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Frequency of *Staphylococcal* Cassette Chromosome *mec* Type IV and Type V in Clinical Isolates of Methicillin Resistant *Staphylococcus aureus*

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Abstract

MRSA is able to generate a modified variety of penicillin binding protein named as PBP2a instead of PBP which makes it resistant against penicillin and methicillin. Production of PBP2a is due to the presence of a gene on *Staphylococcal* cassette chromosome or SCC termed as "*mec-A* gene". SCC is a mobile genetic element carrying many resistance genes. It is because of current antibiotic selection pressure that by now there are eight types (I - VIII) of SCC*mec*. This research has been designed to determine frequency of SCC*mec* type IV and V in clinical isolates of MRSA. A total of 70 presumptive MRSA isolates collected from a tertiary care hospital of Lahore were cultured on blood agar, incubated overnight at 37˚C aerobically. Next day, they were examined for cultural characteristics, colonial morphology, gram stain, and biochemical profile. Confirmation of MRSA was done by phenotypic disk diffusion method according to (CLSI) 2013 guidelines. *mecA* gene was also detected at molecular level. Molecular identification of SCC*mec* type IV and V was done by Nested PCR strategy. A total of 50 isolates were confirmed to be MRSA Molecular detection of SCC*mec* type IV and V revealed that 11 isolates (22%) possess SCC*mec* type IV and only 2 isolate (4%) carries SCC*mec* type V. It is obvious from results that SCC*mec* type IV and V are present in our population too. Larger study (with larger sample size) might be undertaken to find out actual emergence of SCC*mec* type IV and V in our population.

Keywords

MRSA, SCC*mec* Type IV, SCC*mec* Type V

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1. Introduction

Distribution of methicillin resistant *staphylococcus aureus* (MRSA) is worldwide and its incidence varies depending upon geographical location of various regions. A survey report documented the prevalence of MRSA from 42% to 51% in Pakistan [1] [2]. Main causative agent responsible for this resistance is carriage by the MRSA strains *mecA* gene. *mecA* gene encodes an extra penicillin-binding protein (PBP2a). It has low affinity for all *β*-lactam antibiotics [3] [4]. Many techniques have been used for epidemiological typing of MRSA which helps to study evolutionary relationship of different MRSA strains. However, there is very little epidemiological data about frequency of SCC*mec* type IV and V in Pakistan.

Staphylococcal cassette chromosome *mec* is a mobile genetic element which is characterized by presence of two genetic elements *mec* gene complex, *ccr* gene complex and Junkyard regions. *mec* gene complex is composed of IS431*mec*, *mecA*, and its regulatory genes *mecR1*and *mecI*. Two important *ccr* gene complexes have been reported one carrying two adjacent *ccr* genes, *ccrA* and *ccrB*, and the second carrying *ccrC*. The *ccrA* and *ccrB* genes identified have been classified into four and five types respectively resulting in five classes of *mec* complex (A, B, C1, C2, and E), corresponding to which eight SCC*mec* types (I - VII) have been described [5] [6]. *Ccr* gene encodes cassette chromosome recombinase which integrates or excise the SCC*mec* into the recipient chromosome.

Type IV SCC*mec* is one of the most challenging types, which carries *mecA* gene and lack other resistance genes. Its structure is smaller than the other SCC*mec* types and carries functional recombinases, being shortest and most mobile it spreads in community more rapidly than other SCC*mec* types [7]. SCC*mec* type V structurally resembles SCC*mec* type IV as it lacks other resistance genes except *mecA* [6]. It has been reported that SCC*mec* type IV and V are replacing other SCC*mec* types I, II and III in hospital setting [8]. It is reported that 90% of SCC*mec* IV and 62% of SCC*mec* V strains carry PVL gene [9]. PVL cytotoxin is responsible for serious MRSA associated infections. Prevalence of SCC*mec* type IV and V has not yet reported in Pakistan. Herein we report frequency of SCC*mec* type IV and V from clinical isolates of MRSA. To best of our knowledge, this is the first study from our setup.

