Detection and expression of methicillin/oxacillin resistance in multidrug-resistant and non-multidrug-resistant Staphylococcus aureus in Central Sydney, Australia

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Ninety clinical Staphylococcus aureus isolates from separate patients were examined phenotypically and genotypically for susceptibility to methicillin/oxacillin. Thirty were methicillin/oxacillin susceptible and 60 were methicillin and oxacillin resistant (MRSA). The 60 MRSA isolates examined were subdivided into two groups according to their antibiotic profiles and comprised 30 non-multidrug-resistant (NMDR) isolates, resistant to less than two non-β-lactam antibiotics, and 30 multidrug-resistant (MDR) isolates, resistant to three or more non-β-lactam antibiotics. Phenotypic and genotypic analysis of methicillin/oxacillin showed that despite use of the guidelines published by the NCCLS for the testing of S. aureus susceptibility to methicillin/oxacillin, MIC values of some NMDR MRSA isolates fell below the NCCLS-recommended breakpoints. Etest strips failed to detect two NMDR MRSA isolates tested with oxacillin and four tested with methicillin. Lowering the NCCLS-recommended oxacillin screen agar concentration from 6 to 2 mg/L and temperature of incubation to 30°C, improved the specificity and sensitivity of NMDR MRSA detection from 87% to 100%. On PFGE analysis these NMDR MRSA strains were genotypically different. Genotypic tests, such as multiplex PCR for the mecA/nuc genes and DNA hybridization for the mecA gene, or phenotypic monoclonal antibody-based tests to detect penicillin-binding protein 2a (PBP2a) offer advantages for problematic isolates in detecting or confirming low-level phenotypic heterogeneous mecA expression of oxacillin and methicillin resistance in NMDR MRSA.

Introduction

In hospitals, prolonged hospital stay and antibiotic therapy, especially with β-lactam antibiotics, predispose patients to acquisition of methicillin/oxacillin-resistant Staphylococcus aureus (MRSA).¹⁻³ Hospital-acquired MRSA are usually associated with increased expression of multiple antibiotic resistance genes, including those for aminoglycoside resistance.⁴⁻⁷

In Central Sydney, as in other parts of Australia, there has been a recent increase in MRSA susceptible to gentamicin and variably susceptible to other non-β-lactam antibiotics, namely tetracycline, trimethoprim, erythromycin and ciprofloxacin.⁸⁻¹¹ These isolates have been designated ‘non-multidrug-resistant’ (NMDR) MRSA, and, depending on the patient history, some have been termed ‘community-acquired’ MRSA.³ These isolates express heterogeneous resistance to methicillin and oxacillin via penicillin-binding protein 2a (PBP2a), encoded by the mecA gene, in contrast with most hospital-acquired MRSA, which show homogeneous resistance to methicillin even though they possess the same gene.¹²,¹³

The level of heterogeneous expression of resistance to methicillin/oxacillin in vitro and the borderline MICs for some strains of NMDR MRSA are often problematic. Rapid, accurate and simple diagnostic tests that would detect the
expression of resistance to methicillin/oxacillin in MRSA could help clinicians in selection of appropriate antibiotic regimens and in epidemiological surveillance.12,14,15

In this study we investigated several standard techniques, including some recently described genotypic and phenotypic assays, for the detection and identification of methicillin/oxacillin resistance in both MDR and NMDR MRSA clinical isolates. We evaluated the multiplex PCR for the mecA genes,16–18 the Velogene rapid MRSA identification test, a DNA hybridization (cycling probe technology) test for mecA,19 the PBPa latex MRSA screen test and two chromogenic screening agar media,19–22 CHROMagar Staph. aureus21 and the ORSAB media supplemented with oxacillin to detect methicillin resistance. Our aims were to compare (i) antibiotic profiles and screening methods in detecting expression of methicillin/oxacillin resistance and (ii) the accuracy in determining MICs of methicillin and oxacillin for methicillin-susceptible S. aureus (MSSA), and NMDR and MDR MRSA. Rapid and simple diagnostic tests that can detect expression of resistance to oxacillin and methicillin in S. aureus would be an improvement in the diagnosis of staphylococcal infections and would help clinicians to treat them more efficiently.15

