

Chicken Egg Yolk Antibody (IgY) Powder Against *Escherichia coli* O78:K80

¹A.H. Mahdavi, ¹H.R. Rahmani, ¹N. Nili, ¹A.H. Samie and ²S. Soleimani-Zad
¹Department of Animal Science, ²Department of Food Science and Technology,
Isfahan University of Technology, Isfahan, 8415683111, Iran

Abstract: The growth inhibitory effect of egg yolk antibody (IgY) powder for food application was investigated on *Escherichia coli* O78:K80, as a model and the major cause of associated septicemic disease in broiler chickens. Laying hen's hyperimmunized with *E. coli* O78:K80 bacteria for a 12 weeks period and IgY was isolated by the water-dilution method. *E. coli* O78:K80 was incubated with IgY (50, 100 and 150 mg mL⁻¹ specific and non-specific IgY powder) for 6 h during, which samples were taken at 2 h intervals. After the first immunization, the antibody specific activity in serum and egg yolk increased and became plateau on day 7 and 14, respectively. The protein concentration of serum and IgY powder did not change during the immunization period, but the ratio of gamma globulins to total serum protein and IgY purity (total IgY in protein) of IgY powder increased ($p < 0.05$) and remained high throughout the experimental period. Specific IgY powder at the concentration of 150 mg mL⁻¹ decreased bacterial proliferation by 1.18 log CFU mL⁻¹ compared with the control group at 6 h of incubation period ($p < 0.01$). Results of this study suggest that *E. coli* O78:K80 could have long term antigenic properties to induce an immune response in laying hens. The large-scale and long-term production of IgY, with high purity and high specific activity, can be attained by water dilution method. The inhibitory effect of specific IgY powder may encourage IgY powder to be applied as a feed additive to protect against *E. coli* O78:K80.

Key words: Feed additive, egg yolk antibody (IgY) powder, *Escherichia coli* O78:K80, antibacterial activity

INTRODUCTION

Strains of *Escherichia coli* serotype O78 have been found to cause numerous extra- and intrainestinal clinical symptoms in various hosts (Adiri *et al.*, 2003). Enterotoxigenic *E. coli* (ETEC) serotype O78, has been isolated from humans in enteritis, Newborn Meningitis (NBM) and sepsis (Babai *et al.*, 1997; Gophna *et al.*, 2001). In farm animals, this is one of the predominant serogroups in intestinal disease of diarrhaegenic cattle and calves (Ewers *et al.*, 2004a) and sepsis in sheep and poultry (Ron *et al.*, 1991). Colibacillosis is mainly a poultry disease that refers to any localized or systemic infection caused entirely or partly by *E. coli* (Barnes *et al.*, 2003). This subversive disease is the primary cause of morbidity, mortality and condemnation of carcasses in the poultry industry worldwide (La Ragione and Woodward, 2002; Ewers *et al.*, 2004b). In the past few years, both the incidence and intensity of colibacillosis have rapidly increased and may continue to become an even greater problem in the poultry industry (Altekruse *et al.*, 2002). The infection is usually secondary to viral or mycoplasma infections or environmental stresses and most frequently involves Avian Pathogenic *E. coli* (APEC) of serogroups

O1, O2 and O78 (Gomis *et al.*, 2001), with the latter two constituting about 80% of the cases (Adiri *et al.*, 2003). Vandekerchove *et al.* (2004) reported that *E. coli* strains isolated from the majority of the flocks belonged almost exclusively to the O78 serotype. Approaches to prevent and control APEC infections in the poultry industry include improved hygienic methods, vaccination, use of competitive exclusion products and finally antimicrobial chemotherapy, with increase of resistance to antimicrobial agents affecting limited success (Kwaga *et al.*, 1994; Gomis *et al.*, 2003; La Ragione *et al.*, 2004; Miles *et al.*, 2006; Knezevic and Petrovic, 2008; Lampang *et al.*, 2008).

Egg yolk antibody (IgY), as a food grade, has been studied to substantiate the effective use for bacterial passive immunization (Kariyawasam *et al.*, 2004; Wilkie *et al.*, 2006). There are several benefits of using IgY as a feed additive to restrain bacterial infection. IgY can be isolated simply from egg yolk by the water-dilution method on a large-scale (Akita and Nakai, 1992, 1993) and it is relatively stable under various conditions including heat, pressure, acidity, alkalinity and proteolytic enzymes such as trypsin and chymotrypsin (Otani *et al.*, 1991; Shimizu *et al.*, 1992; Tini *et al.*, 2002). The quantitative advantage of raising IgY has been presented by

Gottstein and Hemmeler (1985), who showed that the amount of purified IgY produced in one month is 18 times higher than that of IgG produced in a rabbit. Therefore, large quantities and easily production of IgY with relatively high stability and compatibility with modern animal protection regulations, makes IgY as a potential practical food supplement.

