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ORIGINAL ARTICLE

Thermostabilization of indigenous multiplex polymerase chain reaction reagents for detection of enterotoxigenic *Staphylococcus aureus*

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Stabilization;
Staphylococcus aureus

Abstract *Background/Purpose:* Among DNA-based techniques, polymerase chain reaction (PCR) is the most widely accepted molecular tool for the detection of pathogens. However, the technique involves several reagents and multiple pipetting steps that often lead to error-prone results. Additionally, the reagents entail a cold-chain facility to maintain their stability during storage and transportation. The main aim of the present study was to simplify the utility of a pre-optimized multiplex PCR format that was developed to detect toxigenic strains of *Staphylococcus aureus* by providing stable, pre-mixed, and ready-to-use master mix in a lyophilized formulation.
Methods: Master mix containing all reagents except the template was lyophilized in the presence of an excipient lyoprotectant to achieve long-term stability without altering the sensitivity, specificity and PCR performance. Bromophenol blue was also included in the master mix to reduce the risk of external contamination during gel loading. The stability of lyophilized master mix was analyzed at different temperatures. The PCR performance was also examined after exposure of master mix to notable temperature fluctuations during transportation.
Results: The shelf-life of lyophilized master mix was estimated to be 1.5 months at ambient temperature and 6 months at 4°C. Stability was unaffected by temperature fluctuations during transportation even in cold-chain-free conditions, thus reducing the cost required for cold storage.

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Conclusion: The sensitive, cost-effective, ready-to-use, and ambient temperature stable formulation could be implemented as a detection tool in food analysis and diagnostic laboratories and hospitals and for on-field application outside the laboratories, as well as for detection of toxigenic strains of *S. aureus*.

Introduction

Staphylococcus aureus is a normal part of human flora that often turns into a pathogen due to its toxins, invasiveness, and antibiotic resistance. Owing to its pathogenic potential, the organism is associated with clinical infections including septic shock, endocarditis, osteomyelitis, pneumonia, and bacteraemia.[1–3] Additionally, the food intoxication caused by staphylococcal enterotoxins (SEs) has become a global concern.[4] Timely detection of the organism and its virulence factors is essential to prevent health and safety issues; particularly in areas such as hospitals and food industries where failure or delayed detection may lead to serious consequences. Although culture-based methods are considered as a gold standard for specific detection and profiling of *S. aureus*, they require > 48 hours for result interpretation.[5,6] This delay in identification makes the disease prevention strategies expensive and often unsuccessful. In the past 20–30 years, polymerase chain reaction (PCR)-based methods have been widely exploited for rapid and accurate detection of *S. aureus*, among which, multiplex PCR formats targeting virulence-associated genes are more prevalent.[7–11]

In our earlier work, we reported a multiplex PCR-based system for detection of toxigenic strains of *S. aureus* from various sources.[12] The assay targeted five major enterotoxigenic genes: *sea*, *seb*, *sec*, *seg*, and *sei*; *coa* gene encoding the enzyme coagulase; *mec*A, which confers methicillin resistance; *tst*, which encodes toxic shock syndrome toxin, along with an IAC to rule out false-negative results. An IAC is a nontarget DNA sequence present in the same sample reaction tube which is coamplified simultaneously with the target sequence. The assay was found to be highly specific and efficient in detection of *S. aureus* besides providing information on toxin profile and methicillin-resistance status. However, field application of the developed multiplex PCR was challenging due to various factors reducing its utility as a regular diagnostic tool. PCR being a highly sensitive molecular technique necessitates a clean and contamination-free area for the reaction set-up to avert any false-positive results. Minor pipetting errors also alter the yield of PCR outcome since the reaction has a microvolume. In addition, amplification of multiple genes demands multiple primers that can contribute to error-prone pipetting and inconclusive results. Therefore, a skilled person is required for the reaction set-up. The most important prerequisite for PCR is a cold chain for storage and transportation, which is mandatory to maintain reagent stability.