2. Material and Methods

This descriptive study was carried out in microbiology and resource laboratory at University of Health Sciences, Lahore, Pakistan. A total of 70 presumptive MRSA isolates were collected from a tertiary care hospital of Lahore. These isolates were obtained from various clinical specimens including blood (n = 5), pus (n = 45), sputum (n = 10), cardiovascular tip (n = 4), and tracheal aspirate (n = 6). All the isolates were cultured on blood agar and incubated overnight at 37˚C aerobically. After 24 hours they were examined for cultural characteristics, colonial morphology, Gram stain, and biochemical profile. 50 isolates were identified as MRSA using Gram stain catalase, coagulase and Dnase test. Methicillin resistance was confirmed using (30 µg) disc of cefoxitin by phenotypic disk diffusion method according to CLSI guidelines 2013. Following interpretive criteria was used to identify MRSA as: MRSA = ≤ 27 mm and MSSA = ≥ 27 mm.

3. DNA Extraction

DNA extraction was performed using suspension of isolated colonies prepared in sterile distilled water following protocol of bacterial DNA extraction kit (DNA_{zol} ® BD, USA).Two or three well isolated colonies of each confirmed MRSA isolate were suspended in 0.5 ml autoclaved distilled water. One ml of DNAzol was added in bacterial suspension. The mixture was vortexed vigorously for 15 - 20 seconds and stored at room temperature for 5 minutes then centrifuged for 1 min at 8000 g. 0.4 ml of isopropanol was added to the lysate for precipitation of DNA. The precipitated DNA was sedimented by centrifugation at 6000 g for 5 minutes, supernatant was removed and 0.5 ml of DNAzol was added to the DNA pellet. The DNA pellet was vortexed until it was completely dispersed. It was centrifuged at 6000 g for 5 minutes. The supernatant was removed and washed the DNA pellet by mixing it with 1ml of 75% chilled ethanol. Again it was centrifuged at 7000 g for 5 minutes. The ethanol wash was decanted and stored the eppendrof vertically for 15 minutes to evaporate any residual ethanol. The DNA pellet was dissolved in 50 ul molecular grade water

4. Amplification of SCC*mec* Type IV and V

Amplification of DNA was done by Conventional PCR strategy using specific primers (**Table 1**) for SCC*mec*

Table 1. Sequences of primers used for PCR amplification of loci.

Target gene	Primers	Primer sequences	Amplicon size	References
mecA gene	*mec*A-F	5'-AAAATCGATGGTAAAGGTTGGC-3'	533 bp	[26]
	*mec*A-R	5'-AGTTCTGCAGTACCGGATTTGC-3'		
SCC*mec* type IV	SCC*mec* type IV-F	5'-AGTTTCTCAGAATTCGAACG-3'	311 bp	[27]
	SCC*mec* type IV-R	5'CCGATATAGAATGGGTTAGC-3'		
SCC*mec* type V	SCC*mec* type V-F	5'-TTCTCCATTCTTGTTCATCC-3'	377 bp	(Milheirico *et al*., 2007)
	SCC*mec* type V-R	5'AGAGACTACTGACTTAAGTGG3'		

type IV and V that were commercially synthesized by Gene link. Commercially prepared master mix provided by Fermentas was used in reaction mixture. PCR Reaction was performed in 25 ul reaction volume composition of reaction mixture is given as, Master mix (2x) = 12.5 ul, DNA template = 5 ul, Forward primer (100 pmol) = 1.2 ul, Reverse primer (100 pmol) = 1.2 ul, ddH_2O = Upto 25 ul. PCR was optimized by predanaturation at 94˚C for 3 min followed by 94˚C for 45 s, 35 cycles of 51˚C for 45 s, 72˚C for 1min ending with final extension step at 72˚C for 10min and hold at 4˚C. The products were electrophoresed in 2% agarose stained with ethidium bromide, and video images obtained using gel documentation system (Becton Dickinson, USA).

