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Specific Chicken Egg Yolk Antibodies against Enterotoxigenic *Escherichia coli* K99 in Serum and Egg Yolk of Immunized Laying Hens

Research Article

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ABSTRACT

Enterotoxigenic *Escherichia coli* K99 is one of the dominant pathogens associated with diarrhea of calves. Immunoglobulin Y (IgY), has been used as an inexpensive alternative to antibiotics for the prevention and therapy of several bacterial infections. The study aimed to prepare IgY antibodies against *E. coli* K99 and to investigate its *in vitro* effectiveness. *E. coli* K99 was grown in the tryptic soy broth, and the bacterial suspension was inactivated by formaldehyde. Thirty White Leghorn hens allocated to control and treatment groups. 1 mL of the prepared bacterial suspension or sterilized physiological serum emulsified with Freund's complete adjuvant was injected at two weeks interval to the hens in treated or control group. The total IgY was purified with polyethylene glycol (PEG) 6000 from egg yolks (EY). Purified IgY fractions were processed for protein concentrations by Bradford assay. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to analyze purified IgY fractions. An indirect enzyme-linked immunosorbent assay (ELISA) method was used to measure the specific IgY titers in serum and EY. The ability of non-specific and anti-*E. coli* K99 IgY antibody at 100 and 200 mg/mL concentration was evaluated by growth inhibition assay *in vitro*. According to the ELISA data, total IgY concentration in serum and EY of control was relatively constant, while increased in the treatment group ($P<0.05$). The level of binding activity of specific IgY in serum and EY increased in immunized hens ($P<0.05$). Specific IgY had the highest activity for bacterial inhibiting growth at the level of 200 mg/mL. These results suggested the prepared IgY antibodies could inhibit *E. coli* K99 *in vitro* growth.

KEY WORDS chicken antibodies, *E. coli*, IgY, passive immunity.

INTRODUCTION

Some strains enterotoxigenic *Escherichia coli* (ETEC) that produce both K99 fimbriae and heat stable type I enterotoxin (STa) antigens have been considered as one of the dominant pathogens associated with diarrhea of neonatal calves. *E. coli* K99 fimbriae adhere to receptors on the surface of the small intestinal epithelium to facilitate the bacterial cell binding and colonization, while their STa enterotoxin lead to damage of the intestinal epithelium and disrupt gut fluid osmolality, inducing watery diarrhea, dehydration, and neonatal calf acidosis (Bi *et al.* 2017). Consequently, the diarrheal infection causes high mortality and morbidity rate among neonatal calves and increases the economic cost of cattle production through worldwide (Hashish *et al.* 2016). During a study conducted in Iran, the prevalence of *E. coli* strains in calves with diarrhea was reported 76.45% approximately (Shahrani *et al.* 2014). According to this report, the majority of the isolated *E. coli* strains were resistant to more than one antibiotic.

Strategies to reduce mortality and morbidity after observing scours include mainly therapeutic use of antibiotics. However, with the growing problem of antibiotic resistance, the risk of ineffective treatment of infections has significantly grown, causing alarming situation throughout the world. As a result, laws are regulated to ban the use of antibiotics in livestock production, especially in developed countries. In this context, an urgent need for developing safe and effective alternatives to antibiotics are highly felt to prevent and treat infectious diseases (Bi *et al*. 2017).

Immunoglobulin Y (IgY), main antibody in chicken egg yolk (EY) and a functional counterpart of mammalian IgG, has been recently used as excellent alternative to antibiotics for prevention and therapy of several bacterial and viral infections (Li *et al*. 2017). A quantity of studies have reported that oral administration of IgY provides effective protection against a wide diversity of gastrointestinal pathogens such as ETEC, porcine epidemic diarrhea virus, bovine and human rotaviruses, and bovine coronavirus causing diarrhea in humans and animals (Li *et al*. 2016; Diraviyam *et al*. 2014). Specific IgY against *E. coli* O78:K80 has been shown to reduce bacterial growth by 1.18 log colony-forming unit (CFU)/mL *in vitro* (Mahdavi *et al*. 2010). Also, feeding IgY increased growth performance in piglets, and improved health performance in calves (Owusu-Asiedu *et al*. 2003; Vega *et al*. 2011).