To counteract the aforementioned complications, freeze-drying of PCR reagents have been reported and is being practiced,[13,14] which involves lyophilization of PCR master mix in the presence of a suitable lyoprotectant. In the case of reported freeze-dried PCR formats, only a limited number of target genes are involved. In the present study, the above-mentioned multiplex PCR format involving eight target genes for simultaneous detection of virulence factors of *S. aureus* was refined into a freeze-dried format. The primary objective was to develop a pre-dispensed, ready-to-use, room-temperature-stable lyophilized formulation and its evaluation for field application. The "wet" PCR reagents were lyophilized in the presence of suitable lyoprotectant that preserved the functionality of the dried reagents, and the resultant freeze-dried PCR master mix was assessed for its storage stability, sensitivity, specificity, transportation feasibility, and in-field deployment. Caution was taken to carefully choose lyoprotectant that was readily soluble in water and had no interference with the PCR amplifications. Our format also contained bromophenol blue to achieve contamination-free results. The assay was performed by a single step of reconstitution using PCR grade water and adding suspect DNA template before subjecting it to PCR, thus, removing the requirement for skilled personnel and multiple pipetting steps. The assay was further evaluated on naturally contaminated food samples and presumptive clinical isolates.

Methods

Bacterial strains and growth conditions

S. aureus reference strains ATCC 700699 and FRI-722 were used in the present study. All clinical isolates mentioned in the previous study[12] were also used. All bacterial strains were cryopreserved in 15% glycerol at −80°C and cultured overnight at 37°C using Brain Heart Infusion agar and modified Mannitol Salt Broth (HiMedia, Mumbai, India) containing sodium pyruvate (HiMedia) when required.

Genomic DNA, PCR reagents, and conditions

Genomic DNA (gDNA) of *S. aureus* strains was extracted by conventional phenol:chloroform method,[15] as well as by rapid boil-lysate technique[16] with slight modification. A single colony of each *S. aureus* reference strain from an agar plate was suspended in 200 μL sterile distilled water separately and vortexed vigorously. The cells were lysed in a boiling water bath for 10 minutes followed by centrifugation at 10,000 *g* for 5 minutes to remove debris. The supernatant containing crude DNA was transferred into a fresh tube and used for PCR immediately or stored at −20°C for future use. The design and evaluation of

Table 1 List of genes targeted in the study with their corresponding primer sequences and amplicon size.

Gene	Primer (5′—3′)	Accession No.	Source	Amplicon size (bp)
sea	F — CTGTTCAGGAGTTGGATCTTC R — CTTGAGCACCAAATAAATCG	L22566.1	This study	156
coa	F — CGTTACAAGGTGAAATCGTT R — CCATATTGAGAAGCTTCTGTTG	X16457.1	This study	247
sec	F — CCACTTTGATAATGGGAACTTAC R — GATTGGTCAAACTTATCGCCTGG	AB084256.1	Schmitz *et al*. 1998[26]	270
seb	F — CCAGATCCTAAACCAGATGAGTT R — GTTTTTCGTTTATCAGTTTGATG	AY750900	Shylaja *et al*. 2009[10]	326
sei	F — GGTGATATTGGTGTAGGTAA R — CATATTCTTTGCCTTTACCAG	AB060537.1	This study	451
seg	F — GGTTCATTGTCAAATAGACTG R — CTATTGTCGATTGTTACCTG	DQ778337.1	This study	520
tst	F — CCCTTTGTTGCTTGCGAC R — TGGATCCGTCATTCATTGTTA	AY074881.1	Shylaja *et al*. 2009[10]	569
IAC	F — CAATTCCACACAACATACGA R — CGGATAAGGCGCAGCG	U07164	This study	660
*mec*A	F — TCCAGGAATGCAGAAAGA R — CTGGTGAAGTTGTAATCTGGA	KC243783.1	This study	806

multiplex PCR has been reported previously.[12] The primer sequences and amplicon size of target genes is given in Table 1. No modifications were made in the concentration and volume of the PCR reagents and the amplification conditions. The final formulation containing all PCR reagents excluding the gDNA is termed as "PCR master mix: throughout the manuscript.

