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Characterization of Anti-*E. coli* Antibody Extracted From Immunized Hen Eggs By Polyethylene Glycol (PEG) Precipitation

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ABSTRACT

Background: The aim of this study was to provide a simple and efficient way of producing IgY antibodies against *Escherichia coli* and extract that by polyethylene glycol (PEG) precipitation method.

Methods: Anti- *Escherichia coli* antibody was produced in hens by using formalin-killed *E. coli* and confirmed by ELISA method, applying serum` antibody. The specific IgY was extracted from egg yolk by Polyethylene Glycol (PEG) Precipitation and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Results: The result of ELISA shows that the IgY titer increased from initial immunization and the high titre (≥0.071) persisted after the first immunization. Heavy chain of IgY with ~68 KDa was observed in the Gel electrophoresis pattern.

Conclusion: The results of the ELISA indicate the specificity of the immunoglobulin Y to the target antigen and the result of SDS-PAGE represented the appropriate extraction method. More research must be done on the ability of these antibodies to inhibit the growth of *E. coli*.

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Introduction

Escherichia coli is usually a commensal bacterium of humans and animals. Pathogenic variants cause intestinal and extraintestinal infections, including gastroenteritis, urinary tract infection, meningitis, peritonitis, and septicemia (1).

Because of the indiscriminate use of antibiotics in animal and in humane, *E. coli* has developed resistance to some of these agents that has resulted in failures in the treatment of the infectious diseases caused by *E. coli*. Moreover, *E. coli* can serve as reservoirs of resistance genes which have been efficiently exchanged not only with each other but, also with other enteric pathogens of humans and animals (2).

Another alternative, which might be more attractive, is antibody therapy. Oral administration of antibodies derived from serum and colostrum and even with monoclonal antibodies has been very successful; however, it is prohibitively expensive to obtain the large amounts of antibodies required (3).

Egg yolk immunoglobulin (IgY) can be isolated from the egg yolks of immunized hens by several simple steps without distressing the birds (4). The use of chicken antibody on a large scale offers several advantages, such as low costs, large yield and scalable production and storability (5).

This article therefore provides a simple and efficient way of producing IgY antibodies against *Escherichia coli*, identifying of anti- *E. coli* antibody in serum by ELISA method and extraction of that by polyethylene glycol (PEG) precipitation method. The aim of this study was to provide a simple and efficient way of producing IgY antibodies against *Escherichia coli* and extract that by polyethylene glycol (PEG) precipitation method.

Materials and Methods

Bacteria and Culture Conditions

Escherichia coli (ATCC 7852) were obtained from national center of genetic and biological reserves in Iran.

Experimental Animals

Four 50 weeks old, white highline chickens were obtained from aviculture and kept in animal house, School of Medicine, Alborz University of Medical Sciences, Karaj. They were used in the study for the production of anti- *Escherichia coli* antibodies (IgY).

Preparation of Antigen

Formalin-inactivated *Escherichia coli* were prepared as immunogens for immunization and antigens for ELISA experiment. Bacteria were cultured in Brain Heart Infusion Broth overnight at 37 °C and harvested by centrifugation (15 minutes, 3000 rpm). The pellet was resuspended in PBS at a density of nearly 10^8 cells/mL using 0.5 McFarland. Subsequently, bacteria were killed with 10% formalin (V/V), and suspension was hold at 4 °C for 16 hours. In order to remove the formaldehyde, bacterial suspension was washed twice with PBS and resuspended in sterile PBS at same concentration. Complete killing of the bacteria was tested by culturing the *E. coli* on MHA for 48 hours at 37 °C, and the suspension was stored at 20 °C (6, 7).

Immunization of Chickens

Chickens received four injections, one primary injection and three boosters. For the main injection, 500 µL of antigen was emulsified with an equal volume of Freund's complete adjuvant (FCA) using Bozkir et al. Procedure (8). Then the solution was injected intramuscularly at one site of breast muscle of chickens. Three booster injections of antigen with Freund's incomplete adjuvant (FIA) were given at 14 days interval by the same route of administration.

Collection of serum samples

Two mL blood sample was collected from chickens before immunization as pre-immune sera. Then it was collected at 14 days intervals. Serum was separated by centrifugation for 10 minutes at 3000 rpm and stored at 20 °C until use.

