

# Isolation and Characterization of Antibodies Challenging *Escherichia coli* from Japanese Quail

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**ABSTRACT:** Production of chicken immunoglobulin (IgYs) and their application in prophylactic, therapeutic, detection of microbial contaminants and as a diagnostic tool has been widely studied with limited information from other avians. This study produced Corturnix quail (*Coturnix japonica*) egg yolk IgYs against *E. coli*. Formalin inactivated *E. coli* antigens were intramuscularly immunized in quail via thigh muscles. Then after 7<sup>th</sup> day and 14<sup>th</sup> day of immunization the immunized eggs were collected. Egg yolk was separated and IgY antibodies were isolated and purified by Polson *et al* (1981) and the activity was determined by enzyme linked immunosorbent assay (ELISA). The molecular weight of the IgY antibody was found to be 93KDa using SDS PAGE. The specificity was determined by using immunoelectrophoresis. The highest titre value of antibody against the antigen is determined by ELISA and the precipitation ring was observed by Ouchterlony double diffusion. The concentration of the antigen is determined by single radial immune diffusion. The study validates the presence of antibodies in Japanese quail thereby representing quail to be a alternative source of avian antibodies.

**KEYWORDS:** *E. coli*, antibodies, antigen, ELISA, SDS PAGE

## I. INTRODUCTION

An antibody is a protein produced by the body's immune system when it detects harmful substances, called antigens. Examples of antigens include microorganisms (bacteria, fungi, parasites, and viruses) and chemicals. The ability of animal immune systems to produce antibodies capable of binding specifically to antigens can be harnessed to manufacture probes for detection of molecules of interest in a variety of research and diagnostic applications (Bizhanov *et al.*, 2004). No other current technology allows researchers to design and manufacture such highly specific molecular recognition tools. The common mammals used for the production of polyclonal and monoclonal antibodies include mice and rabbits respectively are obtained from the blood of these mammals by stressing them during the collection of blood by repeated bleeding or heart puncture. One disadvantage of purification of the immunoglobulin from the mammalian blood is that it is expensive and time consuming. Egg yolk antibody (IgY) has been successfully used for protection of experimental animals against infectious diseases and toxemia. Successful development of efficient methods to isolate immunoglobulins from egg yolk was the basic requirement of route application of yolk antibodies in microbiology. Egg yolk antibodies have been used in agriculture and veterinary medicine to control germs of infectious intestinal diseases of piglets. Fowl has been used in immunologic studies for more than 50 years but little is known about the structure of their immunoglobulins.

*Escherichia coli* commonly abbreviated *E. coli* is a Gram negative, facultative anaerobic, rod-shaped bacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm-blooded organisms (endotherms). Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in their hosts, and are occasionally responsible for product recalls due to food contamination. *Escherichia coli* infections are widely distributed among poultry of all ages and categories.

# International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 8, August 2016

The advantages include of quail antibody production are the animal suffering is reduced (no bleeding) as antibodies are obtained directly from the egg and only egg collection is required upon immunization. Antibody isolation is fast and simple. Purification is easy (Gassmann *et al.*, 1990). Very low quantities of antigen are required to obtain high and long-lasting immunoglobulin titers in the egg yolk from immunized bird. A single egg contains as much antibodies as an average bleed from a rabbit. Antibodies production is 18 times higher than mammals. It does not activate the complement system.

## II. MATERIALS AND METHODS

### 2.1 EXPERIMENTAL ANIMAL

2 month old Japanese quails were purchased from SKS poultry farm, Alazhumalai, (near Paladam), Coimbatore. Quails were maintained in animal house Karunya University, Coimbatore and used for the generation of antibodies against *E. coli*.

### 2.2 ANTIGEN PREPARATION

#### 2.2.1 Activation of *E. coli* from lyophilized form.

Lyophilized *E. coli* were bought from PSG Medical College, Coimbatore. These were activated by adding it into the Nutrient broth.

