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ORIGINAL ARTICLE

Isolation and identification of enterotoxigenic *Staphylococcus aureus* isolates from Indian food samples: evaluation of in-house developed aptamer linked sandwich ELISA (ALISA) method

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Abstract *Staphylococcus aureus* is one of the major food contaminants worldwide, and its enterotoxins are documented as food poisoning and bioterrorism agents. In the present study, an attempt was made to account on the incidences of toxigenic *S. aureus* and its antibiotic resistance profiles in ready to eat bakery food products from different parts of Southern India (Andhra Pradesh, Karnataka, Kerala, Tamil Nadu, and Telangana). A total of 100 food samples, including milk, cake, cheese and chicken products were assessed for *S. aureus* and Staphylococcal Enterotoxin B (SEB) by PCR. Among the subjected food samples, a total of 51 isolates belong to genus *Staphylococcus* and out of that, 34 isolates were *S. aureus*. Among 34 *S. aureus* isolates, 14 isolates were found positive for SEB. The PCR results were further co-evaluated with in-house developed aptamer linked immunosorbent assay (ALISA) for the specific and sensitive detection of SEB. The obtained ALISA results were promising and found consistent with PCR analysis. Furthermore, 24%, 47%, 91%, 82%, 59%, and 47% of *S. aureus* isolates were found resistant to chloramphenicol, methicillin, penicillin, ampicillin, erythromycin, and oxacillin, respectively and concluded as a multidrug resistance (MDR). In conclusion, the present study revealed high presence of toxigenic and MDR resistant *S. aureus* species among the studied regions of Southern India. The present study cautions the need of stringent food safety regulations in India to control the toxigenic and MDR *S. aureus* from food sources and to minimize the risks associated with *S. aureus*.

Keywords Ready to eat bakery foods · *Staphylococcus aureus* · Staphylococcal enterotoxin B · Polymerase chain reaction · Multidrug resistance · Aptamer linked immunosorbent assay

Introduction

Staphylococcus aureus is a ubiquitous microorganism, which represents a significant burden on human health causing food poisoning, skin infection, bone infection, and life-threatening toxic shock (Krakauer and Stiles 2013). Staphylococcal enterotoxins are exotoxins produced primarily by *S. aureus* and liable for major food-borne illnesses that usually occur after intake of processed meats or dairy foods (Dinges et al. 2000). Staphylococcal enterotoxins are structurally related globular proteins with the molecular weight of 22–29 kDa (Mitchell et al. 2000). The Staphylococcal enterotoxins are one of the most potent food-borne toxins and requires very low concentration ranging from 20 ng to 1 µg for the onset of foodborne illness, i.e. vomiting, nausea, abdominal pain, and occasional diarrhoea with fever (Le et al. 2003; Normanno et al. 2007). Hence, Staphylococcal enterotoxin food poisoning is considered as one of the most popular forms of food poisoning found in human population worldwide. The Centers for Disease Control and Prevention (CDC) have

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estimated that around 2,40,000 illnesses, 1000 hospitalizations, and six deaths each year worldwide owed to consumption of foods contaminated with Staphylococcal enterotoxins (Scallan et al. 2011).

Among Staphylococcal enterotoxins, SEB is one of the virulence factors produced primarily by *S. aureus* and has a superantigenic activity (Stiles and Krakauer 2005). The toxic effects of super-antigens include pyrogenicity, activation of T-cells and macrophages with massive production of cytokines, and escalation of endotoxin shock (Callahan et al. 1990). The T cell responses normally activate about 0.0001–0.001% of T-cell population. Whereas, superantigens activate about 20–30% of T-cells and in few cases even up to 70% and end in life-threatening toxic shock (Choi et al. 1990). The SEB initiates the T-cell activation by binding to the major histocompatibility complex (MHC) class II at a different site, and it does not have to pre-process like other antigens (Mudili et al. 2015). The SEB remains stable when exposed to extreme temperature, pH, and relatively resistant to proteolytic enzymes, including pepsin, trypsin, and papain, one important characteristic that makes SEB a potential bioterrorism agent (Cunha et al. 2007). Among Staphylococcal enterotoxins, SEB is one of the most prevalent and possess high-level health risks (Omonigho and Ikenebomeh 2002). Therefore, innovative detection platforms are always requisite for quick and reliable detection of SEB in order to endorse the food safety and to safeguard the human population from the ill-effects of toxins.

Currently, several methods based on antibodies and analytical instrumentations are widely used for the identification of Staphylococcal enterotoxins. The availability of the specific antibodies is of vital importance in the immune detection system (DeGrasse 2012). Nevertheless, antibodies are sensitive to temperature variation, pH shift and have limited lifespan. The production of specific antibodies against the toxins has their limitations such as identical antisera cannot be generated consistently and high-cost involvement in maintaining hybridoma cell lines to produce antibodies (Urushibata et al. 2010). To overcome this aptamer based technology can be used.

Aptamers are single-stranded DNA or short RNA sequence generated by SELEX process, binds with the specific target through folding into a complex three-dimensional structure (Stoltenburg et al. 2007). The binding affinity between the aptamer and the toxin is based on individual or combinatorial effects of structural affinity, hydrogen bonding, stacking of aromatic rings, electrostatic and van der Waals interactions (Borisov and Wolfbeis 2008). Aptamers are good alternative to antibodies for sensing specific targets like toxic proteins and drugs and also have a high potential as bioprobes for drug targeting and biosensing (Ng et al. 2006). Advantages of aptamers over antibodies are higher affinity, specificity, ease of synthesis along with their stability to resist denaturation and degradation. The aptamers can be easily modified by chemical synthesis and do not require experimental animals for synthesis. Besides, alternative approach of aptamer against specific targets does not require expensive purification methods to avoid batch to batch variation among the antibodies (Mehta et al. 2012 and Jo et al. 2011). Aptamers have become an increasingly important bioassay material to provide a more realistic alternative for detection of SEB from food and environmental samples.

