

Production and Neutralization Potential of Avian Egg Yolk Antibodies (IgY) Targeted against Foodborne *Staphylococcus aureus*

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ABSTRACT

Staphylococcus aureus is one of the most common food poisoning bacteria. In this study, we successfully developed and characterized IgY antibodies against *S. aureus* in chicken egg yolk. *S. aureus* cells were grown to attain growth of mid-log phase and fixed by formalin. The fixed cell culture was mixed with wheat flour in a 1:10 w/w ratio and was given to laying hens as diet on daily basis for a week. Following immunization, eggs were obtained, and the PEG 8000 precipitation method was used to purify the yolk IgY. The specificity and reactivity of produced IgY against *S. aureus* cells was confirmed by cellular ELISA using *E. coli* cells as control. When the neutralizing efficacy of IgY antibodies against *S. aureus* was examined by quantitative in vitro antibacterial assay, 91 % inhibition in bacterial growth was observed at IgY concentration of 5 µg/mL. The microscopic analysis revealed the successful binding of IgY with *S. aureus* cells coated on slide. These results indicate that naturally preserved antibodies in eggs can be used therapeutically for passive immunization and diagnostic purposes. These egg yolk antibodies (IgY) can also be utilized in improving health of human beings by controlling *S. aureus* gastrointestinal tract infection.

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Authors' Contribution

MSA conceived and designed the study. RS performed the laboratory experiments and data collection. ND and ZA contributed to the IgY purification and ELISA assays. IG conducted the antibacterial activity analysis and microscopic evaluation. ML, MB and RP assisted in data interpretation and literature review. ZQS supervised the overall project and provided critical revisions to the manuscript. All authors read and approved the final version of the manuscript.

Key words

Chicken IgY, *Staphylococcus aureus*, ELISA, In vitro antibacterial activity, Immuno microscopy

INTRODUCTION

Antibodies or immunoglobulins are vital component of our defense mechanisms that identify antigens such as bacteria and viruses. These are used for many purposes including diagnostics, therapeutics and passive immunotherapies. The cost effective production of IgY antibodies in egg yolk is becoming more and more popular as it minimizes the use of animals. These antibodies has been applied to various aspects of human and veterinary health, including immunotherapy (Rahman *et al.*, 2013), neutralizing venomous animal toxins (Mendoza *et al.*, 2012),

and as functional food (Horie *et al.*, 2004).

Leslie and Clem first used the word “IgY” in 1969 to describe poultry antibodies, which include those present in egg yolks (Leslie and Clem., 1969). The primary serum antibody found in reptiles, amphibians, as well as birds is called IgY, and possesses similarities with mammalian IgG (Taylor *et al.*, 2008). Chicken IgY is the avian IgY that has been examined, characterized, and described the most. IgY glycoprotein was recognized for the first time as gamma-globulin in a gamma-livetin fraction of yolk, which is produced in blood (5–6 mg/mL) and egg yolks (10–25 mg/mL) (Williams, 1962). According to Jensenius and his colleagues, the entire activity of antibodies found in hen eggs laid within a month is equivalent to the amount found in half a liter of serum from an inoculated rabbit (Jensenius *et al.*, 1981).

Blood collection is not necessary when antibodies are obtained from egg yolks, as this is a non-invasive approach (Pereira *et al.*, 2019). IgY is the main serum immunoglobulin in the hens. This IgY is transferred to the embryo from mother through egg yolk. This egg yolk contains receptors by which IgY is transferred in the yolk.

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Whereas, IgA and IgM forms components of egg albumin (Schade *et al.*, 2005), that is why the egg yolk contains huge amount of IgY (75 % of total Ig population). Hens generate more antibodies than other animals, such as rats, which reduces the number of animals needed to make antibodies. This approach has various economic benefits over the usage of other animals. For instance, keeping a hen is less expensive than keeping mice or rabbits, yet the amount of antibodies generated by chickens is comparable to that of bigger animals like sheep (Horie *et al.*, 2004).

