



Production, purification, and evaluation of quail immunoglobulin Y against *Salmonella typhimurium* and *Salmonella enteritidis*

Atefeh Esmailnejad^{a,*}, Bahman Abdi-Hachesoo^b, Elhamsadat Hosseini Nasab^a, Maryam Shakoori^a

^a Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran

^b Department of Clinical Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran

ARTICLE INFO

Keywords:

Salmonella enteritidis
Salmonella typhimurium
 IgY
 Quail

ABSTRACT

Salmonella species have been the major foodborne problems in food production systems, with *Salmonella enterica* serovars *typhimurium* (*S. typhimurium*) and *enteritidis* (*S. enteritidis*) being among the more common isolates. The oral administration of chicken egg yolk specific antibodies (IgYs) has been established as an efficient alternative for treatment and prevention of gastrointestinal pathogens including *Salmonella*. The present study was aimed to investigate the possible production of specific IgYs against *Salmonella typhimurium* and *Salmonella enteritidis* in quail egg yolks. *Salmonella* spp.-free female Japanese quails (*Coturnix coturnix japonica*) were intramuscularly immunized with formalin or heat-inactivated *Salmonella* immunogens (1.0×10^9 CFU/mL) emulsified with Freund adjuvants. Egg yolk IgYs were purified using ammonium sulfate precipitation method. Anti-*Salmonella* IgYs titer and specificity were determined using enzyme-linked immunosorbent assay (ELISA) and western blot analysis. *Salmonella* specific IgYs detected in the immunized quails were significantly higher than those of the control group, which confirmed the immunization procedure. Specific IgYs against *S. typhimurium* and *S. enteritidis* were identified in both groups immunized with heat or formalin-inactivated immunogens. However, formalin-inactivated immunogens induced relatively higher immune responses over the heat-inactivated ones. Quail anti-*Salmonella* IgYs showed a high specificity to their corresponding immunogens, with moderate cross-reactivity to other members of *Enterobacteriaceae* family. Quail can be regarded as a valuable and inexpensive source for producing large-scale of specific antibodies that can be used for immunodiagnostic and immunotherapeutic purposes.

1. Introduction

Salmonellosis is one of the most common infectious diseases all around the world, with *Salmonella enterica* serovars *typhimurium* (*S. typhimurium*) and *Salmonella enterica* serovars *Enteritidis* (*S. enteritidis*) being among the more common isolates associated with foodborne diseases and *Salmonella* outbreaks in human and animals (Chalghoumi et al., 2008; Lee et al., 2002). Genetic diversity among *Salmonella* spp. has enabled some serovars to survive against sudden environmental changes including the increases in the temperature, the decreases in pH and water activity, and also other radical changes that commonly occur in food production and processing systems (Herrera et al., 2013). Therefore, development of appropriate detection and control measures is essential to minimize the bacterial dissemination and disease progression.

Passive immunization has been successfully applied as an alternative method of prophylaxis and therapy against gastrointestinal

infections including *Salmonella*, *Escherichia*, *Campylobacter*, *Rotaviruses*, and *Coronaviruses* (Gürtler et al., 2004; Rahimi et al., 2007). The administration of specific antibodies has been prompted by the need to find an alternative to conventional antibiotic therapies, in response to the increasing number of antibiotic-resistant or nonresponsive organisms. Antibodies may exert a kind of antimicrobial activity through binding, neutralizing, and ultimately inhibiting the growth, replication, and dissemination of pathogens (Kovacs-Nolan and Mine, 2004). The oral administration of egg yolks containing specific polyclonal antibodies was successfully tested in mice, chickens, calves, and piglets (Gürtler et al., 2004; Rahimi et al., 2007; Yokoyama et al., 1998).

Chicken egg yolk antibody, referred to as immunoglobulin Y (IgY), is the functional equivalent of mammalian immunoglobulin G (IgG), the major antibody found in chickens' serum with a concentration of about 5.0 mg/mL. Chicken IgYs are transferred in large quantities from serum to the egg yolks (with a concentration of about 25 mg/mL) in order to give passive immunity to the developing offspring (Kovacs-Nolan and

* Corresponding author.

E-mail address: a.esmailnejad@shirazu.ac.ir (A. Esmailnejad).

Mine, 2004). The overall structure of chicken IgYs is somewhat similar to the mammalian IgG, with two heavy (H) and two light (L) chains. IgY possesses four constant domains in heavy chain (C_H1–C_H4) that makes them have a higher molecular weight (180 kDa) than mammalian IgG (159 kDa). The chicken IgY molecule is also more hydrophobic than the IgG, with a lower isoelectric point (Kassim et al., 2011; Kovacs-Nolan and Mine, 2004).

