Evaluation of disc diffusion and Etest for determining the susceptibility of *Staphylococcus aureus* to mupirocin

Marie-France I. Palepou, Alan P. Johnson, Barry D. Cookson, Hamish Beattie, Andre Charlett and Neil Woodford

The susceptibilities to mupirocin of 102 selected clinical isolates of *Staphylococcus aureus* and of control strain *S. aureus* NCTC 6571 were determined by disc diffusion (using discs containing 5, 15, 25, 30, 50 and 200 μg of mupirocin) and Etest and the results were compared with MICs determined using an agar incorporation method. On the basis of agar incorporation MICs, 42 isolates were sensitive to mupirocin (MIC ≤ 4 mg/L), 39 showed low-level resistance (MICs = 8–128 mg/L) and 22 were highly resistant (MICs ≥ 256 mg/L) and contained the *mupA* resistance gene. Using Stokes’ criteria, none of the discs used gave major errors (sensitive isolates classified as highly resistant) or very major errors (highly resistant isolates classified as sensitive) in assigning a category of susceptibility, but minor errors (a difference of one category) were noted with all strengths. The best correlation with agar incorporation MIC was obtained with 25 μg mupirocin discs, which classified correctly 98 (95%) isolates, while worse correlations were noted with 5 μg and 200 μg discs which are the only types currently available commercially, for which there were 47 and 30 minor errors, respectively. The MICs found by Etest were the same as, or lower than, those determined by agar incorporation. Etests classified correctly all 42 mupirocin-sensitive isolates, 19 (49%) low-level resistant isolates and 16 (73%) highly resistant isolates. Two isolates that contained the *mupA* gene and showed agar incorporation MICs of 256 mg/L and 512 mg/L were not classified as highly resistant by any of the diffusion methods used. Agar incorporation MIC determination, possibly supported by detection of the *mupA* gene, offers the most effective means of identifying high-level mupirocin resistance in *S. aureus*, although the Etest also proved to be reproducible. However, we conclude that 25 μg discs warrant further evaluation for possible use in clinical laboratories, as they appear to be more reliable than the discs currently available.

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Mupirocin binds to isoleucyl tRNA synthetase (IRS) and thereby prevents protein synthesis in susceptible bacteria. Two forms of resistance are recognized in staphylococci: low-level resistance (MICs = 8–256 mg/L) which has been attributed to mutations affecting the native, chromosomally encoded IRS protein, and high-level resistance (MICs > 512 mg/L) which results from the acquisition of new DNA (usually a plasmid) that encodes a second, mupirocin-resistant IRS enzyme. This acquired enzyme is the product of a gene variously designated ileS, ileS-2, or mupA. Hereafter, we will also refer to it as mupA. High-level resistance is often transferrable between strains and was first reported among clinical isolates of S. aureus from a hospital in London. This form of resistance is encountered increasingly in the UK, particularly in the south-east of England, and is exhibited by both methicillin-sensitive and -resistant S. aureus. Its association with various epidemic strains of MRSA may cause serious infection control problems.

As topical applications of mupirocin may achieve local concentrations of ca. 20,000 mg/L, low-level resistance may not be clinically significant; however, high-level resistance, encoded by mupA, has been associated with treatment failure. It is therefore important for the clinical laboratory to distinguish these two forms of resistance. The use of 5 μg discs is recommended for testing the susceptibility of staphylococci to mupirocin, but these discs fail to distinguish between low-level and high-level resistance. Discs containing 200 μg mupirocin are also available commercially and their use does discriminate the two forms of resistance, but the large inhibition zones formed cause practical problems for clinical laboratories that wish to screen multiple agents on a single agar plate.

The objectives of the present study were two-fold: (i) to determine an optimum disc strength (chosen from 5, 15, 25, 30, 50 and 200 μg) for determining the susceptibility of S. aureus isolates to mupirocin and (ii) to evaluate the Etest as an alternative means for determining mupirocin MICs. For both objectives, the test methods were evaluated by comparing the results with MICs determined by an agar incorporation method. A PCR assay for the mupA high-level resistance gene was used to ensure correct classification of isolates.