5. Results

5.1. Bacterial Isolates

A total of 70 *S. aureus* isolates were collected out of these 70, 50 were confirmed to be MRSA, 12 strains were confirmed to be MSSA. The rest 8 were neither MRSA nor MSSA and they were not proceeded further. Those 50 MRSA strains were isolated from different sources including Pus (92%); sputum (4%), blood (2%); tracheal aspirate (2%); cardiovascular tip (2%)

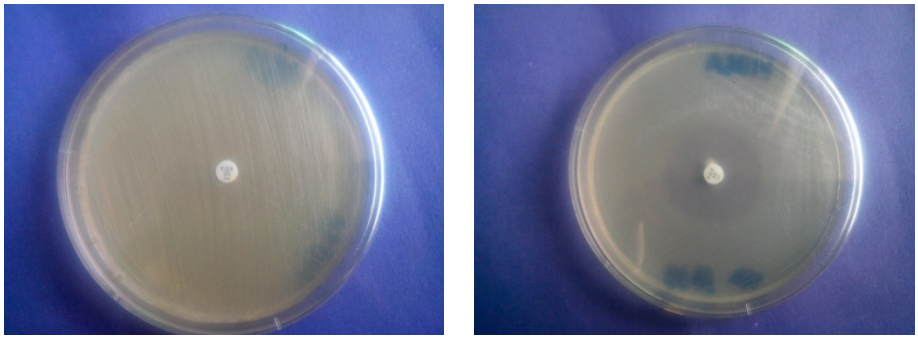
5.2. CLSI Phenotypic Detection of MRSA

- These isolates were methicillin resistant phenotypically (**Figure 1**) and also found to have *mecA* gene by PCR (**Figure 2**).

5.3. Molecular Detection of SCC*mec* Type IV and V

A few PCR amplified representative *mec*A, SCC*mec* type IV and V genes are presented in **Figure 3** and **Figure 4** respectively.

The Molecular detection of SCC*mec* type IV and V revealed that 11 isolates (22%) possess SCC*mec* type IV and only 2 isolate (4%) carries SCC*mec* type V as given in **Figure 5**.



(a) (b)

Figure 1. (a) Disc diffusion test showing no clear zone of inhibition for cefoxitin disc; for *S. aureus* (MRSA). (b) Disc diffusion test showing a clear zone of inhibition for cefoxitin disc 27mm in diameter for *S. aureus* (MSSA).

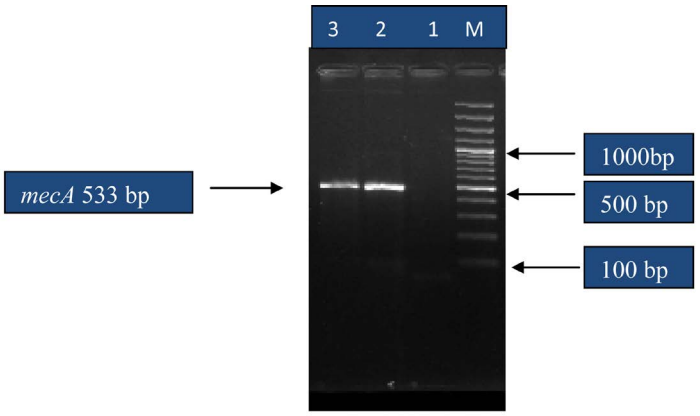


Figure 2. Ethidium bromide stained agarose gel demonstrating the PCR amplification of *mecA* gene. **Lane M**, DNA molecular size marker (100 bp DNA ladder; Biorad) **Lane 1**, negative control. **Lane 2**, positive control. **Lane 3**, *mecA* gene positive.

