Evaluation of disc diffusion methods and Vitek 2 automated system for testing susceptibility to mupirocin in *Staphylococcus aureus*

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**Objectives**: To compare the performance of the automated Vitek 2 system, the disc diffusion method and a home-made mupirocin screen agar (MSA) to detect mupirocin resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) isolates.

**Methods**: A total of 125 MRSA isolates were tested. The level of mupirocin resistance was determined by agar dilution and Etest techniques (gold standard), by the Vitek 2 system, on MSA (Mueller–Hinton + mupirocin 4 mg/L) and with the disc diffusion method using 10 µg mupirocin Neo-Sensitabs (MUP-10) and mupirocin paper discs of 5, 20 and 200 µg (MUP-5, MUP-20 and MUP-200). High-level mupirocin resistance (HLMR) was confirmed by PCR for the *mupA* gene.

**Results**: Thirty-two MRSA isolates showed HLMR (MIC ≥512 mg/L) and harboured the *mupA* gene, 39 strains showed low-level mupirocin resistance (LLMR) (8–32 mg/L) without the *mupA* gene and 54 were susceptible without the *mupA* gene. The sensitivity and the specificity of the Vitek 2 system and the screening medium (MSA) for the detection of mupirocin resistance was 100%. The diffusion method using 5 and 10 µg discs demonstrated a sensitivity of 100% and a specificity of 98.1% and 100%, respectively. Using interpretative criteria of 6 and 17 mm, the MUP-20 disc showed the best classification concordance with reference methods.

**Conclusions**: The diffusion method using low-content discs or the Vitek 2 microdilution system showed excellent agreement with MICs and PCR results to separate mupirocin-susceptible from -resistant MRSA strains. Disc diffusion with MUP-20 or combined use of low and high mupirocin content discs enabled the classification of susceptibility categories (susceptible, LLMR and HLMR) but required overnight incubation compared with 12 h for the Vitek 2 system.

Keywords: MRSA, mupirocin resistance, low-level resistance, topical decolonization therapy

**Introduction**

*Staphylococcus aureus* is a major pathogen responsible for various community-acquired and nosocomial infections, including bacteraemia, pneumonia, skin and soft tissue infections, and osteomyelitis.1

Methicillin-resistant *S. aureus* (MRSA) are implicated in serious infections and nosocomial outbreaks.2 These strains show resistance to a wide range of antibiotics, thus limiting the treatment options to very few agents such as glycopeptides and linezolid.

Mupirocin topical therapy is widely used to eradicate nasal carriage of *S. aureus* including MRSA. It is recommended as part of decolonization regimens used for controlling the spread of these strains.3,4 This antimicrobial agent competitively binds to isoleucyl-tRNA synthetase (IRS) and inhibits protein synthesis. Massive use of this antibiotic in patients colonized with MRSA has been followed by outbreaks of MRSA resistant to mupirocin (first reported in the UK, in 1987).5 Two types of mupirocin resistance have been described. Low-level mupirocin resistance (LLMR) results mainly from alterations in the chromosomal IRS and is found in MRSA strains showing mupirocin MICs ranging from 8 to 256 mg/L.6 High-level mupirocin resistance (HLMR) is due to an additional IRS, resistant to inhibition by mupirocin, encoded by the *mupA* gene located on a transferable plasmid.7,8 It has been previously shown that strains exhibiting HLMR cannot be eradicated with mupirocin. The clinical significance of LLMR remains unclear. It has been suggested that the high concentration achieved locally by mupirocin (20 000 mg/L) could clear MRSA nasal carriage.9

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Harbarth et al.\textsuperscript{10} showed in a mupirocin versus placebo-randomized, double-blind trial for MRSA decolonization in hospitalized adult patients that low-level resistance at the entry of the study was associated with treatment failure in both study arms, mupirocin and placebo.

Mupirocin resistance can be detected by the disc diffusion method using a 5 or 10 \( \mu \)g mupirocin disc. However, this technique cannot discriminate between LLMR and HLMR.\textsuperscript{11} The level of resistance has to be confirmed by MIC determination or by the detection of the \textit{mupA} gene. Recently, a new antimicrobial susceptibility testing card for staphylococci, the AST-P549 card, which includes mupirocin in its panel has been developed for the Vitek 2 system.\textsuperscript{12} The aim of this study was to evaluate the performance of the Vitek 2 system using the new card, the disc diffusion method using discs of mupirocin (5, 10, 20 and 200 \( \mu \)g) and a home-made mupirocin screen agar (MSA) to detect mupirocin resistance in MRSA isolates.

