Molecular Identification of Methicillin Resistant Staphylococcus aureus Isolated from Patients with Wound Infections in Khartoum Teaching Hospital, Sudan

Babiker O. Haroun and Eidha A. Bin Hameed

ABSTRACT: Antimicrobial resistance has become a great public health problem worldwide and multi-drug resistant Staphylococcus aureus was widely reported. The presence or absence of methicillin resistance gene (mecA) in 48 clinical wound isolates of S. aureus was examined by the polymerase chain reaction (PCR). The results were analyzed in relation to the disk diffusion method of oxacillin (1µg). PCR was amplified at a sequence of mecA gene at 1319 bp of 9 (18.75%) isolates, and were identical to those of disk diffusion test. All strains were studied for their susceptibility to traditionally used antibiotics. The results revealed that the drug of choice for the treatment of Methicillin Resistant Staphylococcus aureus (MRSA) and Methicillin Sensitive Staphylococcus aureus (MSSA) was vancomycin and multi-drug resistance was common among MRSA strains. The study concluded that wound infections showed common multiple antibiotic resistant of MRSA and the PCR assay was found to be reliable and more accurate to detect MRSA infection.

KEYWORDS: Staphylococcus aureus, Methicillin resistance gene, Polymerase chain reaction (PCR).

INTRODUCTION
Resistance to methicillin was first described for Staphylococcus aureus in 1960, shortly after the introduction of the drug into clinical practice (Cunha, 2005). Since, then methicillin-resistant S. aureus (MRSA) has become a widely recognized cause of morbidity and mortality throughout the world (Hookey et al., 1998). Usually staphylococcal strains have penicillin-binding proteins (PBPs). In the absence of β-lactam antibiotics the staphylococci utilize usual PBPs to synthesize the cell wall which is composed of peptidoglycan (Goffin and Guysen, 1998). In contrast, an additional low-affinity penicillin binding protein PBP designated PBP 2a is encoded by a unique MRSA-PBP gene and is the main factor responsible for the expression of methicillin resistance (Higashi et al., 1999). The β-lactam resistance of MRSA which is an intrinsic resistance of the cells to all β-lactam antibiotics is mediated by the methicillin resistance determinant (Tokue et al., 1992).

Traditionally, methicillin or oxacillin was tested and results are representative of all β-lactam agents (Brown et al., 2005). In disk diffusion method against microbial isolates was measured routinely for the choice of appropriate chemotherapy. In the case of MRSA strains, the disk diffusion is influenced by growth conditions, pH, and NaCl concentration (Felten et al., 2002). Therefore, it is important to confirm whether S. aureus isolated from patients possesses MRSA-PBP gene in relation to determination by disk diffusion.

The objective of this study was to investigate the presence of MRSA-PBP gene in clinical isolates of S. aureus by PCR and disk diffusion method of oxacillin.

MATERIALS and METHODS
A total of 48 S. aureus isolates were recovered from clinical wound specimens, such as ulcers, burns, abscesses, post-operative wounds, and chronic wounds from Khartoum Teaching Hospital, Sudan. All isolates were collected between September 2005 to August 2007.
S. aureus isolates were identified by standard microbiological methods including Grams stain, catalase, coagulase, DNase and growth on mannitol salt agar. MRSA was determined by the disc diffusion method to oxacillin according to the National Committee for Clinical Laboratory Standard (NCCLS) guidelines. Antimicrobial susceptibility testing was performed by the disc diffusion method for cephalaxine (30 mg), co-trimoxazole (25 mg), clindamycin (2 mg), erythromycin (15 mg), vancomycin (30 mg), tetracycline (30 mg), rifampicin (5 mg), amoxicillin (10 mg), and ciprofloxacin (5 μg). A suspension of the tested organisms was adjusted against 0.5 McFarland standard turbidity and inoculated onto Mueller-Hinton agar (Oxoid), then incubated at 35-37 °C for 16-18 hours, and examined for evidence of inhibition.

