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FOOD MICROBIOLOGY - RESEARCH PAPER

Optimization and evaluation of a multiplex PCR assay for detection of *Staphylococcus aureus* and its major virulence genes for assessing food safety

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Abstract

Staphylococcus aureus is a natural commensal microflora of humans which causes opportunistic infections due to its large arsenal of exotoxins, invasion, immune evasion, and antibiotic resistance mechanisms. The primary goal of this study is to develop a multiplex PCR (mPCR) assay for simultaneous detection of *Staphylococcus aureus* (*nuc*) and its virulence genes coding for prominent exotoxins namely alpha hemolysin (*hla*), enterotoxins A (*sea*), enterotoxin B (*seb*), toxic shock syndrome toxin (*tsst-1*), and the gene coding for methicillin resistance (*mecA*). A competitive internal amplification control (IAC) was included in the assay to exclude the false negative outcomes. Highly specific primer pairs were designed for the target genes using in silico resources. At the outset, monoplex PCRs were standardized using reference *S. aureus* strains. Primer specificity to the target genes was authenticated through restriction digestion analysis of amplified PCR products. Multiplex PCR was optimized in increments of one gene starting with *nuc* and IAC amplified simultaneously using one pair of primers (*nuc*) in a competitive manner. The mPCR assay was found to be highly sensitive with a detection limit of ~10 CFUs per reaction for pure cultures. Multiplex PCR assay was further evaluated on the retail and processed food samples to test the prevalence of *S. aureus* and study their exotoxin profiles. Of the 57 samples examined, 13 samples (22.80%) were found to be contaminated with *S. aureus* whose DNA was extracted after a 6-h enrichment period. Among these, a high percentage of hemolytic and enterotoxin A positive strains were encountered. The mPCR assay developed in this study would be a useful tool for rapid and reliable monitoring of *S. aureus* for food quality testing and from clinical infections.

Keywords Staphylococcal enterotoxin A · Staphylococcal enterotoxin B · Alpha hemolysin · Toxic shock syndrome toxin · Methicillin resistance · Food quality testing

Introduction

Staphylococcus aureus is an opportunistic pathogen associated with several clinical manifestations such as skin and soft tissue infections, and food poisoning to life-threatening complications such as pneumoniae, endocarditis, osteomyelitis, and toxic shock syndrome [1]. *S. aureus* is responsible for nosocomial and community-acquired infections [2]. WHO has categorized *S. aureus* as one of the 6 priority nosocomial pathogens (ESKAPE) of international concern to public health based on their multidrug resistance phenotype and virulence mechanisms [3]. Contamination of food with *S. aureus* followed by secretion of enterotoxins is responsible for staphylococcal food poisoning responsible for several food poisoning outbreaks globally [4–7]. *S. aureus* has been reported from a wide variety of foods such as confectionery items, meat products, fruit juices, ready-to-eat salads, salad dressings, and dairy products [8]. *S. aureus* is capable of proliferating in a wide range of temperature, pH, and salt concentrations thereby enabling it to grow in wide variety of foods [9]. The presence of *S. aureus* in food is an indication of poor hygiene, and efficient detection

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strategies are crucial for detecting this microorganism [10]. Food handlers with poor hygiene and bare handling practices contaminate the food and disseminate the pathogen and thus pose a serious threat of staphylococcal food poisoning outbreaks [1, 11, 12]. In addition to this, contaminated food contact surfaces, gloves, cutting, slicing, or packaging tools could also be the sources of cross contamination [13].

Early and accurate detection of pathogenic microbes and microbial contaminants from food, environment, and clinical samples is crucial for mitigating losses in health and wealth [14]. Although conventional microbiological culture-based methods are considered gold standard, they require nonselective enrichment followed by selective enrichment, plethora of biochemical or serological procedures which consumes > 48 h for the definitive result [15]. Demand for rapid, high-resolution and low-cost diagnostic procedures led to detection systems that have significantly shortened final outcome. Polymerase chain reaction (PCR) is the most widely accepted molecular tool for pathogens and disease diagnosis. PCR revolutionized diagnostics and allowed definite microbial identification down to species and strain levels [16]. Multiplex PCR allows simultaneous amplification of two or more targets in a single reaction which made possible to investigate large number of samples in short duration. Multiplex PCR format is an attractive choice which combines benefit of detecting multiple genes with high specificity and greater taxonomic resolution. Proper controls are crucial in any diagnostic procedures. In order to address the false negatives, inclusion of an internal amplification control in all diagnostic PCR is mandatory [17].

S. aureus harbors a variety of secretory virulence factors such as alpha-hemolysin (HLA), staphylococcal enterotoxins (SEA, SEB etc.) and toxic shock syndrome toxin (TSST-1) which accounts for its severe pathogenesis. Alpha hemolysin is a 33-kDa pore-forming exotoxin secreted by most *S. aureus* strains and is associated with the hemolytic, necrotic, and cytolytic properties in addition to being a major cause for pneumonia [18]. Staphylococcal enterotoxins (SEs) belong to a large family of staphylococcal and streptococcal pyrogenic toxin superantigens. SEs are heat stable and potent gastrointestinal toxins and superantigens responsible for non-specific T cell proliferation resulting in toxic shock [19, 20]. SEA and SEB are the major enterotoxins produced by *S. aureus* among variety of enterotoxins secreted by this microorganism. They are also heat stable gastrointestinal toxins and most potent superantigens produced by *S. aureus* implicated in staphylococcal food-poisoning outbreaks [21]. TSST-1 is an important exotoxin of *S. aureus* which causes systemic illness called toxic shock syndrome (TSS) [20, 22]. TSST-1 stimulates CD4+ T cells, macrophages, and dendritic cells to release large amounts of cytokines (IL-1β, TNF-α, IL-12, IFN-γ, IL-6) which will progress to systemic toxic shock response and multiorgan failure [23, 24]. Methicillin resistance in *S. aureus* is primarily mediated by production of PBP2a, an altered penicillin binding protein with low affinities for β-lactam antibiotics. *mec*A gene is the genetic determinant that codes for PBP2a, and hence the presence of *mec*A gene is considered a useful marker for detecting methicillin resistance [25, 26]. Rapid detection of food pathogens and their virulence genes from food or clinical sources is crucial in reducing the economic and health losses.

