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Heterogeneity in *femA* in the Indian Isolates of *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 *fem* or *nuc* along with the methicillin-resistance determinant, *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 *mecA*, 16S rRNA, *femA* and *nuc* markers. While *mecA*PCR and 16S rRNAPCR results were consistent with other studies, *femA* and *nuc* showed dramatic variation in detection rate (sensitivity) of *S. aureus* 29.6% and 53.7% respectively. Evidences are presented to demonstrate the absence of *femA*. Our attempt to amplify the complete *femA* gene using sequences flanking *femA* further confirmed these results and also indicated that variations exist even in the genomic sequences around *femA*. Our data reveals the need for exercising care while using primers designed on sequences of constitutive genes like *femA* and *nuc* for PCR based identification of *S. aureus* species. Though geographic variations in the genome of *S. aureus* have previously been reported from around the world, we present here evidence for the first time from India for absence of *femA* and also for probable variations in the sequences around the *femA* gene in clinical isolates of *S. aureus*.

Keywords: *femA*; *mecA*; *nuc*; PCR; *S. aureus*

1. Introduction

Staphylococcus aureus is one of the major causes of wide spread 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 *Staphylococcus aureus* to develop resistance to antibiotics and remain non-responsive to treatment [3]. Shortly after the introduction of methicillin, reports of Methicillin-resistant *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 *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 *mec* (SCC*mec*), a mobile genetic element, is composed of the *mec* gene complex which includes *mecA*, its regulatory genes *mecI*, *mecR* and a *ccr* gene complex. The latter encodes site specific recombinases, namely *ccr*A/B, which in-turn regulate the mobility of SCC*mec* [8]. Significant geographic variations have been found in the structural organization of the SCC*mec* and these variations have been used to classify the SCC*mec* types [9]. However, *mecA* alone does not solely confer the methicillin resistance. Studies have shown that *fem* (factors essential for methicillin-resistance) or the auxiliary genes like *fem A/B/X* in addition to *mecA* are also impor-

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tant in the expression of methicillin resistance [10]. The *femABX* operon encodes factors which are responsible for the formation of pentaglycine bridges in the cell wall of *Staphylococci* [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 microbeology laboratory. These tests have limitations and frequently show variations [12]. Although the detection of *mecA* gene remains the gold standard for detecting methicillin-resistance, its detection alone does not confirm the presence of *Staphylococcus aureus* [13] and there is no consensus on the molecular target that could be used to confirm the *S. aureu*s species. Constitutively expressed genes such as *femA*, *fem*B and *nuc* are being used as molecular targets for the identification of *Staphylococcus aureus* [14,15]. Polymorphisms within these constitutive genes have been reported [16] worldwide and also failures to confirm the species of *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*mec* or the genome of *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 molecular markers of MRSA namely, 16S rRNA, *mecA*, *femA* or *nuc*. We are reporting here absence of *femA* gene in several clinical isolates of *S. aureus* (both MRSA and MSSA), poor sensitivity of *nuc* as a target for *S. aureus* identification and probable variation in the sequences flanking the *femA*. These findings have serious implications for molecular diagnosis of *S. aureus* and are reflective of the geographic changes occurring in the genome of *S. aureus*.

2. Materials and Methods

54 isolates of *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 *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.

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.

2.2. Polymerase Chain Reaction

All the primer sequences listed **Table 1** were procured from Eurofins Genomics India Pvt Ltd. Multiplex-PCR mixture for *mecA*, *femA* and 16S rRNA consisted of the following; 1 × PCR mixture (Fermentas Life Sciences/ HiMedia) containing 1.5 mM $MgCl_2$, 50 pM of each 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.

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 *nuc* gene in these isolates. The PCR reaction mixture contained in 20 µL final volume 50 pM of *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 *femA* flanking primers were performed in a volume of 20 µL containing 10 µL of DNA lysate, containing 1.7 mM $MgCl_2$, 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.

3. Results

3.1. 16s rRNA, *femA* and *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 Staphylococci genus, *femA* for *S. aureus* and *mecA* for methicillin resistance. All the isolates tested positive for the genus specific 16S rRNA 886 bp product. The 293 bp *mecA* was detected in all 26 (100%) MRSA isolates, 16 (61.5%) MSSA isolates and 1 CoNS isolate.

Table 1. Information related to the PCRs performed in this study.

Name of the Primers	Sequence 5'-3'	Product Size bp	Reference
mecA-F	ACG AGT AGA TGC TCA ATA TAA	293 bp	[19]
mecA-R	CTT AGT TCT TTA GAG ATT GA		
femA-F	CGA TCC ATA TTT ACC ATA TCA	450 bp	
femA-R	ATC ACG CTC TTC GTT TAG TT		
16s-1	GTG CCA GCA GCC GCG GTA A	886 bp	[20]
16s-2	AGA CCC GGG AAC GTA TTC AC		
*nuc*1	GCG ATT GAT GGT GAT ACG GTT	270 bp	[15]
*nuc*2	AGC CAA GCC TTG ACG AAC TAA AGC		
fem-A1	AGA CAA ATA GGA GTA ATG AT	509 bp	[14]
fem-A2	AAA TCT AAC ACT GAG TAA TGA T		
FSF-*femA*	AAC GAG AGA CAA ATA GGA GTA ATG A	1312 bp	
FSR-*femA*	TCA TGT TTT GAT AAT TCC CTT CC		

mecA-F: *mecA*-Forward Primer, *mecA*-R: *mecA*-Reverse, *femA*-F: *femA*-Forward Primer, *femA*-R: *femA*-Reverse Primer, 16s-1: 16S rRNA Forward Primer, 16s-2: 16S rRNA Reverse Primer, *nuc*1: *nuc* Forward Primer, *nuc*2: *nuc* Reverse Primer, *fem-A*1: *femA* Forward Primer, *fem-A*2: *femA* Reverse Primer, *FSFfemA: Flanking Sequence femA forward Primer*, *FSRfemA: Flanking Sequence femA Forward Primer*.

