ORIGINAL ARTICLE

Use of biotin-labeled IgY overcomes protein A interference in immunoassays involving *Staphylococcus aureus* antigens

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Abstract Staphylococcal protein A (SpA) is an immunoglobulin-binding protein secreted by all *Staphylococcus aureus* strains and is responsible for high false positives during immunoassays. Avian IgY were reported to eliminate SpA interference when used as a capture antibody in a double antibody sandwich format. But this procedure requires generating two antibodies in different animals for capture and revealing antibodies. In the present study, a simple enzyme-linked immunosorbent assay (ELISA) was developed and evaluated for detection of staphylococcal enterotoxin B without any interference by SpA. Biotin-labeled IgY were used for probing for staphylococcal enterotoxin B (SEB) so that streptavidin-horseradish peroxidase (HRP) could be used for color development instead of anti-chicken IgG-HRP, which interferes in the assay by binding to SpA. Western blotting, dot ELISA, and polymerase chain reaction (PCR) were performed to validate the results of the biotin IgY ELISA to show that the assay was free from SpA interference. Large numbers of *S. aureus* strains were screened for SEB production. Sensitivity of the assay was ~10 ng/ml of SEB in spiked milk samples. The ELISA was specific to SEB, with no cross-reactivity to other closely related enterotoxins (SEA, SEC, SED, SEE). The presently described ELISA is highly effective in eliminating SpA interference and this method does not require the need for two different antibodies, as in the sandwich ELISA.

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Introduction

Staphylococcus aureus is an important pathogen responsible for a variety of illnesses such as skin and soft tissue infections, necrosis, osteomyelitis, endocarditis, pneumonia, and toxic shock syndrome (Otto 2014). In addition, certain strains secrete a variety of heat stable staphylococcal enterotoxins (SEs) on food that, if ingested, cause staphylococcal food poisoning (SFP) (Asao et al. 2003; Alibayov et al. 2014). SFP is characterized by a series of gastrointestinal symptoms such as nausea, vomiting, and abdominal cramps. In some instances, SEs act as super antigens and cause severe autoimmune response leading to toxic shock syndrome. Among the SFP cases recorded worldwide, classical SEs (SEA-SEE) are responsible for more than 90 % of cases (Techer et al. 2013). SEB is one of the important classical SEs implicated in food intoxications; it is also a potent inducer of T cells leading to septic shock (Marrack et al. 1990). Thus, it has been listed as a potent category B biological warfare agent. Consequently, rapid and accurate methods are needed to detect SEB in food samples from food poisoning cases and also to test for deliberate attempts at food adulteration.

Numerous techniques are currently available for detection of SEs in food samples, such as ELISAs (Chiao et al. 2013; Kuang et al. 2013) and PCR-based methods (Jeyasekaran et al. 2011). Commercially, many antibody-based kits are available for detection of SEs with good levels of sensitivity. Other sensitive methods, such as immuno-PCR (Rajkovic et al. 2006; Reddy et al. 2014), mass spectrometry (Sospedra et al. 2012), and biosensor techniques such as surface plasmon resonance (SPR) (Tsai and Li 2009) and piezoelectric immunosensor (Salmain et al. 2011) are also reported for SEs detection. However, these methods are costly, requiring technical expertise and, thus, are not feasible for regular food testing. A majority of these methods are immunoassays based on antibody recognition of an antigen. Staphylococcal protein

A (SpA) is a 42 kDa surface-bound and secretory protein produced by most *S. aureus* strains (Goding 1978). SpA is an immunoglobulin binding protein that binds non-specifically to most subclasses of mammalian IgG antibodies during immunoassays, leading to false positive results. In addition, *S. aureus* also secretes another immunoglobulin binding protein called Sbi (*S. aureus* binder of immunoglobulin) that has a mechanism of binding to immunoglobulins very similar to that of SpA (Smith et al. 2011). Various strategies were proposed to circumvent SpA interference during immunoassays. Many of these methods involve additional steps of sample incubation to reduce binding of SpA to IgG molecules. A double antibody sandwich method was proposed in which IgY capture of *S. aureus* antigens was found to eliminate SpA interference (Mizutani et al. 2012; Reddy et al. 2013). However, this sandwich method requires generating antibodies in two different animals and includes an additional antibody incubation step than normal ELISA procedure.