The use of chicken eggs as a source of specific polyclonal antibodies offers several advantages over producing antibodies in mammals. Harvesting antibodies from eggs laid by immunized chickens is much less invasive and only requires the collection of eggs rather than blood sampling in mammals. Furthermore, the sustained high and long-lasting antibody titer in chickens reduces the need for frequent booster injections and is, therefore, more advantageous in the consideration of animal welfare (Gürtler et al., 2004; Najdi et al., 2016; Schade et al., 1992). In contrast to IgG, IgY does not either interact with mammalian Fc receptors or activate the complement system, so it does not mediate inflammatory responses or interfere with immunological assays. Due to the long phylogenetic distance between avian and mammals, chickens can more efficiently produce antibodies against highly conserved mammalian proteins. This also requires much less antigen to prompt an effective immune response (Nilsson et al., 2012; Xu et al., 2011). Chickens, thus, can be considered as convenient, cost-effective, and rich sources of specific antibodies that have been applied successfully for immunodiagnostic, prophylactic, and therapeutic purposes in recent years.

Quails are mid-sized birds belonging to the *Phasianidae* family which are found in Asia, Africa, and some parts of Europe. Japanese quails (*Coturnix coturnix japonica*) are reared worldwide for both meat and egg production purposes (Ainsworth et al., 2010; Kassim et al., 2011). They are well adapted to the laboratory conditions and possess a number of advantages over laying hens, including rapid growth, high egg-laying intensity, early sexual maturity, and lower maintenance costs (Scholtz et al., 2010). However, quail IgY has an overall structure homologous to the chicken IgY and they are similar in several biological aspects (Bae et al., 2009). Based on the above-mentioned advantages, this study aimed to investigate the possible production of specific IgYs against *Salmonella typhimurium* and *Salmonella enteritidis* in Japanese quail egg yolks. Titer and specificity of the produced antibodies were evaluated using ELISA and western blot analysis methods.

2. Material and methods

2.1. Antigen preparation

Salmonella typhimurium and *Salmonella enteritidis* were grown in TSB medium at 37 °C for 24 h; then, subcultured in a fresh broth and incubated at 37 °C for 18 h. Cells were treated with 10% formalin at 37 °C for 3 h to obtain formalin-inactivated immunogens. Heat-inactivated cells were also prepared by heating the bacteria at 100 °C for 20 min. The complete killing of bacteria was confirmed by culturing on nutrient broth medium. Finally, cells were harvested by centrifugation at 9000 × g for 20 min and pellets were washed three times with sterile PBS. *S. typhimurium* and *S. enteritidis* concentrations were adjusted to 1.0 × 10⁹ CFU/mL with PBS and stored at –20 °C. *Escherichia coli*, *Mannheimia haemolytica*, and *Klebsiella pneumonia* were also used for evaluating the cross-reactivity and they were prepared as described above.

2.2. Immunization of quails

A total of 50 newly-hatched *Salmonella* spp.-free female Japanese quails (*Coturnix coturnix japonica*) were purchased from a local hatchery unit and divided into 5 groups, each containing 10 quails. Starting at 7 wk of age, the quails were maintained in 80 × 60 × 30 cm cages under constant temperature (27 ± 2 °C) with full access to water and feed ad libitum. The photoperiodicity was also controlled at 14 h lighting and

10 h darkness. The breeding conditions were approved by the Shiraz University Policy on Animal Care and Use. The birds were adapted to the new environment for two weeks prior immunization. *Salmonella* spp.-free status was confirmed by bacteriological analysis of cloacal swabs (ISO, 2002), bacterial culture, and PCR technique on arrival date. The mentioned tests were repeated every two weeks throughout the experiment. For immunization, 0.25 mL of whole bacterial suspension (1.0 × 10⁹ CFU/mL) was emulsified with an equal volume of Freund's complete adjuvant (Sigma Aldrich, St Louis, MO, USA) in the first immunization and 0.5 ml was injected into the quails' superficial pectoral muscles. Groups 1 to 4 were immunized as follows; group 1, formalin-inactivated *S. typhimurium* (FIST), group 2, heat-inactivated *S. typhimurium* (HIST), group 3, formalin-inactivated *S. enteritidis* (FISE), and group 4, heat-inactivated *S. enteritidis* (HISE). From the second to the fourth immunization, Freund's complete adjuvant was replaced with the Freund's incomplete adjuvant. In the fifth and sixth immunizations, the booster injections were carried out in the absence of adjuvant. Incomplete Freund's adjuvant is considered to be one of the most effective adjuvants that widely used in subsequent immunizations for consistently producing high titer antibodies. However, it may produce severe chronic local inflammation and causing skin ulcerations. Therefore, after four immunizations with Freund's adjuvants and in order to minimize these adverse effects, the adjuvant was removed in the last two immunizations. All immunizations were conducted at two weeks interval. The control group (group 5) was administered with 0.25 mL of sterile PBS plus 0.25 mL adjuvant, except for the last two immunizations that sterile PBS was injected without adjuvant. Eggs from each group were collected daily and stored at 4 °C until IgY purification was reached. The study was approved by Shiraz University Policy on Animal Care and Use.

