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ORIGINAL ARTICLE

IgY antibodies of chicken do not bind staphylococcal binder of immunoglobulin (Sbi) from *Staphylococcus aureus*

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Abstract

The immunoglobulin (Ig) binding proteins of *Staphylococcus aureus* namely staphylococcal protein A (SpA) and staphylococcal binder of immunoglobulin (Sbi) are responsible for false positives during immunoassays. Avian IgY antibodies were reported to have no affinity to SpA and thus are safe for use in immunoassays. However, the behaviour of Sbi with IgY was not reported. The purpose of the present study is to evaluate the interactions between IgY antibodies and Sbi protein from different *S. aureus* strains. Initially, heterologous cloning and expression of complete *sbi* gene in *Escherichia coli* was undertaken. Recombinant Sbi protein was utilized to generate polyclonal anti-Sbi IgY and anti-Sbi antibodies in chicken and BALB/c mice respectively. Indirect ELISA and Western blotting were performed to evaluate the reactivity of anti-Sbi antibodies. Non-reducing PAGE followed by Western blotting and double-antibody sandwich dot-ELISA were performed to analyze the reactivity of IgY antibodies with recombinant Sbi and native Sbi from *S. aureus* strains. To avoid the possible interference of enzyme-conjugated secondary antibodies from mammalian sources, mouse anti-Sbi revealing antibodies were labeled with biotin so that streptavidin-HRP was used as developing reagent for chromogenic reaction. Sbi was highly immunogenic in chicken and mouse with antibody titers of 1:128,000 and 1:64,000 dilutions respectively. We observed that unimmunized IgY antibodies showed no affinity to either recombinant Sbi or native Sbi from *S. aureus* strains in Western blotting and double antibody sandwich ELISA. In view of these observations, we recommend that IgY antibodies are safe and free from false positives due to SpA and Sbi in immunoassays involving detection of *S. aureus* antigens/exotoxins.

Keywords *Staphylococcus aureus* · Immunoglobulin Y (IgY) · Immunoglobulin G (IgG) · Staphylococcal binder of immunoglobulin (Sbi) · Staphylococcal protein A (SpA) · False positives · ELISA

Introduction

Staphylococcus aureus is an opportunistic pathogen widely distributed in a broad host range and the severity of infections range from mild skin and soft tissue infections to serious infections such as endocarditis, septicemia, and toxic shock syndrome (Reddy et al. 2017; Wang et al. 2017). *Staphylococcus aureus* is a major public concern due to outbreaks in hospitals and also in the community (Oliveira Calsolari et al. 2011; Yi et al. 2012; Reddy et al. 2017; Soleimanzadeh et al. 2018). Success of *S. aureus* as a pathogen emerges from possessing unique combination of genetic factors that enable the bacteria to evade the host's immune system (Adhikari et al. 2016; Reddy et al. 2017). Staphylococcal cell wall–associated protein A (SpA) binds Fc fragment of immunoglobulin G and also Fab fragments of certain immunoglobulin classes (Sasso et al. 1991; Burman et al. 2008). Apart from SpA, a second staphylococcal immunoglobulin binding protein namely Sbi has been identified which occurs in majority of *S. aureus* strains (Zhang et al. 1998). Sbi is a 436-amino acid protein containing two immunoglobulin binding domains with sequence similarity to five immunoglobulin-binding domains of SpA. Sbi interacts only with Fc fragment of IgG unlike SpA which can bind both Fc and Fab fragments (Upadhyay et al. 2008). Sbi is a multifunctional protein that interferes with both adaptive and innate immune mechanisms

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by binding Fc fragment of IgG antibodies and interaction with central C3 protein of complement system respectively (Atkins et al. 2008; Burman et al. 2008).

Complement mediates a powerful and immediate innate immune defense mechanism, and is activated within seconds of pathogen entry. Activated complement system cleaves central complement protein C3 into fragments C3a and C3b followed by depositing C3b on to the surface of microbe. This step activates the complement cascade leading to formation of membrane attack complex (MAC) which forms a pore on the membrane followed by destruction of microbe by lysis. Pathogens have acquired several mechanisms to inhibit the complement attack in order to survive and establish an infection. In case of *S. aureus*, several proteins such as Sbi, fibrinogen-binding protein, Efb, Ehp, SCIN, and SSL7 were identified that target the multiple steps in the complement activation pathways (Upadhyay et al. 2008). Immune subversive protein Sbi lacks the typical Gram-positive cell wall anchoring sequence LP*X*TG. Of the four domains of Sbi protein, I and II domains interfere with adaptive immunity by binding to Fc regions of IgG, domains III and IV modulate innate immunity by inducing futile consumption of complement through binding of C3 proteins.

Avian antibodies from chickens (IgY) are increasingly used for developing diagnostic procedures due to low costs and higher yields than from their mammalian counterparts (Reddy et al. 2013, 2014). Antibodies can be harvested from egg yolks without bleeding or using any invasive techniques (Reddy et al. 2015). Heavy chains of IgY are composed of four constant domains unlike three constant domains of mammalian IgG. IgY also differ from IgG in Fc domain and hence they cannot fix complement and shows no affinity to immunoglobulin-binding proteins such as protein A of *S. aureus*, protein G of *Streptococcus* sp., and protein L of *Peptostreptococcus* sp. (Pierce IgY extraction kit 2017a, b). However, the interaction of immunoglobulin Y with Sbi, a second immunoglobulin binding protein from *S. aureus* is not reported. In the present study, we have analyzed the affinity of IgY antibodies from chicken towards Sbi protein of *S. aureus*. We employed various assays to understand the IgY affinities with both recombinant and native forms of Sbi protein.