Avian IgY antibodies are more efficacious than mammalian antibodies for ELISA procedures involving *S. aureus* antigens because they have the least affinity towards SpA (Reddy et al. 2013). However, IgY antibodies are advantageous only when they are used as capture antibodies in a double antibody sandwich ELISA. While in assays involving western blots or plate ELISA, IgY are as susceptible to SpA as IgG due to the fact that the enzyme-conjugated secondary antibodies still react with SpA, leading to false positives (Reddy et al. 2014). However, this cross-reactivity can be overcome by replacing the secondary antibody step with a different signal-generating molecule. Due to their strong binding abilities, streptavidin and biotin interactions are commonly employed in many studies. In this study, we evaluated the efficacy of biotinylated IgY to overcome the non-specific cross-reactivity by using streptavidin-HRP as a signal-generating molecule.

Materials and methods

Materials

Dehydrated media and supplements were procured from Himedia Laboratories, India. Rabbit antibodies against SEB were procured from Sigma-Aldrich (St. Louis, MO, USA). The biotinylation kit, streptavidin-HRP, and anti-rabbit and anti-chicken-HRP conjugates were procured from Bangalore Genei (Bangalore, India). *Taq* DNA polymerase, *Pfu* DNA polymerase, and dNTPs were obtained from Fermentas/ Thermo Scientific (Thermo Fisher, Waltham, MA, USA).

Cloning and expression of the *seb* gene

DNA sequences corresponding to mature SEB protein was PCR-amplified using the primers listed in Table 1. The PCR

Table 1 List of primers used in the study

Primer	Sequence (5′-3′)	Product length
Cloning		
Seb-F	CGCGGATCCGAGAGTCAACC AGATCCTAA	682 bp
Seb-R	CCCAAGCTTCATTTTATTGTCA TTGTACATC	
Detection		
Seb-F	TGTATGGTGGTGTAACTGAGC	269 bp
Seb-R	TGCAGGCATCATGTCATAC	

product was cloned directionally into *Bam*HI and *Hind*III sites of pRSET A vector and transformed into *Escherichia coli* BL21DE3 host cells. Transformed clones were screened for SEB production after inducing with IPTG by SDS-PAGE and western blot analysis with anti-histidine antibodies. Recombinant SEB protein was purified from one liter of induced culture under denaturing conditions using Ni-NTA agarose (Qiagen, Hilden, Germany) by a gravity flow method.

Generation of anti-SEB IgY antibodies

Four-week-old chickens were immunized with 200 μg of denatured recombinant SEB protein in emulsion with Freund's complete adjuvant, followed by three booster doses at ten-day intervals with same amount of protein in emulsion with Freund's incomplete adjuvant. Ten days after the fourth immunization, eggs were collected to isolate the IgY antibody fraction. IgY was isolated using a commercial IgY purification kit (Pierce/Thermo Scientific, Waltham, MA, USA), according to the manufacturer's instructions.

Biotinylation of anti-SEB IgY and IgG

Purified IgY antibodies and commercial rabbit IgG antibodies were labeled with biotin molecules using a commercial antibody biotinylation kit following the manufacturer's instructions. Briefly, 5 mg/ml of IgY and IgG in PBS was mixed with 5 mg of biotin-NHS along with a conjugation buffer for 30 min. The mixture was passed through a 10-ml gel filtration column, the flow-through was collected, followed by elution in PBS in 1-ml fractions. Elutions with absorbance of A_{280} greater than 0.5 were pooled; stabilizing solution was added and stored at -20^0 C.

Western blot analysis

To check the efficacy of biotin-labeled IgY over normal IgY, IgG, and biotin-labeled IgG, western blot analysis was performed separately to test the SpA cross-reactivity. Culture supernatants of SEB-producing and non-producing strains were concentrated by a methanol/chloroform protein precipitation method, resolved on 12 % SDS-PAGE gel, and transferred onto

a nitrocellulose membrane. After blocking, membranes were probed separately with biotinylated and normal IgY and IgG antibodies. Membranes probed with biotinylated antibodies were treated with streptavidin-HRP, whereas the other two membranes were treated with their respective anti-primary-HRP conjugated antibodies. After washing in PBST, blots were developed in diaminobenzidine tetra hydrochloride and 30 % H_2O_2 solution. A control blot was performed with anti-SpA antibodies to test the pattern of SpA cross-reactivity. The band at 28 kDa corresponded to SEB and bands at 40–55 kDa were interpreted as SpA cross-reactivity.