Bacteria that are responsible for microbial spoilage in foods are usually spore-forming bacteria. Among them, acetic acid producing bacteria contribute significantly in microbial spoiling because they can survive at low pH and limited nutrients level. Other key factors that affect microbial spoilage include water activity, pH, temperature and food composition (Rawat, 2015).

S. aureus is one of the most common food poisoning bacteria. It is difficult to eradicate *S. aureus* from the human environment, producing a wide spectrum of diseases, and its salt tolerance allows it to thrive and secrete toxins in meals, especially salty foods (Ibrahim, 2020). Bacterial infections are the major cause of foodborne illness in many countries, and they may survive and reproduce in host cells. *S. aureus* can be found in food animals, including sheep, dairy cattle as well goats with subclinical mastitis, which can contaminate milk (Argudin *et al.*, 2010). *S. aureus* can potentially be transferred to meals via dust, air, or food contact surfaces. To decrease the effect of *S. aureus* causing food poisoning, anti-*S. aureus* IgY can be produced to eliminate these effects and illness.

The primary goal of this study was to generate and purify the necessary immunoglobulin IgY from the yolk of laying hen eggs against the food poisoning bacteria *Staphylococcus aureus*. Many bacteria grow antibiotic resistant over time, therefore passive immunization might seem to improve via manufacturing huge amounts of polyclonal antibodies IgY in egg yolks in labs. This would enable food technology to use egg yolk as a therapeutic agent more successfully while reducing the need for antibiotics to treat infections caused by *S. aureus*.

MATERIALS AND METHODS

Experimental animals and bacterial strain used

Four domestic laying hens (*Gallus gallus*) were obtained from poultry farm and were kept in animal house under standard conditioned of animal health. These chickens were used to produce desired immunoglobulin (IgY) in the egg against food poisoning bacterium *S. aureus*. Glycerol stock of *S. aureus* was received from the School of Biochemistry and Biotechnology (SBB),

University of the Punjab, Lahore and further maintained on minimal salt medium.

Vaccine preparation

The *S. aureus* culture was aseptically transferred into falcon tube containing minimal media and placed in incubator at 37°C overnight. The growing cells were streaked on minimal agar plate to obtain *S. aureus* colonies. A single colony was picked from LB agar plate and inoculated into different flasks containing minimal media of total volume up to 500 mL for the growth of *S. aureus* at large scale to prepare large amount of vaccine. Medium containing inoculated *S. aureus* culture was incubated at 37 °C in shaking incubator. When the OD_{600nm} of culture reached at 0.6, the media was placed on ice for 20 min. 20 mL of 10 % buffered formalin (10 mM Tris-Cl (pH 7.4) with 10 % v/v formalin) was added drop wise in every 500 ml of ice cold *S. aureus* culture. The cells were mixed with buffered formalin for 25-30 min on ice. After fixation, the media was removed via centrifugation at 5000 rpm at 4°C for 15 min. The excessive fixative in pellets was removed by washing 3 times with 1X TBS (pH 7.4). The cells were adjusted to final concentration of 1 X 10¹⁰ cells/mL in 1X TBS and stored at -20°C for oral vaccination.

Production of anti S. aureus IgY in laying chicken eggs

Two domestic laying hens (*Gallus gallus*, weigh 1.5 kg) were chosen for oral vaccination and maintained in animal house under standard conditions after approval of study from the University ethical committee. The food selected for oral vaccination was wheat flour. One gram of prepared *S. aureus* vaccine was mixed thoroughly with 20 grams of wet flour to make fresh wet cake every day for one time and was given to laying hens daily for 6 days. Eggs of laying hens immunized with *S. aureus* were properly marked with dates as well as days. For further processing, these eggs were preserved in storage at 4°C. Control laying hens were fed with wheat flour only. Eggs collected of laying hens immunized orally and intramuscularly were used for purification of IgY. Two more laying chickens were chosen to receive intramuscular vaccinations at various places in her breast muscles. Fixed cells of *S. aureus* were combined with 0.1 mL of 1X PBS (10³ cells /0.1 mL) and was injected in chickens at 5 days interval for 20 days. The chicken received four injections using the same procedure, but the cell concentration increased from 10³ cells /0.1 mL to 10⁶ cells/0.1 mL.

