Heterogeneity in \textit{femA} in the Indian Isolates of \textit{Staphylococcus aureus} Limits Its Usefulness as a Species Specific Marker

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ABSTRACT

Increase in prevalence of MRSA worldwide and hence the need for rapid detection, have led to use of molecular methods for confirmation of the species and also MRSA. Species specific markers like \textit{fem} or \textit{nuc} along with the methicillin-resistance determinant, \textit{mecA}, have been used by several investigators worldwide for the identification of MRSA. In the current study, we have screened 54 microbiologically confirmed (MRSA, MSSA and CoNS) isolates for the presence of \textit{mecA}, 16S rRNA, \textit{femA} and \textit{nuc} markers. While \textit{mecA} PCR and 16S rRNAPCR results were consistent with other studies, \textit{femA} and \textit{nuc} showed dramatic variation in detection rate (sensitivity) of \textit{S. aureus} 29.6% and 53.7% respectively. Evidences are presented to demonstrate the absence of \textit{femA}. Our attempt to amplify the complete \textit{femA} gene using sequences flanking \textit{femA} further confirmed these results and also indicated that variations exist even in the genomic sequences around \textit{femA}. Our data reveals the need for exercising care while using primers designed on sequences of constitutive genes like \textit{femA} and \textit{nuc} for PCR based identification of \textit{S. aureus} species. Though geographic variations in the genome of \textit{S. aureus} have previously been reported from around the world, we present here evidence for the first time from India for absence of \textit{femA} and also for probable variations in the sequences around the \textit{femA} gene in clinical isolates of \textit{S. aureus}.

Keywords: \textit{femA}; \textit{mecA}; \textit{nuc}; PCR; \textit{S. aureus}

1. Introduction

\textit{Staphylococcus aureus} is one of the major causes of widespread gram positive bacterial nosocomial infections, especially the post-surgical wound infections [1]. Its disease manifestations range from minor skin infections to life-threatening diseases such as pneumonia, sepsis etc. The emergence of MRSA in patients with no apparent risk factors seems to be a growing concern. In India the prevalence of MRSA is 51.8% in 2010 [2]. Although the discovery of penicillin proved to be a major breakthrough in treating these infections, with it had also emerged a major concern; the notorious ability of \textit{Staphylococcus aureus} to develop resistance to antibiotics and remain non-responsive to treatment [3]. Shortly after the introduction of methicillin, reports of Methicillin-resistant \textit{Staphylococcus aureus} (MRSA) had begun to surface within hospitals in the early 1960s, which are now increasingly prevalent worldwide [4,5]. Such endemic MRSA infections are difficult to eradicate and remain active reservoirs of infection thereby increasing the hospital costs, length of hospital stays, morbidity and mortality.

Methicillin-resistance in staphylococci is expressed by the \textit{mecA} gene that produces a “Penicillin Binding Protein 2a” (PBP2a) [6], a modified transpeptidase. This PBP-2a has lower affinity to penicillin and its derivatives than the other PBPs [7]. The Staphylococcal Chromosome Cassette \textit{mec} (SCCmec), a mobile genetic element, is composed of the \textit{mec} gene complex which includes \textit{mecA}, its regulatory genes \textit{mecl}, \textit{mecR} and a \textit{ccr} gene complex. The latter encodes site specific recombinases, namely \textit{ccr}A/B, which in-turn regulate the mobility of SCCmec [8]. Significant geographic variations have been found in the structural organization of the SCCmec and these variations have been used to classify the SCCmec types [9]. However, \textit{mecA} alone does not solely confer the methicillin resistance. Studies have shown that \textit{fem} (factors essential for methicillin-resistance) or the auxiliary genes like \textit{fem A/B/X} in addition to \textit{mecA} are also impor-
tant in the expression of methicillin resistance [10]. The \textit{femABX} operon encodes factors which are responsible for the formation of pentaglycine bridges in the cell wall of \textit{Staphylococcus} [11].

