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GENERATION, PURIFICATION AND NEUTRALIZATION POTENTIAL OF CHICKEN EGG YOLK ANTIBODIES (IgY) AGAINST MASTITIS CAUSING *ESCHERICHIA COLI* AND *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

Laying hen when immunized with antigens of *Staphylococcus aureus* and *Escherichia coli*, major etiological agent causing mastitis in dairy industries releases antibodies (IgY) which was purified and dialyzed. The concentration of antibodies increased steadily and reached a high titre by the 49th day of Immunization. The antibody titre was assayed by ELISA with a high OD_{490} value even at 1/10000 dilution. The protein estimation was done for antibodies by Lowry's method and was found to be 3.67 mg/ml for *E.coli* and 3.94 mg/ml for *S.aureus.* Protein profile of IgY antibodies analyzed by SDS- PAGE showed a band with molecular weight of 180 KDa. Growth inhibition assay when performed with 2.5mg/ml of specific IgY against the bacterial antigens showed reduction in growth of *E.coli* and *Staphylococcus aureus*. These results indicate that antibodies generated in chicken can be used for diagnosis and therapeutic purposes in bovine mastitis.

KEY WORDS: **Mastitis, IgY, *Staphylococcus aureus and Escherichia coli***

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INTRODUCTION

Mastitis is basically an inflammation of the mammary gland i.e. the udder and teats. It is therefore potentially susceptible. The inflammatory response consists of an increase in blood proteins and white blood cells in the mammary tissue and the milk. The purpose of the response is to destroy the irritant, repair the damaged-tissue and return the udder to normal function[1]. Whilst over 200 microbial species, sub species and serotypes have been isolated from bovine mammary gland[2] and identified as causative agents of mastitis, many workers from India have reported that *Staphylococcus* sp., is the chief etiological agent of mastitis in cattle[3]. Mastitis causes heavy economic losses to the dairy industry worldwide. The first report on mastitis caused losses in India was about 529 crores annually. These losses increased to 6053.21 crores annually. Nearly 70 % of this loss is a result of reduced milk production caused by sub-clinical mastitis. Bacteria eventually enter the glandular tissues where they affect alveolar cells. Toxins produced by bacteria causes damage or death to milk-secreting epithelial cells, and these cells produce substances to the blood stream that increase blood vessel permeability. This allows leukocytes to move from the blood into the alveolus where they function by engulfing bacteria[4].

Antibiotic treatment will not control this disease but it may, in certain cases, shorten the duration of the infection. Treatment effectiveness decreases as the cow becomes older and even as the first lactation progresses. The almost extreme properties of antibodies to recognize small specific structures on other molecules have made them an indispensable tool in laboratory in various applications such as research, diagnostic and therapy. Antibodies presently available for these purposes are mostly mammalian monoclonal or polyclonal antibodies. In 1893, Klemperer first demonstrated that the immunization of a hen resulted in the transfer of specific antibodies from the serum to the egg yolk. It is a refinement in that the painful and invasive blood sampling or sacrificing the animal are replaced by collecting eggs. It entails a reduction in the number of animals used because the antibodies productivity in laying hens is nearly 18 times greater than that in rabbits[5]. Antigen-specific IgY can be produced on a large-scale from eggs laid by chickens immunized with selected antigens[6]. This study was conducted to generate antibodies in White leghorn chicken against the major mastitis causing pathogens *E.coli* and *Staphylococcus aureus.* The objectives of the study were, to prepare and standardized the whole cell antigens of *Escherichia coli* and *Staphylococcus aureus,* to generate antibodies against the prepared antigens in 21-weeks old white leghorn chickens, to purify and characterize anti-*Escherichia coli* antibodies and anti-*Staphylococcus aureus* antibodies from the egg yolk of immunized chickens, to evaluate the specificity of the purified chicken IgY and to measure the inhibitory activity of the IgY against the pathogens used growth inhibition assay.

