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Development and validation of multiplex polymerase chain reaction assay for concomitant detection of genus *Staphylococcus* and clinically relevant methicillin resistance determinants

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ABSTRACT

The increasing emergence of methicillin-resistant staphylococci (i.e., methicillin-resistant *Staphylococcus aureus* [MRSA] and methicillin-resistant coagulase-negative staphylococci [MRCoNS]) has become a threat globally for both human and animal populace. Phenotypic detection of MRSA and MRCoNS is a less sensitive and time-consuming approach which affects the treatment outcome. Thus, a rapid and accurate method is needed for an early diagnosis of MRSA/MRCoNS infections. The present study aimed at standardization and validation of a multiplex polymerase chain reaction (mPCR) assay to detect genus *Staphylococcus* (16s rRNA gene) and methicillin-resistance determinants (*mec*A and *mec*C genes) simultaneously. The assay characteristics were evaluated against 53 well characterized strains comprising of 40 *Staphylococcus* and 13 non-*Staphylococcus* strains. Among *Staphylococcus* strains, 32 were *mec*A positive and one strain was *mec*C positive. The lower limit of detection of the mPCR assay was 1ng/mL (Genome copies: 16S rRNA = 1.1×10^9; *mec*A = 3.17×10^9; *mec*C = 1.6×10^9), with analytical sensitivity and specificity of 100%. The mPCR assay developed in the study is useful for rapid and accurate diagnosis of MRSA/MRCoNS infections. The assay can be an important diagnostic as well as surveillance tool to investigate the emergence and dissemination of methicillin-resistant staphylococci which is of both clinical and public health significance.

1. INTRODUCTION

Members of genus *Staphylococcus* are widespread globally colonizing 20–30% of the human population [1]. They are known to cause wide array of infections including mild skin infection, sepsis, and even life-threatening bacteremia in certain cases [2]. Genus *Staphylococcus* comprises of two groups, namely, coagulase-positive *Staphylococcus* and the emerging coagulase-negative staphylococci (CoNS) [3]. Apart from human medicine, *Staphylococcus* spp. has also gained importance in veterinary medicine affecting livestock and poultry. The transmission of drug resistant strains from animals to humans and vice-versa can have a potential impact on the public health. There are evidences of clonal transmission of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant CoNS (MRCoNS) between humans and cows, and also these strains may spread clonally within a herd of dairy cows [4,5].

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In *Staphylococcus*, methicillin resistance is primarily as a result of altered penicillin binding protein (PBP2a) that shows very less affinity for all beta-lactam antibiotics. PBP2a is regulated by *mec*A gene. Identification of *mec*A gene among staphylococcal isolates is considered as the gold standard for the presence of methicillin resistance [6,7]. The chromosomal mediated mecA gene is harbored on a large mobile genetic element called SCC*mec* which are the known vectors that transfer the resistant genes among various *Staphylococcus* species. At present, international working group on classification of staphylococcal cassette chromosome has recognized 11 different types of SCC*mec* elements [8]. The existence of closely related SCC*mec* elements in *S. aureus* and CoNS implies horizontal transfer of SCC*mec* [9].

In 2011, a study from England on bovine mastitis reported a novel *mec*A homolog in MRSA isolates carrying SCC*mec* type XI. The homologous gene was initially called *mec*A_{LGA251}, but later renamed as *mec*C [10]. This novel *mec*C showed approximately 69% identity to *mec*A at the DNA level and can colonize, causing diseases in humans and other host species [11]. In contrast to these chromosomally mediated *mec*A and *mec*C genes for methicillin

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resistance, *mec*B gene originally described as *mec*A_m, was identified in transposon *mec* complex (Tn6045) of *Macrococcus caseolyticus* [12]. However, in *Macrococcus canis,* SCC*mec* carrying *mec*B gene independent of Tn6045 was identified [13,14]. In addition, *mec*D gene, which share above 66% of nucleotide identity with *mec*B gene, was recently found in *M. caseolyticus* isolates of bovine and canine origin [15].