Selection of lyoprotectants and lyophilization

To the PCR master mix, 0.01% (w/v) bromophenol blue was added and subjected to lyophilization. In this process, glycerol (4.0%, v/v), trehalose (5.0%, w/v), and sorbitol (5.0%, v/v) were incorporated in separate PCR master mixes as lyoprotectants. Simultaneously, master mix without any lyoprotectant was also included in lyophilization process as a control to demonstrate the inevitability of the lyoprotectant. This formulation was frozen at −80°C in 0.5 mL Cliklok polypropylene micro-centrifuge tube (Tarsons Products, Kolkata, India) and lyophilized for 3—4 hours at 5.0^{-2} mBar pressure using a Heto vacuum concentrator (Thermo Scientific, Hudson, NH, USA). The vials were sealed and stored in sterile, waterproof and light-tight pouches with a desiccant at ambient temperature (25°C) for 7 days. To examine the stability of the PCR master mix upon lyophilization, PCR assay was performed. The lyophilized PCR master mix was reconstituted with 48 μL PCR-grade water (Sigma-Aldrich, St Louis, MO, USA), and mixed well using a pipette. After brief centrifugation at 1000 *g* for 1 min, the entire content was transferred to a 0.2-mL PCR tube (Thermo Scientific) and subjected to PCR amplification by adding 2 μL of boil-lysed gDNA of *S. aureus* ATCC 700699 and FRI 722 as a template. PCR without any template DNA served as a negative control. PCR mixture containing wet reagents was included in the experiment as a positive control. The amplicons were resolved under electrophoresis on 2.0% agarose (Seakem LE, FMC Bioproducts, Rockland, MA, USA) gel and documented using GelDoc system (Syngene, Cambridge, UK).

Storage performance and accelerated aging testing

The stability of the lyophilized PCR master mix was calculated by the Q_{10} method of accelerated aging technique. Accelerated aging estimates the shelf-life of a product by exposing it to aggravated conditions. A set of lyophilized PCR master mix vials was stored at 37°C for 1 month. The performance of lyophilized master mix was tested periodically at 7-day intervals by subjecting it to PCR using boil-lysed gDNA of the *S. aureus* reference strains. Simultaneously, PCR using wet reagents was performed at each interval for comparative analysis of the results. The longest duration that the thermostabilized PCR maintained its activity was calculated as described by Clark.[17]

Age of the thermostabilized PCR tubes = 0.5 months at 37°C
Ambient temperature = 25°C
Q_{10} = 1.8
Acceleration factor (based on 12°C temperature difference): $(1.8)^{1.2}$ = 2 .0
Length of time at elevated temperature = 0.5 months
Estimation of shelf life: Accelerated age = age × acceleration factor
0.5 months × 2.0 = 1.0 month
Shelf life = accelerated age + actual age
1.0 month + 0.5 months = 1.5 months.

In parallel, to compare the accelerated aging calculation with real performance, a set of freeze-dried master mix vials was stored at different temperatures for a period of 6 months. The storage temperatures used in this experiment included 4°C, 25°C, and 37°C and the PCR performance was analyzed as described above.

Determination of limit of detection

To determine the detection sensitivity of lyophilized master mix, PCR was performed using dilutions of purified as well as boil-lysed gDNA of *S. aureus* ATCC 700699 and FRI-722 as template DNA. Purified gDNAs were subjected to 10-fold dilutions ranging from 1 μg to 1 pg and boil-lysed gDNA dilutions were prepared by serially diluting overnight culture suspension of aforesaid *S. aureus* reference strains (McFarland standard concentration = 0.5) up to 10 CFU/mL of saline and further subjection to the boil-lysis technique. Each dilution of purified and boil-lysed gDNA was added to the lyophilized master mix separately and PCR was performed as mentioned in the previous section. In both cases, the lowest concentration showing visible amplification of all eight target genes along with the IAC was considered as the limit of detection. PCR mixture containing wet reagents was included in the experiment as a positive control. A negative control in which no gDNA was added to the lyophilized master mix was also included.

