Title: Analysis of borderline oxacillin resistant *Staphylococcus aureus* (BORSA) isolated in Tunisia

1: Laboratoire de Microbiologie, CHU Habib Bourguiba, Sfax, Tunisie

2: Service de Microbiologie, Université de Caen Basse-Normandie, CHU Cote de Nacre, 14033 Caen cedex, France

Running title: Borderline oxacillin resistant *Staphylococcus aureus*

Keywords: *Staphylococcus aureus*, oxacillin, BORSA, MIC

Corresponding author:

Pr Adnene Hammami

Adresse : Laboratoire de Microbiologie, CHU Habib Bourguiba, Rue El Ferdaous 3029 Sfax, Tunisia.

Téléphone : 216 74 456 450

Fax : 216 74 456 450

E-mail : adnene.hammami@rns.tn
Abstract

Twenty three strains of *Staphylococcus aureus* with borderline resistance to oxacillin were studied. These strains were undetected by the cefoxitin test; PBP2a, *mecA* and *mecA* LGA251 were negative and were genetically unrelated.

To detect all of the strains resistant to oxacillin, laboratories should test in routine both cefoxitin and oxacillin.
Resistance to methicillin in *Staphylococcus aureus* is commonly mediated by PBP2a, an additional low affinity penicillin-binding protein, encoded by the chromosomal *meca* gene (4). In 2011, Garcia-Alvarez et al describe a novel *meca* gene homologue, called *mecaLGA251*, associated with resistance to β-lactam antibiotics. This gene has been presented in clinical strains of methicillin-resistant *S. aureus* (MRSA) which have been isolated in the UK and in Denmark (11). The intrinsic resistance, *meca* mediated, may be both homogenous which is easily detectable or heterogeneous (4). Oxacillin disc diffusion has been the traditional method for methicillin resistance screening. However, this test often fails to detect heterogeneous MRSA populations. Since 2001, the cefoxitin 30 µg disc test has showed to be more efficient in predicting methicillin resistance (10, 24, 25). This test has been recommended by different committees, such as the CLSI: Clinical and Laboratory Standards Institute (6) and the CA-SFM: Comité de l’antibiogramme de la société Française de microbiologie (7), for the purpose of predicting *meca*-mediated resistance in *Staphylococcus spp*. Detection of the PBP2a or the *meca* gene are the reference methods for methicillin susceptibility testing, but these are not feasible in most clinical laboratories (4). Resistance to methicillin may be extrinsic, non *meca* mediated, in *S. aureus* with low-level resistance to oxacillin known for borderline oxacillin-resistant *Staphylococcus aureus* (BORKAS) (16-19). Typically, this borderline phenotype results from excess production of β-lactamase. It was described initially by MC Dougal and Thornsberry in 1986 (17). According to these authors, these strains were neither heteroresistant nor multiple drug resistant, and produced large amounts of normal staphylococcal β-lactamase which partially hydrolyze oxacillin, and became fully susceptible to oxacillin in the presence of β-lactamase inhibitors (17). However, the borderline phenotype has been attributed to other mechanisms: production of an
inductible, plasmid-mediated, methicillinase or different modifications in the PBP genes, due
to spontaneous amino acid substitutions in the transpeptidase domain (19, 21).

In the present report, we analyzed 23 strains of *S. aureus* with reduced susceptibility
to oxacillin that were isolated in Sfax University hospital (Tunisia).

From 2006 to 2011, 1895 clinical strains of *S. aureus* were recovered in Sfax University
hospital. Among these strains, 415 (21.9%) were MRSA and 23 (1.2%) had reduced
susceptibility to oxacillin. These 23 strains were obtained from various clinical specimens: 14
were from pus, four from Blood culture, three from tracheal aspirate, one from urine and
one from ear. Fourteen strains were isolated from the dermatological unit.

