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IN-VITRO EVALUATION OF CHICKEN EGG YOLK ANTIBODIES GENERATEDAGAINST Streptococcus pyogenes

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Abstract: Passive immunization by oral administration of specific antibodies has been an attractive approach against group A streptococcal (GAS) pathogens in both humans and animals. Recently, laying chickens have attracted considerable attention as an alternative source of antibodies for the prevention and treatment of infectiousGAS diseases. Chicken egg yolk antibodies (IgY) were raised in twenty-two weeks old white leghorn chicken against formalin killed whole cell antigen of *Streptococcus pyogenes*. The level of the antibody in serum was monitored and booster doses were given wherever necessary. The antibodies were purified from the egg yolk of immunized chicken using PEG and Ammonium Sulphate precipitation method and further purified by dialysis method. The protein profile of anti-Streptococcus pyogenes IgY antibodies were analyzed by SDS-PAGE and Quantitative Titration of IgY antibodies by ELISA. High titer of specific antibody was found to be 1: 100000 on 91stday detected by modified ELISA and the titer were maintained with booster doses. The protein concentrationof the egg yolk was 39.99 ± 0.79 mg/ml and the total IgY concentration in egg yolk was increased during the immunization period and reached maximum of 15.26± 0.57 mg/ml. In-vitro efficacy of anti-Streptococcus pyogenes IgY was determined by growth inhibition assay. A significant reduction in the growth was observed after 16 hours of incubation. These findings may open the door for significant advances in IgY technology and passive immunotherapy with an alternative high specific nature and low cost effective.

***Keywords: Streptococcus pyogenes,* IgY, ELISA, SDS-PAGE, GAS, Quantitative titration, Microagglutination.**

I.INTRODUCTION

Streptococcus pyogenes is a major human-specific pathogenic bacterium that causes a wide scope of manifestations ranging from mild localized infections to life-threatening invasive infections. Inadequate treatment of *Streptococcus pyogenes* infections can result in the post-streptococcal infection sequelae as acute rheumatic fever and post- streptococcal glomerulonephritis. Additionally, it causes invasive infections like necrotizing fasciitis and toxic shock syndrome that is associated with and high morbidity and mortality. Streptococci are gram-positive cocci, catalase- negative, coagulase-negative cocci that occur in pairs or chains. They are divided into three groups based on the type of hemolysis on blood agar: beta-hemolytic (complete lysis of red cells), a hemolytic (green hemolysis), and gamma-hemolytic (no hemolysis). Beta-hemolytic streptococci are categorized as group A streptococci (*Streptococcus pyogenes*). Streptococcus species are associated with numerous bacterial diseases in both humans and animals. Arthritis, neonatal sepsis, meningitis, and pneumonia are few examples of diseases in humans, while in animals they mainly cause mastitis. *Streptococcus pyogenes* is differentiated as group A streptococcus (GAS) as it contains N- acetyl glucosamine linked to ramose polymer.

S. pyogenes are capable of infecting humans, mainly through adhesion and colonization of the host mucosal surface epithelial cells of the upper respiratory tract. Production of IgY antibodies from hen's egg yolk is an alternative method to cure the disease and also used to neutralize the pathogenic organism in humans. Immunoglobulins (antibodies) can be readily produced in eggs by immunizing hens against specific antigens, serum antibodies of hyper immunized hens are transferred efficiently and accumulated in the egg yolk. These Immunoglobulins can have broad applications from developing immunoassays to treating disease. Researchers have used egg antibodies in passive immunotherapy to treat a range of other diseases from bovine rotavirus in cattle to Mastitis in dairy cattle. Antibody production in eggs is particularly advantageous because hens can be effectively immunized, antibodies are readily accumulated in

the yolk, and eggs are a convenient and inexpensive food source. IgY is successfully used in immune testing,medical diagnosis, heterografts and therapy.

II. OBJECTIVE

- Generation and purification of against Streptococcus pyogenes antigen in 21 weeks old white leghornchickens.
- Characterization of IgY generated against *Streptococcus pyogenes.*
- To check the physiochemical properties of generated anti-Streptococcus pyogenes IgY.
- To check the growth inhibitory activity of generated IgY against *Streptococcus pyogenes*

III. MATERIALS AND METHOD

3.1 Experimental Animal:

Twenty-one weeks old White Leghorn chickens in good health condition were obtained from Chandran poultry, Palladam.

3.2 Sample collection:

Bacterial strain used for the present study is *Streptococcus pyogenes*. The sample was collected from P.S.G Institution of Medical Sciences and Research, Peelamedu, Coimbatore.

3.3 Characteristics of organism:

To check for purity of the culture, microscopic and biochemical characters of the strains were carried out by the following tests.

3.3.1 Gram staining test:

The prepared smear was air dried and heat fixed. Crystal violet was flooded over the smear for one minute and drained. After washing Gram's iodine was added and left for one minute. The smear was then washed in 95%(V/V) ethanol for 30 seconds. It was counter stained with safranin for two minutes. After air drying, the smear was examined under oil immersion objective in a light microscope.