Materials and methods

Materials

Dehydrated microbiological media, supplements, antibiotics, dyes, and stains, were procured from HiMedia, Mumbai, India. Standard *S. aureus* strains were received from Defense Food Research Laboratory (DFRL), Mysore India. *Taq* DNA polymerase, deoxyribonucleotides (dNTPs), mouse monoclonal anti-histidine antibodies, goat anti-mouse IgG conjugated with HRP and goat anti-IgY antibodies conjugated to HRP were purchased from Sigma Aldrich, India. FastDigest restriction enzymes, alkaline phosphatase, T4 DNA ligase, and IPTG was from Thermo Scientific (Fermentas), USA. pRSET vector series was from Invitrogen, USA. Novablue and BL21DE3pLysS strains were from Novagen, USA. Ni-NTA agarose was from Qiagen, Germany. All inorganic chemicals for buffer preparation were purchased from Fisher or Qualigens, India.

Cloning and expression of *sbi* gene

Primer pair spanning the entire length of *sbi* gene was used for amplification followed by cloning directionally into pRSET A vector. For directional cloning, *Bam*HI and *Eco*RI restriction sites were introduced at 5′ ends of *sbi* primers (Table 1). Following restriction digestion, gel purified linear vector and PCR product were ligated together and transformed into chemically competent *Escherichia coli* NovaBlue cloning host. Recombinant pRSET A-*sbi* plasmid was isolated and sequenced in both directions using T7 promoter and terminator primers and the obtained sequences were compared with expected sequences. Recombinant plasmid was further transformed to BL21DE3pLysS strain for expression of recombinant Sbi protein. Expression of r-Sbi protein was confirmed by pilot scale expression studies followed by staining SDS-PAGE gels and Western blot analysis with anti-histidine antibodies.

Purification of r-Sbi protein

Recombinant Sbi protein was purified from 500 ml culture grown in Luria-Bertani broth supplemented with 100 μg/ml ampicillin sodium salt. Hexa histidine-tagged Sbi protein was

Table 1 Primers for amplification of *sbi* gene

Primer	Sequence (5′-3′)	Primer length	Size of product
Sbi-F	[a]CGC***GGATCC***AACACGCAACAAACTTCAAC	29	1167 bp
Sbi-R	[b]CCG***GAATTC***[TTA]TGATGATGCAGTTT CTTGAG	32	

[a] Letters in bold and italics refer to restriction recognition sites

[b] **[TTA]** refers to the end codon placed in the primer

purified under denaturing conditions using urea. Elutions were pooled and dialyzed against 1× PBS in the presence of glutathione oxidized and reduced ratios of 1:10 (1 mM to 10 mM) for protein refolding. Protein quantity was estimated by Lowry's colorimetric assay using BSA as standard.

Immunization and antibody generation

Anti-Sbi antibodies were produced in white leghorn breed of chicken layers and female BALB/c mice. For raising anti-Sbi IgY antibodies, three hens were injected with 150 μg of r-Sbi protein in the breast muscle in emulsion with Freund's complete adjuvant (FCA). This was followed by three booster doses with same quantity of antigen in emulsion with Freund's incomplete adjuvant (FIA) at 10-day intervals. Eggs collected 7 days after last booster was kept separately for IgY isolation. IgY antibodies were isolated by salt precipitation using PEG 6000 as described by Pauly et al. 2011 with slight modifications. IgY quality was evaluated by SDS-PAGE analysis followed by staining with Coomassie Brilliant Blue R-250. IgY isolated from eggs collected 2 days prior to start of immunization served as negative controls.

Mouse anti-Sbi antibodies were produced for using as revealing antibodies during IgY testing. Three female BALB/c mice were immunized with 50 μg of r-Sbi in emulsion with FCA. This was followed by three booster doses with same quantity of protein in emulsion with FIA. Blood was drawn by retro-orbital plexus puncture from immunized mice. Blood drawn 2 days prior to first immunization served as negative controls.

Antibody titer testing of anti-Sbi IgY and anti-Sbi IgG

Antibody titers were tested with serum separated from small quantity of blood drawn from immunized chicken and mouse. Serum from unimmunized chicken and mice served as controls. Antibody titers of anti-Sbi chicken IgY and mouse IgG was tested using r-Sbi protein as antigen in indirect ELISA. Briefly, each well of 96 well Nunc maxisorp microtiter plate was coated overnight with 100 μl of 10 μg/ml concentration of r-Sbi protein at 4 °C. This was followed by blocking in 5% non-fat skim milk solution prepared in PBS and incubated at 4 °C overnight. After washing in PBST (PBS Tween 20, 0.05%), plates were sealed with cellophane tape and kept at −20 °C for further use. For testing antibody titers, mouse and chicken serum samples from three animals were diluted two-fold serially in 1× PBS starting with 1:1000 till 1:128000 dilutions. One hundred microliters of each dilution was added to coated plates and incubated at 37 °C for 1h. Following washing in PBST, wells were loaded with appropriate goat anti-chicken HRP conjugate or goat anti-mouse HRP conjugate. Microtiter plate was developed with 1 mg/ml *o*-phenylenediamine dihydrochloride as chromogen and 0.03% H_2O_2 solution. Absorbance was scanned at 450 nm in BioTek Synergy multimode plate reader.

Biotinylation of IgY and IgG

Enzyme-conjugated secondary antibodies in general are raised from mammalian sources. They can bind non-specifically to immunoglobulin binding proteins such as SpA and Sbi leading to ambiguous results. To avoid this situation, we have labeled the primary chicken IgY and mouse polyclonal antibodies with biotin so that streptavidin-HRP can be used as developing reagent. Biotin labelling of unimmunized IgY, immunized IgY, unimmunized mouse IgG, and immunized mouse IgG were performed using a commercial biotin labelling kit (Roche, Germany). Briefly, immunized and unimmunized IgY (~5 mg) purified from egg yolks and immunized and unimmunized serum from mice (0.5 ml) were subjected to biotin labelling on free amine groups ($–NH_2$) of antibodies using biotin-7-NHS. After labelling, unreacted biotin-7-NHS was separated from labeled antibodies using Sephadex G-25 columns. Labeled antibodies eluted in 1× PBS were stored at −20 °C for further use in Western blots and sandwich ELISA.