Anti-S. aureus IgY isolation

The PEG (polyethylene glycol) method was employed to obtain antibodies (IgY) from the yolks of eggs. Egg spoons were used to separate the yolk from the white, and

any extra egg white was removed by rolling yolk on paper towels. The yolk of the egg membrane was punctured egg yolk was collected in sterile falcon tube with the help of syringe and the volume of yolk was noted as V1. After that, two-fold yolk volume of 1X PBS buffer was added in yolk and again volume (V2) was noted. For the separation of the protein IgY from the lipids in the yolk of the egg yolk, 3.5 % PEG 8000 was added to the mixture's final volume. PEG treated egg yolk was vortexed thoroughly for 30 sec and tubes were placed on rolling shaker for 10 min. The mixture was then centrifuged at 10,000 rpm for 20 min at 4°C, the supernatant was collected and filtered using filter paper. The volume of filtered supernatant was recorded as V3. Again, PEG-8000 was added to a final concentration of 8.5 % and mixture was placed on a rolling shaker for 20 min after vortexing. The mixture was again centrifuged at 10,000 rpm for 20 min at 4°C, the pellet was collected and dissolved in 10 mL of 1 X PBS buffer. After that, PEG-8000 was added to a final concentration of 12 % and mixture was vortexed. Again, the mixture was centrifuged at same conditions and collected pellet was dissolved in 1 mL of 1 X PBS. The mixture was dialyzed against 1 L of 1X PBS for overnight and stored -20°C for further use. The IgY concentration was determined at 280 nm by using 1.33 as extinction coefficient of IgY.

Characterization of anti *S. aureus* IgY

Electrophoretic analysis

The purified IgY partial purity was confirmed on 10 % SDS polyacrylamide gel and 10 % native gel. Protein band was observed after staining and de staining. The IgY from eggs of laying hens immunized with oral vaccine and blood collected after intramuscular vaccination were used separately in immunochemical assays.

ELISA

The specificity and reactivity of IgY was tested by cellular ELISA. *S. aureus* and *E. coli* were grown separately on LB media and cells were adjusted to 10^3 cells/10 μ L. After that, 90 μ L of fresh LB media was mixed with 10 μ L of diluted bacterial culture and added in 96 well micro-titer plate. After an incubation of one hour, 100 μ L of 8 % paraformaldehyde in 1 X PBS was added as fixing solution and incubated for 20 min to fix the cells with plate. The attached cells were washed gently 3 times with 1 X PBS and 200 μ L of blocking solution (5 % skimmed milk in 1X PBS) was added in each well. Plates were incubated for 1 h at 25 °C. After washing three times with PBS, 200 μ L of egg purified diluted antibody (anti *S. aureus* IgY, 1:20 dilution with PBS) was added in each well and incubated for 2 h in humidified chamber. After washings, 200 μ L of diluted antibody (goat anti-IgY

AP-conjugated, 1:1000 dilution) was added in each well. The plates were incubated for 1 h at room temperature and washed 3 times with 1X PBS. After washing, 200 μ L of freshly prepared substrate solution (para-nitrophenyl phosphate) was added in each well and yellow color development was recorded.

Growth inhibition assay

The specific inhibition of *S. aureus* cells by anti *S. aureus* IgY was checked by growth inhibition assay. Anti *S. aureus* IgY antibody was diluted at different increasing concentrations in 1X PBS buffer (1 μ g/mL to 5 μ g/mL of IgY) and added in 5 mL LB media containing 10^5 cells/mL of *E. coli* and *S. aureus* separately. The culture was incubated for 6 h at 37°C. After incubation, OD_{600nm} was measured using 1x PBS as a blank. The absorbance of culture was noted.

