



Storage stability of anti-*Salmonella* Typhimurium immunoglobulin Y in immunized quail eggs stored at 4°C

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ABSTRACT

Chicken egg yolk antibodies (IgYs) are extensively used for immunotherapy and immunodiagnostic purposes. Oral immunotherapy with specific IgYs is established as an efficient alternative to traditional antibiotic therapy in human and animals. Storing immunized eggs in refrigerator for a period of time could provide an inexpensive and convenient source of large volumes of specific antibodies. This study investigated the storage stability of anti-*Salmonella* Typhimurium IgYs in immunized quail egg yolks at 4°C over a period of more than 6 months. *Salmonella* spp.-free female Japanese quails (*Coturnix coturnix japonica*) were intramuscularly immunized with *Salmonella* Typhimurium whole bacterial suspension (1.0×10^9 CFU/ml) emulsified with Freund adjuvants. During a period of 10 days after final immunization, eggs from each group were collected, randomized and stored at 4°C over a period of 200 days. Egg yolk IgY titer and specificity were determined using ELISA technique. *S. Typhimurium* specific IgY antibodies were detected in immunized quails and were significantly higher than the control group which confirmed the immunization procedure. Eggs from immunized quails can be collected and stored in 4°C refrigerator over a period of 2 months without any concern about the antibody degradation. After 80 days of storage at 4°C, although lower antibody titer was obtained in comparison to the first of study, anti-*S. Typhimurium* IgY level remained stable up to the 6 months without more significant declining. This trend will provide economical sources of polyclonal antibodies through reducing the number of immunized animals, management expenses and housing costs.

Key words: Antibody stability, IgY, Quail, *Salmonella* Typhimurium

Immunoglobulin G (IgG) is the major class of produced antibodies in blood circulating system which protects humans and animals from infectious diseases through humoral immunity (Wang *et al.* 2004). Chicken egg yolk immunoglobulin, commonly called immunoglobulin Y (IgY), is the homologue of mammalian IgG and transports in large quantities from the blood of laying birds to the egg yolk (Schade *et al.* 2005). As a means of protecting the offsprings against potential pathogens, serum IgY of laying chickens is transferred to the egg yolk and serves as passive immunity of developing embryos (Kovacs-Nolan and Mine 2012). Therefore, chickens can be artificially immunized against specific pathogens to produce disease-specific antibodies (Cook and Trott 2010, Xu *et al.* 2011). Chicken egg yolk contains 50–150 mg of IgY of which 2 to 10% are pathogen-specific antibodies (Kassim *et al.* 2011). Egg yolk antibodies are extensively used as powerful tools for different diagnostic and therapeutic purposes. Antibodies

extracted from egg yolks are also used to prevent different infections including bacterial, viral and parasitical diseases (Chalghoumi *et al.* 2009, Lee *et al.* 2009, Wang *et al.* 2011, Hotta *et al.* 2013).

Use of chickens for producing egg yolk polyclonal antibodies has lots of advantages over mammals. The most substantial advantage is that the production and collection of egg yolk IgY is non-invasive. The purification of egg yolk antibodies eliminates animal suffering, as it does not need any animal bleeding or sacrificing and the modest stress is applied on laying chickens which are used for production of specific antibodies. Moreover, high and long-lasting antibody titer produced in chickens decrease the frequent booster injection which is advantageous in consideration of animal welfare (Gürtler *et al.* 2004, Najdi *et al.* 2016). Another advantage of chicken IgY is that due to the evolutionary distance between avian and mammals, chickens are more successfully able to produce antibodies against highly conserved mammalian antigens and also require far less antigen to prompt an efficient immune response (Xu *et al.* 2011, Nilsson *et al.* 2012). Avian maintenance costs are also much lower than those for mammals, so birds can be considered as cost-effective,

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convenient and high-yield sources for producing specific antibodies (Gürtler *et al.* 2004, Schade *et al.* 2005).

Oral immunotherapy with chicken egg yolk immunoglobulins has been confirmed as potential alternative to conventional antibiotics due to their high specificity (da Silva and Tambourgi 2010; Mulvey *et al.* 2011). Immunization in chickens will result in stable circulating antibody level and high antibody titer in egg yolk. Immunized eggs can be collected and stored at 4°C during a period when the chickens are expected to have reached a high and stable antibody level. It will provide large volumes of specific antibodies which can be used for therapeutic, preventive and diagnostic purposes (Nilsson *et al.* 2012). Nevertheless, stability of antibodies in immunized eggs stored in refrigerator for a long time may limit the volume of antibody or the storage period. In the current study, we evaluated the storage stability of anti-*Salmonella* Typhimurium IgYs in immunized quail egg yolks, stored at 4°C over a period of 200 days.