Determination of antibody titer by Indirect ELISA

The activity of IgY was monitored by the enzyme linked immunosorbent assay (ELISA) procedure described by Chakravarthi et al. (2013) (9).

Briefly *Escherichia coli* antigens were coated onto microtiter plates in coating buffer (0.05M carbonate bicarbonate buffer, pH 9.6) for overnight at 4 °C. After washing the plates three times with PBST and blocking non-specific sites with blocking buffer (1% BSA in PBST) test sera were serially diluted (in sample diluent) on the plate. preimmune sera was used as control. The total Immunoglobulin titer was determined using rabbit anti-chicken immunoglobulin coupled to Horse Radish Peroxidase (diluted with buffer assay,1:10000). The colorimetric detection was carried out using tetramethyl benzidine (TMB) as a chromogenic substrate of HRP, after washing the plate with PBST. The absorbance of each well was measured at 490nm.

Extraction of total IgY from egg yolk

The antibodies were extracted from egg yolk by the method of Pauly et al (10). Briefly, the yolk (15ml) was carefully separated from the white by "yolk spoon" in order to remove as much egg white as possible. Twice the egg yolk volume of PBS (pH=7.2) was mixed with the yolk, followed by the addition of polyethylene glycol 6000 (PEG 6000) up to 3.5% (w/v) and mixing with magnetic stirrer for 20 min. After centrifugation for 20 min (10000 rpm, 4 °C), the supernatant was poured through a folded filter and 8.5 % PEG 6000 in gram (calculated according to the new volume) was added to the tube. The suspension was centrifuged followed by the past. Finally, PEG 6000 up to 12% (w/v) was added to pellet and after centrifugation the pellet containing IgY was resuspended in 25 ml PBS.

Protein profile by SDS – PAGE

The chicken egg yolk antibodies and its molecular weight were determined by Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gel at 80 V according to the method of Laemmli (11).

Result

Chickens received four injections, one primary injection and three boosters. The result of ELSA shows that the IgY titer increased from initial immunization and the high titer (≥0.071) persisted after the first immunization (Figure 1).

Furthermore comparing serum dilutions with control group (pre-immune sera) indicate that up to 1.10000 dilution antibodies present in serum, which shows high amount of antibodies are produced in immunized chickens (Figure 2).

IgY contains two major bands, one of them about 68 kDa and another one about 25 kDa as the heavy and light chains respectively. The pattern of gel

electrophoresis (Figure 3) shows a single band with a molecular weight of ~68 KDa as heavy chain. Due to low molecular weight, Light chain often cannot be seen in the pattern of gel.

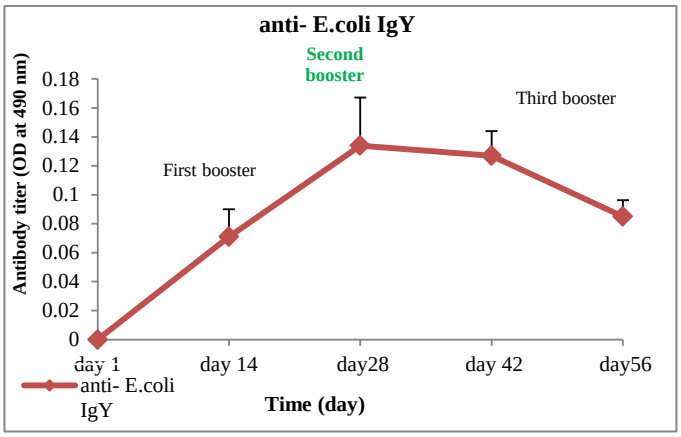

Figure 1. Titer of anti-*Escherichia coli* immunoglobulin (Ig)Y in Serum (0.0001, mean ± STD).