The aim of this study is to develop a multiplex PCR for simultaneous detection of some of the main virulence-associated genes of *S. aureus* namely *sea*, *seb*, *tsst-1*, *hla*, and *mec*A genes. An internal amplification control (IAC) was included to rule out false negatives. To verify the mPCR results, the outcome was correlated with phenotypic confirmation by Western blot analysis of exotoxins or Kirby-Bauer disk diffusion assay for testing antibiotic resistance. The mPCR was evaluated for its specificity and sensitivity of detecting *S. aureus* and its associated virulence factors from pure cultures. The primary objective of this study was tested by application of this assay on natural food samples. The percentage incidence of *S. aureus* and its virulence genes from food samples were determined by mPCR.

Materials and methods

Materials

Dehydrated microbial culture media used in the study was purchased from HiMedia laboratories, Mumbai, India. *Taq* DNA polymerase and dNTPs were purchased from Sigma-Aldrich, USA. Oligonucleotide primers were synthesized at Eurofins facility, Bangalore, India. FastDigest Restriction enzymes were from Thermo Scientific, India. All the other chemicals and reagents were purchased from other sources are presented at their place of mention.

Bacterial strains and growth conditions

A total of 26 reference strains were used in this study. Prior to experimentation, these strains were screened through the appropriate biochemical tests. Pure cultures of these strains were maintained on BHI agar plates for short-term use and as soft agar stabs (4°C) and 15% glycerol stocks (−80°C) for long term storage.

Primer design and restriction mapping

Highly specific oligonucleotide primers were designed for the simultaneous detection of *nuc*, *hla*, *sea*, *seb*, *tsst*-1, and *mec*A genes of *S. aureus* (Table 1). Primers were designed with the help of Gene Runner (version 6.5) and Primer3Plus

Table 1 List of oligonucleotide primer sequences, expected size of PCR products of genes targeted in multiplex PCR

Gene target	Primer sequence (5′–3′)	Product size (bp)	Accession no.
sea	F-GTTATCAATGTGCGGGTG R-CGTCTAGCCATAAATTGATC	120	M18970
seb	F-TGTATGGTGGTGTAACTGAGC R-TGCAGGCATCATGTCATAC	269	M11118
nuc	F-GCTGGCATATGTATGGCA R-GCTTCAGGACCATATTTCTC	389	CP000046
tsst-1	F-CCCTGTTCCCTTATCATCT R-TCCATGTATTTGAGTTAGCT	472	GG730292.1
hla	F-GCGAAGAAGGTGCTAACA R-CAATTGGTAATCATCACGAAC	569	X01645.1
mecA	F-TCCAGGAATGCAGAAAGAC R-CTGGTGAAATTGTAATCTGGA	806	GU227428.1
IAC_pUC19	F-*GCTGGCATATGTATGGCAGGTATC TGCGCTCTGCTG R-*GCTTCAGGACCATATTTCTCGCC ATACCAAACGACGAG	700	L09137.2

*Base sequence highlighted in black indicate *nuc* gene primer sequences. The IAC generated will have *nuc* primer sequence at the termini. Therefore, *nuc* gene primers alone can be used to amplify IAC and *nuc* gene products competitively in a single PCR reaction

(https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). In silico specificity of the primers was determined through the NCBI primer BLAST program. The primers were designed with similar annealing temperatures for simultaneous amplification of all genes in a single PCR reaction. Amplicon sequences of the *nuc*, *hla*, *sea*, *seb*, *tsst*-1, and *mec*A genes were used for in silico restriction mapping to identify the specific restriction markers in the sequences using NEBcutter tool. Amplicon sequences of *nuc*, *hla*, *sea*, *seb*, *tsst*-1, and *mec*A genes were submitted to Gene Runner software (Ver 6.5) and the appropriate restriction sites and the fragment size after restriction were identified for the assessing the authenticity of the developed mPCR assay.

Genomic DNA preparation

Genomic DNA was isolated from all the cultures either by phenol:chloroform:isoamyl alcohol (25:24:1) [27] or boiling lysis methods [28]. Firstly, *S. aureus* (Table 2) and other bacterial strains (Table 3) were grown in BHI

Table 2 List of standard strains used in mPCR study

S. aureus standard strains	*Sea*		*Seb*		*Nuc*		*Tsst*	*Hly*		*mecA*	
	mPCR	WB	mPCR	WB	mPCR	BT	mPCR	mPCR	WB	mPCR	ABST
ATCC 700699	+	+	−	−	+	+	+	+	−	+	R
ATCC 43300	−	−	+	+	+	+	−	+	−	+	R
ATCC 6538p	−	−	−	−	+	+	−	+	+	−	S
ATCC 29213	+	+	+	+	+	+	+	+	+	+	R
NCIM 2079	−	−	+	−	+	+	−	+	+	−	S
NCIM 2492	−	−	+	−	+	+	−	+	+	−	S
NCIM 2122	−	−	+	−	+	+	−	+	+	−	S
NCIM 2654	−	−	+	−	+	+	−	+	+	−	S
NCIM 2794	−	−	+	+	+	+	−	+	+	−	S
FRI 722	−	−	+	+	+	+	−	+	+	+	I
NCIM 2657	−	−	+	+	+	+	−	+	+	−	S
IVRI	−	−	+	+	+	+	−	+	+	−	S
NCIM 2120	−	−	+	+	+	+	−	+	+	−	S
NCIM 2127	−	−	+	+	+	+	−	+	+	−	S

