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Jacobs Journal of Microbiology and Pathology

Research Article

Analysis of the Correlation between Oxacillin/Cefoxitin Susceptibility/Resistance, the Presence of the Gene *MecA* and PBP2a Production in Hospital Infection-Associated Staphylococcal Isolates

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Received: 10-15-2014

Accepted: 01-19-2015

Published: 02-03-2015

Abstract

Oxacillin/cefoxitin-susceptible *mecA*-positive staphylococcal strains present a challenge for clinical and therapeutic management and require great attention from clinical microbiology laboratories, as phenotypical tests for oxacillin/cefoxitin and PBP2a detection might be misleading, and the incorrect identification could lead to treatment with inappropriate antimicrobial therapy. PBP2a/PBP2´ is an alternative, low-affinity penicillin-binding protein (PBP) encoded by the gene *mecA*. The aims of the present study were to determine whether the presence of the gene *mecA* in *Staphylococcus aureus* and *coa*gulase-negative *Staphylococcus* (CoNS) isolates correlates with the oxacillin/cefoxitin-resistance phenotype and PBP2a production, to uncover heterogeneous populations harboring pre-*mec* cells, and to discover whether the pre-*mec* populations under antibiotic exposure become homogeneously resistant. A retrospective analysis through screening and/or MIC tests involving 24 *mecA* PCR-positive *S. aureus* and 15 CoNS hospital infection-associated isolates showed that some *mecA* PCR-positive isolates were oxacillin/cefoxitin-susceptible, and indirect immunofluorescence (IFI) and western blotting (WB) did not detect PBP2a expression in all of the screened *mecA* PCR-positive isolates. A population analysis of randomly selected strains revealed that *S. aureus* strains harbored homogenous cell populations, whereas CoNS strains harbored heterogeneous populations. Oxacillin exposure did not change the susceptibility of the homogenous pre-*mec S. aureus* strains, whereas exposure to cefoxitin induced resistance in these strains, and exposure to antibiotics led to resistance in heterotypic CoNS populations. These results demonstrated that the phenotypical tests employed were unable to detect oxacillin/cefoxitin resistance or PBP2a expression in all *mecA* PCR-positive isolates studied; both the results of the phenotypical resistance detection tests and antibiotic exposure are influenced by the homogenous or heterogeneous characteristics of the isolate cell populations.

Keywords: *Staphylococcus aureus*; CoNS; Hospital Infections; Oxacillin; Cefoxitin; *mecA*; PBP2a.

Introduction

Staphylococcal resistance to the β-lactam class of antibiotics, termed methicillin-resistance, is an important public health issue, as this resistance reduces therapeutic options. Methicillin-resistant *Staphylococcus aureus* (MRSA) has been blamed for a number of hospital-acquired staphylococcal infections worldwide. Furthermore, the incidence of nosocomial infections, particularly those associated with prosthetic devices, and methicillin-resistant *coa*gulase-negative *Staphylococcus* (MRCoNS) are increasing [1].

Methicillin resistance in staphylococci is primarily defined by the presence of the gene *mecA*, located on the mobile genetic element called staphylococcal cassette chromosome *mec* (SCC*mec*). The gene *mecA* codes for an alternative penicillin-binding protein named PBP2a or PBP2', which is essential for cell wall synthesis and bacterial growth in the presence of β-lactam antibiotics [2].

Therefore, the detection of the *mecA* gene and/or PBP2a expression would be more reliable than phenotypical tests of oxacillin/cefoxitin detection. However, the determination of methicillin susceptibility among staphylococci is generally performed with oxacillin or cefoxitin phenotypic tests [2]. Whereas *mecA* is a determinant of oxacillin resistance, the phenotypic expression of resistance among *mecA*-positives may vary according to the population of resistant and susceptible cells originating from different cell populations that display heterotypic (heterogeneous) or homotypic (homogeneous) resistance. Furthermore, there is evidence of conversion from heterotypic to homotypic or homogeneous high-resistance level following exposure to beta-lactams [3-5].