Antibiotic susceptibility was performed using the disc diffusion method on Mueller-Hinton
agar (Bio-Rad). Minimal inhibitory concentrations (MICs) were determined by broth
microdilution method in unsupplemented Mueller-Hinton media. Antimicrobial susceptibility
results were interpreted according to the CLSI guidelines (6). ß-lactamase production was
realized by using nitrocephin discs (cefianse, bioMérieux). All of the strains were ß-lactamase
producer and were borderline resistant to oxacillin: inhibition zones diameters ranged from
10 mm to 13 mm for oxacillin 1 µg in Mueller-Hinton agar after incubation at 35°C and at
30°C, and MICs varied from 2 to 4 µg/ml. these strains were not only susceptible to cefoxitin
(inhibition zone ≥28 mm) but also susceptible to amoxicillin+clavulanic acid (inhibition zone
≥25 mm and MICs <0.125-0.25 µg/ml), to cefotaxime (inhibition zone ≥25 mm and MICs 0.5-
2 µg/ml) and to imipenem (inhibition zone ≥42 mm and MICs <0.125-0.125 µg/ml).

PBP2a production detected using PB2a latex agglutination test as recommended by the
manufacturer (bioMérieux) and detection of the mecA and the mecA_{GA251} genes by PCR (9,
20) were negative for all of the strains. The lack of the mecA gene confirmed that these
strains were not MRSA. The phenotype β-lactamase–hyperproducing BORSA was suggested. To confirm this hypothesis, the activity of clavulanic acid in combination with oxacillin was tested as previously demonstrated by others (5,22). We found that these strains became fully susceptible to oxacillin in the presence of β-lactamase inhibitor. In other words, for all of the strains, a significant increase (≥5 mm) of inhibition zone diameter for oxacillin was showed after the addition of 4 µg of clavulanic acid and a decrease which was more than two fold dilutions in oxacillin MICs was obtained in the presence of 4 µg/ml of clavulanic acid (Table 1). Oxacillin zone diameter and MICs were unaffected by the clavulanic acid in quality control strains (methicillin susceptible *S. aureus* ATCC 25923 and 29213 and MRSA ATCC 43300).

These 23 strains showed different antibiotypes (Table 1). All of these strains were susceptible to fosfomycin, to glycopeptides and to chloramphenicol. The *erm* ABC genes were investigated for by PCR as described previously (1). *ermC* was amplified from 13 strains which were resistant to erythromycin.

The typing of the strains was performed by pulsed-field gel electrophoresis (PFGE) as described previously (3). Genomic DNA was digested with the restriction enzyme Sma I, and the fragments were separated in agarose gels by electrophoresis according to the manufacturer’s recommendations. Image normalization and construction of similarity matrices were carried out using the finger printing II software (Bio-Rad). PFGE revealed that BORSA strains were genetically unrelated in our hospital (Fig 1). 18 genotypically different strains were identified, suggesting that BORSA strains were originated from different ancestors.
Borderline strains of *S. aureus* have been reported to be associated with both nosocomial and community-acquired infections in some institutions, and have been isolated from various infection sites, including skin, surgical wounds, respiratory samples, abscess and blood (2, 12-15, 23). Outbreaks of BORSA infections have been reported in two different dermatological units in Denmark (2, 14). These two outbreaks have been caused by two different clones, based on the same typing methods (PFGE and *spa* typing). Whereas, in our study, strains that were isolated in the dermatological unit were not closely related.

The incidence of BORSA strains is uncommon (19). It’s certainly underestimated given that many of clinical microbiology laboratories use only the cefoxitin test for detection of oxacillin resistance in *Staphylococcus* spp. Indeed, the cefoxitin test is a marker of resistance to oxacillin by acquisition of *mecA* gene and it’s unable to detect BORSA strains (10, 24, 25).

Detection of BORSA strains may influence the choice of antibiotics in the treatment. It has been augured by some authors that there is no apparent reason for BORSA strains to be considered as resistant to all other ß-lactams (5, 18, 22). Therefore, infections caused by these strains can probably be safely and effectively treated with ß-lactam antibiotics including cloxacillin or with the use of a ß-lactam-ß-lactamase inhibitor combination (16, 26, 27). However, other authors have demonstrated that oxacillin hasn’t been effective against BORSA strains (8, 23). As the treatment of infections caused by BORSA with ß-lactams is full of risk, therapy will be conducted according to the MIC of oxacillin and to the severity of the infection. In our study, most of patients were effectively treated with pristinamycin. The five patients who were treated with ß-lactams (cefotaxime or imipenem) were also recovered.