Western blot analysis

Western blot analysis was performed to test the reactivity of mouse anti-Sbi and chicken anti-Sbi antibodies. Both whole cell lysates and concentrated culture supernatants were used to test antibody reactivity. Samples dissolved in 1× Laemmli buffer were separated on 12% SDS-PAGE gels using tris-glycine buffer in Bio-Rad mini Tetra cell. After electrophoresis, the separated proteins were transferred onto nitrocellulose (NC) membrane in Bio-Rad transblot apparatus. Membranes were blocked in 5% non-fat skim milk solution dissolved in PBS and incubated at 4 °C overnight. Blots were washed in PBST solution to remove excess milk proteins from the membrane. Further blots were incubated with 1:2000 dilutions of biotinylated chicken anti-Sbi and mouse anti-Sbi antibodies at room temperature for 30 min on a gel rocker. Blots incubated with unimmunized and biotinylated chicken and mouse immunoglobulins were used as controls. After washing, the blots were incubated in 1:5000 dilutions streptavidin-HRP (Invitrogen, USA) for 30 min at room temperature. Blots were developed after washing step using diaminobenzidine tetrahydrochloride and 0.03% H_2O_2 in PBS. Blots were flooded with tap water to stop excess reaction leading to background.

Non-reducing PAGE for protein-protein affinity drag assay

Protein-protein interactions between recombinant Sbi and IgY antibodies was evaluated by SDS-PAGE (9%) under non-reducing conditions. For this, recombinant Sbi protein

(20 μg) was incubated with unimmunized egg yolk IgY (40 μg) and anti-Sbi egg yolk IgY (40 μg) at room temperature for 30 min followed by PAGE separation. The separated proteins were transferred to nitrocellulose membrane by Western blot analysis in Bio-Rad mini Transblot apparatus. The Sbi protein was probed using biotinylated mouse anti-Sbi antibodies and further detected by streptavidin-HRP. After chromogenic development of blots, the affinity of Sbi with IgY was confirmed by size of Sbi protein on the blots.

IgY capture dot ELISA

A double antibody sandwich dot-ELISA was performed to confirm the interaction between Sbi proteins from *S. aureus* with IgY antibodies from birds. Unimmunized IgY and anti-Sbi IgY were used as capture antibodies in separate tests. Briefly, 10 μl of IgY antibodies in 1:1000 dilutions was spotted on nitrocellulose (NC) membrane strips of 1 cm × 1 cm dimensions and allowed to dry at room temperature for 30 min. Each NC strip was placed in wells of 12-well cell culture plate. Membranes were blocked with 5% non-fat skim milk solution and incubated at 4 °C overnight. Excess milk proteins were washed from membranes using PBST solution. Whole cell lysates containing native Sbi protein from reference *S. aureus* strains were added to the IgY-coated strips in wells and incubated at RT for 1 h. Sbi bound to IgY was detected using 1:1000 dilutions of biotinylated mouse anti-Sbi antibodies. This was followed by incubation of membranes with 1:5000 dilutions of streptavidin-HRP. Membranes were developed with diaminobenzidine tetrahydrochloride and 0.03% H_2O_2 in PBS. Result was interpreted by visual inspection of blots.

Ethics

Chicken and mice were maintained under hygienic conditions in animal housing facility of Vignan's pharmacy college which is adjacent to VFSTR. Permission for using animals was sought during Institutional Animal Ethical Committee (IAEC) meeting held in June of 2017 at Vignan's Pharmacy College, Vadlamudi, Guntur, Andhra Pradesh. Mice were provided with protein rich pellet feed and mineral water. Chicken were provided with commercial poultry feed used locally in most poultry farms. Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines were followed for care and use of animals and the Institutional Animal Ethical Committee (IAEC) of Vignan's Pharmacy College approved the protocols and procedures employed in this study.

Results

Cloning and heterologous expression of *sbi* gene

Complete *sbi* gene without the signal sequence was amplified from *S. aureus* NCIM 2127 strain (Fig. 1a). pRSET A vector (Reddy et al. 2012; Babu et al. 2017) was used for cloning of *sbi* gene for bulk production of Sbi protein. Treatment of plasmid with alkaline phosphatase prevented plasmid recircularization and thus no background colonies with recircularized plasmid were observed. Transformation efficiency in DH5α using ligation mixture was 6×10^2 recombinants/μg of ligated plasmid. Recombinant plasmid (pRSET A-*sbi*) was sent for sequencing to confirm

