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Original Article

Microbiology Section

Use of Triplex PCR for Rapid Detection of PVL and Differentiation of MRSA from Methicillin Resistant Coagulase Negative Staphylococci

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ABSTRACT

Introduction: Methicillin-Resistant *Staphylococcus aureus* (MRSA) has become a major public health problem in both hospitals and communities. Panton – Valentine Leucocidin (PVL) has been reported to be an important marker for the highly pathogenic community acquired *S. aureus* infections. A rapid detection of these MRSA is very important for its treatment. The specific detection of MRSA is always a problem due to the prevalence of methicillin resistance among the coagulase negative Staphylococci. Hence, this study was done to develop a rapid triplex PCR for the detection of *PVL* positive MRSA and for the simultaneous differentiation of MRSA from Coagulase Negative Staphylococci (CoNS).

Materials and Methods: We developed a triplex PCR for the specific detection of *PVL* positive Community Acquired (CA) – MRSA and for its simultaneous differentiation from the coagulase negative Staphylococci. We used PCR for targeting the *fem A* gene which is specific for *S. aureus, mecA* which is specific for methicillin-resistance and *luk - PV* which is specific for the *PVL* toxin. The method was evaluated with a total of 100 clinical isolates of *Staphylococcus spp*.

Results: The triplex PCR was successfully standardized by using the reference strains and it was evaluated by using clinical strains. The method was found to be rapid, highly sensitive (100%), specific (99%) and cost effective.

Conclusion: Triplex PCR can be used as a diagnostic tool for the detection of the highly pathogenic strains of CA-MRSA.

Key Words: *PVL* MRSA, MRCoNS, Triplex PCR, *femA, mecA*

INTRODUCTION

Staphylococcus aureus is a major human pathogen which causes various infections, both in the otherwise healthy individuals and in hospitalized patients. Over 60% of the clinical isolates of *S. aureus* were found to produce the penicillin binding protein 2a(PBP2a)-the factor which is responsible for the methicillin resistance, which is encoded by the gene, *mecA* [1]. The *mecA* gene has also been reported in coagulase negative *staphylococcus* (CoNS). About 62% of the CoNS, (*S. epidermidis* (75%), *S. lugdunensis* (25%), *S.hemolyticus* (12%) and *S. saprophyticus* (5%) have been reported to be methicillin-resistant [2]. *femA* is a chromosomally encoded ubiquitous gene in Staphylococci, which is involved in the formation of the peptidoglycan pentaglycine interpeptide linkages [3]. It also plays a role in the expression of methicillin resistance, the mechanism of which is not clearly understood. *femA* can be used as a marker for the differentiation of *S. aureus* from the coagulase negative Staphylococci. Panton-Valentine leukocidin (*PVL*) has gained much importance in the recent past due to its association with the community acquired methicillin-resistant *S. aureus* (CA-MRSA) infection. PVL is a phage encoded exotoxin of *S. aureus* and it has been found to be cytotoxic to rabbit and human neutrophils as it induces apoptosis. The *S. aureus* isolates with *PVL* are rapidly spreading and they cause serious skin and soft tissue infections such as pyomyositis, abscesses, breast abscesses, necrotizing fasciitis and pneumonia in otherwise healthy individuals [4-8]. Molecular methods are available for the detection of methicillin-resistance among the *Staphylococcus spp*. [9-11]. But, only few methods are available for the detection of *PVL* and for the simultaneous identification and differentiation of MRSA and methicillin-resistant coagulase negative Staphylococci (MRCoNS) [12, 13]. Hence, in this study, a rapid triplex PCR which targetted the *femA, mecA* and the *pvl* genes was used for the specific detection of *PVL* and methicillin-resistance and for the differentiation of MRSA from MRCoNS.

MATERIALS AND METHODS

The bacterial isolates

The method was standardized by using the 10 standard strains of Staphylococci, which included 6 MRSA, 2 *S. aureus* and 2 CoNS [Table/Fig-1].

A total of 100 retrospective clinical isolates of Staphylococci, which were previously confirmed by standard phenotypic methods, were used for the evaluation of the triplex PCR. The bacterial isolates included 25 clinical isolates of methicillin-susceptible *S. aureus* (MSSA), 25 clinical isolates of MRSA, 25 clinical isolates of CoNS and 25 clinical isolates of MRCoNS. The clinical isolates were collected between June to August 2011 from a tertiary care centre in Chennai, from various clinical specimens. An institutional ethical clearance was obtained and an informed consent was obtained from the study participants. The isolates were identified by standard methods [14]. The methicillin-resistance was detected by cefoxitin disc diffusion and oxacillin agar dilution methods [14].

S. aureus ATCC 29213	MSSA	*femA*
S. aureus ATCC 25923	MSSA	*femA, pvl*
S. aureus ATCC 43300	MRSA	*femA, mecA*
S. aureus COL	MRSA	*femA, mecA*
MRSA-Mu50	MRSA	*femA, mecA*
MRSA-Mu3	MRSA	*femA, mecA*
MRSA-USA300 (FPR3757)	MRSA	*femA, mecA, pvl*
MRSA-USA400 (MW2)	MRSA	*femA, mecA, pvl*
S. epidermidis ATCC 12228	MRSE	*mecA*
S. epidermidis ATCC 35984	MRSE	*mecA*

[Table/Fig-1]: List of reference strains used for the standardization of the Triplex PCR.