MATERIALS AND METHODS

(i) Growth and maintenance of standard strain

Standard strains of *Staphylococcus aureus* (MTCC NO. 7405) and *Escherichia coli* (MTCC NO. 14302) procured were grown on nutrient agar. Single bacterial colony from nutrient agar plates was inoculated into 5ml of nutrient broth medium under aseptic condition. This culture was used to inoculate higher volumes of medium for the future analysis. Purity of the strain was maintained by plating the overnight grown culture on respective nutrient agar plates. Isolated colonies were verified microscopically before further sub culturing or storage.

(ii) Milk sample collection

The mastitis infected milk sample collected from Veterinary Hospital in the locality was used for analysis of particular infected pathogens which were compared with the isolated standard strains.

(iii)Characteristics of organism

To check for purity of the culture, microscopic observation was first done. The isolates were sub cultured on Mannitol salt agar, MacConkey agar and Nutrient agar plates. Plates were incubated at 37ºC overnight and cultural characteristics of the colonies were studied after incubation.

(iv) Preparation of whole cell antigen

The whole cell antigen was prepared under standard indigenous conditions. Pure isolated colonies of *Staphylococcus aureus* and *Escherichia coli* were inoculated into trypticase soy broth and incubated at 37ºC overnight. They were then transferred to 250 ml of broth and incubated overnight on a rotator platform at 200 rpm. Cells were harvested by centrifugation at 3000 rpm for 15 to 20 minutes at 4ºC. Supernatant was discarded and the pellet was washed three times with 0.04 M PBS (pH 7.2, 3500 rpm, 15min, 4ºC). The pellet was resuspended in phosphate buffer saline. Subsequently, bacteria were killed with 3% formalin (V/V) for 2hrs at 37º C (continuous shaking at 2000rpm) followed by vigorous vortexing at 1600rpm for 25 hrs at 4º C. Then bacterial suspension was centrifuged at 10,000rpm for 10 minutes. Then supernatant was discarded and the pellet was resuspended in saline and used for immunization after purity check.

(v) 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. Blood was sampled at intervals of two weeks from the initiation of immunization and checked for the presence of antibodies.

(vi) Purification and concentration of egg yolk antibodies

The egg 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 and the yolk without the membrane was allowed to flow into a graduated cylinder. The egg yolk required for the five purification methods were collected in a conical flask and mixed well and made into 5 aliquots each containing 20 ml of egg yolk. This 20ml of egg yolk was subjected to different stages of purification using Polson *et al*., method[7]. The egg yolk antibodies were desalted against 1-2 liter of phosphate buffer for over 16 hours with 2 times replacement of buffer.

(vii) Protein Profile of IgY

Protein profile of IgY antibodies were analyzed by Sodium Dodecyl Sulphate Poly Acryl amide Gel Electrophoresis (SDS-PAGE) as described by Laemmli (1970)[8]. 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 sample was run until they reach the bottom of the gel. The characteristic protein pattern for the IgY antibodies can be visualized after coomassie brilliant blue staining.

(viii) Titration of antibodies by ELISA

The antibody titre was assayed by an ELISA procedure as described by Sunwoo *et al.* (2000) with modifications. The specific binding activity of IgY against whole bacterial cells were tested as follows. A microtitre plate was coated with 100 *µ*L of *S.aureus* (1.11 mg of cells/mL; 10 *µ*g of protein/ mL) and *E.coli* (10^7 cells per well) whole cells in carbonate-bicarbonate buffer (0.05 *M*, pH 9.6). The (diluted 1:1,000 in PBS, 100 *µ*L per well)

specific egg yolk antibodies (IgY) was reacted with coated antigens. The same volume (100 *µ*L) of rabbit anti-chicken IgG conjugated with horseradish peroxidase (diluted 1:1000 in PBS) and freshly prepared substrate solution, 2-2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) in 0.05 *M* phosphate citrate buffer (pH 5.0) containing 30% hydrogen peroxide were used for secondary antibody and substrate, respectively. Absorbance of the mixture was read at 490nm by a kinetic micro plate reader.

(ix) Specificity of antibodies by Growth Inhibition Assay

This assay was conducted to investigate whether the binding activity of anti-*Staphylococcus aureus* IgY and anti-*E.coli* IgY could inhibit *Staphylococcus aureus* and *E.coli* growth in a liquid medium. The IgY solutions were sterilized by using a 0.22-*µ*m membrane filter. Two milliliters of specific or nonspecific IgY solution were then added to the same volume of prepared *S.aureus* and *E.coil* cultures. The bacteria and IgY mixtures were incubated at 37°C with shaking. Aliquots of samples (100 *µ*L) were taken at 0, 2, 4, and 6 h of incubation and OD values were taken at 600nm.

