Original Research Article

Hospital-Acquired Methicillin Resistant *Staphylococcus aureus*: Analysis of meca Gene and Staphylococcal Cassette Chromosome

Ola A.Kader, Gihan A.EL Batouti, Abeer A. Ghazal and Kholoud M. Baraka

1 Professor of Microbiology, Department of Microbiology, Medical Research Institute, University of Alexandria, Alexandria, Egypt
2 Lecturer of Microbiology, Department of Microbiology, Faculty of Pharmacy, Pharos University in Alexandria, Alexandria, Egypt
3 Lecturer of Microbiology, Department of Microbiology, Faculty of Pharmacy, University of Damanhour, Damanhour, Egypt

*Corresponding author email id:

ABSTRACT

Staphylococcus cassette chromosome (SCC) is a variable genetic element that contains the meca and is considered the most reliable method for detection of *Methicillin resistant Staphylococcus aureus* (MRSA). The aim of this study was to characterize hospital acquired MRSA (HA-MRSA), both phenotypically and genotypically. Fifty staphylococci strains were isolated from hospitalized patients, from which 38 (76%) were mannitol fermenters. Twenty strains (40%) were resistant to both oxacillin and cefoxitin, however, 2 (4%) isolates were resistant to oxacillin only and 7 (14%) were resistant to cefoxitin only. The 29 resistant strains were diagnosed as MRSA by detection of meca gene using real time PCR. They were all penicillin-binding protein2a producers. Among these isolates, 26 (89.66%) were typable by using Zhang set of primers. Only 3 (10.34%) of the 29 isolates were Panton-Valentine Leukocidin positive, from which 2 were SCCmec type IV and V, while the third remained untypable. The combination of phenotypic characteristics in conjunction with antibiograms are useful to a certain extent, but further work is required to find a reliable marker to facilitate the recognition of HA-MRSA.

Keywords

Methicillin resistant *Staphylococcus aureus*, Staphylococcus cassette chromosome, meca gene, Penicillin-binding protein, Panton-Valentine Leukocidin

Introduction

*Staphylococcus aureus* (*S. aureus*) continues to be a serious health problem worldwide due to its intrinsic nature of virulence, its ability to cause a wide array of infections, and its capacity to develop resistance to a number of antibiotics with the ability for clonal dissemination, through both mutation and acquisition of exogenous genes (Deurenberg and Stobberingh, 2008).

* S. aureus can cause potentially serious healthcare associated infections. In 1959, methicillin was introduced to overcome the resistance problem. However, the first
MRSA was isolated shortly after the introduction of methicillin (Lee et al., 2011).

Virulence factors are encoded by phages, plasmids, pathogenicity islands and the SCC. Increased resistance for antibiotics is encoded by a transposon (Tn 1546) inserted into a conjugated plasmid that has also encoded resistance to disinfectants (Chambers and Deleo, 2009, Lee et al., 2011).

The resistance to methicillin is not due to β-lactamase production but to the expression of an additional penicillin-binding protein (PBP2a), acquired from other species, which was resistant to the action of the antibiotic, and this protein is coded for by a mobile genetic element termed the methicillin-resistant gene (mecA) (My et al., 2011). PBP2a has low affinity to methicillin rendering the bacteria that produce it; resistant to all beta-lactam antibiotics(Kim et al., 2012). In recent years, the gene has continued to progress so that many MRSA strains are currently resistant to several antibiotics such as penicillin, oxacillin, and amoxicillin(Chambers and Deleo, 2009).

The rates of MRSA infections in hospitals, as well as the community, have continued to rise. The differentiation between the typical HA-MRSA and community acquired MRSA (CA-MRSA) strains based on epidemiologic definitions has become difficult(Yamamoto et al., 2010, Mediavilla 2012).

SCCmec is a variable genetic element that contains the methicillin resistance determinant, meca. SCCmec typing is one of the most important molecular tools available for distinction between HA-MRSA and CA-MRSA (Turlej et al., 2011).

HA-MRSA strains are typically associated with one of three types I–III, which carry additional antibiotic resistance genes. In contrast, CA-MRSA has almost exclusively been associated with type IV. More recently, type V SCCmec is described to be harboured by S. aureus strains which behave similarly to type IV strains, causing typical CA-MRSA infections. (Turlej et al., 2011, Shanshuang et al., 2011)

In 1932, Panton and Valentine described Panton-Va lentine Leukocidin (PVL), a leukocidintoxin as a virulence factor that form pores in the membrane of host defense cells by the synergistic action of two secretory proteins, designated LukS-PV and LukF-PV (Tristan et al., 2007).