The triplex PCR method

The triplex PCR method for the detection of *PVL* and the simultaneous differentiation of MRSA from MRCoNS was designed by using the primer sequences from various published studies [Table/Fig-2] [4, 15, 16]. The concentrations of the forward and reverse primers of each gene (*pvl* – 0.1µM each, *mecA* – 0.2µM each and *femA* – 0.08µM each) were optimized for the multiplex PCR.

Primer sequences	PCR Reaction Mixture (25µl Reaction volume)	PCR cycling conditions (Eppendorf Gradient Mastercycler)	Amplicon separation by 1.5% Agarose gel electrophoresis
femA (Mehrotraet al., 2000) [23] F: 5' – AAAAAAGCAC ATAACAAGCG – 3' R: 5' – GATAAAGAAGA AACCAGCAG – 3'	10X Taq polymerase buffer; 2.5U Taq DNA polymerase (NEB); 200mM dNTPs (Bio-tools); 10pico moles of each primer (Sigma)	Initial Denaturation: 94°C/2min	132bp
mecA (Kondo et al., 2007) [24] F: 5'-TGCTATCCACC CTCAAACAGG-3' R: 5'-AACGTTGTAAC CACCCCAAGA-3'		30 cycles of Denaturation: 94oC/ 45sec; Annealing: 55oC/30sec; Extension: 72oC/45sec	286bp
pvl (Lina et al.,1999)[6] F:5'–ATCATTAGGTAAAAT GTCTGGACATGATCCA–3' R: 5'– GCATCAASTGTATT GGATAGCAAAAGC– 3'		Final Extension: 72oC/ 2min	441bp

[Table/Fig-2]: Multiplex PCR method for detection of pvl , MRSA and differentiation from MRCoNS.

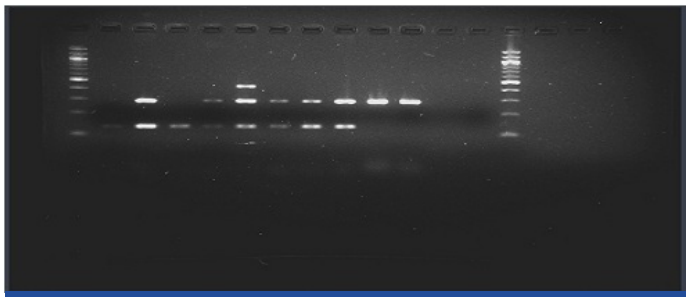
The template DNA for the multiplex PCR was prepared from an overnight culture (Brain Heart Infusion Agar), which was obtained by boiling a few colonies of Staphylococci in 100µl of DNase free water (Qiagen, Germany) for 10 minutes and centrifuging the suspension at 10,000 rpm for 3min. Five micro-litres of the supernatant was used as the template for the PCR. The PCR was carried out in a 25µl reaction mix which contained 200µM of dNTPs (NEB), 1X PCR buffer (NEB) and 0.5U Taq DNA polymerase (NEB). The amplification was done by using a Mastercycler Gradient (Eppendorf, Hamburg, Germany) under the following cycling conditions (one cycle of initial denaturation at 94°C for 4 min, 25 cycles of denaturation at 94°C for 30s, annealing for 30s at 54°C, and extension at 72°C for 30 s, followed by a final extension at 72°C for 5 min. The amplified products were separated on a 1.5% low melting agarose gel (Medox India Pvt Ltd). They were electrophoresed (0.5X TBE buffer at 150V and 90mA for 30 minutes), stained with 0.5% ethidium bromide, visualized and recorded by using gel documentation system (BIO-RAD). A 100bp ladder (RBC Bioscience Corp, Taiwan) was run as a molecular marker.

Standardization of the Triplex PCR for the detection of *PVL* MRSA

In this study, the triplex PCR was optimized to specifically identify the *S. aureus* at the species level *(femA)* and to detect the methicillin-resistance *(mecA)* and the *PVL* toxin *(pvl)* simultaneously by using the previously described primers in three different studies. Standardization of the PCR was done by a stepwise optimization of the individual reaction components: the primers, $MgCl_2$ and the dNTPs. The annealing temperature for the triplex PCR was standardized by a gradient technology by using the Master Cycler Gradient (Eppendorf, Hamburg, Germany). The Triplex PCR was found to produce excellent results with 1.5mM $MgCl_2$, 0.5U Taq DNA polymerase, 200µM dNTPs, 10ng of DNA and a 55^0C annealing temperature. The specificity of the triplex PCR was determined by using 10 reference strains of Staphylococci, which included 6 MRSA, 2 MSSA and 2 methicillin-resistant *S. epidermidis* (MRSE). To test the specificity of the triple PCR assay, the DNA templates from *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were included. As a negative control, the reaction mixture was tested with sterile water.