Dot ELISA and microtiter plate ELISA for SEB analysis

Dot ELISA and indirect plate ELISA were also performed to further analyze the efficacy of biotin-labeled IgY antibodies preventing SpA cross-reactivity. For dot ELISA, concentrated culture supernatants were dissolved in 50-mM carbonate-bicarbonate buffer; 10 μl of sample was spotted onto a nitrocellulose membrane, air dried, and subjected to blocking with 5 % non-fat skim milk solution for 1 h at room temperature. For plate ELISA, 50 μl of concentrated samples in carbonate-bicarbonate buffer were coated onto microtiter plates (Nunc/Thermo Fisher, USA) at 37 °C for 1 h. Plates were blocked in 5 % milk solution at 37 °C for 1 h. Microtiter plates were then treated with biotinylated, as well as non-biotinylated, IgY and IgG antibodies. After washing in PBST, plates were then further treated with streptavidin-HRP and respective anti-primary-HRP antibodies. After development, results of dot ELISA and plate ELISA were interpreted in relation to western blot results.

Evaluation of biotin-labeled IgY ELISA on *S. aureus* isolates

A large number of *S. aureus* isolates collected from various sources (Reddy et al. 2013) was evaluated by biotin IgY ELISA using microtiter plates. Cultures were grown in brain-heart infusion (BHI) broth overnight at 37 °C, with constant shaking, and cell-free culture supernatants were concentrated by methanol/chloroform precipitation. Concentrated culture supernatants dissolved in carbonate bicarbonate buffer were coated on microtiter plates at 37 °C for 2 h followed by blocking with 5 % milk solution at 37 °C for 1 h. Plates were incubated with 50 μl of 1:500 dilutions of biotinylated IgY (2 mg/ml) antibody for 1 h at room temperature followed by washing in PBST and further incubation in streptavidin-HRP solution. Plates were developed with o-phenylenediamine dihydrochloride (Sigma-Aldrich) and H_2O_2 solution in citrate-phosphate buffer. Absorbance was measured at 450 nm. All of the isolates were tested in duplicate, and absorbance was expressed as mean± SD. Absorbance of samples at a rate two times that of the blank value was considered positive,

and these results were further confirmed by PCR for the *SEB* gene.

Sensitivity and specificity evaluation

Sensitivity and robustness of the biotin IgY assay were evaluated by spiking the recombinant SEB protein into pasteurized milk samples at various concentrations ranging from 1 mg/ml to 1 pg/ml. Unspiked milk served as a control. The lowest dilution at which absorbance was two times that of the control sample was considered the limit of detection of the assay. Similarly, the specificity of the assay was assessed by testing other closely related enterotoxin-producing *S. aureus* strains as well as with the culture supernatants of other Gram-positive and Gram-negative organisms.

Evaluation on natural samples

Milk samples (n=20) were tested for the presence of SEB toxin with and without enrichment. For enrichment, 20 ml of milk was added to 80 ml of BHI broth and incubated at 37 °C, with shaking. Samples were collected every three hours and tested for SEB production. These results were also interpreted using conventional methods of isolation and characterization.

Results

Cloning and expression of the SEB gene

The PCR-amplified *seb* gene (Fig. 1a) was cloned into pRSETA plasmid and the recombinant clones were analyzed for SEB production after induction with IPTG by Coomassie staining of SDS-PAGE gels and western blot analysis. All the tested clones were positive for SEB production, as determined by both SDS-PAGE (Fig. 1b) and western blot analysis, with anti-histidine antibodies. Recombinant SEB protein was purified from an induced *E. coli* culture under denaturing conditions using urea as the denaturant (Fig. 1c). Recombinant protein was dialyzed against 1x PBS with 0.5 M urea. Protein was quantified by Lowry's calorimetric assay using BSA as the standard, made up to 2 mg/ml with PBS, and stored at -20 °C for further use.