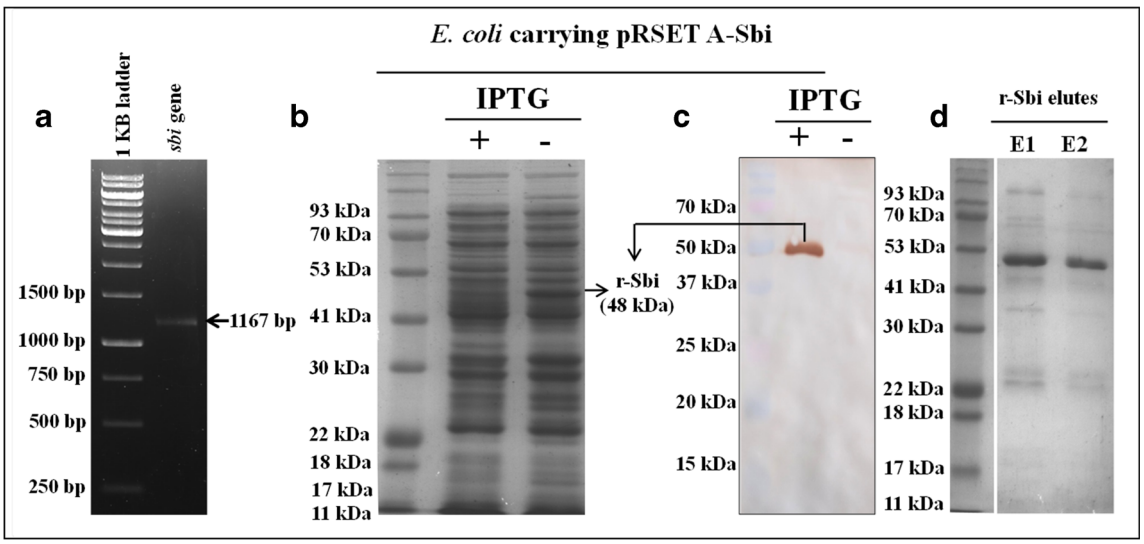

Fig. 1 Heterologous cloning and expression of *sbi* gene of *S. aureus* in *E.coli* and its purification **a**. Agarose gel showing PCR amplified *sbi* gene using *Pfu* DNA polymerase, **b**. Coomassie stained SDS-PAGE gel showing expression of *sbi* gene after IPTG induction, **c**. Western blot probed with anti-6× histidine antibody showing expression of r-Sbi protein, **d**. Coomassie Brilliant Blue stained SDS-PAGE gel showing purified recombinant Sbi protein

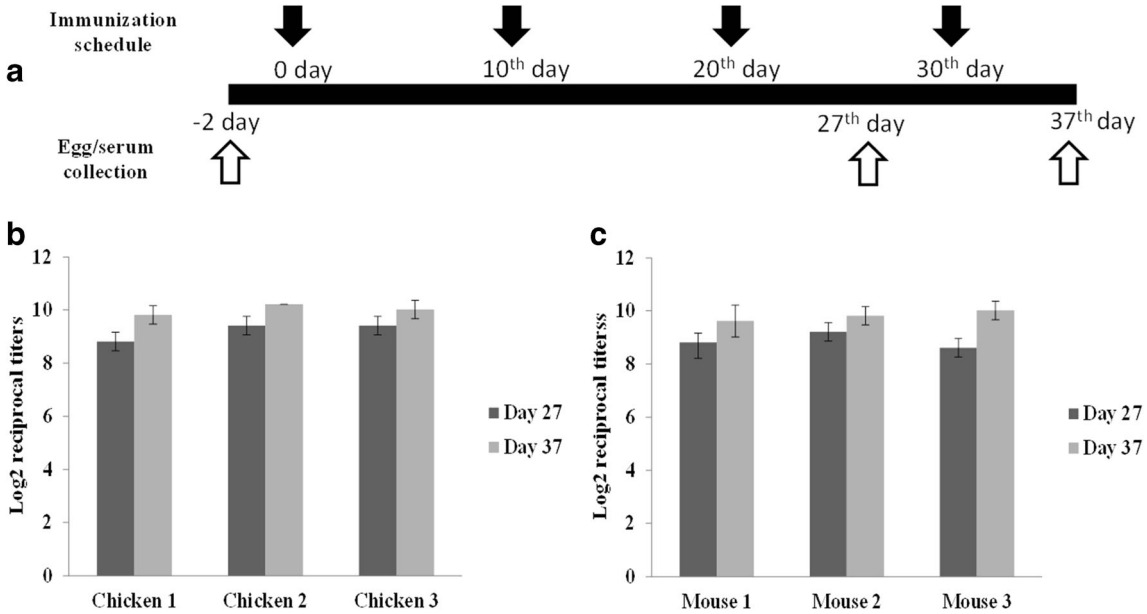

Fig. 2 Estimation of antibody titers from chicken and mouse anti-Sbi serum **a**. Depiction of immunization and blood/egg collection schedules for antibody titer determination, **b**. Estimation of antibody titers of chicken anti-r-Sbi serum induced by r-Sbi protein at different time points of immunization, **c**. Estimation of antibody titers of mouse anti-r-Sbi serum induced by r-Sbi administration at different time points of immunization

the orientation and gene sequence information. Obtained DNA sequence of cloned *sbi* gene was in complete agreement with expected sequence in multiple sequence alignment. During pilot scale expression studies, recombinant Sbi protein was observed at 48 kDa as expected. This was confirmed by both Coomassie stained SDS-PAGE gels and Western blot analysis with anti-histidine (6× his) antibodies (Fig. 1b, c).

Purification of r-Sbi protein

The buffers composition for purification of 6×His-tagged Sbi protein under denaturing conditions is as per the recommendations of QIAexpressionist (Qiagen manual). For effective cell lysis, bacterial pellet was dissolved in urea lysis buffer (pH 8.0) with 2 mg/ml of lysozyme and incubated at room temperature with gentle rocking for 1 h with occasional mixing by vortexing. Cell lysis was reinforced by passing the cell lysate through syringe with narrow gauze needle followed by 4 freeze and thaw cycles at −80 °C and 37 °C respectively. Hexa histidine-tagged Sbi protein was purified by affinity protocol using Ni-NTA resin. Elutions were subjected to SDS-PAGE analysis (Fig. 1d), and fractions containing purified protein were pooled and dialyzed against 1× PBS and glutathione for removal of excess urea. The concentration of dialyzed protein was adjusted to ~2.0 mg/ml and stored at − 20 °C in 1.0 ml aliquots for further use.