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**).

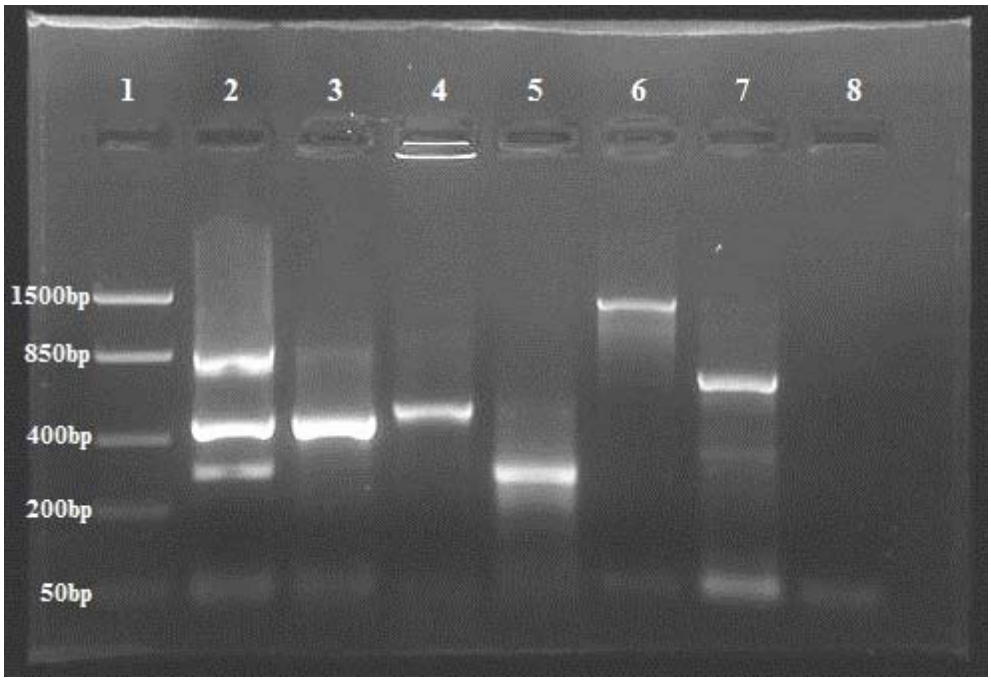
4. Discussion

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

Table 2. List of *femA* gene sequences reviewed from the GenBank Database.

S.No	Strain	GenBank accession	Gene location
A:			
1	MRSA252	NC_002952	1444550 - 1445812
2	USA300_FRP3757	NC_007793	1397007 - 1398308
3	JKD6008	NC_017341	1427188 - 1428456
4	COL	NC_002951	1420810 - 1422111
5	Newman	NC_009641	1417031 - 1418293
6	ED98	NC_013450	1391969 - 1393231
7	USA300_TCH1516	NC_010079	1410993 - 1412255
8	TCH60	NC_017342	1939657 - 1940994
9	RF122	NC_007622	1344801 - 1346102
10	MW2	NC_003923	1381193 - 1382455
11	Mu3	NC_009782	1456933 - 1458195
B:			
12	JKD6159	NC_017338	1365979 - 1367241
13	N315	NC_002745	1379204 - 1380466
14	Mu50	NC_002758	1455533 - 1456795
15	T0131	NC_017347	1428806 - 1430068
16	04-02981	NC_017340	1413795 - 1415057
17	HO 5096 0412	NC_017763	1357669 - 1358931
18	TW20	NC_017331	1470704 - 1471966



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.

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

Table 3. Summary of results phenotype vs genotype.

Genotype	Phenotype						Total (54)*
	MRSA (26)*		MSSA (26)*		CoNS (2)*		
	+	–	+	–	+	–	+
mecA	26 (100%)	0 (0%)	16 (61.5%)	10 (38.5%)	1 (50%)	1 (50%)	43 (79.6%)
16S rRNA	26 (100%)	0 (0%)	26 (100%)	0 (0%)	2 (100%)	0 (0%)	54 (100%)
femA	9 (34.6%)	17 (65.4%)	7 (26.9%)	19 (73.1%)	0 (0%)	2 (100%)	16 (29.6%)
nuc	15 (57.7%)	11 (42.3%)	14 (53.8%)	12 (46.2%)	0 (0%)	2 (100%)	29 (53.7%)
femA full length	7 (26.9%)	19 (73.1%)	6 (23.1%)	20 (76.9%)	0 (0%)	2 (100%)	13 (24%)

*Total Number of Isolates.

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] and there are no reports which specifically investigated the predictive value of *femA* gene for the identification of *S. aureus*. 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 auxillary 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*. Auxillary 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 (4^{th} & 13^{th} 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, could be region specific, 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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