MATERIALS AND METHODS

Antigen preparation: *Salmonella* Typhimurium (ATCC 19586) was 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. Complete killing of bacteria was confirmed by culturing on nutrient broth medium. Finally cells were harvested by centrifugation at 9,000×g for 20 min at 4°C and pellet was washed three times with sterile PBS. *S.* Typhimurium concentration was adjusted to 1.0×10⁹ CFU/ml with PBS and stored at -20°C.

Immunization of quails: Newly hatched *Salmonella* spp.-free female Japanese quails (*Coturnix coturnix japonica*; 20) were purchased from a local hatchery unit. The breeding conditions were approved by the Shiraz University Policy on Animal Care and all institutional and national guidelines for the care and use of laboratory animals were followed. Starting at 7 week of age, quails were maintained as 10 birds per cage of 80 × 60 × 30 cm under constant temperature (27±2°C) with *ad lib.* access to water and feed. The photoperiodicity was also controlled at 14 h lighting and 10 h darkness. The birds were adapted to the new environment for 2 weeks prior immunization. *Salmonella* spp.-free status was confirmed by bacteriological analysis of cloacal swabs as described in Annex D to ISO 6579:2002 (International Organization for Standardization) (ISO 2002), bacterial culture and PCR technique on arrival date and every two weeks throughout the experiment. For immunization, 0.125 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 0.25 ml was injected into the quails superficial pectoral muscles. Immunization continued for 2 months to ensure that the immune response had reached a plateau, and would be stable during the egg collection period. From the second to the forth immunization, Freund's complete adjuvant was replaced

with the Freund's incomplete adjuvant. All immunizations were carried out in 2 weeks intervals. The control group being administered with 0.125 mL of sterile PBS plus 0.125 mL adjuvant. Eggs from each group were collected during a period of 10 days (from day 70 to 80) and stored at 4°C until IgY purification. Upon collecting, eggs were randomized to avoid any bias due to the birds and laying date and allocated to the different storage periods including 0, 10, 20, 50, 80, 140 and 200 days. Egg yolk antibody was purified at each step using proposed method.

IgY purification from egg yolk: The isolation of anti-*S.* Typhimurium IgY from yolk was carried out by the method of Kitaguchi *et al.* (2008) with minor modifications (Kitaguchi *et al.* 2008). Briefly, egg yolks were carefully separated from albumin and then the yolk membranes were punctured and the whole yolks were 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 deionized 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 sulphate. 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 and the supernatant was removed. 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 determination of the anti-*Salmonella* IgY concentration by ELISA. The IgY purity and yield of eggs isolated after varying storage times were analyzed on Tris-glycine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% resolving and 5% stacking acrylamide gel.

Anti- *S.* Typhimurium ELISA: Optimization of the anti-*S.* Typhimurium IgY titer was conducted using a checker board titration of enzyme-linked immunosorbent assay (ELISA). Polystyrene microtiter plates with 96 wells were coated with 100 µl/well of formalin inactivated *S.* Typhimurium at a concentrations range from 10¹⁰ to 10⁴ CFU/ml in 10 mM phosphate-buffered saline (PBS, pH 7.2) and incubated at 4°C overnight. After washing 3 times with washing buffer [0.14 M NaCl, 50 mM Tris-HCl at pH 7.2 with 0.05% (vol/vol) Tween 20], the uncoated surfaces of the wellswere blocked by adding 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 5 times with PBST and 100 µl/well of anti- *S.* Typhimurium IgY diluted 100 times with blocking buffer was added to duplicate wells and incubated at 37°C for 1 h. Plates were washed 5 times with PBST and incubated for 1 h with 100 µl/well of horse radish peroxidase-conjugated rabbit anti-chicken IgG (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:10,000 with blocking buffer. The plates were washed an additional 5 times and a colour reaction was initiated by adding 100 µl/well of TMB substrate and

incubated at room temperature for 15 min. The reaction was stopped by adding 100 µl/well of 3 M H₂SO₄ and the optical density (OD) of each well determined at 450 nm with a microtiter plate reader. A defined chicken anti- *S. Typhimurium* antibody stored at -70°C was used as positive control and the titer of control was determined at the same plate.

Statistical analysis: The imprecisions of the measurements were presented as means±standard deviations (SD) of samples from each group. Analysis of variance (ANOVA) and student's t-test were utilized to test the significant differences of antibody titer between different storage periods (day 0, 10, 20, 50, 80, 140 and 200) and between control and experimental groups. 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).