3.3.2 Cultural characteristics of organism:

The *Streptococcus pyogenes* was cultured on Blood agar, Brain heart infusion broth and Nutrient agar plates Cultural characteristics of the plates were studied after the incubation.

3.3.3 Antibiotic susceptibility test:

Muller hilten agar is most commonly used agar for the antimicrobial activity test. So, the Muller Hilten Agar media was prepared under sterile condition then the media was poured into a petri plate and allowed for solidification. After that agar plate was then inoculated with *Streptococcus pyogenes* by making lawn culture using sterile swab. Then the Antibiotic discs (Co-trimazole, Chloramphenicol, Ampicillin, Azithromycin) were placed on the agar and incubated at37 ^{0} c for 24 hours.

3.4 Preparation of whole cell antigen:

Pure isolated colonies *Streptococcus pyogenes* were grown in 5ml of Selenite F broth at 37ºC for overnight. Then the culture broth was centrifuged at 7000 rpm for 15 mins. Remove the supernatant collect the pellet. Repeat this process at least three times and wash it with PBS until get a clear pellet. Formalin was added to it and kept overnight at room temperature. Again, the pellets were washed with PBS and stored under refrigeration.

3.4.1 Purity & Sterility testing of antigen:

For the sterility checking, complete killing of the bacteria was tested by resuspending an aliquot of the cell pellet in PBS saline and plating of this suspension into nutrient agar. The plates were incubated overnight at 37ºC and examined for the presence of bacterial growth.

3.5 Immunization of chickens:

For first immunization, the five-month old white leghorn chickens were intra muscularly injected at multiple sites of the breast muscles with prepared bacterial antigens. Booster doses were given with two weeks intervals. Eggs were collected at the intervals of three weeks from the initiation of immunization and checked for the presence of antibodies. Further, eggs laid by the chicken under the test were collected regularly and stored at4ºC

3.6 Purification and concentration of anti-Streptococcus pyogenes antibodies from Egg Yolk

3.6.1 Separation of Egg-Yolk

Under strict aseptic techniques the egg yolk was separated from white and was washed with water to remove as much albumin possible. The yolk membrane and any remaining egg white will stick to the tissue paper.The yolk sac was discarded. The amount of yolk obtained was measured. Then a continuous flow of 25mM phosphate buffer was maintained

until all the un-retained protein came out. IgY was eluted with 250mM phosphatebuffer pH 8.0.

3.6.2 Purification of IgY

The egg yolk antibodies were purified by the method of Polson *et al.*, (1980). To the 20ml of egg yolk, an equal amount of buffer "S" (10mM phosphate, 100mM NaCl, pH 7.4 containing 0.01% sodium azide) was added to the yolk and stirred. To this mixture 10.5% PEG 8000 in buffer "S" was added to a final concentration of 3.5%. The mixture was stirred for 30 minutes at room temperature. The stirred mixture was centrifuged at 10,000 rpm for 20 minutes. The supernatant was filtered through Whatman filter paper. The PEG was added to the supernatant for final concentration of 8.5%. The mixture was stirred and centrifuged at 10,000 rpm for 20 minutes. The pellet was collected and the 10ml buffer-"S" was added to make final concentration of 12.5% PEG. The mixture was stirred thoroughly for 30 minutes at room temperature and centrifuged at 10,000 rpm for 20 minutes. The pellet was taken and 800 µl of PBS was added and mixed it well.

3.6.3 Purification of IgY fraction by dialysis

The egg yolk antibodies were collected and mixed well immediately the antibodies were packed in dialysis bags for further purification process. The activation of cellulose membrane was facilitated by the membrane was cut into pieces of required and convenient length and allowed to boil for 10 minutes in a large volume of 2% (w/v) sodium bicarbonate and 1mM of EDTA (pH 8.0). The tubing was rinsed thoroughly in distilled water. Again, it was allowed to boil in 1mM EDTA (pH 8.0). The tubing was cooled down and stored at 4°C. Care was taken that the tubing was thoroughly submerged. After this step, the tubing was handled with gloves. Before use, the tubing was washed inside and outside with distilled water. The pooled IgY fraction obtained from egg yolk was transferred to an active dialysis bag. The contents were pooled into the dialysis bag and clipped with dialysis bag clips after including some air and twisting the open end of the dialysis bag. The bags containing pooled contents were dispensed into the respective NaCl solution and kept overnight for dialysis process. After the bags were transferred into phosphate buffer solution.

3.7 Packing of chromatography column

For purification of immunoglobulin, the column size 2x30cm was used. The burette was first cleaned well and it was packed first with glass wool to form an even bed and a rubber tube with pinch-cock was attached to the tip of the burette. The column was fixed to stand in vertical position. The silica gel was poured into the column along the sides to avoid air bubbles and was allowed to settle. Once the column was set, it was equilibrated with 25mM phosphate buffer (till the out flow of buffer showed pH 8.0).