Materials and methods

Bacterial strains and selection of isolates for analysis

Ninety clinical isolates of S. aureus were examined. This included 60 MRSA isolates and 30 oxacillin- and methicillin-susceptible isolates. Each isolate was from an individual patient whether hospitalized or an outpatient and no repeat isolates were included. Thirty MDR MRSA and 30 MSSA strains were isolated and selected randomly from routine clinical specimens from different infected sites (deep and superficial wounds, blood, urine and sputum) at Concord Repatriation General Hospital between 2000 and 2001. The remaining 30 NMDR MRSA comprised 21 randomly selected clinical isolates, mainly from outpatient clinical sources (superficial wounds and blood) from Concord Repatriation General Hospital and Canterbury Hospital between 2000 and 2001, and nine EMRSA-15 isolates provided by the phage-typing laboratory at Royal Prince Alfred Hospital. Reference strains MSSA ATCC 25923, MRSA ATCC 43300 and an EMRSA-15 isolates provided by the phage-typing laboratory at Royal Prince Alfred Hospital. Reference strains MSSA ATCC 25923, MRSA ATCC 43300 and an EMRSA-15 (supplied by the Central Public Health Laboratory, Colindale, London, UK—from the ‘Harmony Collection’) were used as controls.

Bacterial identification

All S. aureus isolates were identified primarily by routine laboratory procedures.13,14,22 Gram-positive, catalase-positive cocci were tested for mannitol salt fermentation (Oxoid-CM85; Oxoid, Melbourne, Australia) and DNase production on agar plates (Oxoid-CM321). Clumping factor was detected by using rabbit plasma and the Staphaurex kit (Oxoid). Organisms were confirmed to be S. aureus by the tube coagulate test.

Susceptibility screening and antibiotic profiles

Susceptibility of all S. aureus isolates to antimicrobial agents was determined by agar dilution as described previously.23,24,25 Agar dilution plates were inoculated with a MAST replicator device (MAST Diagnostics, Merseyside, UK) designed to deliver 104–105 cfu/inoculum to each spot from suspensions (0.5 McFarland standard) prepared with colony material selected from second stock retrieval plates in accordance with NCCLS guidelines.25 To detect methicillin and oxacillin resistance only, Mueller–Hinton agar plates (Oxoid) supplemented with 2% NaCl and incubated at both 35 and 30°C were used, and read at 24 h. The plates at 30°C were further incubated for another 24 h. All other antibiotic plates without NaCl supplementation were incubated at 35°C for 16–20 h. A list of antibiotics tested appears in Table 1. Antimicrobial breakpoints were interpreted according to NCCLS published guidelines.25

Chromogenic screening media

The chromogenic plate media CHROMagar Staph. aureus (CHROMagar, Paris, France; distributed by Dutec Diagnostics, Sydney, Australia) and the oxacillin resistance screening agar base (ORSAB CM1008; Oxoid) with the selective supplement SR195 (Oxoid) were prepared according to the manufacturers’ instructions.24 The ORSAB medium supplement SR195 was supplied by the manufacturer and contained oxacillin (1 mg) and polymyxin B (25 000 IU) in each vial, sufficient to supplement 500 mL of the medium. CHROMagar Staph. aureus was first supplemented with 4 mg/L and later, on repeat testing, the concentration was decreased to 2 mg/L oxacillin when the agar cooled to 48°C. On CHROMagar Staph. aureus, characteristic S. aureus colonies appeared pink to red, while with the ORSAB medium, which is an alternative medium to mannitol salt agar, using aniline blue to detect mannitol fermentation, presumptive MRSA appeared as intense and diffuse blue colonies. MSSA were inhibited by the oxacillin in the media.