Phenotypical oxacillin-susceptible isolates harboring the gene *mecA* (OS-MRS and OS-MRCoNS) are regarded as pre-methicillin resistant (pre-*mec*) because they may become resistant either by induction or selection in the presence of antibiotics. Oxacillin-susceptible *mecA*-positive isolates may be misidentified as susceptible. This misidentification can lead to inappropriate antimicrobial therapy because many clinical laboratories only assess the oxacillin susceptibility phenotype and not the presence of *mecA* or the expression of PBP2a [2,5,6].

Phenotypically susceptible oxacillin/cefoxitin and *mecA*-positive staphylococci strains are a challenge for clinical and therapeutic management and require great attention from clinical microbiology laboratories because the susceptible phenotype may result from low protein expression or the heterotypic phenotype, which leads to the evasion of susceptibility tests and the incorrect choice of drugs for treatment [3,7].

The aims of the study were to determine if the presence of the gene *mecA* in *S. aureus* and *coa*gulase-negative *Staphylococcus* (CoNS) isolates correlate with the oxacillin/cefoxitin-resistance phenotype and PBP2a production, which would confirm the cell populations to be homogeneously resistant or would reveal heterogeneous populations harboring pre-*mec* cells (*mecA*-positive/oxacillin-negative), and to determine if antibiotic exposure enabled a pre-*mec* population to become homogeneously resistant.

The results showed that *mecA* gene testing is a more reliable test for the detection of methicillin resistance among staphylococci, confirming that all *mecA*-positive isolates should be regarded as resistant, even if these strains show phenotypically susceptible behavior. The phenotypical tests for oxacillin/cefoxitin and PBP2a detection might be influenced by the homogenous or heterogeneous characteristics of the isolate cell populations, and the inappropriate use of antimicrobials could increase resistance.

Material and Methods

Bacterial isolates and growth conditions

Twenty-four *S. aureus* and 15 CoNS *mecA* PCR-positive isolates from the bacteriotheque of the Department of Microbiology, CPqAM/FIOCRUZ-PE were selected for study. The isolates originating from nosocomial infections from Recife, PE, Brazil, from 2002-2004 were stored at -80ºC in brain heart infusion (BHI) broth (HiMedia Laboratories Pvt Ltd 23, Vadhani Industrial Estate, LBS Marg, Ghatkopar West, Mumbai, Maharashtra 400086, India) / 25% glycerol. Among the *S. aureus* 10 isolates originated from hemocultures, one from urine culture, seven from catheter tips and six from pulmonary fluids; among the CoNS (identified as *S. epidermidis*) isolates eight originated from hemocultures, five from catheter tips and two from pulmonary fluids. The source of the strains is shown in Table 1. The isolates were characterized as nosocomial infections according to the Centers for Disease Control and Prevention criteria [8], and were identified by the conventional bacteriological catalase test, the *coa*gulase tube test, the thermonuclease growth test, and the mannitol salt agar test. All of the samples were methicillin resistant as determined by the presence of the *mec* gene by PCR [9]. The absence of the *coa*gulase in the strains was confirmed by a PCR test that was negative for the *coa* gene [10]. The reference strains *S. aureus* ATCC 33591 (methicillin-resistant) and *S. aureus* ATCC 29213 (methicillin-susceptible) were used as positive and negative controls, respectively. Unless otherwise stated, all cultures were grown on sheep blood agar plates overnight at 37º C.

Determination of susceptibility to oxacillin and cefoxitin by *mecA* PCR-positive isolates

The susceptibility to oxacillin was screened using a screening agar plate test (Mueller Hinton agar, HiMedia) supplemented

with 4% NaCl and 6 mg/L oxacillin). The oxacillin and cefoxitin minimum inhibitory concentration (MIC) were determined by microdilution of the cultures in Mueller Hinton broth (HiMedia). The results were recorded after a 24 hours incubation at 35º C and interpreted according to Bard et al. [2], and Clinical and Laboratory Standards Institute [11-13]. *S. aureus* isolates with an oxacillin MIC ≤2 µg/mL were considered susceptible; those with a MIC ≥4 µg/mL were considered resistant; isolates with a cefoxitin MIC ≤4 µg/mL were considered susceptible; and isolates with a MIC ≥8 µg/mL were considered resistant. CoNS isolates with an oxacillin MIC ≤0.25 µg/mL were considered susceptible and those with a MIC ≥0.5 µg/mL were considered resistant; isolates with a cefoxitin MIC ≤0.25 µg/mL were considered susceptible and those with a MIC ≥0.5 µg/mL were considered resistant.