#### 2.2.2 Subculturing of *E. coli*

After reviving of *E. coli* take 50ml of nutrient broth and add 2ml of activated *E. coli* into the nutrient broth. Now keep the broth in 37°C for 18 hours in shaker.

#### 2.2.3 Antigen preparation

Mastitis causing *E. coli* cells were grown overnight in Nutrient broth. Cells were harvested by centrifugation, washed, and resuspended in phosphate-buffered saline (PBS). Formaldehyde was added to a final concentration of 1% (vol/vol), and the suspension was held at 4°C for 16 h. Cells were then washed twice with PBS to remove the formaldehyde and resuspended at the same density in sterile PBS. Complete killing of the *E. coli* suspensions was confirmed by motility test. Suspensions were stored at 4°C for before use.

### 2.3 IMMUNIZATION OF BIRDS

Three birds were taken were 1 bird is control and 2 were test birds. The 2 months old Japanese quails were immunized to obtain *E. coli* specific antibody from the eggs. The antigen solution is injected into the quail through thigh muscles. First immunization was made by intramuscular injection with 0.5ml of antigen into the right thigh of 2 test birds. The second immunization was given on 7<sup>th</sup> day through the left thigh.

Dosage	Days
I Dosage (0.5mL)	0
II Dosage (0.5mL)	7

TABLE 1: Immunization schedule

### 2.4 PURIFICATION OF ANTI-*E. coli* (IgY) ANTIBODIES

#### Purification of venom antibodies from egg yolk

##### (a) Separation of Egg yolk

The eggs were brought to room temperature 30 minutes prior to purification. The egg shell was carefully cracked and the yolk was separated from white to remove albumin as much as possible and rolled on a paper towel to remove adhering egg white. The membrane was punctured and the yolk without the membrane was allowed to flow in to a graduation cylinder and the yolk volume was recorded.

##### (b) Precipitation of lipids from egg yolk

The egg yolk was mixed with twice the volume of PBS buffer. 3.5% Polyethylene Glycol (PEG) 6000 (in grams) in PBS was added to it and vortexed thoroughly. The mixture is centrifuged for 20 minutes at 10000 rpm at 4°C to precipitate the lipids. IgY and other proteins are present in the supernatant.

##### (c) Removal of proteins other than antibodies

# International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 8, August 2016

The colorless translucent was then transferred into a centrifuge tube by filtering through cheese cloth and the pellet was discarded. 8.5% PEG 6000(in grams) was added and vortexed. It was then centrifuged for 20 minutes at 10000 rpm at 4°C to precipitate the lipids. IgY and other proteins are precipitated by PEG. The supernatant was discarded and the pellet is used. It was then dissolved in 1ml of PBS (pH-7.5) by means of a glass rod and vortexed. The final volume was made up to 10ml by PBS. The solution is mixed with 12% of PEG 6000(w/v, 1.2 grams) and centrifuged for 20 minutes at 10000 rpm at 4°C. The supernatant is discarded and the pellet is suspended in 800µl of PBS with a glass rod.

#### (d) **Purification of antibodies by dialysis**

The dialysis bags were cut into pieces of required lengths (7cm) and were boiled for 10 minutes in distilled water. The bags were transferred into boiling 2% sodium carbonate and 1Mm EDTA solution and boiled for 10 minutes. The bags were further washed thrice with distilled water and the process was repeated. Finally the bags were boiled for 10 minutes in distilled water and stored at 4°C in 50% ethanol. Care was taken that the dialysis bag was thoroughly submerged.

The pooled IgY fraction obtained from egg yolk was transferred to the activated dialysis bag and tied on both ends after including some air and twisting the open end of the dialysis bag to develop reasonable pressure. The dialysis bag with pooled IgY fraction is suspended in saline over night. The completion of the dialysis was checked with Nessler's reagent. The contents were then dispersed into eppendorff tubes and centrifuged in order to remove impurities.

