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ORIGINAL ARTICLE

Production of hen egg yolk antibody against zoonotic disease (salmonellosis) and passive immunization in mice

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ABSTRACT

Salmonellosis as joint human and animal diseases is very important, in this sudy; White egg–laying hens were immunized with *Salmonella* (S.) *typhi* and *S. typhimurium*-heat-killed whole cells in Freund's adjuvant. Immunization was done with 10^7 c.f.u of bacteria injection into the breast muscle of each egg-laying hen. Specific antibodies were detected by the enzyme-linked immunosorbent assay in the serum and in eggs. The effect of oral passive immunization was evaluated with egg yolk antibodies against *Salmonella typhimurium* in controlled experimental salmonellosis in mice. Chicken egg yolk antibodies (IgY) were raised against *S. typhimuruim* in the serum and in eggs as well. Anti-*S. typhimurium* IgY cross-reacted by 63%, 25% and 14% with *S. typhi, Shigella dysenteriae* and *E. coli* respectively.Results suggest that egg yolk antibodies specific for salmonella protect mice from experimental salmonellosis. Hence, eggs from hens immunized with *S. typhi* or *S. typhimuruim* have the potential as a useful source for passive immunity.

***Key words*:** salmonellosis, Immunoglobulin Y (IgY), ELISA, zoonotic disease.

Introduction

Salmonella enterica remains a major cause of food-borne gastroenteritis worldwide. It was reported that human salmonellosis reached the level of about 30000 cases per year in 1990s [1]. Many cases of human salmonellosis are attributed to consumption of infected poultry meat and eggs; the majority of cases being the result of *S. enterica*, serovar and *typhimurium*. There is an increasing prevalence of antibiotic-resistant bacteria, which makes traditional antibiotics less effective. Oral administration of specific antibodies is an attractive approach to establish protective immunity against gastrointestinal pathogens in humans and animals [2,3]. One of these antibodies is IgY, the immunoglobulin equivalent to mammalian IgG in birds. Egg yolks from immunized chickens are an important source of immunoglobulins, yielding large amounts of high affinity antibodies easier to obtain than mammal sera. Furthermore, the IgY yield is up to 18 times greater than that of IgG from sera [4]. The availability of large amounts of relatively inexpensive IgY from egg yolks makes it feasible to use these antibodies for passive immunization therapy through oral administration or injection [5]. The efficacy of this approach has been shown in human and veterinary medicine as for rotavirus diarrhea in humans [6,7], Escherichia coli infections in pigs [8,9,10,11], *Aeromonas hydrophila* infection in *Carassius auratus* Gibelio [12] and *Streptococcus mutans*-induced dental caries [3,13]. In aquatic species, IgY against *Edwardsiella tarda* was administered orally to passively immunize Japanese eels [14]. These studies demonstrated that IgY could serve as an effective means against bacterial and viral infections [15]. The objective of the present study was to evaluate the efficacy of chicken egg yolk antibodies against experimentally induced salmonellosis in mice. Efficacy of protection was tested in challenge with the pathogenic bacteria.

Materials and Methods

Bacteria and culture condition:

S. typhimurium (ATCC= 14028), was inoculated in SS agar and incubated at 37°C in an incubator

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overnight. On the following day, the culture was diluted in BHIB and grown as described, at 37°C in an orbital shaker incubator until an optical density of 1 (at 600 nm) was obtained. Cells were harvested by centrifugation at 2000g for 15 min in phosphate buffer saline (PBS, pH 7.2), washed twice and resuspended in PBS. The suspension concentration of 10^7 CFU/ ml was used for immunization. One strain from each of the bacteria S. typhi, *Shigella dysenteriae and* E*. coli* (human isolate from the Lab reference, Bo Ali Sina Hospital, Tehran, Iran) was also used in the enzyme-linked immunosorbent assay (ELISA).

Production of egg-yolk antibodies (IgY):

Immunization: Four adult egg-laying White Leghorn hens were immunized with *S. typhimurium* (ATCC= 14028). The organisms were prepared as described above, boiled for 5 min, and emulsified with an equal volume of Freund's adjuvant. This 1-ml preparation was divided and injected into three separate sites on the breast of each hen on the initial day of immunization. Subsequently, 1-ml booster injections of bacteria prepared in the same fashion but emulsified in Freund's incomplete adjuvant were given in the same manner, four times at 2-weeks intervals. Blood samples were taken from the hens before each booster injection, and eggs for the animal immunization were collected for two months after the completion of hen immunization.