Recombinant PBP2a preparation

The *mecA* gene was amplified from the reference strain *S. aureus* ATCC 33591 DNA using primers previously described by Perez-Roth et al. [14] and Petinaki et al. [15], with some modification by the addition of restriction sites for the enzymes *Bam HI/Xho* I: 5'primer – TAGGGATCCGTAGAAATGACTGAACGTCCGATAA; 3'primer – TTGCTCGAGTTCTGCAGTACCGGATTTGC. The segment was cloned into a pET21A vector (Merck KGaA, Frankfurter Straße 250, 64293, Darmstadt, Deutschland), and the plasmid was transformed into *Escherichia coli* BL21 cells by thermal shock. The cells were then grown in Luria Bertani (LB) broth (HiMedia) and induced with isopropylthio-β-galactoside (IPTG). The growth was harvested, suspended in PBS and lysed by ultrasonication. The lysates were electrophoresed in 15% SDS-PAGE, and then, the 76 kDa protein band of interest was excised and suspended in PBS.

Production of anti-PBP2a antiserum

Anti-PBP2a antiserum was produced in two New Zealand white rabbits. The animals were initially immunized by subcutaneous inoculation with 300 mg of the recombinant PBP2a protein in (v/v) complete Freund adjuvant (Sigma-Aldrich, 3050 Spruce St., St. Louis, MO 63103, USA) followed by three inoculations of 250 mg of protein in (v/v) incomplete Freund adjuvant (Sigma-Aldrich) at 15 day intervals. Sera was collected 14 days after the last inoculation and stored at -80º C.

Rabbits were from CPqAM-FIOCRUZ/PE facilities (Campus da UFPE, s/n. Cidade Universitária, 50740-465 Recife, PE, Brasil), and the experiment was carried out in the Experimental Animal Laboratory of CPqAM-FIOCRUZ/PE. Prior to immunization, the animals were tested serologically to ensure that they had no previous exposure to the antigen.

Extraction of cell wall proteins

Cell wall proteins were extracted following Katayama et al. [16], with modifications. Briefly, exponentially growing cells were harvested and washed by centrifugation for 5 min at 20.000 x g at 4º C with PBS, pH 7.5. The pellet was suspended in 1 mL of 50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 5 mM $MgCl_2$, pH 7.5; 20 µL of lysostaphin (100 mg/mL); 20 µL of RNase (5 mg/mL); and 1 µL of DNase (1 unit/µL) and incubated at 37° C in a water bath for 30 min. After a thermal shock to 4° C for 5 min, the lysates were harvested and washed twice, as described, and resuspended in 60 µL of Laemmli buffer, followed by electrophoresis on a 12% SDS-polyacrylamide gel and blotting onto an Immobilon-P PVDF membrane (Millipore, Merck Millipore Brazil, Rua São Paulo, 30. Barueri – SP, São Paulo. CEP 06465-130, Brasil). The transfer efficiency was determined by staining the membrane with Ponceau.

Western blot (WB) analysis

Western blots (WB) were performed as previously described [3,16], with small modifications. The membrane was blocked for 1 hour with 5% skim milk in TBS-Tween (TBST), washed with TBS and incubated successively with the anti-PBP2a antiserum diluted 1/30,000 in TBST and an anti-rabbit IgG peroxidase conjugate diluted 1/10,000 according to the vendor (Jackson ImmunoResearch Laboratories, Inc. 872 West Baltimore Pike, West Grove, PA, 19390, USA). Bound antibody was detected by electrochemiluminescence (ELC) using Kodak equipment (Kodak Tractor and Equipment, 321 Douglas Dam Road KODAK, TN 37764, Buckner, KY, 40010 USA).

