

# Dissemination Of t437-SCCmecIV And Coagulase-Negative t037-SCCmecIII Types Among Borderline Oxacillin-Resistant *Staphylococcus aureus* Isolated From Skin Infections And Diabetic Foot Ulcers

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**Background:** In a recent decade, the occurrence of *S. aureus* isolates with low-level oxacillin resistance, have been reported increasingly. The aim of this study was to estimate the prevalence of *S. aureus* with low-level of oxacillin resistance and to determine their molecular characteristics, including *spa* types, SCCmec types and presence of toxin genes.

**Methods:** A total of 249 *S. aureus* strains were analyzed. Antimicrobial susceptibility was preliminarily tested by the disk diffusion method, and further was verified with the E-test and agar dilution methods. All borderline oxacillin-resistant strains (BORSAs) were screened for the *mecA* gene and virulence factors, including Panton-Valentine leukocidin (PVL). Staphylococcal cassette chromosome *mec* (SCCmec) typing and *spa* typing were also carried out.

**Results:** Twelve (4.8%) borderline oxacillin-resistant strains with MIC  $\leq 4$   $\mu\text{g/mL}$  were identified. Almost all strains (11/12) were oxacillin-susceptible methicillin resistant *S. aureus* carrying *mecA* gene (OS-MRSA). Among the 12 borderline strains, five *spa* types (t437, t037, t015, t216, t267) and two SCCmec types (III, IV) were identified, with the most prevalent being t437-SCCmecIV *pvl*-positive. The second most frequent *spa* type, t037-SCCmecIII, was *sea*-positive and did not produce coagulase. The majority of borderline strains originated from skin infections and diabetic foot ulcers and were multidrug-resistant (macrolides, lincosamides and chloramphenicol).

**Conclusion:** This study demonstrated that *S. aureus* with borderline resistance to oxacillin represented primarily SCCmecIV *spa* type t437 and coagulase-negative SCCmecIII *spa* type t037 and were isolated from skin infections and diabetic foot ulcers.

**Keywords:** *Staphylococcus aureus*, MRSA, OS-MRSA, borderline oxacillin-resistant *S. aureus*, low-level oxacillin resistance

## Introduction

Infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) constitute a global public health burden. Minimum inhibitory concentration (MIC) of oxacillin for most MRSA strains exceeds 4  $\mu\text{g/mL}$ ; oxacillin-resistance is determined by the presence of the *mecA* gene encoding penicillin-binding protein 2a (PBP2a). However, in recent years, clinical MRSA strains with low-level resistance to oxacillin were isolated in several countries including the Netherlands, Germany, Greece, Taiwan, China, Japan, the USA and Angola.<sup>1-6</sup> The strains with low-level resistance to oxacillin are also referred to as borderline oxacillin-resistant *S. aureus* (BORSA) or oxacillin-

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susceptible MRSA (OS-MRSA). This group includes two subsets of strains, *mecA*-positive and *mecA*-negative. The mechanism of phenotypic resistance to oxacillin in the *mecA*-negative strains that harbor neither *mecA* nor *mecC* gene is still not fully understood. Probably resistance of these strains to oxacillin is associated with overproduction of beta-lactamases or, in some cases, with point mutations in PBP genes.<sup>7,8</sup> Borderline isolates may be easily misidentified as methicillin-sensitive *S. aureus* (MSSA), which may result in treatment failure and spread of these strains within the hospital and community setting.<sup>9</sup> Epidemiology and clinical presentation of infections caused by BORSA seem to be similar to those associated with MRSA; usually, the infections are more severe than those caused by MSSA, even if a larger dose of oxacillin is administered.<sup>10</sup> According to Huang et al,<sup>11</sup> bacteremia caused by BORSA was significantly more often associated with pneumonia or empyema than the systemic infections caused by MSSA and MRSA. Available evidence suggests that the problem of BORSA/OS-MRSA spread is frequently underestimated, and considering their documented emergence in new environments, these strains need to be monitored and accurately distinguished from MSSA.<sup>12</sup>

The aim of this study was to estimate the prevalence of borderline oxacillin-resistant *S. aureus* and to determine their molecular characteristics, including *spa* types, *SCCmec* types and presence of toxin genes.