Specific IgY production using chicken possess many valuable benefits over mammalian IgG, as it is cost-effective, safe, conveniently produced with high yields and non-invasive. Also, oral consumption of IgY antibodies does not cross-react with the mammalian immune system, reducing the chance of antibody capture. Besides, this eco-friendly antibody provides no adverse effects, disease resistance and toxic residue in animal (Li *et al*. 2016). Because of the invariability of IgY during food processing, it can be used in animal supplements (Amro *et al*. 2018). Hence, these benefits allow IgY a valuable and available tool for diagnostic, therapeutic and preventive purposes (Jiang *et al*. 2016). According to IgY benefits, the high prevalence of K99 associated calf diarrhea and the importance of maintaining the health of calves, the purpose of this research was to investigate the possibility of producing specific IgY antibody against *E. coli* K99 and to study its biochemical and immunological properties and antibacterial effects on the bacterial growth *in vitro*. This study is the first step of biomanufacturing an antidiarrheal product on industrial scale.

MATERIALS AND METHODS

Antigen preparation

E. coli strain K99 used in this study was provided by Dr Yahya Tahamtan, Razi Vaccine and Serum Research Institute Shiraz Branch, Iran. K99 strain was cultured in the tryptic soy broth (TSB) medium for 18-24 hr, at 37 ˚C under aerobic conditions. The freshly grown bacterial cultures were adjusted with sterilized PBS to achieve 1.5 × 10^8 CFU/mL (McFarland inde×No 1) and then centrifuged at 3000 × g at 25 ˚C for 15 min. The collected bacterial sediment was washed thrice with PBS (0.1 N, pH 7.0). The bacterial suspension was inactivated by overnight incubation with 0.5% formaldehyde, centrifuged as above, and recovered pellet washed thrice with PBS. The inactivated bacterial pellet was freeze-dried and stored at -20 ˚C until use.

Chicken immunization

A total of 30 White Leghorn hens, 37-38 week old, were kept under room temperature (25±2 ˚C), humidity at 50% and a 16:8 h light:dark oscillations, with water *ad libitum* and food restriction (110 g/hen/day) during the entire study, at the animal lab, Department of Animal Science, College of Agriculture and Natural Resources, University of Tehran, Karaj. The birds were randomly distributed in 6 cages of 5 birds each. After distributing, the cages were allocated to control and treatment groups (3 cages/group). For primary immunization, 1 mL of the prepared bacterial suspension emulsified with 1 mL Freund's complete adjuvant was injected into the breast muscle of the treated hens on both sites (at 0.5 mL each). For the control group, 1 mL of sterilized physiological serum emulsified with 1 mL Freund's complete adjuvant was intramuscularly injected per hen (similar to the treatment group). Two booster immunizations were followed using Freund's incomplete adjuvant with two weeks of the time interval. Blood samples were collected from the wing vein every 2 weeks, and the eggs laid by birds were collected, marked and stored daily at 4 ˚C from the first immunization to the end of the study (10 weeks) until further processing.

Isolation and purification of IgY

The collected EY from immunized hens were pooled, and the total IgY was purified with polyethylene glycol (PEG) 6000 precipitation method (He *et al*. 2015). The protein (IgY) concentrates obtained at the final step were stored with 0.1% Sodium azide at 4 ˚C.

Protein concentrations

The purified IgY fractions were processed for total protein concentrations by Bradford assay (Bradford, 1976), and their approximate molecular weight determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses (Weber and Osborn, 1969).

Electrophoresis

SDS-PAGE was applied to analysis the purified IgY fractions, according to the protocol of Weber and Osborn

(1969). SDS gel electrophoresis (Bio-Rad, CA, USA) was performed on 4% stacking gel and 12% separating gel, at 60 and 120V, respectively. The gels were stained and observed after Coomassie brilliant blue R-250 staining according to standard protocols (Zhen *et al*. 2008).