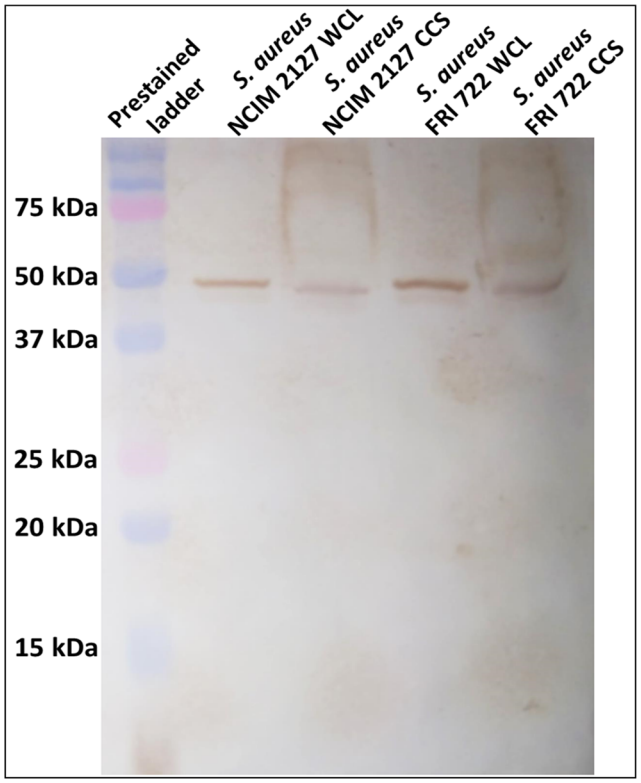

Fig. 3 Comparison of Sbi protein in *Staphylococcus aureus* whole cell lysate (WCL) and concentrated culture supernatant (CCS) fractions by Western blot analysis. Blots were treated with anti-Sbi IgY antibodies. The amount of Sbi protein bound to cell membrane was more than the concentrated culture supernatants

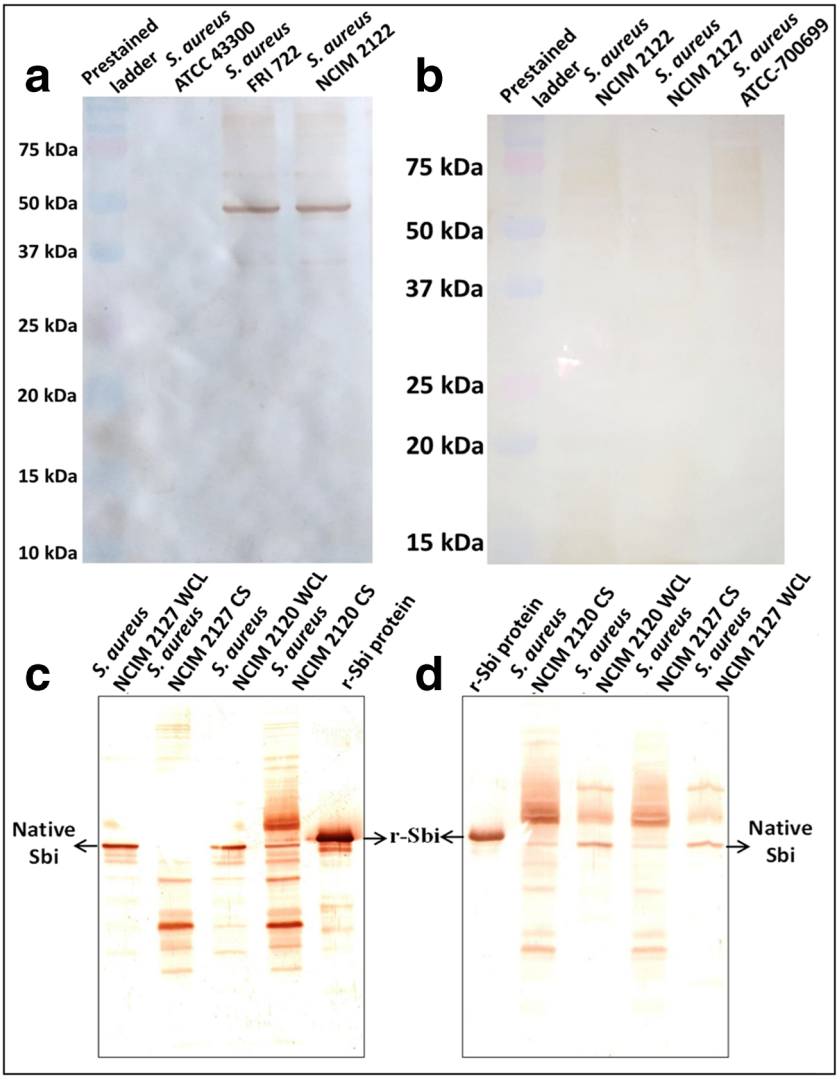

Fig. 4 Reactivity testing of IgY and IgG antibodies with Sbi protein **a**. blot treated with biotin labeled anti-Sbi IgY antibodies, **b**. blot treated with biotin-labeled unimmunized IgY, **c**. blot treated with biotin labeled anti-Sbi IgG, **d**. Blot treated with biotin labeled unimmunized IgG. From Fig. 5b, it was deduced that unimmunized IgY does not bind Sbi or SpA proteins

Immunization, antibody generation, and testing antibody titers

Immunization and blood collection schedules for generating anti-Sbi antibodies in chicken and mouse are provided in Fig. 2a. Small quantity of blood samples were drawn after second and third booster doses from chicken through venipuncture of brachial veins (the wing veins) with a sterile syringe needle. Serum antibodies showed antibody titers of 1:128,000 dilutions in indirect ELISA indicating a strong immunogenic nature of the antigen (Fig. 2b). The maximum antibody dilution showing absorbance at least twice the value of unimmunized serum was considered as endpoint titer. Titers were tested in three replicates from three different chickens. Eggs were collected 7 days after last immunization and IgY was extracted from egg yolk by salt precipitation. The yield of IgY was found to be approximately 20 mg/egg yolk. The IgY concentrations was adjusted to 2.0 mg/ml and used in further assays. The quality of IgY preparation was tested by SDS-PAGE analysis and only minor traces of contaminating proteins were found.