Although, there are numerous conventional phenotypic approaches for unmasking the methicillin resistance among staphylococci [16,17] like-oxacillin agar screen method, determination of minimum inhibitory concentration using agar dilutions, broth dilutions and E tests, cefoxitin, and oxacillin disk diffusion tests; however, discrepancies in the accuracy of these methods make them unreliable. Apart from being less sensitive and time consuming, the phenotypic expression of drug resistance depends on various conditions, i.e., growth media, temperature, and osmolarity. These variables further add to the limitation of these methods [18,19]. Conversely, molecular approach offers a rapid, accurate strategy with increased sensitivity and specificity for the identification of these drug resistant pathogens. Herein, we developed a novel multiplex polymerase chain reaction (mPCR) for the concurrent detection of *Staphylococcus* genus, and the methicillin resistance determinants *mec*A and *mec*C. The timely detection of methicillin resistance among staphylococci will be valuable for the quick diagnosis of the ailment caused by drug-resistant strains.

2. MATERIALS AND METHODS

2.1. Bacterial Strains

The selection of strains was done in such a way so that our assay is tested against multiple positive and negative controls for the genus *Staphylococcus* and methicillin resistance. The panel included 40 well characterized strains which served positive control for genus staphylococci. Out of these strains, 32 were used as a positive control for *mec*A gene and one *S. saprophyticus* strain (BH32) identified in our laboratory previously was used as a positive control for *mec*C gene. (GenBank Accession No: MH448900). The *mec*A positive staphylococcal strains were isolated from cattle milk (*n* = 23), cattle nasal (*n* = 1), cattle wound (*n* = 1), extramammary site (*n* = 2), and hand swab of animal handlers (*n* = 3). The additional two *mec*A positive strains were acquired from ATCC (American type culture collection), namely, ATCC 33591 (MRSA), and ATCC 43300 (MRSA). The *mec*C positive strain was isolated from buffalo milk.

In addition, we tested our assay with a negative control panel (*n* = 13), which covered different genera of Gram-positive and Gram-negative bacteria, namely, *Streptococcus agalactiae, Streptococcus uberis, Streptococcus dysgalactiae, Lactobacillus acidophilus, Lactococcus lactis, Leuconostoc mesenteroides, Micrococcus aurantiacus, Enterococcus faecalis, Aerococcus viridans, Streptococcus bovis, Escherichia coli*, *Shigella sonnei,* and *Klebsiella pneumoniae.* This panel was used a negative control for all the targets included in the mPCR assay. The other ATCC strains involved in the standardization of mPCR assay are detailed in Table 1.

2.2. DNA Extraction and Precipitation

The DNA from bacterial isolates were extracted from overnight grown staphylococcal isolates by QIAamp DNA minikit (Qiagen, Duesseldorf, Germany) as per manufacturer's recommendations. The purity and concentration of the extracted DNA were tested by NanoDrop 2000c (Thermo Fischer Scientific Inc, USA). Absorbance 260 nm/280 nm ratio of ~1.8 was accepted as pure for DNA.

Table 1: Evaluation of multiplex PCR against nine staphylococcal and 13 non-staphylococcal ATCC strains