Land application of poultry manure can contaminate water sources by introducing pollutants such as nutrients, organic matter, sediment, pathogens, heavy metals, hormones and antibiotics (Mishra *et al.*, 2008). It is demonstrated that uncontrolled disposal of raw manure can potentially spread pathogens to non-target environments (Bach *et al.*, 2002). For example, the abilities for *E. coli* to survive, replicate and move downwards in soil for up to two months through manure spreading has been indicated by Gagliardi and Karns (2000). Also, Kudva *et al.* (1998) reported the survival of *E. coli* O157:H7 for >1 year in hoarded non-aerated ovine manure pile that was exposed to environmental conditions. Therefore, by using IgY powder as a potential food supplement not only resistant pathogenic colonies and harmful microbial products are reduced in the intestinal content of birds, but also due to reduction in antibiotic needs in poultry production system, antibiotic residues decrease in poultry manure, soil and water. Furthermore, Kim and Patterson (2003) reported that specific IgY inhibited microbial uricase activity in broiler litter and laying hen manure leading to reduction in NH₃ generation and decreased environmental NH₃. Therefore, IgY powder as a potential antimicrobial agent may also be used as an important bio-environmental factor for protection of vital factors.

The aim of the present study was to investigate the *in vitro* reactivity and growth inhibitory effect of IgY powder on *E. coli* O78:K80 as a model. This study may result in the prospective future application of IgY powder as an effective measure for restraining the spread of enterotoxigenic *E. coli* infection from animals and birds to human.

MATERIALS AND METHODS

Antigen preparation: A strain of *E. coli* O78:K80 was obtained from Razi Vaccine and Serum Research Institute (Karaj, Iran). To ensure viability of the culture, bacteria were grown in Tryptic Soy Broth (TSB) (Merck, Darmstadt, Germany) for 6 h (until late log phase) at 37°C with shaking at 180 rpm. Cells were harvested by centrifugation at 5000x g for 15 min at 4°C (Sigma 6K15, Laboratory Centrifuges). The pellet was washed three times with phosphate-buffered saline

(PBS, pH 7.2) and resuspended in PBS to a concentration of 2×10^9 CFU mL⁻¹. To prepare the killed vaccine, 100 mL of the bacterial suspension was sonicated in an ice bath using a microtip (Ultraschallprozessor, Dr. Hielscher, UP200H) at maximum amplitude for 15 min and the lysate was filtered through a 0.45 µm sterile filter (Acrodisc Gelman Sciences, Mississauga, Ont.). Sterility of the filtrate was confirmed by plating it on tryptic soy agar. The vaccine was stored at -20°C, until use.

Immunization of hens: Forty, 36 weeks old Single Comb White Leghorn (SCWL) chickens were immunized intramuscularly at two different sites (0.5 mL per site) of breast muscles. Antigen was produced as described above with or without cells as the control. The bacteria were emulsified with an equal volume of Freund's complete adjuvant for the first immunization, Freund's incomplete adjuvant for booster immunization at 2 weeks and without adjuvant at 4 weeks after the initial immunization, as described by Ruan *et al.* (2005). The immunization dose was approximately 1.0×10^9 CFU of *E. coli* O78:K80 in a volume of 1.0 mL equally divided between two injection sites. Eggs were collected daily for 12 weeks starting from the day of initial immunization, stored at 4°C and processed within 7 days after laying.

Total serum protein and gamma globulins concentrations: Hens were bled every week for 12 weeks starting at primary immunization for determination of serum total protein and gamma globulins concentrations. Total serum protein concentration was determined by biuret method. The share of individual protein fractions was determined by electrophoresis in tapes of gelled cellulose-acetate (Cellologel®, MALTA Chemetron, Milan, Italy). Interrelation and absolute concentration of protein fractions were determined by related software.

Isolation of egg yolk antibody (IgY): The Water-Soluble Fraction (WSF) of egg yolk was isolated as described by Akita and Nakai (1992) with minor modifications. Briefly, egg yolk was separated from egg white and the yolk membrane was punched. Yolk without membrane was transferred into a graduated cylinder and mixed with six volumes of cold acidified distilled water (pH 2.5 adjusted with 0.1 M HCl). The mixture was then adjusted to pH 5.0-5.2 and incubated at 4°C for 12 h. Following centrifugation at 12000x g and 4°C for 20 min, the supernatant was considered as WSF and stored at -20°C, until use.

IgY powder preparation: For wide food application of IgY, IgY powder as a dried, concentrated and stable form for

long-term storage, may be suitable form for these intentions. To ensure that the results would not be confounded by the acidity, the WSF containing specific IgY with high activity levels and non-specific IgY as the control was neutralized with 0.1 M NaOH (adjusted to pH 7.0) and lyophilized by a freeze dryer (Freeze Dryer, DW 8030, Heto holten, Denmark) to obtain IgY powder.

Protein assay: Total protein concentration of WSF and IgY powder was determined by the Bradford method using Bovine Serum Albumin (BSA) (Sigma Chemical Co.) as the reference protein.

