International Journal of Life science & Pharma Research

ISOLATION AND CHARACTERIZATION OF ANTIBODIES CHALLENGING *STAPHYLOCOCCUS AUREUS* USING JAPANESE QUAIL

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ABSTRACT

This study focus on isolation and characterization of antibody (IgYs) which were isolated from the Japanese quail (*Coturnix japonica*) egg yolk, and its effect against *S.aureus*. Formalin inactivated *S.aureus* antigen were intramuscularly immunized in quail via thigh muscles. Then after 7th and 14th day of immunization the immunized eggs were collected. Eggs are used based on the conventional bleeding procedure of mammals. Egg yolk was separated and IgY antibodies were isolated, purified. Enzyme-Linked Immunosorbent Assay (ELISA) was used to analyze the activity of the highest antibody. The molecular weight of the IgY antibody is found to be 93KDa using SDS PAGE. The specificity was determined by using Immunoelectrophoresis. The presence of antibody against the particular antigen is observed by the Ouchterlony Double Diffusion method. The concentration of the antigen is determined by Single Radial Immuno Diffusion. The study validates the presence of antibodies in Japanese Quail thereby representing quail to be an alternative source of avian antibodies.

Keywords: Japanesequail, IgY, *S.aureus*, ELISA, SRID, ODD.

INTRODUCTION

Antibodies are also called as immunoglobulins, they are large Y-shaped proteins which was responsible for the identification and neutralization of foreign objects that enter in to our body. Antigens are mostly harmful or toxic or other foreign substance, that motivate the immune system to build antibodies against it.The animal immune system has the ability to construct its own antibodies for a specific antigen this was further used to makeup probes for detecting the molecules of interest for various research and diagnostic purposes. This was seems to be very specific for specific antigen. Till now there was no technology to device to fabricate 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. Japanese quail (*Coturnix japonica*) possesses at least two distinct classes of immunoglobulin based upon the antigenic differences in the H chains. Egg yolk antibody (IgY) has been successfully used for protection of experimental animals against infectious diseases and toxaemias. In microbiology, there are various efficient methods to isolate immunoglobulins from egg yolk was the basic requirement for the application of yolk antibodies. Egg yolk antibodies have been used in agriculture and veterinary medicine to control germs. Other applications of yolk antibodies includes, protection of low birth weight, immunodeficient infants, the treatment of diarrhea of AIDS patient and women during pregnancy.The advantages include of quail antibody production are: Considering the conventional bleeding procedures of mammals[1] we

are using the eggs to obtain antibodies and the eggs are collected based up on immunization. Antibody isolation was fast and simple. Very low quantities of antigen are required to obtain high and long-lasting immunoglobulin titres in the egg yolk from immunized bird. A single egg contains as much antibodies as an average bleed from a rabbit. Compared to other mammals in quail antibodies production was 18times higher. It does not activate the complement system.Specific antibodies including immunoglobulins from egg yolk have increasing applications in virus diagnosis in detection and estimation of specific molecules in food analysis and as an alternative approach in determination of structure and function of complex molecules.*S.aureus* was a gram-positive facultative anaerobic coccal bacteria and it was reproduced asexually by binary fission. Found mostly in Human respiratory tract and on the skin. Even though it was not pathogenic, it was responsible for many infections. *S. aureus* can survive from hours to weeks, or even months, on dry environmental surfaces, depending on strain[2]. In birds, Economic losses may result from decreased weight gain, decreased egg production[3], lameness, mortality, and condemnation at slaughter.*Staphylococcus aureus*, was found to be a veterinary importance of approximately 20 species which has been isolated in broiler breeders. The most common form of infection involves inflammation of the tendon sheaths known as tenosynovitis and arthritis of the hock[4]. Treatment of staphylococcosis varies in efficacy, but may be cost-effective, as well as advantageous for bird welfare.

MATERIALS AND METHODS

Experimental Bird

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 *S.aureus.*

ANTIGEN PREPARATION

Activation of S.aureus from lyophilized form

Lyophilized S.aureus were bought from Dr.Appalaraja, PSG medical college, Coimbatore. This lyophilized culture is revived by adding the Nutrient broth in to it.