3.8 Sample application and elution

Once the column was equilibrated (25mM phosphate buffer, pH 8.0) the level of buffer in column was allowed to run down to the matrix and the outlet was closed. The immunoglobulin IgY sample layered on the top of the column and was allowed to run till all the sample had entered the bed.

3.9. Collection of blood

The blood collection was performed after the second dosage of antigen was given. By using sterile syringe, 2ml of blood was collected from the hen for the comparison of IgY antibody concentration in Egg yolk and serum

3.10. Separation of serum

The collected blood was undergone centrifugation. The serum was present in the top of the layer and other cells were settled in a tube. Then the serum alone transferred into new Eppendorf tube by using micropipette.

3.11. Rapid agglutination test

The specificity of anti-Streptococcus antibodies of the chicken egg yolk was determined by Rapid Slide Agglutination Test (RSA). Test was done on the plastic strip; 20µl of antigen and 20µl of IgY were placed on a strip and mixed thoroughly by stirring with the help of applicator stick. Then the slide was observed for the appearance of agglutination within 2 minutes.

3.12. Estimation of protein content in IgY fraction by Lowry et al., (1951)

The total protein content was estimated by the method described by Lowry et al., (1951). [14] A quantity of 10mg Bovine Serum Albumin (BSA) was dissolved in 10ml of distilled water and used as Protein stock solution. To a series of clean test tubes 0.2 – 1.0 ml of BSA (Protein stock solution) was added and made up to final concentration of 5 ml with distilled water. From these dilutions 0.2ml was taken in to different test tubes and 2ml of Alkaline Copper Sulphate solution was added to every test tube and incubated for 10 minutes at room temperature. After incubation, 2ml of Folin-Ciocalteau reagent was added followed by 1 ml of test sample was poured in same each test tubes incubated under dark condition for 30 minutes at room temperature. Finally, the optical density (OD) was measured at 660nm. The OD values of IgY were compared with standard graph. After this total protein content was estimated by UV-Vis spectrophotometer at 260nm.

3.13. Protein Profile of IgY

Protein profile of IgY antibodies were analysed by Sodium Dodecyl Sulphate Ploy Acrylamide Gel Electrophoresis (SDS-PAGE) as described by Laemmli (1970). According to Laemmli (1970) the proteins are resolved with 10% (W/V) polyacrylamide separating gel and 4% (W/V) poly acryl amide stacking gel at 250V and 10mA.Equal ratio of prepared bacterial antigens (30µl) and sample treating buffer (30µl) were mixed well and loaded into sample wells. A wide range molecular weight (6.5-205 KDa) marker was also run along with the proteins. The characteristic protein pattern for the IgY antibodies can be visualized after sliver staining. The same protocol followed for the Serum.

3.14. Concentration of antibodies

The eluted IgY fractions were pooled together and concentrated. The dilutions were made from 1:10 to 1:1, 00,000. In 1:10 dilution 200 µl of Crude IgY was mixed with 800µl of PBS buffer. From this mixture 200µl was taken and transferred into next dilution tube which contains 800µl of PBS. This is known as 1:100 dilution repeat this process up to 1, 00,000 dilution from that mixture 200µl was taken and discarded.

3.15. Titration of antibody by ELISA

The antibody titer potency of each serums and IgY fractions obtained above was determined by the following modified ELISA. The particulate *Streptococcus pyogenes* antigen was dispensed at a concentration of 5µg/100µl in phosphate buffer. The resultant dispersion was coated into the individual wells of a 96-well plate (Polyvinyl ELISA plates), at a rate of 100µl per well and incubated at 4°c overnight. The solution was then discarded and unbound antigens in the wells were removed by washing with PBS-T for 3 times. The nonspecific binding sites were blocked by adding 200µl per well of1% bovine serum albumin in PBS and incubating the plates at 37°C for 1 hour. Plates were subsequently washed with PBST and the individual wells of each plate were then added with 100µl aliquots of Egg yolk antibodies (IgY) sat appropriate dilutions, followed by reaction at37°C for 1 hour. In control wells PBS were served as respective control. After the reaction the plates were washed three times with PBS-T. As a secondary antibody biotinated rabbit anti chicken IgY coupled to horseradish peroxidase (Genie Pvt. Ltd, Bangalore) was added at the rate of 100µl per well and the plates were incubated for 1 hour at 37°C. The plates were then washed 3 times with PBST. The antibody titer was determined by adding 100µlof freshly prepared substrate solution to wells of every plate and followed by reaction at room temperature in dark for fifteen min. The reaction was terminated by adding 50µl of terminating solution (4N H2SO4). The absorbance of the well was measured in an ELISA reader at OD490.The same protocol followed for the Serum.