Generation of anti-SEB IgY antibodies

Chickens were immunized with r-SEB protein along with Freund's complete and incomplete adjuvants. Anti-SEB IgY was found in blood of immunized chickens when tested by indirect ELISA after the fourth immunization and a titer of 1:16,000 was observed with indirect ELISA using r-SEB as the antigen. Egg collection was carried out ten days after the
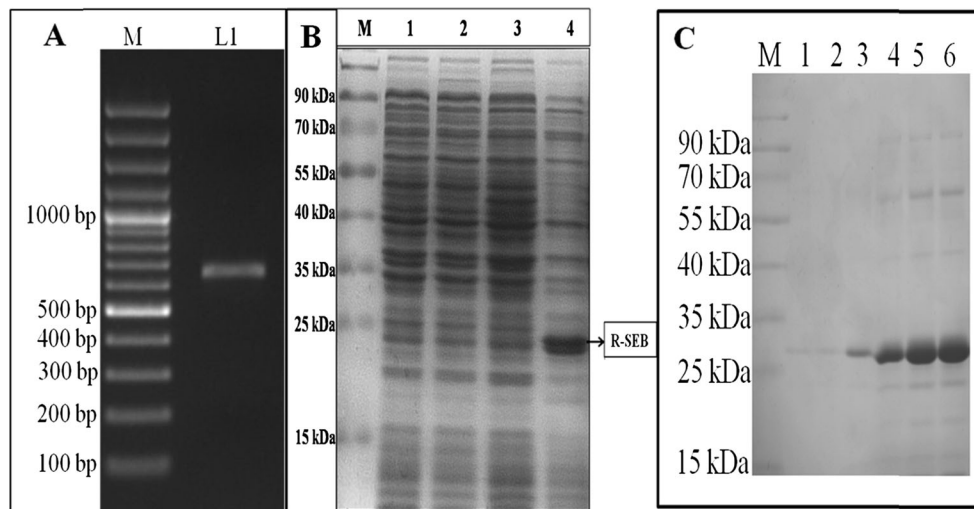
Fig. 1 Cloning, expression, and purification of the SEB gene of *S aureus*. **a** Agarose gel showing the PCR-amplified SEB gene (L1); M- 100 bp DNA ladder. **b** Coomassie-stained SDS-PAGE gel showing the expression of recombinant SEB protein. M- Prestained protein ladder; Lane 1- Uninduced BL21DE3; Lane 2- Induced BL21DE3; Lane 3- Uninduced BL21DE3 clone carrying a pRSET A-*seb* plasmid; Lane 4- Induced BL21DE3 clone carrying a pRSET A-*seb* plasmid showing expressed SEB protein. **c** Coomassie-stained SDS-PAGE gel showing purified recombinant SEB protein

fourth immunization. IgY were isolated by using a commercial IgY purification kit and the yield was more than 20 mg/egg yolk (Fig. 2). The concentration of IgY was adjusted to 5 mg/ml in PBS and stored at -20 °C for further use.

Biotinylation of IgY and IgG

Various combinations of primary antibodies and enzyme-conjugated secondary reagents were tested for SpA cross-reactivity. For this, in-house-raised anti-SEB-IgY and commercial rabbit IgG were labeled with biotin using a commercial labeling kit. The extent of biotinylation was estimated by streptavidin-HABA assay and is found to be approximately 2.17 and 3.0 biotin molecules per IgY and IgG molecules, respectively. Biotinylation was confirmed by dot ELISA by coating labeled antibodies and unlabelled antibodies as controls and probed with streptavidin-HRP solution after blocking in 1 % BSA. Color development was observed only in spots corresponding to biotin antibodies (Fig. 3).

Western blot analysis

SpA reacts with all major classes and subclasses of mammalian immunoglobulins used frequently as primary or enzyme-conjugated secondary antibodies. Hence, western blot analysis helps in testing the reactivities of various antibodies at different regions based on the resolving power of the western blot procedure. Biotinylated and normal IgY and IgG
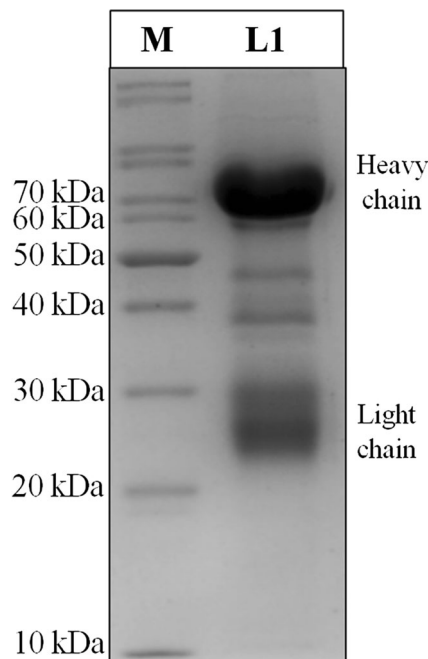