RESULTS

FemA was amplified in all the standard strains of *S. aureus,* which included 6 MRSA and 2 MSSA, whereas no amplification was seen in *S. epidermidis*. All the methicillin-resistant Staphylococci, which included 6 MRSA and 2 MRSE, were found to be positive for the *mecA* gene, whereas both the MSSA isolates were negative for the *mecA* gene. The reference strains of *S. aureus,* ATCC25923, MRSA USA300 FPR3757 and MRSA MW2 were positive for *pvl*, whereas all the other reference strains were found to be negative. The representative picture of the agarose gel electrophoresis has been shown in [Table/Fig-3].



[Table/Fig-3]: PCR for detection of *PVL* and differentiation of MRSA from MRCoNS.

Evaluation of the Triplex PCR for the detection of *PVL* MRSA

After the standardization with the reference strains, the triplex PCR method was evaluated with 100 clinical strains of Staphylococci, which included 25 isolates of MRSA, 25 isolates of MSSA, 25 isolates of MRCoNS and 25 isolates of CoNS with a known methicillin resistance status. All the 25 isolates of MRSA were found to be

positive for both *femA* and *mecA*. Also, 10/25 (40%) MRSA isolates were found to harbour the *pvl* gene, which is the marker for the highly pathogenic community acquired MRSA (USA300, MW2). Of the 25 MSSA isolates, *femA* was detected in all the isolates and 3/25 (12%) isolates were found to be positive for *pvl*. One *S. aureus* isolate which was identified as MSSA by the phenotypic method was found to be positive for the *mecA* gene. The *mecA* gene alone was detected in all the 25 MRCoNS isolates which were included in the triplex PCR, whereas none of the genes was amplified for the methicillin-sensitive CoNS [Table/Fig-4]. There was a good correlation between the results of the triplex PCR assay and those of the traditional phenotypic methods.

Various genes detected	Number (%) of isolates			
	MRSA	MSSA	MRCoNS	CoNS
femA	25 (100)	25 (100)	0 (0)	0 (0)
mecA	25 (100)	1 (4)	25 (100)	0 (0)
pvl	10 (40)	3 (12)	0 (0)	0 (0)

[Table/Fig-4]: Genes detected among the isolates used for evaluation of the Triplex PCR.

DISCUSSION

The genus, *Staphylococcus* comprises about 34 different species and methicillin-resistance was reported in most of the species, which included the most pathogenic species-*S. aureus* and other commensal species. The use of rapid molecular methods, which included PCR for the specific identification of *S. aureus* and the detection of methicillin-resistance, had been described previously [9, 10, 12, 13]. Most of the previous studies had used anyone of the following genes viz., *nuc,* 16S rDNA, *coag* and *femA* with *mecA* for the rapid and the specific detection of MRSA at the species level. The emergence of highly pathogenic CA-MRSA infections in healthy individuals [17] and their recent emergence as nosocomial pathogens [18, 19] with multidrug resistance, had necessitated the development of a rapid diagnostic technique for the specific detection of MRSA. The presence of *pvl* was considered as the marker for the CA-MRSA infection in most of the cases. Even though there are reports on CA-MRSA without *pvl*, its association with CA-MRSA has been reported to cause life threatening infections which include necrotizing pneumonia, necrotizing fasciitis, septicaemia, pyomyositis, brain abscess and pyogenic abscesses [4-8]. *PVL* is a necrotizing cytotoxin which is specific for the human and rabbit polymorphonuclear cells [20], which may be responsible for the Staphylococcal invasiveness and virulence. In developing countries like India [19, 21], Africa [22] and other Asian countries [23], the prevalence rate of the *pvl* positive CA-MRSA is very high as compared to those at other geographic locations [17, 24]. Even in this study, about 12% of the MSSA and 40% of the MRSA were found to be positive for *PVL*, which was slightly higher than that which was reported from other geographical regions and lower than the reports from Africa and India. Although *pvl* was extensively studied and considered as a major factor in the CA-MRSA infection, only few molecular methods employed *pvl* for the detection of the pathogenic CA-MRSA isolates [12,13]. We carefully selected the published primers from various studies and designed a triplex PCR which targetted *pvl*, *mecA* and *femA*. The above PCR method was standardized by using the reference strains and it was evaluated with 100 clinical isolates of *Staphylococcus spp*. Negative controls without the template were run and the DNA from other genera were included to rule out the false positive results which were due to the non specific amplification. The PCR successfully amplified all the 3 target genes and it was found to be highly specific (99%) and sensitive (100%). Only one *S. aureus* isolate which was identified as MSSA by the phenotypic method was found to be positive for *mecA*. This may be due to the negative expression of the gene *in-vitro*, as has been reported in earlier studies [25]. The current method which targetted *femA, mecA* and *pvl* can be used in resource-limited settings as a simple, rapid and a highly sensitive molecular tool for the detection of the life threatening CA-MRSA infections.

CONCLUSION

We successfully developed a new triplex PCR which can be used as a simple, rapid and a highly specific molecular diagnostic tool for the detection of the highly pathogenic strains of CA-MRSA, especially in developing countries like India.

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