Kirby-Bauer antibiotic testing (disc diffusion antibiotic sensitivity test), oxacillin agar screen test, agar dilution and the Epsilometer test (E-test) are commonly used to determine the MRSA phenotype in the clinical microbiology laboratory. These tests have limitations and frequently show variations [12]. Although the detection of \textit{mecA} gene remains the gold standard for detecting methicillin-resistance, its detection alone does not confirm the presence of \textit{Staphylococcus aureus} [13] and there is no consensus on the molecular target that could be used to confirm the \textit{S. aureus} species. Constitutively expressed genes such as \textit{femA}, \textit{femB} and \textit{nuc} are being used as molecular targets for the identification of \textit{Staphylococcus aureus} [14,15]. Polymorphisms within these constitutive genes have been reported [16] worldwide and also failures to confirm the species of \textit{S. aureus} using these genes as targets for PCR detection [17]. However, from India, there are no reports about any variations in the sequences of these constitutive genes yet and for that matter SCC\textit{mec} or the genome of \textit{S. aureus} are yet to be characterised in India. The aim of our study was to optimise and establish a multiplex PCR protocol to screen clinical isolates for MRSA phenotype in the clinical microbe -20

\section{2. Materials and Methods}

54 isolates of \textit{S. aureus} were collected from Jan 2012 to March 2012 in Global Hospital, Hyderabad. These strains were isolated on Mueller Hinton Agar (MHA) either from blood culture bottles or directly from different clinical specimens (pus, drain fluids/secretions etc). Coagulase and catalase tests were used to confirm \textit{S. aureus} in the isolates. Standard disc diffusion test using the antibiotic oxacillin were used to assess the drug sensitivity of these isolates. Epsilometer test (E-test) was used to find out the MIC of the isolates.

\subsection{2.1. Preparation of Bacterial DNA Lysates}

A single bacterial colony was scrapped off the agar and the cells washed by suspending in 100 µl of TEX buffer (10 mM Tris-HCl pH 8.5, 1 mM EDTA, 1% (w/v) Triton X-100). The suspension was vortexed to achieve a uniform suspension and was centrifuged. The pellet was subjected to another wash in TEX buffer. Finally the pellet was resuspended in 100 µl of TEX buffer and was lysed by heating in a dry bath at 95°C for 15min [18]. The lysate was used as the DNA template for PCR. This lysate could be stored at 2°C - 8°C for 2 - 3 months and at -20°C for > 1 year.

\subsection{2.2. Polymerase Chain Reaction}

All the primer sequences listed Table 1 were procured from Eurofins Genomics India Pvt Ltd. Multiplex-PCR mixture for \textit{mecA}, \textit{femA} and 16S rRNA consisted of the following; 1 × PCR mixture (Fermentas Life Sciences/HiMedia) containing 1.5 mM MgCl2, 50 pM each of primer, 200 µM of each dNTP along with 1 U of Taq (HiMedia) and 5 µL of DNA template in 20 µL final volume. PCR reactions were initiated with a denaturation at 94°C for 5 mins, 40 cycles of denaturation (94°C, 30 s), annealing (55°C, 40 s) and primer extension (72°C, 50 s), with a final extension at 72°C for 10mins.

\textit{A femA} monoplex PCR was performed under the reported conditions [14,19] but with 10 µl of the DNA template. Monoplex PCR was also optimized for the identification of the \textit{nuc} gene in these isolates. The PCR reaction mixture contained in 20 µL final volume 50 pM of \textit{nuc} primers and 5 µl of DNA lysate in addition to 200 µM of dNTPs, and 1 unit of Taq DNA polymerase.

The PCR reactions with \textit{femA} flanking primers were performed in a volume of 20 µL containing 10 µl of DNA lysate, containing 1.7 mM MgCl2, 100 pM of primers, 0.2 mM dNTPs along with 2.5 units of Taq DNA polymerase. A 30-cycle amplification followed an initial denaturation at 94°C for 5 min, with denaturation (94°C, 30 s), annealing (45°C, 1 min) and primer extension (72°C, 45 s), and final extension at 72°C for 10 mins.