Evaluation of the rabbit anti-PBP2a antiserum

The activity and specificity of the polyclonal rabbit antiserum produced against the recombinant PBP2a protein was assessed through WB as described above. The optimal dilution of the sera (1:30,000) was determined through titration using serial dilutions (1:10,000 to 1:40,000) of the sera and *S. aureus* strains ATCC 29213 (MSSA) and ATCC 33591 (MRSA).

Indirect immunofluorescence analysis (IFI)

Indirect immunofluorescence was performed using an IFI-Chagas kit (Biomanguinhos/FIOCRUZ, Av. Brasil, 4365 – Manguinhos, Rio de Janeiro/ RJ, Brazil) adapted for this work. Optimal bacterial suspension and sera concentration were determined by titration following instructions provided with the kit. From each strain, 15 µL of bacterial cell suspensions (OD_{600} = 0.08 to 0.10) were dropped onto each well of a glass slide and air-dried. Twenty microliters of anti-PBP2a antiserum diluted 1:1,000 in PBS were dropped onto the dried bacterial suspensions, and the slides were incubated in a humid chamber at 37° C for 30 min. After two washes in PBS, 15 µL of anti-rabbit IgG FITC (Biomanguinhos) diluted 1:800 (according to the kit instructions) in PBS was added and incubated in the dark for 30 min. The slides were washed in PBS, dried at room temperature in the dark and analyzed on a fluorescence microscope.

Analysis of PBP2a production in *mecA* PCR-positive *S. aureus* and CoNS isolate cell populations

A population analysis was performed on three randomly selected *S. aureus* (121/02, 126/02, 666/03), and three CoNS (124/02, 525/04, 976/04) *mecA*-positive isolates that were found to be oxacillin-susceptible on a screening test. Source of isolation and characteristics of these strains are shown in Table 1. After a 24 hours growth at 37º C on sheep blood agar plates, 10 colonies from each isolate were individually analyzed for PBP2a production by IFI as described above.

Evaluation of *mecA* PCR-positive *S. aureus* and CoNS isolate responses to serial antibiotic exposure

The experiment was carried out as described by Kampf et al. [17], with modifications. Three randomly selected *S. aureus* (121/02, 126/02, 666/03) and three CoNS (124/02, 525/04, 976/04) isolates that were determined to be *mecA*-positive and oxacillin-susceptible on screening tests were exposed to increasing concentrations of oxacillin (0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128 and 256 mg/L) and cefoxitin (0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256 and 512 mg/L) in BHI broth. The source of the strains is shown in Table 1. For each strain, 100 µL of an overnight culture was mixed with each oxacillin and cefoxitin concentration and incubated at 37° C for 24 hours. From the test tube with the highest antibiotic concentration showing visible growth, a loopful of culture was plated on sheep blood agar to confirm the culture purity, and a 1:100 dilution in 0.9% saline was re-exposed to oxacillin and cefoxitin as previously described. The procedure was repeated seven times consecutively.

Results

Susceptibility to oxacillin and cefoxitin among the *S. aureus* and CoNS *mecA* PCR-positive isolates

Of the 24 *S. aureus* analyzed, 17 were susceptible and seven were resistant on both oxacillin agar plate screening and oxacillin MIC (≤2 µg/mL) tests. Among the oxacillin-susceptible strains, 14 were also susceptible to cefoxitin (MIC ≤4 µg/Ml), and three were cefoxitin-resistant (MIC ≥16 µg/mL). All the oxacillin-resistant strains (MIC ≥32 µg/mL) were also cefoxitin-resistant (MIC ≥128 µg/mL).

Of the 15 CoNS analyzed, eight were susceptible and seven were resistant on the oxacillin agar plate screening test. Of the susceptible strains, six were oxacillin-susceptible (MIC <2 µg/mL) and two were resistant (MIC 64 µg/mL). The 15 strains were all cefoxitin-resistant (MIC ≥4 µg/mL). Accordingly, all the oxacillin-resistant strains on the screening test were also oxacillin- (MIC 0≥0.5 µg/mL) and cefoxitin-resistant (MIC ≥8 µg/mL).