Subculturing of S.aureus

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

Antigen preparation

To prepare the antigen, *Staphylococcus aureus* was cultured in nutrient broth for 18 hrs at 37ºC. Using centrifugation (10000 rpm for 10 min) the cells were harvested, discard the supernatant and washed twice the pellet with 0.9% NaCl (normal saline). The cells were diluted with sterile normal saline to108 cfu/ml and inactivated using 0.5% formaldehyde for 18 hrs.Cells were then washed twice with PBS to remove the formaldehyde and resuspended at the same density in sterile PBS. Complete killing of the *S.aureus* suspensions was confirmed by motility test. Suspensions were stored at 4°C for before use.

IMMUNIZATION OF BIRDS

3 birds were taken 1 bird is control and 2 were test birds. The 2 months old Japanese quails were immunized to obtain *S.aureus* 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 the 7th day through the left thigh. Adjuvants were not advised to be included.

Table 1
Immunization Schedule.

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

PURIFICATION OF ANTI-S.aureus (IgY) ANTIBODIES

Purification of venom antibodies from egg yolk

Separation of Egg yolk

To separate the egg yolk from the albumin, the egg shell was cracked and separation was done. Using a tissue paper the adhering egg white was removed. To extract the yolk, the membrane was puncture and the yolk was allowed to flow in to round bottom centrifuge tube and the yolk volume was recorded.

Precipitation of lipids from egg yolk

To the collected egg yolk PBS buffer will be added twice the volume of yolk. In this mixture add 3.5% PEG 6000 (in grams) and vortexed completely. To precipitate the proteins centrifuge the mixture at 10000 rpm at 4°C for 20 minutes. Discard the pellet, the supernatant contains IgY and other proteins.

Removal of proteins other than antibodies

The colourless 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. Using PEG the IgY and other proteins are precipitated and the pellet was used for further process. By means of a glass rod and vortex dissolve the pellet in 1ml of PBS (pH-7.5) and make up to 10 ml using PBS. The solution was mixed with 12% of PEG 6000(w/v, 1.2 grams). Centrifuge the mixture at10000 rpm for 20 minutes at a maintaining temperature of 4°C. Discard the supernatant and using glass rod resuspend the pellet in 800µl of PBS.

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 2% sodium carbonate and 1Mm EDTA solution and boiled for 10 minutes. Wash thrice the bag with distilled water and. Finally the bags were boiled in distilled water for 10mins and stored at 4°C in 50% ethanol. The fractionated IgY was separated and transferred in to the activated dialysis bag, then twist the open end of the dialysis bag and tie both the ends of the bag to develop reasonable pressure. Care was taken that the dialysis bag was thoroughly submerged and the IgY fraction is suspended in saline over night. The completion of the dialysis was checked with Nesseler's reagent. The contents were then dispersed into eppendorff tubes and centrifuged in order to remove the impurities.

IMMUNOELECTROPHORESIS

Prepare 10 ml of 1.5% agarose and pour it in a glass plate and allow the gel to set for 30 minutes. Using gel puncher, punch the well with the help of template. Add 10 µl of the antigen to the well and place the glass plate in the electrophoresis tank. Pour 1X Electrophoresis buffer into the electrophoresis tank such that it just covers the gel. Electrophorese until the antigen along with the blue dye travels 3-4 cms from the well. After electrophoresis take the slide out and remove the remaining gel from the troughs (A & B). Add 80 µl of antiserum A in one of the trough and antiserum B in the other. Place the glass plate in a moist chamber and incubate overnight at 37°C. We will find the specificity of antigen and antibody.

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

CASTING OF VERTICAL SLAB GEL

Separating or lower gel.

Assemble the glass plates with spacers of required thickness. Prepare a 10% separating polyacrylamide gel. Mix filter and degas for 5 min under vacuum (presence of dissolved oxygen inhibits polymerization of Acrylamide). Add the catalysts APS and TEMED. Pour the gel solution into the gap between the glass plates without any bubble formation. If any bubbles are formed, they has to be removed. Then overlay the separating gel solution with water saturated isobutanol. Leave the gel undisturbed for 45-50 minutes. Polymerization was completed when the interface between the gel and the overlaid water becomes distinct. Then drain off the overlaid water, wash the top layer with distilled water to remove any unpolymerized Acrylamide. Drain the excess water completely.

