Identification and Discrimination of Methicillin Resistant *Staphylococcus aureus* Strains Isolated from Burn Wound Sites Using PCR and Authentication with MALDI-TOF-MS

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Abstract The present study demonstrates isolation and identification of methicillin resistance *Staphylococcus aureus* (MRSA) strains in the samples collected from burn patients. About 106 swab samples were collected from burn patients of >40% burn injury and were subjected to isolation using nutrient agar followed by screening using Me Re Sa selective medium agar. A total of 10 isolates with identity to *S. aureus* were obtained and further authenticated using Polymerase Chain Reaction and matrix assisted laser desorption/ionization time of flight mass spectrometry analysis. Presence of *mec A* gene and the peak pattern observations suggested seven of the 10 isolates are MRSA. Thus, the present study emphasizes the process of identification of MRSA using two different bio-analytical techniques, which authenticate the presence of MRSA.

Keywords Burn injury · Me Re Sa selective medium · *S. aureus* · PCR · MALDI-TOF-MS · Methicillin resistance *Staphylococcus aureus*

Staphylococcus aureus is a major pathogen associated with hospital and community acquired infections around the world. Before availability of antibiotics, invasive infections caused by *S. aureus* were often fatal [1]. However, introduction of penicillin greatly improved the prognosis for patients with severe staphylococcal infections, but after a few years of clinical use, resistance appeared owing to the

production of β -lactamases [2]. Methicillin was designed to have resistance towards β -lactamase, but methicillin resistant *S. aureus* (MRSA) strains that were resistant to β -lactam antibiotics was identified soon after methicillin introduced into clinical practice [3] and which was predominant in hospital acquired infections and community-acquired infections as well [4].

In clinical practice infection was a major drawback and infection related mortality was encountered in burn patients. Thus, measures to prevent and treat infections were essential for the survival of patients with extensive burns [5]. In order to avoid mortality in burn patients, accuracy and promptness in detection of MRSA for suitable antibiotic therapy for patients infected with these strains was of high demand [6].

The major problem associated with MRSA infection lies in identification of strains. The delay in process of identification worsens the situation [7]. With reference to the detection of methicillin resistance in *S. aureus*, several methods including classic methods of determining minimum inhibitory concentrations (MIC), screening techniques with solid culture medium containing oxacillin or ceftioxin [8], methods that detect *mec A* gene or its protein product (PBP 2 protein) [9] and identification using whole cell protein profiles by matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) was also found advisable [10].

Thus, to facilitate, ease and to authenticate MRSA strains from samples obtained from burn patients, two different test methods were attempted, viz., PCR based *nuc* and *mec A* gene identification and MALDI-TOF-MS. In brief, present approach involves isolation of microbes from external wound sites of burn patients from various hospitals in Chennai, Tamil Nadu. Identification/differentiation and characterization of MRSA was made using regular microbial

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Fig. 1 a Phylogenetic tree constructed for *S. aureus* based on 16S rDNA analysis (Clinical strain of MRSA S1). **b** Phylogenetic tree constructed for *S. aureus* based on 16S rDNA analysis (Clinical strain

of MRSA S2). **c** Phylogenetic tree constructed for *S. aureus* based on 16S rDNA analysis (Clinical strain of MRSA S3)

Fig. 1 continued

methods (phenotypic and genotypic) and also using MALDI-TOF-MS. The results were compared and authenticated.

Materials and Methods

Collection of Specimens

A total of 106 burn wound infected samples were collected using sterile swabs from external wound sites of burn patients from hospital wards, labeled properly and transported to the Microbiology laboratory of Central Leather Research Institute, Chennai within 2–3 h of collection using a cool pack and were cultured in Nutrient broth medium for 24 h at 37°C. The cultures were then serially diluted and spread on to Nutrient agar medium (Hi Media, India) and were incubated at 37°C for 24 h.