In the present study, an attempt was made to account on the incidences of toxigenic *S. aureus* and its antibiotic resistance profiles from India. Several food matrices were subjected to isolate *S. aureus* strains which were further characterized by biochemical and molecular methods. Enterotoxigenic S. *aureus* species were confirmed by gene-specific PCR and results were cross-checked with in-house developed ALISA method.

Materials and methods

Materials and reagents

Culture media (brain heart infusion broth, mannitol salt agar, Baird-Parker agar, Simmons citrate agar, Christenson's urea agar, and Muller-Hinton agar), sterile polypropylene containers, and reagents (Gram staining kit, sulfanilic acid, and α-naphthylamine) were obtained from HiMedia laboratories, India. Ampicillin, erythromycin, chloramphenicol, ciprofloxacin, methicillin, oxacillin, penicillin, vancomycin, nitrocellulose membrane, streptavidin, horse radish peroxide, 3,3′,5,5'-tetramethylbenzidine, and hydrogen peroxide were procured from Sigma-Aldrich, India. Taq DNA polymerase and dNTPs were procured from Thermo Scientific, India. The aptamer library and primers were synthesized at 1 mM and 250 nM, respectively from Integrated DNA Technologies, USA. The other chemicals used in the study were fine grade and obtained from Merck Millipore, India.

Sample collection

A total of 100 ready to eat (RTE) bakery products, including milk (25), cake (25), cheese (25), and chicken (25) samples were collected from different regions of Southern India during the month of March and April 2016. The sample collection sites were located by spatial data of latitude and longitude coordinates using Diva-Geographic Information System software (Hijmans et al. 2004) and showed in Fig. 1. The samples were packed in a sterile polypropylene container and safely transported to the

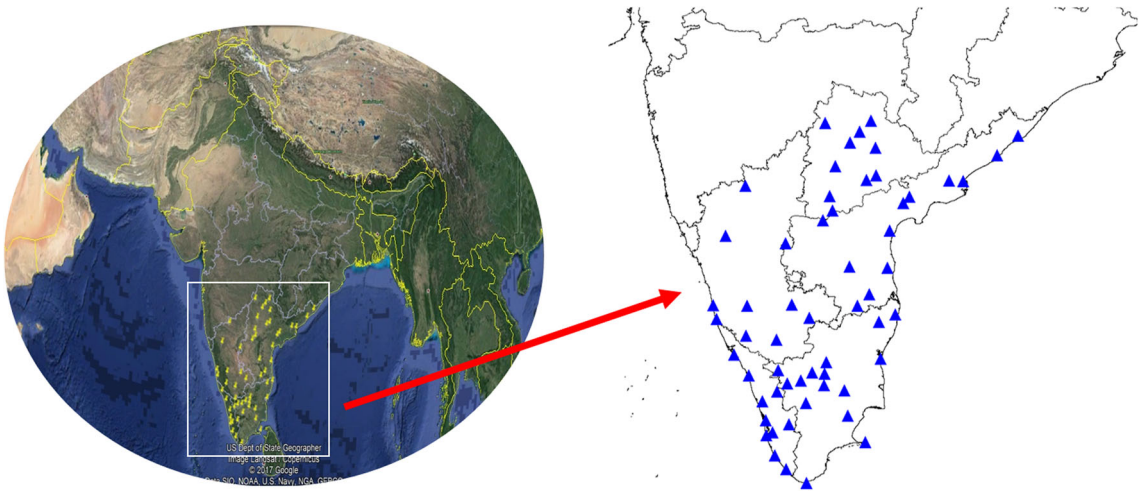

Fig. 1 The sample collection sites were located by spatial data of latitude and longitude coordinates using Diva-Geographic Information System software

laboratory in the icebox and examined for *S. aureus* and SEB. The polymerase chain reaction was carried out to detect *S. aureus* and its enterotoxin serotypes (*sea*, *seb*, and *sec*). While, aptamer linked immunosorbent assay (ALISA) was preferred to detect SEB serotype alone.

Isolation of *Staphylococcus* species

Samples weighing 1 g was suspended in brain heart infusion broth (BHI) media and homogenized at 120–140 rpm for 5 min. A serial dilution of 10^{-6} was performed, 0.1 mL of suspension was spread plated on mannitol salt agar, and incubated under aerobic conditions at 37 °C for 24 h. The presumptive isolates of *S. aureus* with yellow colonies and zones were picked and sub-cultured to obtain a pure culture. The isolated colonies were further streaked on Baird-Parker agar and observed for the exclusive characteristic feature of *S. aureus*, i.e. black, convex and shiny colonies, fine white rim, opaque, and clear zone.

Biochemical characterization

Traditional biochemical characteristics of isolated *S. aureus* were tested by catalase, coagulase, nitrate reduction, citrate, urease, glucose and mannitol fermentation after confirmation with Gram staining. The isolates positive for biochemical tests aimed at *S. aureus* were further assessed by PCR.

Detection of *S. aureus* and enterotoxin genes by PCR

PCR method was used for specific detection of genus *Staphylococcus*, *S. aureus,* and enterotoxin genes by targeting the thermonuclease (n*uc*), hemolysin (*hly–A*), and enterotoxin genes (*sea, seb, sec*), respectively. Briefly, food samples were enriched in BHI broth media at 37 °C overnight, the culture pellets were collected by centrifugation at 8000 rpm for 2 min. The pellets were dissolved in 100 µl of sterile nuclease-free water and boiled at 95 °C for 15 min. Following, centrifugation was carried out at 12,000 rpm for 5 min and supernatant containing genomic DNA was collected and used as a template for PCR. The primers specific for *nuc, hly-A, sea, seb, and sec* were designed using Gene Runner software version 4.0 (Hastings Software Inc., NY, USA) and sequences are given in Supplementary Table 1. Briefly, PCR reactions mixture contains 1 × Taq buffer, 100 µM dNTPs, 1.5 mM $MgCl_2$, 5 pmol forward and reverse primers, 10-50 ng template, and 1 U Taq DNA polymerase. The PCR amplification was carried out with initial denaturation at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 30 s for *nuc, hly-A*, and 1 min for *sea, seb, sec*, and the final extension at 72 °C for 8 min. After successful amplification, electrophoresis analysis of PCR products (5 µL) was carried out in 1% agarose gel under a constant current at 100 V for 45 min. The DNA marker of 100 bp was used to compare the amplified PCR product length with the target amplicon sizes of *nuc* (389 bp), *hly—A* (569 bp), *sea* (200 bp), *seb* (400 bp), *and sec* (546 bp). The gel images were documented using Vilber Lourmat gel imaging system, France.

