Development of anti-*Helicobacter pylori* immunoglobulins Y (IgYs) in quail

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Summary

Helicobacter pylori (*H. pylori*) is a gram-negative, microaerophilic bacterium that cause the stomach infection in more than 50% of human population worldwide. The aim of this study was to examine the possibility of anti-*H. pylori* immunoglobulins Y (IgYs) production in quails and evaluate the effect of the different methods of immunization on titers of IgY in egg yolks. Whole cell bacterial antigen was used for immunization of quails. Forty Japanese quails (*Coturnix japonica*) were divided into four groups. The first group intramuscularly immunized with one dose of antigen (3×10^8 inactivated bacteria) whereas the second group injected with half dose. Third group administered orally. Yolk IgY was isolated using precipitation method of water dilution combined with chloroform. Dot-blot and ELISA (enzyme-linked immunosorbent assay) were used for determining the specificity and quantifying the titer of IgY in egg yolks. Results showed that quails as well as chickens are able to produce anti-*H. pylori* IgY. Quails antibodies have high titer and specificity that can be used in therapeutic and research purposes. This study indicated that higher amounts of antigen can not develop higher titer of IgY and injection is not necessary for efficient immunization of the quail against *H. pylori*.

Key words: ELISA, *Helicobacter pylori*, IgY, Quail

Introduction

Helicobacter pylori (*H. pylori*) is a gram-negative, spiral, microaerophylic bacterium that cause the stomach infection in more than 50% of human population worldwide. It causes gastritis and gastric ulcers that play a pivotal role in the development of gastric carcinoma (Blaser, 1992; Parsonet *et al*., 1994; Uemura, 2001). Current treatments of infection often exploit multidrug therapy, including amoxicillin, clarithromycin, metronidazole and a proton pump inhibitor like bismuth (Gibaldi, 1995). However antibiotic therapy fails in 10-15% of cases due to the appearance of antibiotic resistance (Peitz *et al*., 1998). Resistances and the challenge of re-treatment after first failure requests that novel approaches including vaccines (Del Giudice *et al*., 2001; Kuster, 2001), probiotics (Aiba *et al*., 1998), natural extracts (Mabe *et al*., 1999), mucosal protective agents and anti-adhesion components should be further studied (Mysore *et al*., 1999).

Passive immunization upon oral administration of anti-*H. pylori* immunoglobulin may be effective in prevention of infection. However, oral administration of large amounts of antibodies is expensive. Egg yolk has been recognized as an excellent source of polyclonal antibodies. Using birds as the immunization host for producing egg yolk antibodies (IgY), instead of IgG from mammalians, has a number of advantages:

a) The animal suffering is reduced (no bleeding)

b) Antibody isolation is simple and fast

c) A single egg contains as many antibodies as an average bleed from a rabbit (Schade *et al*., 1996; Davalos *et al*., 2001; Tini *et al*., 2002)

IgY also has the advantages in that, it avoids the interference with immunological assays caused by the complement system, rheumatoid factors, anti-mouse IgG antibodies, or human and bacterial Fc receptors (Carlander *et al*., 1999). These differences in molecular interaction bring advantages to the application of IgY antibodies and they have been used successfully in different areas of treatment, research, diagnostics, medical application and biotechnology.

Chicken anti-*H. pylori* IgY could significantly inhibit the growth and urease activity of *H. pylori in vitro*. It has been orally administered for infected BALB/c mice and 70% of animals showed a significant decrease in degrees of gastritis one week after oral immunization (Suzuki *et al*., 2004). Functional drinking yogurt containing specific *H. pylori* IgY, if administered three times daily for 4 weeks, can decrease urea breath test values (Kazimierczuk *et al*., 2005). It has been demonstrated that chicken *H. pylori* IgY at 16 mg/ml dose fully inhibited the growth of *H. pylori in vitro* and was identified as minimal inhibitory concentration (Koo *et al*., 1999).