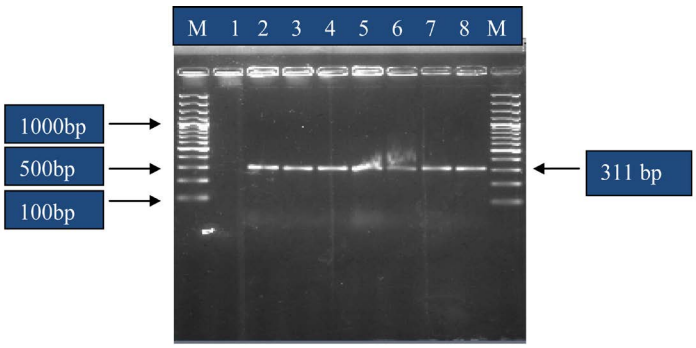


Figure 3. Ethidium bromide stained agarose gel demonstrating the PCR amplification of SCC*mec* type IV gene. **Lane M**, DNA molecular size marker (100 bp DNA ladder; Biorad) **Lane 1**, negative control. **Lane 2**, positive control. **Lane 3-8**, SCC*mec* type IV gene positive.

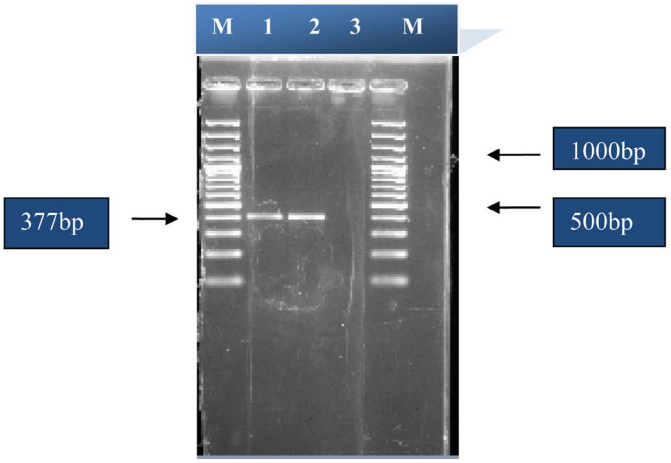


Figure 4. Ethidium bromide stained agarose gel demonstrating the PCR amplification of SCC*mec* type V gene. **Lane M**, DNA molecular size marker (100 bp DNA ladder; Bio-Rad) **Lane 1**, positive control. **Lane 2**, SCC*mec* type V gene positive, **Lane 3** negative control.

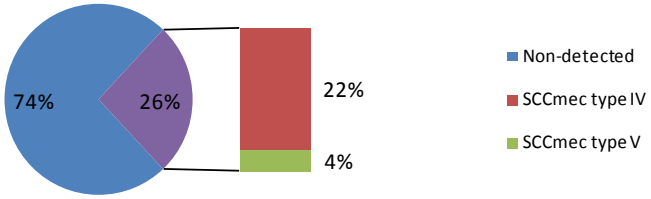


Figure 5. Frequency of SCC*mec* type IV and Type V amongst MRSA strains n = 50.

6. Discussion

In many countries including Pakistan there is an increasing trend towards the usage of antibiotics [10]. Improper use of antibiotics has resulted in the evolution of many resistant pathogens like MRSA. *mecA* gene which encodes an extra penicillin-binding protein (PBP2a) leads towards the resistance to *β*-lactam antibiotics. The *mecA* gene is located on the Staphylococcal cassette chromosome *mec* (SCC*mec*), which carries methicillin-resistance gene and other antibiotic resistance genes integrated in the *S.aureus* chromosome. Detailed molecular characterization of SCC*mec* types has not been done in Pakistan; therefore, this study is designed to detect the genotypes SCC*mec* type IV and V. In this study, the frequency of SCC*mec* type IV and V is evaluated and the results showed SCC*mec* type IV = 12 (21%) and V = 2 (4%) in our 50 clinical MRSA isolates. Frequency was determined by PCR strategy as it is more sensitive and specific method used for the detection of different microbiological and viral pathogens.