Cold-chain-free transportation assay

To ascertain the effect of temperature fluctuation on stability, the lyophilized PCR master mix containing the selected lyoprotectant stored at 4°C was transported to two different destinations in cold-chain free conditions and evaluated for performance. The lyophilized master mix stored at 4°C for 4 months was transported from Mysore to SDM College of Medical Sciences and Hospital, Dharwad (481 km) through a cold-chain-free postal service, and PCR was performed by technical personnel of the institute. Lyophilized master mix stored at 4°C for 6 months was transported from Mysore to Composite Food Laboratory (CFL) of Army Service Corps (ASC), Delhi (2300 km). PCR was performed as mentioned in the above section. Total duration of transportation and temperature fluctuations exposed on to the lyophilized vials were noted.

Probing on food samples and clinical isolates

To evaluate the field application of the freeze-dried PCR master mix, naturally contaminated samples, including milk (n = 16), ice-cream (n = 13), cake (n = 10), and pastries (n = 10), were pre-enriched with modified Mannitol Salt Broth containing sodium pyruvate, and template DNA was prepared as described previously.[12] Concurrently, *S. aureus* strains were isolated and biochemically confirmed from each pre-enriched sample. PCR was carried out with lyophilized PCR master mix using the same template DNA as mentioned in previous sections. Isolates of *S. aureus* (n = 91) from our laboratory repository were also included in this study. Reference strains of *S. aureus* were used as positive controls and reactions with no gDNA as negative controls.

Results

Excerption of desirable lyoprotectant

The amplification pattern of pre-optimized multiplex PCR format is depicted in Fig. 1. Excipients such as glycerol (4.0% v/v), trehalose (5.0% w/v), and sorbitol (5.0% v/v) were examined for their individual potential in preserving the stability of PCR reagents when incorporated in the pre-optimized PCR master mix. Each lyoprotectant containing freeze-dried PCR master mix was stored at 25°C for 7 days and evaluated for its performance. Gel electrophoresis of the resultant PCR is depicted in Fig. 2. In the case of glycerol, the lyophilized master mix stored showed no PCR amplification at all. However, simultaneous PCR with wet reagents resulted in successful amplification of all the target DNA fragments. In the case of sorbitol-containing PCR master mix, amplification of all nine fragments was recorded during the study period. However, the amplicons were hazy and smearing was noticed throughout the lane reproducibly. Comparatively, the addition of trehalose had no negative effect on amplification performance of PCR master mix as recorded by gel electrophoresis (Fig. 2) in which the quality of amplification was in complete coherence with that of the wet PCR mixture. Also, the inevitability of lyoprotectant was demonstrated when the negative control freeze-dried PCR master mix without lyoprotectant showed no PCR amplification.

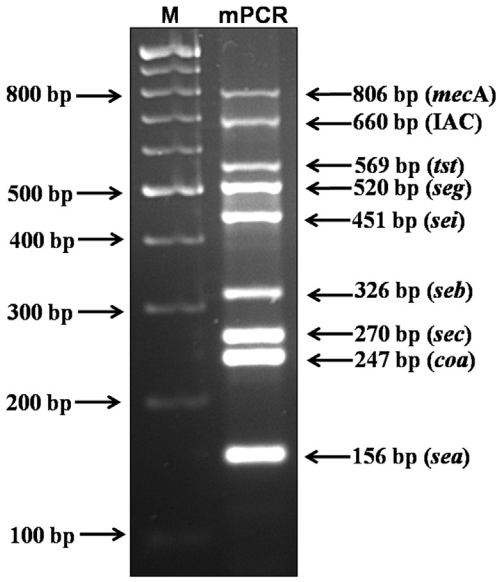

Figure 1. Agarose gel electrophoresis showing PCR amplification of pre-optimized multiplex PCR format. mPCR = multiplex polymerase chain reaction.