\section{3. Results}

\subsection{3.1. 16s rRNA, \textit{femA} and \textit{mecA} Multiplex PCR}

Of the 54 isolates which were included in the study, 21 (38%) were isolated from blood, 13 (24%) from pus, 9 (16.6%) from wound swabs and the rest from other sources such as sputum, tissue, endo-tracheal secretions and others. These isolates were identified as MRSA (26 isolates), MSSA (26 isolates) and CoNS (2 isolates). Multiplex-PCR was performed targeting 16S rRNA for the \textit{Staphylococcus} genus, \textit{femA} for \textit{S. aureus} and \textit{mecA} for methicillin resistance. All the isolates tested positive for the genus specific 16S rRNA 886 bp product. The 293 bp \textit{mecA} was detected in all 26 (100%) MRSA isolates, 16 (61.5%) MSSA isolates and 1 CoNS isolate.
It was interesting to note that only 16 (29.6%) isolates showed amplification of the 450 bp femA gene (9 MRSA, 7 MSSA) with both the CoNS isolates were negative for femA.

### 3.2. Monoplex PCR of the Species-Specific femA and nuc Genes

As femA failed to show up in the multiplex PCR, femA monoplex-PCR was done using the primers reported by Al-Talib et al. [19] and Kobayashi et al. [14]. Both these sets of primers failed to identify the femA in those isolates, thereby confirming our results of multiplex PCR.

As femA is generally accepted as a species specific marker, we wanted to corroborate femA results with another species specific marker, namely the thermostable nuclease gene, nuc, to confirm S. aureus and compared the sensitivities of femA and nuc for detecting S. aureus. Out of the 54 isolates, 29 (53.7%) showed the 270 bp nuc that included 52% MRSA and 48% MSSA, while both the CoNS isolates were negative for nuc amplification.

### 3.3. Amplification of the Full Length femA Using Primers from the Flanking Regions

To further investigate the reason for the non-amplification of the femA gene in most of the isolates, assuming internal sequence variations, we designed primers flanking the femA gene (27 bp upstream and 22 bp downstream to femA) to amplify the full length femA gene of 1263 bp, giving a PCR product of 1312 bp. This was done as follows. 11 femA gene sequences of Staphylococcus aureus submitted in the GenBank data base were downloaded and a ClustalW analysis was performed. Primers for femA gene flanking sequences were designed using the online tool Primer3 and the expected product size was 1312bp. The designed primers were blasted against all the 11 femA sequences from the GenBank database and the primers showed 100% alignment with all the femA sequences (Table 2).

13 of the 54 isolates, including 7 MRSA and 6 MSSA showed amplification of full length femA gene. Twelve of them, showing the expected 1312 bp product were the femA positive isolates and one was about 700 bp truncated product (Figure 1). All the other femA negative isolates failed to show any amplification using these primers (see also Table 3).

### 4. Discussion

In this study, during our screening for methicillin resistance markers in clinical isolates, we observed that the femA species specific marker failed to amplify in several isolates. We present evidence to show that femA cannot be used as a reliable marker for S. aureus in this geographical region. We demonstrated this using both multiplex and monoplex PCR.

There has been a tremendous increase in the number of MRSA cases in the past few years. Therefore, several efforts have been made for quicker and early detection of MRSA. Molecular methods have now become the gold-standard for rapid detection of MRSA [12,21]. Several studies have reported the use of mecA as marker for detection of methicillin resistance, fem genes and nuc for
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Table 2. List of *femA* gene sequences reviewed from the GenBank Database.

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Figure 1. PCR amplification of molecular targets for MRSA and *S. aureus*.

Lane 1: Low Range Ladder, Lane 2: Multiplex-PCR [16S rRNA (886bp), femA (450 bp), mecA (293bp)], Lane 3: femA-Al-Talib (450 bp), Lane 4: femA-Kobayashi (509 bp), Lane 5: nuc (270 bp), Lane 6: femA full length (1312 bp), Lane 7: femA full length (mutated, approx. 700 bp), Lane 8: Negative Control.
identification of S. aureus species. Good correlation of phenotype with genotype tests were reported for MRSA isolates, which harboured mecA and femA genes [19,22]. In the study by Kobayashi et al., mecA was detected in 100% of MRSA, 16.7% of MSSA isolates. 10.6% of their S. aureus isolates did not amplify femA gene PCR product [14]. Thermostable nuclease gene nuc was reported to have 100% sensitivity and specificity for the identification of S. aureus isolates [15,23]. In India, only a few studies have reported the use of femA and nuc along with mecA as molecular targets for identification of S. aureus and characterisation of MRSA [24,25].