Stacking or upper gel

Prepare 5% Acrylamide gel solution. Mix and degas under vaccum for five minutes and add the TEMED and APS. Mix in such a way that eliminates the formation of air bubbles. Pour the solution between the glass plates on the top of the separating gel. Insert the Teflon comb into the stacking gel solution without trapping any air bubbles. Carefully remove the comb and wash the wells with distilled water using a syringe fitted with a fine hypodermic needle.

ELECTROPHORESIS

Mount the gel assembly on the vertical gel tank and pour Tris glycine buffer to the upper and the lower tanks. Care was taken to ensure that no bubbles are trapped in the gel wells. Connect the wire leads from the gel tank to the power supply. Load the protein samples to be electrophoresed into the well using a micro syringe (usually 20µl of sample containing 40µg of sample will be adequate). Apply current (20 mA constant for a 15 CM x 15 cm x 0.6 mm slab gel) till the dye enters the separating gel. At that time increases the current to 25mA (constant) and run till the bromo phenol dye reaches the bottom of the gel. After the run was over, the gel assembly was disassembled and the glass plates was removed carefully. The orientation of the gel was marked by cutting one of the corners of the gel.

FIXING AND STAINING OF THE GEL

Remove the gel from the glass plate and transform it to a suitable tray containing 5 volumes of the CBB solution (gel may be fixed in 5-10% TCA before staining). Leave overnight in the stain at room temperature. Next morning remove the stain. Add the gel destaining solution. Replace the destaining solution 3 times at 10-15 minutes intervals. After the final removal of the destaining solution, give two changes in 15% acetic acid at 10 minute intervals and finally leave the gel in 7% acetic acid when the proteins appear bright purple against an almost clear background. The gel may be stored in 7% acetic acid or may be dried before storage.

SINGLE RADIAL IMMUNODIFFUSION

Prepare 1.0% agarose solution in 1x assay buffer. Add 120ul of antiserum to agarose solution. Mix thoroughly for uniform distribution of antibody. Pour agarose containing the antiserum on to a glass plate. Leave it undistributed till it get solidify. Cut wells using a gel puncher, with the help of template. Add 20ul of the given standard antigens and test antigens to each wells. Keep the gel plate in moist chamber and incubate overnight at room temperature. Measure the circle diameter and mark their edges.

OUCHTERLONY DOUBLE DIFFUSION FOR ANTIBODY TITRATION

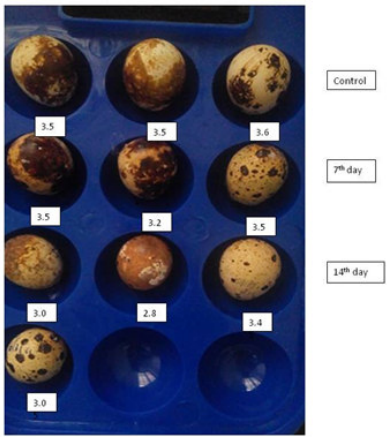
Prepare 1.0% agarose solution in 1X assay buffer. Pour 5ml of the gel solution onto a clean glass plate placed on a horizontal surface. Allow the gel to set. Punch wells in the gel with the help of gel puncher. Serially dilute the test antiserum up to 1:16 dilution. Take 20µl of 1X assay buffer in each of the vials. Mix well by adding 20µl of test antiserum in the first vial. The dilution of antiserum in this vial was 1:1. Transfer 20µl of 1:1 diluted antiserum into the second vial. The dilution in this vial was 1:2. Repeat the dilutions up to fifth vial for 1:16 dilutions. Add 10µl of the antigen to the centre well and 10µl each of undiluted, 1:1, 1:2, 1:4, 1:8, 1:16, dilutions of antiserum into the wells. Place the plate in a moist chamber and incubate at room temperature, overnight. An opaque precipitin line between the antigen and antisera wells were observed. Note down the highest dilution at which the precipitin line was formed. This was the titre value of the antiserum.