Storage stability

The PCR success of freeze-dried samples might be diminished by several factors such as absorption of moisture by dried reagents and possible contamination during storage. These possibilities were avoided by sealing the freeze-dried vials and their subsequent storage in sterile, waterproof pouches along with a desiccant.

The accelerated aging technique was used to calculate the shelf-life of lyophilized master mix by exposing it at 37°C for 1 month. Stability performance was consistent up to 15 days of storage at elevated temperature (Fig. 3). Through the calculation, the shelf-life of the master mix

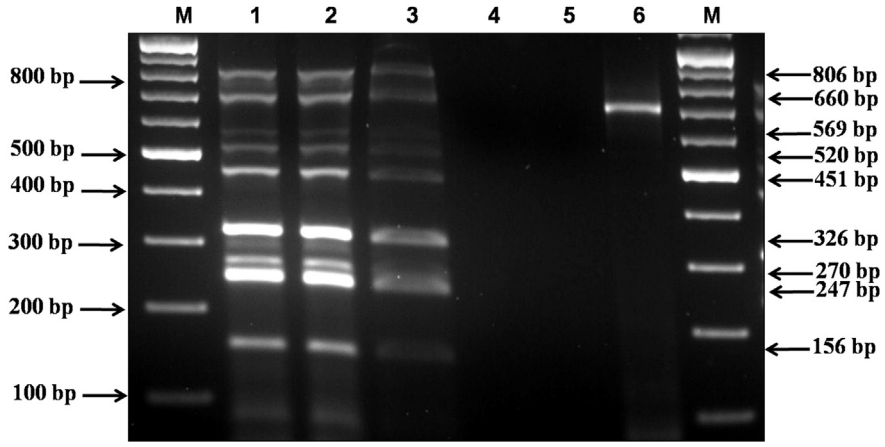

Figure 2. Agarose gel electrophoresis representing analysis of polymerase chain reaction performance of lyophilized master mix after 7 days incubation with the incorporation of different lyoprotectants. Lanes M, 100-bp DNA ladder (Fermentas, GMBH, Germany); 1, "wet" master mix; 2, Lyophilized master mix with trehalose; 3, Lyophilized master mix with sorbitol; 4, Lyophilized master mix with glycerol; 5, Lyophilized master mix without lyoprotectant; 6, Negative control (without DNA).

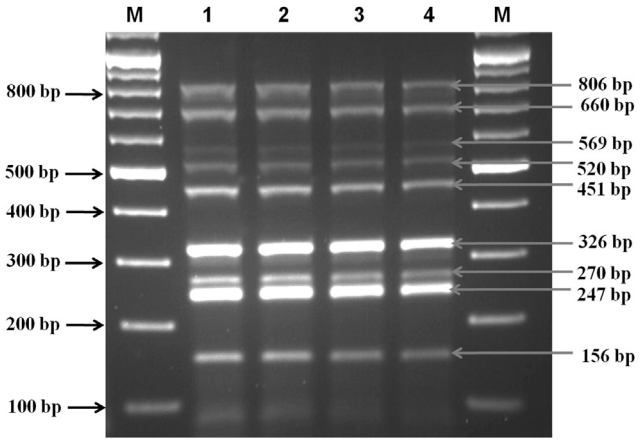

Figure 3. Agarose gel electrophoresis pattern showing polymerase chain reaction amplification of lyophilized master mix after incubation at different temperatures for a period of 15 days. Lanes M, 100-bp DNA ladder (Fermentas); 1, "wet" master mix; 2, Master mix stored at 4°C; 3, Master mix stored at ambient temperature; 4, Master mix stored at 37°C.

was estimated to be 1.5 months at 25°C. In parallel, stability was analyzed at three different temperature conditions for a period of 6 months. PCR performance of lyophilized master mix as analyzed at weekly intervals suggested that the master mix was stable and showed successful amplification when stored at 4°C for $>$ 6 months (Fig. 4). However, at elevated temperatures, the intensity of amplicons was found to be diminished with increase in time. Consequently, master mix stored at room temperature showed a consistent stability performance for up to 1.5 months, whereas at 37°C storage, it had a short-term stability of only 15 days.