These proteins are encoded by two cotranscribed genes of a prophage integrated in the S.aureus chromosome and are secreted before they assemble into a pore-forming polymorphonuclear leukocytes membranes, leading to their lysis (Tristan et al., 2007, Shallcross et al., 2012).

PVL is mostly associated with CA-MRSA infections and is distinguishable from HA-MRSA by nonmultidrug resistance and the carriage of SCCmec type IV. (12) PVL is widely associated with the presence of SCCmec IV and sporadically with SCCmec V but not SCCmec types I, II or III (David and Daum, 2010).

This study aimed to characterize HA-MRSA both phenotypically and genotypically.

**Material and Methods**

A total of 50 staphylococci strains were isolated from hospitalized patients whom had developed infections 48 hours after their admission. Specimens included pus from wounds, catheters, blood, sputum and vaginal swabs.
All samples were inoculated on blood agar and MacConkey agar (Oxoid Ltd, Wade Road, Basingstoke, Hampshire, UK) plates. Staphylococcal isolates were identified by their colonial appearance. Colonies suspected as staphylococci were Gram stained and tested for catalase, oxidase, slide coagulase and tube coagulase tests (Baird, 1996).

Susceptibility of staphylococci was determined by the disc diffusion method that included oxacillin and cefoxitin discs. (Clinical and Laboratory Standards Institute, 2009).

PBP2a Latex Agglutination Test (OXOID®) was performed according to the manufacturers’ instructions to detect the presence of PBP2a responsible for methicillin resistance. Agglutination seen with the test but not the control, within 3 minutes was considered PBP2a positive (Hussain et al., 2000).

Genotypic identification of MRSA was carried out by detecting mecA gene encoding the low affinity PBP2a. Molecular typing of MRSA was carried out using Zhang et al., (2005) scheme targeting mec gene (Class A and B), ccr gene complexes and individual SCCmec types and subtypes using real time PCR by SYBR Green1 technique. Real time PCR was used to determine the occurrence of PVL as a virulence factor (Jones et al., 2007, Enany et al., 2010).

**Staphylococcal DNA Extraction**

Staphylococcal isolates were subcultured overnight at 37°C on blood agar media. Few colonies were emulsified in 200 µl sterile distilled water to produce a heavy suspension. The bacterial suspension was heated at 100°C for 15 min. The suspension was then centrifuged at 14, 000 rpm for 5 min.

**Reagents**

SYBR Green universal PCR master mix 2-fold was purchased from (Fermentas life sciences®). All Primers were purchased from metabion international AG and were reconstituted with sterile distilled water to obtain a concentrate of 100 picomoles/µl.

**Protocol of amplification**

Each PCR tube contained the following:

12.5 µl SYBRGreen universal PCR master mix 2-fold, 0.5 µl forward and reverse primer, 5 µl DNA extract, and 6.5 µl PCR grade water bringing the reaction volume to 25µl. A negative control was prepared by the addition of the same contents to the tube with water instead of the extract.

The tubes were placed in the thermal cycler for amplification. The thermal profile included 1 Cycle of activation at 95 ºC for 10 min., followed by 35 Cycles of denaturation at 95 ºC for 15 sec, annealing at 55 ºC for 30 sec., and extension at 72 ºC for 30 sec. This was followed by the melting curve analysis of 1 Cycle at 95 ºC for 1 min., 55ºC for 30 sec., and 95 ºC for 30 sec.

Amplification was done for SCCmec typing or detection of PVL gene using the same protocol and thermal profile as for mecA amplification except that the annealing temperature varied according to the amplified gene. Annealing temperature was 50ºC for Zhang et al primers amplifying SCCmectype II, III, IVb, IVc class A and B 53ºC for SCCmec type IVa and IVd, 55ºC for SCCmec type V and 60ºC for PVLgene.

**Results and Discussion**

The 50 (94.34%) staphylococci isolates were positive for both the clumping factor
(detected by the slide coagulate test) and free coagulate (detected by the tube coagulate test ) and were thereby diagnosed as S. aureus.