Genomic DNA was isolated by using chloroform DNA purification protocol. With a 1 l loop, a small quantity of growth equivalent to about two small colonies was scraped from the top of the culture and placed into 100 l of sterile distilled water in a microcentrifuge tube. Hundred μl of Chloroform (Sigma) were added, and the mixture was vortexed for about 10 seconds. The mixture was heated at 80°C for 20 minutes, after that time it was held at -20°C for at least 20 minutes. The sample was allowed to thaw but while still cold was centrifuged at 12,000xg for 3 minutes in a minicentrifuge. The sediment layer that contains the DNA was stored at -20°C till used (Yates et al., 2002).

Two 20-mer PCR primers of methicillin resistance (mecA) gene were chosen. These oligonucleotides are complementary to the target segment of the MRSA-PBP gene sequence, as follows: primer MR1, 5'-GTG GAA TTG GCC AAT ACA GG-3' (478 to 497) and primer MR2, 5'-TGA GTT CTG CAG TAC CGG AT-3' (1816 to 1797) (Tokue et al., 1992).

The PCR reaction mixture comprising of reaction buffer (10 l), 500U Go Taq DNA polymerase (Promega) (0.3 l), 10mM dNTPs mix (1 l), 25mM MgCl2 (3 l), 10mM each primer (10 l), template DNA (1 l), and distilled water (14.7 l) was brought to a final volume of 50 l.

The amplification procedural steps were as follows: an initial step at 94°C for 30 second; (94°C for 30 second, 55°C for 30 second, and 72°C for 1 minute, each step in this cycle was repeated 40 times), and a final extension step at 72°C for 4 minutes. The PCR-amplified samples were subjected to electrophoresis on 0.4% agarose gel with 3 l ethidium bromide incorporated for DNA staining in Tris-borate EDTA (1x TBE) buffer. The PCR products were visualized and photographed on an UV transilluminator and the sizes of the PCR products were determined by comparison to the 1kb ladder DNA marker (Promega).

RESULTS
Phenotypic resistance showed no inhibition zone to oxacillin in 9 (18.75%) S. aureus strains out of 48 isolates. These results were identical to genotypic results whereas the amplified DNA fragment of MRSA-PBP gene resulted the predicted size, that 1319 bp was detected in 9 strains and no DNA amplification was seen in 39 strains. (Fig. l).

The antibiotype was conducted for 48 S. aureus isolates against 10 antimicrobial agents by disc diffusion method. All isolates showed 100% sensitivity to vancomycin. For the other antibiotics, the degree of sensitivity was as follows; co-trimoxazole 27 (56.25%), rifampicin 35 (72.9%), clindamycin 33 (68.75%), tetracyclen 16 (33.3%), erythromycin 31 (64.6%), cephalaxine 29 (60.4%), oxacillin 39 (81.25%), amoxicillin 19 (39.6%), and ciprofloxacin 32 (66.7%).

Twenty (41.7%) of isolates were multiple antibiotic resistant to four or more of antimicrobial agents, nine of them were also MRSA. MRSA and MSSA strains were found to be highly resistant to tetracycline and amoxicillin. (Table 1 and 2).
Figure 1. Agarose gel electrophoresis of PCR products amplification of mecA gene. Lane M (1 kb ladder) DNA marker. Lanes 1-4 methicillin resistant S. aureus (1319 bp) DNA fragment. Lanes 5-7 methicillin sensitive S. aureus.

Table 1: Frequency of multi-drug resistance among MRSA (n=09) and MSSA (n=39) isolates

<table>
<thead>
<tr>
<th>Resistance pattern</th>
<th>MRSA</th>
<th>MSSA</th>
<th>Total</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fully sensitive</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>(0.00)</td>
</tr>
<tr>
<td>Resistant to 1 agent</td>
<td>0</td>
<td>6</td>
<td>6</td>
<td>(12.05)</td>
</tr>
<tr>
<td>Resistant to 2 agents</td>
<td>0</td>
<td>9</td>
<td>9</td>
<td>(18.75)</td>
</tr>
<tr>
<td>Resistant to 3 agents</td>
<td>0</td>
<td>13</td>
<td>13</td>
<td>(27.01)</td>
</tr>
<tr>
<td>Resistant to 4 agents</td>
<td>0</td>
<td>6</td>
<td>6</td>
<td>(12.05)</td>
</tr>
<tr>
<td>Resistant to 5 agents</td>
<td>1</td>
<td>5</td>
<td>6</td>
<td>(12.05)</td>
</tr>
<tr>
<td>Resistant to 6 agents</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>(06.25)</td>
</tr>
<tr>
<td>Resistant to 7 agents</td>
<td>5</td>
<td>0</td>
<td>5</td>
<td>(10.04)</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>39</td>
<td>48</td>
<td>(100.0)</td>
</tr>
</tbody>
</table>