Strain name	Species	16S rRNA	mecA	mecC
ATCC 25923	*Staphylococcus aureus*	+	−	−
ATCC 25904	*Staphylococcus aureus*	+	−	−
ATCC 29740	*Staphylococcus aureus*	+	−	−
ATCC 43764	*Staphylococcus chromogenes*	+	−	−
ATCC 29970	*Staphylococcus haemolyticus*	+	−	−
ATCC 29062	*Staphylococcus sciuri*	+	−	−
ATCC12228	*Staphylococcus epidermidis*	+	−	−
ATCC 33591	*Staphylococcus aureus*	+	+	−
ATCC 43300	*Staphylococcus aureus*	+	+	−
ATCC 13813	*Streptococcus agalactiae*	−	−	−
ATCC 9927	*Streptococcus uberis*	−	−	−
ATCC 43078	*Streptococcus dysgalactiae*	−	−	−
ATCC 4356	*Lactobacillus acidophilus*	−	−	−
ATCC 11454	*Lactobacillus lactis*	−	−	−
ATCC 8293	*Lactobacillus mesenteroides*	−	−	−
ATCC 11731	*Micrococcus aurantiacus*	−	−	−
ATCC 19433	*Enterococcus faecalis*	−	−	−
ATCC 11563	*Aerococcus viridans*	−	−	−
ATCC 33317	*Streptococcus bovis*	−	−	−
ATCC 25922	*Escherichia coli*	−	−	−
ATCC 25931	*Shigella sonnei*	−	−	−
ATCC 70063	*Klebsiella pneumoniae*	−	−	−

Precipitation was carried out to concentrate the extracted DNA. Briefly, 1/10th volume of 3 M sodium acetate (pH = 5.2 with glacial acetic acid) was added to the extracted DNA. About 2–3 volume of 100% ice cold ethanol was added and the suspension was stored at −20°C for 1 h. After centrifuging at 12,000 rpm for 15–20 min, the ethanol was decanted, and the pellet was washed with 70% ethanol. The pellet was allowed to air dry and finally resuspended in TE buffer to get concentrated DNA.

2.3. Oligonucleotide Designing and Multiplex PCR Assay

The mPCR was designed to target three genes simultaneously, namely, 16S rRNA gene for the identification of genus *Staphylococcus*; *mec*A, and *mec*C genes for the detection of methicillin resistance. The oligonucleotide sequences for 16S rRNA and *mec*A genes were obtained from previously published reports [20,21]. The oligonucleotide sequences for *mec*C gene were designed from the sequences available in the GenBank database (GenBank Accession No: MH448900) [Table 2]. The oligonucleotide sequences were examined for specificity and PCR suitability using the National Centre

Table 2: Oligonucleotide sequences used in multiplex PCR

Gene targeted	Primer name	Oligonucleotide sequence (5'-3')	Amplicon size (bp)	Annealing temp
16S rRNA	16S rRNA_F	GTGATCGGCCACACTGGA	842 bp	52°C
	16S rRNA_R	CAACTTAATGATGGCAACTAAGC		
*mec*A	*mec*A_F	ACGAGTAGATGCTCAATATAA	293 bp	
	*mec*A_R	CTTAGTTCTTTAGCGATTGC		
*mec*C	*mec*C_F	GCTCCTAATGCTAATGCA	584 bp	
	*mec*C_R	GGCTTAGAACGCCTCTATGA		

PCR: Polymerase chain reaction

for Biotechnology Information Primer-BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The mPCR reaction mixture comprised PCR grade water, template DNA, PCR buffer, $MgCl_2$, dNTPs, primers, Bovine serum albumin, and Taq DNA polymerase at working concentrations, as described in Table 3. The thermocycling conditions include initial denaturation at 94°C for 5 min, followed by 35 cycles with denaturation at 94°C for 1 min, annealing at 52.5°C for 1 min, and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. The PCR amplicons were examined on 1.5% agarose gel containing ethidium bromide (10 µg/mL). A mixture of DNAs from the reference strain ATCC 33591 (positive for *Staphylococcus* genus and *mec*A) and from strain BH32 (positive for *Staphylococcus* genus and *mec*C) was used as a positive control.

2.4. Analytical Sensitivity of Multiplex PCR Assay

The DNA extracted from strain ATCC 33591 was spiked with the known concentration of the DNA extracted from strain BH32. Serial ten-fold dilutions equivalent to 1000 ng/mL (3.32×10^8 genome copies/mL)–0.1 ng/mL (3.32×10^4 genome copies/mL) of purified DNA was made and examined by the 16SrRNA, *mec*A, and *mec*C specific primer sets in a multiplex reaction. Each standard dilution was run in triplicates.