Fig. 2 SDS-PAGE analysis of anti-SEB IgY purified from egg yolks. Gel was stained with Coomassie brilliant blue to visualize the proteins. Lane M- Unstained protein ladder; Lane 1- IgY purified by salt precipitation
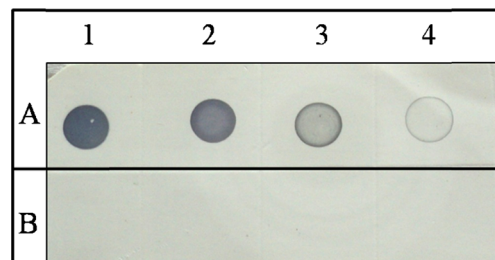

Fig. 3 Dot ELISA to test the biotinylation of IgY antibodies using streptavidin-HRP. A1-A4- Different dilutions of biotin-labeled IgY coated on a nitrocellulose membrane. B1-B4- Normal IgY coated on a nitrocellulose membrane. Color development was observed only with lanes corresponding to biotin-labeled antibodies

antibodies were tested separately for SpA cross-reactivity. In experiments performed with IgG, SpA reactivity was observed with both normal as well as biotinylated IgG antibodies (Fig. 4a and b). Though, theoretically, normal IgY does not bind SpA, reactivity with SpA was observed, owing to the HRP conjugated anti-IgY-IgG antibodies raised in rabbits (Fig. 4c). The only case where SpA cross-reactivity was not observed is when blots were treated with a primary biotinylated IgY and secondary streptavidin-HRP reagents (Fig. 4d). Cross-reactivity observed in Fig. 4a, b, and c matched the reaction observed with the control blot treated with anti-SpA antibody (Fig. 4e), indicating that the cross-reaction is caused mainly by SpA, by binding either the primary or secondary antibody, or both.

Dot ELISA and microtiter plate ELISA for SEB analysis

Dot and plate ELISAs were performed to relate the western blot results for SpA cross-reactivity and also for rapid detection of SEB toxins from samples. For this, methanol/chloroform-concentrated culture supernatants dissolved in carbonate-bicarbonate buffer were used as analytes. In case of dot ELISA, samples negative for SEB were also reactive with all combinations except with biotin IgY and secondary streptavidin-HRP combination (Fig. 5a). All the tested strains were reactive with IgG-treated blots irrespective of SEB production (Fig. 5b).

Evaluation of biotin-labeled IgY ELISA on *S. aureus* isolates

Biotin-labeled IgY was used in an indirect ELISA to test the production of SEB toxins in overnight cultures. The assay was able to clearly differentiate the SEB-producing strains from non-producers (Fig. 6). The results of the IgY ELISA were compared with the IgG ELISA, western blotting for the SEB
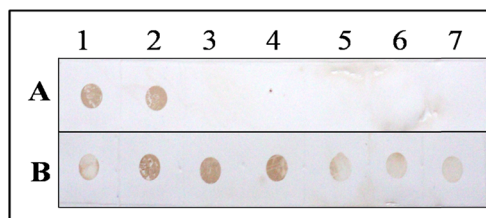

Fig. 5 Dot ELISA performed with biotin-labeled IgY and IgG for detection of SEB, from concentrated culture supernatants grown over night. Membranes treated with IgY (Fig. 5a) were better at reducing protein A interference as compared to IgG-treated membranes (Fig. 5b). Lane 1- Fri 722; Lane 2- ATCC 29213; Lane 3- ATCC 43300; Lane 4- ATCC 700699; Lane 5- ATCC 6538p; Lane 6- ATCC 19095; Lane 7- E 2155

toxin, and PCR for the presence of SEB gene (Table 2). When the assay was evaluated on isolated *S. aureus*, 10 strains were found to be SEB producers out of a total 62 strains. These results were confirmed by PCR screening for the presence of the *seb* gene.