Immunochemical methods:

ELISA, sodium dodecyl sulfate polyacrylic acid gel (SDS-PAGE) and purification of IgY in serum as well as in eggs were performed as described elsewhere [paper IgY botulism and paper pasteurella] and previously.

Egg yolk separation and purification of IgY:

All eggs were collected on the day of oviposition, labeled with date and hen number and stored at 4°C for further use. The egg yolk was separated from the egg white by gently lifting it out of the egg white, while insuring that the egg yolk membrane remained intact. The egg yolk membrane was washed with distilled water using a water bottle, punctured and 10 ml of yolk was collected into a 50 ml polypropylene tube. The yolk sample was diluted to a final volume of 40 ml with distilled water acidified to the pH of 2.5 with 0.1N HCl and stored at 20°C overnight. The samples were thawed, centrifuged (25* 1000 rcf) in a Sorvall SS-34 rotor for 15 min at 4°C. The supernatant was collected and an equal volume of 100% saturated ammonium sulfate was added dropwise, with shaking, to the sample. After overnight storage at 4°C, the samples were centrifuged (25* 1000 rcf), the supernatant was discarded and the pellet was re-suspended in PBS at (pH 7.4). The IgY purification procedure was performed by a method previously described (4), with some modifications. Briefly, the egg yolk was carefully separated from the white and washed. The content was filtrated through a 0.45 mm membrane, up to 100 ml isopropanol was added and after centrifugation at 25*1000 rcf for 15 min, supernatant was discard and up to 50 ml acetone was added to the pellet. The yolk content was mixed with 100 ml cold chloroform (-20 C). After centrifugation at 10 000 *rcf for 10 min at 4°C, the supernatant was discarded and the pellet was dissolved in a volume of PBSS equal to the volume of the yolk as measured previously and stored at –20°C. The protein concentration of the purified IgY was determined (Protein Assay; BioRad). A random sample of 10 IgY preparation was examined for purity by comparison to an IgY standard (Promega) using polyacrylamide gel electrophoresis (PAGE). A precast 12% polyacrylamide mini gel (NuPAGE Bis-Tris SDS-PAGE; Invitrogen) using an Xcell SureLock Mini-Cell (Invitrogen) was run at 200V for 45 min. The bands were visualized by staining the gel (SimplyBlue SafeStain; Invitrogen), according to the manufacturer's instruction.

The pellet was easily resuspended in acetone after 18 hours at 4° C and the solution was centrifuged at 4° C, 5000 rcf for 45 minutes. Finally, the pellet was resuspended with PBS and dialyzed against PBS.

Cross-Reactivity of IgY:

The cross-reactivity of IgY was determined by using ELISA and the following bacterial cells: *S. typhimurium, S.typhi, Escherichia coli* and S*higella dysenteriae*. Wells of the microplate were coated with 150 µl of lyophilized whole cells in carbonate-bicarbonate buffer. IgY specific for *S. typhimurium* was serially diluted with PBS and added (100 µl per well) to react with coated antigens. The cross-reactivity of anti-*S. typhimurium* IgY against selected bacteria was determined by comparing the activities against those bacteria to the activity against *S. typhimurium*.

Utility egg- yolk antibody:

For this purpose, first sixteen mice were obtained and then randomly divided into four groups. All mice were acclimatized for a minimum of 1 week prior to the experiment. Group IV containing four mice was used as the negative control (treated with antibodies). The other three groups each containing four mice were treated with placebo (egg yolk with no antibodies, as a positive control called group III), and egg-yolk antibodies (treatment, group I). Mice in group II were fed with a commercial diet. After 24 h, groups I and IV were treated with egg-yolk antibodies, group II was fed with the commercial diet and group III was treated with placebo. Then mice in

groups I, II and III were administrated with 5 ml of *S. typhimurium* at a dose of 10^9 CFU. The bacterial suspension was delivered orally to each mouse with a syringe attached to a polyethylene tube held in the mouse's oral cavity. At the same time, mice in group I were treated with egg-yolk antibodies. After this phase, the mice in group I and II were treated with egg-yolk antibodies and group III with placebo on the first day and once a day for the following two consecutive days. The clinical response of each mouse was monitored throughout the experiment in terms of occurrence of diarrhea, fecal consistency score, weight loss and mortality.