Blood was drawn from mouse by retro-orbital sinus puncture and serum was separated. Mouse serum was tested for antibody titers after second and third booster doses. Hyperimmune serum showed endpoint titers of 1:64000 dilutions in PBS by indirect ELISA after 3rd booster (Fig. 2c). Readings were taken with three biological replicates from three different mice. Serum collected after 7 days past last booster was used in all the experiments.

Biotinylation of antibodies

The biotin labelling of antibodies was estimated by streptavidin-HABA assay and the mole to mole ratio was found to be approximately 3.5 and 2.7 biotin molecules for immunized and unimmunized IgY antibodies respectively.

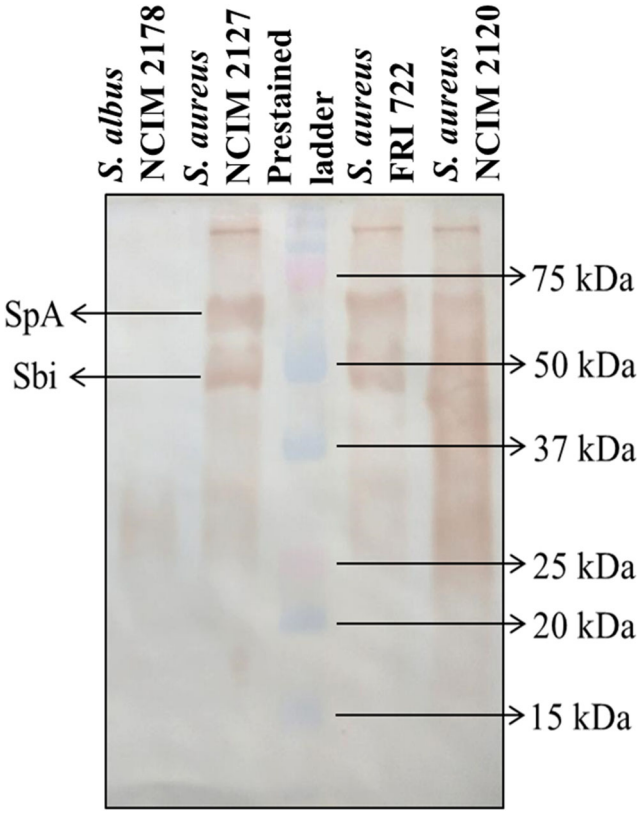

Fig. 5 Treatment of blots with anti-staphylococcal protein A antibody from a commercial source. SpA (54 kDa) and Sbi (45 kDa) are variable molecular weight immunoglobulin binding proteins clearly distinguishable on Western blot

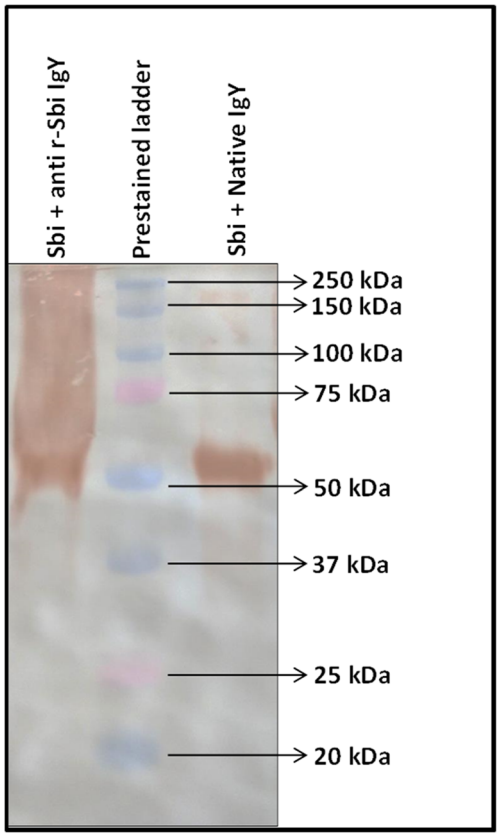

Fig. 6 PAGE analysis for testing the affinity of IgY antibody with Sbi protein. Anti-Sbi IgY and unimmunized IgY were incubated with r-Sbi protein and subjected to native PAGE. Unimmunized IgY did not bind Sbi protein and hence no shift in molecular weight was observed

Similarly the rate of biotinylation was 2.9 and 2.3 biotin molecules for immunized and unimmunized mouse antibodies respectively. Further, biotinylation was confirmed by dot-ELISA by coating 10 μl of different elutions followed by probing with streptavidin-HRP. After development only biotin labeled antibodies produced chromogenic reaction indicating successful labelling. The control antibodies did not produce any chromogenic reaction.

Western blot analysis

Whole cell lysate and concentrated culture supernatant fractions were initially tested to check for the presence of Sbi protein by Western blotting. *S. aureus* stationary phase broth cultures (14–15 h) were used for testing Sbi quantity. The amount of Sbi protein present in whole cell fractions was far more than the concentrated culture supernatants indicting that Sbi is localized more in membrane form than the secretory form (Fig. 3). This observation was in agreement with the observations made by Burman et al. 2008.