Antibiotic susceptibility test

The *S. aureus* isolates were subjected to antibiotic sensitivity testing by standard disc diffusion method on Muller-Hinton agar (Merck, Germany) according to

Table 1 Biochemical and molecular characterization of *Staphylococcus aureus*

Biochemical tests	Milk isolates	Cake isolates	Cheese isolates	Chicken isolates
Enzymatic activity				
Catalase	16	10	12	13
Coagulase	13	4	7	10
Nitrate reduction	16	10	12	13
Citrate utilization	13	4	7	10
Urease production	16	10	12	13
Sugar fermentation				
Glucose fermentation	16	10	12	13
Mannitol fermentation	16	10	12	13
Molecular characterization				
PCR (*nuc*) gene	16	10	12	13
PCR (*hly-A*) gene	13	4	7	10

recommendation of Clinical and Laboratory Standards Institute (CLSI) (Wang et al., 2018). The antibiotic resistance of *S. aureus* isolates was tested against ampicillin (10 µg), erythromycin (15 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), methicillin (10 µg), oxacillin (1 µg), penicillin (6 µg), and vancomycin (30 µg). The isolates were divided into two groups based on the zone of inhibition and expressed as sensitive and resistant. *S. aureus* ATCC 29213 (Wound isolate, Micro-Media Systems, Inc.) was referred as a control.

Detection of SEB by aptamer linked immunosorbent assay (ALISA)

S. aureus isolated from food samples (milk, cake, cheese, and chicken) and standard cultures were enriched in BHI broth for 4 h at 37 °C. Following, the culture supernatants were recovered by centrifugation at 10,000 rpm for 3 min and filtered through 0.22 µm syringe filters and filtrate was used for SEB detection with our previous in-house developed IgY aptamer hybrid system (Mudili et al., 2015). Briefly, anti-SEB IgY (500 $ngmL^{-1}$) generated in chicken egg yolk was coated on nitrocellulose membrane and blocked with 5% skim milk solution (prepared in PBS pH 7.4) for 1 h at 45 °C. Subsequently, 100 µL of filtrate obtained from *S. aureus* culture supernatant was added and incubated for 1 h at 37 °C. The membrane washed twice with PBST and PBS to remove loosely bound non-specific antigens. The SEB specific biotinylated ssDNA aptamer (250 $ngmL^{-1}$) was added and incubated for 1 h at room temperature (25 ± 2 °C) and washed twice with PBST and PBS to remove unbound aptamers. The Streptavidin-HRP conjugate was added to the membrane and incubated for 30 min at room temperature. The unbound Streptavidin-HRP was washed as above and the substrate TMB-H_2O_2 was added for color development, and the results were documented.

The sensitivity of the anti-SEB aptamers was tested with different concentrations of SEB toxin ranging from 10 μgmL^{-1} to 0.01 μgmL^{-1}. The specificity of the anti-SEB aptamers was tested with other toxin-producing (SEA, SEC, and HLA) close relatives of *S. aureus* using standardized ALISA method. The standard cultures used in the study were *S. aureus* ATCC—700699 (Pus isolate, National Center for Research Resources), *S. aureus* ATCC—29213 (Wound isolate, Micro-Media Systems, Inc), *S. aureus* NCIM—2657 (National Chemical Laboratory), *S. aureus* NCIM—5021 (Clinical isolate, Food and Drug Administration), *S. aureus* NCIM—2654 (National Chemical Laboratory), *S. epidermidis* ATCC—12228 (Food and Drug Administration), *S. aureus* ATCC—13565 (Ham isolate, Food and Drug Administration), *S. aureus* ATCC—19095 (Leg abscess isolate, University of Chicago), *Escherichia coli* ATCC—10536 (American Cyanamid Co), and *Salmonella typhimurium* ATCC—14028 (Animal tissue, Centers for Disease Control and prevention). The obtained ALISA results were cross-evaluated with PCR for its specificity against SEB.

Results

Isolation and characterization of *Staphylococcus aureus*

Among 100 RTE bakery food products tested, 51 bakery products were noticed as contaminated with genus *Staphylococcus* by cultural and biochemical methods. A total of 51 isolates were positive for catalase, urease, nitrate, mannitol and glucose fermentation and negative for coagulase and citrate, which determined these isolates as genus *Staphylococcus* (Table 1). The highest rate of *Staphylococcus* species incidence was observed in milk (16) samples followed by chicken (13), cheese (12), and cake (10)

samples (Supplementary Table 2). From 51 *Staphylococcal* isolates, 34 isolates were black and shiny with narrow white margins and surrounded by clear zone on Baird Parker agar medium. These colonies were found to be Gram-positive cocci in clusters on microscopic examination. Moreover, these 34 isolates were found positive for coagulase, citrate, catalase, nitrate, urease, mannitol and glucose fermentation, and determined as *S. aureus*. A number of 13 milk isolates, 10 chicken isolates, 7 cheese isolates, and 4 cake isolates were constituted to total of 34 *S. aureus* isolates (Supplementary Table 2). Furthermore, 51 *Staphylococcus* positive isolates were subjected to molecular confirmation by PCR (Table 1).