ENZYME LINKED IMMUNO-SOBENT 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 was maintained. The assays are very sensitive and give accurate results can be either made visually or photometrically. The procedure of ELISA varies from Antigen and Antibody detection against any viral diseases. Indirect ELISA technique was used.Coat ELISA plates with antigens at a predetermined concentration in carbonate buffer pH 9.6, 100µl/well. Incubate overnight at 4°C. Discard and wash the plates thrice with PBST. Incubate for 2 hours at 37°C. Discard, wash the plate thrice with PBST. Incubate positive, negative controls and blanks. Discard, wash thrice with PBST. 100µl/well with peroxidise conjugate. Incubate for 2 hours at 37°C. Discard, wash thrice with PBST. Add 100µl/well of a solution containing OPD (4mg/10ml of PCB, pH 5) with 10µl of H_2O. Incubate in the DARK at room temperature for 20 minutes. Stop the reaction with 50µ/well of 4N H_2SO_4. Read the plate at 492nm on an ELISA reader. A sample was considered to be positive if the difference in OD values was >0.1 between the HSV antigen and Vero at that particular dilution. Antigen was considered significant at that dilution.

RESULTS AND DISSCUSSIONS

EGG SIZE REDUCTION

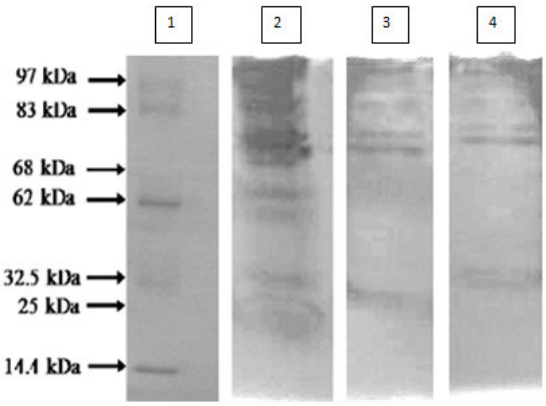


The eggs immunized with the anti *S.aureus* antibobies were collected on the 14th day from the day of immunisation "25.02.2015- 03.03.2015". It was observed that as the days passed by the size of the egg decreased, this noticed that according to "Storage Stability of Japanese Quail *(Coturnix coturnix japonica)* Eggs at Room Temperature"[5] showed the maturity of the egg and presence of antibodies.

IMMUNOELECTROPHORESIS

Immunoelectrophoresis was performed for the days of 7th and 14th. 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 14th day.

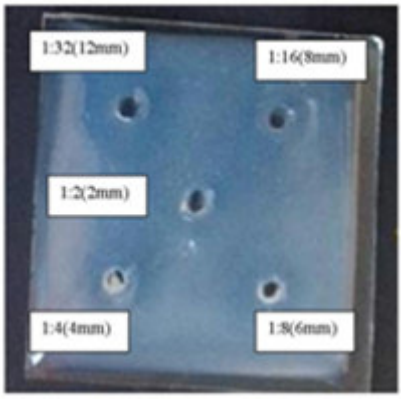
SDS PAGE



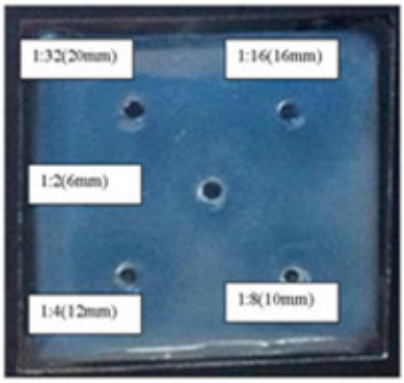
Lane 1- ***marker (100 kDa)(20µl)***
Lane 2- ***control (20µl)***
Lane 3- ***7th day sample(20µl)***
Lane 4- ***14th day sample(20µl)***

SDS-PAGE was performed using the samples of the control, 7th and 14th days along side ran a protein marker of100kDa.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*)[6] 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.