Immuno microscopy

A smear of pure culture of *S. aureus* was made on a marked area of microscopic slide coated with albumin. The smear was air dried and heat fixed using flame. After that, a drop of blocking solution (1% BSA) was placed on the marked area of slide and slide was incubated for 30 min at room temperature. The slide was then incubated with anti-*S. aureus* IgY as primary antibody (1:20 dilution) for one hour after washing slide three times with 1X TBS buffer. After that, the slides were again washed with 1X TBS buffer and 50 μ L of diluted (1:1000) enzyme conjugated goat anti-IgY antibodies was added and incubated for 45 min in humidified chamber. Finally, 50 μ L of substrate solution was added on slide after three washings with 1X TBS buffer and slides were incubated in humidified chamber for 15 min. The slides were observed under the microscope.

RESULTS

Electrophoretic analysis of anti-*S. aureus* IgY isolated from egg yolk

The eggs yolks were collected and anti-*S. aureus* IgY antibodies were isolated via PEG precipitation method. The concentration of anti-*S. aureus* IgY antibodies from egg yolk was determined and it was found nearly 18.5 mg/mL. When the isolated IgY antibodies were analyzed by SDS-PAGE, two bands of ~ 66 kDa and ~ 26 kDa were observed which indicate the presence of two heavy chains of IgY in ~ 66 kDa band and two light chains in ~ 26 kDa band while in case of native-PAGE analysis, a single band of MW equal to ~180 kDa was observed as shown in Figure 1A, B.

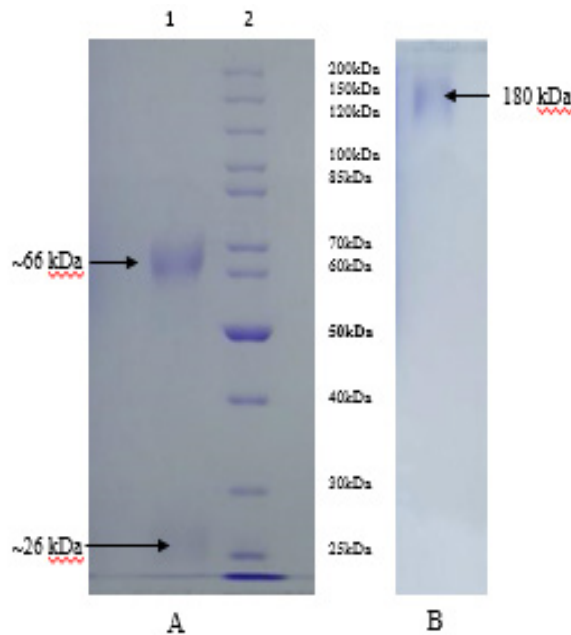


Fig. 1. Analysis of isolated anti-*S. aureus* IgY antibodies by SDS-PAGE and Native-PAGE, A) Lane 1 bands indicate the presence of IgY heavy (~66 kDa) and light chains (~26 kDa), Lane 2 represent protein ladder, B, a single band of IgY equal to 180 kDa was observed on native-PAGE gel.

Characterization of anti *S. aureus* IgY by ELISA

For the purpose of confirming the existence of specific anti-*S. aureus* IgY in the sample against *S. aureus*, an ELISA was conducted. The development of color in test wells and absence of color in control wells confirmed the presence of anti *S. aureus* IgY antibodies in the sample. It was observed that the reactivity of IgY did not show reactivity with *E. coli* which indicated the specificity of the antibody. Similar ELISA results were observed with purified IgY from yolk of egg from laying hen immunized by intramuscular or by oral vaccination process (Fig. 2).