2.5. Specificity of the Multiplex PCR Assay

The mPCR specificity was determined by amplifying 10 ng/mL of DNA from *Staphylococcus* field isolates ($n = 40$), of which 32 isolates were *mec*A positive and 1 isolate was *mec*C positive). The remaining seven *Staphylococcus* isolates were only genus positive. None of the isolates were positive for both *mec*A and *mec*C genes. In addition, specificity of the mPCR assay was further evaluated by testing 10 ng/mL of extracted DNA from 13 ATCC non-*Staphylococcus* isolates and nine ATCC *Staphylococcus* isolates. A negative template control was incorporated in each run.

3. RESULTS AND DISCUSSION

The emergence of multidrug-resistant MRSA clones poses an ongoing challenge for infection control specialists. Hence, it is important to track the epidemiology of MRSA to limit their transmission within the community [22]. The traditional methods used for epidemiological surveillance of MRSA are expensive, time-consuming, with less sensitivity, and robustness. PCR based assays are known for immense flexibility, higher efficiency, increased sensitivity, and specificity than conventional methods. The detection of methicillin resistance by PCR based methods has been described in several reports [23-25]; however, these studies targeted only *mec*A gene individually or in association with virulence factors like *pvl* [21,26]. Herein, we developed a mPCR assay for concurrent detection of genus *Staphylococcus* (16S rRNA gene) and its methicillin resistance determinants, namely, *mec*A and *mec*C genes [Figure 1].

Table 3: Multiplex PCR assay reaction mixture setup

Reagent	Stock concentration	Volume (µL)	Working concentration
Sterile $H2_O$		11.75	
PCR Buffer	10×(25 mM)	2.5	1×(2.5 mM)
dNTP's	10 mM	0.75	0.3 mM
$MgCl_2$	25 mM	2.5	2.5 mM
16S rRNA (FP+RP)	5 pmoles/µL each	0.75*2	0.15 pmoles/µL each
*mec*A (FP+RP)	5 pmoles/µL each	0.75*2	0.15 pmoles/µL each
*mec*C (FP+RP)	20 pmoles/µL	0.75*2	0.6 pmoles/µL
Template	Variable	2	Variable
Taq DNA polymerase	5 U/µL	0.5	2.5 U
Bovine serum albumin	20 mg/ml	0.5	0.4 mg/ml
Total reaction volume		25	

PCR: Polymerase chain reaction, FP: Forward primer; RP: Reverse primer; dNTP's: Deoxynucleotide triphosphates (dNTPs: dATP, dCTP, dGTP, dTTP)

In the previous publications, misidentification of some important CoNS was reported, as these studies possibly did not aim fully conserved region in the 16S rRNA gene of *Staphylococcus* [23,27]. In the present study, *Staphylococcus* genus-specific primer targeting conserved 16S rRNA gene [15] was used which allowed to detect *Staphylococcus* precisely without any ambiguity. The mPCR detected 16S rRNA gene with precisely among staphylococcal strains. The detection of methicillin resistance is further complicated with the recent discovery of the $mecALGA_{251}$ (*mec*C) gene within a novel SCC*mec* XI element in *S. aureus* [28] and CoNS [29]. In view of *mec*C harboring *Staphylococcus* spp., *mec*A negative results alone can no longer be a final conclusive report to exclude methicillin resistance. Genotyping methods with exclusion of *mec*C gene for methicillin resistance would lead to contradictory outcomes when compared to phenotypic methods [30]. Though, demonstration of *mec*A gene by PCR is known to be a gold standard method for the identification of methicillin resistance; however, the detection of *mec*C gene has become equally important in ascertaining methicillin-resistant staphylococci [31]. The strength of the present study was the incorporation of *mec*C specific primers in addition to the most commonly targeted *mec*A gene. Although, the inclusion of recently discovered methicillin-resistant determinants,

*i.e., mec*B and *mec*D would have increased the significance of the study [13-15]. However, it was not practically possible for us to target these genes in view of the absence of confirmed *mec*B and *mec*D positive strains (positive controls). Considering the priority, this will be our future strategy to incorporate *mec*C and *mec*D genes as an advancement of our mPCR assay.