Indirect ELISA

In order to measure the specific IgY titers in serum and EY, an indirect ELISA method was used as explained by Zhang *et al.* (2016). In brief, a 96-well ELISA microplate (Biomat, Italy) was coated with 100 µL of *E. coli* K99 whole-cell suspension in 0.05 M carbonate-bicarbonate buffer (pH 9.6). After incubating for 18 h at 4 ˚C, the wells were rinsed thrice with 10 mM PBS solution and each well was filled with 150 µL solution of 2.5% BSA (Sigma-Aldrich Co., Missouri), as a blocker and incubated for one hour at room temperature (20 to 25 ˚C). 100 µL of a serially diluted serum, EY and IgY fractions in PBS- Tween 20 solution (0.05%; PBS-T) were added to the wells and the plates then incubated at room temperature for 30 min. After re-washing the plates with PBS-T, 100 µL of horseradish peroxidase conjugated goat anti-chicken IgG (Abcam-UK) was added and incubated at room temperature for 30 min. Finally, the plates were re-rinsed with PBS-T and 100 µL of the TMB (3,3′,5,5′-tetramethylbenzidine, 1%, w/v) was added as a substrate and incubated at 25 ˚C for 10 min. After that, 100 µL of 1N of HCl was added into the wells to end the reaction, and the optical density (OD) at 450 nm was read on a microplate reader (BioRad, USA).

Growth inhibition assay

The modified method of Lee *et al.* (2002) was used to evaluate the ability of non-specific and anti-*E. coli* K99 specific IgY antibody to prevent the bacterial growth *in vitro*. The specific IgY antibodies purified from the EY were adjusted at the concentrations of 0, 100, 200 mg/mL and filter sterilized using 0.22 um millipore filter membranes. Nonspecific IgY and PBS were applied as negative or blank control. 1 mL of the prepared IgY fractions were added to the same volume of the freshly grown *E. coli* K99 culture (1.5×10^8 CFU/mL) and the suspensions were incubated at 37 ˚C with shaking. 100 µL of samples were taken at 0, 2, 4, 8, and 24 hr time interval, and their optical density (ODs) were read at 600 nm. The number of viable bacteria in CFU/mL was determined by the standard pour plate method.

Statistical analysis

Normality of distribution was tested using the Shapiro-Wilk test of SAS 9.4 software (Razali *et al*. 2011). All data were analyzed using the general linear model procedure of SAS 9.4 software (SAS, 2004), and $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Biochemical properties of serum and egg yolk of immunized hens

The total IgY concentrations in the EY and serum of both control and treatment were estimated and compared. According to the results, the total IgY concentration in the serum was relatively constant (P>0.05) in the control group compared with treatment group where the IgY concentrations were significantly increased (P<0.05) 6 week after the initial immunization. At 4 and 8 weeks of immunization, there were significant differences between groups in the concentration of total serum IgY (P<0.05). As shown in Figure 1, the total protein concentration of EY in the control group had no changes over the experimental period (P>0.05). As expected, the total protein concentration of EY was statistically increased in the treatment group than the control group (P<0.05). Immunization of hens led to increase this variable at week 6 and 8 versus week 2 and 4, respectively (P<0.05). Similarly, significant differences in the total IgY concentration of EY were detected in the control group at different time, with significantly (P<0.05) increased concentrations 4 weeks after the immunization. However, no difference in the total IgY concentrations of EY in the control group in week 0, 2, and 8 (P>0.05) was recorded.

As shown in Figure 1, the total IgY concentration of EY reached a peak in the treatment group at 6th week after immunization of the hens, and this concentration remained relatively constant thereafter. Lowest concentration of IgY was found in the EY of treatment group at the first week of experiment than the following weeks (P<0.05). Injection of inactivated *E. coli* K99 to the hens led to significantly increase the IgY concentration of EY at week 6, 8, and 10 (P<0.05).

The results of our study indicated that immunizing hens with formaldehyde inactivated *E. coli* K99 whole cells can not only lead to increased total protein concentrations in the EY but also results in high total IgY yield. These results indicated that inactivated bacterial whole cells is able to active the humoral immunity for producing specific IgY in the immunized chickens.

Thus, the activated humoral immunity could be a reason for an increased total protein and IgY concentrations in the serum and EY in this group. However, the EY total protein level increased at week 6 and 8 after the first and second booster immunizations and then decreased to its level at week 0. These results disagree with those of Sunwoo *et al.* (2002) and Lee *et al*. (2002), who observed no difference in the total IgY concentration of EY from chickens immunized with *E. coli* O157:H7, and *Salmonella enteritidis* and *typhimurium* spp. whole cells in their experiments respectively.