Quails are comparatively sturdy birds which require minimum floor space and require low investment. Quails start laying eggs in about six to seven weeks of age as opposed to the 20 to 24 weeks required for a chicken to begin producing and they have a high rate of egg laying

(280 eggs per year). Quail eggs are rich in HDL cholesterol and choline, a chemical essential for brain function. The egg of Japanese quail contains 158 Cal. of energy, 74.6% water, 13.1% protein, 11.2% fat, 1.1% total ash. The mineral content includes 0.59 mg calcium, 220 mg phosphorus and 3.8 mg iron. The vitamin content is 300 IU of vitamin A, 0.12 mg of vitamin B1, 0.85 mg of vitamin B2 and 0.10 mg nicotinic acid.

According to the above mentioned advantages, we aimed to produce anti-*H. pylori* IgY in quail egg. In the present study, the specificity of produced quail anti-*H. pylori* IgY as well as comparison of different dose and rout of administration of *H. pylori* as an antigen for the immunization of quails were examined. The data also suggests the possibility of developing quail IgY antibodies against *H. pylori* as an alternative to hen egg antibodies and vaccine development in quail.

Materials and Methods

Antigen preparation

Helicobacter pylori was cultured in a Jar fermentator by repeated batch culture using chocolate agar with 5% fetal blood serum and incubated with 5% CO_2 at 37°C for 24 h. Cells were harvested by centrifugation at 5000 RPM for 15 min in 4°C. Bacterial pellet was washed three times with PBS (Phosphate-buffered saline, pH = 7.2) and a suspension of *H. pylori* was made with sterile PBS. The bacterial concentration was adjusted to 12×10^8 CFU/ml. The suspension was heated for 20 min in 80°C. Complete killing of *H. pylori* suspension was confirmed by culture.

Immunization of quails

Forty female Japanese quails of seven weeks old were obtained from local breeding unit and divided into four groups containing 10 birds each. The birds were hosed as 10 birds per cage of 80 × 60 × 30 cm. The quails were raised in floor pens and given diet "*ad libitum*" based on a complete feed mix containing 21% total protein and 11.7 MJ ME designed for adult birds at this stage. Killed *H. pylori* suspension (12×10^8 CFU/ml) emulsified with Freund's complete adjuvant (FCA) at the ratio of 1:1. First group of quails were injected with one dose of 3×10^8 inactivated bacteria plus FCA (0.5 ml) at multiple sites of pectoral muscle. Second group were injected with half dose (0.25 ml) in the same manner. For third group, 0.25 ml (3×10^8 CFU/ml) of killed bacterial solution with 0.25 ml of sterile distilled water was administered orally. Forth group was the control group and injected with 0.5 ml of sterile distilled water plus FCA. Quails in the first and second groups received subsequent booster injections in the same way and amounts except with Freund's incomplete adjuvant. The third group received the antigen orally the same as the first time. Control group was injected with 0.25 ml of sterile distilled water plus 0.25 ml Freund's incomplete adjuvant (FIC). In the third immunization the first group was injected with 0.5 ml of antigen, the second group received 0.25 ml of antigen and control group injected with 0.5 ml of sterile distilled water without adjuvant. The third group was administered 0.5 ml of oral solution as in the first time. All immunizations were carried out in 2 week intervals. Bleeding was done frequently to determine the amounts of antibodies in the serum. Eggs were collected from the first day until the end of the test and stored at 4°C.

Isolation of IgY and dot-blotting

Quails egg yolks were separated from the albumin and homogenized with equal volume of PBS (pH = 7.2). It was then mixed with an equal volume of chloroform and incubated at room temperature for 2 h. Following centrifugation at 700 g for 10 min, the supernatant was collected and filtered using a membrane filter (0.45 µm pore size) and stored at -20°C until use.