## Materials And Methods

### Bacterial Strains

A total of 249 non-duplicate *S. aureus* methicillin-resistant strains from various clinical samples were analyzed. The study was based on a retrospective analysis of methicillin-resistant strains isolated within the last 21 years and archived at the Department of Medical Microbiology, and the strains from the Laboratory of Clinical Microbiology, University Clinical Center in Gdansk, isolated during routine clinical laboratory procedures. The identity of *S. aureus* isolates was verified with conventional methods and with Pastorex Staph-Plus latex agglutination kit (Bio-Rad, Marnes-la-Coquette, France) and confirmed based on the polymerase chain reaction (PCR) of *S. aureus*-specific region of the thermonuclease gene, *nuc*.<sup>13</sup> The isolates were stored at  $-80^{\circ}\text{C}$  in trypticase soy broth (TSB) (Oxoid, Basingstoke, England) supplemented with 15% glycerol.

### Antimicrobial Susceptibility

The antimicrobial susceptibility of *S. aureus* strains was tested by disk diffusion method using penicillin G, erythromycin, clindamycin, ciprofloxacin, tetracycline, gentamicin, sulfamethoxazole/trimethoprim, chloramphenicol and fusidic acid discs from Becton Dickinson (Franklin Lakes, NJ, USA) and was interpreted according to the Clinical and Laboratory Standards Institute (CLSI).<sup>14</sup> MIC for vancomycin was determined by E-tests, in line with the manufacturer's instructions (AB Biodisc, Solna, Sweden).

### Detection Of Methicillin Resistance

All archival strains that were previously labeled as methicillin-resistant were subjected to repeated analysis. Susceptibility to oxacillin and cefoxitin was preliminarily determined by the disk diffusion method on Mueller-Hinton agar (Becton Dickinson, Franklin Lakes, NJ, USA). The plates were incubated for 24 h at  $35^{\circ}\text{C}$ , and the results were interpreted according to the CLSI.<sup>14</sup> In the case of the strains with discordant results of oxacillin and cefoxitin susceptibility tests, oxacillin MIC was additionally determined with the agar dilution test, in line with the CLSI guidelines,<sup>15</sup> and by means of E-test (Becton Dickinson, Franklin Lakes, NJ, USA). In brief, selected *S. aureus* strains were plated on Mueller-Hinton agar supplemented with 4% sodium chloride containing 1, 2, 4, and 6  $\mu\text{g/mL}$  oxacillin. The results were recorded after 24-h and 48-h incubation at  $35^{\circ}\text{C}$ . The strains with oxacillin MICs  $\leq 4 \mu\text{g/mL}$  on agar dilution test were considered BORSA. All selected strains were tested for the presence of *mecA* gene using the method described previously elsewhere.<sup>16</sup> Moreover, one *mecA*-negative strain was tested for the carriage of *mecC* gene.<sup>17</sup> The reference strains, *S. aureus* ATCC 43300 and *S. aureus* ATCC 29213, were used as positive and negative controls, respectively.

### Coagulase Production

For all borderline oxacillin-resistant strains free coagulase test was performed under standard conditions by incubating 0.8 mL of TSB culture with 0.2 mL rabbit plasma for 2, 4, 6 and 24 h at  $37^{\circ}\text{C}$ .<sup>18</sup> The reference strains, *S. aureus* ATCC 29213 and *S. epidermidis* PCM 2118, were used as positive and negative controls, respectively.

### Molecular Typing

Total DNA of *S. aureus* isolates was purified using Genomic DNA kit (A&A Biotechnology, Gdynia, Poland), in line with the manufacturer's instructions.

Typing of the staphylococcal chromosomal cassette *mec* (SCC*mec*) was carried out as described previously by Milheirício et al.<sup>16</sup> Genes encoding enterotoxins (*sea*, *seb*, *sec*, *sed*), exfoliative toxins (*eta*, *etb*), toxic shock syndrome toxin-1 (*tst*) and Panton-Valentine leukocidin (*lukS-PV/lukF-PV*) were detected by means of multiplex PCR, as described elsewhere.<sup>19,20</sup> The presence of penicillinase encoded by *bla<sub>Z</sub>* gene was determined according to Wang et al.<sup>21</sup> The PCR products were electrophoretically resolved in 1.5% agarose containing 0.5 µg/mL ethidium bromide.

### spa Typing

*spa* typing was performed according to Harmsen et al.<sup>22</sup> Nucleotide sequencing of the repeat-containing region of the *spa* gene was conducted on both DNA strands of the PCR product from Genomed (Warszawa, Poland), using BigDye Terminator Ready Reaction Cycle Sequencing kit (St Louis, MO, United States). *spa*-types were identified with Ridom Staph-Type software v.2.1.1 (Ridom GmbH, Münster, Germany).<sup>22</sup>

### Statistical Analysis

Distributions of quantitative variables were presented as numbers and percentages and compared between groups using the Pearson chi-square or Fisher exact test. All calculations were carried out with Statistica 10 package (StatSoft, Tulsa, OK, United States), with the threshold of statistical significance set at  $p \leq 0.05$ .