Sensitivity and specificity testing

The sensitivity of the assay was tested on samples of milk, a common source of staphylococcal food poisoning. For this, recombinant SEB was used, and the assay was able to reproducibly detect a minimum quantity of ~10 ng/ml of SEB toxin. During specificity testing with other closely related enterotoxin producing strains, none of the strains positive for classical SEs were reactive, with the exception of SEB. Since many *S. aureus* strains are multiple enterotoxin producers, care was taken during specificity testing such that each strain was representative of only one enterotoxin type. Additionally, many other non-staphylococcal bacterial strains were tested for cross-reactivity, and none of them were found positive with this assay (Table 3).
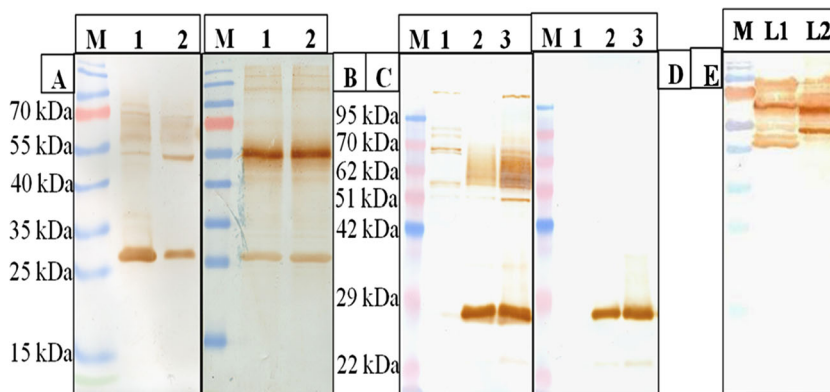

Fig. 4 Western blots showing SpA reactivity with normal and biotinylated IgG and IgY antibodies. **a** Blot treated with normal IgG. Lane M- Prestained protein ladder; Lane 1- FRI 722; Lane 2- NCIM 2122. **b** Blot treated with biotinylated IgG. Lane M- Prestained protein ladder; Lane 1- FRI 722; Lane 2- NCIM 2122. **c** Blot treated with normal IgY. Lane M- Prestained protein ladder; Lane 1- ATCC 6538p; Lane 2- FRI 722; Lane 3- NCIM 2122. **d** Blot treated with biotinylated IgY and streptavidin-HRP. SpA cross-reactivity is not observed with this combination. Lane M- Prestained protein ladder; Lane 1- ATCC 6538p; Lane 2- FRI 722; Lane 3- NCIM 2122. **e** Blot treated with anti-SpA antibody to test the SpA reactivity. Lane M- prestained protein ladder; lane 1- NCIM 2657; lane 2- FRI 722

Fig. 6 Indirect ELISA with biotin-labeled IGY on standard culture supernatants grown overnight. Biotin-labeled IgY was able to clearly differentiate SEB-producing *S. aureus* strains (FRI 722, ATCC 29213, NCIM 2122, NCIM 2127) from non-producers (ATCC 43300, ATCC 700699, ATCC 6538p, ATCC 19095)
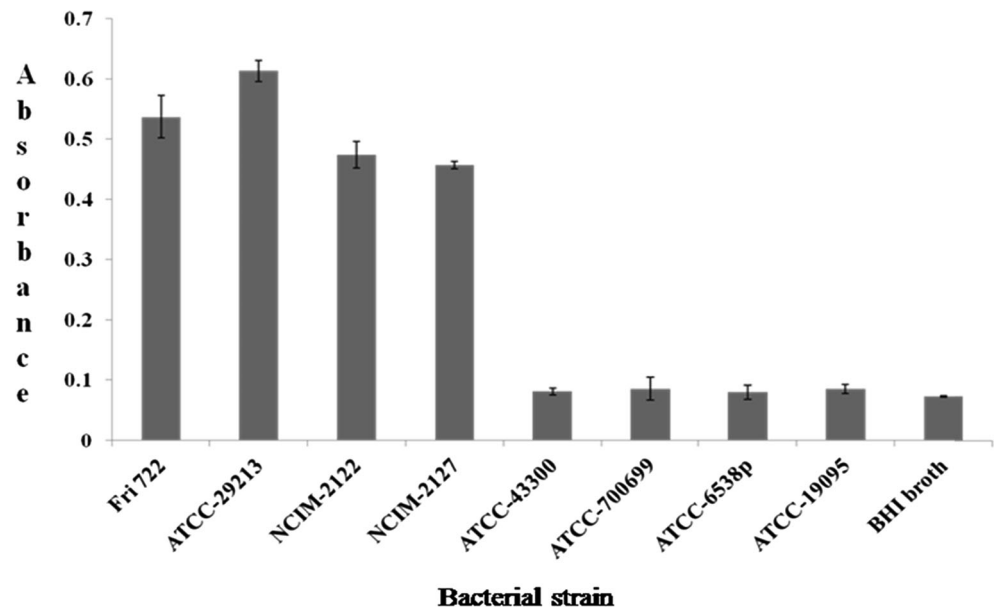
Evaluation on natural samples