PBP2a detection in *mecA* PCR-positive *S. aureus* and CoNS isolates

Seventeen *S. aureus*-susceptible and two oxacillin screening-resistant strains were analyzed for PBP2a production through WB and IFI using in-house polyclonal rabbit antiserum produced against recombinant PBP2a protein. PBP2a protein was detected by IFI and WB in all but three samples. One of the PBP2a-negative samples was cefotoxin-resistant by MIC, although it was oxacillin-susceptible (Table 1). Figure 1 shows the detection of PBP2a in *S. aureus* strain ATCC 33591 (MRSA) through IFI and the absence of detection in strain ATCC 29213 (MSSA).

Eight CoNS-susceptible and one oxacillin screening-resistant sample were analyzed by WB and IFI for PPB2a production. PBP2a protein was detected in the oxacillin-resistant strains but in only two oxacillin-susceptible strains. Whereas PBP2a was undetectable by IFI and WB among six *mecA*-positive/oxacillin screening-susceptible CoNS strains despite the fact that two had been found to be oxacillin-resistant (MIC <2 µg/mL) and all were cefoxitin-resistant (MIC ≥4 µg/mL) (Table 1).

Table 1. The hospital infection-associated *Staphylococcus aureus* and *coa*gulase negative *Staphylococcus* isolates analyzed.

Identification	Source	Testing				
		screen	MIC		IFI	WB
			Oxacillin	Cefoxitin		
S. aureus						
666/03	Pulmonary fluids	S	0,015	2	+	+
827/04	Hemoculture	S	0,015	2	+	+
599/04	Surgical wound	S	0,03	2	+	+
202/02	Hemoculture	S	0,03	2	+	+
687/03	Hemoculture	S	0,06	2	+	+
722/03	Catheter	S	0,06	2	-	-
835/03	Hemoculture	S	0,12	2	+	+
47/02	Catheter	S	0,015	4	+	+
741/03	Hemoculture	S	0,03	4	-	-
990/04	Hemoculture	S	0,03	4	+	+
121/02	Hemoculture	S	0,03	4	+	+
126/02	Catheter	S	0,06	4	+	+
749/03	Hemoculture	S	0,06	4	+	+
995/04	Pulmonary fluids	S	0,06	4	+	+
862/04	Surgical wound	S	0,25	16	-	-
807/03	Pulmonary fluids	S	2	128	+	+
808/03	Pulmonary fluids	S	2	128	+	+
236/02	Hemoculture	R	32	128	+	+
794/03	Catheter	R	64	128	+	+
969/04	Catheter	R	64	128	ND	ND
853/03	Catheter	R	64	128	ND	ND
038/04	Catheter	R	32	256	ND	ND
464/04	Hemoculture	R	128	256	ND	ND
710/04	Urine culture	R	128	256	ND	ND
CoNS						
124/02	Catheter	S	0,06	4	-	-
522/04	Catheter	S	0,12	8	-	-
976/04	Hemoculture	S	0,12	8	+	+
525/04	Catheter	S	0,25	16	-	-
942/04	Catheter	S	0,015	128	+	+
105/02	Hemoculture	S	0,015	128	-	-
81/02	Hemoculture	S	64	128	-	-
109/02	Hemoculture	S	64	128	-	-
09/04	Hemoculture	R	32	64	+	+
237/02	Surgical wound	R	0,5	8	ND	ND
804/03	Surgical wound	R	0,5	8	ND	ND
162/02	Hemoculture	R	8	32	ND	ND
992/04	Hemoculture	R	16	64	ND	ND
070/04	Catheter	R	4	128	ND	ND
012/04	Hemoculture	R	16	256	ND	ND

CoNS: coagulase negative staphylococci. MIC: minimum inhibitory concentration. IFI: indirect Immunofluorescence. WB, western blotting. +: positive. -: negative. ND: not done. The oxacilin MICs from the susceptible S. aureus isolates ranged from 0.015 to 2 and from the resistant's from 32 to 128. The oxacilin MICs from the susceptible CoNS isolates ranged from 0.015 to 0.25 and from the resistant's from 0.5 to 64. The cefoxitin MICs from the susceptible S. aureus isolates ranged from 2 to 4 and from the resistant's ranged from 16 to 256. The cefoxitin MICs from the resistant CoNS isolates ranged from 4 to 256. Shadows mean resistance.