WB Western blotting, *BT* biochemical testing, *ABST* antibiotic sensitivity testing, (+) positive, (−) negative, *ATCC* American Type Culture Collection, *NCIM* National Collection of Industrial Organisms, *FRI* Food Research Institute, *IVRI* Indian Veterinary Research Institute, *R* resistant, *S* sensitive, *I* intermediate

Table 3 List of non-*Staphylococcus aureus* strains tested by multiplex PCR

S. no.	Organism name	Strain	*sea*	*seb*	*nuc*	*tsst*	*hla*	*mecA*	IAC
1.	*Staphylococcus epidermidis*	ATCC 13518	–	–	–	–	–	–	+
2.	*Staphylococcus haemolyticus*	ATCC 29970	–	–	–	–	–	–	+
3.	*Listeria monocytogenes*	ATCC 19115	–	–	–	–	–	–	+
4.	*Escherichia coli*	ATCC 10536	–	–	–	–	–	–	+
5.	*Streptococcus pyogenes*	ATCC 21059	–	–	–	–	–	–	+
6.	*Salmonella typhimurium*	ATCC 14028	–	–	–	–	–	–	+
7.	*Bacillus cereus*	ATCC 10876	–	–	–	–	–	–	+
8.	*Salmonella paratyphi*	ATCC 9150	–	–	–	–	–	–	+
9.	*Shigella flexneri*	ATCC 9199	–	–	–	–	–	–	+
10.	*Shigella boydii*	ATCC 9207	–	–	–	–	–	–	+
11.	*Klebsiella pneumoniae*	ATCC 10031	–	–	–	–	–	–	+
12.	*Proteus vulgaris*	ATCC 13315	–	–	–	–	–	–	+

ATCC America type culture collection, (+) positive, (−) negative

broth at 37°C overnight. Overnight cultures were allowed for centrifugation at 10,000 *g* for 10 min. Genomic DNA was isolated from 1.5 mL of the harvested cells using phenol:chloroform:isoamyl alcohol (25:24:1) method. For cell lysis, 10 µl of 5 mg mL^{-1} lysostaphin (Sigma Aldrich) was used for *Staphylococcus* cultures and 10 µl of 20 mg mL^{-1} Lysozyme (HiMedia, India) for non-staphylococcal bacterial strains. DNA was dissolved in 100 µl of 10 mM Tris-Cl, pH 8.0, and quantified using Nanodrop UV-VIS spectrophotometer (Thermo Scientific, USA). For boil lysis method, overnight cultures were allowed for centrifugation at 10,000 *g* for 10 min and cell pellet was resuspended in sterile double-distilled water. Cell pellet was subjected for boil lysis in a dry bath at 100 °C for 2 min followed by immediate freezing at −80 °C. This procedure is repeated two more times. Immediately, the suspension was centrifuged at 10,000 rpm for 10 min and supernatant containing the genomic DNA was used for the PCR experiments. Genomic DNA isolated by phenol:chloroform:isoamyl alcohol method was used for mPCR standardization. DNA isolated by freezing-boiling method was used for evaluation of mPCR. Final concentration of the genomic DNA was adjusted to ~50 ng μl^{-1} for PCR experiments.

Standardization of monoplex PCRs

Initially, monoplex PCRs were standardized using annealing temperature as variable parameter in a final reaction volume of 20 µl containing 1× PCR buffer, 100 µM dNTPs, 1.5 mM $MgCl_2$, 5 pmol each of forward and reverse primers (Table 1), 10–25 ng of DNA template, and 1 U of *Taq* DNA polymerase (Sigma, India). All the reactions were performed in Eppendorf Master cycler gradient (Germany) thermal cycler with an initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 52–58°C for 30 s, extension at 72°C for 1 min and a final extension at 72°C for 5 min. Each round of PCR was always performed with a negative control without DNA to rule out carry over contamination. PCR products were visualized on 1.5% agarose gel prepared using 0.5× TBE containing 0.5 µg/mL of ethidium bromide. After solidification, agarose gels were placed in horizontal electrophoresis unit (Bangalore Genie, India), and PCR products were mixed with 6× DNA loading dye and loaded into the wells. PCR products were resolved at 100 V and the stained gels were visualized under UV transilluminator and the images were captured using SynGene gel documentation system.

Restriction profile analysis

To authenticate the primer pairs, the gel-purified amplicons of *hla*, *sea*, *seb*, *tsst*-1, *nuc*, and *mec*A genes were subjected to restriction digestion analysis. Restriction digestion was carried out using FastDigest enzymes (Thermo Scientific) (Table 4) at 37°C as recommended by manufacturer. Appropriate restriction enzymes determined during in silico restriction mapping experiment were used for the restriction profile analysis of amplicons. Digested products were electrophoresed on 2% agarose gel and documented with GeneSys gel documentation system. Restriction enzymes

Table 4 Restriction mapping of amplified PCR products

Gene	Amplicon size	Restriction enzyme	Observed band sizes
sea	120	RsaI	20, 100
seb	269	RsaI	103, 166
nuc	389	AluI	146, 243
tsst	472	HaeI	18, 35, 419
hla	569	AluI	35, 161, 373
mecA	806	AluI	472, 334

used for restriction profile analysis and their expected band sizes are given in Table 4.