## Results

### Prevalence Of Borderline Oxacillin-Resistant *S. aureus*

The analyzed group of 249 *S. aureus* included 12 (4.8%) identified as borderline oxacillin-resistant strains based on oxacillin MIC  $\leq 4$  µg/mL determined with the agar dilution method. The strains were isolated from wounds (n=5), foot ulcers (n=3), furuncle (n=1), catheter (n=1), blood (n=1) and nose (n=1) (Table 1).

### Antimicrobial Susceptibility

MICs of oxacillin for the 12 borderline strains varied between 2 µg/mL and 4 µg/mL when determined with agar dilution method and between 1 µg/mL and 4 µg/mL if estimated using the E-test. 9 out of 12 BORSA strains were susceptible to oxacillin but resistant to cefoxitin, whereas 3 out of 12 isolates were susceptible to cefoxitin but resistant to oxacillin. Moreover, BORSA strains turned out to be resistant to erythromycin (11/12), clindamycin (7/12), chloramphenicol (6/12), tetracycline (4/12), ciprofloxacin (3/12), doxycycline (3/12) and gentamicin (3/12). Most BORSA (10/12) were identified as multidrug-resistant strains, and aside from beta-lactams showed also resistance to other antibiotics (Table 1).

### Molecular Characteristics

Five *spa* types were identified among the analyzed borderline strains. Half (6/12) of them represented type t437, one-fourth

**Table 1** Characteristics Of Borderline Oxacillin-Resistant *S. aureus*

Patient No.	<i>spa</i> Type	Year	Origin	MIC Of Oxacillin (µg/mL)		Resistance To (DD, mm)		Phenotype Of Resistance	Coagulase Production
				AD	E-Test	OX	FOX		
1	t037	1998	Wound	4	2	12 R	25 S	OX, E, CC, DX, GE, CIP, C	–
2	t037	2000	Foot ulcer	2	1	12 R	26 S	OX, E, CC, DX, GE	–
3	t037	2000	Wound	2	1	11 R	23 S	OX, E, DX, GE	–
4	t437	2013	Furuncle	4	2	14 S	19 R	FOX, E, CC, TE, C	+
5	t437	2013	Wound	4	2	15 S	19 R	FOX, E, CC, TE, C	+
6	t437	2013	Wound	4	2	14 S	19 R	FOX, E, CC, TE, C	+
7	t437	2016	Foot ulcer	2	1	15 S	18 R	FOX, E, CC, TE, C	+
8	t437	2017	Wound	4	2	13 S	17 R	FOX	+
9	t437	2018	Foot ulcer	4	1.5	17 S	19 R	FOX, E, CC, C	+
10	t216	2013	Nose	4	1.5	14 S	16 R	FOX, E, CIP	+
11	t267	2016	Blood	4	4	15 S	19 R	FOX, E	+
12	t015	2018	Catheter	4	2	18 S	18 R	FOX, E, CIP	+

**Abbreviations:** AD, agar dilution; DD, disk diffusion; OX, oxacillin; FOX, cefoxitin; E, erythromycin; CC, clindamycin; DX, doxycycline; TE, tetracycline; GE, gentamicin; CIP, ciprofloxacin; C, chloramphenicol; R, resistant; S, sensitive.

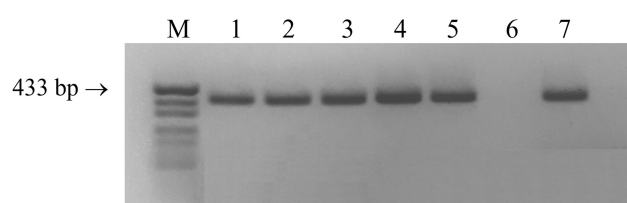
(3/12) belonged to type t037, and single strains were identified as types t015, t216 and t267. Almost all strains (11/12) carried *mecA* and *blaZ* genes. The strains carried SCCmec type IV (9/12) or SCCmec type III (2/12). The list of identified toxin genes included *lukF-lukS-PV* (5/12), *seb* (5/12), *sea* (3/12) and *tst* (1/12) (Table 2, Figure 1).