Protein Assay:

The Bio-Rad protein assay (Micro titer Plate Protocol), based on the method of Bradford (1976).

Statistical analysis:

Data were registered and analyzed using the statistical package for social science (SPSS for windows; 13.00 version). All data were expressed as the mean ± SD or SE. Person's correlation was used for describing statistical significance.

Results:

Production of specific antibody in hen serum and eggs:

An increase in *S. typhimurium* specific immunoglobulin was detected in the serum of immunized hens 20 days after the initial immunization by the ELISA (Figure 2, data are shown only for weeks 6 to 12). This response reached a peak at about day 140 (Figure 2). The antibody response was mirrored in the eggs of immunized hens (data not shown). The antibody response had increased through weeks 6 to 12 and persisted after this period. The antibody response persisted in the egg yolks throughout the period that the eggs were monitored. Data are shown only for weeks 6 to 12, as the antibody level remained constant thereafter. The amount of antibody in serum was higher than in eggs as shown in (Figure 3).

Purity of egg yolk and serum IgY:

The purity of egg yolk and serum IgY was analyzed by SDS-PAGE. (Figure 1) shows an electrophoretic pattern of the purity of IgY in egg yolk (line 1) compared with that of the serum (line 2). Our preparations showed fewer bands in serum than in egg yolk (line 2 and line 1). Addition of 2ME to IgY showed a 60 kDa band corresponding to the heavy chain and a 26 kDa band corresponding to the light chain (line 3). A 30 kDa band was present in the purified serum from the hens immunized with *S. typhimurium* with 2ME probably corresponding to a degraded product of the IgY (line 3).

Lane1, purified egg yolk immunoglobulin Y (IgY) immunized with *S. typhimurium* without 2ME. Lane 2, purified serum from hens immunized with *S. typhimurium* without 2ME. Lane 3, purified serum from hens immunized with *S. typhimurium* with 2ME. Lane 4, molecular weight marker.

Cross-Reactivity of IgY:

The antibody raised against *S. typhimurium* shows cross-reactivity with other enteric bacteria as well. The anti- *S. typhimurium* IgY cross-reacted by 63% with S. typhi as shown in Figure 4. Anti-*S. typhimurium* IgY also showed a low cross-reactivity with both S. dysenteriae and *E. coli*, by 25% and 14% respectively. The high cross-reactivity of anti-*S. typhimurium* IgY with S. typhis compared to *S. dysenteriae* and *E. coli* can be explained by the fact that both Salmonella spp. share somatic antigens and common epitopes on the different flagellin. Anti-Salmonella IgY used in this study was a polyclonal antibody against bacterial whole cells, and, thus, it may be able to raise antibodies against cross-reacting antigens. The cross-reactivity of IgY can add more value to antibacterial properties of IgY in that IgY might have an antibacterial effect on bacteria with cross-reacting antigen as well as target bacteria.

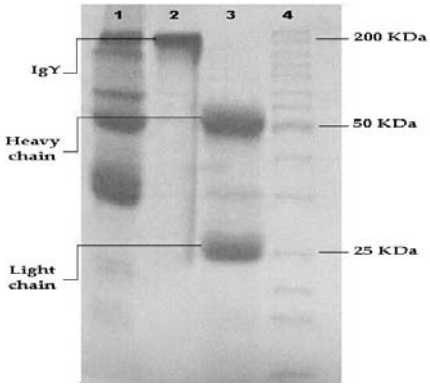

Fig. 1: SDS-PAGE of the immunoglobulin purified from egg yolk and serum.