MICs of oxacillin and methicillin (Etest)

The MICs of oxacillin and methicillin were determined using the Etest system (AB Biodisk, Solna, Sweden; distributed in Australia by Australian Laboratory Services, Sydney). The Etest was performed on all S. aureus using Mueller–Hinton agar supplemented with 2% NaCl.26,27 These plates were inoculated by swabbing the surfaces with a direct colony suspension of the test strain equivalent to a 0.5 McFarland standard, and the MIC was read after incubation at 35°C for 24 h. MICs of methicillin/oxacillin were also determined by agar dilution
Detection of methicillin/oxacillin resistance

methodology recommended by the NCCLS. Breakpoints published by the NCCLS were used: oxacillin susceptible (S) < 2 mg/L and resistant (R) ≥ 4 mg/L; methicillin S < 8 mg/L and R ≥ 16 mg/L, on Mueller–Hinton agar with 2% NaCl incubated at 35°C in ambient air for 24 h.

Oxacillin agar screen test

All MRSA isolates were spot inoculated on to Mueller–Hinton agar supplemented with 6 mg/L oxacillin and 4% NaCl, from a 0.5 McFarland standard suspension as described previously. The plates were incubated at 35°C for 24 h as recommended by the NCCLS and simultaneously at 30°C for 24 h. If any growth (more than one colony) was detected, the isolate was considered oxacillin or methicillin resistant.

PBP2a latex MRSA screen test

The test was performed according to the manufacturer’s instructions (Oxoid); a 1 µL loopful of the test isolate was emulsified in four drops of an extraction reagent and boiled for 3 min. The suspension was cooled to room temperature and one drop of a second extraction reagent was added and mixed. The suspension was centrifuged at 1500g for 5 min. A 50 µL aliquot of the supernatant was added to each of two circles on a disposable test card and mixed with one drop of the anti-PBP2a monoclonal antibody-sensitized latex or one drop of the negative control latex. The samples were then mixed for 3 min (or up to 5 min) on a shaker. Any agglutination was observed visually.

DNA extraction

A single colony was taken from a nutrient agar or horse blood agar plate (Oxoid) that had been incubated overnight and emulsified into 50 µL of lysostaphin (100 mg/L; Sigma, Sydney, Australia). After incubation for 10 min at 37°C, 50 µL of proteinase K (100 mg/L; Sigma) and 150 µL of TE buffer (1 mM EDTA/10 mM Tris, pH 7.5) were added to the suspension and incubated for a further 20 min at 37°C before incubation at 95°C for 5 min to deactivate the proteinase K. Five microlitres were then taken from the suspension and used directly for the multiplex PCR.

Multiplex PCR for mecA and nuc gene detection

The multiplex PCR procedure was based on a modification by Unal et al. and this was used as the ‘gold standard’ for all isolates. Oligonucleotides used were mecA F primer 1282 and mecA R primer 1793, which gives a PCR product of 533 bp (Figure 1). Primers nuc

![Figure 1. Multiplex PCR analysis for the mecA and nuc genes in MDR MRSA, NMDR MRSA and MSSA. Arrows indicate the 533 and 367 bp amplicons. Lane 1, DNA marker; lane 2, MRSAAATCC 43300 control; lane 3, S. aureus ATCC 25923 (MSSA); lanes 4–10, MDR MRSA; lanes 11–16, NMDR MRSA; lanes 17 and 20, MSSA; lanes 18 and 19, NMDR MRSA.](image)
(5′-GCGATTGATGGTACGCTTT-3′) and nuc2 (5′-AGCCAGCCTTGACGAACTAAAGC-3′) were as described by Brakstad et al.,16 giving a PCR product of 278 bp. All primers were supplied by Sigma Genosys (Sigma). Multiplex PCR was performed on a Corbett research PC-960 air cooled thermocycler using a reaction mixture of 50 µL, consisting of 1.25 U of AmpliTaq DNA polymerase (Perkin Elmer, Foster City, CA, USA); 200 µM dNTPs (Pharmacia, Sydney, Australia); 20 pmol each primer, 1.5 mM MgCl2, 100 mM Tris–HCl (pH 8.3) and 500 mM KCl. The extracted DNA was amplified for 30 cycles consisting of 60 s at 94°C for denaturation, 30 s at 50°C for annealing and 90 s at 72°C for primer extension. Twenty microlitres of the PCR product was then analysed by agarose gel electrophoresis [2% agarose prepared in TAE (1 mM EDTA/40 mM Tris acetate, pH 8) buffer]. Gels were stained with ethidium bromide and photographed under UV light as shown in Figure 1.