Identification and Phenotypic Study of Bacterial Strains

About one hundred and six bacterial strains of hospital origin obtained from Nutrient agar plates from the above

experiment and four *S. aureus* type strains obtained from MTCC (Microbial Type Culture Collection, Chandigarh, India) were used for identification. *S. epidermidis* (MTCC 3382, MTCC 6810) was used as negative control in PCR amplification. *Escherichia coli* (MTCC 521) was used as negative control for biochemical tests. The strains were identified based on growth characteristics on Barid Parker Agar Base with Egg Yolk Tellurite Emulsion (Hi Media, India), Gram staining, 3% potassium hydroxide (KOH) and catalase test [11] and spot inoculation on Mueller–Hinton agar (Hi Media, India) containing 1% of Methicillin (Sigma Chemical Co., St. Louis, Mo.) [12]. Further to identify the strains with methicillin resistance, cultures obtained were grown on Me Re Sa selective agar medium (HiMedia, India) with 1% methicillin and incubated at 37°C for 24 h. To observe the morphological features of microbes isolated, scanning electron microscopy was attempted as per procedure [13] using Jeol 840 scanning electron microscope. The isolated cultures were maintained in 10% glycerol at –20°C until further use. Further authentication of *S. aureus* was done using polymerase chain reaction (PCR) according to the methods summarized by Ghebremedhin et al. [14].

Fig. 2 **a** Phylogenetic tree constructed for *mecA* gene based on gene sequencing (Clinical strain of MRSA S1). **b** Phylogenetic tree constructed for *mecA* gene based on gene sequencing (Clinical strain

of MRSA S2). **c** Phylogenetic tree constructed for *mecA* gene based on gene sequencing (Clinical strain of MRSA S3)

Fig. 2 continued

Preparation of Chromosomal DNA for PCR (Genotypic Identification)

Genomic DNA from the microbial cultures was prepared as reported earlier [15].

PCR for *mec A* and *nuc* Gene Detection

PCR was performed using extracted genomic DNA for simultaneous detection of *nuc* [16] and *mec A* [17] according to Merlino et al. [18]. The control organisms include *S. aureus* MTCC 3610, *S. aureus* MTCC 7443, *S. epidermidis* MTCC 6810 and *S. epidermidis* MTCC 3382. Ten µl of PCR product was analyzed by agarose gel electrophoresis [2% agarose prepared in TAE (1 mM EDTA/40 mM Tris acetate, pH 8.0)] at 50 V for 60 min. Gels were stained with ethidium bromide and were photographed under ultraviolet light. The obtained PCR product was purified and was sequenced according to the procedure described elsewhere [19].

Sample Preparation and Analysis for MALDI-TOF-MS of Intact Bacterial Cells

α -Cyano-4-hydroxycinnamic acid was dissolved in a 2:1 (H₂O: ACN) solution containing 0.1% TFA for the preparation of saturated matrix [20] and the sample was prepared according to Edwards-Jones et al. [21].

Spectra were obtained using a Voyager-DE PRO Biospectrometry Workstation MALDI-TOF-MS in positive-ion mode with an acceleration voltage of 26 kV and analyzed using Powerful Voyager Version 5 Software with Data Explorer software and calibrated with Bovine serum albumin as an external standard at *m/z* ratio of 100–7,000 kDa. Mass spectrum was collected in positive and negative modes and each spectrum was the sum of ions obtained from 100 laser shots performed in five different regions of same well. Only peaks with intensities 0.4 mV after baseline subtraction was considered in analysis and the results were analyzed using Origin Pro statistical software version 8.0.

Fig. 3 **a** Strain specific MALDI-TOF-MS profile of clinical isolate S1. **b** Strain specific MALDI-TOF-MS profile of clinical isolate S2. **c** Strain specific MALDI-TOF-MS profile of clinical isolate S3.

d Strain specific MALDI-TOF-MS profile of clinical isolate S10. **e** Strain specific MALDI-TOF-MS profile of reference strain of *S. aureus*

Antibiotic Susceptibility Tests

Disk diffusion assay was performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Experiments were done in triplicates and the zone of inhibition was measured using antibiotic zone scale (Hi-Media, India) and antibacterial activity expressed in terms of inhibition zone (mm).