2.3. IgY purification from eggs yolk

The isolation of anti-*S. typhimurium* and anti-*S. enteritidis* IgYs from egg yolks was carried out using a method proposed by Kitaguchi et al. (2008) with minor modifications (Kitaguchi et al., 2008). Briefly, egg yolk was carefully separated from albumin; then, the yolk's membrane was punctured and the whole yolk was allowed to drain into a glass dish and homogenized completely. Five gram of the well-mixed yolk was transferred into a sterile tube and diluted with 9 volumes of distilled dionized water acidified to pH 5.1 with 0.1 N HCl and stored at 4 °C overnight. After incubation, the mixture was centrifuged at 10,000 × g for 25 min at 4 °C and the supernatant was collected and gently mixed with an equal volume of 40% saturated ammonium sulfate. The mixture was left to stand for 4 h at room temperature and then centrifuged at 12,000 × g for 30 min at 4 °C. The supernatant was removed later on. The pellets were re-suspended in 5 ml sterile PBS and dialyzed against 20 mM phosphate buffer at 4 °C overnight. The dialyzed samples were stored at –20 °C and used for the determination of the anti-*Salmonella* IgYs concentration by ELISA. The IgY purity and yield of eggs were analyzed on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% resolving and 5% stacking gels. Gel staining was carried out with Coomassie blue solution (Bio-Rad Laboratories) and size standards were determined using Prestained Protein Ladder (Cat. No. PR901641, Cinna Clon, Iran) in the gel.

2.4. ELISA

The optimization of the anti-*S. typhimurium* and anti-*S. enteritidis* IgYs titers and specificity were conducted using a checkerboard titration of enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated with 100 µL/well of formalin or heat-inactivated *S. typhimurium* and *S. enteritidis* at concentrations ranging from 10¹⁰ to 10⁴ CFU/mL in 10 mM phosphate-buffered saline (PBS, pH 7.2) and incubated at 37 °C overnight. The uncoated surfaces were blocked using 200 µL/well of blocking solution [0.14 M NaCl, 50 mM Tris-HCl at pH

7.2 with 0.1% (vol/vol) Tween 20 and 3% (vol/vol) bovine serum albumin] and incubated at 37 °C for 2 h. The plates were washed five times with washing buffer PBST [0.14 M NaCl, 50 mM Tris-HCl at pH 7.2 with 0.05% (vol/vol) Tween 20] and incubated with 100 μ L/well of anti- *S. typhimurium* and anti-*S. enteritidis* IgYs diluted 100 times with blocking buffer at 37 °C for 1 h. Then, the plates were washed 5 times with PBST and incubated for 1 h with 100 μ L/well of horseradish peroxidase-conjugated rabbit anti-chicken IgG (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:10,000. The plates were washed five additional times and a color reaction was initiated by adding 100 μ L/well of tetramethylbenzidine (TMB) (Sigma Aldrich, St Louis, MO, USA) substrate and incubated at room temperature for 15 min. The reaction was stopped by adding 100 mL/well of 3 M H₂SO₄ and the optical density (OD) of each well was determined at 450 nm with a microtiter plate reader (Immunoskan BDSL, Thermo Lab. Systems, Finland). Defined chicken anti- *S. typhimurium* and anti- *S. enteritidis* antibodies stored at –70 °C were used as positive controls in each plate.

2.5. Cross-reactivity of IgY

The cross-reactivity of anti- *Salmonella* IgYs was determined using ELISA method as described by Kassim et al. ELISA plates were coated with formalin-inactivated cells of *Escherichia coli*, *Mannheimia haemolytica*, and *Klebsiella pneumonia* as antigens and anti- *Salmonella* IgYs prepared at 1:100 dilution were added to react with the coated antigens and determine any binding possibility. The cross-reactivity was evaluated by comparing the optical density obtained when the produced anti- *Salmonella* IgYs were used against above-mentioned non-corresponding antigens coated with those of corresponding antigens (*S. typhimurium* and *S. enteritidis*) which were considered as 100%.