Enzyme-Linked Immunosorbent Assay (ELISA): Antibody specific activities in the sera and WSF were measured by ELISA as described by Sunwoo *et al.* (2002) with minor modification. A 96-well microtitre plate (Nunc MaxiSorp C Immunoplate) was coated overnight at 4°C with either 200 µL of 10⁶ CFUs of sonicated *E. coli* O78:K80 in carbonate-bicarbonate buffer (Sigma Chemical Co.) (0.05 M, pH 9.6). The plate was washed 3 times with washing solution containing 50 mM Tris buffered saline, pH 8.0 and 0.05% Tween 20 (TBS-T) (Sigma Chemical Co.). After washing, 200 µL of TBS-T was added to each well and incubated at 37°C for 30 min as a blocking step. Then, plate was washed with TBS-T and samples (100 µL) were added. Sera were diluted 1000x and the WSF, obtained by six times dilution of yolk was further diluted 167 times to obtain 1000x dilution. Diluted samples were added to the wells and incubated at 37°C for 90 min. Following incubation, the plate was washed 3 times with TBS-T and 100 µL of goat anti-chicken IgG conjugated with horseradish peroxidase (Sigma Chemical Co.), diluted 1:100000 with TBS-T, was added and incubated at 37°C for 90 min. The plate was then washed with TBS-T and incubated for 30 min with 100 µL of substrate solution (TMB/H₂O₂, 1.0:1.0) (Kirkegaard and Perry, Gaithersburg, MD). The reaction was stopped with addition of 100 µL of 2M H₂SO₄ and absorbance was measured at 450 nm using an automated spectrophotometer (Ultramicroplate Reader, ELx808, Bio-Tek instruments Inc. USA). Titres were reported as the mean ± standard error of the log₁₀ of the dilution in the OD of samples versus controls containing non-immunized hen's serum or WSF.

To measure total IgY concentration of the WSF and IgY powder, ELISA experiment was performed as described above with chicken IgG ELISA quantitation kit (Bethyl Laboratories, Inc. USA), except that microtitre plate was coated with 100 µL of goat anti-chicken IgG at a final concentration of 10 µg mL⁻¹. Samples of WSF were diluted 1:20000 with TBS-T. Specific or non-specific IgY powder was reconstituted (10 µg mL⁻¹) and diluted with

TBS-T (1:20000). Two-fold serial dilutions of chicken reference serum (6.25 mg mL⁻¹ IgG) in TBS-T (200-3.12 ng mL⁻¹) were used as standards to provide a relative measurement of total IgY concentration.

Growth inhibition assay: Experimental conditions utilized in our growth inhibition assays were standardized in preliminary experiments by modifications of previous reports (Lee *et al.*, 2002a; Sunwoo *et al.*, 2002).

The same strain of *E. coli* O78:K80 used as an antigen for immunizing chickens was subcultured on MacConkey agar (Merck, Darmstadt, Germany) plate at 37°C overnight and then suspended in TSB. The *E. coli* were grown with shaking at 180 rpm in TSB at 37°C overnight and the volume of 30% (v v⁻¹) of glycerol in TSB was added and stored at -80°C, until used. After subculturing of the bacteria on TSB at 37°C with shaking overnight, 0.5 mL of prepared bacterial culture were mixed with 50 mL of TSB and optical density was adjusted to 0.05 at 600 nm, corresponding to a cell density of approximately, 2.5 × 10⁷ CFU mL⁻¹. The turbidity of culture (OD at 600 nm) was measured by spectrophotometer (UNICO Spectrophotometer, 2100UV-vis USA) at 30 min intervals. The growth curve was plotted until reaching stationary phase (6 h).

Specific or non-specific IgY powder was reconstituted to 100, 200 and 300 mg mL⁻¹ with TSB. IgY solution was then centrifuged at 1500x g at 4°C for 20 min. The supernatant was taken and sterilized by using a 0.45-µm membrane filter. Two milliliters of specific or non-specific IgY solution were then added to the same volume of prepared *E. coli* O78:K80 culture and the mixtures were incubated at 37°C with shaking. Aliquots of the samples (100 µL) were taken at 0, 2, 4 and 6 h. Plate counts were performed by the Miles and Misra (1938) method on TSB agar plates in triplicate. The inoculated plates were incubated at 37°C overnight and colonies were counted to determine total number of Colony Forming Units (CFU) per mL of samples.

Statistical analysis: Bacterial growth inhibition was analyzed using the Student's t-test. Statistical analyses of measured traits on serum, WSF and IgY powder were done using the General Linear Models (GLM) procedure of Statistical Analyses Systems (SAS, 2001). A probability level of p<0.05 and p<0.01 were considered statistically significant and high significant, respectively.

RESULTS AND DISCUSSION

Although, the antimicrobial effects of IgY against numerous microbial species have been demonstrated,

Table 1: The alterations of Total Protein (TP) and gamma Globulins (G) concentrations of serum and gamma Globulins to Total serum Protein ratio (G/TP) during the immunization period