3.16. Micro Agglutination Method

In this micro agglutination method 50µl of antigen was taken in each tube from 1:20–1:640 concentration. Before taking the antigen, it was mixed with 20µl of methylene blue. Then the 50 µl IgY antibodies were taken added into first tube and from that first tube 50µl was taken and added to second tube. Continue this process until last tube. From that tube 50µl was taken and discarded. Then the OD value was taken at 470nm under Spectrophotometer.

3.17. Growth inhibition assay for *Streptococcus pyogenes*

The fresh *Streptococcus pyogenes* culture broth was prepared and kept for incubation at 37^0 c for 24 hours. Then the 24 hours culture was taken and 1 ml is poured into every 11 tubes. First tube is control tube without antibody. In second tube 0.1µl of IgY was added, in third tube 0.2µl of IgY was added. Continue this up to last tube. In last tube 1.0µl of IgY was poured. After the addition of antibodies, the tubes were kept in incubation at 37^0 c for 24 hours. The OD value was taken at 470nm

3.18. Preparation of IgY yolk powder

3.18.1 Freeze-drying:

The egg shell was carefully cracked and the yolk was separated from white, washed with distilled water to remove as much albumin as possible and rolled on a paper towel to remove adhering egg white. The membrane was punctured with a lancet. Then the yolk was poured into a measuring cylinder. The same procedure was repeated for all the eggs and the final volume of the egg yolk was measured. From the pooled yolk, 15ml was taken and subjected for PEG extraction method to purify IgY. The egg yolk mixture was then frozen overnight and freeze dried in ice condenser at -40ºC and at a vacuum of 0.12 mbar to obtain Freeze dried Yolk Powder.

3.18.2 Determination of physicochemical properties and stability of IgY:

The stability of freeze dried IgY-*Sp* powder in different physicochemical conditions was determined by ELISA using purified IgY as control.

3.18.2.1. Heat stability:

Freeze dried IgY-*Sp* powder was incubated at 4ºC, 10ºC, 28ºC, 37ºC, 60ºC, 70ºC, 80ºC and 90ºC for 30 minutes. The heat treated IgY-Sp powder was cooled in an ice bath. The remaining antibody activity was measured by ELISA.

3.18.2.2. pH stability:

The pH of freeze dried IgY-*Sp* powder was modulated to the desired pH 2, 4, 6, 8 and 10 with 0.1 M sodium phosphate buffers. These solutions were incubated at 37°C for 2 hours. After incubation the activity of antibody was estimated by ELISA.

3.18.2.3. Stability of IgY in liquid yolk:

The yolks were separated from immune eggs and pooled together. 15ml of yolk was sampled from the pooled yolk and subjected for purification of IgY by PEG extraction method (Polson et al., 1980); the purified IgY was used as untreated control. Then the pooled yolk was equally dispersed in to different containers and exposed to various temperature and pH separately for the desired incubation time. After incubation, the IgY from each sample was purified and the stability of IgY was determined by ELISA.

IV. RESULTS AND DISCUSSION

4.1 Characterization of *Streptococcus pyogenes*

4.1.1 Gram staining

The gram stained smear of the strain *Streptococcus pyogenes* showed Gram-positive concise, non-motile, and non-spore forming; *Streptococcus pyogenes* characteristically is a round to ovoid coccus 0.6-1.0 µm in diameter.

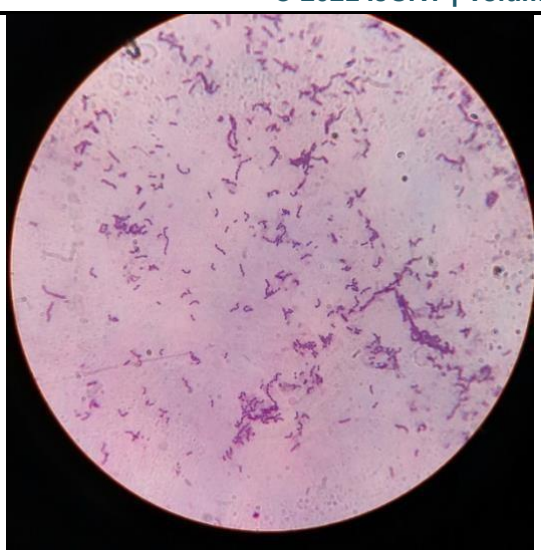

Figure 1. Gram staining- *Streptococcus pyogenes*

4.1.2 Cultural characteristics

Streptococcus pyogenes

The isolated bacterial cultures were grown on Blood agar. *Streptococcus pyogenes on blood agar* showing Small (0.5-1mm), circular, semi-transparent colonies. Produce wide zone of β- haemolysis Mucoid colonies are formed by strains that produce large capsules.

4.1.3 Biochemical characteristics

Biochemical characteristics of the bacterial strain were determined by using various biochemical tests like catalase, carbohydrate test, MR-VP test, Indole test, Urease test, TSI test etc., their results were recorded in the following table.