Detection of *Staphylococcus* and *S. aureus* by PCR

Among the 100 food isolates, 51 isolates which were confirmed as *Staphylococcus* by biochemical characterization remained positive for genus specific gene *nuc* (thermostable nuclease) of *Staphylococcus* (Fig. 2a). Among 51 *Staphylococcus* isolates, 34 isolates were shown positive for species-specific gene *hly—A* (hemolysin A) of *S. aureus* (Fig. 2b). The accomplished results of molecular detection of *Staphylococcus* and *S. aureus* were identical with results of biochemical characterization.

PCR based detection of *S. aureus* enterotoxin genes

The isolated *S. aureus* was distinguished into enterotoxigenic serotypes (*sea*, *seb*, and *sec*) by PCR analysis (Fig. 3). Among 13 *S. aureus* isolates of milk samples, 6 were positive for *seb*, 3 were positive for *sea*, and 4 were positive for *sec*. Among 4 *S. aureus* isolates from cake samples, 2 were positive for *sec* and each one was positive for *seb* and *sea*. Out of 7 *S. aureus* isolates from cheese samples, 4 were positive for *seb*, 3 were positive for *sea*, and none of them were positive for *sec*. Among 10 isolates from chicken samples, 4 were positive for *sea* and 3 each was positive for *seb* and *sec* (Supplementary Table 3).

Antibiotic susceptibility test

The antibiotic resistance of the isolated *S. aureus* to various antibiotics was analyzed by zone of inhibition assay. The isolates showed no resistance to vancomycin and ciprofloxacin and slight resistance to chloramphenicol (24%) and high resistance to β-lactams such as methicillin, penicillin, ampicillin, and oxacillin with 47, 91, 82 and 47%, respectively. Also, showed 59% resistance to macrolide antibiotic erythromycin (Table 2). The data confirm the potential role of RTE bakery foods in spreading MDR *S. aureus* strains in India and cautions the health risks of consumers.

Detection of SEB toxin by aptamer linked immunosorbent assay (ALISA)

The attained *S. aureus* isolates from original RTE bakery food products and standard strains, including SEA, SEB, SEC, and HLA toxins producing strains, and other Gram positive and negative strains were enriched in BHI broth media for 4 h at 37 °C and culture supernatant was recovered by centrifugation at 10,000 rpm for 3 min and used in the study.

The specificity of the anti-SEB aptamers aimed at SEB toxin was validated by dot-ELISA along with standard toxin producing strains, SEA (*S. aureus* ATCC—13565), SEB (*S. aureus* ATCC—700699), SEC (*S. aureus* ATCC—19095), and HLA (*S. aureus* ATCC—10832, Food and Drug Administration). The culture supernatants of SEB strains were alone reactive to anti-SEB aptamers. The other strains producing SEA, SEC, and HLA toxins were not cross-reactive towards the anti-SEB aptamers (Supplementary Fig. 1A).

Moreover, specificity of anti-SEB IgY was evaluated by indirect plate ELISA, and it was found to be highly reactive to SEB (*S. aureus* ATCC—700699) with slight cross-reactivity towards SEA (*S. aureus* ATCC—13565) and SEC (*S. aureus* ATCC—19095), and no cross-reactivity was observed with HLA (*S. aureus* ATCC—10832). (Supplementary Fig. 1B).

Furthermore, sensitivity of developed ALISA technique was tested against various concentrations of SEB. The optimal concentration of 250 $ngmL^{-1}$ anti-SEB aptamer was found to detect lowest concertation of 25 $ngmL^{-1}$ SEB. The limit of detection (LOD) of ALISA was concluded as 25 $ngmL^{-1}$ (Supplementary Fig. 2).

Finally, culture supernatants of standard strains for SEA (*S. aureus* ATCC—13565), SEB (*S. aureus* ATCC—700699, *S. aureus* ATCC—29213, *S. aureus* NCIM—2657, *S. aureus* NCIM—5021, and *S. aureus* NCIM—2654), SEC (*S. aureus* ATCC—19095), other Gram positive (*S. epidermidis*, ATCC—12228), and Gram negative (*E. coli* ATCC—10536, and *S. typhimurium* ATCC—14028) bacterial strains were tested for cross-reactivity with ALISA intended for SEB detection. The developed ALISA technique was found only reactive with SEB and no cross-reactivity was observed with SEA, SEC, and other Gram positive and negative strains (Fig. 4a).

In the present study, developed ALISA technique was further tested with culture supernatants of *S. aureus* isolates from original RTE bakery food products to distinguish SEB serotype. The culture supernatants of SEB producing *S. aureus* isolates were displayed purple dots (Fig. 4b). A total of 14 *S. aureus* isolates were found positive for SEB, which included 6 from milk samples, 4 from cheese samples, 3 from chicken samples, and one from cake samples

