Amplification of mecA gene in multi-drug resistant Staphylococcus aureus strains from hospital personnel

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Abstract
Background: Antibiotic resistance is common among bacterial pathogens associated with both community acquired and nosocomial infections. In view of the present problem of drug resistance we investigated the prevalence of methicillin resistant Staphylococcus aureus (MRSA) and amplified the mecA gene in the isolates from the hand swabs of the hospital personnel.

Methodology: The nuc gene was amplified to characterize these isolates at species level. The S. aureus isolates were analyzed for their susceptibility to different classes of antibiotics using the disk diffusion method. The spot inoculation test was performed to detect methicillinase production in these isolates.

Results: In the screened isolates of S. aureus, 14.2 and 15 kb of plasmids were present. These isolates showed pronounced resistance against β-lactam antibiotics including second- and third-generation cephalosporins, aminoglycosides, macrolides and fluoroquinolone. Some of the isolates included in this study were resistant to three or more antibiotics. Expression of methicillinase was detected by spot inoculation test, and a few of the isolates were found to produce methicillinase. Moreover, mecA gene was also amplified. Of 17 isolates only 7 showed presence of mecA gene.

Conclusion: This study highlights the emerging trend of multiple drug resistance in S. aureus strains isolated from hospital personnel working in a premier hospital in North India.

Key Words: methicillin resistance, Staphylococcus aureus, plasmid, mecA gene, hospital personnel.


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Introduction
Antimicrobial resistance is steadily rising among bacterial pathogens associated with both community and health care-associated infections [1,2]. Among the most important of these pathogens are vancomycin-resistant enterococci (VRE) and methicillin-resistant Staphylococcus aureus (MRSA) [3]. The majority of MRSA strains have been associated with hospital-acquired colonization and infections [4]. MRSA strains in nursing homes and long-term care facilities are usually of nosocomial origin [5], and most MRSA strains isolated from patients upon admission to hospitals or nursing homes can be traced to a previous stay in a similar setting [6]. In the mid-1990s, however, infections with MRSA began occurring in communities in patients who had no history of previous hospital stays and known risk factors for acquisition of MRSA infection [7]. These MRSA isolates are referred to as community MRSA (cMRSA) and have been reported from many parts of the world, including Canada [8], the United States of America, [7,9], Australia, the Southwest Pacific [10], and different European countries [11].

Risk for colonization or infection with S. aureus is highest in patients with diabetes mellitus, intravenous drug users, and patients undergoing hemodialysis, surgical patients, and patients with acquired immunodeficiency syndrome [12]. In the last few years, however, reports of patients with serious MRSA infections who had no known risk factors or exposure to a health care setting have been increasing [13-19]. The distinctive properties of the community acquired MRSA strain compared to nosocomial strains include a much more susceptible antimicrobial phenotype because of the presence of a much smaller staphylococcus cassette chromosome mecA (type IV) [20] and the
presence of different exotoxin gene profiles, including Panton-Valentine leukocidin [6,11].

In view of the background outlined above, we initiated our investigations to study the resistance pattern of *S. aureus* strains isolated from health workers in a hospital environment. Moreover, we intended to characterize MRSA strains among these isolates by amplifying *mecA* gene and methicillinase assay.

**Materials and Methods**

*Sample collection*

Seventeen *S. aureus* isolates were obtained from hand-swab samples of thirty-four hospital personnel (doctors, nurses and ward boys) of the general ward of in-patients at the Post Graduate Institute of Medical Education and Research, Chandigarh, India.

**Identification and molecular characterization of *S. aureus***

The isolates were identified as *S. aureus* morphologically on culture, by Gram staining and by using traditional biochemical tests, including catalase, coagulase, slide agglutination and phosphatase test [21].

Acid production was also detected from glucose, D-maltose, D-trehalose, D-mannitol, sucrose and β-D-fructose. Species identification was confirmed by polymerase chain reaction (PCR) amplification of thermostable nuclease gene (*nuc*) using the primers, *nuc*-F (5’GCGATTGTGATGTTACGGTT-3’) and *nuc*-R (5’-AGCCAAGCCTTGACGAACTAAAGC-3’) as described by Brakstad et al. (22).

PCR conditions for *nuc* gene comprised a thermal temperature of 94°C for 5 minutes, followed by 34 cycles of 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute, followed by final extension for 10 minutes at 72°C.