We first identified the isolates on the basis of morphological and biochemical characteristics followed by the detection of MRSA by disc diffusion method using 30 µg disc of cefoxitin as recommended by CLSI. As molecular methods were found to be 94% sensitive and specific in detecting MRSA isolates [11] therefore, MRSA was also tested for the presence of *mecA* gene by using PCR. Results showed 533-bp fragment of *mecA* gene which was amplified from DNA of all the isolates. PCR results were consistent with phenotypic detection method of MRSA and in complete agreement with a similar study [12]. These isolates were used for subtyping SCC*mec* by using specific primers. We isolated type IV and V solely from pus specimens. This finding is similar to a study conducted in India where highest number of these types was isolated from patients suffering from Skin and Soft Tissue Infections (SSTI) [13]. Ali and co workers also had reported highest number of these strains from pus specimen [14].

Among eleven Asian countries, nine have reported the most prevalent type SCC*mec* type III while SCC*mec* type IV has been reported in Korea (n = 2), Philippine (n = 2) and Taiwan (n = 2) [15]. A study in Iran reports prevalence of type IV or V about 14.8% [16]. One of the first reports about MRSA in Pakistan, from AKUH, Karachi, showed the prevalence of two SCC*mec* types *i.e.* type III from HA-MRSA and type IV from CA-MRSA [15]. A combined study on MRSA isolates from India and Pakistan revealed that 38% isolates belonged to SCC*mec* type IV, which were solely isolated from Pakistan while Indian isolates carried SCC*mec* type III and IIIa [17]. In our results, PCR based genotypic detection of SCC*mec* type IV and V revealed that SCC*mec* type IV was more prevalent.

SCC*mec* type V isolated in present study is not yet reported in other Asian countries such as Pakistan, Philippines, Singapore, Thailand, Vietnam and Saudi Arabia, Japan, Taiwan and Indonesia [15]. However in Mumbai, India, SCC*mec* V has also been documented along with SCC*mec* IV as SCC*mec* V = 41% and SCC*mec* IV = 34% [9]. A study from Switzerland has reported 78% SCC*mec* type IV and 15% SCC*mec* type V [18]. In USA, Japan and Korea, the most prevalent SCC*mec* types were I and II [19] [20].

The number of SCC*mec* IV and V MRSA strains isolated in the laboratory have been increasing every year from 2006 to 2009; it is apparent that these clones may be replacing other clones in the hospital setting [21]-[23]. Many studies conducted in hospital setting show that MRSA strains SCC*mec* IV and V which were thought to be associated predominantly with CA-MRSA are also prevailing in hospitals [24]. Just as SCC*mec* IV has spread over Europe and Asia, SCC*mec* V may also turn out to be a powerful global epidemic MRSA strain in the next few years [9]. So, for these reasons, our study was very significant not only to detect MRSA, but also to identify SCC*mec* type V.

It might be investigated whether patients with type IV and V strains had some risk factors for MRSA infections or not. As reported by De'souza, patients having type IV strains, 24% had risk factors and 76% had no

documented risk factors. 32% of the patients with SCC*mec* type V strain carried MRSA infections because of some risk factor while the rest had no risk factors [25]. Keeping in view, the increasing rate of SCC*mec* type IV and V in Pakistan and neighbouring countries it should be investigated that either these types are circulators or persisting invaders. Sub typing of SCC*mec* type IV needs to be done to identify the most frequent subtype native to Pakistan. Further molecular analysis of these MRSA isolates by pulsed field gel electrophoresis or MLST (Multi Locus Sequence Typing) may provide much useful information regarding origin and epidemiology of local isolates.

New strains which seem to be imported from expanding community or from other countries are adding up in our SCC*mec* pool. Infections caused by these pathogens lead to prolonged period of illness, financial burden, less number of drugs as choice for treatment and increased risk of mortality. Although antibiotic resistance is a natural phenomenon, yet this process is accelerated by misuse of antibiotics. Unnecessary recommendation of antibiotics should be avoided so that we can somehow reduce the emergence of pan-resistance pathogens like VRSA, otherwise we would not be left with any choice of antibiotics [10].

7. Conclusion

SCC*mec* type IV and V are present in our population too. A larger study (with larger sample size) might be undertaken in future to find out actual emergence of SCC*mec* type IV and V in our population.

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