RESULTS

1. Characterization of Escherichia coli & Staphylococcus aureus

The gram stained smear of the strain *E.coli* showed gram negative short rods. The colony morphology of *Escherichia coli* were large, thick, grayish, moist, smooth, opaque or partially translucent discs on Nutrient agar and the colonies showed metallic green sheen on EMB agar (Fig 1).

Morphological and Cultural Characterization of E.coli

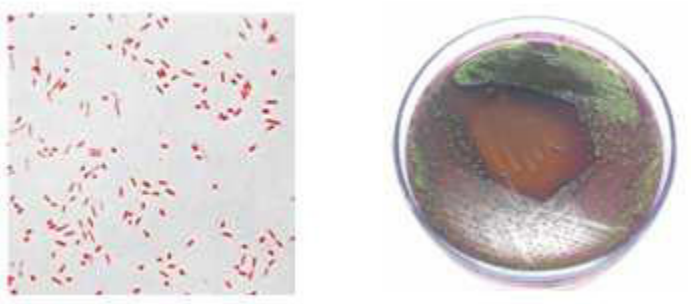


Figure 1
Gram's reaction of E.coli and Colony morphology on EMB Agar

Whereas the *staphylococcus aureus* showed the characteristic grape like clusters of gram-positive cocci and on Nutrient agar the colonies were large, circular 2-3 mm in diameter, low convex and smooth and yellow halo is typically seen around the colonies due to the fermentation of mannitol and the production of an acid on Mannitol salt agar (Fig2).

Morphological and Cultural Characterization Staphylococcus aureus

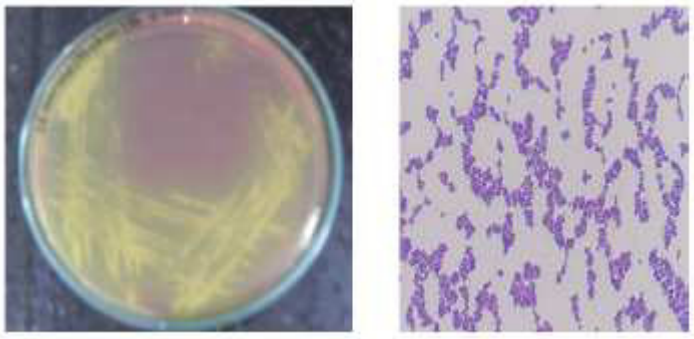


Figure 2
Gram's reaction of Staphylococcus aureus and Colony morphology on MSA

The biochemical characteristics of the bacterial strain were determined by using various biochemical tests. The catalase reaction was determined by application of 2-3 drops of 3 % hydrogen peroxide on to a portion of a colony in a glass slide or directly on colonies on the culture media and the result was recorded (Fig 3).

Biochemical Characterization of Staphylococcus aureus

Figure 3
Catalase positive for S.aureus

2. Purification of (IgY) Egg yolk Antibodies

White Leghorn Chicken were immunized intramuscularly with prepared bacterial antigens of *Staphylococcus aureus* and *E.coli* (1 x 10^9 cells/ml) to generate anti-*Staphylococcus aureus* and anti-*E.coli* antibodies with two week intervals. Eggs were collected from fourth week of immunization and stored at 4°C. The method used for purification of chicken egg yolk antibodies were PEG and ammonium sulphate precipitation described by Polson *et al* (1980). The precipitate was desalted by dialysis to remove ammonium sulphate. The recovered antibodies were detected by Protein estimation and ELISA.