**Antibiotic susceptibility testing**

Antimicrobial resistance of the isolates was determined by the disk diffusion method. The following antimicrobial agents at the indicated concentrations were tested: amikacin 30 µg/ml; ceftriaxone 30 µg/ml; ciprofloxacin 10 µg/ml; gentamicin 10 µg/ml; tobramycin 30 µg/ml, amoxycillin 30 µg/ml, methicillin 5 µg/ml, oxacillin 1 µg/ml, penicillin G 10 µg/ml, kanamycin 30 µg/ml, cefoxitin 30 µg/ml, cefotaxime 30 µg/ml, doxycyclin 30 µg/ml, erythromycin 10 µg/ml, chloramphenicol 30 µg/ml. Throughout this study, results were interpreted according to the criteria of the Clinical Laboratory Standards Institute [23].

**Determination of minimum inhibitory concentration (MIC)**

The four strains of *S. aureus* were inoculated into Brain Heart Infusion (BHI) broth in test tubes and grown to stationary phase for 24 hours at 37°C up to 108-109 CFU/ml. 50 µl of overnight growth culture diluted to 105-106 CFU/ml was inoculated into fresh BHI (50µl) containing varying concentrations, serially diluted (512-2 µg/ml) antibiotics listed in Table 1.

**Table 1. MIC of some selected antibiotics against MRSA strains, MIC (µg/ml).**

<table>
<thead>
<tr>
<th>MRSA Strains</th>
<th>M</th>
<th>P</th>
<th>Ox</th>
<th>Ci</th>
<th>Ce</th>
<th>Tb</th>
</tr>
</thead>
<tbody>
<tr>
<td>S13</td>
<td>8</td>
<td>-</td>
<td>&gt;64</td>
<td>-</td>
<td>&gt;16</td>
<td>-</td>
</tr>
<tr>
<td>S14</td>
<td>16</td>
<td>&gt;64</td>
<td>32</td>
<td>16</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S19</td>
<td>&gt;32</td>
<td>-</td>
<td>32</td>
<td>&gt;16</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>S20</td>
<td>16</td>
<td>16</td>
<td>64</td>
<td>&gt;32</td>
<td>16</td>
<td>64</td>
</tr>
</tbody>
</table>

-Methicillin (M), Oxacillin (Ox), Penicillin G (P), Tobramycin (Tb), Ceftriaxone (Ci), Cephalosporin (Cp). - indicates not detected.

**DNA purification and PCR methods**

Plasmid DNA was prepared using the methods described by Birnboim and Doly, [24] and Ish-Horowicz and Burke, [25] and genomic DNA by the method described by Boom et al. [26]. PCR amplification of *mecA* gene was performed on 0.5 µg of genomic DNA as described earlier [27]. Primers for the detection of *mecA* were 5’-AGTTGTAAGTTGCTCGGTTT-3’ and 5’-AGTGGAACGAAGGTATCATC-3’.

PCR conditions for *mecA* comprised a thermal temperature of 94°C for 5 minutes, followed by 34 cycles of 94°C for 1 minute, annealing at 54°C for 1 minute, and extension at 72°C for 1 minute, followed by a final extension for 10 minutes at 72°C.

**Spot inoculation test to detect methicillinase production in *S. aureus* isolates**

The lawn culture of *S. aureus* Oxford (ATCC 29013) strain, which is sensitive to oxacillin, was prepared on Mueller-Hinton agar plates by
swabbing the plates with bacterial culture corresponding to 0.5 McFarland units.

This was left to dry for 10 minutes followed by division of the plate into four sections. An oxacillin disc (1µg) was placed in each section by means of a sterile blunt needle, and 4-5 colonies of each isolate were picked with the help of a sterile wire loop and placed as spots around each disc at a distance of 7-8 mm.

Four isolates were spotted per disc. The plates were incubated overnight at 37°C as described earlier [28].

Results

Identification and characterization of Staphylococcus aureus

The S. aureus isolates were first identified by morphological and biochemical characteristics. These strains were further characterized by amplifying thermostable nuclease (nuc) gene using the primers defined in the methodology section for characterization and confirmation up to the species level (Figure 1).

Antibiotic susceptibility testing

Of 17 isolates, methicillin resistance was shown by four (SA 13, SA 14, SA 19 and SA 20). SA 6 was the only isolate susceptible to all the antibiotics (Table 2).

Table 2. Antibiotic susceptibility and resistance pattern of different strains of S. aureus.