Results

The isolation and identification procedures, viz., gram-positive cocci; catalase positive; positive KOH test and growth on Barid Parker Agar Base with Egg Yolk Tellurite Emulsion without supplementation of methicillin [22], growth in methicillin spot inoculation test provided 10 microbial isolates as *S. aureus* (S1–S10). Further, the sequence of 3570 bases of 16s rDNA analysis of three MRSA strains (S1, S2 and S3) used for MALDI studies were 99–99.8% similar to the 16s rDNA sequences of *S. aureus*. Phylogenetic tree constructed showed that the isolated strains belong to *S. aureus* (Fig. 1a–c).

Genotypic and phenotypic expression for methicillin resistance in the isolated clinical strains of *S. aureus* was examined using PCR. All isolates tested showed positive for *nuc* gene confirmed *S. aureus* and further the presence of *mec A* gene in seven isolates (S1–S7) confirmed as MRSA. Negative control strains do not contain *mec A* gene. The amplified *mec A* gene is sequenced and the phylogenetic tree (Fig. 2a–c) results showed that the gene sequence was found similar to *S. aureus* strain penicillin binding protein 2a (*mec A*) gene, using NCBI BLAST search (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi).

Followed by PCR analysis, strains confirmed as MRSA were subjected to MALDI-TOF-MS analysis for additional confirmation. Figure 3a–e illustrates MALDI-TOF-MS spectrum of clinical and reference bacterial species and most spectral peaks were observed in the representative mass range [23].

Fingerprint observations of *S. aureus* strains studied showed three common peaks with m/z of 5514, 6013 and 6587 respectively, which were observed in all *S. aureus* isolates and peaks of m/z 1729, 2288, 5514, 6004, 6576 and 6885 found only in MRSA strains [21]. Presence of group peaks around 2300 in S1, S2 and S3 suggests the presence of *mec* element, which contributes to this group of peaks and is an agreement with previous studies [1].

Careful observation in the spectral profiles of MRSA in the present study does not provide a distinctive fingerprint (spectral profiles of S1 and S2 looks similar and S3 dissimilar) (Table 1) which suggests that the isolated MRSA strains S1 and S2 may be of same clonal origin and the

Table 1 Summary of the mass/charge values found in Staphylococcal isolates tested

S1	S2	S3	S10	Reference MRSA strain
708	703			673
796	749			704
	792			741
				793
1734	1729	1729		1731
		1835		
2204	2182	2195		2199
2292	2282	2289		2285
3451		3435	3441	
			4994	
5504	5514	5001	5057	5510
		5031	5121	
		5124	5444	
		5514	5510	
		5817	5734	
			5817	
6004	6013	6013	6010	6014
6576	6587	6133	6140	6552
6885	6903	6570	6590	6898
		6707	6703	
		6813	6816	
			6866	
		7100	7093	

S1 clinical isolate of MRSA1; S2 clinical isolate of MRSA2; S3 clinical isolate of MRSA3; S10 clinical isolate of MSSA10

strain S3 may differ in its clonal origin. Peak profile for all the isolates were summarized in Table 1. Over 90% of the peaks were reproduced in all replicates and relative intensities (but not absolute intensities) were closely comparable.

The results of the susceptibility tests for cultures obtained from clinical samples and type strains of *S. aureus* against 10 antibiotics were summarized in Table 2. Antibiotic inhibition zone diameters against the strains were in compliance with CLSI guidelines.

Discussion

Staphylococcus aureus was a major cause of both health-care associated and community acquired infections throughout the world [24]. Recently, emergence of MRSA despite extensive infection controls dominates and needs special attention [25]. Oxacillin has been used to replace methicillin since it was no longer the agent of choice for treatment of *S. aureus* infections. Detection of *mec A* gene by PCR was considered to be the “gold standard method” because of its simplicity and reproducibility [26]. In the

Table 2 Antibigram profiles of *Staphylococcus aureus* (methicillin resistant) clinical isolates and MTCC strains against selected antibiotics. Disk diffusion break points are based on CLSI (Clinical Laboratory Standards Institute, formerly NCCLS) guidelines