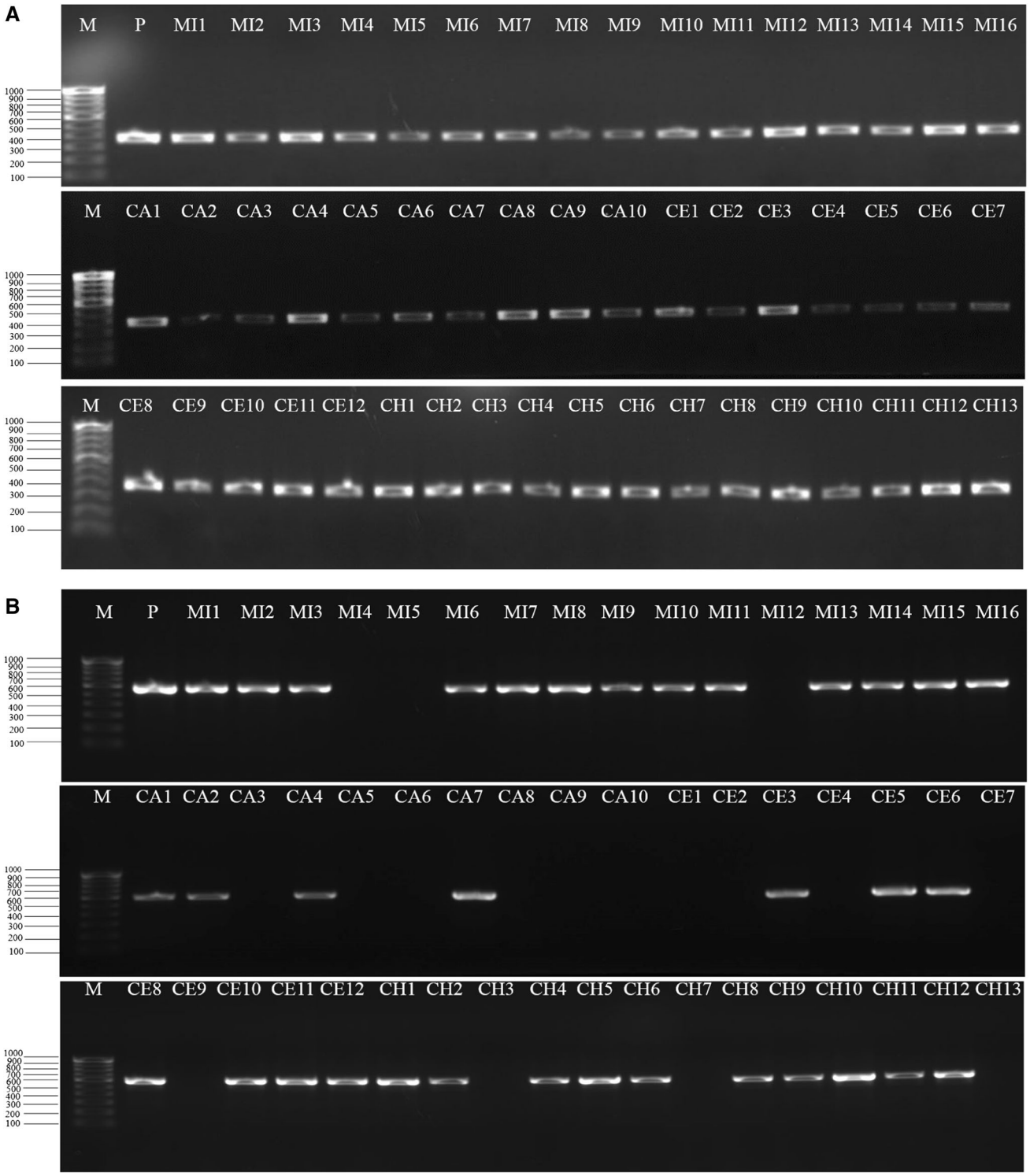

Fig. 2 **a** Detection of genus *Staphylococcus* by PCR targeting *nuc* gene. M (Marker)-100 bp DNA marker, P-Positive control (*Nuc* gene), MI1—CH13 isolates of *Staphylococcus* (389 bp *nuc*). **b** Detection of genus *Staphylococcus* by PCR targeting *hly-A* gene. M (Marker)-100 bp DNA marker, P-Positive control (*hly-A* gene), MI1—CH13 isolates of *S. aureus* (569 bp *hly-A*)

(Table 3). Several *S. aureus* strains are multiple enterotoxin producers, but the isolates from the RTE bakery food samples were represented only one enterotoxin type. The obtained ALISA results were promising and found consistent with PCR analysis. Though, ALISA technique is found rapid and cost-effective comparable to PCR method.