2.6. Western blotting

The specificity of the produced anti-*S. typhimurium* and anti-*S. enteritidis* IgYs were also evaluated using Western blot analysis. The formalin-inactivated immunogens were electrophoresed on SDS-PAGE polyacrylamide gel using 12% resolving and 5% stacking gels; protein bands were transferred to nitrocellulose membrane using semi-dry blotting. Non-specific binding sites on the blotting membranes were blocked with sterile PBS containing 3% w/v skimmed milk and incubated at 4 °C overnight; then, the membranes were washed 3 times with PBST. Blotting membranes were incubated individually with either anti-*S. typhimurium* or anti-*S. enteritidis* IgYs diluted 20 times with blocking buffer for 1 h at 37 °C. The membranes were then washed 3 times with PBST to remove the excess antibody and incubated with horseradish peroxidase-conjugated rabbit anti-chicken IgG (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:10,000 for 30 min at 37 °C. Finally, the membranes were washed 3 additional times and binding of specific IgYs to the corresponding bacteria was visualized by staining the membranes with 0.05% diaminobenzidine (DAB) (Sigma Aldrich, St Louis, MO, USA) in 50 mM Tris pH 7.4 containing 0.05% H₂O₂.

2.7. Statistical analysis

All data were expressed as means \pm standard deviations (SD). Data were analyzed statistically by one-way analysis of variance (ANOVA) and Tukey's post hoc test to determine the degree of significance between the antibody titer of control and experimental groups, reactivity of specific antibodies (anti-*S. typhimurium* and anti-*S. enteritidis* IgYs) to their respective antigens (*S. typhimurium* and *S. enteritidis*), and other non-corresponding bacteria (*E. coli*, *M. haemolytica*, and *K. pneumonia*). A probability of $P < 0.05$ was considered statistically significant. Data were analyzed with SPSS software, version 16 for Windows (SPSS Institute, Chicago, IL, USA).

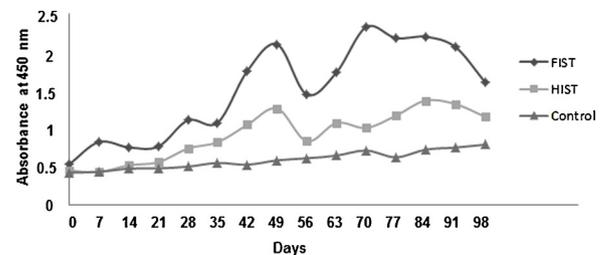


Fig. 1. Profile of anti- *S. typhimurium* formalin- (FIST) and heat- (HIST) inactivated IgYs in quail egg yolk.

3. Results

3.1. Production of specific IgYs

SDS-PAGE analysis of anti- *S. typhimurium* and *S. enteritidis* IgYs revealed high purity and quantity of the products at about 25 kDa as a light chain and 68 kDa as a heavy chain. The immunoreactivity and specificity of the purified IgYs were determined using ELISA and Western blot analysis methods. According to the checkerboard titration of ELISA, quail anti-*Salmonella* IgYs diluted 100 times and the best concentrations of *S. typhimurium* or *S. enteritidis* antigens were found to be 1.0×10^9 CFU/mL. Western blot analysis also confirmed the specificity of produced anti- *Salmonella* IgYs in all immunized groups.

Specific IgYs against *S. typhimurium* and *S. enteritidis* resulted from either heat or formalin-inactivated immunogens toward the second week after the first immunization (Figs. 1 and 2). Anti-*S. typhimurium* or *S. enteritidis* IgYs were detected in all immunized quails and were significantly higher than those found in the control group throughout the experiment which confirmed the immunization procedure ($P < 0.001$).

In the case of *S. typhimurium* immunogens, anti-*S. typhimurium* antibody responses increased steadily from day 14 to 49 in both groups immunized with heat or formalin-inactivated antigens. Antibody level reached a peak at day 49, following the 4th immunization. However, there was a remarkable decrease in antibodies production in both groups at day 56. The increase in antibodies titer was recovered from day 63 following the 5th immunization and reached a peak at day 70 in the group immunized with formalin-inactivated *S. typhimurium* (FIST) and day 84 in the group immunized with heat-inactivated *S. typhimurium* (HIST). Antibody level remained constant for about two weeks (from day 70 to 84) in FIST group, then continued to decrease until the end of study (day 98). In HIST group, the second peak (day 84) was followed by an irreversible decline until the end of study (day 98). Comparing the two groups, antibody titer expressed as optical density was higher in FIST group than in HIST immunogen ($p < 0.05$) (Fig. 1).

In the case of *S. enteritidis* immunogens, anti-*S. enteritidis* antibody responses started to increase from the 7th day after the first immunization in both groups immunized with heat or formalin-inactivated antigens. Antibody level increased steadily from day 14 to 49 in heat-inactivated *S. enteritidis* (HISE) group, where as that of formalin-inactivated *S. enteritidis* (FISE) group increased slowly. In both groups,

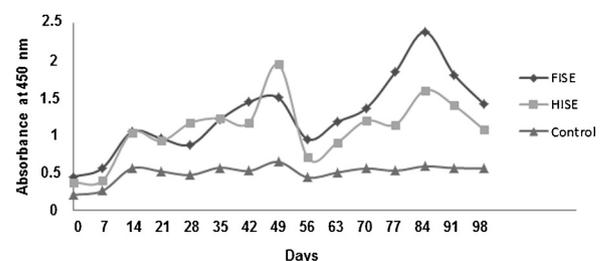


Fig. 2. Profile of anti-*S. enteritidis* formalin- (FISE) and heat- (HISE) inactivated IgYs in quail egg yolk.