Table 1. Bio chemical test results

S.NO	BIOCHEMICAL TEST	RESULT
1.	Indole test	Negative
2.	Methyl Red test	Positive
3.	Voges-Proskauer test	Negative
4.	Citrate test	Positive
5.	Catalase test	Positive
6.	Urease test	Negative
7.	Triple Sugar Iron test	Ferments sugars producing acid, but no gas
8.	Carbohydrate test	
	1)Glucose	Positive
	2)Lactose	Positive

	3)Sucrose	Positive
	4)Maltose	Positive

4.1.4 Antibiotic susceptibility test

Table 2. Antibiotic susceptibility test results

Antibiotic disc	Zone of inhibition (Radius)
Cefotxime	0.75cm
Tetracyclin	0.5cm
Chlaromphenical	0.75cm
Azythromycin	1.8cm

4.2 Generation of Antibodies in Hen

The 21 week- old white leghorn Hens were immunized intramuscularly with prepared bacterial antigensto generate *anti-Streptococcus pyogenes* with two -week intervals. The eggs were collected and antibodies were separated from the egg yolk. Eggs were collected from fourth week of immunization and stored at 4°C.

4.3 Isolation and Purification of Antibodies

The purification method of chicken egg yolk antibodies facilitated by PEG. The pellets were desalted bydialysis for purification. The egg yolk antibodies were further purified by column chromatography and immunoglobulin fractions were recovered. The recovered antibodies were detected by Protein estimation

4.4 Protein estimation:

The total protein concetnration of purified IgY was estimated by UV visible spectrophotometer. Total protein concentration of serum antibody was found to be 0.46 ml and protein concentration of egg yolk IgY was found to be 0.38ml. Compare serum and egg yolk antibody with control serum and yolk IgY will be higher than the control egg. It shows that it produce high amount of antibodies in immunized egg against *Streptococcus pyogenes* antigen.

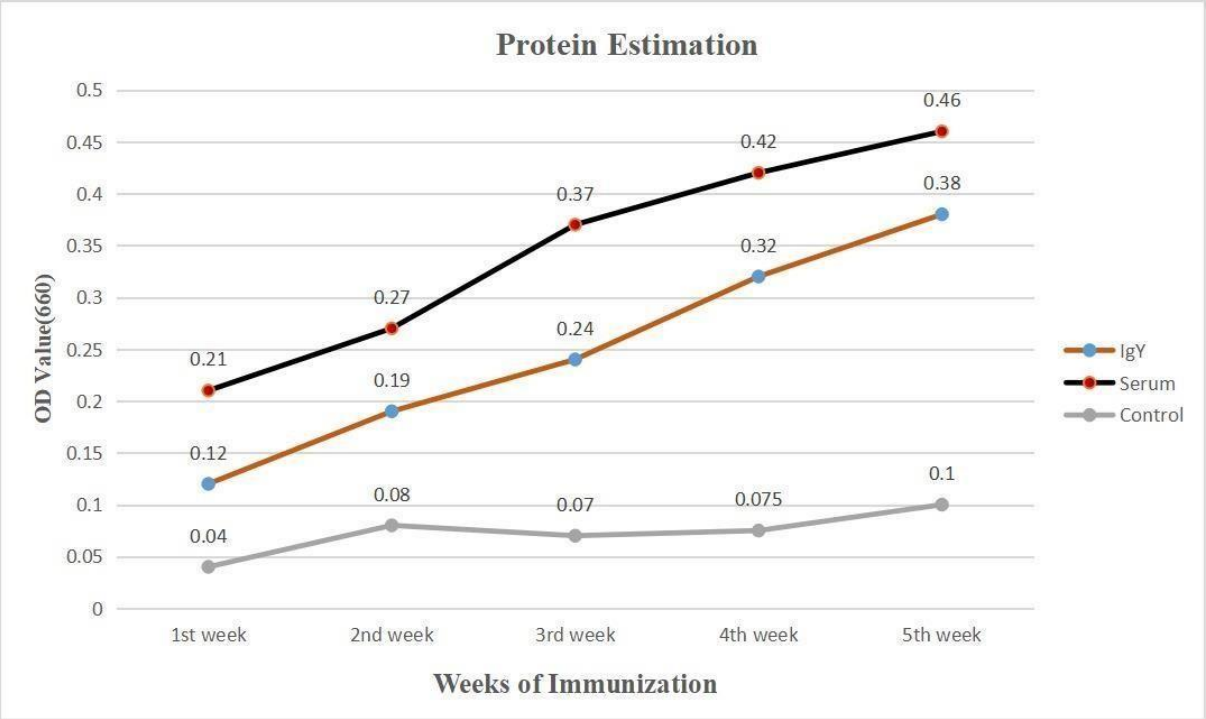

Figure 2.Protein estimation

4.5 **Rapid slide agglutination test:**

Specificity of anti-Streptococcus pyogenes antibodies in the egg yolk from immunized laying chickens was determined by Rapid slide agglutination Test (RSA). Appearance of agglutination within 2minutes, when the antigen was mixed with the corresponding IgY on plastic strip, revealed that the antibody generated in the immunized chicken which was purified as IgY-extracts from eggs which were specific against to their respectiveantigens.