#### **2.5 IMMUNOELECTROPHORESIS**

Immunoelectrophoresis is a powerful qualitative technique for the characterization of anti *E.coli* antibody. In immunoelectrophoresis, 1.5% agarose gel is prepared. Place the glass plate on the template provided. Punch a well with the help of the gel puncher corresponding to the markings on the template at the negative end. Add 10 µl of the antigen to the well and place the glass plate in the electrophoresis tank. Electrophorese at 80-120 volts and 60-70 mA, until the blue dye travels 3-4 cms from the well. The antiserum containing the antibodies added into the troughs diffuses with a plane front to react with the antigens. Due to diffusion, density gradient of the antigen and antibody are obtained and at a specific antigen/antibody ratio (equivalence point) huge macromolecules are formed. They form a visible white complex which precipitates as arcs in the gel. The arc is closer to the trough at the point where the antigen is in highest concentration. The method is very specific and highly sensitive because distinct zones are formed.

#### **2.6 SODIUM DODECYL SULPHATE - POLY ACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)**

Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis, is a technique widely used to separate proteins according to their electrophoretic mobility. The SDS gel electrophoresis of samples having identical charge to mass ratios results in fractionation by size and is probably the world's most widely used biochemical method. The electrophoresis proteins can be conveniently visualized by staining the gel with stains like coomassie blue or silver nitrate etc.

#### **2.7 SINGLE RADIAL IMMUNODIFFUSION**

Single immunodiffusion (RID) is used extensively for the quantitative estimation of antigen. The antigen-antibody precipitation is made more sensitive by the incorporation of antiserum in the agarose. Antigen is then allowed to diffuse from wells cut in the gel, in which the antiserum is uniformly distributed. Initially, as the antigen diffuses out of the well, its concentration is relatively high and soluble antigen-antibody complex reacts with more amount of antibody resulting in a lattice that precipitate to form a precipitation ring. By running a range of known antigen concentrations on the gel and by measuring the diameters of their precipitin rings, a calibration graph is plotted. Antigen concentrations of unknown samples, run on the same gel can be found by measuring the diameter of precipitin rings and extrapolating this value on the calibration graph.

#### **2.8 OUCHTERLONY DOUBLE DIFFUSION FOR ANTIBODY TITRATION**

Ouchterlony double diffusion (ODD) or immunodiffusion technique is one of the simplest techniques extensively used to check antisera for the presence of antibodies for a particular Ag and to determine its titre value. In ODD assays, solutions of Ag and Ab are placed in adjacent wells cut in agarose gel and are allowed to diffuse radially. The Ag and Ab concentration are relatively higher near their respective wells. As they diffuse farther from the wells, their concentration decreases. An antigen will react with its specific antibody to form an Ag-Ab complex. At one point their concentrations become equivalent and the Ag-Ab complex precipitates to form a precipitin line.

#### **2.9 ENZYME LINKED IMMUNO-SORBENT ASSAY (ELISA)**

ELISA also called as Enzyme – Immuno Assay (EIA) employs antibody conjugate to enzymes in such a way that the immunological and the enzymatic activity of each component is maintained. The assays are very sensitive and give

# International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 8, August 2016

accurate results can be either made visually or photometrically. The procedure of ELISA varies from Antigen and Antibody detection against any viral diseases. In this we have done indirect ELISA. The test has been widely used in the diagnosis of viral diseases (Ag-Ab) of human and animals

## III. RESULT AND DISCUSSION

### 3.1 EGG SIZE REDUCTION

It was observed that as the days passed by the size of the egg decreased, this noticed that according to “Storage Stability of Japanese Quail (*Coturnixcoturnix japonica*) Eggs at Room Temperature” showed the maturity of the egg and presence of antibodies.