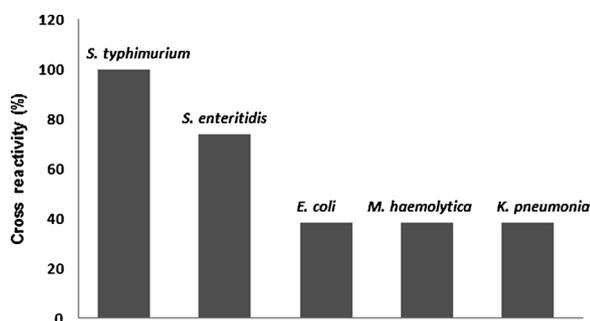


Fig. 3. Cross-reactivity of anti- *S. typhimurium* IgYs against *S. enteritidis*, *E. coli*, *M. haemolytica*, and *K. pneumonia*.

antibody level reached a peak at day 49 although the optical density was higher in HISE group. There was a remarkable decline in antibodies titer at day 56, especially in HISE group. The increase in antibodies titer was recovered from day 63, following the 5th immunization. The second antibody production peak was observed at day 84 for both groups. Subsequently, antibody levels continued to decrease for about 2 weeks until the end of study (day 98). Comparing the two groups, the level of antibody was significantly higher in FISE group during the second antibody production peak (day 84) ($p < 0.05$) (Fig. 2).

3.2. Cross-reactivity of IgYs

The cross-reactivity of produced anti-*S. typhimurium* and *S. enteritidis* IgYs were evaluated by assessing their binding possibilities with other *Salmonella* serovars or other members of *Enterobacteriaceae* family including *Escherichia coli*, *Mannheimia haemolytica*, and *Klebsiella pneumonia* bacteria coated as antigens on the microtiter plates.

As shown in Fig. 3, a cross-reactivity of about 74, 38.5, 38.3, and 38.25% were observed between FIST IgYs to *S. enteritidis*, *E. coli*, *M. haemolytica*, and *K. pneumonia*, respectively, in comparison with that of *S. typhimurium* which was considered as 100% cross-reaction. FISE IgYs also showed a cross-reactivity of about 65.1, 46.3, 12.05, and 46% to *S. typhimurium*, *E. coli*, *M. haemolytica*, and *K. pneumonia*, respectively (Fig. 4).

4. Discussion

Salmonella species have been the major foodborne pathogen problems in food production industry and, therefore, sensitive detection and control strategies are highly recommended for food safety (Herrera et al., 2013). The potential applications of IgYs have been approved in different scientific areas including immunodiagnostic procedures, the detection of pathogenic microorganisms, and also for preventive and therapeutic purposes (da Silva and Tambourgi, 2010; Kovacs-Nolan and Mine, 2004; Sunwoo et al., 2006). Because of the differences in

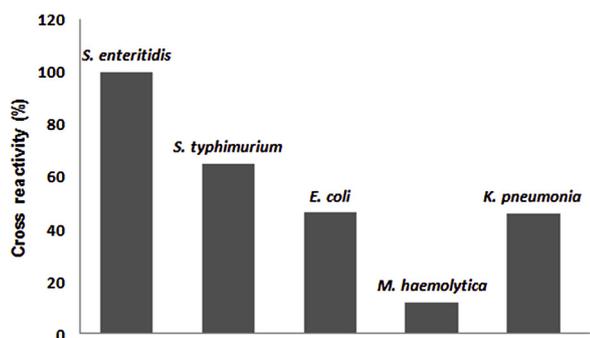


Fig. 4. Cross-reactivity of anti-*S. enteritidis* IgYs against *S. typhimurium*, *E. coli*, *M. haemolytica*, and *K. pneumonia*.

molecular interactions between mammalian and chicken antibodies, the use of egg yolk IgYs has been also recommended in recombinant-immunoglobulin technologies to improve the sensitivity and accuracy of antibody-based immunological assays such as ELISA or Blotting approaches (Bae et al., 2009; Somowiyarjo et al., 1990; Xu et al., 2011). The oral administration of specific IgYs has been shown to be an alternative method for the treatment and prevention of gastrointestinal pathogens including *Salmonella* SPP. (Chalghoumi et al., 2009; Pavic et al., 2012), *Escherichia coli* (Sunwoo et al., 2006), *Bovine coronavirus* (Fu et al., 2006), *Bovine rotavirus* (Vega et al., 2011), *Pseudomonas* (Xu et al., 2011), and *Staphylococcus* (Wang et al., 2011).