4.6. IgY profiling by SDS PAGE:

In this SDS Page it forms ladder with ranges. Each band in the ladder is known molecular weight. The samples can be determined from these known weights. The chicken egg yolk antibodies and its molecular weight was determined by Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gel at 100V and 10 mA according to the method of Laemmli (1970). The purity of IgY increasedto 95% after several purification steps. The electrophoretic pattern of gel filtration fraction with the standard IgY, which was 90% pure . IgY is composed of heavy chains about 205KDa and light chains about 50KDa.

- **Lane 1-Marker**

- **Lane 2- IgY fraction by 28th day of *S. pyogenes***

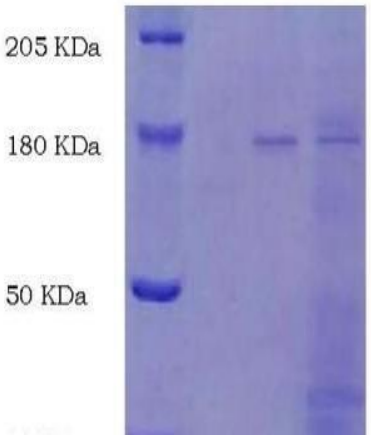

Figure 3. SDS PAGE

4.7 Estimation of antibody titer by ELISA

The antibody titer of each IgY fractions obtained above was determined Indirect ELISA as described byLee *et al.*, (2002). The antibody titer increases at the time of booster injections, even a minute increase in antibodytiter can be traced by this assay. The comparative results show that the antibody titer potencies changes in the courses of immunization. Antibody titer was very low at 0th day egg, then there was a steady increase in titer at consecutive booster immunization and reached its peak at 49th day (Plate 2b & Figure 4). After 49th day there wasa steady plateau in antibody titer till 70thday.

Figure 4. IgY against Streptococcus pyogenes ELISA result

4.8 Growth inhibition assay for *Streptococcus pyogenes*

The Minimum inhibition concentration depends upon the level or concentration of antibody which we added to test sample. The smallest concentration of an antibody that inhibits the growth of *Streptococcus pyogenes*. This value is known as Minimum inhibitory rate.

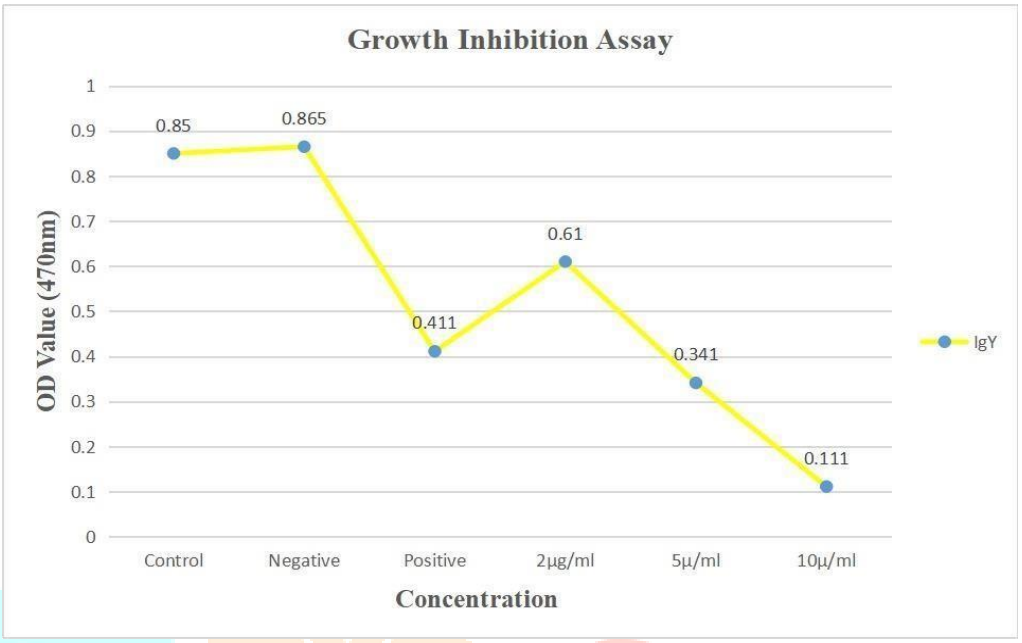

Figure 5. Growth inhibition assay

4.9 Micro agglutination

Micro agglutination test was performed in a microtiter plate. There are six different concentration was taken in those concentrations 1:20 gives a high rate of agglutination. when the dilution rate was increased agglutination rate was decreased. In 1:640 the level of agglutination is low.