| Variables | Days | | | | | | | | | | | | |
|--------------------------|--------------------|--------------------|---------------------|---------------------|---------------------|--------------------|---------------------|---------------------|--------------------|--------------------|---------------------|---------------------|--------------------|
| | 0 | 7 | 14 | 21 | 28 | 35 | 42 | 49 | 56 | 63 | 70 | 77 | 84 |
| TP (g dL ⁻¹) | 5.80 | 6.15 | 6.00 | 5.77 | 5.85 | 6.40 | 5.80 | 5.68 | 5.85 | 5.94 | 5.83 | 5.55 | 5.75 |
| SE | 0.04 | 0.06 | 0.07 | 0.20 | 0.04 | 0.07 | 0.18 | 0.21 | 0.20 | 0.04 | 0.02 | 0.07 | 0.25 |
| G (g dL ⁻¹) | 1.35 ^a | 1.74 ^b | 1.90 ^{ab} | 1.75 ^b | 1.90 ^{ab} | 2.18 ^a | 1.90 ^{ab} | 1.81 ^b | 1.68 ^b | 1.68 ^b | 1.74 ^b | 1.67 ^b | 1.62 ^c |
| SE | 0.02 | 0.03 | 0.05 | 0.10 | 0.06 | 0.02 | 0.05 | 0.06 | 0.10 | 0.01 | 0.10 | 0.05 | 0.03 |
| G/TP | 23.30 ^a | 28.30 ^d | 31.30 ^{bc} | 30.30 ^{bc} | 32.50 ^{ab} | 34.10 ^a | 33.10 ^{ab} | 31.90 ^{bc} | 28.70 ^d | 28.30 ^d | 29.80 ^{bc} | 30.10 ^{bc} | 28.20 ^d |
| SE | 0.60 | 0.21 | 0.56 | 0.53 | 0.76 | 0.07 | 0.46 | 0.28 | 0.39 | 0.38 | 0.60 | 0.14 | 0.32 |

Chickens were injected IM with sonicated *E. coli* O78:K80 on days 1, 14 and 28. Values are the mean of ten samples. **Means with different superscript letters are significantly different (p<0.05)

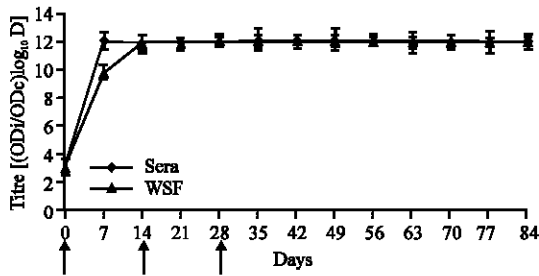


Fig. 1: The changes of antibody's activity in serum and egg yolk (both at 1:1000 dilution: D) of immunized laying hens with *E. coli* O78:K80, during the immunization period. The arrows indicate the immunization times. ODi/ODc means immunized sample's optical density to control's optical density ratio. Values are the mean±SE of forty samples from each week

however, information on using IgY powder as an effective and stable form of antibacterial agent is limited to its application against some bacterial species (Lee *et al.*, 2002b; Sunwoo *et al.*, 2002). Therefore, the investigation of antibacterial properties of IgY powder against specific dangerous bacterial species such as *E. coli* O78:K80, as an important cause of economically devastating diseases, is necessary.

After the first immunization, the concentration of gamma globulins and gamma globulins to total serum protein ratio increased (p<0.05) and did not decrease to pre-immunization level (day 0) throughout the experimental period (Table 1). As a similar trend, the antibody specific activity in serum and egg yolk increased and became plateau on day 7 and 14, respectively (Fig. 1). Specific activity of antibodies is affected by antigen immunogenicity, which in turn is influenced by several factors, such as antigen properties, dosage, the route of administration and the adjuvant used. Surface structures of *E. coli* O78:K80, as a serotype of APEC, such as Lipopolysaccharide (LPS), type 1 pilus adhesin (FimH), P pilus adhesin (PapG) and aerobactin outer membrane

receptor (IutA) are capable to produce high level of antibody (Kariyawasam *et al.*, 2004). Additionally, it has been reported that Freund's adjuvant has the capability of keeping high levels of serum titre for a longer period when laying hens were immunized with antigen compared to using antigen without adjuvant (Sun *et al.*, 2008). Therefore, conforming to previous reports, we assumed that *E. coli* O78:K80 assisting with Freund's adjuvant could have long term antigenic properties to induce an immune response in SCWL chickens.

Serum and egg yolk specific activity remained on the highest titre, from day 7 and 14, respectively, throughout the experimental period. Sunwoo *et al.* (2002) suggested that the change in specific activity is not remarkable after the second booster immunization, thus the need for the second booster injection is alleviated. However, in the present research, the ratio of gamma globulins to total serum protein was significantly increased after the second booster injection (Table 1) and reached to the highest level on day 35. Therefore, it was appeared to be exigent for development of high levels of antibody activity during the immunization period. The immune specific antibody appearance in the yolk was delayed compared to that in the serum after the first immunization. This delay is likely due to the gradual accumulation of IgY during the yolk formation period by selective active transport which was postulated by Kitaguchi *et al.* (2008). Despite a gradual increase in the IgY specific activity in egg yolk, this fourfold increase remained consistently high for 10 weeks.