The analytical sensitivity of our mPCR assay was determined by testing the serial ten-fold dilutions equivalent from 1000 ng/mL (3.32×10^8 genome copies/mL) to 0.1 ng/mL (3.32×10^4 genome copies/mL) of purified DNA known to be positive for 16S rRNA, *mec*A, and *mec*C genes. The primer sets were tested in three different combinations (*Staphylococcus* + *mec*A; *Staphylococcus* + *mec*C; and *Staphylococcus* + *mec*A + *mec*C). The mPCR assay could detect 1 ng/ml of positive DNA, which corresponds to 1.1×10^9genome copies/ml for 16S rRNA gene, 3.17×10^9 genome copies/ml for *mec*A gene, and 1.6×10^9 genome copies/ml for *mec*C gene [Figure 2]. A study from Malaysia reported the analytical sensitivity of the pentaplex PCR to be 10 ng/mL [21]. Similarly, Rocchetti *et al*., [3] in 2018, showed the sensitivity up to a dilution of 10^{-2} for *S. aureus* and 10^{-5} for CoNS. This difference in the analytical sensitivity among the studies is inevitable, as it depends on the complexity of multiplexing, the sequences targeted, primer pair efficiency, and assay conditions.

The expression of *mec*A and *mec*C genes is variable and relies on several factors such as pH, temperature, media composition, inoculum size, salt concentration, and incubation time of cultures. Although, the phenotypic detection methods are widely accepted, they have their own limitations as the phenotypic expression of methicillin resistance is often heterogeneous, which describes a cell population wherein a small fraction of cells display high-level methicillin resistance [19]. All of these features highlight the necessity to develop a fast, precise, and sensitive method for the detection of methicillin resistance in staphylococci, which is independent of all these variables [32,33]. Evaluation of our mPCR assay with thirty known *mec*A positive and *mec*C negative staphylococci showed specific amplification for genus staphylococci and *mec*A gene among all thirty isolates tested. As expected, no amplification for *mec*C gene was observed among all thirty *mec*A positive *Staphylococcus* strains. Among nine ATCC *Staphylococcus* strains, amplification was observed with all genus specific primers while two of them, ATCC 33591 and ATCC 43300 turned positive for *mec*A gene as well. However, none of the non-staphylococcal ATCC reference strains (n = 13) showed amplification to any of the three genes tested. Thus, the mPCR assay exhibited 100% specificity [Table 1].

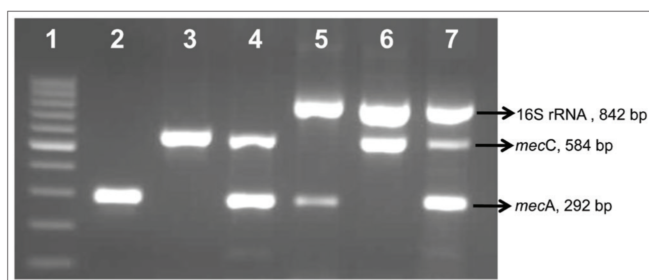

Figure 1: Standardization of multiplex polymerase chain reaction. Lane 1: 100 bp marker; Lane 2: *mec*A positive; Lane 3: *mec*C positive; Lane 4: *mec*C and *mec*A positive; Lane 5: *Staphylococcus* and *mec*A positive; Lane 6: *Staphylococcus* and *mec*C positive; Lane 7: *Staphylococcus*, *mec*C and *mec*A positive