**Velogene rapid MRSA identification assay, cycling probe technology and DNA meca hybridization**

The Velogene assay was performed in accordance with the manufacturer’s instructions with the exception that the S. aureus colonies were taken from a 5% horse blood agar plate (HBA–Columbia base; CM331, Oxoid) instead of the recommended tryptic soy agar (TSA) with 5% sheep blood. Most laboratories in Australia tend to use 5% HBA for the isolation of S. aureus. In the probe-based test, a 1 µL loopful of growth from a HBA plate was suspended in 50 µL of lysis buffer and incubated at 55°C for 20 min. The suspension was then incubated at 95°C for 5 min. A 50 µL aliquot of cycling reagent was added, and the suspension was incubated at 55°C for a further 25 min. Cycle stop reagent was added, and the suspension was transferred to streptavidin-coated microtitre wells incubated at room temperature for 10 min. After two washes, the detection substrate reagent was added. Development of a blue colour was indicative of a methicillin-susceptible isolate (meca negative) and a colourless reaction indicated the presence of a methicillin-resistant strain (meca positive). Once the colour developed the detection stop reagent was added. The manufacturer gives either a visual (by colour) or spectrophotometric option to read the colour development. We used visual inspection for determination of the assay’s performance as described previously by Louie et al.19

**PFGE**

Chromosomal DNA fragments cut with SmaI were separated on a CHEF-DR II apparatus (Bio-Rad, Sydney, Australia) using the following pulse-field parameters: 120° constant angle, two linear ramps of 4–8 s for 15 h followed by 10–15 s for 15 h at a field strength of 6 V/cm. Gels were stained with ethidium bromide and photographed under UV illumination. Isolates that differed by one to three bands, consistent with a single differentiating genetic event, were assigned a numbered subtype. The gel pictures were inspected visually by two different investigators. The PFGE patterns were interpreted according to Tenover et al.,29 i.e. four or more band differences between two strains defined different genotypes.

**Results**

**Antibiotic profiles of MSSA, NMDR MRSA and MDR MRSA**

The antibiotic profiles of all 90 clinical S. aureus isolates examined are shown in Table 1. NMDR MRSA had variable antibiotic profiles. One large subgroup included EMRSA-15, which was later confirmed by PFGE analysis. All MDR MRSA were resistant to gentamicin and trimethoprim, whereas all NMDR MRSA were susceptible to gentamicin and trimethoprim. The EMRSA-15 isolates were all susceptible to gentamicin and trimethoprim, and resistant to ciprofloxacin. All 90 staphylococcal isolates were susceptible to vancomycin and teicoplanin.

**Comparison of genotypic and phenotypic assays for detection of the expression of methicillin resistance of MSSA, MDR MRSA and NMDR MRSA**

The genotypic and phenotypic expression of methicillin resistance of 90 clinical S. aureus isolates was examined in this study. Results are shown in Table 2. All 90 isolates tested positive for the nuc gene and hence were all genetically confirmed to be S. aureus isolates (Figure 1). Of the 60 MRSA (30 MDR and 30 NMDR) tested, all were positive for the meca gene by multiplex PCR. The remaining 30 MSSA were negative for the meca gene. The probe-based Velogene MRSA assay and the monoclonal-based PBP2a latex MRSA screen gave 100% correlation with the multiplex PCR in detecting oxacillin and methicillin resistance in both MDR and NMDR MRSA. No problems were encountered with the performance of any of the assays when the isolates were taken from the 5% HBA medium instead of the recommended 5% sheep blood TSA medium. The MSSA isolates were all negative for the probe-based Velogene rapid MRSA identification test and the monoclonal-based PBP2a latex MRSA screen test. All 90 isolates grew on CHROMagar Staph. aureus without supplementation and were easily identified at the species level as S. aureus by their red to pink colony colour at 35°C in ambient air after 24 h incubation. When the CHROMagar Staph. aureus medium was first supplemented with 2 mg/L and later 4 mg/L oxacillin, all the MSSA were inhibited and the plate showed no growth. The oxacillin screen agar (with 6 mg/L oxacillin) also inhibited the growth of all MSSA isolates. All MDR MRSA grew on CHROMagar Staph. aureus when supplemented with 2 and 4 mg/L oxacillin, as well as on the
Detection of methicillin/oxacillin resistance