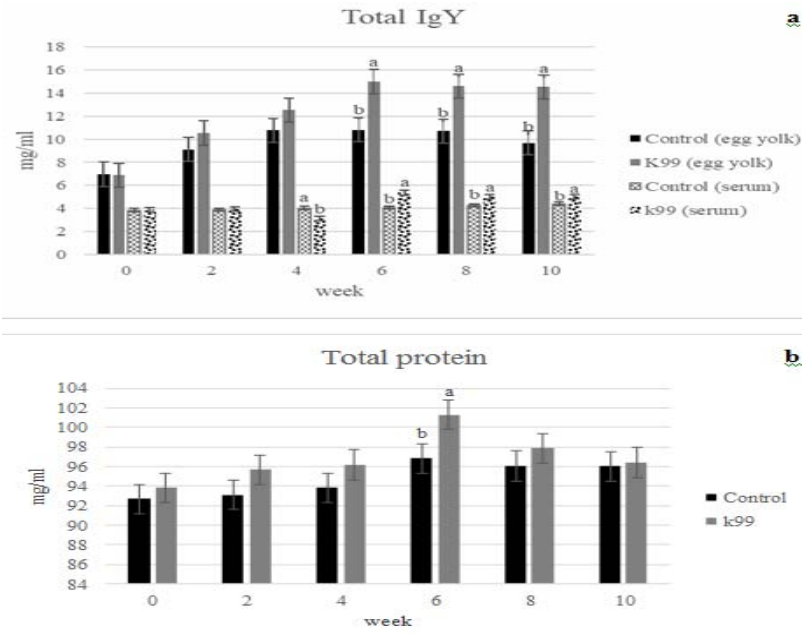

Figure 1 The concentrations of protein and total IgY
(a) Total IgY in the egg yolk and serum obtained from chickens immunized with *E. coli* K99 and (b) Total protein in the egg yolk of control and treatment groups
a, b: Means with different superscript letters are significantly different (P<0.05)

Liou *et al*. (2010) also compared the IgY production efficiency of commercial vaccine (CM) or isolated from a local farm (AF) antigen of *E. coli* and reported that the serum IgY titers induced by these antigens significantly increased 2 weeks after 1st injection and reached to a peak level after 3rd immunization, which is consistent with our results. Several factors including the initial immune situation of chickens, differences in the bird's genetic lines or strains, the percentage of hen-day production and yolk total protein, the differences among individuals in the same strain and the poor immunogenicities of inactivated *Salmonella enteritidis and typhimurium* maybe involve in these discrepancies. It has been recently shown that laying chicken breed has an important role in acquiring the highest level of IgY production (Amro *et al.* 2018). According to previous studies, the specific serum antibodies are transported to the EY with a delay of about 5-6 days (Schade *et al*. 2005). So, the lag between total protein levels in EY with another result, possibly related to transfer protein to the EY. It is noteworthy that the effect of an antigen immunogenicity is depended on several aspects such as animal species/strain, the antigen intrinsic attributes and frequency, amount and method of use of antigen or adjuvant type (Kuby, 1997). In our study, intramuscular injection of formaldehyde inactivated *E. coli* K99 whole cells which was emulsified with Freund's adjuvant led to stimulate immune system in White Leghorn chickens and produce specific antibodies.

In addition, it has been reported that Freund's adjuvant has the capability of keeping high levels of serum titer for a longer period when laying hens were immunized with antigen compared to using antigen without adjuvant. In our study, the concentration of total IgY was approximately 12.51 mg/mL in the EY of eggs obtained from treatment group, which is similar to its range (8-25 mg/mL of EY) as reported by previous studies (Schade *et al*. 2005). The total IgY concentration in EY of non-immunized chickens (control group) was also increased by the injection of Freund's adjuvant, which might be because of inducing an immune response in this group.

