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Full Length Research Paper

Discriminative multiplex (hexaplex) PCR strategy for the detection of methicillin resistance and virulence factors in *Staphylococcus aureus*

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In clinical microbiology, phenotypic characterization is laborious as well as time consuming strategy and remains less discriminative among high virulent to less virulent clinically important strains. Induction of molecular techniques, allow more accurate and quick way out for identification of *Staphylococcus aureus* along with its virulence capabilities. This study describes a multiplex (hexaplex) strategy for a rapid detection of methicillin resistance, simultaneously discriminating *S. aureus* from coagulase-negative staphylococci (CoNS) and occurrence of virulence factors. It targets the *nuc* (Specific for *S. aureus*), *mec A* (methicillin resistance determinant), *fem A* and *fem B* (*S. aureus* specific factors essential for methicillin resistance), *Luk S/F PV* (encodes for Panton Valentine Leukocidin-PVL), and *spa* (encodes protein A). Validation of this strategy was performed using previously characterized clinical isolates of methicillin susceptible *S. aureus* (MSSA), methicillin resistant *S. aureus* (MRSA), and CoNS from different hospital facilities. Amplification results were consistent and perfectly accurate in accordance to the biochemical and resistance properties of the isolates. This molecular approach renders clinical microbiology a feasible, rapid, simple and reliable technique, discriminating MSSA, MRSA, and CoNS and provides an early and accurate way of detection. This technique will add significant contribution to prevent the wide spread dissemination of the infections as well as facilitate the designing of improved ways of treatment and cure.

Key words: MSSA, MRSA, *mec A*, panton valentine leukocidin, *nuc* gene, *spa* gene.

INTRODUCTION

Staphylococcus aureus has been a versatile organism due to its adaptability as both a commensal and a pathogenic organism. It is frequently involved in community and hospital acquired infections, associated in septicemia, pneumonia, wound sepsis, septic arthritis, endocarditic, and post surgical infections with a high rate of mortality and morbidity (Sara et al., 2003; Lowy, 1998; Laupland et al., 2003). The extensive use of antibiotics accounted for the hasty dissemination of antibiotic resistance in *S. aureus* has become a global concern; the most notable example of this phenomenon is methicillin resistant *S. aureus* (MRSA). The first clinical isolate of MRSA was reported in 1961, just one year after the launch of methicillin and in 1980s it became widespread health-care concern (Chambers, 1997; Jevons, 1961; Livermore, 2000). Methicillin resistance is mainly arbitrated to PBP 2a, a Penicillin Binding Protein with an altered β-lactam affinity (Georgopapadakou and Liu, 1980; Hartman and Tomasz, 1984; 1986). *mec A* gene encodes for PBP 2a protein, which is carried by a mobile element “Staphylococcal chromosomal cassette (SCC)” and used as a molecular marker for MRSA (Hiramatu et al., 2001; Wu et al., 1996). Simultaneously, there are certain factors essential for methicillin resistance (*fem*),

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found in both methicillin-susceptible and methicillin-resistant *S. aureus*. These *fem* factors are physically distinct from SCC, affecting level of resistance (Berger, 1983; Murakami and Tomasz, 1989; Berger et al., 1989). Two of the *fem* factors are co-transcribed and exist as *fem AB* operon, and are important discriminative markers for *S. aureus* distinguishing it from other coagulase negative staphylococci (CoNS) (Perez et al., 2001; Yamashita and Kagawa, 1992).

Virulence features of MRSA have made its pathogenesis more threatening for health-care concerns, as first was emerged in late 1990s, producing Panton-Valentine Leukocidin resulting from the expression of *lukF-PV* and *lukS-PV.* The PVL is widely associated with community acquired MRSA epidemiology; reported 77 to 100% in various studies (Hidron et al., 2009; Naas et al., 2005; Lina et al., 1999; Naimi et al., 2003). Similarly, *S. aureus* have several "microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)" that play important role in evading and destroying host defenses. Protein A is one of its examples that bind to Fc portion of immunoglobulin, structurally determined by *spa* gene, which shows high intra-specie polymorphism (Patti et al., 1994; Gordon and Lowy, 2008; Deisenhofer, 1981; Frenay et al., 1996).

It has been found problematic to detect MRSA accurately and rapidly, along with the severity of infection in an effective manner. Phenotypic characterization techniques are growth condition dependent, limiting the discriminative features. This study describes a multiplex PCR (M-PCR) strategy for the detection of methicillin susceptible *S. aureus* (MSSA) and MRSA, discriminating from CoNS by analyzing various loci including *nuc, fem A, fem B* (specific for *S. aureus*) and *mec A* (for methicillin resistance). Simultaneously, small number of virulence factors can also be identified with in the population by using this type of M-PCR.