Neutralizing efficacy of chicken anti-*S. aureus* IgY antibodies against *S. aureus*

To validate the neutralizing efficacy of anti-*S. aureus* IgY antibodies against *S. aureus*, quantitative in vitro antibacterial assay was performed using different increasing concentrations of IgY antibodies (1-5 µg/mL). Simultaneously, the inhibitory effect of IgY antibodies was also determined against *E. coli* cells to confirm the specificity of anti-*S. aureus* IgY antibodies. It was observed that at IgY concentration of 5 µg/mL, 91 % inhibition in bacterial growth was observed as compared to controls. In addition, anti-*S. aureus* IgY antibodies did not show any inhibitory effect on the growth of *E. coli* (Fig. 3).

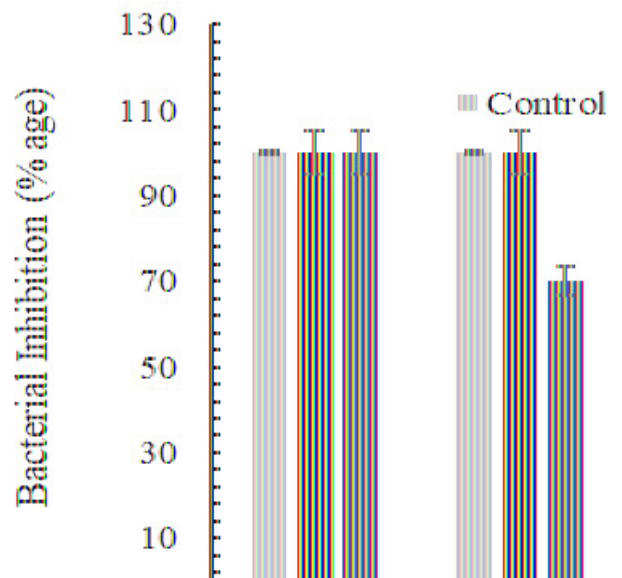


Fig. 2. ELISA, (A) analysis of anti *S. aureus* IgY antibodies isolated from egg yolk of hens immunized by intramuscular injections by ELISA, well 0, no color development was observed in well coated with *E. coli* cells as control and anti *S. aureus* IgY antibody was used as primary antibody, wells 1-12 color development was observed in wells coated with *S. aureus* and anti *S. aureus* IgY antibody was used as primary antibody, (B) analysis of anti *S. aureus* IgY antibodies isolated from egg yolk of hens immunized by oral vaccination, well 1-11, color development was observed in wells coated with *S. aureus* and anti *S. aureus* IgY antibody was used as primary antibody, well 11, no color development was observed in well coated with *E. coli* cells as control and anti *S. aureus* IgY antibody was used as primary antibody.

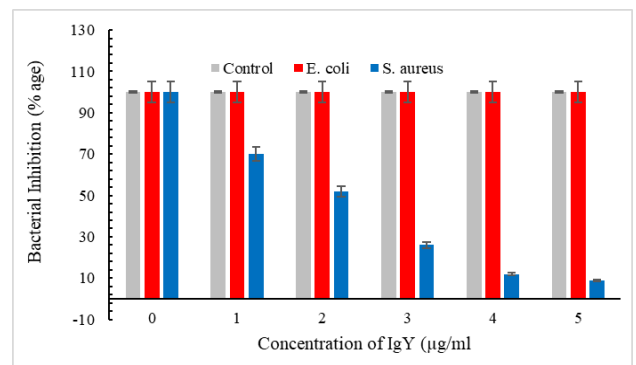


Fig. 3. Percentage Inhibition of *S. aureus* and *E. coli* growth incubated with different concentrations of anti-*S. aureus* IgY antibodies in a liquid medium. The growth of *S. aureus* was significantly inhibited with the increase in the concentration of IgY antibodies, but the growth of *E. coli* cells was not inhibited.

Binding of anti-S. aureus IgY antibodies with S. aureus using immuno microscopy

To confirm the direct binding of IgY antibodies with *S. aureus* cells, the cells from *S. aureus* culture were spotted on slide and incubated with anti-*S. aureus* IgY antibodies followed by treatment with enzyme conjugated secondary antibody and substrate. The presence of colored colonies on slide confirmed the binding of anti-*S. aureus* IgY antibodies with *S. aureus* cells as shown in Figure 4.