Limit of detection

The lyostabilized PCR master mix supplemented with trehalose (5.0% w/v) was evaluated for detection sensitivity using purified gDNA of *S. aureus* ATCC 700699, FRI-722 and whole bacterial cell load of known CFU. For comparison, wet PCR was also included in the experiment. Both freeze-dried and wet reaction mixtures reproducibly revealed a detection sensitivity of 10 pg/reaction with purified gDNA and 10^6 CFU/mL of bacteria per reaction; similar to the pre-optimized format (data not shown).

Stability studies during transportation

To assess the stability of freeze-dried master mix for field application, the lyophilized PCR master mix supplemented with trehalose (5.0% w/v) stored at 4°C was transported in a cold-chain-free condition. The time interval as well as ambient temperature during transportation was recorded. Due to transportation for 48—72 hours, the lyophilized reaction mixtures were exposed to temperature fluctuations. Slight or negligible temperature fluctuation was observed between Mysore and Dharwad, whereas a significant fluctuation was recorded between Mysore and New Delhi (Table 2). PCR was performed in the respective institutions and the agarose gel analysis of the same showed that all nine target DNA fragments were successfully amplified (Fig. 5).

In situ deployment

The lyophilized master mix was assessed for its efficiency to amplify the targets from direct food samples. Several natural samples were tested for the presence of toxigenic *S. aureus*. When PCR was carried out with lyophilized master mix using DNA extracted from pre-enriched samples, the format was able to amplify the genes without showing any inhibition due to food matrix. The conventional isolation and biochemical assays confirmed the presence of *S. aureus* (data not shown). The isolates of *S. aureus* tested by lyophilized master mix also showed virulence profiling as recorded by gel electrophoresis (Fig. 6). The virulence profiling data of isolates is given in Table 3.

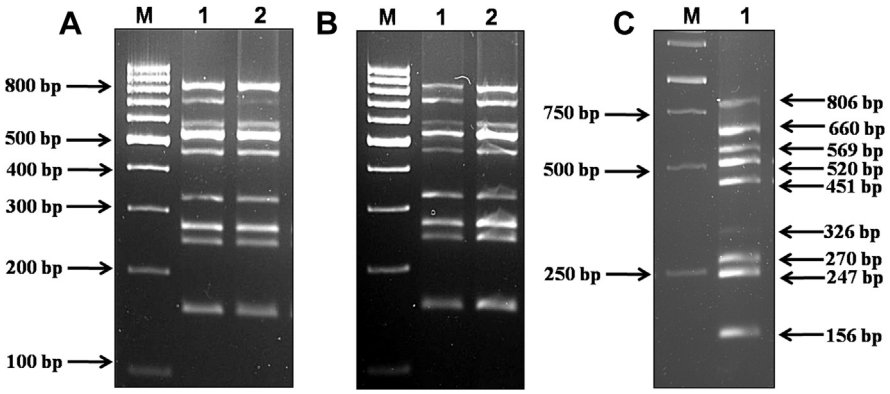

Figure 4. Agarose gel electrophoresis showing amplification pattern after long term storage at different temperatures. (A) After 1 month storage. Lanes M, 100-bp DNA ladder (Fermentas); 1, Master mix stored at 4°C; 2, Master mix stored at ambient temperature. (B) After 1.5 months storage. Lanes M, 100-bp DNA ladder (Fermentas); 1, Master mix stored at ambient temperature; 2, Master mix stored at 4°C. (C) After 6 months storage. Lanes M, 1 kb DNA ladder; 1, Master mix stored at 4°C.