SINGLE RADIAL DIFFUSION



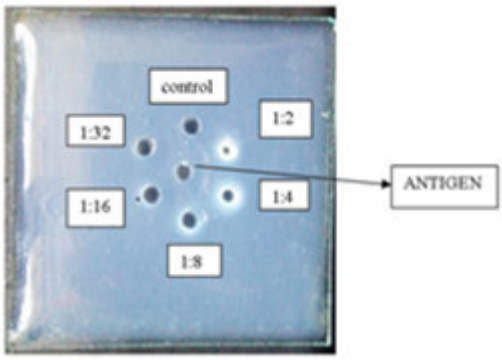
SINGLE RADIAL IMMUNODIFFUSION 7th DAY



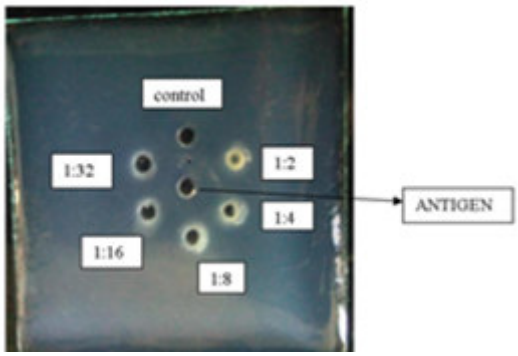
SINGLE RADIAL IMMUNODIFFUSION 14th DAY

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.

OUCHTERLONY DOUBLE DIFFUSION (ODD)



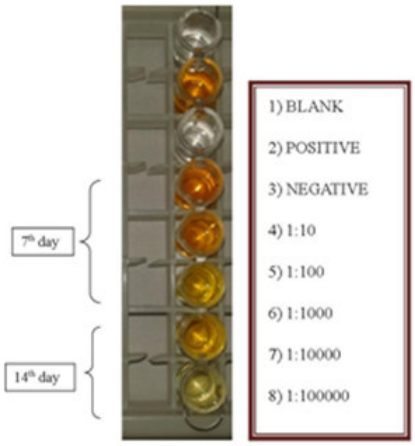
OUCHTERLONY DOUBLE DIFFUSION (ODD) 7th day



OUCHTERLONY DOUBLE DIFFUSION 14th day

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

ELISA

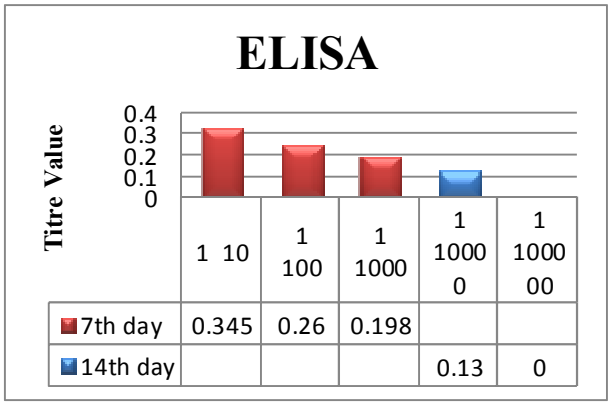


As the days increased the titre value also increased from 1:10- 1:100000.

Table 2
ELISA Titre value for 7th and 14th day.

DILUTION	7th Day Titre Value	14th Day Titre Value
1:10	0.345	-
1:100	0.260	-
1:1000	0.198	-
1:10000	-	0.130
1:100000	-	0.000

ELISA GRAPH



CONCLUSION

The avian egg yolk antibody was 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 S.aureus antigen through intramuscular vaccination (injection volume 0.5ml).The eggs were collected on 7th and 14th day. Purification of IgY antibodies from egg yolk involves:

- Separation of egg yolk
- Precipitation of lipids from egg yolk.
- Removal of proteins other than antibodies.

Purification of antibodies was done by dialysis. The purified antibodies were stored at -20°C.The molecular weight of antibody was conformed to be 93kDa. using 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 indicates the specificity between the antigen and antibody. 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 *S.aureus* quail antibody had increased. The study therefore claims the presence of specific polyclonal antibodies produced and all the objectives was standardised.

ACKNOWLEDGEMENT

I whole heartedly thank my guide, Mr. Levin Anbu Gomez Assistant Professor, Karunya University, for his valuable guidance, suggestion, motivation and constant support. I thank Dr.C.Vani**,** Assistnat Professor, Karunya University for her guidance and technical support.My heartfelt Thanks to Dr.A.Michael**,** Associate Professor & Head , PSG CAS, for his support and constant guidance at every stage of the work.Last but not the least I thank Dr.Aiyavu**,** Assistant Professor**,** Government College of Arts & Science , Pudhukottai for streamlining and reviewing the manuscript and for his valuable suggestions in this regard.

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