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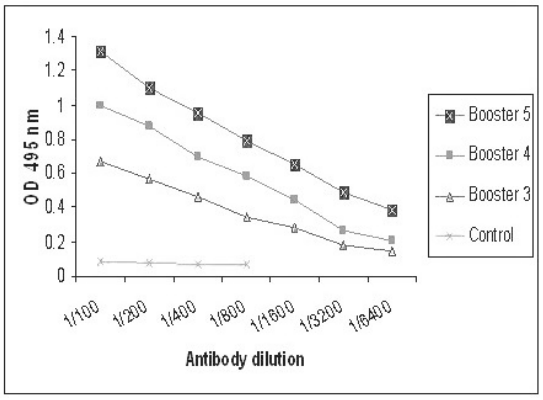

Fig. 2: Immunoglobulin-related antibody activity in sera collected from hens after 3, 4 and 5 booster injections. Hens were treated with *S. typhimurium* five times intramuscularly at two weekly intervals. Blood was collected from the hens at 2-week intervals from the third immunization and antibody activity in sera was determined by ELISA. The OD values are the means of two replicates.

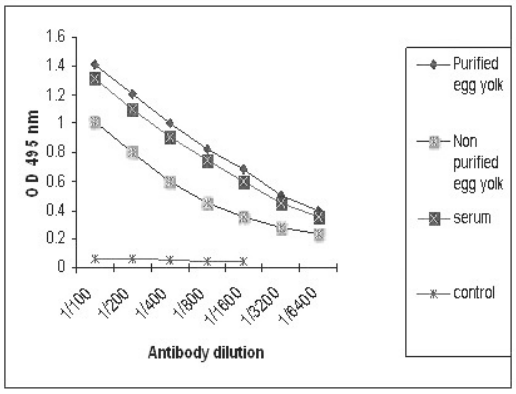

Fig. 3: Evaluation of IgY production raise against *S. typhimurium* in the serum and egg yolk by the Enzyme-linked immunosorbent assay. IgY was purified from eggs yolk (---■---), from serum (---×---) and non purified egg yolk antibody (---XX---). Placebo was used as a negative control.

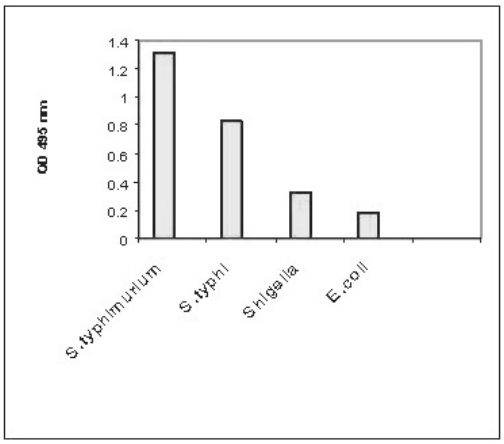

Fig. 4: The cross reaction of monoclonal antibody with some structurally related molecules such as codein and apomorphine was determined. The cross-reactivity of anti-*S.typhimurium* IgY with S.typhi, Escherichia coli and *Shigella dysenteriae*. Values are the mean of triplicate samples.

Protection of immunized mice against lethal challenge with *S. typhimurium* One the following day after oral consumption of IgY, mice were challenged with 10^7 CFU of *S. typhimurium*, and subsequent test-animal survival was recorded daily for the subsequent 30-day period. No animal developed any sign of clinical illness or died by day 5 after challenge with *S. typhimurium* being fed with either the control or immune eggs. After 5 days, reduction in appetite, movement and tremor were observed in group III (table 1). This group was treated with the placebo and developed severe illness and died within 10 days. Mice in group II treated orally with IgY, during the challenge and thereafter, showed 50% protection against a lethal dose of *S. typhimurium* challenge. In contrast, the mice treated with egg-yolk antibodies from the immunized hens exhibited no signs of illness and none of the mice died (Table 1). There was no difference in mortality between group I and the unchallenged control group.

Discussion:

The clinical application of orally administered antibodies covers the treatment and prevention of gastrointestinal infections caused by enteric pathogens in susceptible individuals, including those with immunodeficiency diseases [2]. Human antibodies would be the most physiologically adequate forms of passive immunization, but there are several limitations to their use, such as low specific titers, risk of transferring disease and high costs. By using non-human antibodies, these limitations can be overcome. Passive immunization is already an established strategy in veterinary medicine, for which there are commercial bovine colostrum and avian antibody products available [16]. IgY performs well in the intestinal mucosa, since it can be recovered from the intestine after oral administration and has a good resistance to enzymatic activity and gastric acid pH [17]. There are reports of the therapeutic utilization of IgY in veterinary enteric diseases, and its utilization in human medicine has been suggested [8,17].