The presence of mecA genes detected by real time PCR was considered to be the gold standard in the diagnosis MRSA (figs. 1 and 2). Out of the 50 S. aureus isolated from hospitalized patients, 29 (58%) isolates were positive for mecA gene and were PBP2, producers; hence were diagnosed as MRSA; among which 27 (93.01%) were resistant to cefoxitin while only 22 (75.86%) isolates were found to be resistant to oxacillin. The antibiotic sensitivity of HA-MRSA ranged from 58.62% for cotrimoxazole, erythromycin or tetracycline to 96.55% for rifampicin.

Molecular typing:

Regarding the 29 HA-MRSA strainsthat were initially tested with SCCmec class A and class B primers (Zhang et al., 2005); 11 (37.93%) strains were SCCmec class A positive, 6 (20.69%) strains were SCCmec class B positive, while, 12 (41.38%) strains could not be classified as class A nor B.

When testing the 11 class A positive cases with SCCmec ccr2 and SCCmec ccr3 primers (Zhang et al., 2005), only 4 cases were positive: 2 cases (18.18%) were positive with SCCmec ccr2 primers, and were thereby considered as SCCmec type II. The 2 (18.18%) other cases were positive with SCCmec ccr3 primers and were considered as SCCmec type III. The 7 (63.64%) remaining class A positive cases were negative for both SCCmec ccr2 and ccr3 primers.

When testing the 6 class B positive cases with SCCmec ccr2 and SCCmec ccr1 primers, only 3 cases (50%) were positive, 2 cases (33.33%) were positive with SCCmec ccr2 primers and were thereby considered as SCCmec type IV. The other case (16.67%) was positive with SCCmec ccr1 primers and was considered as SCCmec type I.

The remaining 22 untypable HA-MRSA strains (10 class A or B positive, ccr negative strains and 12 class A and B negative strains) were tested with an individual set of Zhang et al., (2005) primers for SCCmec type I, SCCmec type II, SCCmec type III and SCCmec type V.

Out of the 10 class A or B positive cases, 7 cases (70%) were SCCmec type I and 3 (30%) cases were SCCmec type III. Out of the 12 class A and B negative strains, 6 cases (50%) were SCCmec type V, 3 cases (25%) were SCCmec type I and 3 cases (25%) remained untypable.

Fig. 3 shows the distribution of the 29 HA-MRSA, where 26 (89.66%) were typable by Zhang et al., (2005); set of primers' as follows: 11 (37.93%) were SCCmec type I, 2 (6.90%) were type II, 5 (17.24%) were type III 2 (6.90%) were type IVa and 6 (20.69%) were type V and 3 (33.34%) remained untypable.

S. aureus has a remarkable ability to develop antibiotic resistance. MRSA first emerged as a healthcare associated pathogen in the 1960s. Since the 1980s, the emergence of MRSA has become widespread in hospitals and long-term care facilities, accounting for numerous healthcare associated infections. It is a global public health problem and represents the most commonly identified antibiotic-resistant pathogen (Rayet et al., 2011 and Tong et al., 2015).

A total of 50 S. aureus isolates were collected from infected wounds of
hospitalized patients in the Medical Research Institute, Alexandria University. They were identified as Gram-positive, catalase positive, oxidase negative and coagulase positive cocci.

The accurate and early determination of methicillin resistance is of key importance in the prognosis of infections caused by S. aureus. Identification of the mecA gene is the most reliable method of detecting MRSA isolates (Turlej et al., 2011, Shanshuang et al., 2011).

In the present study the presence of mecA gene was detected by real time PCR using the SYBR Green I dye. This method obviates the need for target-specific fluorescent probes, reducing assay setup and running cost, but its specificity is determined entirely by the primer sequence. However, melting curve analysis, depending on GC content and the size of the amplicon, was carried out to identify the amplification product and to determine its purity and specificity. Melting curve analysis of the mecA positive strains gave excellent results regarding specificity, where the position of the peaks were identical for all strains.

Salimnia and Brown (2005) evaluated oxacillin and cefoxitin disc diffusion utilizing mecA as the gold standard, the category agreement for oxacillin disc was 93.3% and for cefoxitin disc 99.7%.