<table>
<thead>
<tr>
<th>S. aureus strains</th>
<th>Types of antibiotic(s) against which each strain is resistant</th>
<th>Strains of types based on antibiogram</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA 1</td>
<td>Cl</td>
<td>A</td>
</tr>
<tr>
<td>SA 2</td>
<td>Cl</td>
<td>A</td>
</tr>
<tr>
<td>SA 3</td>
<td>Cl</td>
<td>A</td>
</tr>
<tr>
<td>SA 4</td>
<td>Tb, Cl</td>
<td>B</td>
</tr>
<tr>
<td>SA 5</td>
<td>Cl</td>
<td>A</td>
</tr>
<tr>
<td>SA 6</td>
<td>None</td>
<td>C</td>
</tr>
<tr>
<td>SA 7</td>
<td>E</td>
<td>D</td>
</tr>
<tr>
<td>SA 8</td>
<td>Am, D</td>
<td>E</td>
</tr>
<tr>
<td>SA 9</td>
<td>P, Tb, K, G</td>
<td>F</td>
</tr>
<tr>
<td>SA 10</td>
<td>Cl</td>
<td>A</td>
</tr>
<tr>
<td>SA 11</td>
<td>Ox, Tb, Cl, G</td>
<td>G</td>
</tr>
<tr>
<td>SA 12</td>
<td>P, T, M</td>
<td>H</td>
</tr>
<tr>
<td>SA 13</td>
<td>A, M, Ox, P, Cl, Do, J</td>
<td>I</td>
</tr>
<tr>
<td>SA 14</td>
<td>P, Cl</td>
<td>H</td>
</tr>
<tr>
<td>SA 15</td>
<td>Cl</td>
<td>A</td>
</tr>
<tr>
<td>SA 16</td>
<td>Am, M, Ox, P, G, Tb, K</td>
<td>J</td>
</tr>
<tr>
<td>SA 19</td>
<td>Cl, Ce, D, E</td>
<td>J</td>
</tr>
<tr>
<td>SA 20</td>
<td>Am, M, Ox, P, G, Tb, K</td>
<td>J</td>
</tr>
</tbody>
</table>

All the isolates were susceptible to chloramphenicol and amikacin (Figure 1). Ciprofloxacin, the only fluoroquinolone used, was next in terms of efficacy, with only 6% of the isolates being resistant. Twelve percent of the isolates were found to be resistant to cefoxitin. In the cephalosporin group, most of the isolates were found to be sensitive to cefotaxime (only 6% resistant) while ceftriaxone was the least effective among all the antibiotics used, as 80% of isolates were resistant to it. In the other β-lactam group, 18% of isolates exhibited resistance to methicillin, 24% to oxacillin and 40% to penicillin G. Intermediate resistance was found to erythromycin (Figure 2).

Figure 1. PCR amplification of nuc gene.

Lane 1 is the positive control; lanes 2 and 3 are test isolates; lane 4 is the negative control. M is the 100 bp marker.

Figure 2. The bar diagram explains the percent resistance against each antibiotic.

Antibiotics used in this study

Abbreviations of the antibiotics are as follows: Amoxycillin (Am), Methicillin (M), Oxacillin (Ox), Penicillin G (P), Amikacin (Ak), Gentamicin (G), Tobramycin (Tb), Kanamycin (K), Cefoxitin (Cn), Cefotaxime (Ce), Erythromycin (E), Chloramphenicol (C), Ciprofloxacin (Cf).

Among the 9 isolates exhibiting multidrug-resistance to the antibiotics used in this study, only
three (SA20, SA19 and SA14) showed pronounced resistance against different classes of antibiotics such as β-lactams including second- and third-generation cephalosporins, aminoglycosides, macrolides and fluoroquinolone. SA20 was resistant to 86%, while SA19 and SA14 were resistant to 73% and 40% antibiotics respectively (Table 2). Moreover, these strains were classified into 10 different types on the basis of antibiotic sensitivity profile (Table 2).

Minimum inhibitory concentration (MIC) was also determined for selected antibiotics among the four MRSA strains. The resistance data is provided in Table 1.

Detection of mecA gene by PCR amplification

The mecA gene is responsible for methicillin resistance in S. aureus. PCR amplification of mecA gene was positive in the following isolates: SA1, SA3, SA6, SA8, SA11, SA12, and SA20 (Figure 3).

Figure 3. PCR amplification of mecA gene.

Analysis of plasmid DNA

Out of 17 isolates, 16 had plasmids of comparable size (14.2-15 kb), some of which are shown in Figure 4. Molecular weights were calculated by comparing Hind III digested λ-phage DNA marker and were analyzed by using Photocapt MV software.