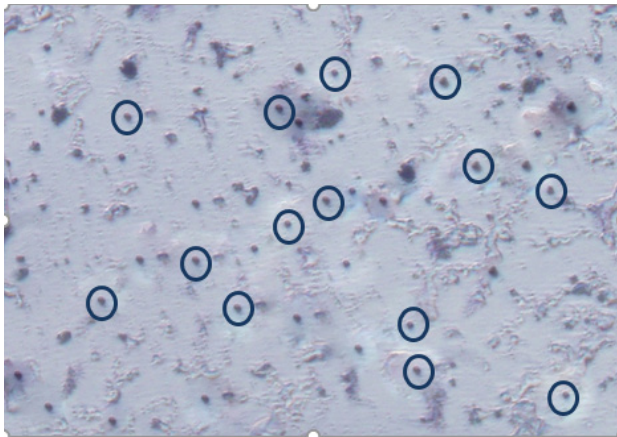


Fig. 4. Microscopic analysis of *S. aureus* cells coated on slide and treated with anti-*S. aureus* IgY antibodies and enzyme conjugated goat anti-IgY antibodies. The treatment of cells with substrate solution further stained the cells as encircles.

DISCUSSION

The production of desired IgY in egg laying hens and extraction of specific antibodies from egg yolk is an attractive approach in the field of diagnosis and therapy. Inexpensive and easy housing of chickens, non-stressful collection of eggs and non-invasive, high yield and easy isolation of antibodies make chickens as an ideal alternative to produce antibodies for passive immunization. The eggs can be considered as a good source for isolation of microbe specific antibodies. Of the three types of antibodies found in birds, namely IgY, IgM and IgA, IgY antibody is the most prominent and comparable to the IgG antibody found in mammals (Taylor *et al.*, 2009). Effective protection against bacterial or viral infections has been observed with the oral delivery of certain yolk immunoglobulin (IgY) (Ebina *et al.*, 1990; Hatta *et al.*, 1997; Kim *et al.*, 2000).

According to microbiologists, the growth of bacteria that produce by-products and enzymes significantly contributes to food spoilage. Bacteria are generally the most problematic pathogens in the decomposition of protein-rich meals such as meat, chicken, fish, and certain

dairy products. During the Covid-19 pandemic, food safety emerged as a crucial component among the four pillars of the food system (Selamoglu and Memon, 2021).

S. aureus is a notable and widely recognized food-borne pathogen because of its capacity to generate enterotoxin strains in food. Improper handling or storage at high temperatures might lead to the ingestion of the tainted food (Argudin *et al.*, 2010). On the other hand, ingestion of milk that is raw as well as cheese made with raw milk from dairy livestock having *S. aureus* mastitis has also been connected to epidemics (Johler *et al.*, 2015). Staphylococci are naturally resistant to the majority of antibiotics (Lindberg *et al.*, 2004; Livermore, 2000), although residues of antibiotics in dairy products are unfavorable for a variety of causes (McEwen *et al.*, 1992). Antibiotic usage in animal production adds to microorganism resistance, and these residues could prove harmful to the production of some cultured milk products (Kirk *et al.*, 1997). The prohibition of antibiotics has led to the emergence of a wide range of substitutes, including probiotics, organic acids, and others. Because of the high specificity, passive immunization with antibodies is one strategy that has a lot of promise (Sol *et al.*, 1997).