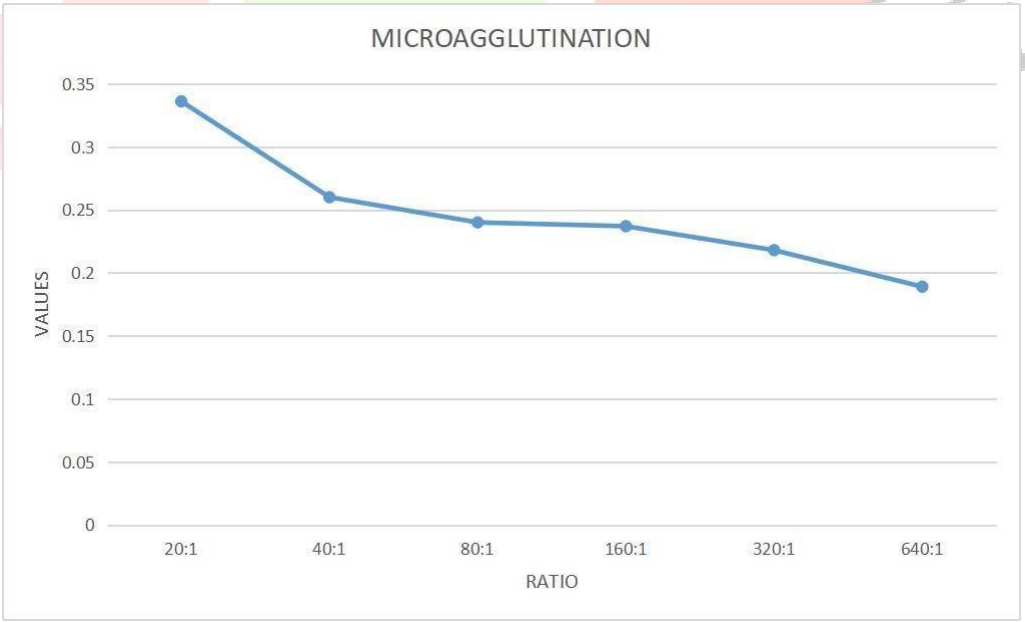

Figure 6. Micro agglutination result

4.10 Physicochemical properties and stability of IgY:

The stability of IgY in different physicochemical conditions was determined by the methods prescribedby Shin et al., 2002.

4.10.1 Heat stability

Specific reactivity of IgY after treatment with different temperatures such as 4ºC, 10ºC, 28ºC, 37ºC, 60ºC, 70ºC, 80ºC and 90ºC for 30 minutes was assessed by determining the difference between the specific reactivity of treated IgY and untreated control IgY using ELISA. The study revealed that the IgY solution was stable at 4ºC, 10ºC, 25ºC and 37ºC. Approximately 25% of the reactivity was lost at 60ºC when compared to the reactivity of untreated control. At 70ºC there was a significant decrease in the antibody activity and the complete loss of activityobserved at 80ºC and 90ºC.