As shown in Fig. 2, after the first immunization, protein concentration in WSF did not change significantly, but total IgY concentration and IgY purity (total IgY in protein) in the WSF, with increasing of gamma globulins to total serum protein ratio, were increased about 25% (p<0.05) and remained relatively constant until the end of the experiment. The average (±Standard Deviation) concentrations of protein and total IgY and IgY purity in the nonimmunized WSF were 4.341±0.2, 1.035±0.1 mg mL⁻¹ of WSF and 23.84%, respectively. During the immunization period, protein and total IgY concentration of immunized WSF were 4.46±0.22

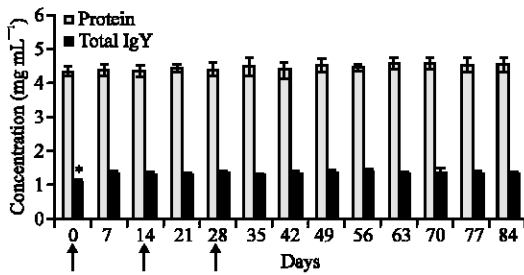


Fig. 2: Protein and total IgY concentrations of Water Soluble Fraction (WSF) obtained from non-immunized (day 0) and immunized egg yolk with *E. coli* O78:K80 during the experimental period. The arrows indicate the immunization time. Values are the mean±standard deviation of ten eggs in each week. *Indicates significantly lower concentration ($p < 0.05$)

and 1.31 ± 0.074 mg mL⁻¹ of WSF with 29.37% purity, indicating that 70.63% of the immunized WSF was from other livetins and lipoproteins. In contrast to the previous reports (Lee *et al.*, 2002a; Sunwoo *et al.*, 2002) suggesting total IgY concentrations in the WSF was relatively constant before and after the first immunization, the findings showed significant increase of IgY concentration in the WSF derived from serum gamma globulins. As already postulated this result indicates that all livetins of egg yolk correspond to proteins of serum, reported by Williams (1962).

Additionally, the concentration of total IgY in the immunized egg yolk was approximately, 7.86 mg mL⁻¹ of egg yolk. It has been indicated that the range of total IgY concentration in egg yolk from the immunized chicken is $8-25$ mg mL⁻¹ of egg yolk (Sunwoo *et al.*, 1996; Li *et al.*, 1998; Sunwoo *et al.*, 2002). In this research, the concentration of total IgY in immunized egg yolk was close to the lowest level reported. This variations reported may be due to the various methods of isolating IgY from egg yolk and measuring IgY concentration (Akita and Nakai, 1992), differences in the birds genetic lines or strains for some traits such as egg weight, egg yolk weight, the percentage of hen-day production and yolk total protein (Li *et al.*, 1998) and finally the differences among individuals in the same strain (Carlander *et al.*, 2001). The IgY purity of immunized WSF in the study was similar to Akita and Nakai (1993), who reported that the water-dilution method yielded IgY in the highest purity (31%) and with the similar activity compared to other methods. Results suggest that the simplicity and cost-effectiveness of water dilution method make this process as a promising way for a large-scale and long-term production of high purity IgY.

Table 2: Characteristics of IgY powder prepared from the Water-Soluble Fraction (WSF) containing *E. coli* O78:K80-specific and non-specific IgY

| IgY powder | Protein (mg g ⁻¹) | Total IgY (mg g ⁻¹) | Purity (%) |
|---|-------------------------------|---------------------------------|--------------------|
| Anti- <i>E. coli</i> O78:K80 specific IgY | 475 | 96.40 ^a | 20.30 ^a |
| SE | 7.28 | 2.63 | 0.02 |
| Non-specific IgY | 463 | 72.50 ^b | 15.70 ^b |
| SE | 5.7 | 1.45 | 0.01 |

Values are the mean of ten samples. ^{a, b}Means with different superscript letters in each column are significantly different ($p < 0.05$)

Protein concentration of specific and non-specific IgY powder were similar (Table 2), however, total IgY concentration and IgY purity of specific IgY powder were approximately 30% higher than the non-specific IgY powder ($p < 0.05$). Furthermore, the purity of specific IgY powder was approximately 9% lower than that of the immunized WSF. In reconstruction step of the assay, IgY powder was not dissolved completely. Therefore, the reduction in the purity of IgY powder resulted from freeze-drying that decreased the solubility and ELISA readings of IgY, as reported by Sunwoo *et al.* (2002). Nevertheless, freeze-drying appears to be more appropriate for production of IgY powder, as it yields higher powder with lower moisture and higher antibody titer than spray drying (Yokoyama *et al.*, 1992). Results of the present study postulate that the increase of gamma globulins to total serum protein ratio, after the first immunization and the reflection of this raising on WSF purity (Fig. 2) could be reason of higher specific IgY powder purity than non-specific IgY powder.

The growth curves of *E. coli* O78:K80, which was plotted under the same condition with the growth inhibitory assay, consisted of lag (0-2 h of incubation time), exponential (2-6 h of incubation time) and stationary phase. As shown in Fig. 3, lower levels of specific IgY powder had no inhibitory effect on bacterial growth compared to the non-specific (control) group. At the concentration of 100 mg mL⁻¹ of specific IgY powder, bacterial growth slightly decreased after 6 h of incubation (0.23 log CFU mL⁻¹). During 2-4 h of incubation time, the number of *E. coli* O78:K80 increased by 0.5 log CFU mL⁻¹ and 1.3 log CFU mL⁻¹ in the presence of 150 mg mL⁻¹ of the specific and non-specific IgY powder, respectively, indicating that bacteria in the specific treatment group proliferated 2.6 times less than the control group ($p < 0.01$). Also, in this level, cell counts of the specific group were reduced ($p < 0.01$) by 1.18 log CFU mL⁻¹ in comparison to that of the control group at 6 h of incubation time. Similarly, Sunwoo *et al.* (2002) reported that in the presence of 180 mg mL⁻¹ of specific IgY powder, the number of *E. coli* O157:H7 decreased significantly, but non-specific IgY powder had no inhibitory effects. Binding of specific IgY to bacterial surface components