Table 2. Comparison of genotypic and phenotypic assays in detecting MSSA, NMDR MRSA and MDR MRSA

<table>
<thead>
<tr>
<th>Assay type</th>
<th>Methods</th>
<th>Total no. of isolates tested</th>
<th>No. of S. aureus type positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotypic</td>
<td>multiplex PCR <em>meca</em></td>
<td>90</td>
<td>30 30 30</td>
</tr>
<tr>
<td></td>
<td><em>nuc</em></td>
<td>90</td>
<td>30 30 30</td>
</tr>
<tr>
<td></td>
<td>Velogene probe-based <em>meca</em> DNA hybridization</td>
<td>90</td>
<td>30 30 30</td>
</tr>
<tr>
<td>Phenotypic</td>
<td>latex screen: PBP2a monoclonal based oxacillin screening agar 6 mg/L at 35°C</td>
<td>90</td>
<td>0 26 30 30</td>
</tr>
<tr>
<td></td>
<td>oxacillin screening agar 2 mg/L at 30°C</td>
<td>90</td>
<td>0 30 30 30</td>
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<tr>
<td></td>
<td>ORSAB + oxacillin 2 mg/L at 35°C</td>
<td>90</td>
<td>0 30 30 30</td>
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<td></td>
<td>CHROMagar Staph. aureus + oxacillin 4 mg/L at 35°C</td>
<td>90</td>
<td>0 26 30 30</td>
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<td></td>
<td>CHROMagar Staph. aureus + oxacillin 2 mg/L at 30°C</td>
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*Four NMDR MRSA strains had very poor or no growth at 35°C but grew at 30°C after 24 h of incubation.

Recommended NCCLS oxacillin screen agar at 35°C in ambient air after 24 h of incubation. However, of the NMDR MRSA isolates tested on the CHROMagar Staph. aureus medium containing 4 mg/L oxacillin and the recommended oxacillin screening agar with 6 mg/L oxacillin, only 26 (87%) of the isolates grew at the recommended incubation temperature of 35°C in ambient air after 24 h (Table 2). There was no advantage to incubating the methicillin or oxacillin plates beyond 24 h. By reducing the concentration of oxacillin to 2 mg/L and lowering the incubation temperature to 30°C for both CHROMagar Staph. aureus and the recommended screen agar, 100% growth was achieved for all NMDR MRSA. The chromogenic ORSAB medium containing 2 mg/L oxacillin gave 100% correlation with all MDR and NMDR MRSA at 35°C. Improved growth was observed at 30°C for four NMDR MRSA. On ORSAB medium all of the NMDR and MDR MRSA isolates appeared as diffuse blue colonies. None of the 30 MSSA isolates tested on ORSAB medium showed growth.