## Comparison Between *spa* Types t437 And t037

Borderline strains belonging to type t437 were identified twice as often as those from type t037 and were isolated from more recently archived clinical material. Strains belonging to both *spa* types originated from skin infections and diabetic foot ulcers. Type t437 and t037 differed in terms of their molecular characteristics; while the strains of type t437 carried toxin genes *pvl* (5/6), *seb* (4/6), and SCCmec type IV, those representing type t037 harbored *sea* (3/3) and *tst* (1/3) genes and SCCmec type III. Most of the t437 strains were resistant to clindamycin (5/6), erythromycin (5/6) and chloramphenicol (5/6), whereas all isolates belonging to the *spa* type t037 showed a distinct pattern of resistance to doxycycline and gentamycin (3/3). Unlike the t437 strains, none of the t037 isolates produced coagulase (Table 3).

## Discussion

In a recent decade, the occurrence of *S. aureus* isolates with low-level oxacillin resistance, especially *mecA*-positive strains, have been reported increasingly. Published



**Figure 1** Agarose gel image of *lukS-PV/lukF-PV* PCR amplicon (433 bp). Lane M – molecular size marker (*pUC19* DNA/*MspI* enzyme, Fermentas, Lithuania); lanes 1–5 – representative PVL-positive *S. aureus* strains of *spa* type t437, lane 6 – negative control; lane 7 – positive control.

data about the prevalence of these isolates differ considerably depending on the country and clinical material, from slightly above 1% (1.1% in Taiwan, 1.25% in Japan, 1.6% in China),<sup>3,4,23</sup> to a few percent (5.8–9.8%),<sup>2,24</sup> or even more than 10% or 20%.<sup>5,25</sup> Considering these data, the prevalence of borderline strains in our material should be considered moderately high.

According to literature, borderline *S. aureus* are isolated from skin and soft tissue infections, bacteremia, pneumonia and urinary tract infections.<sup>11</sup> The majority of these strains identified in our study originated from skin infections; to the best of our knowledge, our study was the first to document the presence of borderline resistant strains in diabetic foot ulcers. In recently published paper, Lin et al<sup>26</sup> did not specify whether the t437 strains isolated from diabetic foot ulcers showed low-level oxacillin resistance or were fully resistant to this antibiotic.

Most previously isolated OS-MRSA were identified as community-acquired MRSA (CA-MRSA) strains. Based on

**Table 2** The Presence Of Virulence And Resistance Genes To  $\beta$ -Lactams Antibiotics In Different *spa* And SCCmec Types Of Borderline Oxacillin-Resistant *S. aureus*

Patient No.	<i>spa</i> Type	Repeat Succession	SCCmec Type	Genetic Profile										
				<i>mecA</i>	<i>mecC</i>	<i>blaZ</i>	<i>lukF-lukS-PV</i>	<i>sea</i>	<i>seb</i>	<i>sec</i>	<i>sed</i>	<i>tst</i>	<i>eta</i>	<i>etb</i>
1	t037	15-12-16-02-25-17-24	III	+	–	+	–	+	–	–	–	–	–	–
2	t037	15-12-16-02-25-17-24	–	–	–	+	–	+	–	–	–	+	–	–
3	t037	15-12-16-02-25-17-24	III	+	–	+	–	+	–	–	–	–	–	–
4	t437	04-20-17-20-17-25-34	IV	+	–	+	+	–	–	–	–	–	–	–
5	t437	04-20-17-20-17-25-34	IV	+	–	+	+	–	+	–	–	–	–	–
6	t437	04-20-17-20-17-25-34	IV	+	–	+	+	–	+	–	–	–	–	–
7	t437	04-20-17-20-17-25-34	IV	+	–	+	–	–	–	–	–	–	–	–
8	t437	04-20-17-20-17-25-34	IV	+	–	+	+	–	+	–	–	–	–	–
9	t437	04-20-17-20-17-25-34	IV	+	–	+	+	–	+	–	–	–	–	–
10	t216	04-20-17-20-17-31-16-34	IV	+	–	+	–	–	+	–	–	–	–	–
11	t267	07-23-12-21-17-34-34-34-33-34	IV	+	–	+	–	–	–	–	–	–	–	–
12	t015	08-16-02-16-34-13-17-34-16-34	IV	+	–	+	–	–	–	–	–	–	–	–