Table 2 Variable factors applied on lyophilized master mix during transportation.

Transit of lyophilized vial	Distance (km)	Transportation time (h)	Temperature fluctuation recorded (°C)
From Mysore to Dharwad	481	48	24—28
From Mysore to New Delhi	2300	72	25—39

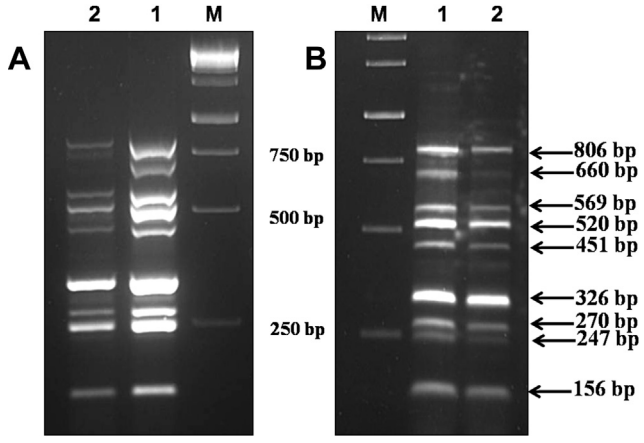

Figure 5. Representative agarose gel electrophoresis indicating transportation stability result of lyophilized master mix. (A) Gel for PCR result performed at SDM, Dharwad. Lanes M, 1-kb DNA ladder (Fermentas); 1, "wet" master mix; 2, Lyophilized master mix stored at 4°C for 4 months; (B) Gel for PCR result performed at CFL, Delhi. Lanes M, 1-kb DNA ladder (Fermentas); 1, "wet" master mix; 2, Lyophilized master mix stored at 4°C for 6 months. PCR = polymerase chain reaction.

Discussion

The present study described the strategic development of a freeze-dried, pre-dispensed, ready-to-use PCR master mix based on our previously reported multiplex PCR for specific detection of antibiotic-resistant *S. aureus* with simultaneous toxin profiling.[12] The multiplex PCR format was stabilized in lyophilized form to make it compatible for field-based diagnostic applications. Primary care was taken to retain the specificity, sensitivity, and reproducibility of the assay unaltered. As an initial challenge, the lyophilization step of the amplification mixture resulted in sample loss during lyophilization in 0.2-mL PCR tubes because of more volume of master mix (50 μL) and subsequent loss of PCR performance in comparison with wet PCR mixture (data not shown). The loss was visible as an exudation of contents from the tube that was indicated by bromophenol blue. The tubes were replaced with 0.5-mL Cliklok polypropylene microcentrifuge tubes (Tarsons Products Pvt. Ltd., Kolkata, India) to overcome this challenge, and whole PCR master mix content was retained inside the tubes without any sample loss during or after lyophilization. PCR performance should not be altered due to the addition of lyoprotectants and freeze-drying process. Hence, selection of a suitable lyoprotectant that does not interfere with the amplification performance of standardized format and in turn should maintain long-term stability of PCR reagents was another task. Therefore, commonly used lyoprotectants such as trehalose, sorbitol and glycerol were selected based on previous studies in which successful results were obtained.[13,18—20] They were further assessed for their suitability in our optimized assay. Of the three lyoprotectants examined, trehalose (5.0% w/v) was found to be highly promising in effectual freeze-drying of the master mix without any loss in functionality of the PCR reagents. Glycerol-supplemented (4.0% v/v) freeze-dried PCR master mix showed complete loss of functionality of PCR reagents and thus no amplification was recorded. The high hygroscopic nature of glycerol and its increased concentration due to lyophilization would likely have affected the