Western blot analysis revealed the reactivity of anti-Sbi antibodies with native Sbi protein. Biotin labeled chicken anti-Sbi antibodies reacted strongly with native Sbi protein from different *S. aureus* strains (Fig. 4a). Blots treated with unimmunized biotin labeled IgY did not show any reactivity with Sbi protein from *S. aureus* strains (Fig. 4b). Both blots were developed after treatment with streptavidin-HRP. This indicates that IgY antibodies from unimmunized chicken do not have affinity to Sbi proteins and hence no reactivity was observed.

As for controls, we tested the reactivity of mammalian antibodies from mice. Biotin-labeled mice anti-Sbi antibodies showed strong reactivity in blots with native Sbi protein from *S. aureus* strains and r-Sbi (Fig. 4c). Blot treated with biotin-labeled unimmunized mouse polyclonal antibodies also produced reactivity to Sbi proteins (Fig. 4d). This experiment indicates that antibodies from mammalian sources have affinity to immunoglobulin-binding Sbi and SpA proteins.

To differentiate the reactivities of both Sbi and SpA proteins, a control blot was performed by treating with rabbit polyclonal anti-protein A antibodies (Sigma Aldrich, Israel). This blot clearly differentiated the Sbi protein from SpA protein (Fig. 5) and confirmed that we are not interpreting SpA reactivity with Sbi. Reactivity of SpA and Sbi at different regions on blots was clearly distinguished with this experiment.

Affinity testing by non-reducing SDS-PAGE

After performing PAGE and development of Western blots, we observed a shift in size of Sbi protein only when anti-Sbi IgY antibodies used for testing affinity. On the other hand, no shift in size was observed with Sbi protein incubated with unimmunized IgY. In case of r-Sbi incubated with anti-Sbi IgY the Sbi reactivity appeared as a smear as a result of IgY interacting with Sbi through the bivalent Fab region possibly leading to large antibody-antigen complexes (Fig. 6). Biotin-labeled mice anti-Sbi antibodies was used to detect Sbi protein. The results from this experiment indicated that Sbi and IgY antibodies did not interact with each other.

Double antibody sandwich dot ELISA

A double antibody sandwich ELISA was performed to confirm the interaction between IgY and Sbi protein (Fig. 7a). When anti-Sbi IgY was used as capture antibody, chromogenic reaction was observed as expected (Fig. 7) with Sbi protein from different *S. aureus* strains. When unimmunized IgY was used as capture antibody and incubated with Sbi proteins from the whole cell lysates of *S. aureus* strains, no chromogenic reaction was observed indicating a possibility that Sbi did not bind the IgY (Lig. 7c). In both cases biotin labeled mouse anti-Sbi antibodies were used for the detection of Sbi proteins bound to the IgY antibodies.

Discussion

S. aureus is an important pathogen responsible for variety of diseases ranging from mild skin infections to serious diseases such as endocarditis, osteomyelitis and toxic shock syndrome. The pathogen has developed several immuno-regulatory proteins for immune evasion and achieves this by regulating innate and adaptive immune systems (Reddy et al. 2017). Among them Sbi binds with C3d in a 1:1 ratio but can bind