MATERIALS AND METHODS

Bacterial isolates

The previously collected clinical bacterial isolates including MSSA, MRSA, and methicillin resistant *Staphylococcus epidermidis* (MRSE) (coagulase negative *Staphylococcus*) were collected for this study. All of these isolates were biochemically screened in duplicates after collection from Civil Hospital Karachi and Jinnah Postgraduate Medical Centre, Karachi, Pakistan.

Screening and identification

Screening for *S. aureus* was performed on Baird Parker agar (BPA) (Oxoid, UK) and mannitol salt agar (MSA) (Oxoid, UK). Coagulase activity and lecithinase activity were tested on BPA, whereas mannitol fermentation and salt tolerance were analysed by using MSA. Methicillin resistance was ascertained by 1 µg oxacillin disc (Oxoid, UK) diffusion, achieved on Muller Hinton agar (Oxoid, UK) and incubated at 35°C for 24 h. To avoid any ambiguity, cefoxitin disc (Oxoid, UK), 30 µg was also used to determine methicillin resistance (CLSI, 2006; 2007).

DNA isolation

Enzyme based method was adopted for DNA isolation. Overnight grown culture (1 ml) in Brain Heart Infusion broth (Oxoid, UK) was used, following MasterPure™ Gram Positive DNA purification protocol (Epicentre Biotechnologies, Cat# MGP04100).

Oligonucleotide primers

The previously reported oligonucleotide primers were used, which were obtained commercially from Eurofins mwg operon (USA), as given in Table 1.

Multiplex-PCR (M-PCR) amplification

DNA suspension (3 µl) was used for each 50 µl PCR mixture, containing 1X buffer (50 mM KCl, 15 mM Tris HCl, pH 8.8), 2.5 mM $MgCl_2$, 0.3 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP and dTTP) (Gene DireX, Cat# DN001025), different concentrations of each primer, as given in Table 1, and 2.5 U of *Taq* polymerase (GeneAid, Cat#TQ251). All M-PCR amplifications were carried out with a reagent control, containing all the reagents except the template DNA and a negative control (MRSE). MBS 0.2S Thermo*Hybaid* (ThermoElectron Corporation) was used for DNA amplification with the following PCR cycle: initial denaturation at 94°C for 5 min, followed by 10 amplification cycles, denaturation at 94°C for 40 s, annealing at 60°C for 40 s, and extension at 72°C for 45 s, and another 25 amplification cycles denaturation at 94°C for 60 s, annealing at 50°C for 60 s, and extension at 72°C for 1.5 min. Final extension was done at 72°C for 15 min, and cycle was terminated to hold at 4°C. M-PCR product (10 µl) was subjected to gel electrophoresis, performed on 2% agarose in 1X Tris-borate-EDTA (TBE) buffer at 100 V for 75 min, stained with 0.5 µg/ml ethidium bromide. For the reference of amplicon sizes of the PCR products, 100 bp Gene Ruler™ (fermentas, Cat# SM0242) was also used. Gel was visualized using GelDoc 2000 system (BioRad, Italy).

Validation of multiplex-PCR

All M-PCR reactions were performed in duplicates to eliminate any doubts. For each set, separate PCR mixture was prepared, used and then obtained results were compared. Following these findings, this technique was adopted for routine laboratory research for the screening of *S. aureus*. A total of 150 samples collected from different teaching hospitals were screened. These clinical isolates included nasal samples, blood samples, pus samples, body fluid, wounds and CSF samples, and urine samples.

RESULTS

Single target PCR

Prior to the M-PCR, single target PCR was performed to ensure whether each gene is satisfactorily amplified by the adopted strategy resulting in expected sizes. For the isolate MBSA-206, the single target amplification resulted into same fragment length, as obtained in M-PCR,

Table 1. List of oligonucleotide primers and their concentrations.

Primer	Sequence (5' to 3')	Conc. (µM)	Amplicon size (bp)	Gene	Reference
nun1	GCG ATT GAT GGT GAT ACG GTT	0.16	279	*Nuc*	[Brakstad et al., 1992]
nun2	AGC CAA GCC TTG ACG AAC TAA AGC	0.16			
mec1	GTA GAA ATG ACT GAA CGT CCG ATA A	0.24	310	*mec A*	[Zhang et al., 2004]
mec2	CCA ATT CCA CAT TGT TTC GGT CTA A	0.24			
fem A1	AGA CAA ATA GGA GTA ATG AT	0.24	509	*fem A*	[Kobayashi et al., 1994]
fem A2	AAA TCT AAC ACT GAG TGA TA	0.24			
fem B1	TTA CAG AGT TAA CTG TTA CC	0.22	651	*fem B*	[Kobayashi et al., 1994]
fem B2	ATA CAA ATC CAG CAC GCT CT	0.22			
Luk-PV1	ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A	0.16	433	*Luk S/F PV*	[Lina et al., 1999]
Luk-PV2	GCA TCA AGT GTA TTG GAT AGC AAA AGC	0.16			
spa1	ATC TGG TGG CGT AAC ACC TG	0.20	1150-1500	*spa*	[Shakeri et al., 2010]
spa2	CGC TGC ACC TAA CGC TAA TG	0.20			

Figure 1. Single target PCR for each marker gene.

corresponding to the expected sizes, as shown in Figure 1.