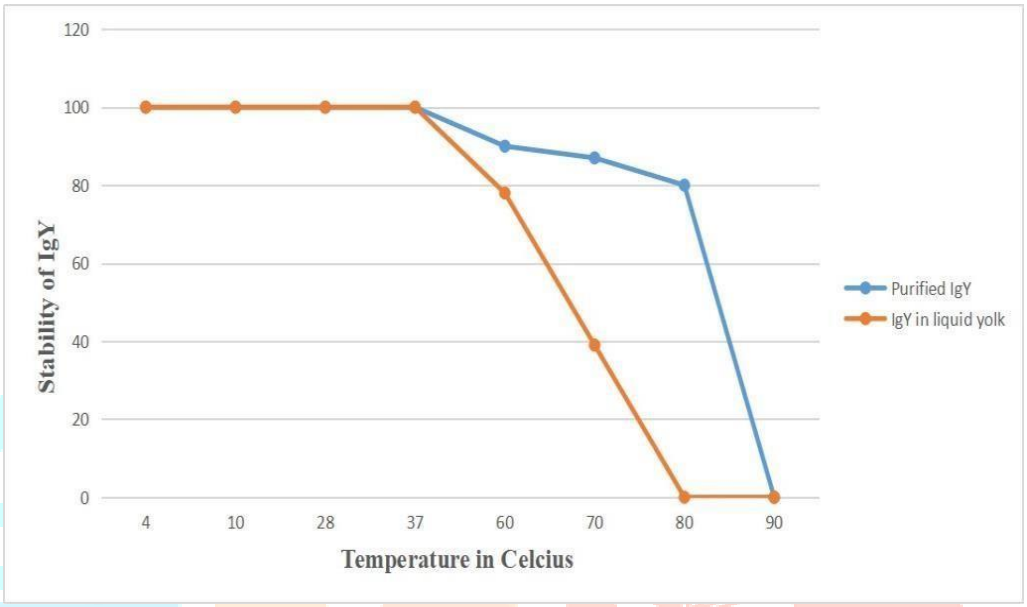

Figure 7. Thermal stability of IgY

4.10.2 pH Stability

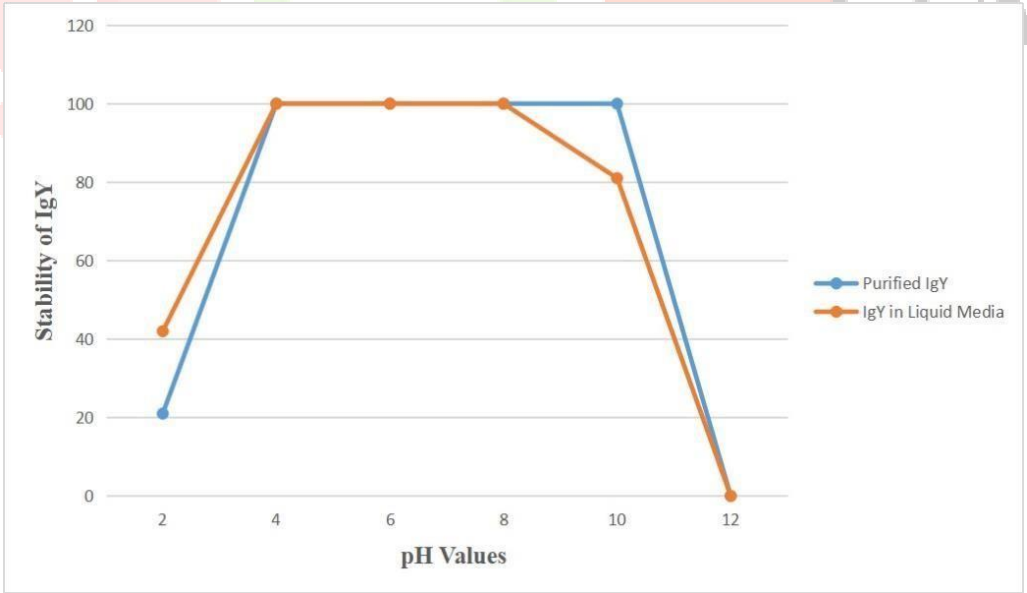

Figure 8. pH Stability of IgY

The stability of IgY when incubated at different pH such as 2, 4, 6, 8 and 10 for 2 hours at 37ºC was assessed by determining the difference between the specific reactivity of treated IgY and untreated control IgY using ELISA. The study results showed that the IgY was stable at pH ranges of 4 and 8. At pH 10 there was 70%activity and 20% activity at pH 2. There was significant decrease in IgY activity and complete loss was found at pH 12

4.10.3 Stability of IgY in liquid yolk

The stability of IgY at different temperature and pH treatments was determined when it was in its originalform such as liquid yolk. After incubating the yolk for desired time intervals at different temperatures and pH ranges (as studied previously for purified IgY-extract), the IgY was extracted from the treated egg yolk by Polsonet al., (1980) method and then the residual activity of IgY was determined by ELISA. IgY with liquid yolk was relatively stable to high temperature, at 80°C and complete loss of activity at 90°C. The IgY with liquid yolk wasfound to be stable at a pH range of pH 3.0 to pH 11.

V. CONCLUSION

Standard strains of Streptococcus pyogenes were obtained from P.S.G Institution Medical Sciences and Research. The strains were characterized morphologically and biochemically. *Streptococcus pyogenes* whole cellantigen were prepared and inactivated by 0.3% formalin to immunize in twenty-one-week-old white leghorn hens. The chickens were intramuscularly immunized with antigen of Streptococcus pyogenes. Periodically the booster doses were given at every 2 weeks of interval till the antibody level reaches a plateau. The antibodies generated from the chicken immunized with the antigen were purified from the egg yolk by Polson et al., (1980) and furtherdialyzed. Specific reactivity of anti-Streptococcus pyogenes antibodies with respect to antigen was assessed by rapid slide agglutination test.

The protein content of the egg yolk was assayed by the method of (Lowry et al., 1951) and was found to be 37.34mg/ml and 14.99mg/ml respectively and it was found to be increasing with subsequent immunization. The purity of the harvested antibodies were checked by SDS-PAGE and it was found to 180 KDa. The specific antibody level in the egg yolk was very weak on 21st day and gradually increased and reached the peak on 77th and 84th day. The titre of specific antibody was found to be 1:100000 on 84th Day by performing ELISA.

Stability of IgY in different temperature and pH was evaluated to determine its ability to withstand the gastrointestinal tract environment. Anti-*Sp* IgY was stable at 4oC, 10oC, 25oC, 37oC and its activity was lost at 60oC and then significantly decreased at 70oC. The purified IgY was stable between pH 4.0 and pH 8.0. It was noticed that the ability of IgY was relatively stable when it was with its natural form (yolk) when compared to purified lyophilized form.

In-vitro efficacy of anti-Streptococcus pyogenes -IgY was analyzed by growth inhibition assay. The binding activity of specific IgY with bacterial cells was resulted in inhibiting the growth of *Streptococcus pyogenes* in liquid medium. A significant reduction in the growth was observed after 16 hours of incubation.

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