No milk samples were positive for SEB prior to enrichment. However, one sample was clearly found to be positive for SEB production after enrichment for 10 h. This was further confirmed by isolation of the *S. aureus* strain positive for SEB production from the enriched sample.

Discussion

Staphylococcus aureus is one of most common causes of food-borne illness in many parts of the world. It secretes many extracellular toxins that are responsible for its pathogenicity (Abdou et al. 2012). SEs are a class of pyrogenic exotoxins secreted by many strains and are implicated in staphylococcal food poisoning (SFP) and toxic shock in humans (Balaban and Rasooly 2000; Pinchuk et al. 2010). SFP is associated with symptoms of diarrhea, vomiting, and abdominal cramps lasting for 24 to 48 hours. SEs are superantigens, molecules that trigger proliferation of lymphocytes regardless of antigen processing (Tang et al. 2012). SEB is a classical enterotoxin and a potent superantigen implicated in food poisoning, and it s also considered a potential bioweapon. Several methods have been reported for detection of SEB in food samples, including PCRs (Abdou et al. 2012; Thapa et al. 2013), ELISAs (Thompson et al. 1986), western blotting, reversed passive latex agglutination (RPLA) testing (Di Pinto et al. 2004), quantitative I-PCR, and several biosensor methods. The majority of methods employ antibodies from mammalian sources which react with some of the *S. aureus* proteins, such as SpA, leading to false positive results. The double antibody sandwich assay reported

IgY antibody capture that was free from interference by these immunoglobulin binding proteins. However, this method requires antibodies from two animal sources. Several other methods were reported to reduce SpA interference in single antibody ELISAs by employing several modifications, such as incubation in diethylpyrocarbonate (DEPC; Nguyen et al. 2010), biotinylation of rabbit IgG to mask the protein A binding site (Hahn et al. 1986), or removal of protein A from the sample by immobilized IgG (Hjelm et al. 1972). However, we have observed that most of these methods are not reliable, given the reduction in sensitivity by DEPC treatment and persistent cross-reaction of protein A with biotin-labeled IgG.

The exclusion of mammalian antibodies from immunoassays can overcome SpA interference, instead using avian IgY antibodies, which have been shown to have the least affinity to SpA. Since antibody generation from egg yolks of chickens is easier, without the need of invasive methods, we sought to develop an ELISA with a single antibody based on IgY. However, the secondary antibody (mammalian source) used in the test was a major obstacle, since this was binding to protein A and giving false positive results. Therefore, the novelty of the study involved replacing the secondary antibody (anti-chicken IgG-HRP) with another signal-generating molecule (streptavidin-HRP). Further, we presume that another immunoglobulin binding protein, Sbi, may also have very little affinity for IgY, given its similar mechanism of action to that of SpA, although this will require further experimental evidence. Additionally, direct labelling of IgY with enzymes such as HRP or alkaline phosphatase, or with fluorophores such as fluorescent isothiocyanate (FITC), rhodamine, or green fluorescent protein (GFP), can be employed to circumvent the problem of immunoglobulin-binding proteins. Streptavidin and biotin molecules are frequently used in immunoassays

Table 2 Comparison of the biotin-IgY ELISA with the biotin-IgG ELISA, western blotting, and PCR on standard and isolated *S. aureus* strains