Materials and methods

Bacteria

One hundred and two clinical isolates of S. aureus were selected from the collection held in the PHLS Laboratory of Hospital Infection, together with S. aureus strain NCTC 6571 as a control. The isolates chosen had previously been classified as sensitive to mupirocin or displaying low-level or high-level resistance based upon their MICs. Each category included methicillin-sensitive (MSSA) and methicillin-resistant (MRSA) isolates of diverse phage types. Care was taken to exclude multiple isolates of epidemic MRSA strains.

Medium

Diagnostic Sensitivity Test (DST) agar (Unipath, Basingstoke, UK) supplemented with 2% lysed horse blood was used throughout.

Agar incorporation MIC determination

For the agar incorporation susceptibility tests, the medium contained two-fold increasing concentrations of mupirocin in the range 4–512 mg/L. Five colonies of each isolate were inoculated into 5 mL nutrient broths (Unipath), which were incubated overnight at 37°C. The undiluted broth cultures were applied to mupirocin-containing plates with a multipoint inoculator (Mast, Bootle, UK) to give a final inoculum of ca. 10⁶ cfu/spot. The MIC for each isolate was read as the lowest concentration that inhibited visible growth after 18 h incubation at 37°C.

Disc diffusion

Susceptibility tests were performed using discs of six mupirocin contents, namely 5, 15, 25, 30, 50 and 200 μg (kindly supplied by Mast Group Ltd). The inoculum for disc testing was prepared by diluting two drops (ca. 70 μL) of the overnight cultures used for MIC determination in 5 mL of fresh nutrient broth. Using a rotary plater, two plates were then inoculated with each test isolate using a swab dipped into the diluted broth; this inoculum was previously determined to be sufficient to give semi-confluent growth after overnight incubation. In all tests, the test isolate was applied around the edge of the plate and S. aureus NCTC 6571 was applied to the centre. The 5 μg and 200 μg mupirocin discs were placed opposite each other on the first plate, with the other four discs (15, 25, 30 and 50 μg) on the second plate. All agar plates were incubated for 18 h at 37°C after which time the radii of the zones of inhibition were measured with callipers from the edge of the zone to the edge of the mupirocin disc. In addition to measuring these radii, the zones of inhibition for the test isolates were also interpreted using Stokes’ criteria.

MIC determinations with E test

The diluted broths used for disc diffusion tests were also used for the Etest studies. A gain, a swab was dipped into the broth and then used to inoculate one half of an agar plate. S. aureus NCTC 6571 was inoculated on to the other half of the plate. A mupirocin Etest strip (Cambridge Diagnostics, Cambridge, UK) was applied carefully to the middle of the plate. All plates were incubated for 18 h at 37°C.
37°C and the MIC for each isolate was read as the point where the zone of inhibition intersected with the graduated strip, then ‘rounded up’ to the nearest equivalent log dilution.

Detection of the mupA gene by PCR
Isolates were examined for the mupA gene by PCR. A 190 bp intragenic fragment was amplified with forward and reverse primers mupA_F (5'-TGACAATAGAAAAAGGACAGG-3') and mupA_R (5'-CTCTAATTCAACTGGTAAGCC-3'), equivalent to nucleotide positions 554–743 of the published sequence. The method for preparing the template DNA and the amplification conditions were as described previously for amplification of sections of the mecA and 16S rRNA genes of S. aureus. Indeed, the two mupA primers may be used with these other pairs in a multiplex assay (N. Woodford, unpublished observation). All amplifications were achieved using Touchdown (Hybaid, Teddington, UK) or Genius (Techne, Cambridge, UK) thermal cyclers.

Statistical methods
The extent of the overlap of the distributions of radii for isolates showing sensitivity, low-level or high-level resistance to mupirocin were compared for selected disc contents (see Results). Pairwise logistic regressions were performed either with sensitivity and low-level resistance, or with low- and high-level resistance as the dependent variable, and zone radius as the predictor. From these regression models, the predicted probability of each isolate being in the higher category was calculated. By choosing any particular predicted probability as a cut-off, isolates with a larger predicted probability were classified as having the higher level of resistance. This enabled isolates to be placed in a classification table from which the sensitivity and specificity of the classification could be estimated. A plot of the sensitivity vs. (1 - specificity) was obtained as the predicted probability cut-off was varied; this plot is also known as a ROC curve. The areas under the ROC curves were used to indicate the discriminatory ability of the discs; a value of 1.0 indicated no overlap in distributions of radii, while a value of 0.5 indicated that the distributions of radii were indistinguishable.