M-PCR based gene detection

Conditions were optimized to ensure the amplification of each target gene for the M-PCR. Each primer set was optimized to variable primer concentration along with different amount of template DNA and $MgCl_2$ concentration, in order to overcome the problem of product amount for different gene varying in their sizes. Such problems usually occur during M-PCR, favoring the shorter length products over the longer ones. Two amplification cycles were included in the M-PCR protocol of variable annealing temperatures, in order to reduce the possibility of non-specific amplification plausible, due to different annealing temperature of recruited primers.

M-PCR conditions were optimal at 2.5 mM $MgCl_2$, 3 µl DNA, and listed primer concentrations (Table 1), producing best amplified products for *nuc, mec A, fem* A,

Table 2. M-PCR profile.

Strain	Genes amplified					
	nuc	***fem A***	***fem B***	***mec A***	***Luk S/F PV***	***Spa***
MBSA-173	+	+	+	-	+	+
MBSA-174	+	+	+	-	+	+
MBSA-175	+	+	+	-	+	+
MBSA-177	+	+	+	-	+	+
MBSA-176	+	+	+	+	+	+
MBSA-186	+	+	+	+	-	-
MBSA-206	+	+	+	+	-	+
MBSA-33	+	-	+	+	+	-
MBSA-203	+	+	+	+	+	-
MBSA-204	+	+	+	+	+	-
MBSE-185	-	-	-	+	-	-
MBSE-205	-	-	-	+	-	-

+, Gene amplified; -, gene not amplified.

Table 3. Comparison of phenotype-genotype characteristics.

Strain	Biochemical result		Genotyping results				Remark
	Coagulase	**Antimicrobial susceptibility**	***nuc***	***fem A***	***fem B***	***mec A***	
MBSA-173	+	MS	+	+	+	-	MSSA
MBSA-174	+	MS	+	+	+	-	MSSA
MBSA-175	+	MS	+	+	+	-	MSSA
MBSA-177	+	MS	+	+	+	-	MSSA
MBSA-186	+	MR	+	+	+	+	MRSA
MBSA-206	+	MR	+	+	+	+	MRSA
MBSA-176	+	MR	+	+	+	+	MRSA
MBSA-203	+	MR	+	+	+	+	MRSA
MBSA-204	+	MR	+	+	+	+	MRSA
MBSA-33	+	MR	+	-	+	+	MRSA
MBSE-185	-	MR	-	-	-	+	MR-CoNS
MBSE-205	-	MR	-	-	-	+	MR-CoNS

MS; Methicillin susceptible (oxacillin and cefoxitin susceptible); MR, methicillin resistant (oxacillin and cefoxitin resistant); MR-CoNS, methicillin resistant coagulase negative staphylococcus.

fem B, Luk S/F PV, and *spa*. A total of 12 Staphylococcal species were screened using this hexaplex PCR strategy, and resulted in 10 *nuc* positives (*S. aureus*) and 2 *nuc* negatives, confirmed by the amplification of *fem* factors (*fem A* and *fem B*) in all 10 *S. aureus*. Among these 10 *S. aureus*, 6 were *mec A* positive (MRSA) and 4 were *mec A* negative (MSSA). Virulence profile for *spa* and *Luk S/F PV*, 100% MSSA were positive to both, 66% MRSA were positive for *spa*, 66% MRSA were positive for *Luk S/F PV,* and 50% MRSA were positive to both genes (Table 2).

Reproducibility of results

Amplifications of *nuc, mec A, Luk S/F PV, fem A, fem B* and *spa* were well reportable due to lucid differences in their band sizes (279, 310, 433, 509, 651, 1150 to 1500, respectively), and well observed on ethidium bromide stained gel. Results were consistently reproducible, as duplicate M-PCRs resulted with the same genes amplifications. Obtained results were strongly confirmed by the biochemical characterization as well as comparison between phenotype-genotype characteristics performed prior to the M-PCR (Table 3).