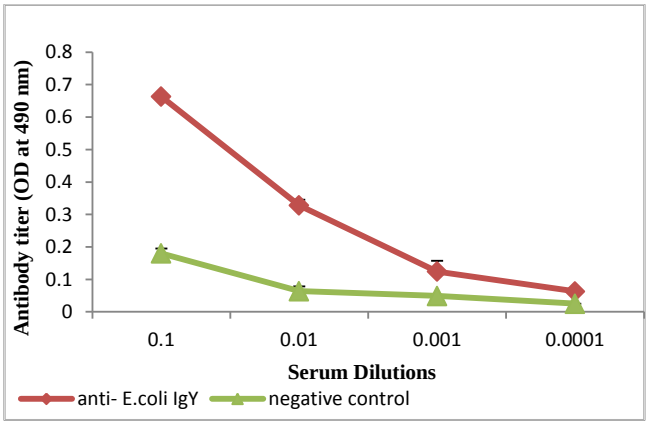

Figure 2. Titer of antibody against *Escherichia coli* in immunized chicken serum with the bacteria after injections, Pre-immune sera was served as control (mean ± STD).

Discussion

Several studies have been carried out to explore the usefulness of avian immunoglobulin Y in food, drug, microbial, and residual analyses (12), and its roles in immunodiagnostics (13), passive immunization, and therapeutic functions (14). The advantages of using avians such as chicken and quails as a source of immunoglobulins have also been Soltan Dallas (15).

In the present study, the activity and titer of specific immunoglobulin Y in serum, which were determined by ELISA, shows the presence of antigen specific antibodies for the specific pathogenic bacteria. The titer of the specific antibody produced in immunized hens increased rapidly from the first injection and reached a peak after second booster injection at the fourth week and was maintained stable for two weeks. However it decreased after last booster injection that this subject shows in order to achieve the stability in the titer of antibody, require repeated booster injections. Clifton-Hadley (2002) reported that the specific IgY against *Salmonella typhimurium* increased after booster injections and was stable for some weeks (16).

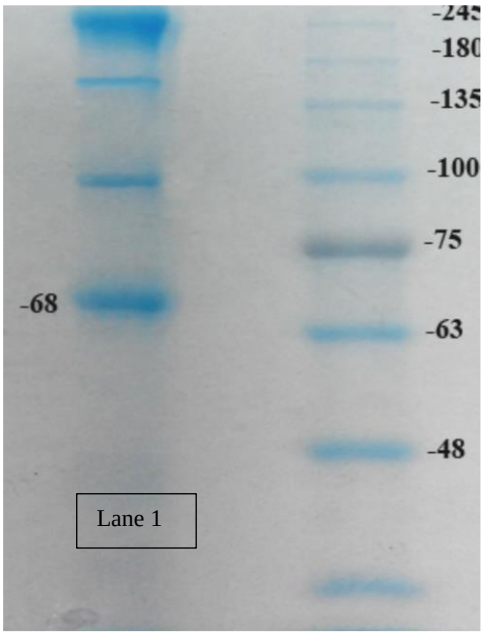

Figure 3. SDS-PAGE Profile of IgY. Lane-1: anti-Escherichia coli antibody exteracted from egg yolk by polyethylene glycol precipitation with molecular weight about 68 kDa.

The extraction of antibodies from egg yolk was done by precipitation with polyethylene glycol based on method of Paulson. The method involves two important steps. The first one is the removal of lipids and the second is the precipitation of total IgY from the supernatant of step one. Observation of antibody's heavy chain in the Gel electrophoresis pattern, represented the appropriate extraction method. However some other bonds were seen too. This fact indicates that in order to achieving purified immunoglobulin Y, purification methods must be used with this method. Pauly et al (2011) reported that the IgY-sample that was obtained by PEG-precipitation, worked very well in a lot of different immunological assays (17). In another research that was done by Ren (2016), the new method, called the chloroform-polyethylene glycol procedure was used. It was found that the chloroform - polyethylene glycol method yielded 2.57 times more IgY than the conventional polyethylene glycol method (18).

The extraordinary amount of antibody obtained by IgY-technology opens the door also for using IgY in human and veterinary medicine for therapeutic/prophylactic purposes.

Conclusion

The results of the ELISA indicate the specificity of the immunoglobulin Y to the target antigen. In order to find a viable alternative to antibiotic treatments, more research must be done on the ability of these antibodies to inhibit the growth of bacteria and infections.

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Ethics approval and consent to participate

Not needed.

Conflict of interest

The authors declare that they have no conflicts of interest.

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