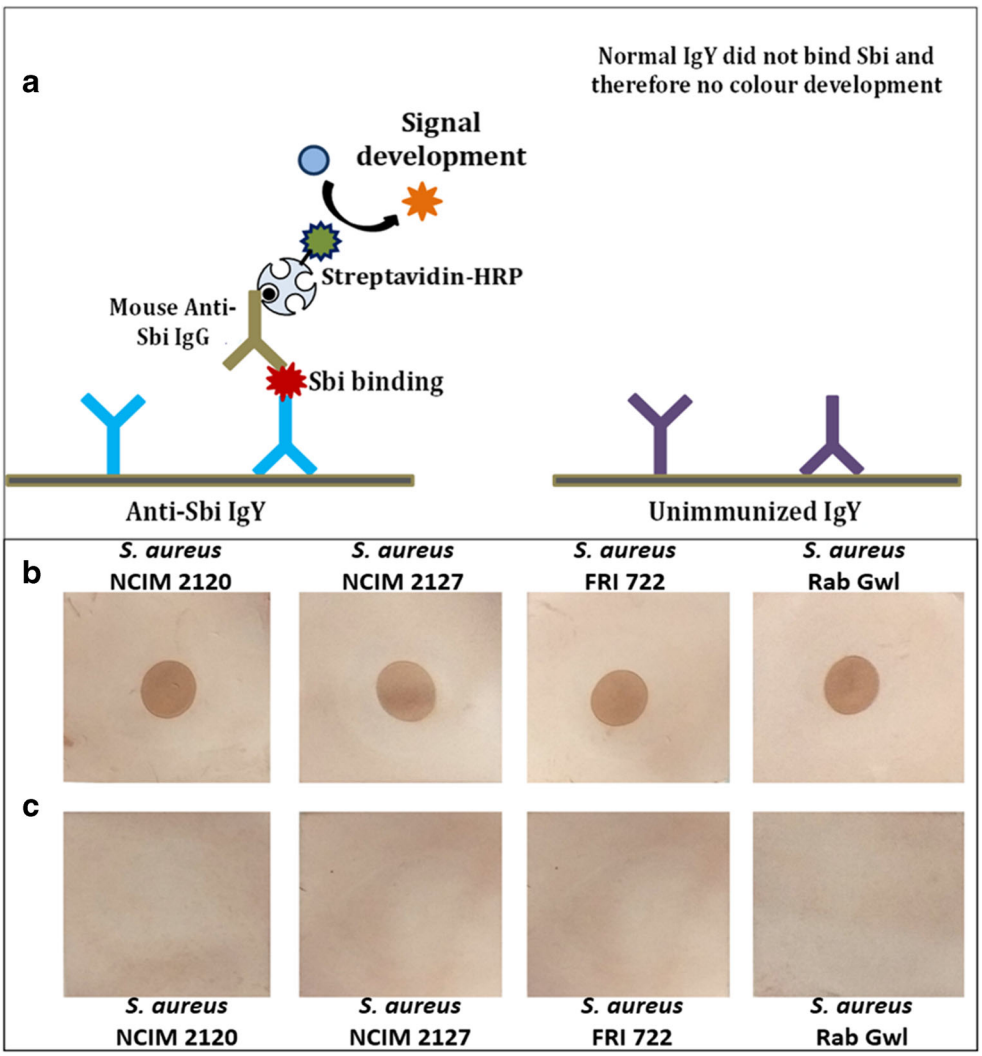

Fig. 7 Sandwich dot-ELISA for studying IgY interaction with Sbi protein **a**. Schematic representation of sandwich ELISA with anti-Sbi IgY and unimmunized IgY as capture antibodies, **b**. dot-ELISA performed with anti-Sbi IgY as capture antibodies, **c**. dot-ELISA performed with unimmunized IgY as capture antibody showing no chromogenic reaction

to C3d at two complexes namely complex 1 and complex 23. In classical pathway of complement action, Sbi interacts and interferes with residues involved in thioester bond formation between C3d and bacterial surface blocking the formation of convertases (Clark et al. 2011). Sbi shares homology of antibody-binding domains with SpA and binding similarities at complex 1 with Efb-C and Eph another two proteins also secreted by *S. aureus*. Efb-C and Eph also interact with C3d to disarm the complement system function against bacteria. As Sbi inhibits complement, it helps in *S. aureus* resistance against complement-mediated killing by phagocytosis. Therefore, timely detection of pathogens aids in mitigating the infection and reducing the spread of pathogen. Immunoassays are powerful tools which aids in detection of pathogens, analytes and many other substances. During testing, immunoassays are often encountered with hurdles such as cross-reactivity from closely related antigenic substances. In case of *S. aureus*, these hurdles are in the form of immunoglobulin binding proteins namely SpA and Sbi. SpA and Sbi are surface-anchored, highly conserved proteins present in all *S. aureus* strains. Both are known to block opsonophagocytosis in the presence of host antibodies (Hong et al. 2016). Several procedures were recommended to reduce the interference of SpA such as preincubation of sample with serum antibodies, and treatment of blots with diethyl pyrocarbonate (DEPC). In our earlier work, we reported the efficacy of immunoglobulin Y over immunoglobulin G in eliminating false positives due to SpA during detection of *S. aureus* exotoxins (Reddy et al. 2013). Efficacy of IgY antibodies over IgG was demonstrated by IgY capture ELISA (Reddy et al. 2013) and IgY immunocapture PCR (IC-PCR) (Reddy et al. 2014) in double antibody sandwich ELISAs and by using biotin labeled IgY in single antibody assay (Reddy et al. 2015). Additionally, a hybrid Aptamer Linked ImmunoSorbent Assay (ALISA) procedure was developed using IgY capture antibody and anti-staphylococcal enterotoxin B (SEB) aptamer for detection of SEB (Mudili et al. 2015).

IgY antibodies of birds are similar with IgG of mammals by being the major immunoglobulin-providing defense to infectious agents and produced in high concentrations in blood following the decline in IgM levels. However, IgY is strikingly different from IgG with higher molecular weight and an extra constant domain (CH4). Large quantities (100–150 mg) of IgY can be harvested from a single egg yolk without using invasive procedures (Reddy et al. 2013). IgY are advantageous over mammalian IgG since they are not recognized by immunoglobulin binding proteins such as protein A of *S. aureus*, protein G of group G, and C *Streptococcus* or protein L of *Peptostreptococcus magnus* (thermofisher.com).

Results from our experiments indicate that Sbi protein was present both in culture supernatants and whole cell lysates. Since Sbi does not have cell wall binding motif it could be possible that the protein may be lodged in the plasma membrane as indicated by Burman et al. 2008. When we tested with stationary phase *S. aureus* cultures grown in BHI broth, more Sbi was detected in whole cell lysates than in concentrated culture supernatants. Results from experiments such as Western blotting, native PAGE and sandwich ELISA indicated that neither recombinant Sbi nor native Sbi from *S. aureus* strains showed any interaction with IgY antibodies. Biotin labelling of IgY and IgG helped in elimination of using mammalian secondary antibodies and thus aids in determining the actual reactivity of naïve IgY and IgG with Sbi. When IgY was incubated with r-Sbi followed by native PAGE and Western blot analysis, there was no shift in position of Sbi protein indicating the absence of any affinity between both the molecules. Even sandwich ELISA with native and r-Sbi suggested the same observation that Sbi protein has no affinity with IgY antibodies.

In view of the above observations, it is evident that neither SpA nor Sbi have affinities to IgY immunoglobulins and due to the simplicity of IgY extraction, higher yields, and low maintenance costs IgY could serve as better alternatives over mammalian counterparts especially with detection of *Staphylococcus aureus* antigens.

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Compliance with ethical standards

Conflicts of interest Authors declare no conflicts of interest.

Research involving human participants and/or animals Chicken and mice were maintained under hygienic conditions in animal housing facility of Vignan's pharmacy college which is adjacent to VFSTR. Necessary permissions for using animals were sought from Institutional Animal Ethical Committee (IAEC) of Vignan's Pharmacy College, Vadlamudi, Andhra Pradesh. Mice were provided with protein rich pellet feed and mineral water. Chicken were provided with commercial poultry feed used in local poultry farms. Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines were followed for care and use of animals and the Institutional Animal Ethical Committee (IAEC) of Vignan's Pharmacy College approved the protocols and procedures employed in this study.

Informed consent N/A

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