1. Protein Estimation

Table 1
Estimation of concentration of antibody fraction by Lowry's Method

Egg collection (Days)	Total Protein Concentration (mg/ml)	
	Anti-*E.coli* IgY	Anti-*S.aureus* IgY
Pre immune Egg yolk	0.30	0.35
Day 1	0.35	0.48
Day 7	0.54	0.69
Day 14	0.74	0.83
Day 21	0.92	1.12
Day 28	1.09	1.39
Day 35	1.32	1.68
Day 42	1.77	2.22
Day 49	2.41	2.59
Day 56	2.68	2.86
Day 63	2.84	3.45
Day 70	3.36	3.56
Day 80	3.42	3.62
Day 90	3.67	3.94

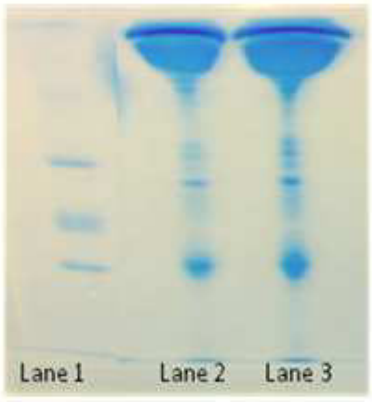
The total protein concentration of purified IgY was estimated by UV – Visible Spectrophotometer (Table 1).

2. Protein profile by SDS – PAGE

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 100 V and 10 mA according to the method of Laemmli (1970). The SDS- PAGE shows a single band with a molecular weight of 180 KDa in both lane 2 and 3. A standard molecular protein marker was also run in parallel along with IgY fraction in the lane 1. (Fig 4)

Protein profile of Antibody Fraction by SDS-PAGE

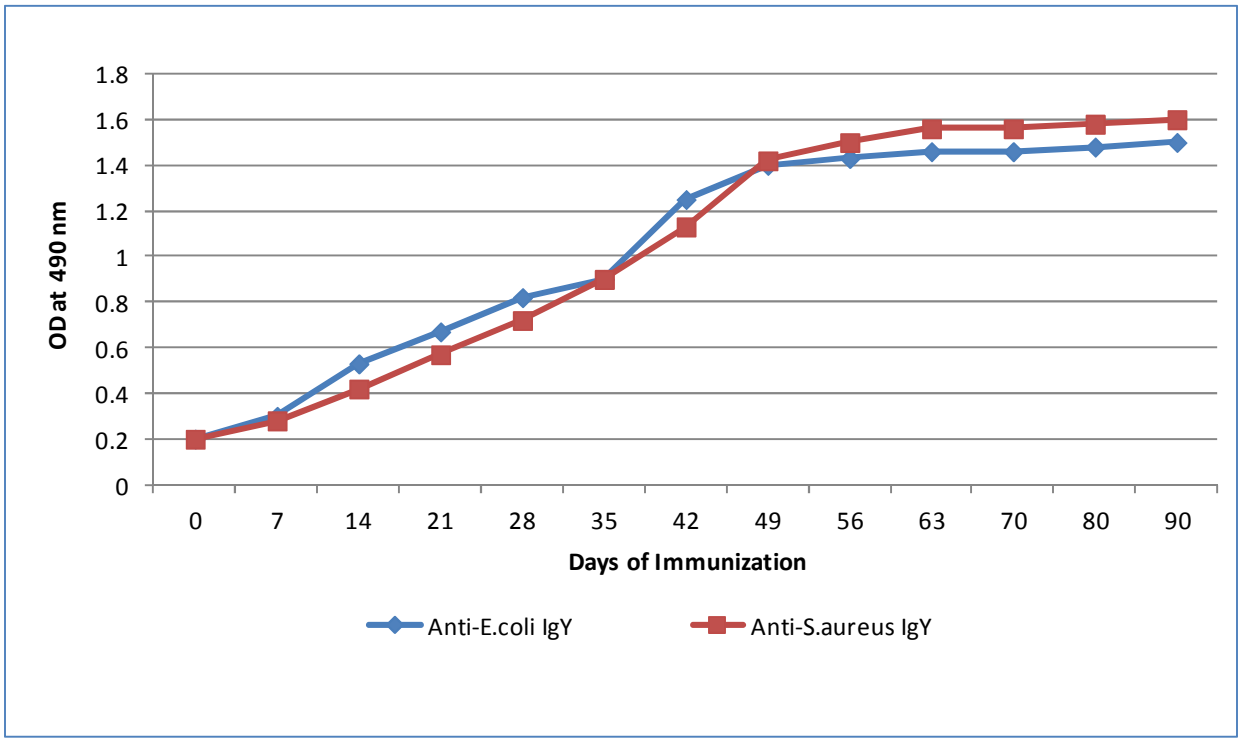
Figure 4
Electrophoretic pattern of SDS-PAGE subjected to Coomassie Blue Staining



