{Absorbance without antigen}} \times 100$$

Total IgY Concentration

To quantify the total IgY concentration in the lyophilized form ELISA method was performed. Specific IgY powder were reconstituted and serially diluted in PBS (2 to 0.125 µg/ml). The standard curve was then used to estimate the total IgY concentration in the samples.

Purity of IgY

Purity of the IgY was calculated as follows

$$\text{Purity of the IgY} = \frac{\text{Concentration of IgY}}{\text{Concentration of protein}} \times 100$$

Stability of IgY

Heat Treatment

Antibody solutions (1mg/ml) were heated at 50, 60, 70, 80 or 90°C in a water bath for 30min in the presence of 30% sucrose, lactose or trehalose; 5% cyclodextrin or dextran; 20% water as a control.

Acid treatment

The pH of the 0.2% IgY solution in saline in the presence of 0.50% polyol was adjusted to pH 2.7 with HCl. The resulting solution was incubated at 37°C. After incubation at each pH, the solution was neutralized with PBS that contained 0.05% tween 20 (PBS-T). ELISA examined the antibody activity.

Results

The protein concentration in the egg yolk did not change significantly after the first immunization but the concentration of specific IgY in terms of total protein content has increased after the subsequent booster doses, the highest titre was remained throughout the experimental period. Purification of the egg yolk was done by polson method using ammonium sulphate as the precipitating agent. The concentration of total IgY in the egg yolk was approximately 4.9mg/ml of each egg. This concentration may differ while using other types purification methods, and also depends on breeds of birds and weight of the egg yolk.

The purity of chicken egg yolk antibodies and their molecular weight were determined by Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE), using 12% gel according to the method of Laemmli. IgY was found to be of high molecular weight about 180 KDa.

The titrations of specific antibody raised were measured through optical density at 492 nm. Periodic immunization of chickens raises the antibody concentration in egg yolk, constantly. The specificity of the antibody to the antigen was also monitored using ELISA which shows the antibody titre to a maximum dilution of 1:10000.

The specific IgY(%) of *C.jejuni* was calculated as 25.1% and the purity of *C.jejuni* antibodies was calculated as 21.0% . The specificity of *C.jejuni* antibodies was determined by ELISA. 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. The rate of dilution of antibodies given an OD490 value in 1/1000 dilution.

The heat stability of chicken IgY was evaluated by ELISA after incubating the antibody for 30min at 50, 60, 70, 80 or 90°C in an aqueous solution in the presence of sugars. Very little loss of IgY activity was observed with any of the treatments when the antibody was exposed to 50°C except for the control (water). However, at 60°C the highest recovery of IgY activity was observed in the presence of egg yolk and sucrose with the recovery being 100 and 96%, respectively. A moderate loss of IgY activity at 60°C was observed in the presence of all sugars except the control where half of IgY activity was lost. IgY retained about 70% of its activity when incubated at 70°C in the presence of sugars, while only about 20% of its activity was recovered when heated in the presence of dextran. It is noteworthy that the recovery of IgY in the presence of sugars increased with increasing temperature. Aggregation of the sugars at the higher temperatures may have provided a physical shield for the IgY antibodies. IgY activity was completely lost at 80°C and 90°C.

The stability of IgY at each pH is shown in Fig. 2. IgY showed good stability at pH 5-7 after incubating 24 h at each respective pH. IgY was inactivated irreversibly at pH below 4. IgY inactivation was irreversible. The time-dependent changes of IgY activity at acidic pH are presented in Fig.3. When incubating at each pH for 4 h, IgY lost antibody activity at pH 4 or below. The antibody activity was significantly reduced (up to 50%) at pH below 3. IgY almost completely lost its activity by incubating for 4 h at pH 2. The most noticeable loss in antibody activity was observed during the first 30 min. The ability of IgY to survive digestion in the gastrointestinal tract is further supported by the observation that egg yolk antibodies can provide complete passive protection against *C.jejuni* infection in chickens.

Discussion

Commercial poultry are the major natural reservoirs of *C. jejuni*, and up to 100% of broilers at slaughter-age may harbor the organism. Once some birds become infected, *C. jejuni* spreads rapidly to most of the birds in the flock, which remain colonized up to slaughter, leading to carcass contamination at the processing plants The prevalence of *Campylobacter* contamination of carcasses and poultry products can vary greatly, depending on the sensitivity of the cultural procedures utilized and by the point along the process chain at which sampling is being Disease caused by *Campylobacter* usually manifests at diarrhoea, fever and severe

abdominal pain. Although, most human cases are sporadic and outbreaks are relatively rare (Ketly, 1997), more serious consequences of campylobacteriosis include the autoimmune-mediated demyelinating neuropathies Guillain-Barre and Miller Fisher syndromes.

Certain types of antimicrobials have also been used for disease control in human medicine. There is increasing microbiological and clinical evidence that resistant bacteria may pass from animals to humans, resulting in infections that are more difficult to treat. It is unlikely that a single viable alternative to antimicrobials will be implemented successfully. IgY technology, including the production and use of polyclonal IgY antibody (Ab), is a highly innovative and an expanding branch in human and veterinary medicine

Hence, the present investigation is focused to generate chicken antibodies against *Campylobacter jejuni* to inhibit its shedding in broiler chickens by passive immunization using chicken IgY as feed additives**.** Binding of specific IgY to 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. The specific IgY powder was dose dependent and the remarkable increase in the antibody specific activity of immunized WSF. Using the simple and practical immunization protocol, it is suggested that IgY could be a suitable alternative in food applications to promote antibacterial effects.

The assessment of stability of IgY was done by treating the IgY with various sugars and acidic pH with the help of HCL. And this work was also done with IgY powder which is used to find the stability of IgY in the gastric environment of the birds intestinal tract.