**MICs and the expression of oxacillin and methicillin resistance in MSSA, MDR MRSA and NMDR MRSA**

Results of all MIC determinations for MSSA, NMDR MRSA and MDR MRSA isolates are displayed graphically in Figure 2. All MSSA had Etest MICs in the range 0.19–1.5 mg/L oxacillin and 0.75–1.5 mg/L methicillin. All 30 MSSA were considered to be susceptible to oxacillin and methicillin according to published NCCLS guidelines.26 All MDR MRSA isolates had oxacillin and methicillin Etest MICs >256 mg/L, exhibiting homogeneous resistance using NCCLS breakpoints.25 However, Etest MICs for NMDR MRSA varied. MICs of oxacillin and methicillin ranged from as low as 1.5–>256 mg/L and 4–>256 mg/L, respectively (Figure 2). All of the NMDR MRSA isolates showed heterogeneous resistance. These strains showed a double zone of inhibition or a hazy mixed population of bacterial growth at the point of intersection between the zone edge and the Etest strip. The same four NMDR MRSA isolates that grew poorly at 35°C on the NCCLS-recommended screening agar also gave MICs below or borderline to the recommended oxacillin and methicillin breakpoints. Two NMDR MRSA isolates were susceptible to oxacillin with MICs of 1.5 mg/L, while four isolates each had methicillin MICs of 4, 6, 6 and 8 mg/L, at 35°C in ambient air after 24 h incubation. These isolates were all found to be resistant to oxacillin with MICs of 16 and 24 mg/L and to methicillin with MICs of 32, 24, 24 and 24 mg/L, on retesting at 30°C on Mueller–Hinton agar with 2% NaCl after 24 h of incubation in ambient air. The presence or absence of NaCl had little effect on the MICs for these isolates (data not shown). PFGE analysis of the four isolates concerned showed four or more band differences between strains indicating that they were not genotypically related to each other (Figure 3). One of the NMDR MRSA strains (Figure 3, lane 4) showed identical banding patterns to the EMRSA-15 control strain (Figure 3, lane 2).

**Discussion**

Rapid and accurate detection of methicillin resistance in *S. aureus* isolates is imperative for appropriate patient treatment and implementation of institutional programmes for recognition and management of MRSA outbreaks and cross-infection.4,12,15 The multidrug resistance pattern exhibited by most Australian hospital-acquired MRSA has for many years assisted the bench technologist and clinical microbiologist to discriminate *meca*-positive strains of *S. aureus* from *meca*-negative strains. However, variations in antibiotic susceptibility profiles (Table 1) and the increased local frequency of
isolation of NMDR MRSA in Central Sydney makes phena-
typic interpretation difficult. These strains are usually resist-
ant to β-lactam antibiotics but are often susceptible to agents
such as gentamicin, tetracycline, trimethoprim and variably
erythromycin and ciprofloxacin.3,6,8,15 Mis-identification of
MSSA as MRSA will promote unnecessary or inappropriate
use of vancomycin. But also, as pointed out by Boyce,4 mis-
classification of mecA-positive MRSA will result in costly
implementation of special MRSA isolation and barrier
precautions, which are not warranted for patients with sus-
ceptible strains.

In clinical practice the drug of choice for mecA-positive
MRSA strains is either vancomycin or teicoplanin.15 NMDR
MRSA (mecA positive) in our area are increasingly associated
with soft tissue infections and abscesses in patients outside
hospitals in the community. As shown in Table 1, all clinical
MSSA, and NMDR and MDR MRSA isolates examined
in this study were susceptible to vancomycin and teicoplanin
at the breakpoints recommended by the NCCLS.25 The
EMRSA-15 isolates examined in this study were all resistant
to ciprofloxacin. Previous studies reported variable resistance
to erythromycin.11,12

In this study, both the genotypic assays used, i.e. the multi-
plex PCR and the DNA hybridization Velogene rapid MRSA
identification test for mecA, demonstrated clearly that the
expression of methicillin resistance in all MDR and NMDR
MRSA examined was due to the mecA gene (Table 2). We
also demonstrated for the first time that S. aureus colonies for
the probe-based Velogene rapid MRSA identification test can
be successfully taken from 5% HBA rather than the recom-
manded 5% blood TSA. The genotypic results correlated
(100%) with the monoclonal-based PBP2a latex screen test in

Figure 2. Distribution of oxacillin and methicillin MICs for MSSA, NMDR MRSA and MDR MRSA isolates determined by Etest after incubation
at 37°C for 24 h. Circles, NMDR MRSA; squares, MDR MRSA; triangles, MSSA.
Detection of methicillin/oxacillin resistance

detecting the presence of PBP2a in all MDR and NMDR MRSA strains. In comparison with the genotypic tests, the slide latex agglutination method was rapid (10 min per test), sensitive and specific.