For each disc content, the optimal radius to distinguish each pair of categories was taken to be the lowest observed radius above that which theoretically gave an equal predicted probability of an isolate being in either category. The estimated sensitivity and specificity were obtained using this classification. Exact binomial 95% confidence intervals around these estimates were calculated with Stata Statistical Software, release 5.0 (Stata Corporation, College Station, TX, USA).

R esults
Agar incorporation MICs and detection of mupA
The distribution of MICs for the 103 isolates is shown in Figure 1. Isolates resistant to mupirocin (MICs > 4 mg/L) showed a bimodal distribution. Thirty-nine isolates with MICs in the range 8–64 mg/L inclusive did not contain the mupA gene and were therefore classified as showing low-level resistance. Twenty-two mupA-positive isolates and with MICs of ≥256 mg/L were classified as showing high-level resistance. Forty-two isolates were sensitive to mupirocin (MICs ≤ 4 mg/L).

Effect of disc content on mupirocin susceptibility determination
Figure 2 shows the radii of the inhibition zones recorded for the commercially available 5 µg and 200 µg discs (panels a and f, respectively), together with those for the other discs manufactured for use in this study. None of the disc contents allowed all isolates of the three susceptibility categories to be distinguished clearly. The discrimination between low-level and high-level resistance was poor with 5 µg discs but, with this exception, the discrimination obtained with most other discs was generally good. This was confirmed by statistical analysis of the distributions of radii obtained with 5, 25 and 200 µg discs (Table). The value for the ‘area under the ROC curve’ indicates the degree of overlap between the radii for isolates in each category, ranging from 0.5 for indistinguishable distributions to 1.0 for those distinguished completely.

Lowering the interpretative radii for 25 µg discs gave increased sensitivity without affecting specificity. Radii from the disc of >10 mm to indicate sensitivity and <2 mm to indicate high-level resistance therefore appeared to be suitable ‘working criteria’ for further evaluation. In this study, the application of these criteria would have resulted in four isolates being classified incorrectly, two with low-level resistance and two mupA-positive isolates with high-
Figure 2. Radii of the zones of inhibition noted for 103 isolates of *S. aureus* when diffusion tests were performed with (a) 5 µg, (b) 15 µg, (c) 25 µg, (d) 30 µg, (e) 50 µg and (f) 200 µg mupirocin discs. Isolates were sensitive to mupirocin (●), or exhibited either low level (■) or high-level (▲) resistance.
Susceptibility testing of *S. aureus* against mupirocin

**Table.** Comparison of 5, 25 and 200 μg mupirocin discs for determining the susceptibility of *S. aureus*

<table>
<thead>
<tr>
<th>Disc content (μg)</th>
<th>Categories from disc (mm)</th>
<th>% Sensitivity</th>
<th>95% CI</th>
<th>% Specificity</th>
<th>95% CI</th>
<th>Area under the ROC curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>S, LL</td>
<td>4</td>
<td>100</td>
<td>92.0–100</td>
<td>100</td>
<td>91.0–100</td>
</tr>
<tr>
<td></td>
<td>LL, H L</td>
<td>3</td>
<td>94.9</td>
<td>87.2–99.4</td>
<td>18.2</td>
<td>5.2–40.3</td>
</tr>
<tr>
<td>25</td>
<td>S, LL</td>
<td>12</td>
<td>97.7</td>
<td>88.0–99.9</td>
<td>97.4</td>
<td>86.5–99.9</td>
</tr>
<tr>
<td></td>
<td>LL, H L</td>
<td>4</td>
<td>92.3</td>
<td>79.1–98.4</td>
<td>90.0</td>
<td>70.8–98.9</td>
</tr>
<tr>
<td>200</td>
<td>S, LL</td>
<td>15</td>
<td>93.2</td>
<td>81.3–98.6</td>
<td>94.9</td>
<td>82.7–99.4</td>
</tr>
<tr>
<td></td>
<td>LL, H L</td>
<td>9</td>
<td>94.9</td>
<td>82.7–99.4</td>
<td>90.0</td>
<td>70.8–98.9</td>
</tr>
</tbody>
</table>

Abbreviations: S, sensitive; LL, low-level resistance; HL, high-level resistance.