Before the discovery of *mec*C gene, targeting *mec*A gene alone was considered as criteria for the detection of methicillin resistance. Hence, duplex PCR targeting *nuc* gene, a species-specific marker for *S. aureus* and *mec*A gene with altered PBP2a was considered the gold standard for detection of MRSA isolates. In 2018, Rocchetti *et al*. [3] standardized mPCR for the identification of genus *Staphylococcus* and *mec*A gene, further differentiating *S. aureus* from CoNS using *coa* gene. Previously, Maes *et al*. [23] in 2002 developed a triplex PCR which included 16S rRNA, *mec*A and *nuc* gene-specific primers for the rapid characterization of staphylococci in positive blood cultures. However, the discovery of the *mec*C gene has challenged the microbiological laboratories and clinicians in terms of the detection of methicillin resistance and prophylaxis/treatment options. Thus, it is imperative to have a diagnostic assay targeting *mec*C resistant determinant as well, which will provide the exact burden of the methicillin-resistant staphylococci without any ambiguity.

4. CONCLUSION

Inclusion of *mec*A and *mec*C specific gene targets is an attempt to narrow down the gap in the detection of methicillin resistance. The mPCR assay was able to identify the genus *Staphylococcus*, methicillin resistance determinants with 100% sensitivity and specificity. This

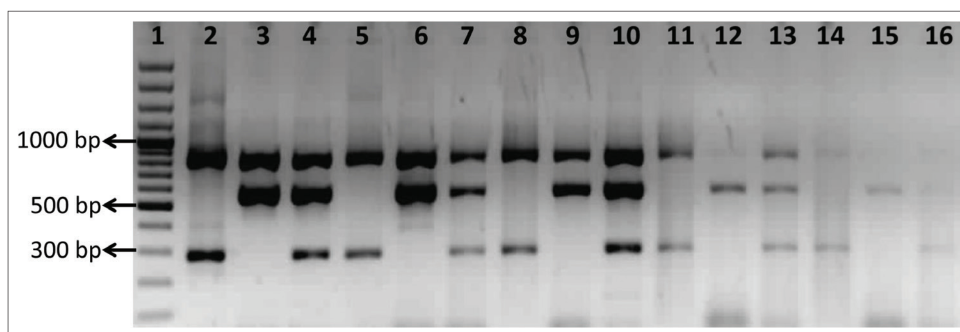

Figure 2: Analytical sensitivity of the multiplex polymerase chain reaction. Lane 1: 100 bp ladder; Lane 2-4: 1000 ng/mL (Lane 2: *Staphylococcus* and *mec*A, Lane 3: *Staphylococcus* and *mec*C, Lane 4: *Staphylococcus*, *mec*A, and *mec*C); Lane 5-7: 100 ng/mL (Lane 5: *Staphylococcus* and *mec*A, Lane 6: *Staphylococcus* and*mec*C, Lane 7: *Staphylococcus*, *mec*A, and *mec*C); Lane 8-10: 10 ng/mL (Lane 8: *Staphylococcus* and *mec*A, Lane 9: *Staphylococcus* and*mec*C, Lane 10: *Staphylococcus*, *mec*A, and *mec*C); Lane 11-13: 1 ng/mL (Lane 11: *Staphylococcus* and *mec*A, Lane 12: *Staphylococcus* and*mec*C, Lane 13: *Staphylococcus*, *mec*A, and *mec*C); Lane 14-16: 0.1 ng/mL (Lane 14: *Staphylococcus* and *mec*A, Lane 15: *Staphylococcus* and *mec*C, Lane 16: *Staphylococcus*, *mec*A, and *mec*C)

assay could be successfully used in clinical laboratory settings for rapid identification of methicillin-resistant staphylococci, which in turn may help in adopting strategies for timely and effective treatment and control options.

5. CONFLICT OF INTEREST

Authors declared that they do not have any conflicts of interest.

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None.

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