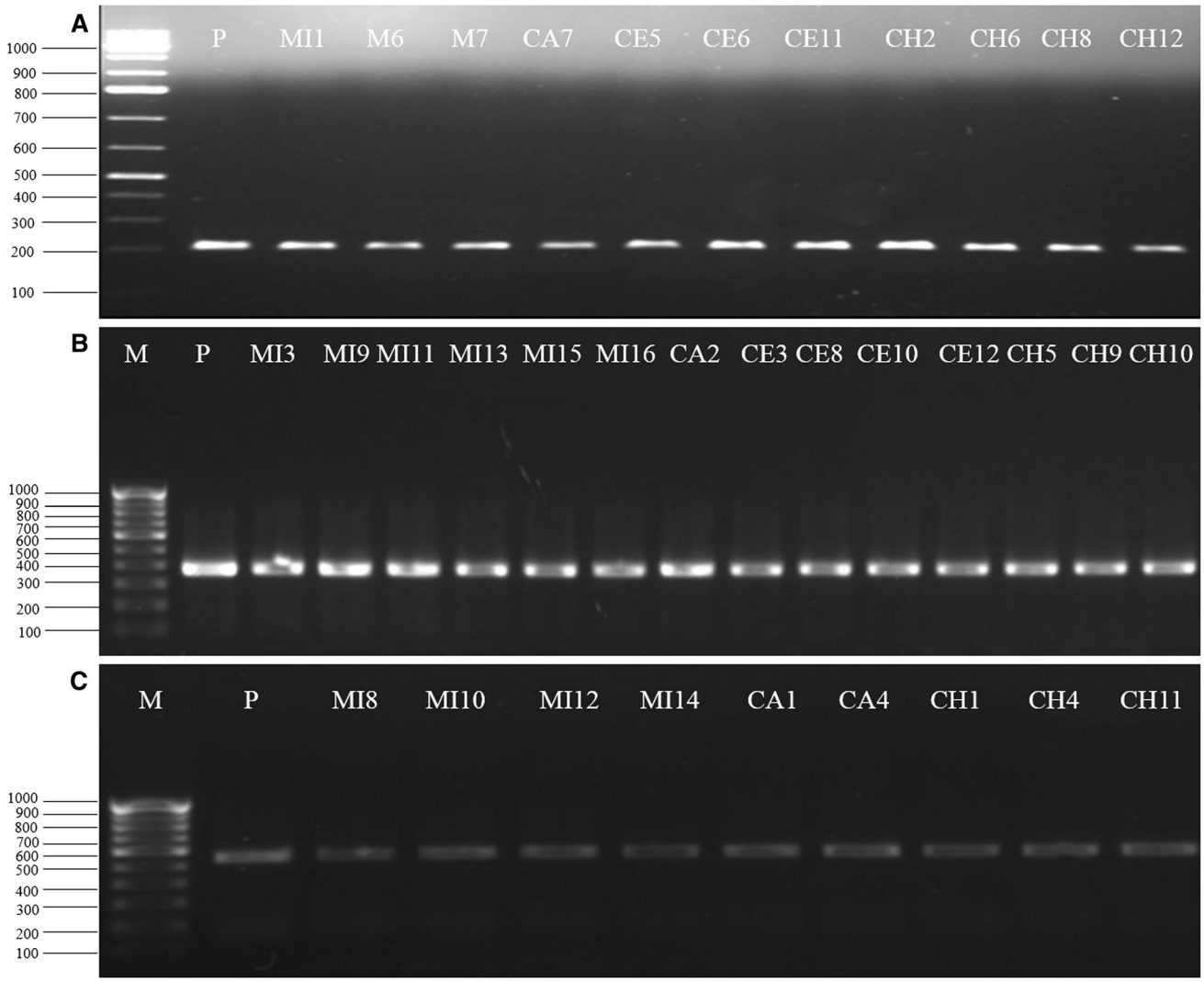

Fig. 3 PCR based detection of *S. aureus* enterotoxin genes. **a** Evaluation of SEA specific PCR assay; M (Marker)-100 bp DNA marker, P-Standard SEA Positive *S. aureus* (200 bp), MI1—CH12 SEA producing isolates. **b** Evaluation of SEB specific PCR assay M (Marker)-100 bp DNA marker, P-Standard SEB Positive *S. aureus* (400 bp), MI3—CH10 SEB producing isolates. **c** Evaluation of SEC specific PCR assay M (Marker)-100 bp DNA marker, P-Standard SEC Positive *S. aureus* (546 bp), MI8—CH11 SEC producing isolates

Table 2 Antimicrobial susceptibility of *S. aureus* isolates

Antibiotics	*S. aureus* isolates (n = 34)	
	Sensitive (%)	Resistant (%)
Methicillin	18 (53%)	16 (47%)
Penicillin	3 (9%)	31 (91%)
Ampicillin	6 (18%)	28 (82%)
Erythromycin	14 (41%)	20 (59%)
Oxacillin	18 (53%)	16 (47%)
Ciprofloxacin	34 (100%)	–
Chloramphenicol	26 (76%)	8 (24%)
Vancomycin	34 (100%)	–

Discussion

Bakery food business is a growing sector worldwide and plays a momentous role in catering for urban area inhabitants with unique flavor, accessible, and nutritious foods. Bakery food assures food security and employment for a considerable proportion of urban area inhabitants in both developed and developing countries (Smith et al. 2004). Regardless of plausible interests, RTE bakery foods have been documented to be unhygienic and concerned with food-borne outbreaks worldwide (Smith et al. 2004). Microbiological spoilage is frequent issue in restricting the shelf life of high and intermediate moisture bakery food products and is also a chief factor of economic loss for the bakery business. It has been appraised that in the United