Fig.1.Egg size reduction

### 3.2 ELISA

As the days increased the titre value also increased from 1:10 to 1:1000000

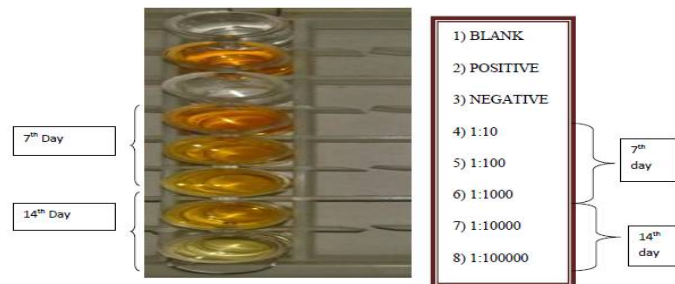


Fig.2. ELISA

Dilution	7 <sup>th</sup> day	14 <sup>th</sup> day
<b>1:10</b>	<b>0.340</b>	-
<b>1:100</b>	<b>0.254</b>	-
<b>1:1000</b>	<b>0.190</b>	-
<b>1:10000</b>	-	<b>0.150</b>
<b>1:100000</b>	-	<b>0.000</b>

Table 2: ELISA titre values for 7<sup>th</sup> and 14<sup>th</sup> day.

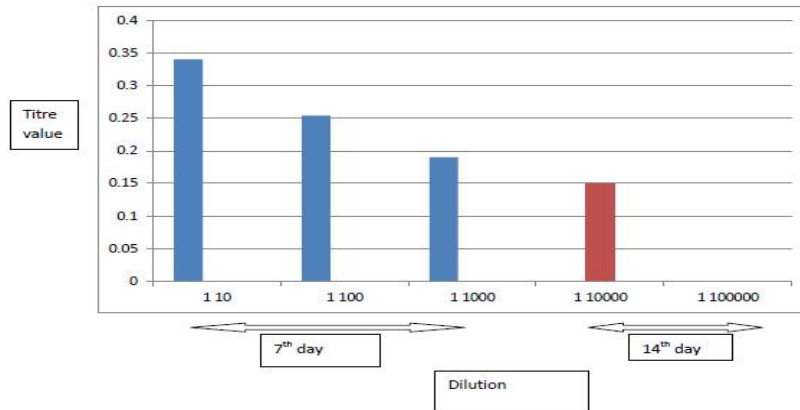


Fig.2.1. ELISA graph

### 3.3 IMMUNOELECTROPHORESIS

Immuno-electrophoresis was performed for the days of 7<sup>th</sup> and 14<sup>th</sup>. From the gel it was observed a thin precipitin line was formed at both the trough side of the gel. A thick precipitin line was found at the day 14 since antibody concentration is more on the 14<sup>th</sup> day.



Fig.3. Immuno-electrophoresis slide

### 3.4 SDS PAGE

SDS-PAGE was performed using the samples of the control, 7<sup>th</sup> and 14<sup>th</sup> days along side ran a protein marker of 100kDa. From the gel picture it was observed that the bands fell in place for both the days. From the literature (**Production of Coturnix Quail Immunoglobulins Y (IgYs) against *Vibrio parahaemolyticus* and *Vibrio vulnificus***) it was evident that the light chain was 25kDa and the heavy chain was 68kDa. The light chain had a band at 25kDa and the heavy chain at 68kDa.

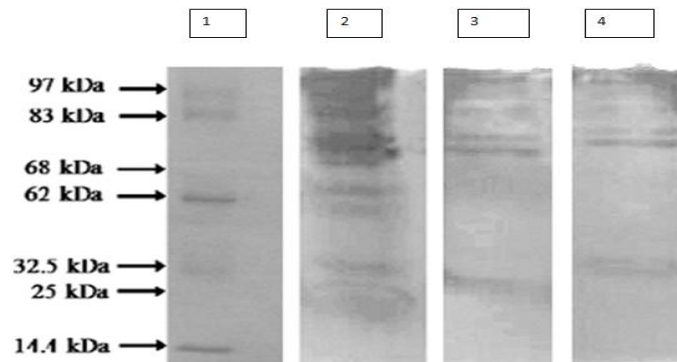


Fig.4. SDS PAGE gel picture

Lane 1-marker (100 kDa)(20µl)  
Lane 2-control (20µl)  
Lane 3- 7<sup>th</sup> day sample(20µl)  
Lane 4- 14<sup>th</sup> day sample(20µl)

# International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 8, August 2016

### 3.5 Single radial immunodiffusion

The zone was observed in all the concentration. The antigen concentration is directly proportional to the diameter of the zone. It shows the specificity of the antibody.