### Materials and methods

#### Collection of bacterial isolates

MRSA isolates \((n = 125)\) from two national surveillance programmes conducted in 2001 and 2003 in Belgian hospitals\textsuperscript{13,14} were included in this study: (i) HLMR isolates \((n = 32)\) with MICs \(\geq 512\) mg/L and harbouring the \textit{mupA} gene; (ii) LLMR isolates \((n = 39)\) with MICs ranging from 8 to 32 mg/L without the \textit{mupA} gene; and (iii) susceptible isolates \((n = 54)\) with MICs \(\leq 4\) mg/L and without the \textit{mupA} gene. All MRSA strains were confirmed by multiplex PCR for \textit{mecA} and \textit{nuc} genes as described previously.\textsuperscript{15} Isolates were stored at \(-80\) °C and sub-cultured for 2 consecutive days on Columbia blood agar before testing.

Four \textit{S. aureus} reference strains were included in each test run: ATCC 29213 (mupirocin-susceptible), Carter (LLMR and \textit{mupA}-negative), Eagles and F89 (both HLMR and \textit{mupA}-positive).

#### Antimicrobial susceptibility testing methods

Disc diffusion testing was performed by streaking a Mueller–Hinton II agar plate (Becton-Dickinson, Heidelberg, Germany) with an inoculum equivalent to a 0.5 McFarland standard. The following discs were applied on the dried surface of the agar: 10 \( \mu \)g Neo-Sensitabs (MUP-10) (Rosco, Taastrup, Denmark) and paper discs with 5, 20 and 200 \( \mu \)g of mupirocin (MUP-5, MUP-20, MUP-200) (Oxoid, Basingstoke, UK). For MUP-5 and MUP-10, we used the interpretative zone diameter criteria published by Fuchs et al.\textsuperscript{11}: susceptibility corresponding to \(\geq 14\) mm for the 5 \( \mu \)g disc and \(\geq 16\) mm for the 10 \( \mu \)g disc (Table 1). There are no interpretative criteria for MUP-20 and MUP-200.

### Table 1. Categorization of MRSA strains \((n = 125)\) into mupirocin susceptibility categories by Vitek 2 system, disc diffusion tests and MSA

<table>
<thead>
<tr>
<th>Test categories</th>
<th>mupirocin-susceptible ((n = 54)) MIC (\leq 4) mg/L</th>
<th>low-level mupirocin-resistant ((n = 39)) MIC 8–256 mg/L</th>
<th>high-level mupirocin-resistant ((n = 32)) MIC (\geq 512) mg/L</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitek 2 (AST-P549)</td>
<td>54</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>susceptible ((\leq 2) mg/L)</td>
<td>0</td>
<td>39</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>resistant ((\geq 8) mg/L)</td>
<td>0</td>
<td>39</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disc diffusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MUP-5</td>
<td>53</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>98.1</td>
</tr>
<tr>
<td>susceptible ((\geq 14) mm)</td>
<td>1</td>
<td>39</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>resistant ((\leq 13) mm)</td>
<td>0</td>
<td>39</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MUP-10</td>
<td>54</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>100</td>
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<tr>
<td>susceptible ((\geq 16) mm)</td>
<td>0</td>
<td>39</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>resistant ((\leq 15) mm)</td>
<td>0</td>
<td>39</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MUP-20</td>
<td>53</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>susceptible ((\geq 17) mm)</td>
<td>1</td>
<td>34</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>LLMR ((7–16) mm)</td>
<td>0</td>
<td>5</td>
<td>32</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>HLMR ((\leq 6) mm)</td>
<td>0</td>
<td>3</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MUP-200</td>
<td>54</td>
<td>36</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>susceptible or LLMR ((\geq 7) mm)</td>
<td>0</td>
<td>3</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLMR ((\leq 6) mm)</td>
<td>0</td>
<td>3</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSA</td>
<td>54</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>susceptible (no growth)</td>
<td>0</td>
<td>39</td>
<td>32</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MUP-5, 5 \( \mu \)g mupirocin disc; MUP-10, 10 \( \mu \)g mupirocin disc; MUP-20, 20 \( \mu \)g mupirocin disc; MUP-200, 200 \( \mu \)g mupirocin disc; LLMR, low-level mupirocin resistance; HLMR, high-level mupirocin resistance.
Mupirocin MICs ranging from 0.06 to 128 mg/L were determined by using the agar dilution method on Mueller–Hinton II agar using a 0.5 McFarland standard inoculum as recommended by the CLSI. For strains with MICs above 4 mg/L, the level of resistance was tested by the Etest method on Mueller–Hinton II agar according to the manufacturer’s procedure. The following breakpoints, published by the BSAC, were used: ≤4 mg/L, susceptible; 8-256 mg/L, low-level resistance; ≥512 mg/L, high-level resistance.17

Vitek 2 susceptibility testing

Susceptibility testing with the Vitek 2 system was performed according to the manufacturer’s instructions, using the AST-P549 card (bioMérieux, Marcy l’Étoile, France). The interpretative categories were as follows: susceptibility, ≤2 mg/L; resistance, ≥8 mg/L.