Specificity of anti-*H. pylori* IgY was investigated by immune blotting system. Two µL of heat inactivated *H. pylori* as target antigen and *E. coli* as a control for possible cross reactivity were spotted on nylon membrane (Roche, Germany). The spots were allowed to dry and membrane was blocked with 5% skim milk for 1 h. The membrane was exposed to anti-*H. pylori* IgY diluted 1:10 at 25°C for 1 h. After washing steps including 3 times for 5 min with PBS-T 5% (phosphate-buffered saline and Tween 20), membrane was incubated with goat anti-chicken antibody conjugated with HRP (IDEXX GmbH, Switzerland) diluted 1:10. After three washes with PBS-T and then once with PBS (5 min), the membranes were incubated with TMB substrate (3, 3', 5, 5' -Tetramethylbenzidine) for 1 min. Stained dots were observed as colored spots on the membrane, confirming production of specific anti-*H. pylori* IgY in the egg yolks. IgY obtained from the egg yolk of a non-immunized quail was used as a negative control. Positive samples from immunized quails were pooled and then used for checkerboard titration.

Titration of anti-*H. pylori* IgY

Optimization of the antibody titer was conducted using a checker board titration of ELISA (enzyme-linked immunosorbent assay). In each microplate well, 100 µL of heat inactivated diluted antigen, ranging from 1:4 to 1:1024 (serial 4 fold of 12×10^8 CFU/ml) was coated overnight at 4°C. Unbound antigens were removed by washing (3 times) with PBS-T. Then the wells were blocked with 100 µL skim milk 5%. After three additional washing steps, 100 µL of quail egg yolk antibodies (anti-*H. pylori* IgY) was added to wells using two fold dilutions starting at 1:25 and left for 30 min at 37°C. The plate was then washed and 100 µL of horse radish peroxidase-labeled goat anti-chicken IgY (IDEXX GmbH, D-55286 Wörrstadt, Switzerland) was added to each well and left for 30 min at 37°C. After washing, TMB substrate was added and the plate was incubated in a dark place for 15 min at room temperature. Finally, the reaction was stopped by the addition of 100 µL/well of stopper solution (1% SDS). Absorbance at 450 nm was measured using a microplate reader (Stat FAX 2000, Awareness Technology, Inc., USA). The optimum

results were determined according to the highest absorbance difference between the known positive and known negative serum samples with a minimal background.

ELISA

The titer of IgY antibodies against *H. pylori* was determined by indirect ELISA. According to data obtained from checker board titration (Fig. 1), 96-wells polystyrene plates were coated with 50 µL of heat inactivated *H. pylori* diluted 1:4 (3×10^8 CFU/ml) in PBS (pH = 7.2) and incubated in 4°C overnight. The next day plates were washed with PBS-T 3 times. For blocking the empty sites, skim milk 5% was used and incubated for 1 h at room temperature. Plates were subsequently washed and incubated with 50 µL of 1:25 diluted quail anti-*H. pylori* IgY antibodies and incubated at room temperature for 45 min. Reactive egg yolk extracts were used as positive and non-immunized quails' egg yolk extracts were used as negative control. After washing steps, 50 µL of goat anti-chicken IgG conjugated with horseradish peroxidase was added to each well and incubated at room temperature for 45 min. The plate was then washed with PBS-T and incubated with 50 µL of TMB substrate solution for 30 min at room temperature. The reaction was stopped with addition of 50 µL of 1% SDS and absorbance values were measured at 450 nm using a microplate reader (Stat FAX 2000, Awareness Technology, Inc., USA).