Lane 1: Molecular Weight Protein Marker (14-200kDa)
Lane 2: Anti-E.coli IgY Fraction
Lane 3: Anti-S.aureus IgY Fraction
The molecular weight of the IgY fraction was determined to be 180kDa

3. Estimation of antibody titre by ELISA

Graph 1
Quantification of antibody titre in chicken Egg yolk using indirect ELISA



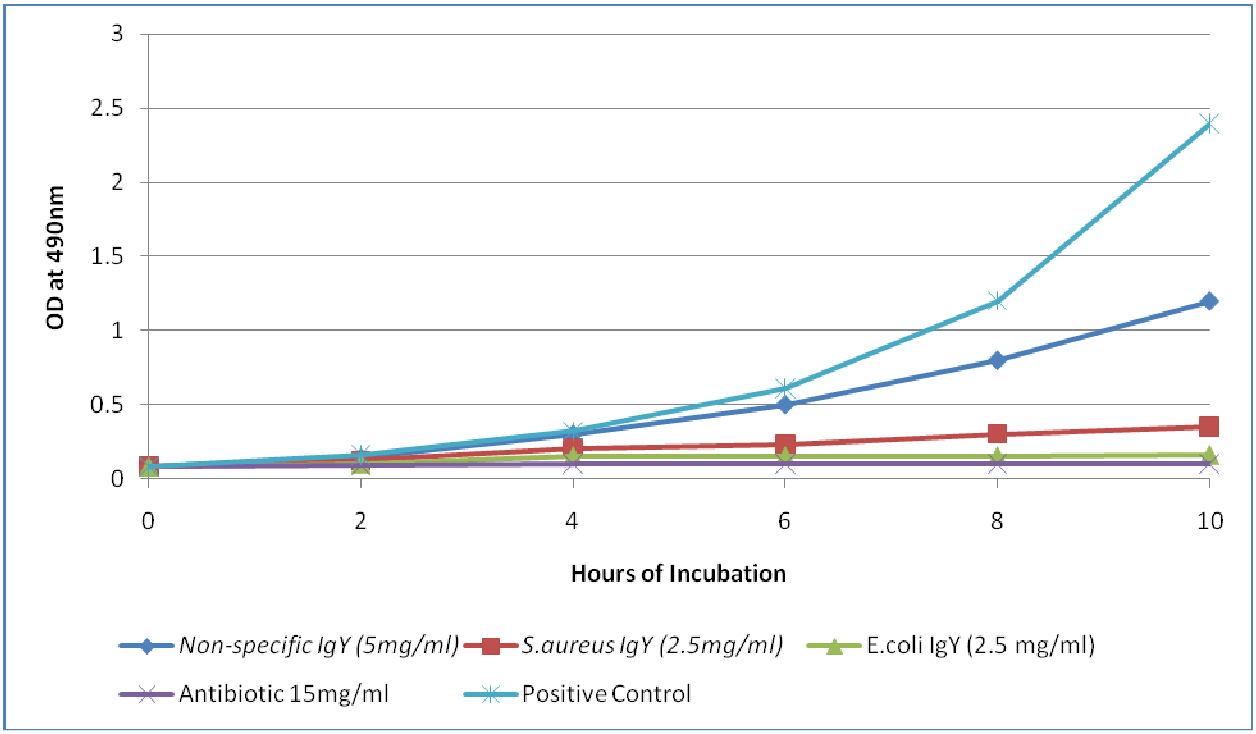
The antibody titre increases at the time of booster injections. In ELISA a highest titre of 1.514 was observed during 60th day at OD_{490}.