Detection of the mupA gene

A multiplex PCR assay was performed to detect staphylococcal 16S rRNA and mupA genes. The sets of primers were previously reported by Maes et al.,15 and Ramsey et al.,16 respectively. Briefly, DNA was extracted from the colonies after lysostaphin cell lysis for 30 min at 37°C by using the QiaGen kit DNeasy Blood & Tissue (Westburgh, USA). The 25 μL PCR mixture contained 1 × PCR buffer (Perkin-Elmer Applied Biosystems, Foster City, USA), 2 mM MgCl₂, 16S rRNA-specific primers (0.09 mM) and mupA-specific primers (1.2 μM) (MWG Biotech, Ebersberg, Germany), deoxyribonucleoside triphosphates (250 μM each; Promega, Madison, USA) and AmpliTaq DNA polymerase (1.5 U; Perkin-Elmer Applied Biosystems). A DNA sample of 3 μL was used as the target in the PCR. Amplification conditions consisted of 30 cycles of 30 s at 94°C, 30 s at 53°C and 2 min at 63°C with a final step of 10 min at 63°C. The DNA fragments were separated by electrophoresis on a 1.5% agarose (Invitrogen, Merelbeke, Belgium) gel stained with ethidium bromide.

Sensitivity and specificity

The sensitivity and specificity were determined as the ability of a test to separate susceptible from resistant strains. In addition, the ability of each test method to classify resistant strains into LLMR and HLMR categories was evaluated.

Results

Among the 125 MRSA isolates tested, 32 (25.6%) strains showed an MIC of mupirocin ≥512 mg/L and harboured the mupA gene (HLMR category). Thirty-nine (31.2%) isolates were low-level resistant to mupirocin with MICs ranging from 8 to 32 mg/L and did not carry the mupA gene (LLMR category). The 54 (43.2%) remaining isolates were mupirocin-susceptible with MICs ≤4 mg/L and no mupA gene detected.

Disc diffusion results

The disc diffusion method using MUP-5 and MUP-10 showed excellent accuracy for the detection of mupirocin resistance in MRSA isolates (100% sensitivity for both; 98.1% and 100% specificity for MUP-5 and MUP-10, respectively) (Figure 1). All HLMR and the majority of LLMR strains had no inhibition zone around the MUP-5 disc (Figure 1). All HLMR and only five LLMR strains grew up to the edge of the MUP-20 disc. Based on the distribution observed, we proposed the best tentative breakpoints for the MUP-20 disc: by using interpretative breakpoints of susceptibility ≥17 mm and resistance ≤16 mm, the MUP-20 disc demonstrated sensitivity and specificity of 100% and 98.1%, respectively. As shown in Figure 1, no clear cut-off value could be defined with the MUP-200 disc to distinguish between susceptible and resistant strains.

All HLMR MRSA strains also showed no zone of inhibition with MUP-200 and only three LLMR strains grew in contact with the disc. MUP-200 showed a sensitivity of 100% and a specificity of 92.3% to separate HLMR from LLMR.

A zone diameter breakpoint of ≤6 mm (no zone of inhibition) effectively separated HLMR from LLMR MRSA isolates for both MUP 20 and MUP 200 discs.

Vitek 2 results

All HLMR and LLMR strains were found resistant by the Vitek 2 system with MICs higher than 8 mg/L (Table 1). All mupirocin-susceptible strains were correctly classified with MICs lower than 2 mg/L. The Vitek 2 test thus demonstrated excellent sensitivity and specificity (100%) for the detection of mupirocin resistance in this collection of MRSA isolates.