Methicillinase expression assay

Spot inoculation test performed to detect the expression of methicillinase showed four isolates producing methicillinase, SA13, SA14, SA19 and SA20.

Discussion

Recent research has revealed that colonization of MRSA poses a substantial threat for the hospital environment, resulting in nosocomial infections. The word colonization here does not mean the normal sites of colonization (nostrils and in the skin of the axilla/groin.), but rather unhygienic surfaces, such as the hands of the hospital personnel. According to NNIS data for the year 2004, 59.5% of S. aureus strains causing infections in hospitals were MRSA. This is not only true for Europe and United States but for India as well. Initially, occasional reports on MRSA were available, but now it has become one of the established nosocomial pathogens [29]. Although no surveillance system exists, the figures obtained from some large medical care facilities including tertiary care hospitals is alarming, with percentages as high as 51.6% to 54.8% [29,30]. This is much higher than the range of 20 to 32.8% shown by earlier reports [31,32]. Since our sample size is very limited, we can not represent the entire situation; however, at least in the premise of this hospital, our data has significance and it can be said that MRSA are emerging in the hospital environment.

Health care workers usually act as asymptomatic carriers of multiple drug resistance organisms, especially MRSA, and help in its transmission to patients [4]. This study was conducted using isolates from hand swab samples, keeping in mind the spread of MRSA in the hospital environment through the hospital personnel.
Reports of patients with serious MRSA infections, who had no known risk factors or previous exposure to a health care setting, have been increasing [13-19]. The analysis of the antibiotype of S. aureus clones isolated in this study showed resistance against different groups of antibiotics including β-lactams of second- and third-generation of cephalosporin. Of the 17 isolates, 5 showed multiple drug resistance. Moreover, a common feature shown by these strains is a high resistance against ceftriaxone. Three isolates (SA20, SA19 and SA14) showed resistance against multiple antibiotics (5 to 13 antibiotics), while the remaining ones were resistant to one or more antibiotics (Table 2). The only isolate susceptible to all the antibiotics used in the study was SA6. Furthermore, these strains were classified as different types based on their antibiogram (Table 2).

Plasmid profiling showed that these isolates (SA14, SA19 and SA20) had plasmids of similar molecular weights; however, their restriction fragment analysis revealed their diversification in band patterns (data not shown). This observation might be a result of these plasmids carrying different gene cassettes for resistance against different classes of antibiotics [33].

Although high methicillin resistance in isolates of S. aureus was not found in our study, its emerging profile (18% exhibiting methicillin resistance) is a sign of danger for both community acquired and hospital-associated infections. Methicillin resistance is either due to expression of mecA gene or the synthesis of methicillinase or due to both. [34]. All the isolates were subjected to PCR amplification of mecA gene. PCR results revealed that seven isolates were carrying mecA gene. Of the mecA positive isolates, only SA20 was also found to be phenotypically resistant to methicillin. The remaining isolates did not show phenotypic resistance probably due to the over-expression of mecR and mecI genes which are co-repressors of mecA gene [35, 36]. Methicillin resistance (MR) shown by SA19 and SA14 is not a result of mecA expression. The expression of MR in these isolates was further studied by spot inoculation test to detect methicillinase. In addition to the above, SA13 and SA20 were also expressing methicillinase. Therefore, SA 20 might be showing a dual mechanism for resistance against methicillin, i.e. the expression of both PBP 2a and methicillinase.

The repression of mecA gene and the resulting absence of MR in some of the isolates could be due to several factors. Both genetic and environmental factors play a significant role in the expression of MR. The genetic factor could be repression of mecA by mecI and mecR1, which are its co-repressors [37]. The induction of mecA gene occurs through a signaling pathway initiated by the interaction of β-lactams with mecR1, a trans-membrane protein [38]. Therefore, selective pressure generated by indiscriminate use of antibiotic therapy is an important environmental factor in the induction of mecA gene [37].

The present study demonstrates the production of methicillinase in some of the MRSA strains isolated from hospital workers. It also suggests that S. aureus, suspected to be carriers of mecA, should be the subject for both phenotypic and genotypic analysis to confirm their MR status. According to this study carried out in Chandigarh, the least effective antibiotics against MRSA are ceftriaxone, tobramycin, and penicillin, whereas the most effective ones are chloramphenicol, amikacin, and ciprofloxacin. It is highly recommended that hospital personnel dealing with patients should be trained for hand washing in order to help control nosocomial infections.

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**Conflict of interests:** No conflict of interest is declared.