By analysing phenotypic growth patterns and MICs (Figure 2) of methicillin and oxacillin resistance we were able to subdivide the MDR and NMDR MRSA into two separate bacterial populations. All MDR MRSA examined showed high-level resistance to both oxacillin and methicillin with MICs > 256 mg/L and they all expressed homogeneous resistance at 37°C. This type of homogeneous resistance is usually linked to antibiotic tolerance. It has been suggested previously that high homogeneous resistance and antibiotic tolerance traits may be related to the complexity of antibiotic pressure operating in the clinical environment.30

The NMDR MRSA examined in this study showed interstrain variability in the expression of methicillin and oxacillin resistance, which was reflected in the different MIC ranges shown in Figure 2. This variability in MIC occurred even though all NMDR MRSA isolates examined were mecA positive. Two NMDR MRSA isolates tested against oxacillin and four NMDR MRSA isolates tested against methicillin gave MICs below or borderline to the NCCLS-recommended breakpoints of <2 mg/L oxacillin and <8 mg/L methicillin. However, the resistance of these isolates to both oxacillin and methicillin increased when the isolates were incubated at 30°C for 24 h in ambient air. Variation in methicillin and oxacillin resistance due to temperature changes has been described previously by Thornsberry et al.31 Changing the temperature to 30°C for heterogeneously resistant MRSA sometimes results in a large proportion of cells appearing resistant. Tomasz30 has also demonstrated that in certain heterogeneous MRSA strains, expression of methicillin resistance may be thermosensitive. When staphylococcal strains are plated on methicillin-containing agar and incubated at 30°C the majority of cells may appear as highly resistant bacteria, whereas the same experiment with 37°C incubation yielded heterogeneous culture, with only very few cells capable of growing on the methicillin plates.

The low MICs for these NMDR MRSA strains also affected their growth patterns on the different screening agars examined when incubated at the recommended temperature of 35°C. The ORSAB medium containing a low oxacillin concentration of 2 mg/L gave the best results with 100% correlation at 35°C incubation for 24 h. However, more growth was observed at 30°C for four isolates. Lowering the oxacillin concentration in the oxacillin screen agar from 6 mg/L and in the CHROMagar Staph. aureus from 4 mg/L, as described previously, to 2 mg/L and temperature of incubation to 30°C improved the specificity and sensitivity from 87% to 100% of all screening agars in detecting all NMDR MRSA.31 PFGE analysis of the four NMDR MRSA isolates with low or borderline resistance to methicillin/oxacillin showed four or more band differences, indicating that they were unrelated genotypically (Figure 3), according to the criteria established by Tenover et al.29

In conclusion, NMDR MRSA isolation has been reported with increased frequency in Australia and worldwide, and, despite universal guidelines published by the NCCLS for the susceptibility testing of S. aureus to methicillin/oxacillin, MICs for some NMDR MRSA isolates fall within a ‘grey zone’. Low-level resistant MRSA have also been described in Switzerland, showing difficulties in detection when conventional phenotypic methods recommended by the NCCLS were used.32 These strains express resistance to methicillin through the mecA gene. Laboratories without genotypic or monoclonal-based assays may have problems in detecting or confirming low-level mecA phenotypic heterogeneous expression of oxacillin and methicillin resistance in NMDR MRSA. The degree of heterogeneity is variable in NMDR MRSA strains and modification of growth conditions can increase or decrease the proportion of cells that express resistance to various degrees. We have shown in this study that multiplex PCR for the mecA/nuc genes, the Velogene rapid cycling probe technology for mecA identification and the PBP2a latex MRSA screen test are excellent assays as con-

Figure 3. PFGE profiles from SmaI macrorestriction fragments of the four NMDR MRSA isolates with low-level resistance to methicillin/oxacillin, showing that they are not genotypically related. Lane 1, DNA marker; lane 2, EMRSA-15 control; lanes 3–6, NMDR MRSA; lane 7, internal MRSA control.
firmatory methicillin resistance tests for these NMDR MRSA showing heterogeneous resistance. The direct implementation of such assays will allow more rapid establishment of effective antibiotic therapy and should help control the spread of MRSA in hospitals and the community. Further investigative research is in progress to elucidate factors involved in heterogeneous expression of methicillin resistance in NMDR MRSA and the effects of different non-β-lactam antibiotics on these strains as an alternative to vancomycin use.

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Detection of methicillin/oxacillin resistance