Internal amplification control

IAC was designed and prepared using pUC19 plasmid as template and pUC19 primers. pUC19 primers were flanked by *nuc* gene primers at 5′ ends. Internal amplification control DNA was prepared using PCR program with an initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension of 1 min at 72°C followed by a final extension of 5 min at 72°C with 5 pmol each of forward and reverse pUC-19_IAC primers (Table 1). PCR-amplified IAC was prepared in bulk and the entire product was resolved on agarose gel. The separated IAC was excised from agarose gel cleaned by PCR/Gel cleanup kits (Macherey-Nagel, India). Minimum quantity of above IAC template required for the amplification of both *nuc* gene and IAC template competitively using *nuc* primer pair was determined and subsequently used in all PCR reactions.

Standardization of multiplex PCR

Multiplex PCR conditions were standardized at an annealing temperature of 58 °C. The PCR reaction was standardized in a final volume of 40 µl with 1× PCR buffer, 300 µM dNTPs, approximately 10–25 ng of template, and 2 U of *Taq* polymerase at an initial denaturation at 94°C for 4 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing in the gradient temperatures of 54 °C to 60 °C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. For eliminating false negatives, 0.01 ng of IAC DNA carrying *nuc* gene primer overhangs was added to each PCR reaction. Primers and $MgCl_2$ were used in varying concentrations during standardization to determine ideal concentration for sufficient amplification of all six genes and IAC. The PCR products were run on 1.5% agarose gel as described earlier and the results were documented using Syngene gel documentation system.

Phenotypic evaluation of mPCR

Thermonuclease activity which is a characteristic feature of *S. aureus* was determined biochemically on DNase test agar with toludine blue. A zone of clearing or halo around the colonies is taken as indication of thermonuclease activity. Antibiotic sensitivity for determining methicillin resistance was done by Kirby-Bauer disk diffusion test using Oxacillin disks [29]. The secretion of alpha hemolysin, SEA, and SEB by *S. aureus* strains in the culture supernatants was determined by Western blot analysis using in-house-raised mouse antibodies. Exotoxins in culture supernatants were concentrated 10-fold using methanol-chloroform precipitation method. Concentrated samples were separated on 12% SDS-PAGE analysis followed by Western blotting [30] on to nitrocellulose membrane (Pall Life sciences, USA). Blots were treated with primary antibodies against α-hemolysin, SEA, and SEB followed by probing with HRP-conjugated secondary antibodies. The blots were developed with 0.1 mg/mL diaminobenzidine tetrachloride (Sigma) and 0.03% H_2O_2.

Sensitivity and specificity of mPCR

Sensitivity and specificity of the mPCR assay was assessed using staphylococcal and non-staphylococcal cultures listed in Tables 2 and 3. Overnight cells of the cultures were serially diluted up to 10^{-10} dilutions in saline (0.8% NaCl). One milliliter of the culture dilution was plated on plate count agar by pour plate method to determine the CFU count of the dilutions. Similarly, 1 mL of all dilutions was processed for mPCR assay by centrifuging at 10,000 *g* for 10 min. Cell pellets were suspended in 30 µl of sterile ddH_2O and lysed the cells by boiling lysis method. The supernatant containing DNA was collected after centrifugation and the entire supernatant containing DNA was used in mPCR reaction as described above. The least dilution of CFU at which the PCR amplification occurred was considered limit of detection of the mPCR assay. The specificity of primer pairs was tested with genomic DNA from other staphylococcal and non-staphylococcal species.

Artificial spiking of *S. aureus* in food samples

The mPCR was tested and evaluated on to artificially spiked food samples according to a method followed by Kota et al. [31] with slight modifications. Briefly, the procedure involves testing three sets each of raw chicken meat, cake samples, and boiled rice samples which were macerated with sterile BHI broth and boiled for 10 min to kill the residual bacterial cells. Samples were cooled and 10-fold serially diluted *S. aureus* culture was inoculated in to the macerated sample for enrichment and incubated at 37°C for 6 h. After enrichment, 1 mL of enriched sample was collected from different dilutions and centrifuged at 1500 *g* for 30 s. The purpose of this low RCF spinning is to sediment the food particles or debris from the food matrix post enrichment. The supernatant obtained from the above step was subjected to second spinning at 10,000 *g* for 5 min to sediment the bacterial cells enriched from the food sample. DNA was extracted from the cells by boiling lysis method and subjected to mPCR as described earlier. Direct DNA extraction from enriched samples without removing the particulates might interfere with the PCR outcome.

Evaluation of mPCR on natural samples

Different retail food samples (milk, food from street vendors, chicken, eggs from local stores, and cake/pastries from local bakeries) were collected in and around Guntur and Tenali towns of Andhra Pradesh, India. Sampling data is tabulated in Table 5. Food samples were collected aseptically in an air-tight screw-capped sterile plastic bottles (HiMedia) and were carefully transported to laboratory immediately for sample processing and mPCR analysis. Samples were evaluated by mPCR with and without enrichment. Enrichment and evaluation of natural samples was carried out as per protocol of Kota et al. [31] described in the earlier method. Briefly, 1 g or 1 mL of sample was macerated in 9 mL BHI broth and the entire content was added to 90 mL broth. After 6 h of enrichment, 1 mL of enriched sample was collected from each sample, clarified by centrifugation at 1500 *g* for 30 s. The supernatant was again centrifuged at 10,000 *g* for 5 min to harvest the cells for mPCR. The supernatant was discarded and the pellet containing *S. aurues* was dissolved in 30 µl of sterile double-distilled water, DNA harvested by boil lysis methods, and the entire content was used in mPCR assay.