In this study, the possible production of immunoglobulin Y specific to *Salmonella typhimurium* and *Salmonella enteritidis* in quail egg yolks and its utilization on ELISA and Western blot as a diagnostic tool were investigated. The SDS-PAGE analysis of purified antibodies revealed high purity and quantity of the product at about 25 kDa as a light chain and a 68 kDa as a heavy chain. These results were confirmed by the findings reported by Kassim et al. (2011) and Bae et al. (2009) who obtained two bands of light and heavy chains of quail IgYs at the same molecular weight. Therefore, egg yolk antibody purification method proposed by Kitaguchi et al. (2008) can achieve high yield and purity of IgYs in quail. Specific IgYs against *S. typhimurium* and *S. enteritidis* resulted from either heat or formalin-inactivated immunogens were started to increase in egg yolks from the second week after the first immunization. Since chickens transfer the maternal antibodies to the egg yolks for the protection of the embryos as a passive immunity, the lag time observed in the first two weeks may stand for the time it takes specific antibodies to be produced and transferred from the serum to the egg yolks. Antibody responses increased steadily from day 14 to 49 in all groups and reached a peak at day 49, following the fourth immunization. Second antibody production peak was observed at day 84 in FISE, HISE, HIST groups and day 70 in FIST group. Comparing the groups immunized with heat or formalin-inactivated antigens, relatively stronger immune responses were obtained against formalin-inactivated immunogens. Subjecting the bacteria to the 100 °C for 20 min for producing heat-inactivated immunogen would denature the outer membrane protein (OMP) which may serve as one of the main antigenic factors, involved in stimulating the immune system, as well as an epitope for the antibody. Therefore, heat may affect the antibody responses and also antigen-antibody binding possibilities by changing the antigenic epitopes. In accordance with our results, previous researches also reported the higher immunogenicity of formalin-inactivated antigens over the heat-inactivated ones regarding different bacteria (Kassim et al., 2011).

Few studies have explored the production, purification, and utilization of quail egg yolk specific antibodies. Quail IgYs have been produced against *Influenza HIV-1* viruses (Kovgan et al., 1989), *Plant potyviruses* (Somowiyarjo et al., 1990), *Vibrio parahaemolyticus*, *Vibrio vulnificus* (Kassim et al., 2011), and *Helicobacter pylori* (Najdi et al., 2016). Kassim et al. (2011) investigated the possible production of specific egg yolk IgY against *Vibrio* SPP. in *Coturnix* quail. Specific IgYs started to increase in egg yolks from the 14th day after the first immunization and the highest titer was reached at day 35 for *V. vulnificus* and day 42 for *V. parahaemolyticus*. The second antibody production peak was observed at day 63 for both immunogens; then, the peaks were followed by an irreversible decline in spite of the booster immunization. Formalin-inactivated immunogens induced higher immunoresponses in both quails immunized with *V. vulnificus* or *V. parahaemolyticus* over those immunized with heat-inactivated immunogens (Kassim et al., 2011). This result is in agreement with the pattern we observed in quails immunized with *Salmonella* SPP.

Najdi et al. (2016) also examined the production and specificity of quail anti-*Helicobacter pylori* IgY and showed that specific IgYs started to rise in egg yolk from the 28th day after the first immunization. The first and the second peaks of anti-*H. pylori* IgY were observed at days 35 and 56, respectively. This study revealed that higher doses of antigen

could not definitely develop higher immune responses and that the oral administration of antigen could generate an effective immunization in quail (Najdi et al., 2016). In contrary to this research, a reduction in antibody level was observed at day 56 in all groups immunized with *S. typhimurium* or *S. enteritidis*. Subsequently, antibody titer was recovered from day 63 following the 5th immunization. Variations in antibody production patterns among different studies might be due to the differences in individual immune responses, type and dose of antigens, route of antigen administration as well as the specificity and sensitivity of the tools used for assessing the antibody level.

Cross-reaction analysis of quail anti-*S. typhimurium* IgY showed the highest cross-reactivity with *S. enteritidis* (74%) and the lowest with *K. pneumoniae* (38.3%). In the case of anti-*S. enteritidis* IgY, the highest and lowest cross-reactivity were observed with *S. typhimurium* (65.1%) and *M. haemolytica* (12.05%), respectively. Generally, quail anti-*Salmonella* IgYs showed a high specificity to their corresponding immunogens, with moderate binding affinity to other members of *Enterobacteriaceae* family ($p < 0.05$). The high cross-reactivity of anti-*Salmonella* IgYs observed between *S. typhimurium* and *S. enteritidis* can be explained by the fact that *Salmonella* serovars share many epitopes (Kudrna et al., 1985). Chalghoumi et al. (2008) used the outer membrane protein of *S. typhimurium* and *S. enteritidis* as a target of immunization and reported a high cross-reactivity of up to 78.8% for anti-*S. typhimurium* IgY to *S. enteritidis*, and up to 71.7% for anti-*S. enteritidis* IgY against *S. typhimurium* (Chalghoumi et al., 2008). The observed values of cross-reactivity of quail anti-*Salmonella* IgYs were lower than those reported in hens. We used the inactivated whole bacteria cells for the immunization in quails while hens were immunized with purified OMP. It is more likely that the degree of cross-reactivity directed against a purified immunogen like OMP, with more shared epitopes among *Salmonella* spp., is higher than that directed against a whole bacteria cell.