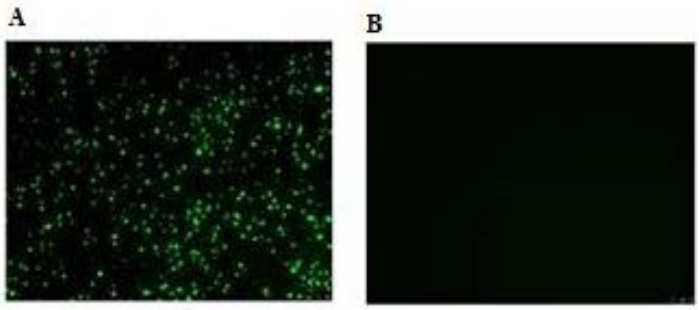

Figure 1. Specificity of the polyclonal rabbit antiserum produced against the recombinant PBP2a protein. A: PBP2a detection in *S. aureus* strain ATCC 33591 (MRSA) using IFI. B: absence of PBP2a in the strain ATCC 29213 (MSSA).

Analysis of *mecA* PCR-positive *S. aureus* and CoNS isolate cell populations

An analysis of individual colonies from three *mecA*-positive *S. aureus* strains (121/02, 126/02, 666/03) revealed that 10 of 10 colonies analyzed from each strain were PBP2a-positive, as detected by WB and IFI. Figure 2 shows the PCR amplification of the gene *mecA* and PBP2a detection through WB and IFI in five colonies from the strain *S. aureus* 121/02.

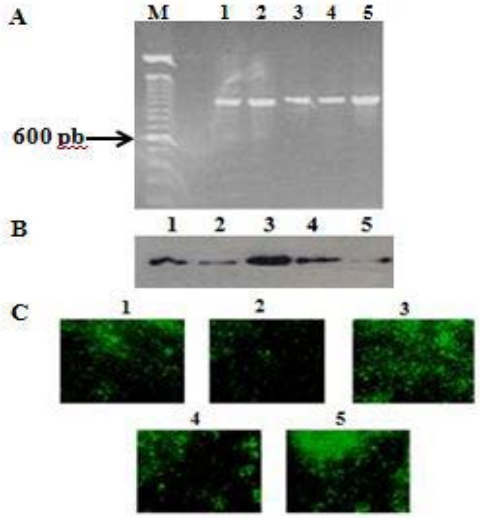

Figure 2. Results of the analysis of individual colonies from the homogeneous 121/02 *mecA*-positive *S. aureus* strain. A: PCR amplification of the *mecA* gene. B: PBP2a detection through WB. C: PBP2a detection through IFI.

Among the three *mecA*-positive CoNS strains analyzed (124/02, 525/04, 976/04), PBP2a production was detected through WB and IFI in only three of 10 colonies from strains 124/02 and 976/04 and in four of 10 colonies from strain 525/04. Figure 3 shows the results of the WB and IFI assays for PBP2a detection in 10 colonies from the strain *S. aureus* 525/04.

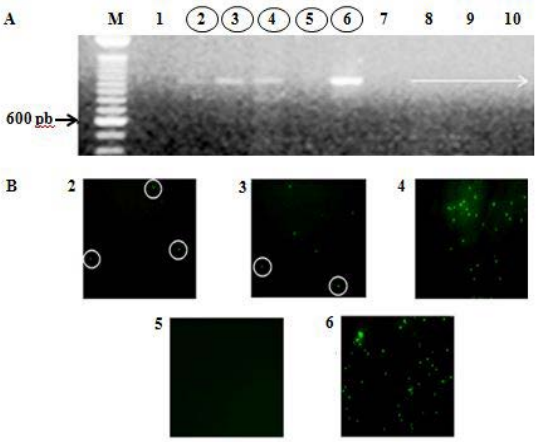

Figure 3. Results of the analysis of individual colonies from the heterogeneous 525/04 *mecA*-positive CoNS strain. A: PBP2a detection through WB. B: PBP2a detection through IFI.