Results

Primer design and restriction mapping

Utmost care was taken during primer designing such that a minimum of 70–100 bp gap was maintained between successive amplicon. Primer properties such as hair-pin loops, dimers, GC content, and Tm values of the *sea*, *seb*, *tsst*-1, *nuc*, *mecA*, and *hla* genes were evaluated with Gene Runner software (Ver 6.5.52). All the designed primers have shown optimal GC content (40–60%) and Tm values (56–60 °C) without the chances of loop formation, and self- and cross-dimerization. Primer pairs were identified to be highly specific to target sequences through BLAST analysis with none or very little specificity to unintended targets. Expected PCR amplicon sizes are provided in Table 1. The restriction enzyme sites located within the amplicons and the estimated restricted fragment sizes are represented in Table 4.

Standardization of monoplex PCRs

The monoplex PCRs were standardized using different annealing temperatures and the annealing temperature of 58 °C gave optimum amplification products for all genes at the expected regions (Table 1, Fig. 1), while no amplification products were detected in negative controls. Thermonuclease (*nuc*) gene amplification was used for specific identification of *S. aureus*. The *nuc* gene–amplified products were observed in all *S. aureus* isolates tested, but the same primers did not show any amplicons when tested with the genomic DNA isolated from non-*S. aureus* cultures.

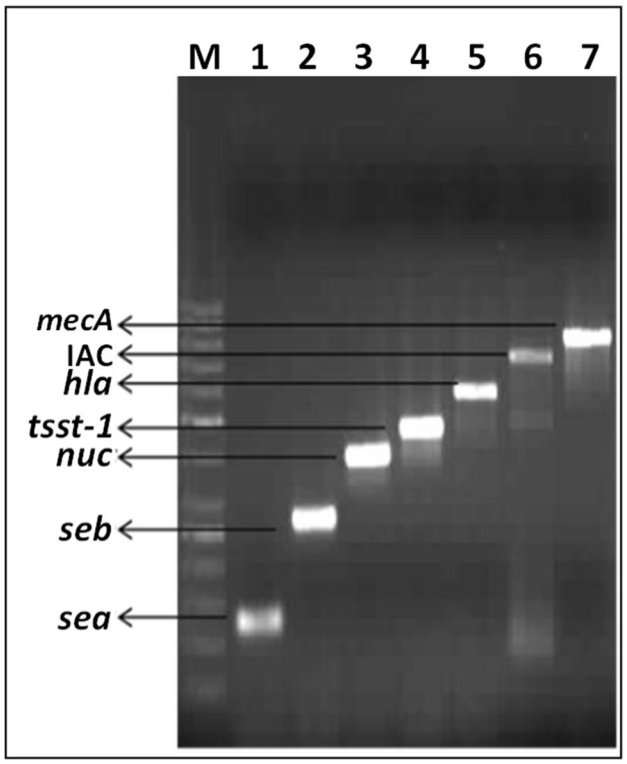

Fig. 1 Standardization of monoplex PCR. Agarose gel showing individual PCR amplified products of monoplex PCRs after standardization. Lane M, 50 bp ladder; lane 1, *sea* gene; lane 2, *seb* gene; lane 3, *nuc* gene; lane 4, *tsst*-1 gene; lane 5, *hly* gene; lane 6, IAC; lane 7, *mec*A gene

Table 5 Evaluation of mPCR on natural food samples

Sample	Source	No. of samples	Multiplex PCR result					
			nuc	*sea*	*seb*	*hla*	*tsst*	*mec*A
Milk	Local stores	8	2	0	0	2	0	1
Chutney	Street vendors	10	2	0	0	2	0	0
Eggs	Local stores	6	1	0	0	0	0	0
Paanipuri water	Street vendors	13	2	0	0	2	0	0
Pastries	Local bakeries	20	6	2	1	5	0	1
Total		57	13	2	1	11	0	2

Restriction analysis

After the in silico restriction mapping of the amplified sequences of *sea*, *seb*, *tsst*, *nuc*, *mecA*, and *hla* genes, restriction digestion was performed for the gel purified products. Upon restriction, bands of the digested fragments were resolved in 2% agarose gel and their band sizes were observed. The size of in silico restricted fragments and restriction enzyme cleaved fragments were in complete agreement with each other indicating the correct gene amplification by the designed PCR primers. The results of restriction digestion analysis for the purified PCR products of *sea*, *seb*, *tsst*, *nuc*, *mecA*, and *hla* genes are presented in Table 4 and Fig. 2.

Internal amplification control

IAC was included for ruling out false negative results which is mandatory for all detection PCRs [17]. A 700-bp-long PCR product was amplified from pUC19 vector with the help of pUC19 forward and reverse primers with *nuc* gene primer overhangs at the 5′ ends. Therefore, the *nuc* gene serves as IACs' competitive partner both competing for the *nuc* primers during amplification. The IAC product length was kept higher than the *nuc* gene product to provide competitive edge [17]. Initially, IAC and *nuc* gene were co-amplified using *nuc* primers to determine the minimum concentration necessary for the PCR reaction. A minimum of 0.01 ng of IAC was used in each PCR reaction so that it does not hinder the amplification of other genes.