Velasco et al., (2005) found that 5.9% out of their strains that were positive for the mecA gene yielded false negative results with the oxacillin disc diffusion. They explained the lower sensitivity by the absence of, or reduced expression of, the mecA-encoded protein, PBP2a; suggesting that antibiotics, able to induce expression of methicillin resistance such as cefoxitin, are the most appropriate for detecting MRSA isolates, as all mecA-positive isolates were detected with the cefoxitin disc with a sensitivity of 100%. (Gupta et al., 2009)

Out of the 50 S. aureus strains isolated from hospitalized patients 29 (58%) were positive for the mecA gene and were PBP2a producers, 27 (93.01%) out of them were resistant to cefoxitin disc while only 22 (75.86%) isolates were resistant to oxacillin, disconfirming that identification of MRSA is more accurate by either directly detecting the gene encoding the methicillin resistance determinant (mecA) or its product, penicillin-binding protein 2a (PBP2a).

In the present study, out of the 29 HA-MRSA 14 (48.28%) isolates were found to be multidrug resistant. These isolates were resistant to 2 or more antibiotic classes including quinolones, co-trimoxazole, erythromycin and tetracycline. A 41.38% were found resistant to co-trimoxazole, 31.03% to quinolones and only 3.45% to rifampicin. All isolates were sensitive to vancomycin.

Zhang et al., (2005) typing scheme targets the individual regions of the classes of the mec complex (IS431-mecA, IS1272-mecA, and mecI-mecRI). Four primers were used to identify class A and B of the mec gene complex, the allotypes of the ccr complex were identified using 6 primers. SCCmec types I to V were identified by the combination of the mec classes and ccr allotypes.

Out of the 29 HA-MRSA, 11 strains were positive for class A, 6 strains were positive for class B and 12 strains were negative for both class A and B. By ccr typing, only 4 strains out of the 11 class A positive strains were positive for ccr genes: 2 strains were positive for ccr2 and were classified as SCCmec type II and 2 strains were positive
for ccr3 and were classified as SCCmec type III. The remaining 7 class A positive strains were ccr negative & thereby couldn't be assigned to any SCCmec type. Also, only 3 strains out of the 6 class B positive strains were positive for ccr genes: 2 strains were positive for ccr2 and were classified as SCCmec type IV and 1 strain was positive for ccr1 and was classified as SCCmec type I. The remaining 3 class B positive strains were ccr negative & thereby couldn't be assigned to any SCCmec type.

Table 1: Initial set of primers were used to identify mecA, SCCmec and PVL gene

<table>
<thead>
<tr>
<th>Primer Type</th>
<th>Primers</th>
<th>Oligonucleotide sequence (5'-3')</th>
<th>Ampl- icon size (bp)</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>mecA</td>
<td>M1-F M2-R</td>
<td>885-TGGCTATCGTGTCACAATCG-904 1194-CTGGAACCTTTGTTAGCAGAG-1175</td>
<td>310</td>
<td>mecA</td>
</tr>
<tr>
<td>Zhang et al., (2005)</td>
<td>Type I-F Type I-R</td>
<td>GCTTTAAAGAGTGTCGTTACAGG GTTCTACTCAGATGACGTCC</td>
<td>613</td>
<td>SCCmec I</td>
</tr>
<tr>
<td></td>
<td>Type II-F Type II-R</td>
<td>CGTTAAGATGATGAAGCG CGAAATTTGTTATGACGTCC</td>
<td>398</td>
<td>SCCmec II</td>
</tr>
<tr>
<td></td>
<td>Type III-F Type III-R</td>
<td>CCATATTGTGTACGATGCG CCTAGTTGTGCAGATCAG</td>
<td>280</td>
<td>SCCmec III</td>
</tr>
<tr>
<td></td>
<td>Type IVa-F TypeIVa-R</td>
<td>GCCTTATTGCAAGAAACCG CTACTCCTGAAAGACGTCC</td>
<td>776</td>
<td>SCCmec Iva</td>
</tr>
<tr>
<td></td>
<td>Type IVb-F TypeIVb-R</td>
<td>TCTGGAATTACTCAGCTGC AAACAATTTGCTCTCCCTC</td>
<td>493</td>
<td>SCCmec IVb</td>
</tr>
<tr>
<td></td>
<td>Type IVc-F TypeIVc-R</td>
<td>ACAATATTTGTTATCGGAGAGC TTGATGAGGTATTGCTGG</td>
<td>200</td>
<td>SCCmec IVc</td>
</tr>
<tr>
<td></td>
<td>Type IVd-F TypeIVd-R</td>
<td>CTCAAAATACGGACCCAATACA TGCTTCACTCTATGCTAAAG</td>
<td>881</td>
<td>SCCmec IVd</td>
</tr>
<tr>
<td></td>
<td>Type V-F Type V-R</td>
<td>GACATTTGTTACCTAAATGAGCGTG AAAGTGTTACCTGTACAC</td>
<td>325</td>
<td>SCCmec V</td>
</tr>
<tr>
<td></td>
<td>mecI-F mecI-R</td>
<td>CCCTTTTTATACAAATCGTTATATCA TCTCAGAATGGG</td>
<td>146</td>
<td>Class A mec</td>
</tr>
<tr>
<td></td>
<td>IS1272-FmecR1-R</td>
<td>TATTTTGGGTTCACTCAGGCTCCACG TTAATCTTACATAGG</td>
<td>1, 305</td>
<td>Class B mec</td>
</tr>
<tr>
<td>PVL</td>
<td>luk-PV-1 luk-PV-2</td>
<td>ATCACTAGTAAAAATGTCTGGACATG ATCCAGCATCAATGTATTGAGC</td>
<td>433</td>
<td>PVL</td>
</tr>
</tbody>
</table>
**Fig. 1** Shows amplification plots (fluorescence against cycle number) of the mecA positive strains with different Ct.