Quails are relatively sturdy birds which are resistant to several infectious diseases and environmental stresses. They are less expensive, easily manageable in terms of housing, and experimentally convenient due to their small size (Bae et al., 2009; Scholtz et al., 2010). Quails require fewer volumes of antigens for immunization which is an advantage for producing antibodies against viruses and recombinant proteins that can be purified at low concentrations (Somowiyarjo et al., 1990). They achieve sexual maturity at seven weeks of age as opposed to the 20–24 weeks required for a hen to start laying eggs. Moreover, quails have a high rate of egg laying (280–300 eggs per year), and immunization does not result in the reduction of egg production (Bae et al., 2009; Najdi et al., 2016). However, despite having several advantages, only few studies have utilized quails for investigating the immune responses against pathogens.

5. Conclusion

In conclusion, the present study demonstrated the possibility of production of specific immunoglobulin Y against *Salmonella typhimurium* and *Salmonella enteritidis* in Japanese quail egg yolks. Quail anti-*Salmonella* IgYs were significantly specific to their corresponding immunogens, with moderate cross-reactivity to other members of *Enterobacteriaceae* family. Quail can be considered as an inexpensive source of specific antibodies that can be used for immunoassay and immunotherapy purposes.

Conflict of interest

The authors declare that they have no conflicts of interest to disclose.

Funding

This work was supported by Shiraz University [Grant No. 94GCU1M271548 and 95GCU1M271548]. We also thank the

Microbiology Department, Faculty of Veterinary Medicine, University of Tehran; for providing the *Salmonella typhimurium* and *Salmonella enteritidis* bacteria.