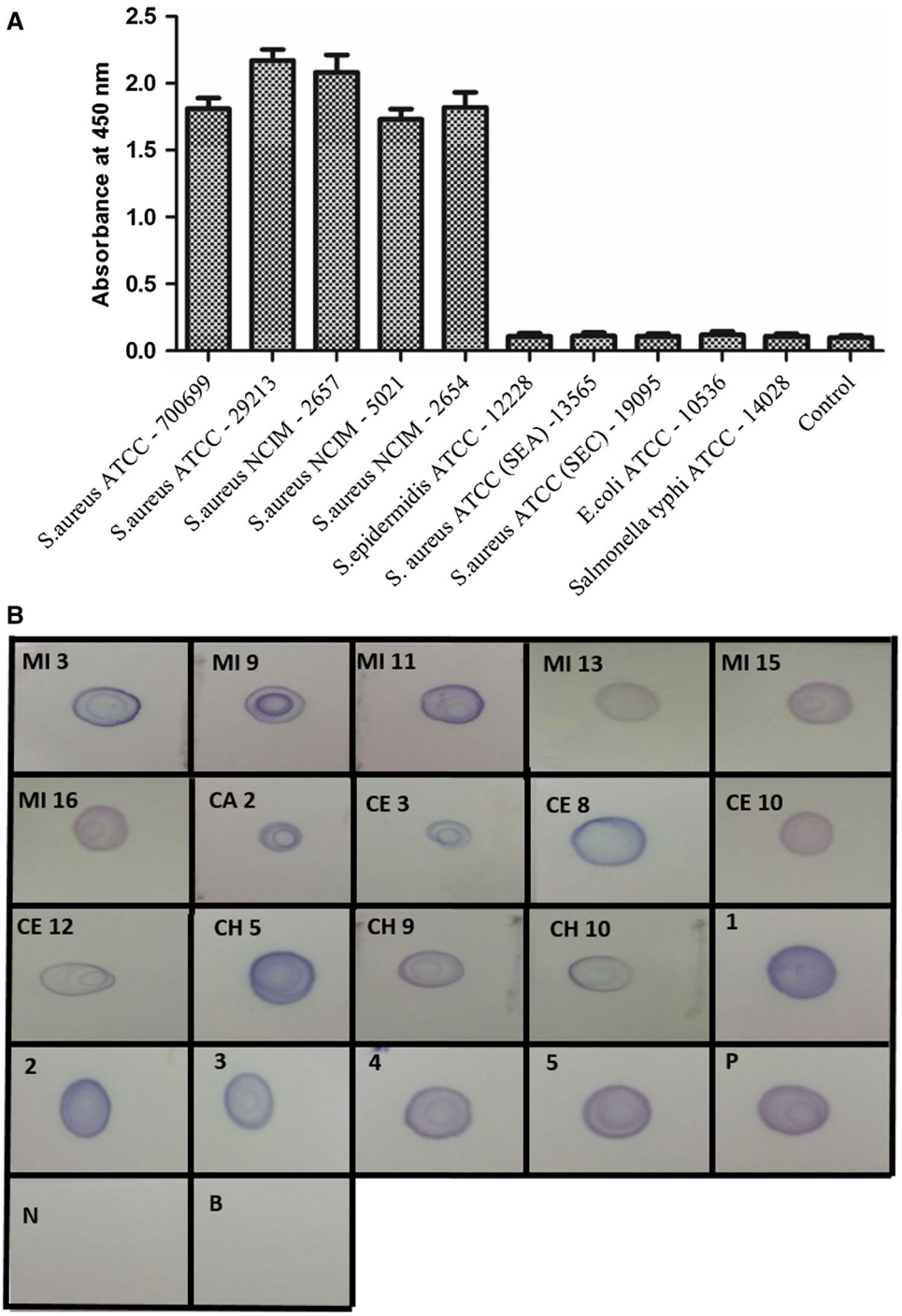

Fig. 4 **a** Specificity of ALISA on different culture supernatants. Culture supernatants of various strains of *S. aureus* and other bacterial pathogens were added to anti-SEB IgY pre-coated wells of microtitre plate and ALISA was carried out using biotinylated anti-SEB aptamer. PBS was used as a blank. The experiment was performed in triplicate and results were expressed in mean ± SEM. **b** Evaluation of ALISA on RTE bakery food samples. The cultures were added to anti-SEB IgY pre-coated nitrocellulose membrane and the food samples were evaluated with biotinylated aptamer. Milk isolates 3, 9, 11, 13, 15, 16, cake isolate 2, cheese isolates 3, 8, 10, 12, and chicken isolates 5, 9, 10 were SEB positive, 1—*S. aureus* ATCC (American Type Culture Collection, USA) 700699, 2—*S. aureus* ATCC 29213, 3—*S. aureus* NCIM (National Collection of Industrial Organisms, India)—2657, 4—*S. aureus* NCIM—5021, 5—*S. aureus* NCIM—2654, P-Positive, N-Negative (SEC), B-Blank (PBS)

States alone, economic loss due to microbiological spoilage is about 1% to 3% per annum and measured as a great economic obstacle in food industry. The significant factor influencing the microbiological spoilage of RTE bakery foods is water activity (a_w). The minimal a_w support the growth of spoilage microbes in RTE bakery foods is 0.60. The high moisture RTE bakery foods (a_w 0.94–0.99) support almost all bacteria, molds, and yeasts. As most bacteria need a high a_w for growth and therefore, bacterial contaminations are often occurred in RTE bakery foods with a high moisture content, i.e. pizza, cake, pastries, soft cookies, milk products, chicken and meat products, and egg products (Smith et al. 2004). Furthermore, unhygienic methods in preparation and selling, and multiple routes of entrance have been reported as the chief factors accountable for assisting the entry of microbial pathogens (Mankee et al. 2003). The most probable bacterial contaminants of RTE bakery foods are *S. enteritidis*, *S. typhi*, *S.*

Table 3 Detection of SEB by ALISA and PCR

Bacterial strains	ALISA	PCR
Standard strain		
S. aureus ATCC—700699	+	+
S. aureus ATCC—29213	+	+
S. aureus NCIM—2657	+	+
S. aureus NCIM—5021	+	+
S. aureus NCIM—2654	+	+
S. aureus ATCC—13565	–	–
S. aureus ATCC—19095	–	–
S. epidermidis ATCC—12228	–	–
E. coli ATCC—10536	–	–
S. typhimurium ATCC—14028	–	–
RTE bakery food isolates		
Milk isolate (3,9,11,13,15, and 16)	+	+
Milk isolate (1,2,6,7,8,10, and 14)	–	–
Cake isolate (2)	+	+
Cake isolate (1,4, and 7)	–	–
Cheese isolate (3,8,10, and 12)	+	+
Cheese isolate (5,6, and 11)	–	–
Chicken isolate (5,9, and 10)	+	+
Chicken isolate (1,2,4,6,8,11, and 12)	–	–

typhimurium, *S. aureus*. *C. botulinum*, *B. cereus*, *B. licheniformis*, *B. subtilis*, and *L. monocytogenes*. Among them, *S. aureus* is one of most considerable causes for food poisoning outbreaks in bakery products. Humans harbouring *S. aureus* are a foremost cause of contamination of RTE bakery foods throughout preparation or post preparation. Post preparation contamination is also probable from surfaces, air, and cross-contamination. Constituents of bakery foods might also serve as sources of contamination of *S. aureus*.