![Amplification Plots](image1)

**Fig. 2** Melting curve analysis of the mecA positive strains to identify the amplification product and to determine its purity and specificity.

![Dissociation Curve](image2)
By this method of typing, out of 29 HA-MRSA, only 7 (24.14%) strains were typable: SCCmec type I [1 isolate (3.45%)], SCCmec type II [2 isolates (6.90%)], SCCmec type III [2 isolates (6.90%)] and SCCmec type IV [2 isolates (6.90%)].

Zhang et al., (2005) designed 8 sets of specific primers based on analyses and alignments of the MSSA and MRSA genomes and variable SCCmectype and subtype sequences in the GenBank database.

In the present study, these sets of primers were used to type the 22 strains which were not typable otherwise: class A or B positive, ccr negative (10 strains) and class A and B negative (12 strains): 19 (86.36%) out of these 22 strains could be classified by this method. 10 (45.45%) strains were SCCmec type I, 3 (13.64%) strains were SCCmec type III, 6 (27.27%) strains were SCCmec type V and 3 (13.64%) strains remained untypable.

By using Zhang et al., (2005) scheme, 26 (89.66%) out of the 29 HA-MRSA were typable. SCCmec type I: 11 (37.93%), type II: 2 (6.90%), type III: 5 (17.24%), type IVa: 2 (6.90%) and type V: 6 (20.69%), while 3 (10.34%) remained untypable.

Type V SCCmec is structurally similar to type IV SCCmec, in that it contains mecAas the only gene encoding antibiotic resistance. Its size (28 kb) was also comparable to that of type IV SCCmec (21 Kb) and was much smaller than those of the type I to III SCCmec elements (34 to 67 kb), (Valsesiaet al., 2015).

There has been much interest in PVL, due to its involvement in severe disease among children and young adults with no known exposure to healthcare establishments. CA-MRSA has been reported to carry the loci for PVL in high frequency, and to be associated with the SCCmectype IV. (Katopodiset al., 2010, Sobhy Net et al., 2012, Valsesiaet al., 2015)

However, only 12% out of 108 CA-MRSA isolated in Finland (Fang et al., 2008) contained the PVL gene, moreover 6.7% of isolates from Irish patients (Rosseyetal., 2011) with an epidemiological history of CA-MRSA carried PVL genes. Similarly, 78% of CA-MRSA isolates, referred to a
central reference facility for investigation of the carriage of PVL, were negative.

However, 3 (10.34%) out of the 29 HA-MRSA isolates were PVL positive and 2 (6.9%) were SCCmec type IVa. These findings confirm that molecular typing for HA and CA-MRSA differentiation cannot be used as a sole tool.

In this study, the combination of PVL, SCCmec type IVa and a few untypable strains were not sufficient to identify all presumably HA-MRSA strains isolated from the hospital.

The combination of phenotypic characteristics in conjunction with antibiograms are useful to a certain extent, but further work is required to find a reliable marker or combinations of markers to facilitate the recognition of HA-MRSA.

References