In conclusion, the clinical laboratories should be in position to recognize BORSA strains by routine susceptibility testing and especially for differentiating them from truly methicillin...
resistant or susceptible *S. aureus*. Hence, we recommend the utilization of both the cefoxitin disc as a marker of resistance to oxacillin by acquisition of the *mecA* gene, and oxacillin disc on Mueller Hinton agar incubated at 30°C or on Mueller Hinton agar with NaCl for detection of BORSA strains.
References


FIG. 1. Dendrogram of PFGE pattern of 23 BORSA Strains
<table>
<thead>
<tr>
<th>Strain</th>
<th>Specimen</th>
<th>Date of isolation</th>
<th>Ward</th>
<th>Inhibition zone diameter (mm)</th>
<th>MIC (µg/ml)</th>
<th>Other resistance markersa</th>
<th>erm</th>
<th>PFGEb</th>
</tr>
</thead>
<tbody>
<tr>
<td>V44</td>
<td>Eye</td>
<td>01/07/2006</td>
<td>Dermatology</td>
<td>10 20 30 30 30 44</td>
<td>2</td>
<td>0.52 0.125 1 &lt;0.125</td>
<td>ERY, TET, KAN, TOB, GEN, ERY, L, PT, TET, OFX, RIF, FUC</td>
<td></td>
</tr>
<tr>
<td>V451</td>
<td>Pus</td>
<td>03/10/2006</td>
<td>Dermatology</td>
<td>11 20 30 30 30 44</td>
<td>2</td>
<td>0.52 0.125 1 &lt;0.125</td>
<td>ERY, TET, KAN, TOB, GEN, ERY, L, PT, TET, OFX, RIF, FUC</td>
<td></td>
</tr>
<tr>
<td>RJ56</td>
<td>Tracheal aspirate</td>
<td>10/01/2006 Intensive care unit</td>
<td>7</td>
<td>20 20 28 27 46</td>
<td>4</td>
<td>0.25 0.125 1 &lt;0.125</td>
<td>ERY, TET, KAN, TOB, GEN, ERY, L, PT, TET, OFX, RIF, FUC</td>
<td></td>
</tr>
<tr>
<td>V47</td>
<td>Pus</td>
<td>23/01/2007</td>
<td>Dermatology</td>
<td>13 22 32 30 30 48</td>
<td>2</td>
<td>0.125 0.125 1 &lt;0.125</td>
<td>ERY, TET, KAN, TOB, GEN, ERY, L, PT, TET, OFX, RIF, FUC</td>
<td></td>
</tr>
<tr>
<td>V184</td>
<td>Pus</td>
<td>28/05/2005</td>
<td>Dermatology</td>
<td>13 20 30 28 27 46</td>
<td>2</td>
<td>0.25 0.125 0.5 &lt;0.125</td>
<td>ERY, TET, KAN, TOB, GEN, ERY, L, PT, TET, OFX, RIF, FUC</td>
<td></td>
</tr>
<tr>
<td>V277</td>
<td>Blood</td>
<td>09/07/2006</td>
<td>Dermatology</td>
<td>12 22 33 30 30 48</td>
<td>2</td>
<td>0.25 0.25 1 &lt;0.125</td>
<td>ERY, TET, OX, RIF, FUC</td>
<td>ermC</td>
</tr>
<tr>
<td>L170</td>
<td>Ear</td>
<td>21/08/2008</td>
<td>Maxillofacial</td>
<td>12 21 30 30 30 48</td>
<td>4</td>
<td>0.25 0.25 2 &lt;0.125</td>
<td>ERY, TET, OX, RIF, FUC</td>
<td></td>
</tr>
<tr>
<td>V132</td>
<td>Pus</td>
<td>05/09/2008</td>
<td>Dermatology</td>
<td>12 22 30 26 34 52</td>
<td>2</td>
<td>0.25 0.125 1 &lt;0.125</td>
<td>ERY, TET, OX, RIF, FUC</td>
<td></td>
</tr>
<tr>
<td>V184</td>
<td>Pus</td>
<td>17/10/2008</td>
<td>Dermatology</td>
<td>12 21 32 32 30 48</td>
<td>4</td>
<td>0.125 0.125 1 &lt;0.125</td>
<td>ERY, TET, OX, RIF, FUC</td>
<td></td>
</tr>
<tr>
<td>C4557</td>
<td>Pus</td>
<td>01/12/2008</td>
<td>Infectious diseases</td>
<td>11 19 31 29 29 46</td>
<td>4</td>
<td>0.125 0.125 1 &lt;0.125</td>