In our study, 51% of tested bakery products (milk, cake, cheese, and chicken) have been contaminated with genus *Staphylococcus* and it could be mainly due to unhygienic handling techniques and high moisture content of bakery products. In support of our study, great number of *S. aureus* incidences were noticed in RTE bakery foods worldwide. Briefly, Colombari et al. (2007) in Brazil has noticed an outbreak of foodborne illness owed to consumption of *S. aureus* contaminated RTE bakery foods, especially salads and sandwiches, and determined that RTE food is often prepared by hand, which may increase the incidence of contamination. Most frustratingly, 122 illness cases were reported in Brazil by eating tainted bakery cream puffs contaminated with *S. aureus*. Along with, an outbreak of 215 staphylococcal food poisoning cases was reported in Caribbean cruise ship sailing from the United States owed to consumption of *S. aureus* contaminated cream pastries (Juneja et al. 2002; Waterman et al. 1987). One survey found that 55% of cream products from commercial retailers in Brazil were contaminated with SEA serotype of *S. aureus* (Anunciacao et al. 1995). Sumner et al. (1993) in USA has reported the incidence of enterotoxigenic *S. aureus* in RTE bakery foods, including long johns, apple muffins, oatmeal raisin cookies, and cream puffs. Santos et al. (1981) has reported the occurrence of *S. aureus* in raw and pasteurized milk intended for preparation of commercial Brazilian minas cheese. In India, Desai and Kamat have reported that 85.7% of pastry and creams from commercial bakery retailers were contaminated with *S. aureus* (Desai and Kamat 1998). Also, Leela et al. (1981) perceived enterotoxigenic *Staphylococcus* in bakery cream products in India. Moreover, Sankaran and Leela (1983) has noticed SEA, SEB, and SEE enterotoxigenic *S. aureus* in buns, puffs, and cakes from Indian bakeries. The SEA is more detected in 75% of *S. aureus* incidences and though, occurrences are sporadically attributed to SEB (Argudin et al. 2010). These occurrences concluded that RTE bakery foods are often contaminated by *S. aureus* due to high moisture content and unhygienic practices.

Furthermore, food act as a vital factor for the transfer of antibiotic resistance and it occurs by antibiotic remains in food and resistant foodborne pathogenic microorganisms (Pesavento et al. 2007 and Gundogan et al. 2006). In our study, *S. aureus* isolates were found to be multidrug resistant based on the antibiotic susceptibility studies. A proportion of 24%, 47%, 91%, 82%, 59%, and 47% of *S. aureus* isolates were shown resistant to chloramphenicol, methicillin, penicillin, ampicillin, erythromycin, and oxacillin, respectively. It warns the prevalence of MDR *S. aureus* in RTE bakery foods, and it could be a risk to humans and causes severe health issues. *S. aureus* is resistant against most of the commonly used antibiotics and limit the activity of the drugs due to their ability to produce an exopolysaccharide barrier within the microabscesses (Gundogan et al. 2006).

Though, *S. aureus* could eliminate by heating, enterotoxins are heat resilient and is not possible to inactivate by heating. Thus, *S. aureus* food poisoning outbreaks may still ensue, even in the nonappearance of viable bacteria and if pre-formed enterotoxin is already present in the food (Capparelli and Mata 1975). Among Staphylococcal enterotoxins, SEB posed high-level health risk. In our study, 14% of *S. aureus* isolates were capable to produce SEB and, which is utmost apprehension. Up till now, there is no FDA-affirmed antibody or therapeutic agents to prevent or treat SEB-intoxication. There is always a possibility to use SEB as a biological warfare agent such as an aerosol, food or water contaminant hence categorized under a category B biothreat agent by CDC. Therefore, there is always a prerequisite of appropriate practice for its detection and treatment.

There are several methods available for specific detection of SEB that includes antibody-based detection (ELISAs, lateral flow systems, and immuno-affinity assays), nucleic acid-based detection (PCRs, real-time PCRs, and multiplex PCRs). Immuno-based detection systems are found to be highly sensitive and specific platform for rapid pathogen detection in raw and processed foods. These methods are cost-efficient and reliable than conventional nucleic acid-based methods in detection of the toxin. Furthermore, the conventional nucleic acid method involves complex sample purification and the sensitivity can be easily compromised by several interfering substances (Mudili et al. 2015).

In status quo, aptamers are excellent alternatives for monoclonal antibodies that offer several advantages due to their smaller size and functional similarities to antibodies (Sun and Zu 2015). They strongly penetrate into the tissue and act on the target cells efficiently and increase the therapeutic indices (Xiang et al. 2015). The functional similarities to antibodies lead to extensive use in various biomedical applications, including targeted therapy, cell detection, bioassays, food safety and environmental monitoring (Yang et al. 2015). Aptamers have advanced clinical applicability than antibodies in several aspects like non-immunogenic, non-toxic (Eyetech 2002 and Ireson and Kelland 2006), low or nil side effects (Lao et al. 2015) and successful development of specific aptamers against the limitless range of targets, including peptides, proteins inorganic ions and tissues (Liu et al. 2012 and Stoltenburg et al. 2012). The aptamers may be freely distributed throughout the world, making their use in assays as an attractive alternative to immunoaffinity assays (DeGrasse 2012).

The enterotoxin producing strains from food isolates were validated with aptamer system for the specific and sensitive detection of SEB. The aptamer system showed positive reactions only with SEB and no cross-reactivity was observed against other Staphylococcal enterotoxins. Further, the aptamer specificity was tested on SEB producing standard cultures of *S. aureus* and exhibited positive reactions in dot-ALISA. In this study, Staphylococcal enterotoxins was detected by PCR and aptamer and the results were reliable in both methods. However, aptamer based detection was more sensitive, thermally stable, and cost-effective compared to that of PCR assays and also aptamers could be efficiently developed to molecules for which there are no available antibodies.

Conclusion

The present study revealed the prevalence of MDR and SEB serotype *S. aureus* in RTE bakery foods from southern part of India. The data concludes that RTE bakery foods perform a crucial role in spreading MDR *S. aureus* strains. Moreover, a total of 14% *S. aureus* isolates were found capable to produce SEB and, which is a potential health risk to consumers. The number of samples and time for data collection is limited. However, future studies that involves larger number of RTE bakery food products from all over India should be considered to address the prevalence of *S. aureus.* The study concluded that both PCR and ALISA were equally efficient in identifying the pathogen and its toxin. Due to its ease of access, rapidity and cost-effectiveness in-house developed ALISA method could be an alternative detection method for Staphylococcal enterotoxins from food sources. The present study further warrants the policymakers and other regulatory agencies to maintain hygienic practices and microbiological safety of the food and food products intended for human consumption.

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