From the results it appeared that the IgY is stable in the presence of sugars, at 75°C. Presumably these sugars did not interact with the amino groups of the IgY under these conditions. Certain sugars, especially the non-reducing sugars like sucrose and egg yolk are good thermo Protestants of IgY, while dextran was less effective. The IgY stability at pH 3 was considerably improved using high concentrations of sucrose in solution. These results suggest that sucrose may be useful in stabilizing IgY at low pH. Therefore, IgY could potentially be applied in high acid food products and extensively used for protection against *C.jejuni* infection. This emphasis the application of chicken IgY as feed additives in broilers production.

Conclusion:

Present study concluded that the generated IgY was specific against *Campylobacter jejuni* whole cell antigen and it could effectively bind with that. Then, the generation of antibodies in the chicken is an economic and feasible alternative way to obtain large quantity of polyclonal antibodies for passive immunization against enteric infections in poultry. Therefore the passive immunotherapy with Specific IgY antibodies against *Campylobacter jejuni* infections results in the protection of broiler chickens from harbouring pathogens and shedding of post-mortem lesions. It might facilitate the poultry sector to use

chicken IgY as an effective therapeutic agent, by which they can provide safe and healthy poultry meat, which will be free from pathogenic microbial contamination.

Table 1: Immunization schedule

Dosage Schedule	Days	Antigen dose (10^3cells/m)	Site of Injection
1st Dose	0th	1ml	Intramuscular
1st Booster	14th		
2ndBooster	21st		
3rdBooster	28th		
4thBooster	35th		

Table 2: Estimation of protein content of Partially Purified IgY powder

Tube. No	Volume of BSA standard (ml)	BSA concentration (µg)	Optical Density (OD) at 640 nm.	Protein content (mg/ml)
1	Blank	0	0.00	0.00
2	0.2	20	0.26	10.5
3	0.4	40	0.48	20.0
4	0.6	60	0.78	30.5
5	0.8	80	0.92	36.0
6	1.0	100	1.38	60.0
7(Sample)	0.2	_	1.26	4.9

Figure:1

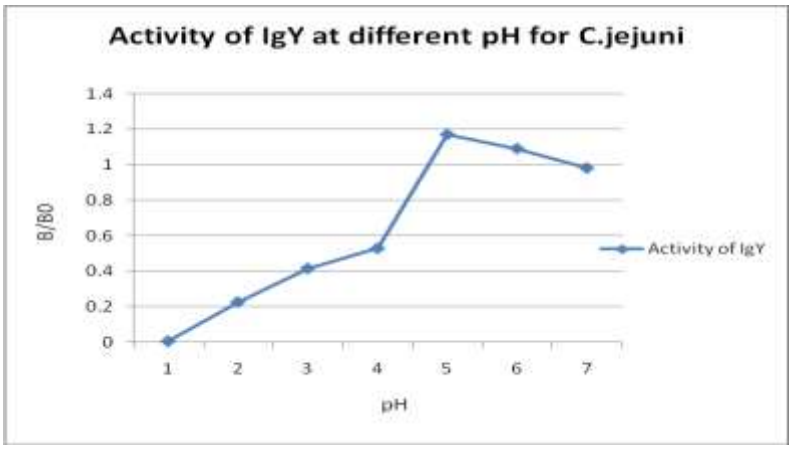


Figure:2

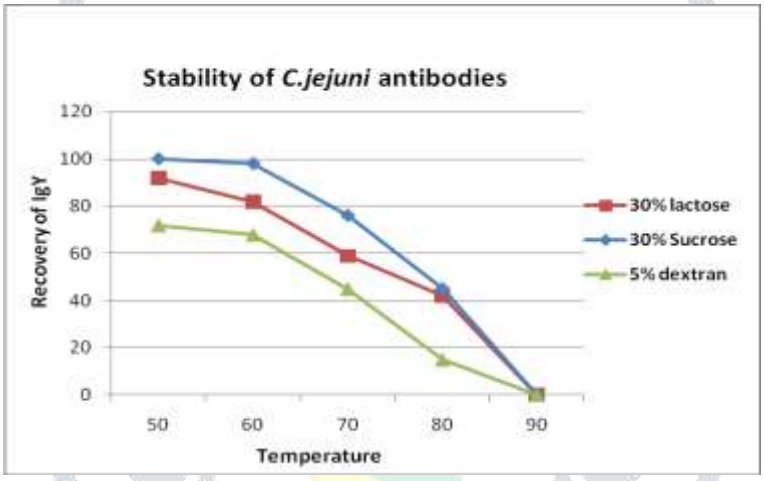
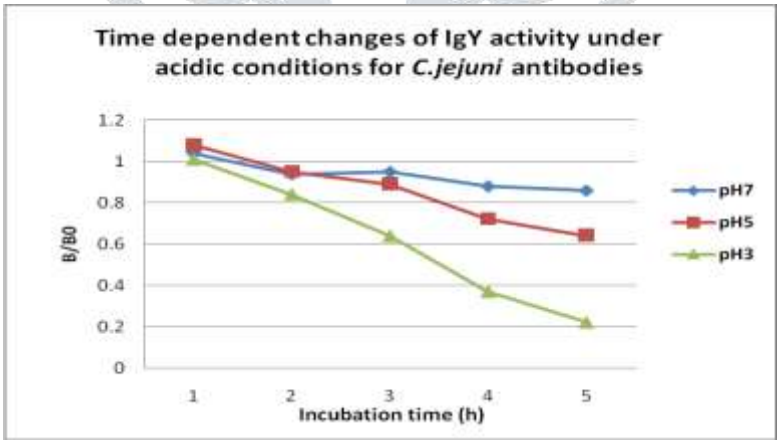


Figure:3



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