<td>ERY, TET, OX, RIF, FUC</td>
<td></td>
</tr>
<tr>
<td>PL140</td>
<td>Urine</td>
<td>19/12/2008</td>
<td>Internal Medicine</td>
<td>12 20 30 26 29 46</td>
<td>4</td>
<td>0.125 0.125 1 &lt;0.125</td>
<td>ERY, TET, OX, RIF, FUC</td>
<td></td>
</tr>
<tr>
<td>V12</td>
<td>Pus</td>
<td>10/01/2009</td>
<td>Dermatology</td>
<td>12 23 30 40 38 56</td>
<td>4</td>
<td>0.25 0.25 2 &lt;0.125</td>
<td>ERY, TET, OX, RIF, FUC</td>
<td></td>
</tr>
<tr>
<td>UB15</td>
<td>Blood</td>
<td>25/01/2009</td>
<td>Burn unit</td>
<td>12 21 28 29 30 49</td>
<td>4</td>
<td>0.125 0.125 1 &lt;0.125</td>
<td>ERY, TET, OX, RIF, FUC</td>
<td></td>
</tr>
<tr>
<td>UB46</td>
<td>Tracheal aspirate</td>
<td>13/04/2009 Intensive care unit</td>
<td>7</td>
<td>20 20 28 30 30 48</td>
<td>2</td>
<td>0.125 0.125 1 &lt;0.125</td>
<td>ERY, TET, OX, RIF, FUC</td>
<td></td>
</tr>
<tr>
<td>V92</td>
<td>Burn</td>
<td>27/08/2009</td>
<td>Dermatology</td>
<td>12 21 30 32 34 50</td>
<td>4</td>
<td>0.125 0.125 1 &lt;0.125</td>
<td>ERY, TET, OX, RIF, FUC</td>
<td></td>
</tr>
<tr>
<td>UV101</td>
<td>Tracheal aspirate</td>
<td>27/05/2009 Intensive care unit</td>
<td>7</td>
<td>20 20 30 32 34 50</td>
<td>4</td>
<td>0.125 0.125 1 &lt;0.125</td>
<td>ERY, TET, OX, RIF, FUC</td>
<td></td>
</tr>
<tr>
<td>V26</td>
<td>Pus</td>
<td>04/11/2009</td>
<td>Dermatology</td>
<td>11 20 30 26 25 46</td>
<td>2</td>
<td>0.25 0.125 1 &lt;0.125</td>
<td>TET, OX</td>
<td></td>
</tr>
<tr>
<td>V134</td>
<td>Pus</td>
<td>16/10/2010</td>
<td>Dermatology</td>
<td>13 21 30 30 26 46</td>
<td>2</td>
<td>0.25 0.125 1 &lt;0.125</td>
<td>TET, OX</td>
<td></td>
</tr>
<tr>
<td>V284</td>
<td>Blood</td>
<td>04/05/2010</td>
<td>Dermatology</td>
<td>13 20 30 29 26 46</td>
<td>2</td>
<td>0.125 0.125 2 &lt;0.125</td>
<td>TET, OX, RIF, FUC</td>
<td></td>
</tr>
<tr>
<td>V14</td>
<td>Blood</td>
<td>30/05/2010</td>
<td>Dermatology</td>
<td>13 20 30 25 26 50</td>
<td>2</td>
<td>0.125 0.125 2 &lt;0.125</td>
<td>TET, OX, RIF, FUC</td>
<td></td>
</tr>
<tr>
<td>ATCC 25923</td>
<td>-</td>
<td>-</td>
<td>22 23 28 16 31 40</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ATCC 29213</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ATCC 43100</td>
<td>-</td>
<td>-</td>
<td>12 13</td>
<td>-</td>
<td>-</td>
<td>0.25 0.25 1 &lt;0.125</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
| S. aureus ATCC 25923 (MSSA): quality control for disc diffusion; S. aureus ATCC 29213 (MSSA): quality control for MIC; S. aureus ATCC 43100 (MRSA): quality control for oxacillin disc diffusion and MIC

| Table 1. Characteristics of Borderline oxacillin-resistant S. aureus (BORSA) strains


- PFGE: Pulsed field gel electrophoresis

- S. aureus ATCC 25923 (MSSA): quality control for disc diffusion; S. aureus ATCC 29213 (MSSA): quality control for MIC; S. aureus ATCC 43100 (MRSA): quality control for oxacillin disc diffusion and MIC