Standardization of multiplex PCR

Multiplex PCR was standardized at annealing temperature of 58 °C by adjusting primer and $MgCl_2$ concentration using genomic DNA from standard *S. aureus* strains (Fig. 3). A concentration of 1.5 mM $MgCl_2$ gave better amplification of all six genes. The forward and reverse primers of each gene were used in following quantities: *sea*, 5 pmoles each; *seb*, 1 pmoles each; *tsst*, 2.5 pmoles each; *hla*, 2.5 pmoles each; and *mec*A, 10 pmoles each. mPCR was evaluated on to a total of 14 well characterized standard and isolated strains of *S. aureus*. The results of mPCR for standard reference *S. aureus* strains used in this study are given in Table 2. These virulence determinants spanning various classes of toxins were selected on the basis of their high prevalence, their

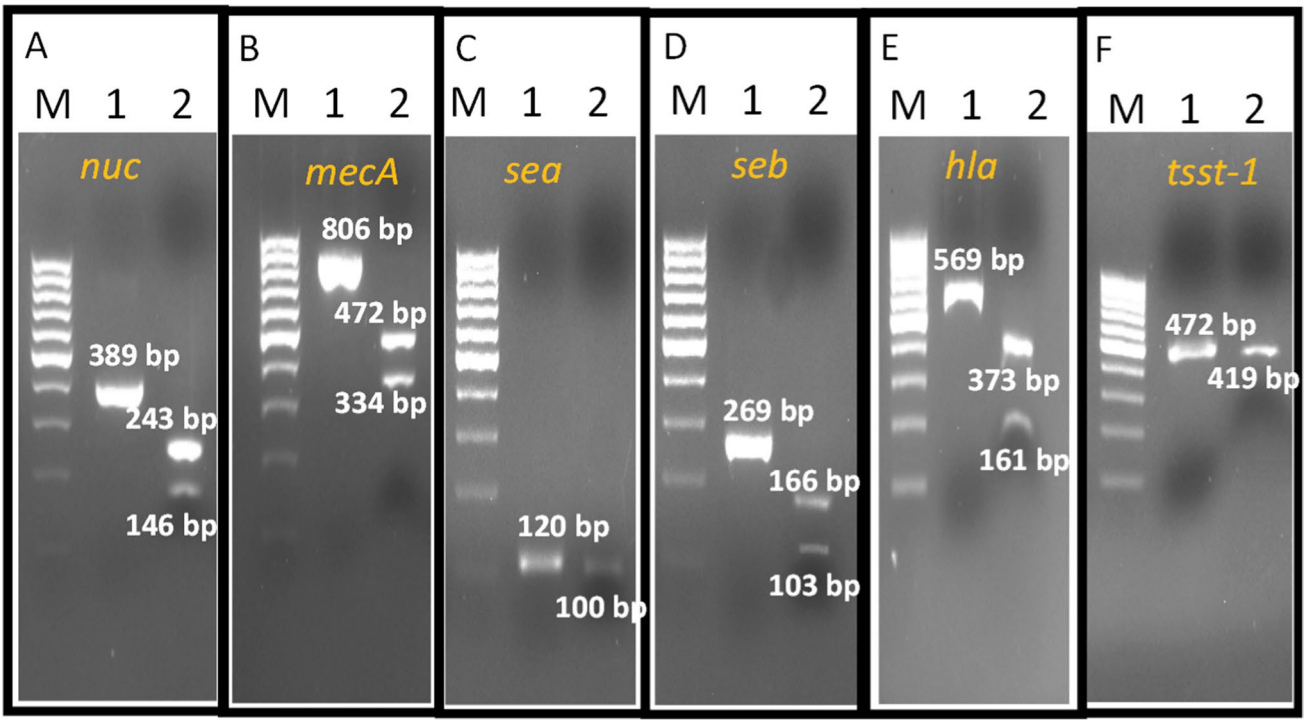

Fig. 2 Validation of monoplex PCR products through restriction digestion analysis. (**A**) *nuc* amplicon digested with AluI. Lane 1, 100 bp ladder; lane 2, *nuc* amplicon (389 bp); lane 3, *nuc*+AluI digestion (146, 243 bp). (**B**) *mec*A amplicon digested with AluI. Lane 1, 100 bp ladder; lane 2, *mec*A amplicon (806 bp); lane 3, *mec*A+AluI digestion (472 bp and 334 bp). (**C**) *sea* amplicon digested with RsaI. Lane 1, 100 bp ladder; lane 2, *sea* amplicon (120 bp); lane 3, *sea* +RsaI digestion (100 bp). (**D**) *seb* amplicon digested with RsaI. Lane 1, 100 bp ladder; lane 2, *seb* amplicon (269 bp); lane 3, *seb*+AluI digestion (103 bp and 166 bp). (**E**) *hla* amplicon digested with AluI. Lane 1, 100 bp ladder; lane 2, *hla* amplicon (569 bp); lane 3, *hla* +AluI digestion (161, 373). (**F**) *tsst*-1 amplicon digested with HaeI. Lane 1, 100 bp ladder; lane 2, *tsst*-1 amplicon (472 bp); lane 3, *tsst*-1+HaeI digestion (419 bp)

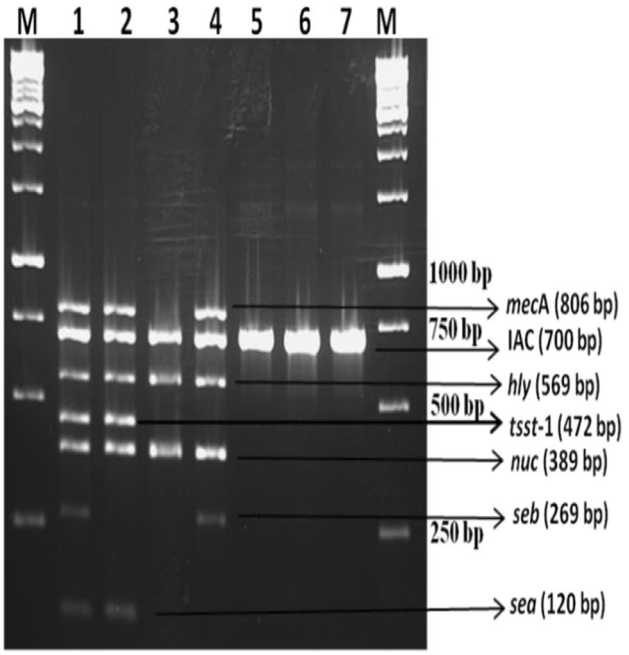

Fig. 3 Agarose gel showing PCR amplified products after mPCR standardization. Lane 1, 1 kb DNA ladder; lane 2, PCR products with genomic DNA of ATCC-700699 and FRI-722; lane 3, PCR amplified products with ATCC-700699; lane 4, amplified products with ATCC-6538p; lane 5, with FRI-722; lanes 6–9, negative controls; lane 10, 1 kb DNA ladder

role in invasive infections, staphylococcal food poisoning, and toxic shock syndrome cases.