SDS-PAGE analysis

SDS-PAGE analysis under reducing conditions showed two main protein bands, corresponding to the molecular weights of approximately 66 and ~30 kDa, which are the heavy and light chains of IgY, respectively. Slight impurities were detected below 35-45 kDa and above 65-70 kDa (Figure 2). The specific IgYs produced against rabies virus (Sun *et al*. 2001), human rotavirus (Hatta *et al*. 1993), *Staphylococcus aureus* (Reddy *et al*. 2014) and *Schistosoma japonicum* (Cai *et al*. 2012) have been reported to vary in the molecular weights of light and heavy chains (in range of 15-30 and 55-70 KDa, respectively), which are similar to our results. These findings demonstrate a specific variability in molecular weights of anti-pathogen IgYs.

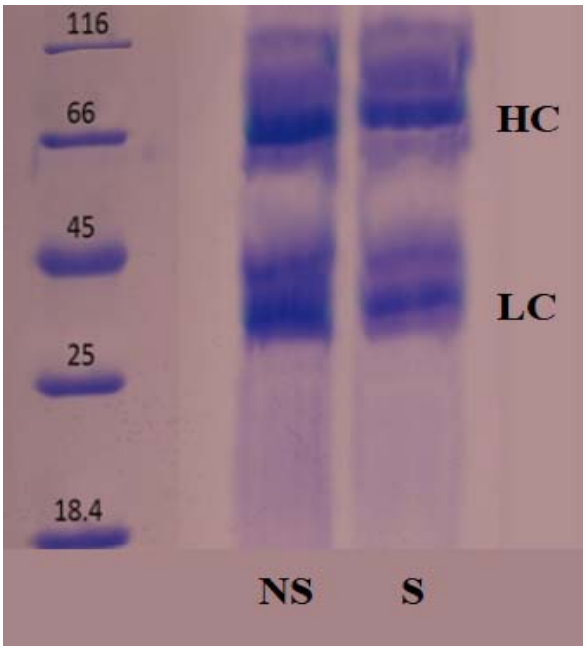

Figure 2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of anti-*E. coli* K99 IgY antibody after purification by PEG 6000 Lanes M: molecular weight marker (Fermentas IV,); 1 and 2: purified specific (S) and non-specific (NS) IgY antibody, respectively (HC: heavy chain and LC: light chain)

Specific IgY activity against K99

ELISA results (Figure 3) showed that the level of IgY-binding activity in blood serum and EY increased one week after the primary injection of inactivated bacteria and constantly elevated after the first and second booster injections. Figure 3 shows that the binding activity of anti-*E. coli* K99 IgY increased with high slope until 7th week and reached a peak (OD of 0.75 in blood serum and OD of 0.68 in EY) at 10th week.

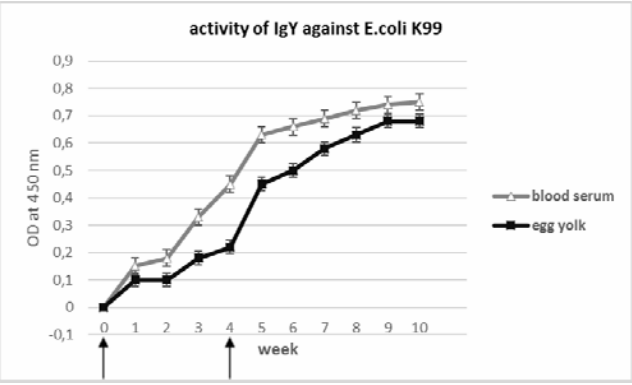

Figure 3 Specific activities of serum and egg yolk antibodies in *E. coli* K99 immunized chickens during the immunization period
Arrows indicate the time of injections

The initial immunization of chickens with inactivated *E. coli* K99 led to induce the primary response and increase the level of binding activity of IgY in EY characterized by a one-week lag phase. As previously stated (Li *et al*. 1998; Lee *et al*. 2002), the transfer and accumulation of antibodies generated against pathogens from serum to egg yolk take a while, demonstrating this lag. The secondary response occurred rapidly, and a greater level of binding activity of IgY obtained after the first and second booster immunizations. After an exponential rise, the OD value of 0.63 in the binding activity of IgY was attained. This IgY activity pattern can be due to the memory B cell activation after booster immunization, because the memory B cells than innate B cells produce antibodies rapidly and more robustly in face of the same antigen (Kuby, 1997). In addition, these results suggest that in order to have higher levels of antibody activity, it would be necessary to use booster doses in hens.