In the present research, IgY immunoglobulin was produced in domestic chickens as a defense against the *S. aureus* bacterium that causes food poisoning. Chickens were chosen because they are able to create a sufficient quantity of antibodies that are cost-effective, sanitary, and easy to obtain, unlike mammals. An advantage of utilizing birds for IgY synthesis is that these antibodies can be extracted from the yolks rather than serum (Gassmann *et al.*, 1990). After being stored at -20 °C for 6 months, the antibody retains its capacity to specifically bind with a particular antigen. An egg can be stored at a temperature of 4°C for a minimum of 6 months with minimal decrease in activity (Carlander, 2002). The *S. aureus* whole cell antigen was fixed using formalin, with a final concentration of 1×10^3 CFU/mL. The generated vaccination was given to chickens to induce the production of targeted antibodies. Once the dosing and injections were finished, the eggs were gathered and the yolks were separated. After purification of IgY by PEG method, its profile was characterized by SDS-PAGE and Native-PAGE which confirmed the presence of IgY in isolated sample as shown in Figure 1. In a similar study, IgY has been extracted and purified by PEG method and purified IgY antibody shows stability for years under specific conditions (Schade *et al.*, 2005).

By utilizing *E. coli* cells as controls in cellular ELISA, the specificity of egg yolk extracted anti-*S. aureus* IgY antibodies against the *S. aureus* bacterium was investigated in this study. It was observed that anti *S. aureus* IgY antibodies binds with *S. aureus* and did not show reaction

with *E. coli* cells which confirmed that IgY antibodies are specific to *S. aureus* cells as shown in Figure 2. These results indicated that highly purified IgY could potentially be employed as a treatment for food-borne illnesses by either combining the egg yolk harboring IgY with any diet or via passive immunotherapy. In another study, indirect ELISA was used to track the specific activity of IgY from the chickens immunized with *S. aureus*. The hens immunological response to *S. aureus* was comparatively strong. Following the hens initial inoculation, anti-*S. aureus* antibodies were generated in the yolk of the egg. Western blotting verified that the IgY remained specific to the antigen, as demonstrated by ELISA (Guimarães *et al.*, 2009).

Furthermore, quantitative *in vitro* antibacterial assay was performed to further investigate the specificity and neutralizing efficacy of anti-*S. aureus* IgY antibodies against *S. aureus*. It was observed that at IgY concentration of 5 µg/mL, 91 % inhibition in *S. aureus* growth was observed and no inhibitory effect of anti-*S. aureus* IgY antibodies on the growth of *E. coli* cells was observed as shown in Figure 3. Simultaneously, the direct binding of anti-*S. aureus* IgY antibodies with *S. aureus* was confirmed by using immuno microscopy as shown in Figure 4.

IgY technology can be applied to mouthwash, body lotions, cosmetics, nose sprays, yogurt, and other helpful foods. It can also be utilized as powder supplements that can help prevent or treat human diseases. IgY utilization in the management of bacterial infections in the digestive system presents the potential to decrease the reliance on antibiotics. The IgY therapy possesses the capacity for passive immunization in neonates and individuals with weakened immune systems. Scientists in South America have developed egg powders as an addition for milk substitutes. These inventions are necessary to safeguard children from life-threatening intestinal diseases in less developed nations (Schade *et al.*, 2005). IgY has a significant promise for the treatment of numerous diseases and an even greater potential in the future. Chicken egg yolk antibodies will have significant use in immunotherapy, diagnostics, as well as research in coming years.

CONCLUSION

This study successfully demonstrated the production and functional validation of immunoglobulin Y (IgY) antibodies specific to *Staphylococcus aureus*, generated through dietary immunization of laying hens. The antibodies, isolated from egg yolks using PEG 8000 precipitation, showed strong reactivity and specificity toward *S. aureus*, achieving up to 91% inhibition of

bacterial growth at a concentration of 5 µg/mL *in vitro*. These findings underscore the potential of IgY as a safe and natural agent for passive immunotherapy against gastrointestinal infections caused by this pathogen. As a non-invasive and sustainable alternative to traditional antimicrobials, IgY offers promising prospects for improving public health. However, additional studies in mammalian systems are necessary to further assess its therapeutic viability and application in real-world clinical or food safety contexts.

DECLARATIONS

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IRB approval

Institutional Ethics Review Board of The University of Punjab approved this study (No.D/93/FIMS, Dated 05/09/2024).

Ethical statement

All animal ex-periments were conducted in accordance with institutional and national ethical guidelines.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon request

Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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