The logistic regression analysis between predicted probability and radius was reversed in this case, i.e. the larger the radius, the higher the probability of the isolate showing low-level resistance. This gives rise to high sensitivity, but low specificity.

Use of E tests for mupirocin susceptibility determination

Figure 4 shows the relationship between the MICs determined by agar incorporation and the E test. Values determined by E test were always equal to, or less than, those determined by agar incorporation. However, for isolates sensitive to mupirocin this resulted from the lower concentrations tested on E test strips. E tests classified correctly all 42 mupirocin-sensitive isolates, but only 19 of 39 (49%) low-level resistant isolates (the others appeared sensitive). Sixteen of 22 (73%) highly resistant isolates gave E test MICs of ≥256 mg/L and three others had MICs of 128 mg/L. Three highly resistant *mupA*-positive isolates, including the two not classified correctly using any disc method, were classified as intermediate (MIC = 32 mg/L, two isolates) or sensitive (MIC = 4 mg/L, one isolate) to mupirocin by E test. Despite these discrepancies with agar incorporation MICs, E test MICs appeared reproducible; *S. aureus* NCTC 6571 was tested on 102 occasions (as a control) and there was only a two-fold difference in MICs (0.06–0.12 mg/L). Similarly, minimal variation in the MIC by E test was noted when tests with low-level or high-level resistant isolates were undertaken (data not shown).

Discussion

A accurate characterization of mupirocin-resistant *S. aureus* in the clinical laboratory is problematic with currently available discs. Interpretative criteria for use with low-
content (5 μg) discs have been proposed, but these only differentiate between sensitive and resistant isolates, and do not distinguish between the low-level and high-level forms of resistance. No criteria exist for use with 200 μg discs and, furthermore, the large inhibition zones obtained with these cause practical problems when testing multiple agents on a single agar plate. Therefore, we assessed the potential of four other disc strengths to solve this problem, and evaluated the Etest as an alternative means of determining the mupirocin MICs for S. aureus isolates.

The 25 μg mupirocin discs, which were prepared especially for this project, gave the best concordance with MICs determined by agar incorporation. Moreover, the zones obtained with these discs had a maximum radius of 17 mm (measured from the edge of the zone to the edge of the disc) and are less likely to interfere with those of other discs than are those obtained with 200 μg discs. In agreement with previous studies, the Etest also proved to be a reproducible method for determining mupirocin MICs, although the values were usually lower than those determined by the agar incorporation method. The Etest classified as sensitive 20 of the 39 isolates shown by agar incorporation to have low-level mupirocin resistance and also classified as sensitive or low-level resistant, six of the 22 highly resistant (by agar incorporation) isolates. Although debatable, it seems likely that mupirocin would be effective against isolates with MICs of 256 mg/L, with or without mupA. We identified two further isolates that contained the mupA gene and were classified as highly resistant by the agar incorporation method (MICs of 256 mg/L and 512 mg/L), but which were not classified as highly resistant by any of the diffusion methods used. We cannot explain these discrepancies, but the expression of mupA and growth rates in these isolates, compared with more typical highly-resistant isolates, warrants further study.

In conclusion, we have confirmed that the commercially available 5 μg and 200 μg discs are not reliable either for detecting all high-level mupirocin-resistant S. aureus or for distinguishing low-level from high-level resistance. A gar incorporation MIC determination, possibly supported by detection of the mupA gene, offers the most effective means of identifying high-level mupirocin resistance in S. aureus. The Etest was reproducible, but we suggest that it requires further evaluation to achieve more reliable interpretations of susceptibility categories in comparison with agar incorporation. We conclude that 25 μg discs warrant further evaluation for possible use in clinical
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laboratories. These discs gave the best agreement with agar incorporation MIC and they appeared to be more reliable than the discs currently available.

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References


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