Table 2: Comparison between MRSA and MSSA in relation to their resistance to different antibiotics

<table>
<thead>
<tr>
<th>No. of isolates</th>
<th>Antibiotic resistance disks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
</tr>
<tr>
<td>MRSA (9)</td>
<td>5</td>
</tr>
<tr>
<td>MSSA (39)</td>
<td>8</td>
</tr>
</tbody>
</table>


DISCUSSION

This study demonstrated that 18.75% of MRSA were common in wound infections with clinical characteristics of patients infected with this organism, and we suggested that some reasons like poor quality and misuse of antibiotics, inadequate hospital infection control, inadequate drug-resistance surveillance, undergoing surgical procedure during the current hospital stay, or acquiring the infection in hospital, and widespread contamination of hospital environment, may be associated with presence of MRSA strains in hospital, although the frequency of MRSA in Sudan is still low. MRSA now is commonly accounted for 20% to 40% of all S. aureus isolates (Rubin et al., 1999). Generally high rates had been reported in the United States.
71% (Boyce, 1990), Malaysia 84% (Hanifah et al., 1992), and Peru 68% (Seas et al., 2006). In Japan it was 56.9% (Oguri, 1992), while the rates of MRSA in Iran were 53.6% (Rahbar et al., 2001), and in Latin America 31% (Gales et al., 2000). A study carried out in Egypt, revealed that MRSA was present in 16.3% of patients nostrils (Shehab El-din et al., 2003), whereas in India, methicillin resistance among S. aureus isolates was 39.5% (Mulla et al., 2007). Another important geographic difference for MRSA prevalence was also found in France, Spain and Italy, where the rates of 30-35% of MRSA were reported (Voss et al., 1994).

The oxacillin disc diffusion test was found to be specific for MRSA under conditions tested in this study. This implies that the oxacillin is an available alternative to the methicillin disc method for routine MRSA susceptibility testing at 37°C. Staphylococcal strains reported as oxacillin resistant are also methicillin resistant (Cunha, 2005). Previous studies showed a relationship between oxacillin resistance and PBP 2a. The MRSA-screen latex agglutination test for detection of PBP 2a was comparable to the mecA PCR with respect to sensitivity, specificity and accuracy for the detection of MRSA (Sakoulas et al., 2001). The MRSA-screen test, the oxacillin disc diffusion and the oxacillin-salt agar screening test showed sensitivities of 100%, 61.3%, and 82.5%, and specificities of 99.2%, 96.7%, and 98.3%, respectively (Cavassini et al., 1999).

S. aureus resistance to methicillin/oxacillin occurs when an isolate carries an altered PBP 2a which is encoded by the mecA gene. The new PBP 2a binds β-lactamas with lower affinity which results in resistance to this class of antimicrobial agents.

In this study, the PCR mixture containing the S. aureus mecA gene primers which has the amplicon size of 1319 bp was assayed to isolate the mecA gene from clinical S. aureus isolates. The PCR products revealed the predicted amplicon size as obtained by Tokue et al. (1992). We have compared PCR assay for the detection of antibiotic resistance mecA gene with conventional method for the determination of susceptibility to oxacillin. Nine S. aureus isolates initially classified as methicillin resistance based on disc diffusion method, and PCR confirmed the presence of mecA gene in these isolates. Therefore, detection of mecA gene by PCR was considered to be the gold standard assay, and these results were similar to those achieved by Tokue et al., 1991), who reported that the PCR assay was found to be sensitive and reliable procedure for rapid diagnosis of mecA gene. Other PCR-based tests were developed for the detection of mecA in staphylococci, and the results obtained were generally consistent with those of standard microbiological assays (Unal et al., 1992, Sakoulas et al., 2001).