**Table 3** Characteristics Of Borderline Oxacillin-Resistant *S. aureus* *spa* Types t037 And t437

Parameter	<i>spa</i> Type t037	<i>spa</i> Type t437	p-Value
Year of isolation	1998–2000	2013–2018	
Prevalence	3/12	6/12	0.400
Skin infection	2/3	4/6	1.000
Foot ulcer	1/3	2/6	1.000
SCCmecIII	3/3	0/6	<b>0.012</b>
SCCmecIV	0/3	6/6	<b>0.012</b>
<i>sea</i>	3/3	0/6	<b>0.012</b>
<i>seb</i>	0/3	4/6	0.167
<i>lukF-lukS-PV</i>	0/3	5/6	<b>0.048</b>
<i>tst</i>	1/3	0/6	0.333
Clindamycin/erythromycin resistance	2/3	5/6	1.000
Gentamicin/doxycycline resistance	3/3	0/6	<b>0.012</b>
Production of coagulase	0/3	6/6	<b>0.012</b>

**Note:** Bold values indicate statistical significance.

their molecular characteristics, also the strains identified in our study were probably CA-MRSA. Similar to many previous reports from Asia, most of our OS-MRSA strains represented *spa* type t437 classified among the international CA-MRSA clones.<sup>24</sup> This implies that the type t437 staphylococci have already expanded onto the European continent. CA-MRSA strains typically harbor staphylococcal cassette chromosome *mec* type IV (SCCmecIV), along with the *pvl* gene which is considered a genetic marker for this group.<sup>27</sup> Nearly half of the strains identified in our study were SCCmecIV-*pvl*-positive, which is consistent with the reports from other European centers,<sup>6</sup> and distinguishes these clones from those isolated in Asia, more often being SCCmecV-*pvl*-negative.<sup>11</sup> Presence of Panton-Valentine toxin was shown to be associated with furunculosis and necrotizing pneumonia, but as emphasized by Gillet et al,<sup>28</sup> this relationship was observed primarily in healthy children and young adults.

To the best of our knowledge, our study was the first to identify coagulase-negative *S. aureus* isolates belonging to *spa* type t037 and showing borderline resistance to oxacillin. Previously, type t037 strains were found among both MRSA and OS-MRSA,<sup>24,26,29</sup> but none of the reported isolates was coagulase-negative. Skinner et al<sup>30</sup> reported on a patient in whom endocarditis was caused by a strain that showed borderline resistance to oxacillin and lacked either thermonuclease or coagulase, i.e. the two basic taxonomic characteristics of *S. aureus*. Aside from difficulties in the selection of appropriate antibiotic therapy,

another challenge in that case was the identification of the isolate at a species level.<sup>30</sup> Missing expression of species-specific proteins has been reported primarily in the case of MRSA; this phenomenon may result from the insertion of a transposon (Tn917) or integration of plasmid genes into the chromosome.<sup>12</sup> According to Duval-Iflah et al,<sup>31</sup> the loss of ability to coagulate plasma may result from a lysogenic conversion with LS1 and LS2 phages.

Borderline *S. aureus* isolates are usually multidrug-resistant; this was also confirmed in our present study in which most of the strains showed resistance to macrolides and lincosamides, but unlike the isolates from Asia, were relatively often susceptible to fluoroquinolones.<sup>11</sup> Treatment of infections caused by OS-MRSA may be challenging; according to Ho et al,<sup>3</sup> nearly half of patients infected with these strains received inadequate treatment due to misidentification of the etiological factor as MSSA, which in two cases had fatal consequences.

It is still unclear which epidemiological and/or clinical factors contributed to the spread of the *S. aureus* with low-level resistance to oxacillin across all continents. One may stipulate if a likely cause of BORSA spread is their better adjustment to environmental conditions with lesser antibiotic selective pressure than in the case of typical MRSA and whether these strains can be even more widespread in future.<sup>32</sup> Recent findings suggest that the MRSA clones might simultaneously co-evolve in different geographic regions.

## Conclusion

This study demonstrated that *S. aureus* with borderline resistance to oxacillin represented primarily SCCmecIV *spa* type t437 and coagulase-negative SCCmecIII *spa* type t037 and were isolated from skin infections and diabetic foot ulcers. Future studies should monitor the spread of these strains and related risks associated with their involvement in other infections.

## Abbreviations

BORSA, borderline oxacillin-resistant *S. aureus* (BORSA); CA-MRSA, community-acquired methicillin-resistant *S. aureus*; CLSI, Clinical and Laboratory Standards Institute; MIC, minimum inhibitory concentration (MIC); MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*; OS-MRSA, oxacillin-susceptible *S. aureus*; PBP, penicillin-binding protein; PCR, polymerase chain reaction; *S. aureus*, *Staphylococcus aureus*; SCCmec, staphylococcal cassette chromosome *mec*; TSB, trypticase soy broth.

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## Disclosure

The authors report no conflicts of interest in this work.

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