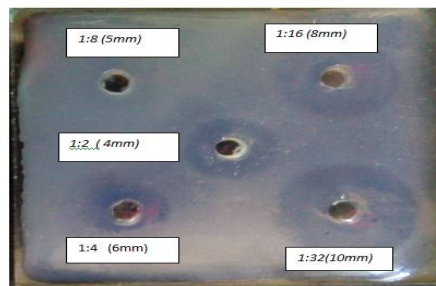


Fig.5. Single radial immunodiffusion 14<sup>th</sup> day

Concentration	14 <sup>th</sup> day diameter (mm)
1:2	4
1:4	6
1:8	5
1:16	8
1:32	10

Table 3: Single radial immuno diffusion data

### 3.6 Ouchterlony double diffusion

The antigen and antibody interaction takes place and a precipitin line will be formed. The highest titre value of the antibody against the antigen is observed in 1:2 dilution.

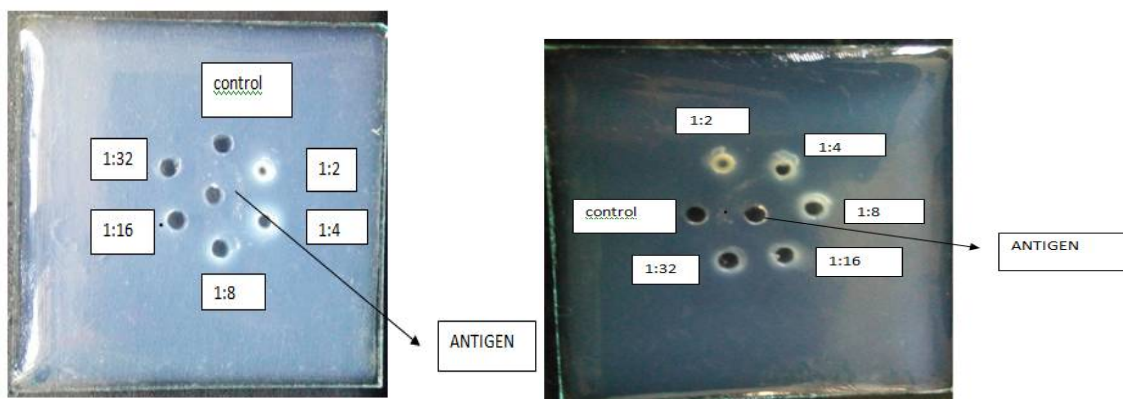


Fig.6.1. Ouchterlony double diffusion (odd) 7<sup>th</sup> day

Fig.6.2. Ouchterlony double diffusion 14<sup>th</sup> day

## IV. CONCLUSION

The avian egg yolk antibody is a new trend in the field of immunology where specific IgY antibodies produced by raising the antigen in two months old Japanese Quail. The two months old Japanese Quail were immunised with *E.coli* antigen through intramuscular vaccination. The eggs were collected on 7<sup>th</sup> and 14<sup>th</sup> day. Purification of IgY antibodies from egg yolk involves :

- Separation of egg yolk
- Precipitation of lipids from egg yolk

# International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 8, August 2016

➤ Removal of proteins other than antibodies.

Purification of antibodies is done by dialysis. The purified antibodies were stored at -20°C.

The molecular weight of antibody was confirmed to be 93kDa by SDS-PAGE. By SRID it proved the presence of zone of precipitation and the antibody concentration is directly proportional to the ring diameter of the antigen. In immunoelectrophoresis the presence of precipitin line between the antigen and antibody indicates its specificity. Specificity of antigen antibody interaction was also observed using Ouchterlony double diffusion. The antibody titre level and its specificity was analyzed by ELISA and it proved the titre level of the anti *E.coli* quail antibody had increased. The study therefore claims the presence of specific polyclonal antibodies produced and all the objectives was standardized.

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