Antimicrobial and multi-drug resistant S. aureus has become a great public health problem worldwide. This study determined the pattern of resistance to commonly used antibiotics of MRSA and MSSA. Antimicrobial and multi-drug resistant to different antimicrobial agents among MRSA strains were significantly higher than those which were MSSA, and this is perhaps due to the differential clonal expansion and random used of drugs in the hospital. However, MRSA which causes nosocomial infections is among the most important multi-resistant pathogens worldwide (Boyce, 1994).

The frequency of antibiotic resistance among S. aureus in current study was in accordance with other studies. The results from intensive care units showed multiple antimicrobial resistance by 78% of 32 S. aureus isolates; 12% co-trimoxazole, 25% teicoplanin, 46% erythromycin, 50% clindamycin, 68% gentamicin, 71% ciprofloxacin, 81% oxacillin and 100% were penicillin resistant (Iseri-Abut et al., 2003). Another study in Nigeria showed
highly resistant *S. aureus* isolates to ampicillin 91.7%, clindamycin 78.3%, cephalaxine 75%, methicillin 71.7% and vancomycin 68.3%, but had very low resistance to gentamicin 3.3%, ciprofloxacin 3.3%, ofloxacin 3.3%, sparfloxacin 3.3% and pefloxacin 10.0%. As many as 71.7% of the isolates showed multi-drug resistance (Onanuga et al., 2005), whereas in Peru 25% of MRSA strains were resistant to multiple drugs (Seas et al., 2006).

MRSA strains were also resistant to multiple antibiotics in Algiers Hospital. 97.6% Many of the isolates were resistant to kanamycin, 86% to fusidic acid, 73% to tetracycline, 25% to erythromycin, 16% to ofloxacin, 11.3% to clindamycin, 7% to gentamicin, 4.5% to pristinamycin, 2.3% to chloramphenicol, and 2.3% to rifampicin (Ramdani-Bouguessa et al., 2006). On the other hand, the rate of antibiotic resistant strains of MRSA was highest for penicillins and cepharosporins in Japan, where over 95% of MRSA were resistant to ampicillin, amoxicillin and piperacillin, and more than 90% of MRSA strains were resistant to cephalosporins (Tanaka et al., 1999), as well in India, the resistance to all antibiotics tested among MRSA and MSSA was found to be 23.2% and 6.6%, respectively, and higher resistance to multiple antibiotics in methicillin resistant strains showed to penicillin followed by gentamicin, tetracycline, erythromycin and ciprofloxacin (Majumder et al., 2001). In Egypt, the resistance of MRSA and MSSA to antibiotics were; oxacillin 100%, 0.0% respectively, amoxicillin+clavulamic acid 25%, 100%, clindamycin 100%, 12.5%, penicillin 100%, 25%, gentamycin 58.3%, 50%, erythromycin and ciprofloxacin 33.3%, 25%, tetracycline and cotrimoxazole 25%, 25%, and rifampicin 16.7%, 12.5% (Shehab El-din et al., 2003), whereas the resistance among MRSA isolates recovered from skin and soft-tissue infections were 5% to clindamycin, 94% to erythromycin, 40% to fluoroquinolones, and 8% to tetracycline (Moran et al., 2006).

Since the emergence of the MRSA in 1960s, teicoplanin and vancomycin have been the drugs of choice and commonly the antimicrobial agents available for the treatment of nosocomial MRSA and other Gram-positive infections (Jarvis, 1998, Nashev et al., 2000). In this study, the only antibiotic to which all MRSA and MSSA strains were still sensitive was vancomycin. These results are similar when compared to a study carried out in Scotland, where both MRSA and MSSA with reduced susceptibility to teicoplanin, but were sensitive to vancomycin (Mackenzie et al., 2002). On the other hand, the isolates of MRSA and MSSA showed 100% sensitivity to vancomycin in Bulgaria (Nashev et al., 2000) and Egypt (Shehab El-din et al., 2003).

**CONCLUSION:**

Wound infections showed common multiple antibiotic resistant of MRSA, and injudicious use of antibiotics will lead to development of more drug resistance. The PCR assay appears to be more reliable and accurate for detection of MRSA-PBP.

**REFERENCES**

of methicillin resistance in *Staphylococcus aureus*. J. Clinic. Microbiol. 37(5); 1591-1594.