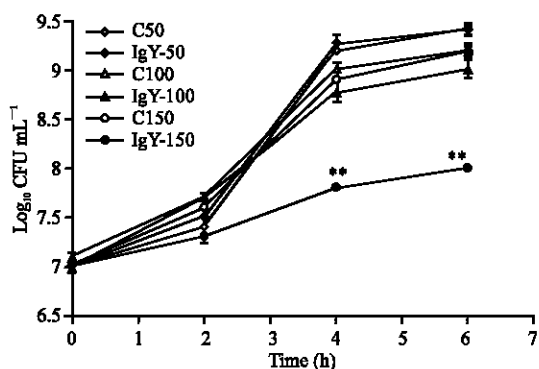


Fig. 3: Growth inhibition curves of *E. coli* O78:K80 (approximately, 1×10^7 CFU mL⁻¹) in the presence of the different concentrations of specific and non-specific (control: C) IgY powder (50, 100 and 150 mg mL⁻¹). Values are the mean \pm SE of triplicate samples. **Indicates high significantly lower counts ($p < 0.01$)

and structural alterations of the bacterial surface have been suggested as the mode of action of specific IgY to inhibit bacterial growth (Lee *et al.*, 2002b; Sunwoo *et al.*, 2002). The findings also, suggest that the inhibitory effect of specific IgY powder was dose-dependent and the remarkable increase in the antibody specific activity of immunized WSF and a considerable increase in the total IgY concentration and IgY purity of specific IgY powder caused significant decrease in proliferation of bacteria, in comparison to the control group. Using the simple and practical immunization protocol, it is suggested that IgY could be a suitable alternative in food applications to promote antibacterial effects.

CONCLUSION

The present study used *E. coli* O78:K80 as a model and the major cause of associated septicemic disease in broiler chickens and it may be useful to evaluate effectiveness against a wider range of pathogenic species. *E. coli* O78:K80 assisting with Freund's adjuvant could have long term antigenic properties to induce an immune response in laying hens. The large-scale and long-term production of IgY, with high purity and high specific activity, can be attained by water dilution method as a simple, practical and economical process. Specific IgY powder at the concentration of 150 mg mL⁻¹ decreased bacterial proliferation significantly. This inhibitory effect may assure IgY powder to be applied as a feed additive to confer novel protection against *E. coli* O78:K80 in protecting humans, chickens and farm animals from gastrointestinal infection, associated septicemia and bacterial resistance to antibiotics caused by *E. coli* O78:K80.

REFERENCES

- Adiri, R.S., U. Gophna and E.Z. Ron, 2003. Multilocus Sequence Typing (MLST) of *Escherichia coli* O78 strains. FEMS Microbiol. Lett., 222: 199-203. DOI: 10.1016/S0378-1097(03)00295-7.
- Akita, E.M. and S. Nakai, 1992. Immunoglobulins from egg yolk: Isolation and purification. J. Food Sci., 57: 629-634. DOI: 10.1111/j.1365-2621.1992.tb08058.x.
- Akita, E.M. and S. Nakai, 1993. Comparison of four purification methods for the production of immunoglobulins from eggs laid by hens immunized with an enterotoxigenic *E. coli* strain. J. Immunol. Methods, 160: 207-214.
- Altekruse, S.F., F. Elvinger, K.Y. Lee, L.K. Tollefson, E.W. Pierson, J. Eifert and N. Sriranganathan, 2002. Antimicrobial susceptibilities of *Escherichia coli* strains from a turkey operation. J. Am. Vet. Med. Assoc., 221: 411-416. DOI: 10.2460/javma.2002.221.411.
- Babai, R., G. Blum-Oehler, B.E. Stern, J. Hacker and E.Z. Ron, 1997. Virulence patterns from septicemic *Escherichia coli* O78 strains. FEMS Microbiol. Lett., 149: 99-105. DOI: 10.1111/j.1574-6968.1997.tb10315.x.
- Bach, S.J., T.A. McAllister, D.M. Veira, V.P.J. Gannon and R.A. Holley, 2002. Transmission and control of *Escherichia coli* O157:H7: A review. Can. J. Anim. Sci., 82: 475-490. <http://ginkgo.cisti.nrc.ca/RPAS/tpv?hm=HInit&journal=cjas&volume=82&afpf=A02-021.pdf>.
- Barnes, J.H., J.P. Vaillancourt and W.B. Gross, 2003. Colibacillosis. In: Saif, Y.M., H.J. Barnes, J.R. Glisson, A.M. Fadly, L.R. McDougald and D.E. Swayne (Eds.). Diseases of Poultry. Iowa State University Press, Ames, IA, pp: 631-652.
- Carlander, D., M. Wilhelmson and A. Larsson, 2001. Limited day to day variation of IgY levels in eggs from individual laying hens. Food Agric. Immunol., 13: 87-92. DOI: 10.1080/09540100120055657.
- Ewers, C., C. Schuffner, R. Weiß, G. Baljer and L.H. Wieler, 2004a. Molecular characteristics of *Escherichia coli* serogroup O78 strains isolated from diarrheal cases in bovines urge further investigations on their zoonotic potential. Mol. Nutr. Food Res., 48: 504-514. DOI: 10.1002/mnfr.200400063.
- Ewers, C., T. JanBen, S. KieBling, H.C. Philipp and L.H. Wieler, 2004b. Molecular epidemiology of Avian Pathogenic *Escherichia coli* (APEC) isolated from colisepticaemia in poultry. Vet. Microbiol., 104: 91-101. DOI: 10.1016/j.vetmic.2004.09.008.
- Gagliardi, J.V. and J.S. Karns, 2000. Leaching of *Escherichia coli* O157:H7 in diverse soils under various agricultural management practices. Appl. Environ. Microbiol., 66: 877-883. <http://aem.asm.org/cgi/content/abstract/66/3/877>.