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Discussion

MRSA represents a major public health challenge in many healthcare institutions worldwide. Since the end of the 1980s, topical mupirocin has been widely used to eradicate the nasal carriage of MRSA and control the spread of these strains, and it has been proved to be the most effective among available decolonization regimens.19 The first mupirocin-resistant MRSA strain appeared shortly after the introduction of this antimicrobial.5 In national MRSA surveys in Belgian hospitals, HLMR increased from 0.8% in 1995 to 3.5% in 2003. LLMR ranged from 4.3% to 2.9% but has tended to decrease since 2001.15,14,20,21 HLMR strains are strongly associated with high rates of decolonization treatment failure.22 The clinical significance of LLMR is more dubious given that the concentration of mupirocin in the 2% ointment exceeds 20 000 mg/L. However, Harbarth et al.10 and Walker et al.22 found that patients colonized with LLMR isolates and receiving nasal decolonization were at increased risk of treatment failure compared with those colonized with fully
Figure 1. Mupirocin zone diameters of inhibition and MIC distribution for 125 MRSA isolates. A broken line marks the zone diameter breakpoint as proposed by Fuchs et al.\textsuperscript{11} A vertical line indicates the newly proposed interpretative zone diameter breakpoint from this study. The horizontal line indicates MIC breakpoints published by the BSAC to separate LLMR from susceptible and HLMR isolates.
susceptible isolates. A recent systematic review indicated the need for larger controlled studies to confirm whether mupirocin remains useful in clearing carriage of LLR S. aureus isolates.23 Because alternatives to mupirocin for eradicating MRSA carriage are limited and knowledge of the level of mupirocin resistance is important in the management of decolonization, it is essential for clinical laboratories not only to discriminate between susceptible and resistant strains but also to determine the level of resistance.

Accurate characterization of the level of mupirocin resistance of MRSA isolates in the laboratory is problematic with currently available discs. The only phenotypic method that discriminates between LLR and HLR is the MIC determination. However, MIC determination is either time-consuming (agar dilution) or expensive (Etest). Detection of the mupA gene by PCR should be an alternative, but access to this technique in routine laboratories is limited by the cost and need for specially trained technologists. In the present study, the results obtained by the Etest and the PCR assay were 100% concordant with all HLR MRSA isolates harbouring the mupA gene and showing MICs ≥512 mg/L. The mupA gene was not detected in LLR or in mupirocin-susceptible strains. Recently, mupirocin was included in the antimicrobial panel of the Vitek 2 system. In our evaluation, the new card correctly identified mupirocin-resistant MRSA isolates. However, the level of resistance is not determined by this method. Likewise, our in-house MSA accurately discriminated susceptible from resistant strains but did not distinguish between LLR and HLR.

As found by Fuchs et al.,11 diffusion methods using low-content discs (5 μg paper disc and 10 μg Neo-Sensitabs tablets) appeared to be reliable to detect mupirocin resistance but, again, did not differentiate between LLR and HLR isolates even though all HLR strains showed no inhibition zone around the discs.24 Indeed, no inhibition zone was observed with the majority of LLR strains. In 2005, the BSAC published interpretative zone diameters for MUP-5 and MUP-20.17 In our collection, the use of a zone diameter breakpoint of ≥22 mm for MUP-5 would have led to the misclassification of 21 (16.8%) susceptible strains as falsely resistant to mupirocin. Likewise, a breakpoint of ≥26 mm for MUP-20 would result in a 22% rate of major errors. No zone diameter criteria exist for 200 μg discs. As shown in Figure 1, all HLR but few LLR strains grew up to the MUP-200 discs. Using interpretative criteria of no zone of inhibition to discriminate between HLR and LLR forms of resistance, MUP-20 and MUP-200 showed sensitivities of 100% and specificities of 87.1% and 92.3%, respectively. With MUP-20, a second cut-off value of 17 mm should distinguish susceptible from resistant strains (Table 2). As outlined by Palepou et al.,25 larger inhibition zones were obtained on Diagnostic Sensitivity Test agar with MUP-200 and many susceptible and LLR strains showed overlapping inhibition zone diameters.

Recently, the concomitant use of MUP-5 and MUP-200 discs was proposed to better distinguish among the three levels of mupirocin susceptibility.26 From the results in our study, we propose that a breakpoint of 6 mm for the MUP-200 disc test can be used to accurately categorize all HLR strains and distinguish them from the majority of LLR and susceptible strains (Table 2).

In conclusion, all phenotypic methods tested in this study accurately discriminated mupirocin-susceptible from -resistant strains, except for MUP-200. The most accurate disc diffusion test results were obtained with the MUP-20 disc test by using two tentative interpretative breakpoints (proposed in the present study) or with the concomitant use of MUP-5 and MUP-200 discs. Further studies are warranted to evaluate the accuracy of MUP-20 disc testing in routine laboratories. The only completely reliable technique discriminating between LLR and HLR mupirocin resistance in S. aureus was MIC determination and detection of the mupA gene. An alternative could be the development of a new card for the Vitek 2 system, which not only detects resistant mupirocin strains but also distinguishes between LLR and HLR.

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References

Evaluation of mupirocin susceptibility testing