DISCUSSION

Suitable, rapid and accurate methods for the identification and detection of bacterial strains are essential for better management of infectious diseases. Subspecies identification is conducted within hospitals and research institutions for many applications; including for identifying an outbreak source during epidemiological investigations, for routine surveillance of hyper invasive, multi-resistance or simply carriage strains, and for small or large scale population structure analyses. Infectious disease genotyping is also utilized for biodefence, in order to identify the source of biological agent.

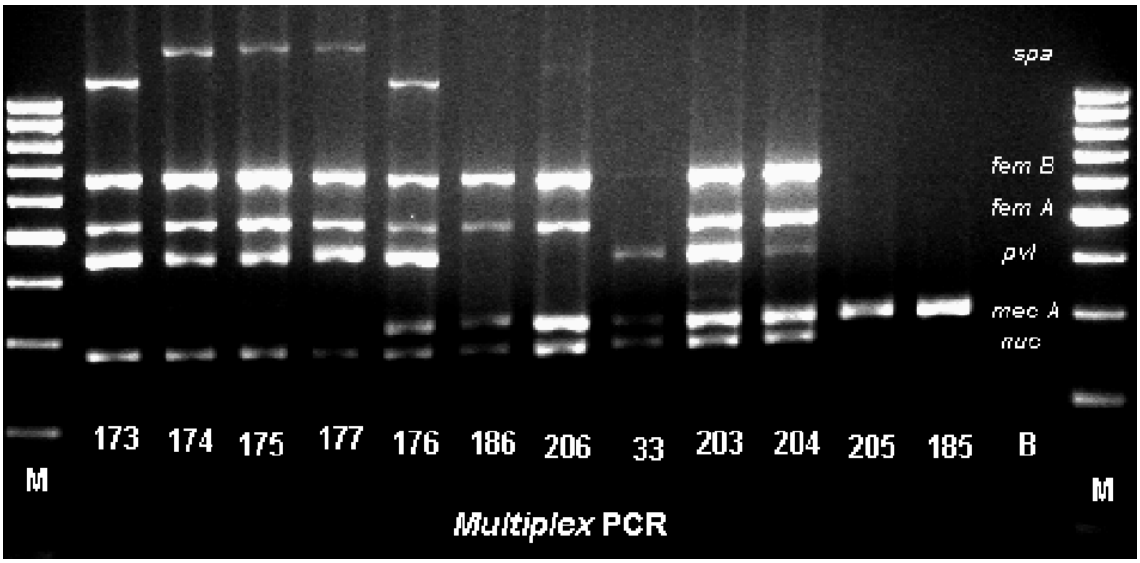

Figure 2. Gel Electrophoretic resolution of different molecular marker (genes).

The bacterial strain identification methods vary in their resolving power, cost effectiveness and the technological platform required for employing them. Based on these factors, the most appropriate genotyping method for any given application can be determined. The detailed investigations of complex traits may require whole genome sequencing or microarray analyses, which are time consuming, laborious and expensive, whereas the routine clinical analyses need genotyping methods that are quick, cost effective and generate portable results of appropriate resolution that can be easily interpreted.

During last few years, a number of studies have established an extremely high capacity of identification and detection of specific bacterial strains as well as the genes of interest. This ability revealed PCR as a powerful tool for the investigations of clinical microbiology. The potential of the PCR methodology for the identification of *S. aureus* strains and for antibiotic resistance genes has already been carried out by many workers (Perez et al., 2001; Cockeril, 1999; Jonas et al., 1999; Vannuffel et al., 1989).

By keeping in mind the fact that the detection of MRSA, along with the severity of infection in an effective manner, has found to be problematic. Phenotypic characterization techniques are growth condition dependent, limiting the discriminative features. This study thus describes a M-PCR strategy for the detection of MSSA and MRSA, discriminating from CoNS by analyzing various loci included *nuc, fem A, fem B* (specific for *S. aureus*), and *mec A* (for methicillin resistance). Simultaneously, small number of virulence factors can also be identified with in the population by using this type of M-PCR (Figure 2). The major alternative to simplex genotyping method was the simple multiplex method, which was first developed by Oliviera and de Lancastre (2002). For Staphylococci, this technique has been used to specifically detect MRSA (Vannufell et al., 1995) and to discriminate between *S. aureus* and CoNS with the detection of methicillin resistance (Schmitz et al., 1997). Simple and rapid identification and discrimination of *S. aureus* and gene profiling (Table 2) are essential for prompt institution of effective antimicrobial chemotherapy and for limiting the unnecessary use of certain classes of antibiotics.

The hexaplex (M-PCR) molecular genotyping approach, described in this study, renders clinical microbiology of *S. aureus* a feasible, rapid, simple and reliable technique discriminating MSSA, MRSA and CoNS and provides an early and accurate way of detection. This technique also contributes in the prevention of the resistant strains of the organism from widespread dissemination and facilitates the antibiotic therapy design.

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