- Gomis, S.M., C. Riddell, A.A. Potter and B.J. Allan, 2001. Phenotypic and genotypic characterization of virulence factors of *Escherichia coli* isolated from broiler chickens with simultaneous occurrence of cellulitis and other colibacillosis lesions. *Can. J. Vet. Res.*, 65: 1-6. <http://www.ncbi.nlm.nih.gov/pubmed/11227188>.
- Gomis, S., L. Babiuk, D.L. Godson, B. Allan, T. Thrush, T.H. Ownsend, P. Willson, E. Waters, R. Hecker and A. Potter, 2003. Protection of chickens against *Escherichia coli* infections by DNA containing CpG motifs. *Infect. Immun.*, 71: 857-863. DOI: 10.1128/IAI.71.2.857-863.2003.
- Gophna, U., T.A. Oelschlaeger, J. Hacker and E.Z. Ron, 2001. Yersinia HPI in septicemic *Escherichia coli* strains isolated from diverse hosts. *FEMS Microbiol. Lett.*, 196: 57-60. DOI: 10.1111/j.1574-6968.2001.tb10540.x.
- Gottstein, B. and E. Hemmeler, 1985. Egg yolk immunoglobulin Y as an alternative antibody in the serology of echinococcosis. *Z. Parasitenkd.*, 71: 273-276.
- Kariyawasam, S., B.N. Wilkie and C.L. Gyles, 2004. Resistance of broiler chickens to *Escherichia coli* respiratory tract infection induced by passively transferred egg-yolk antibodies. *Vet. Microbiol.*, 98: 273-284. DOI: 10.1016/j.vetmic.2003.10.022.
- Kim, W.K. and P.H. Patterson, 2003. Production of an egg yolk antibody specific to microbial uricase and its inhibitory effects on uricase activity. *Poult. Sci.*, 82: 1554-1558. <http://ps.fass.org/cgi/content/abstract/82/10/1554>.
- Kitaguchi, K., K. Osada, F. Horio and A. Murai, 2008. Exclusion of polymeric immunoglobulins and selective immunoglobulin Y transport that recognizes its Fc region in avian ovarian follicles. *Vet. Immunol. Immunopathol.*, 121: 290-299. DOI: 10.1016/j.vetimm.2007.10.006.
- Knezevic, P. and O. Petrovic, 2008. Antibiotic resistance of commensal *Escherichia coli* of food-producing animals from three Vojvodinian farms, Serbia. *Int. J. Antimicrob. Agents.*, 31: 360-363. DOI: 10.1016/j.ijantimicag.2007.10.020.
- Kudva, I.T., K. Blanch and C.J. Hovde, 1998. Analysis of *Escherichia coli* O157:H7 survival in ovine or bovine manure and manure slurry. *Appl. Environ. Microbiol.*, 64: 3166-3174. <http://aem.asm.org/cgi/content/abstract/64/9/3166?maxtoshow=&HITS=10&hits=10&RESULTFORMAT=&fulltext=cfu&searchid=1&FIRSTINDEX=680&resourcetype=HWFIF>.
- Kwaga, J.K., B.J. Allan, J.V. Van Der Hurk, H. Seida and A.A. Potter, 1994. A carAB mutant of avian pathogenic *Escherichia coli* serogroup O₂ is attenuated and effective as a live oral vaccine against colibacillosis in turkeys. *Infect. Immun.*, 62: 3766-3772. <http://iai.asm.org/cgi/content/abstract/62/9/3766>.
- Lampang, K.N., V. Chongsuvivatwong, R. Teanpaisan and V. Kitikoon, 2008. Concordance of antibiotic resistance and ERIC-PCR DNA fingerprint pattern in *Escherichia coli* isolated from farmer and broiler in the same farm. *World J. Microbiol. Biotechnol.*, 24: 1741-1746. DOI: 10.1007/s11274-008-9667-9.
- La Ragione, R.M. and M.J. Woodward, 2002. Virulence factors of *Escherichia coli* serotypes associated with avian colisepticaemia. *Res. Vet. Sci.*, 73: 27-35. DOI: 10.1016/S0034-5288(02)00075-9.
- La Ragione, R.M., A. Narbad, M.J. Gasson and M.J. Woodward, 2004. *In vivo* characterization of *Lactobacillus johnsonii* FI9785 for use as a defined competitive exclusion agent against bacterial pathogens in poultry. *Lett. Appl. Microbiol.*, 38: 197-205. DOI: 10.1111/j.1472-765X.2004.01474.x.
- Lee, E.N., H.H. Sunwoo, K. Menninen and J.S. Sim, 2002a. *In vitro* Studies of Chicken Egg Yolk Antibody (IgY) Against *Salmonella enteritidis* and *Salmonella typhimurium*. *Poult. Sci.*, 81: 632-641. <http://ps.fass.org/cgi/content/short/81/5/632>.
- Lee, K.A., S.K. Chang, Y.J. Lee, J.H. Lee and N.S. Koo, 2002b. Acid stability of anti-*Helicobacter pylori* IgY in aqueous polyol solution. *J. Biochem. Mol. Biol.*, 35: 488-493.
- Li, X., T. Nakano, H.H. Sunwoo, B.H. Paek, H.S. Chae and J.S. Sim, 1998. Effects of egg and yolk weights on yolk antibody (IgY) production in laying chickens. *Poult. Sci.*, 77: 266-270. <http://ps.fass.org/cgi/content/abstract/77/2/266>.
- Miles, A.A. and S.S. Misra, 1938. The estimation of the bactericidal power of the blood. *J. Hygiene.*, 38: 732-749.
- Miles, T.D., W. McLaughlin and P.D. Brown, 2006. Antimicrobial resistance of *Escherichia coli* isolates from broiler chickens and humans. *BMC Vet. Res.*, 2: 7-9. DOI: 10.1186/1746-6148-2-7.
- Mishra, A., B.L. Benham and S. Mostaghimi, 2008. Bacterial Transport from Agricultural Lands Fertilized with Animal Manure. *Water. Air. Soil. Pollut.*, 189: 127-134. DOI: 10.1007/s11270-007-9561-3.