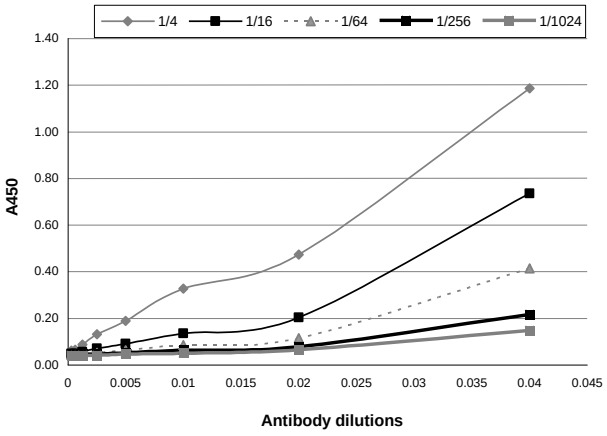

Fig. 1: Using checkerboard titration, optimal working dilutions of antibodies and antigen (*H. pylori*) were determined. Concentration of antigen ranged from 1/4 to 1/1024 and egg yolk extract was serial-diluted7 times. The best results for concentrations of antigen and antibody were found to be 1:4 and 1:25, respectively

Statistical analysis

All experiment results were included in the analysis, and data were analyzed with the SPSS version 14 for Windows (SPSS Institute, Chicago, IL, USA). Descriptive statistics (mean, standard deviation, and median) were calculated for all samples. Student's t-test for paired samples was used to compare means of variables including oral administration, IM injection (complete and half dose) and controls. All statistics were regarded as significant for probability values of ≤0.05.

Results

Dot-blot assays were carried out to check the specificity of anti-*H. pylori* IgY and reactive egg yolk samples were used for ELISA checkerboard titration. *Helicobacter pylori* specific IgY antibodies were observed for all immunized groups and no cross reactivity was observed for *E. coli*. IgY obtained from the egg yolk of non-immunized quails was found to be negative. According to the checkerboard titration, the best concentrations of quail anti-*H. pylori* IgY and *H. pylori* antigen were found to be 1:25 and 1:4, respectively (Fig. 1).

Antibody reactivity in quail egg yolk was measured by indirect ELISA. The antibody levels of immunized quails were significantly higher than control group ($P<0.003$). As seen in Fig. 2, amounts of IgY increased in all groups and reached the peak value in the 35th day of first immunization and then decreased gradually until day 42 and then increased to second peak at day 56 of experiment. There were significant differences between the control and other three groups hroughout the experiment ($P<0.05$). As seen in the results, second group has shown high titers on day 21 of experiment but on day 28, all test groups had almost identical titers. In the following days, anti-*H. pylori* titers in first and second groups had continued to increase and on day 56 (four weeks after the third injection of antigen) they reached the maximum titers of antibody but in the third group, titers fell earlier than others. There was not a significant difference ($P=0.823$) in the titer of specific anti-*H. pylori* IgY between the first group and the second group that received complete dose (3×10^8 bacteria) and half dose of bacterial antigen, respectively. According to Fig. 2 which shows the differences between groups, in the second group that received less inactivated bacteria, IgY amount increased quickly and during the experiment it was higher than the first group that received full dose of antigens. These results also indicated that in third group of quails that received antigen orally with fourteen day intervals, appropriate immune response was created.

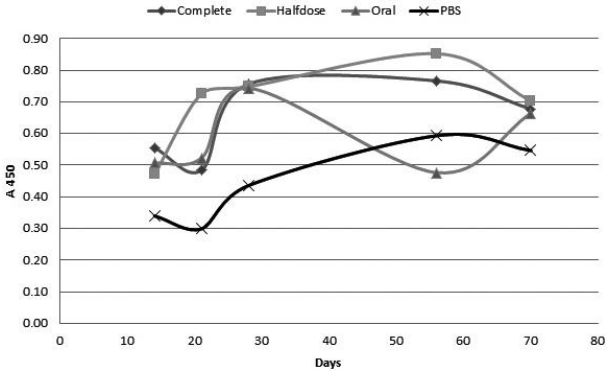

Fig. 2: ELISA results of comparison between anti-*H. pylori* IgY produced in quail by oral administration and IM injection (complete and half dose) of *H. pylori*. Control group only received sterile PBS and adjuvant, intramuscularly