Growth inhibition assay of the generated IgYs

According to the obtained results (Figure 4), the growth of bacteria was affected by the concentration of specific and non-specific IgY antibodies. The specific IgY at level of 200 mg/mL statistically limited the *E. coli* growth in comparison with others over the period of incubation ($P<0.05$). Overall, viability of the bacteria in the presence of 200 mg/ml anti-*E. coli* K99 IgY was decreased by 1.0 log CFU/mL in comparison with that of the control group. Besides, 200 mg/mL non-specific IgY compared to the control led to decrease the bacterial growth after 6 hr of incubation ($P<0.05$). Also, at the level of 100 mg/mL, the non- and specific IgY did not statistically impact on the growth of *E. coli* K99 during incubation.

In our study, the anti-*E. coli* K99 IgY at level of 200 mg/mL had a higher inhibitory effect on the pathogen growth in a liquid medium.

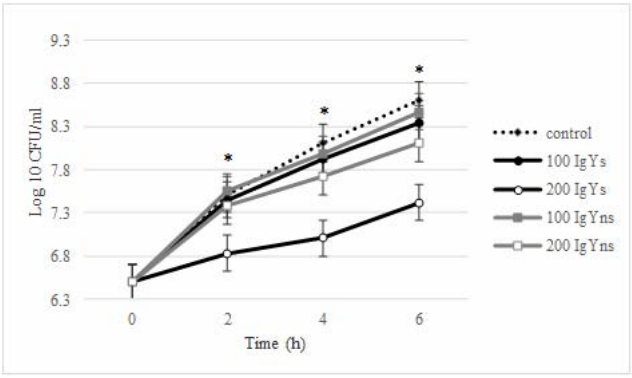

Figure 4 Growth inhibition curves of *E. coli* K99 in the presence of different concentrations (0, 100, and 200 mg/mL) of specific (s) and nonspecific (ns) IgY
* Indicates significantly lower counts ($P<0.05$)

This finding was similar with that of Sunwoo *et al.* (2002), who indicated that anti-*E. coli* O157:H7 IgY (180

mg/mL), but not non-specific IgY, decreased significantly the bacterial growth. Also, Mahdavi *et al*. (2010) showed that 150 mg/mL specific IgY reduced *E. coli* O78:K80 proliferation by 1.18 log CFU/mL compared with non-specific IgY at 6 h of the incubation period. In order to block or impair bacterial function, it is necessary that antibodies attach to the bacterial surface appendages responsible for motility and growth (Mahdavi *et al*. 2010). Since inactivated *E. coli* K99 whole cells was injected to the hens, IgY extracted in this study had the polyclonal properties against several surface epitopes. Thus, growth inhibition of pathogenic *E. coli* may be related to IgY-attaching activities against appendages of bacterial surface. Pereira *et al.* (2019) reported that IgY could inhibit bacterial growth and biofilm creation *in vitro* by binding to the pathogen. The bacterial function also can be limited by clumping of bacteria in reacting with the corresponding antibodies, a process called agglutination (Sădziene *et al.* 1992). Through this mechanism, the proliferation and motility of pathogen will be reduced because of the limited access to nutrients. It can be also noted that only media with high salt levels and or low pH lead to occurred IgY agglutinating feature (Kubo *et al*. 1973). Hence, it does not seem that the agglutination be a mechanism to limit bacterial growth. Finally, the IgY-attaching activity to bacteria as the major antibacterial property may involve in bacterial growth inhibition.

CONCLUSION

The injection of inactivated E. coli K99 to the hens induced humoral immunity and increased IgY antibodies against the pathogen level in serum and egg yolk. Moreover, booster doses were necessary to have higher levels of IgY activities. The specific IgY produced in this study at the level of 200 mg/mL reduced the *E. coli* K99 growth by 1.0 CFU/mL and had the highest inhibitory activity. These data suggested the prepared IgY antibodies could inhibit *in vitro* *E. coli* K99 growth and also the possibility of using IgY antibodies as a feed additive to confer protection against *E. coli* K99 that induced diarrhea in claves.

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