Response of *mecA* PCR-positive *S. aureus* and CoNS isolates to serial antibiotic exposure

Three *S. aureus* (121/02, 126/02, 666/03) and three CoNS (124/02, 525/04, 976/04) *mecA*-positive/oxacillin screening-susceptible isolates were repeatedly exposed to antibiotics. The strains were exposed seven times to 11 crescent concentrations of oxacillin (0.25 to 256 mg/L) and 11 cefoxitin concentrations (0.5 to 512 mg/L) in BHI broth. The results are displayed in Figure 1. Among the *S. aureus* strains (Figure 4A), the susceptibility was not altered after repetitive oxacillin exposure; cefotoxin exposure resulted in increasing resistance until the 3rd exposure and then stabilized, maintaining the same MIC until the end of the experiment at the 7th exposure (Figure 4C). Among the CoNS strains, both oxacillin and cefotoxin exposure led to increasing resistance until the 3rd exposure and then stabilized, maintaining the same MIC until the end of the experiment at the 7th exposure (Figure 4B and D).

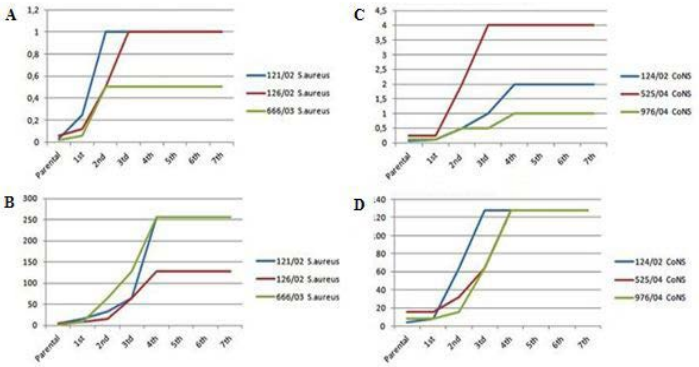

Figure 4. Response of PCR *mecA*-positive *S. aureus* and CoNS isolates to serial antibiotic exposure. A: *S. aureus* strains exposed to oxacillin. B: *S. aureus* strains exposed to cefoxitin. C: CoNS strains exposed to oxacillin. D: CoNS strains exposed to cefoxitin.

Discussion

The *mec*hanisms of staphylococcal resistance to β-lactams are still not completely elucidated, and diagnostic laboratories must consider this characteristic, as the incorrect reporting of the isolates could negatively influence the correct choice of drugs for treatment. Indeed, *mecA*-positive low-oxacillin MIC isolates initially misidentified as methicillin susceptible might emerge as highly resistant strains upon subsequent exposure to β-lactam agents [5]. In addition to *mecA* expression, chromosomal genes and other alternative *mec*hanisms might be involved. In some isolates, particularly borderline oxacillin-resistant *S. aureus* (BORSA), resistance has been attributed to chromosomal mutations or the presence or over-expression of β-lactamase enzymes [5, 6, 18].

Large-scale studies showed that cefoxitin is more reliable than oxacillin for the detection of *mecA*-mediated resistance in staphylococci. In addition, oxacillin heteroresistance is more precisely detected through cefoxitin, reflecting the stronger induction of PBP2a compared with oxacillin [2]. However, discrepant results between oxacillin and cefoxitin disk susceptibility in staphylococci have been observed [19-21].

In the present study, discrepant results were also observed in the assessment of oxacillin/cefoxitin resistance and PBP2a detection through IFI and WB among the *mecA*-positive staphylococcal strains studied. All *mecA*-positive CoNS isolates with oxacillin MIC-susceptibility revealed cefoxitin-MIC resistance (Table 1), and PBP2a expression was detected in only a few strains using IFI and WB. However, among the *S. aureus*, most of the strains displayed oxacillin/cefoxitin susceptibility or a borderline cefoxitin MIC of 4 μg (Table 1), and PBP2a expression was detected in most of the *S. aureus* strains through IFI and WB (Table 1).

The oxacillin/cefoxitin-susceptible phenotype observed among *mecA*-positive staphylococci might reflect the low levels of PBP2a protein expression or the heterotypic resistance phenotype, where only some of the cells harboring the *mecA* gene express PBP2a, and the remaining cells do not [5,17].