References

- Ainsworth, S.J., Stanley, R.L., Evans, D.J., 2010. Developmental stages of the Japanese quail. *J. Anat.* 216, 3–15.
- Bae, H.D., Kitaguchi, K., Horio, F., Murai, A., 2009. Higher incorporation of heterologous chicken immunoglobulin Y compared with homologous quail immunoglobulin Y into egg yolks of Japanese quail (*Coturnix japonica*). *Poult. Sci.* 88, 1703–1711.
- Chalghoumi, R., Thewis, A., Portetelle, D., Beckers, Y., 2008. Production of hen egg yolk immunoglobulins simultaneously directed against *Salmonella enteritidis* and *Salmonella typhimurium* in the same egg yolk. *Poult. Sci.* 87, 32–40.
- Chalghoumi, R., Marcq, C., Thewis, A., Portetelle, D., Beckers, Y., 2009. Effects of feed supplementation with specific hen egg yolk antibody (immunoglobulin Y) on *Salmonella* species cecal colonization and growth performances of challenged broiler chickens. *Poult. Sci.* 88, 2081–2092.
- da Silva, W.D., Tambourgi, D.V., 2010. IgY: a promising antibody for use in immunodiagnostic and in immunotherapy. *Vet. Immunol. Immunopathol.* 135, 173–180.
- Fu, C.-Y., Huang, H., Wang, X.M., Liu, Y.G., Wang, Z.G., Cui, S.-J., Gao, H.L., Li, Z., Li, J.P., Kong, X.G., 2006. Preparation and evaluation of anti-SARS coronavirus IgY from yolks of immunized SPF chickens. *J. Virol. Methods* 133, 112–115.
- Gürtler, M., Methner, U., Kobilke, H., Fehlhaber, K., 2004. Effect of orally administered egg yolk antibodies on *Salmonella enteritidis* contamination of hen's eggs. *J. Vet. Med. B* 51, 129–134.
- Herrera, P., Aydin, M., Park, S.H., Khatiwara, A., Ahn, S., 2013. Utility of egg yolk antibodies for detection and control of foodborne *Salmonella*. *Agric. Food Anal. Biotechnol. (AFAB)* 3, 195–217.
- ISO, 2002. ISO 6579:2002 Annex D: Detection of *Salmonella* spp. in Animal Faeces and in Environmental Samples from the Primary Production Stage. International Organization for Standardization, Geneva, Switzerland.
- Kassim, N., Mtenga, A.B., Lee, W.-G., Kim, J.-S., Shim, W.-B., Chung, D.-H., 2011. Production of Coturnix quail immunoglobulins Y (IgYs) against *Vibrio parahaemolyticus* and *Vibrio vulnificus*. *Food Sci. Biotechnol.* 20, 1577–1583.
- Kitaguchi, K., Minoura, M., Noritake, M., Mizutani, M., Kinoshita, K., Horio, F., Murai, A., 2008. Determination of immunoglobulin Y concentration in yolk extract prepared by water dilution method: comparisons among three strains of chickens. *J. Poult. Sci.* 45, 82–87.
- Kovacs-Nolan, J., Mine, Y., 2004. Passive immunization through avian egg antibodies. *Food Biotechnol.* 18, 39–62.
- Kovgan, A., Isaeva, Y., Rovnova, Z., Shilov, A., 1989. Specific immunoglobulins from egg yolks of Japanese quail immunized with influenza and HIV-1 viruses. *Dokl. Akad. Nauk SSSR* 307, 229–233.
- Kudrna, D.A., Teresa, G.W., Arnzen, J.M., Beard, K.S., 1985. Immunoglobulin M and immunoglobulin G responses in BALB/c mice to conjugated outer membrane extracts of four *Salmonella* serotypes. *Infect. Immun.* 49, 598–608.
- Lee, E., Sunwoo, H., Menninen, K., Sim, J., 2002. In vitro studies of chicken egg yolk antibody (IgY) against *Salmonella enteritidis* and *Salmonella typhimurium*. *Poult. Sci.* 81, 632–641.
- Najdi, S., Brujeni, G.N., Sheikhi, N., Chakhkar, S., 2016. Development of anti-*Helicobacter pylori* immunoglobulins Y (IgYs) in quail. *Iran. J. Vet. Res.* 17, 106–110.
- Nilsson, E., Stalberg, J., Larsson, A., 2012. IgY stability in eggs stored at room temperature or at +4 °C. *Br. Poult. Sci.* 53, 42–46.
- Pavic, A., Groves, P.J., Cox, J.M., 2012. Control of *Salmonella* in poultry through vaccination and prophylactic antibody treatment. *Biochemical Testing*. InTech <https://doi.org/10.5772/29630>.
- Rahimi, S., Shiraz, Z.M., Salehi, T.Z., Torshizi, M.A.K., Grimes, J.L., 2007. Prevention of *Salmonella* infection in poultry by specific egg-derived antibody. *Int. J. Poult. Sci.* 6, 230–235.
- Schade, R., Schniering, A., Hlinak, A., 1992. Polyclonal avian antibodies extracted from egg yolk as an alternative to the production of antibodies in mammals-a review. *Altex* 9, 43–56.
- Scholtz, N., Halle, I., Dänicke, S., Hartmann, G., Zur, B., Sauerwein, H., 2010. Effects of an active immunization on the immune response of laying Japanese quail (*Coturnix coturnix japonica*) fed with or without genetically modified *Bacillus thuringiensis*-maize. *Poult. Sci.* 89, 1122–1128.
- Somowiyarjo, S., Sako, N., Nonaka, F., 1990. Production of avian antibodies to three potyviruses in coturnix quail. *J. Virol. Methods* 28, 125–132.
- Sunwoo, H.H., Wang, W.W., Sim, J.S., 2006. Detection of *Escherichia coli* O157:H7 using chicken immunoglobulin Y. *Immunol. Lett.* 106, 191–193.
- Vega, C., Bok, M., Chacana, P., Saif, L., Fernandez, F., Parreño, V., 2011. Egg yolk IgY: protection against rotavirus induced diarrhea and modulatory effect on the systemic and mucosal antibody responses in newborn calves. *Vet. Immunol. Immunopathol.* 142, 156–169.
- Wang, L.-H., Li, X.-Y., Jin, L.-J., You, J.-S., Zhou, Y., Li, S.-Y., Xu, Y.-P., 2011. Characterization of chicken egg yolk immunoglobulins (IgYs) specific for the most prevalent capsular serotypes of mastitis-causing *Staphylococcus aureus*. *Vet. Microbiol.* 149, 415–421.
- Xu, Y., Li, X., Jin, L., Zhen, Y., Lu, Y., Li, S., You, J., Wang, L., 2011. Application of chicken egg yolk immunoglobulins in the control of terrestrial and aquatic animal diseases: a review. *Biotechnol. Adv.* 29, 860–868.
- Yokoyama, H., Umeda, K., Peralta, R.C., Hashi, T., Icatlo Jr, F.C., Kuroki, M., Ikemori, Y., Kodama, Y., 1998. Oral passive immunization against experimental salmonellosis in mice using chicken egg yolk antibodies specific for *Salmonella enteritidis* and *S. typhimurium*. *Vaccine* 16, 388–393.