Variations (polymorphism) in the genomic sequences are not uncommon in S. aureus, even highly conserved and widely used species specific markers like coagulase (coa), Staphylococcal Protein A (spa) genes have shown polymorphisms. Variations in the sequence of the coa and spa genes and the hyper variable region adjacent to the mecA gene, have been the basis for the most widely used forms of PCR typing of MRSA [26-28].

Further, it is also known that sequence variations culminate in changes in virulence properties of S. aureus which influence clinical disease manifestations in humans [29].

The mecA PCR in our study showed absolute correlation to MRSA phenotype and we observed a rather high rate (58.3%) of detection of mecA among MSSA isolates. An intriguing finding in this study was the non-amplification of femA gene in most (70.4%) of the isolates when screened with two different sets of primers for the femA gene and confirmed by non-amplification of the full length femA gene with the primers flanking the femA gene. Though nuc PCR was more sensitive (57.4%) compared to femA PCR (29.6%) in the detection of S. aureus we could not achieve 100% sensitivity with nuc PCR also.

The femA PCR and nuc PCR showed good correlation in 15 out of 16 isolates. However, 22 S. aureus isolates were negative for both femA and nuc genetic markers. This is suggestive of likely mutations or deletions in the nuc gene also. However, further investigations are being carried out to understand this observation.

Mutations in the auxiliary genes such as the fem, could explain the absence of any phenotypic expression of resistance in MSSA isolates though these isolates contained mecA gene [30,31]. We propose to examine this phenomenon in future in several MSSA isolates. All the MRSA isolates that showed positive femA PCR, had MICs greater than 256 µg/mL; yet there were 3 isolates with MICs > 256 µg/mL that showed no femA gene amplification. It is known that methicillin resistance could manifest with or without mecA. Auxiliary genes like femA influence the extent of resistance [14] implying that there could be other mechanisms that circumvent absence of femA gene to confer methicillin-resistance.

Assuming that mutations in the femA gene sequence at the primer annealing sites could have resulted in non-amplification of PCR product in some isolates, we attempted to amplify the full length femA gene by designing primers flanking femA as described previously. Surprisingly, amplification of the whole gene was seen in only in some of those isolates which were positive with femA primers. One MSSA isolate which was negative for femA showed amplification of a truncated (smaller) (approximately 700 bp instead of 1312 bp) product, suggesting possible deletions in the femA gene. We are sequencing this smaller PCR product. Four femA positive isolates did not amplify the full length PCR product indicating that there are variations in primer annealing sites flanking the femA gene.

Re-visit to the GenBank database for any new entries of femA gene, we found 7 new sequences of S. aureus that have recently been submitted in April/May 2012 (Table 2, B) which showed significant variations in their sequences compared to the 11 femA gene sequences (Table 2, A) which were used in the design of the PCR primers that we evaluated in this study. Of these sequences, a strain JKD6159 showed two nucleotide variations in the sequence flanking the femA gene (4th & 13th base downstream of femA) which could probably explain the non-amplification of the full length femA gene sequence in our isolates also. Significant polymorphisms in the recently submitted femA gene sequences suggest the
femA gene variations are quite common, may play important role in the expression of MRSA and in the identification of S. aureus in clinical isolates. Our study shows that polymorphisms in femA gene sequences are present in both MRSA and MSSA isolates in India and requires further investigations such as sequencing to characterise the genome in general and femA in particular. We have initiated a detailed investigation of genetic variations in the femA gene of S. aureus, its relevance to the drug resistance phenotype and to examine any role for these genomic variations in molecular epidemiology of clinical isolates of S. aureus.

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