The antibody titre potency of each IgY fractions obtained above was determined by the following modified ELISA as described by Lee *et al*., (2002)[9]. The antibody titre increases at the time of booster injections, even a minute increase in antibody titre can be traced by this assay. The comparative results show that the antibody titre potencies changes in the courses of immunization. As it has been found that the antibodies against bacterial antigens move efficiently from serum to an egg yolk and concentrated in the egg yolk. The rate of dilution of antibodies given λ_{490} value in 1/10000 dilution.

4. Specificity of antibodies by Growth Inhibition Assay

Growth inhibition assay was performed to check the specific activity of IgY against the bacterial antigens. The growth curves of *E.coli* and *Staphylococcus aureus* were plotted for the growth of normal bacterial cells and the growth of bacterial cells with IgY fraction separately. Significant reduction in bacterial growth was observed in the cells incubated with IgY fraction.

Growth Inhibition Assay



Graph 2

The inhibitory concentration of IgY necessary to act against E.coli and Staphylococcus aureus is 2.5mg/ml. There was about ~80% reduction of growth when compared to that of the positive control.

DISCUSSION

Bovine mastitis is an inflammation of the mammary gland. The two major bacterial pathogen, *Staphylococcus aureus* and *Escherichia coli* lead to considerable economic losses for the dairy industry. At present antibiotics such as penicillin, methicillin, erythromycin etc., are primarily used for the therapy of mastitis. The vaccine is not very specific for the treatment of disease. The mammalian antibodies IgG plays a major role in the diagnosis of diseases in dairy industry. But laboratory production of antibodies involves immunization and bleeding of animals, causing distress to them. Whereas, the antibody production from chicken egg yolk is simple, inexpensive and non – invasive. IgY can be easily produced and this antibody has received much attention and was found to efficiently prevent or control pathogen infections in animals. On the basis of the advantages of IgY over the mammalian antibodies entitled in the previous report, the present study focused to develop egg yolk antibodies to control the morbidity and mortality of the dairy industry from the infection and diseases caused by the predominant bacterial pathogens such as E*scherichia coli* and *Staphylococcus aureus,* instead of the treating the infected cattles using antibiotics.

The eggs were collected, stored and antibodies were purified from chicken egg yolk by Polson *et al*., (1980) method. The molecular weight of the purified IgY's were confirmed as 180KDa through SDS PAGE. The total protein concentration of purified IgY was estimated by UV–Visible Spectrophotometer. The concentration of antibody was found to be 3.46 mg /ml for *Escherichia coli* and 2.94 mg/ml for *Staphylococcus aureus.* The antibody titre of egg yolk antibodies were determined by ELISA

showed that the presence of antigen specific antibodies for the specific bacterial pathogens. In ELISA a highest titre of 1.514 was observed during the 60th day at OD_{490}. Growth inhibition assay was performed to check the specific activity of IgY against the bacterial antigens which showed reduction in growth of *E.coli* and *Staphylococcus aureus* when treated with 2.5mg/ml of specific IgY. In conclusion, specific IgY antibodies were produced by immunizing hens with a mastitis associated *Staphylococcus aureus* and *E.coli.* IgY as a complement or alternative to antibiotics offers a possibility to avoid development of antibiotic resistance. Passive immunotherapy with specific IgY may be a promising alternative with high specific nature and low cost effective.

CONCLUSION

Antibiotics help our current medical systems by eradicating bacterial infections in the quickest way possible. Though this seems to be the positive scenario, there is also a drawback behind the use of antibiotics. An effective alternative to antibiotics should have a significant and sustainable beneficial impact on health, be proven safe for humans; be easy to apply and store, and provide a substantial return on investment. One such qualifier is egg yolk antibodies[10]. The yolks of eggs laid by immunized chicken have been recognized as an excellent source of polyclonal antibodies. Hens therefore produce a more hygienic, cost efficient, convenient and a plentiful source of antibodies as compared to traditional method of obtaining antibodies from mammalian serum [11.] The antibody generated by an alternative mean was potent enough to inhibit the growth of *E.coli* and *S.aureus*. These highly purified chicken egg yolk antibodies could be used to treat bovine mastitis, economically important disease hampering desired progress in the dairy industry[12] and play an increasing role in research, diagnostics and immunotherapy in future.

ACKNOWLEDGEMENT

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