Phenotypic evaluation of mPCR results

All *S. aureus* strains were found positive for *nuc* gene by PCR. They were further tested for thermonuclease activity on DNase test agar plates with toluidine blue. A 100% correlation was observed with PCR and biochemical test result for nuclease activity. The PCR results for *sea*, *seb*, and *hly* were evaluated by testing the presence of these toxins in the culture supernatants by Western blot analysis. Results of mPCR, Western blotting, biochemical tests, and antibiotic diffusion testing for standard strains are given in Table 2 and Fig. 4. When the mPCR was tested against 37 *S. aureus* isolates from clinical sources, 29 strains were positive for alpha hemolysin gene, of which alpha toxin was detected in culture supernatants of 25 strains. A total of 8 strains were positive for *sea* gene and SEA toxin by multiplex PCR and Western blot analysis respectively. Six strains positive for *seb* were recovered by PCR of which all 6 were tested for presence of SEB toxin in culture supernatants. The *tsst*-1 gene was present in one strain. A high amount of non-specific reaction was observed in all blots due to certain immunoglobulin proteins of *S. aureus* namely staphylococcal protein A and SBI proteins that react nonspecifically with HRP conjugated antibodies. A total of 13 strains were positive for *mecA* gene and when these were tested for oxacillin resistance, they showed intermediate to high resistance by Kirby-Bauer disk diffusion testing.

Sensitivity, specificity testing, and spiking studies

The mPCR assay was highly sensitive showing reproducible amplification with minimum 10 CFUs per PCR reaction. The reason for high sensitivity (10 CFUs) could be that the sensitivity and specificity were tested with pure cultures diluted in saline. Primer pairs used in this study were highly specific and they did not show any amplification with closely related bacterial species (Table 3). No amplification products were observed when genomic DNA of non-specific cultures was used with mPCR conditions except IAC.

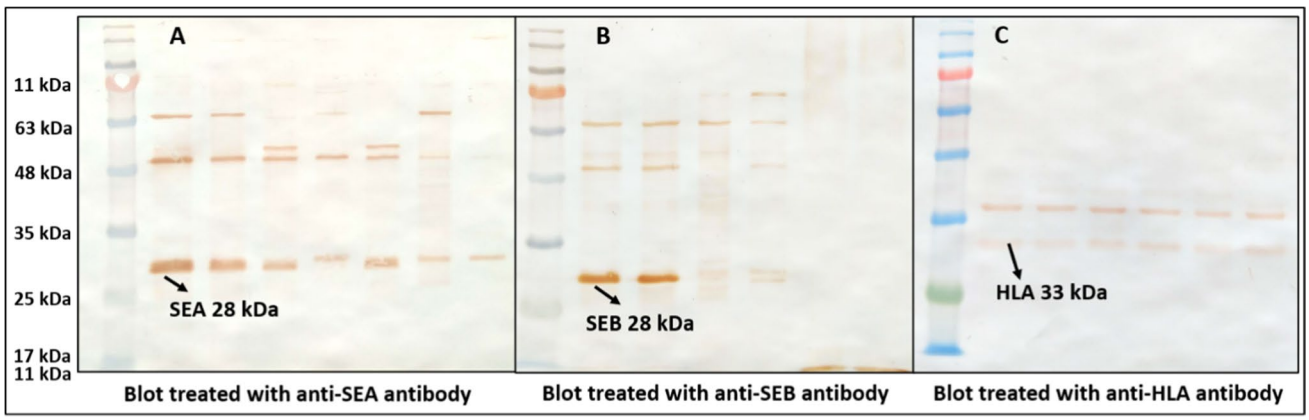

Fig. 4 Phenotypic evaluation of mPCR outcome by Western blot analysis. Western blot analysis was carried out for 3 exotoxins namely SEA, SEB, and HLA toxins. Panel A shows SEA toxin showing chromogenic reaction at 28 kDa. Panel B shows SEB toxin reactive at 28 kDa. Panel C shows Hla toxin showing reaction at 33 kDa

The robustness and sensitivity of mPCR was evaluated on to artificially spiked food samples. Multiplex PCR showed high detection sensitivity with initial count of ~10^2 CFU g^{-1} in chicken, cake samples and ~10–10^2 CFU mL^{-1} in the case of boiled rice samples and milk after an enrichment of 6 h. The CFU count reported for artificial spiking study is the initial CFU count added to the sample which would have increased exponentially post enrichment. To avoid low counts and contaminants from food samples, a 6-h enrichment was suggested.

Evaluation of mPCR on natural samples

Outcomes of the evaluation of mPCR on the food samples are represented in Table 5. The present mPCR assay was able to detect the presence of *S. aureus* in 13 (22.80%) samples out of 57 food samples of 5 different types each collected from different source or vendor. Out of the 13 food samples detected as positive for *S. aureus*, one each from milk and pastries obtained from the local stores were identified as methicillin-resistant *S. aureus*. Among the 13 positive samples, majority of them (11 samples) were detected as positive for alpha hemolysin. Only one sample each from the poultry egg and pastries did not show the presence of alpha hemolysin. The *tsst*-1 gene was absent in all the 13 positive samples, whereas 2 and 1 samples of pastries obtained from the local bakeries were positive for staphylococcal enterotoxin A and B genes respectively.