RESULTS AND DISCUSSION

In this study, we investigated the stability of anti-*Salmonella* Typhimurium IgY in the eggs of immunized quails, stored at 4°C over a period of more than six months. *Salmonella* Typhimurium specific IgY antibodies were detected in immunized quails and were significantly higher than the control group which confirmed the immunization procedure. Egg yolk antibody titers at different storage periods (day 0, 10, 20, 50, 80, 140 and 200) are shown in Fig. 1. Storage of immunized eggs at 4°C for up to 50 days did not produce any significant reduction in antibody level rather than the start of the study (day 0) ($P = 0.091$). After 80 days of storage, slightly lower antibody titer was obtained in comparison to the day 50 ($P = 0.021$). Storing immunized eggs at 4°C up to 140 and 200 days also showed significant decreasing in antibody levels ($P = 0.001$). In spite of significant declining in anti- *S. Typhimurium* IgY titer during a 200-day storage period at 4°C, level of antibody remained stable from day 80 to 140 ($P = 0.99$) and 200 ($P = 0.75$).

The potential applications of IgYs have been approved in different scientific areas including immuno diagnostic procedures, detection of pathogenic microorganisms, and also for preventive and therapeutic purposes (Sunwoo *et al.* 2006, da Silva and Tambourgi 2010, Kovacs-Nolan and Mine 2012). Because of the differences in molecular interactions between mammalian and avian antibodies, use of egg yolk IgYs was also recommended in recombinant-immunoglobulin in technologies to improve the sensitivity and accuracy of antibody-based immunological assays (Somowiyarjo *et al.* 1990, Aae *et al.* 2009, Xu *et al.* 2011). Oral administration of specific IgYs was shown to be an alternative method for treatment and prevention of intestinal pathogens including *Escherichia coli* (Sunwoo *et al.* 2006), *Salmonella* SPP (Chalghoumi *et al.* 2009), *Bovine coronavirus* (Fu *et al.* 2006), *Bovine rotavirus* (Vega *et al.* 2011), *Pseudomonas* (Xu *et al.* 2011) and *Staphylococcus* (Wang *et al.* 2011).

Japanese quails are mid-size birds belonging to the

Phasianidae family which are found in Asia, Africa and some parts of Europe. Quails are reared worldwide for both meat and egg production (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 homologues to the chicken IgY and they are similar in several aspects, such as molecular weight, sedimentation coefficient and yielding patterns of their light and heavy chains under reducing condition (Bae *et al.* 2009). Few studies explored the production, purification and utilization of quail's specific antibodies against pathogens. Quail IgYs were produced against *Influenza H1N1* viruses (Kovgan *et al.* 1989), *Plant potyvirus* (Somowiyarjo *et al.* 1990), *Vibrio parahaemolyticus*, *Vibrio vulnificus* (Kassim *et al.* 2011) and *Helicobacter pylori* (Najdi *et al.* 2016).

Results of this research showed that quails immunized with *S. Typhimurium* can produce high level of stable circulating antibody for two months (data not shown). Due to the high egg laying intensity in quails, high volume of antibody-rich yolks can be obtained during this period. If it is possible to store immunized eggs in a 4°C refrigerator for a long period, it will allow the preparation of large specific antibody batches which could be used for therapeutic and diagnostic purposes. However, declining the antibody titer and specificity during the storage period has been a cause of concern. In order to provide a stable source of antibody, the number of batches and batch-to-batch variation should be minimized. Data obtained in this study demonstrated that immunized quail eggs can be pooled and stored at 4°C up to 50 days without significant reduction in antibody titer and activity. After 80 days of storage, although lower antibody titer was obtained in comparison to the first of study (day 0), anti- *S. Typhimurium* IgY titer remained stable up to day 200. Therefore, in spite of the reduction in antibody titer after about 3 months, it can be stored up to 6 months without more significant declining. The advantage of pooling large number of eggs from different quails is that it decreases the individual effect of low- or high-responding birds. Moreover, by pooling different egg yolks, time and costs of laboratory activities will be reduced.

Nilsson *et al.* (2012) investigated the IgY stability in the eggs of immunized hens. Three laying White Leghorn hens were immunized with *Pseudomonas aeruginosa* and eggs from each hen were collected during a period of 21 days. Finally, eggs were stored at room temperature (18–25°C) or refrigerator (4°C) over a time period of up to 6 months. Result of this study demonstrated that storage for up to 30 days at room temperature and 180 days at 4°C showed no clear trend in antibody titer and activity, although no statistical analysis was conducted to determine the probability level (Nilsson *et al.* 2012). Our results were somewhat different with the data reported in hens. Based on the data obtained in this study, immunized quail eggs

can be stored at 4°C for a shorter period than hens. It might be due to the differences in content of hen and quail eggs which influence the storage stability.

Storage over a period of two months in 4°C refrigerator showed no significant declining in quails anti-S. Typhimurium IgY titer and activity. Despite the reduction in antibody titer after about 80 days, no clear association was observed between storage time and more decreasing in antibody titer during a 200-day storage period. Therefore, eggs from immunized quails can be collected and stored in refrigerator for a long time without any concern about the antibody degradation. This trend will reduce the number of immunized animals and the costs of their management and housing. Furthermore, pooling egg yolks from different immunized birds will lessen batch-to-batch antibody variation and provide reproducible assay performance over the time.

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