Microbial isolates	Am	A	C	E	G	M	Of	Ox	S	T
S1 ^a	S	R	S	R	R	R	R	S	R	R
S2	R	R	S	S	R	R	R	R	R	R
S3	R	R	S	S	R	R	R	R	R	R
S4	R	R	S	R	R	R	R	R	R	R
S5	S	R	S	R	R	R	R	S	R	R
S6	R	R	R	R	R	R	R	R	R	R
S7	R	R	S	R	R	R	R	R	R	R
S8	R	R	S	S	S	S	S	S	S	R
S9	S	S	S	S	S	S	S	S	S	S
S10	R	R	S	S	S	S	S	S	S	R
MTCC ^b 3610	S	S	S	I	I	S	I	S	I	I
MTCC 7443	S	S	S	I	S	S	I	S	I	I
MTCC 6810	S	S	S	I	I	S	I	S	I	I
MTCC 3382	S	S	S	I	I	S	I	S	I	I

Degree of susceptibility: *R* resistant, *I* intermediate; *S* susceptible

Antibiotics used in this study: *Am* amoxicillin, 30 µg; *A* ampicillin, 10 µg; *C* chloramphenicol, 30 µg; *E* erythromycin, 10 µg; *G* gentamicin, 10 µg; *M* methicillin, 5 µg; *Of* ofloxacin, 5 µg; *Ox* oxacillin, 5 µg; *S* streptomycin, 10 µg; *T* tetracycline, 10 µg

^a *Staphylococcus aureus*

^b Microbial type culture collection

present study, out of 106 isolates tested, 10 strains were found to be *S. aureus* based on gram staining, morphological features and 3% KOH test and growth in Me Re Sa agar supplemented with 1% methicillin and methicillin spot agar test further confirmed 7 of the 10 isolates as MRSA. From the 16s rDNA analysis and phylogenetic tree it was confirmed that the strains used in the present study belongs to *S. aureus*.

PCR results demonstrate 10 out of 106 isolates contain *nuc* gene and the presence of *nuc* gene was in complete agreement with the species determinations of *S. aureus* [27]. These strains were further tested for their methicillin resistance using PCR and seven strains had *mec A* gene which confirms their methicillin resistance. Results from PCR amplifications were in good agreement with growth in Me Re Sa agar supplemented with 1% methicillin and methicillin spot inoculation tests used in this study. The detection of both genes in an isolate confirms the identity of an isolate as MRSA [28]. Further, the gene sequence of purified PCR products and their phylogenetic tree confirms the identity of *mec A* gene.

According to Liang et al. [28] direct analysis of proteins from bacterial cell extracts by MALDI-TOF-MS has been a potential means for the identification of bacteria. Thus, in the present study the bacterial samples obtained from PCR

were reconfirmed using MALDI-TOF-MS and the mass spectral data was compared with reported literature [29]. The spectral profiles of MRSA and *S. aureus* exhibited the difference in their mass range of 100–7000 kDa under the experimental conditions as illustrated in the Fig. 3a–e. At a finer level of discrimination, particularly in the peak pattern differences between MRSA and *S. aureus* isolates can be observed within *m/z* ranges of 1000–2500, 5000–5500, 6000–6500 kDa.

In the present study, resistance observed for MRSA strains isolated from burn wound site was on par with previous reports [30]. However, isolates S8 and S10, showed resistance against ampicillin, amoxicillin and tetracycline and this observation was supported by the previous observations [31] implying resistant *S. aureus* traits were most common in hospital community. Apart from these, MTCC type strains did not exhibit any resistance towards the antibiotics tested. S8 exhibits intermediate resistance to erythromycin, ofloxacin, streptomycin and tetracycline. Layer et al. [31] observed a similar findings working with *S. aureus* strains in the hospital premises at Germany and found that most of *S. aureus* isolates were resistant to penicillin, ciprofloxacin, clindamycin, erythromycin and gentamicin.

Conclusion

In the present study, microbial colonies isolated from burn wound site subjected to screening for MRSA and *S. aureus* using standard procedures and the MRSA strains were further authenticated by their phenotypical and genotypical methods followed by MALDI-TOF analysis. We found among ten *S. aureus* isolates, seven isolates exhibited positive response to the said methods followed and suggests both the methods were suitable for identification of MRSA species.

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