In this study, the antibody raised against *S. typhimurium* shows cross-reactivity with other enteric bacteria. The anti-*S. typhimurium* IgY showed cross-reactivity with *S. typhi, S. dysenteriae* and *E. coli* by 63%, 25% and 14% respectively, Whereas Lee *et al* [18] showed that the anti-S. enteritidis IgY cross-reacted by 55.3% with *S. typhimurium* in contrast to significantly low cross-reactivity of anti-S. enteritidis IgY with *E. coli* O157:H7 or *E. coli* 987P. Anti-*S. typhimurium* IgY also showed a low cross-reactivity with both *E. coli* strains, although it did cross-react by 42.4% with *S. enteritidis*. The high cross-reactivity of anti- *S. typhimurium* IgY with *S. typhis* compared to *S. dysenteriae* and *E. coli* can be explained by the fact that both Salmonella spp. share somatic antigens and common epitopes on the different flagellin. Anti-Salmonella IgY used in this study was a polyclonal antibody against bacterial whole cells, and, thus, it may be able to raise antibodies against cross-reacting antigens. The cross-reactivity of IgY can add more value to the antibacterial properties of IgY in that IgY might have an antibacterial effect on bacteria with cross-reacting antigens as well as target bacteria.

These results indicate that mice treated with antibodies from the hens immunized against *S. typhimurium* were protected against this organism (rPirson, $P<0.01$). Previous studies have demonstrated that the egg yolk of the chickens is an excellent source of an abundant supply of relatively inexpensive antibodies [19]. It is clear that these antibodies are suitable for use in ELISA, prophylaxis and therapy of infectious intestinal diseases [20]. obtained significant protection against vibriosis up to a month post IP injection of anti-V. anguillarum antibodies in rainbow trout, while nonimmune sera did not confer any protection.

Our study confirms that specific IgY was more effective against *S. typhimurium* infections in mice when used 24 h before challenge with the virulent strain than when applied after the challenge. The antibody might function to prevent the bacterial adhesion or attachment to the host cells, an essential step for the establishment of bacterial infections. However, the protection rate was generally low in mice when used 15 min before challenge. It also indicates that protection is due to pathogen-specific IgY, not any other components, nor a nonspecific reaction between IgY and the bacterial pathogen. The results are consistent with the findings of other researchers [21]. found that chicken egg-yolk antibodies were as successful as a common antibiotic therapy in rainbow trout and chicken. [22] showed that the *E. coli* K88+ MB-induced diarrhea in 3-day-old piglets was cured 24 h after treating with egg-yolk antibodies. IgY antibodies are a promising alternative suitable for passive immunization in the treatment of viral and bacterial infections.

Our results point to the viability of the immunization of laying hens and the use of egg yolk as a source of antibodies that react to virulence factors of *S. typhimorium*. The efficacy of animal passive immunization by the oral administration of IgY encourages studies concerning its use in human therapy. However, there are still a number of issues remaining to be addressed before it can be used in the human, such as the mechanisms behind the effects of egg-yolk antibodies [23], persistence of the antibodies within the body, its efficacy after oral administration and formulations suitable for oral application through foods.

Table 1: Passive immunization in mic groups.

Time Group	G* I	G II	GIII	G IV
1	egg-yolk antibodies	egg-yolk antibodies	Challenged with 10^9 CFU + egg-yolk antibodies	egg-yolk antibodies
2	—	—	Challenged with 10^9 CFU	egg-yolk antibodies
3	placebo	placebo	Challenged with 10^9 CFU	Placebo
4	egg-yolk antibodies	egg-yolk antibodies	—	—

GI: pre-treatment at one day before challenge
GII: pre-treatment at 15 min before challenge
GIII: time of challenge
G IV: times after challenge (the first day and once a day for the following two consecutive days)
(Placebo: egg yolk with no specific antibodies)
(G*: Group)

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