A population analysis was subsequently performed to elucidate whether the rate of susceptible/resistant cells among *S. aureus* and CoNS cell populations harboring *mecA* is implicated in the oxacillin-cefoxitin susceptibility phenotype. The results revealed that the analyzed *S. aureus* strains were PBP2A-positive, as the PBP2a protein was detected through WB and IFI in 10 of 10 colonies analyzed from each of the three strains analyzed. The CoNS strains presented a heterogeneous population, in which PBP2 production was detected through IFI and WB in only three of the 10 colonies from two strains and four of the 10 colonies from another strain. Therefore, it is reasonable to assume the occurrence of two genetically distinct populations within the same culture: one population comprising resistant cells expressing *mecA* and the other population comprising susceptible cells without *mecA* expression.

Surprisingly, increased heterogeneity was observed among the CoNS isolates compared with the *S. aureus* strains, and the basis of this difference remains unknown. The strains analyzed were obtained from common biological samples (hemoculture, catheter and pulmonary fluids) received from the bacteriological routine of the hospital. Although the origin of MRSA is not fully understood, it has been suggested that MSSA acquires the *mecA* gene through horizontal transfer from CoNS, and CoNS serves as a donor of SCC*mec* to *S. aureus* [22, 23].

The absence of PBP2a protein expression could indicate non-*mecA*-mediated methicillin resistance [6]. However, this assumption is unlikely in the present study, as PBP2a detection was higher among homogenous *S. aureus* than among heterogeneous CoNS populations, suggesting that PBP2a detection depends on the number of protein-producing cells. Indeed, among the heterogeneous CoNS populations, only a few cells expressed the *mecA* gene.

S. aureus and CoNS isolates heterogeneous *mecA*-positive oxacillin/cefoxitin-susceptible cell populations can acquire homogenous high-level resistance after the inappropriate use of antimicrobials. Thus, antibiotic exposure might select or induce a highly homogeneous (homotypic) population of resistant cells [17].

Kampf et al. [17] induced resistance among *mecA*-positive oxacillin-susceptible *S. aureus* through serial exposure to oxacillin and cefotaxime. We therefore examined whether susceptible isolates harboring the *mecA* gene (pre-methicillin-resistant) become resistant after serial antibiotic exposure. Three *S. aureus* cultures, containing susceptible homotypic cell populations, were subjected to serial oxacillin and cefoxitin exposure. Susceptibility to oxacillin did not change throughout the experiment, but the exposure to cefoxitin induced resistance in the three strains tested. In heterotypic CoNS populations in which only three to four of the 10 colonies expressed the *mecA* gene, exposure to antibiotics led to resistance, most likely through the selection of *mecA*-positive cells and the elimination of *mecA*-negative cells.

According to Plata et al. [7] the conversion from a heterogeneous (HeR) to a highly homogeneous (HoR) resistant phenotype among oxacillin-susceptible, *mecA*-positive MRSA strains reflects an oxacillin-induced SOS response.

In the present study, we analyzed a limited number of strains from a narrow background. The strains were selected based on PCR analyses showing *mecA* positivity associated with hospital infections, as most of these bacteria were obtained from hemocultures and catheters, which are prevalent sources of staphylococcal cultures in the bacteriological routine of the hospital. Given the small number of strains studied, analyses conducted with a larger collection of strains from a larger spatio-temporal origin would clarify some of the discrepancies observed in the present study.

In conclusion, an analysis of *mecA* gene expression remains a reliable test for the broad detection of methicillin resistance among staphylococci, as screening and/or MIC tests showed that many *mecA* PCR-positive isolates are also susceptible to oxacillin/cefoxitin, whereas IFI and WB did not detect PBP2a expression in all of the screened *mecA* PCR-positive isolates. Furthermore, IFI and WB techniques are laborious and time consuming. Therefore, all *mecA*-positive isolates should be regarded as resistant, even if these bacteria exhibit phenotypically susceptible behavior.

Ethical considerations

This study was approved through the Research Ethics Committee of the Oswaldo Cruz University Hospital (Protocol number 17.7.0052 CEP/HUOC). The animal procedures were approved through the Ethics Committee on Animal Use of FIOCRUZ (CEUA-FIOCRUZ: L-021/07).

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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