Discussion

Egg yolk antibodies have been used as powerful and specific tools in many diagnostic and biomarker discovery applications. Our previous results suggest that anti-*H. pylori* IgY produced in hens could be recruited as a specific probe for detection of *H. pylori*-specific proteins (Saniee *et al*., 2013). In this study, we aimed to develop quail anti-*H. pylori* IgY using whole bacteria. The results reported here indicate that polyclonal quail IgY can be produced with a desirable yield and specificity. While chicken antibodies have been raised to *H. pylori* (Schade and Hlinak, 1996; Carlander *et al*., 1999; Koo *et al*., 1999; Tini *et al*., 2002; Shin *et al*., 2003; Horie *et al*., 2004; Suzuki *et al*., 2004; Malekshahi *et al*., 2011), they have not been compared to the other species. Along with quail egg samples, egg yolk extracts from immunized hen with the same antigen (Saniee *et al*., 2013) were used for making a comparison between quail and hen antibody titers (data not shown). Quantitative comparision between anti-*H. pylori* developed in quail and hen eggs has revealed a higher titre of specific antibody in quail egg after second immunization (A 0.902/A 0.852). Compared to hen that lays about 250 eggs per year, each quail can lay 300 eggs per year. Although the egg of hen contains more yolk (15 ml per egg) than quail egg yolk (5 ml per egg), quail is a small bird with great benefits such as minimal requirements of feed and breeding space, high resistance against diseases and is therefore economical. Consequently, it is a viable alternative for chickens in production of IgY for medical and research purposes. To the best of our knowledge, this is the first report of using quail as a host for immunization. Quail IgY has the ability to identify the *H. pylori* antigens and it can react with these antigens like chicken's IgY.

We have also investigated possible differences in antibody response that maybe exist for the different route of immunization. Factors affecting antibody production and titer development in quails has not been reported before. Our results indicated that for effective production of immune response and anti-*H. pylori* IgY production in quails, injection is not necessary and we could generate passive immunity orally in the quail. The only important point is that, the intervals must be shorter and number of boosters should be increased. Decreasing IgY titer in oral group started from 28th day, that might be due to rapid removal of antigen from the body of the birds and its inability to stimulate immune system well. Comparision of different routes of immunization for producing the IgY in hen or other birds has not been previously described. Nevertheles, a quantitative comparison on chicken and duck antibody IgY directed against *H. pylori* has been reported by Kalaigandhi *et al*. (2011). Accordingly, when the whole bacteria has been used for the intramascullary immunization of chicken and duck, specific IgY antibodies started to rise from 28th day after first immunization and the highest titer was reached at day 35. Our result is in accordance with chicken and duck and showed the same pattern in quails. We further showed a steady state of antibody titre at 42 days, gradually decreasing from 56 days onwards (Fig. 2). These simillarities in antibody production against *H. pylori* might be the result of using the same antigen and the same route of immunization and closely related species of birds as host.

There have been tremendous efforts for preventing bacterial infections without drug resistance problems. *Helicobacter pylori* infection is widespread in humans, and it can be cured with antibacterial drugs, but because of the appearance of antibiotic-resistant strains, the treatments are not successful in all cases. For this reason, oral adminstration of anti-*H. pylori* IgY can be an alternative and complementary drug with routine antibiotic therapy. It has been demonstrated that several pathogen-specific IgYs could be effective in passive immunization and treatment of human and animal diseases, such as *Rotavirus* diarrhea (Vega *et al*., 2011), *Salmonella* poisoning (Lee *et al*., 2002), Cholerae (Hirai *et al*., 2010) and gastrit ulcers caused by *H. pylori*. Different animal models can be immunized with antigens of *H. pylori* and their antibodies can protect against infections. Although prophylactic and therapeutic effects have been shown in animal models, the mechanisms of protection were not clarified. The quail anti-*H. pylori* IgY can be a usefull tool for the prevention and treatment of *H. pylori* infections. Its effect on the *H. pylori* needs to be investigated in further studies.

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