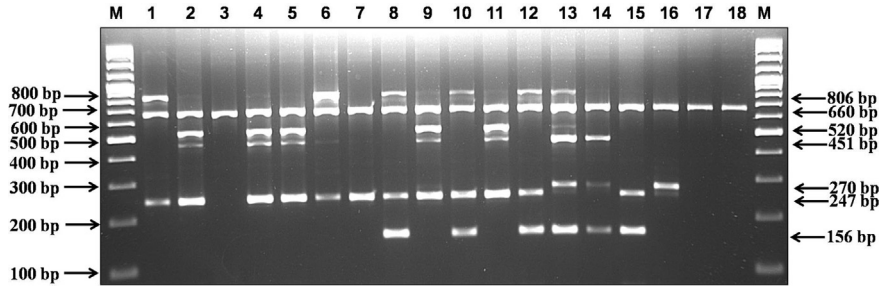

Figure 6. Agarose gel electrophoresis showing PCR amplification based virulence profiling analysis of clinical isolates tested with lyophilized PCR master mix. Lanes M, 100-bp DNA ladder (Fermentas); 1–17, Clinical isolates; 18, Negative control without DNA. PCR = polymerase chain reaction.

Table 3 Virulence profiling of *S. aureus* isolates as analyzed by lyophilized polymerase chain reaction master mix.

Genes targeted	No. of positive strains (n = 91)	Rate of prevalence (%)
sea	33	36.26
seb	26	28.57
sec	56	61.53
seg	54	59.34
sei	53	58.24
coa	87	95.60
tst	3	3.29
*mec*A	25	27.47

stability of the PCR reagents.[13] The smearing of amplicons that was exclusively recorded in the case of sorbitol-containing master mix clearly inferred the interference of sorbitol in the amplification reaction, although the stability of reagents was unaffected. Finally, trehalose was selected as the best suitable lyoprotectant for the multiplex PCR format. We predicted that the low hygroscopicity of trehalose[21] played a major role in preserving the PCR reagents in lyophilized conditions. Although Qu and co-workers[20] have used 40% trehalose in the master mix successfully, we observed an inverse relation between the band intensity and the increased trehalose concentration beyond 5.0%. The master mix was also supplemented with bromophenol blue, which aided proper mixing of all contents and subsequent completion of lyophilization. In addition, any sample loss during lyophilization can be easily detected because of visible color. It also removes the need for loading dye prior to agarose gel electrophoresis, thus avoiding any risk of external/carry-over contamination.[22,23]

Owing to the challenges in stockpiling of PCR-based diagnostic kits and having screened for compatible lyoprotectant, the lyophilized master mix was subjected to storage stability studies. The shelf-life was found to be 1.5 months at ambient temperature as calculated by accelerated aging technique, which was correlated with the real-time performance studies. Although the storage stability of lyophilized master mix was transient at 37°C, a satisfactory performance was obtained consistently at ambient temperature for > 1.5 months. Long-term stability was achieved at 4°C without any loss in amplification performance. In addition, the transportation studies validated appreciable performance of the format even after exposure to marked temperature fluctuations. The detection sensitivity remained consistent in terms of gDNA and CFU even after addition of trehalose, lyophilization process, and storage. These results supported the real-time utility of lyophilized master mix described in the present study as a highly reliable diagnostic tool in point-of-care laboratories as well as in-field investigations, wherein the specificity, sensitivity, and rapidity of the assay will not be compromised.

Considering the commercial value of the lyophilized assays, Kamau and co-workers[24] estimated > $10 per reaction of a lyophilized quantitative PCR assay, which might be an expensive approach. However, design and development of lyophilized multiplex PCR formats facilitates detection of multiple targets within a single reaction in a cost-effective mode. Our format is an illustration for this with an approximation of < $2 per reaction. Dry reagent-based multiplex PCR assays have been reported for the detection of toxigenic *S. aureus*,[25] nevertheless a limited number of gene targets might narrow down their applicability. Comparatively, a wide range application is foreseen for the lyophilized multiplex PCR format developed in this study, as it can simultaneously detect major toxigenic genes as well as methicillin resistance of *S. aureus*. Additionally, cost savings in terms of storage and transportation without the need for cold chains signifies possible field application of the format in an affordable manner for developing countries.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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