- Otani, H., K. Matsumoto, A. Saeki and A. Hosono, 1991. Comparative studies on properties of hen egg yolk IgY and rabbit serum IgG antibodies. *Lebensm. Wiss. Technol.*, 24: 152-158.
- Ron, E.Z., Z. Yerushalmi and M.W. Naveh, 1991. Adherence Pili of *E. coli* O78 Determine Host and Tissue Specificity. In: Ron, E.Z. and S. Rottem (Eds.). *Microbial Surface Components and Toxins in Relation to Pathogenesis*. Plenum Press, New York, pp: 61-68.
- Ruan, G.P., L. Ma, X.W. He, M.J. Meng, Y. Zhu, M.Q. Zhou, Z.M. Hu and X.N. Wang, 2005. Efficient production, purification and application of egg yolk antibodies against human HLA-A*0201 heavy chain and light chain (β 2m). *Protein Exp. Purif.*, 44: 45-51. DOI: 10.1016/j.pep.2005.03.013.
- SAS, 2001. *Statistical Analysis Systems User's Guide*. Version 8.02 Edn. SAS Institute Inc., Cary, NC, USA.
- Shimizu, M., H. Nagashima, K. Sano, K. Hashimoto, M. Ozeki, K. Tsuda and H. Hatta, 1992. Molecular stability of chicken and rabbit immunoglobulin G. *Biosci. Biotech. Biochem.*, 56: 270-274.
- Sun, J.H., Z. Jiang and S. Hu, 2008. Effect of four adjuvants on immune response to F4 fimbriae in chickens. *Vet. Immunol. Immunopathol.*, 121: 107-112. DOI: 10.1016/j.vetimm.2007.09.021.
- 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*. *Poult. Sci.*, 75: 342-345.
- Sunwoo, H.H., E.N. Lee, K. Menninen, M.R. Suresh and J.S. Sim, 2002. Growth inhibitory effect of chicken egg yolk antibody (IgY) on *Escherichia coli* O157:H7. *J. Food Sci.*, 67: 1486-1494. DOI: 10.1111/j.1365-2621.2002.tb10310.x.
- Tini, M., U.R. Jewell, G. Camenisch, D. Chilov and M. Gassmann, 2002. Generation and application of chicken egg-yolk antibodies. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.*, 131: 569-574. DOI: 10.1016/S1095-6433(01)00508-6.
- Vandekerchove, D., P. De Herdt, H. Laevens and F. Pasmans, 2004. Colibacillosis in caged layer hens: Characteristics of the disease and the aetiological agent. *Avian Pathol.*, 33: 117-125. DOI: 10.1080/03079450310001642149.
- Wilkie, D.C., A.G. Van Kessel, T.J. Dumonceaux and M.D. Drew, 2006. The effect of hen-egg antibodies on *Clostridium perfringens* colonization in the gastrointestinal tract of broiler chickens. *Prev. Vet. Med.*, 74: 279-292. DOI: 10.1016/j.prevetmed.2005.12.004.
- Williams, J., 1962. Serum proteins and the livetins in hen's-egg yolk. *Biochem. J.*, 83: 346-355.
- Yokoyama, H., R.C. Peralta, R. Diaz, S. Sendo, Y. Ikemori and Y. Kodama, 1992. Passive protective effect of chicken egg yolk immunoglobulins against experimental enterotoxigenic *Escherichia coli*. *Infect. Immun.*, 60: 998-1007. <http://iai.asm.org/cgi/content/abstract/60/3/998>.