Strain	Biotin-IgY ELISA	Biotin-IgG ELISA	Western blot	PCR
Staphylococcus aureus FRI 722	+	+	+	+
S. aureus ATCC 29213	+	+	+	+
S. aureus ATCC 700699	-	+	-	-
S. aureus ATCC 43300	-	+	-	-
S. aureus ATCC 6538p	-	+	-	-
S. aureus NCIM-2122	+	+	+	+
S. aureus NCIM 2654	+	+	+	+
S. aureus NCIM 2127	+	+	+	+
S. aureus NCIM 2122	+	+	+	+
S. aureus NCIM 2120	+	+	+	+
S. aureus NCIM 2657	+	+	+	+
S. aureus NCIM 5021	-	+	-	-
S. aureus NCIM 2794	+	+	+	+
S. aureus NCIM 2492	+	+	+	+
S. aureus NCIM 2079	-	+	-	-
S. aureus IVRI	+	+	+	+
S. aureus E-2215 (Dharwad)	-	+	-	-
S. aureus E-2233 (Dharwad)	-	+	-	-
S. aureus E-1933 (Dharwad)	-	+	-	-
S. aureus E-1903 (Dharwad)	-	+	-	-
S. aureus E-2447 (Dharwad)	-	+	-	-
S. aureus E-2147 (Dharwad)	+	+	+	+
S. aureus E-2459 (Dharwad)	+	+	+	+
S. aureus E-2277 (Dharwad)	-	+	-	-
S. aureus E-2282 (Dharwad)	-	+	-	-
S. aureus E-2393 (Dharwad)	-	+	-	-
S. aureus E-2295 (Dharwad)	-	+	-	-
S. aureus E-1898 (Dharwad)	+	+	+	+

ATCC- American Type Culture Collection, Manassas USA; FRI- Food Research Institute, USA; NCIM- National Collection of Industrial Microorganisms, Pune; Dharwad- Clinical isolates received from SDM medical college, Dharwad, Karnataka, India; IVRI- Indian Veterinary Research Institute, Izzatnagar, Uttar Pradesh, India

Table 3 List of bacterial strains used for specificity testing with biotin IgY-ELISA

Organism	WB	ELISA
Staphylococcus aureus FRI 722 (Positive control)	+	+
Staphylococcus epidermidis ATCC 12228	-	-
Bacillus cereus ATCC 10876	-	-
Bacillus subtilis NCIM 2124	-	-
Listeria monocytogenes ATCC 19115	-	-
Salmonella typhimurium ATCC 14028	-	-
Salmonella paratyphi ATCC 9150	-	-
Shigella flexneri ATCC 9199	-	-
Shigella sonnei ATCC 9290	-	-
Shigella boydii ATCC 9207	-	-
Klebsiella pneumoniae ATCC 10031	-	-
Klebsiella oxytoca MTCC 3030	-	-
Escherichia coli K-12	-	-
Clostridium perfringens ATCC 13124	-	-

MTCC- Microbial Type Culture Collection, Chandigarh, India

chicken IgY and probing with streptavidin-HRP appear to be a better choice for removing SpA interference.

In this study, an indirect ELISA procedure was developed and evaluated for detection of SEB in food samples without SpA interference. IgY antibodies were generated against recombinant SEB protein in chickens and labeled with biotin molecules. To circumvent cross-reactivity due to SpA binding to enzyme-conjugated secondary antibodies of mammalian origin, avian IgY antibodies were labeled with biotin and streptavidin-HRP was used for color development for detecting SEB. In contrast, biotin-labeled IgG counterparts were still showing cross-reactivity in both western blotting and plate ELISA. Results of dot and plate ELISA with biotin-labeled IgY were in complete agreement with western blotting and PCR analysis for the seb gene. A large number of clinical isolates were positive for SEB production when screened using the novel ELISA procedure. The assay was sufficiently sensitive to detect ~10 ng/ml of SEB from spiked food samples. A large number of milk samples were also tested for SEB contamination using IgY ELISA, and the test was able to detect SEB after a 10 h enrichment period.

IgY antibodies from chickens are better alternatives to mammalian antibodies given the low cost of production and the use of less invasive methods for their purification. IgY antibodies have numerous advantages over mammalian antibodies, and as such, they are increasingly used in immunodiagnostics (Juliarena et al. 2007; Veerasami et al. 2008) and as therapeutic antibodies (Hou et al. 2014; Revathy et al. 2014; Wang et al. 2014). IgY antibodies have shown their efficacy in double antibody sandwich ELISAs where SpA interference is involved. In the present study, we employed biotin-labeled IgY as a probe for detection of SEB toxins, without SpA

due to the strong nature of their interactions, the availability of easier methods for labelling proteins with biotin residues, and to their dramatic signal enhancement. Hence, biotinylation of

interference, in a single antibody ELISA system. This assay was highly specific, and a similar strategy can be applied for detection of other *S. aureus* toxins where SpA might cause false positive results.

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