Discussion

Increasing demand for rapid detection and identification procedures for pathogens and their virulence components to understand prognosis of disease has led to the development of various systems that have considerably reduced the analysis time as compared to conventional culture techniques. For *S. aureus*, methods based on biochemical and immunoassays (latex agglutination tests) are either tedious or less sensitive or may require elaborate sample preparation steps [32]. DNA-based methods on the other hand are rapid and highly sensitive and can have universal applications [33]. Rapid systems based on conventional PCRs and RT-PCRs have been developed for detection of *S. aureus*, and its associated toxin genes and antibiotic resistance genes. Several multiplex PCR assays were reported to detect the *S. aureus* along with other common foodborne pathogens. A recent study reports the development of a multiplex PCR for simultaneous detection of four foodborne pathogens viz., *Escherichia coli*, *Listeria monocytogenes*, *S. aureus*, and *Salmonella enterica* from ready-to-eat food (15). Out of the four pathogens, two are Gram positives and their side effects are due to the exotoxins released into the food by them. This study does not report the toxigenic status of pathogens. Further, the IAC is not included in the assay which is mandatory for diagnostic PCRs. In another report, a multiplex PCR assay was reported which can detect 10 staphylococcal species from bovine mastitis samples [34]. The study claims that this assay can be applied to detect staphylococcal species from human origin. However, for food and clinical applications, this assay is not suitable since it does not provide any information of the virulence status of the staphylococcal species. A triplex PCR was developed for simultaneous detection of *Bacillus cereus* and *S. aureus* using teicoplanin antibiotic functionalized magnetic beads to capture pathogen from the samples. This study does not report the enterotoxin status of the *S. aureus* which is crucial to understand its outcome in food poisoning cases [35]. In a different study, a multiplex PCR was designed for simultaneous detection of five foodborne pathogens namely, *S. aureus*, *L. monocytogenes*, *Shigella flexneri*, *Yersinia enterocolitica*, and *Clostridium difficile* [36] and applied this for meat quality testing. The potential of Gram-positive pathogens in causing food intoxications through enterotoxins is not revealed from this system. A study from Egypt reported a multiplex PCR to detect enterotoxigenic *S. aureus* from bovine and human milk samples [37]. Though this study is promising in reporting enterotoxin status of *S. aureus* from milk samples, the lack of an internal control could compromise the false negative results. Another multiplex PCR which is closely related the present study was reported which detects 5 *S. aureus* enterotoxin genes, *tsst*-1, and methicillin resistance along with an IAC [38]. Though this study is useful for food applications, the target gene chosen for identifying *S. aureus* is coagulase gene which could be found in 11 other staphylococcal species thus prone to misleading conclusions. In our assay, the thermonuclease gene was targeted which is the most promising marker for specific identification of *S. aureus* unambiguously [35–37]. Also, this study mPCR assay utilizes an IAC which is very important in PCR-based molecular assays to rule out the false negatives.

In general, food processing steps would have injured or weakened the microorganisms which regenerate and multiply upon finding suitable growth conditions [38]. Processing of food samples for mPCR without enrichment may yield a false negative outcome. Therefore, majority of studies recommend an enrichment step in general media or a selective media for bringing the bacterial cell count to detectable levels [15]. Similarly, this study found a 6 h enrichment in general enrichment media suitable for most types of food samples. Further, the targeted genes matter most in any mPCR assay. Keeping this in view, SEA and SEB which are most commonly encountered classical SEs in food SE intoxications are included as targets in this assay [39]. Majority of studies reported incidence of MRSA from food samples [40, 41]. These strains spread

toxigenic and antibiotic-resistant traits by horizontal transfer through staphylococcal cassette chromosome. Therefore, *mec*A responsible for methicillin resistance is incorporated in this assay. The presence of thermonuclease activity is the hallmark of all *S. aureus* strains [42]. Therefore, the *nuc* gene was included as a target. Alpha hemolysin gene is a prominent virulence marker and the amount of Hla toxin produced is correlated with the virulence of *S. aureus* strain. TSST-1 target included in this study is critical from clinical point of view and the toxin effects are typically manifested in menstruating women who are using tampons. Finally, an IAC was included which verify the quality of PCR reagents and thermal cycler. The assay was able to meet the sensitivity and specificity satisfactorily.

The oligonucleotide primers designed in the study were found to be highly specific through monoplex and multiplex PCR assay outcomes. No amplification was observed in the non-*S. aureus* cultures used in the study showing that the assay is highly reliable for its specificity. The developed mPCR assay could detect up to 10 CFU per reaction and therefore can be utilized for satisfactory detection of very low population of bacteria in the food and clinical samples. Direct evaluation of mPCR assay on the natural food samples may not give the detectable amplification because of the presence of low count of the pathogens or the presence of PCR inhibitory components. Therefore, dilution of natural food samples in sterile broth followed by enrichment for a period of 6 h will provide sufficient nutrients for the propagation of the bacterial population and dilution of PCR inhibitors. We observed that 6-h enrichment is sufficient for the detection of 10^1 to 10^2 CFU/mL initial *S. aureus* cells in the food samples. We claim that the multiplex PCR assay developed in the study is highly specific and sensitive and a reliable method for the detection of prevalence of MRSA and its associated toxins. This mPCR assay could be used in clinical and food microbiology laboratories as a rapid, definitive, and adjunct *S. aureus* detection system in addition to the routine microbial analysis.

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Author contribution P.N.R designed the study and planned experiments. S.N. and P.N.R performed the experiment and prepared figures and tables. H.B.K. and P.N.R. analyzed the data and wrote the manuscript.

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Declarations

Ethics approval Not applicable.

Conflict of interest The authors declare no competing interests.

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