Interpretive criteria for mupirocin susceptibility testing of *Staphylococcus* spp. using CLSI guidelines

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ABSTRACT

Mupirocin is an antimicrobial agent commonly used to treat staphylococcal infection or to eliminate persistent carriage. To date, interpretive criteria have not been established to define susceptibility or resistance when performing mupirocin susceptibility testing. In this evaluation, using CLSI guidelines, a total of 502 staphylococci comprising 219 methicillin-sensitive *Staphylococcus aureus*, 222 methicillin-resistant *S. aureus* and 61 coagulase-negative staphylococci are tested by broth microdilution, disc diffusion and E-test. Disc diffusion using 5 µg mupirocin discs was found to be a reliable method to distinguish susceptible and resistant strains. Minimum inhibitory concentration (MIC) determination was required to differentiate low-level and high-level resistance to mupirocin. E-test was found to be an accurate alternative to broth microdilution for the routine determination of MIC values of staphylococci to mupirocin. Broth microdilution and disc-diffusion results were plotted on a scattergram, and error rates were calculated. No errors were found using susceptibility criteria of <4 µg/mL (MIC) and >19 mm (zone diameter).

KEY WORDS: Microbial sensitivity tests.
Mupirocin.
Interpretive criteria.
Staphylococcus.

Introduction

Mupirocin (Bactroban) is a bacteriostatic antibiotic used exclusively as a topical agent. It exerts its antimicrobial effect by specifically and irreversibly binding to bacterial isoleucyl-tRNA synthetase (IleS), thus preventing protein synthesis.[1] It has been used widely for the clearance of nasal methicillin-resistant *Staphylococcus aureus* (MRSA) carriage during outbreaks and has been recommended for the decolonisation of methicillin-sensitive *S. aureus* (MSSA) in healthcare personnel.[2] Intranasal application of mupirocin ointment is effective in reducing surgical site infections and the likelihood of bronchopulmonary infection.

Mupirocin-resistant MRSA was first isolated from the skin of patients treated with mupirocin for long periods of time.[3] Nasal application of mupirocin at clinically effective concentrations may result in the presence of low levels of the antibiotic in the pharynx, which could induce or select for the emergence of mupirocin-resistant MRSA.[4] High-level resistant strains are more likely to be associated with clinical and microbiological failure.[5,6]

High-level mupirocin resistance differs from low-level resistance in the location and/or copy number of *mup*A, the plasmid-encoded gene.[7] Due to increased use of mupirocin, staphylococcal strains exhibiting both types of resistance have been reported widely.[1]

Currently, there are no Clinical and Laboratory Standards Institute (CLSI) guidelines (formerly National Committee for Clinical Laboratory Standards [NCCLS]) for outlining interpretive criteria for mupirocin. An increasing prevalence of mupirocin resistance among staphylococci, together with the clinical significance of high-level resistance, necessitates the development of accurate methods and interpretive criteria for determination of susceptibility.

Previous studies suggest a minimum inhibitory concentration (MIC) value of <2 µg/mL and a corresponding zone diameter of >14 mm for susceptibility to mupirocin 5 µg discs.[8,9] However, neither of these studies incorporated mupirocin-resistant isolates and it is suggested that the validity of using mupirocin 5 µg discs for the detection of resistance requires evaluation.

The aims of this study are to determine the interpretive criteria for mupirocin susceptibility testing of *Staphylococcus* spp., to establish correlation between broth microdilution and disc-diffusion methods, and finally to observe the range of mupirocin MIC values in a large population of randomly isolated strains.

Materials and methods

A total of 502 non-duplicate clinical strains of staphylococci were collected. The collection comprised 219 MSSA, 142 MRSA and 61 coagulase-negative staphylococci (CNS) isolated by the microbiology department of Cork University Hospital. In addition, 40 MRSA isolates were obtained from Waterford Regional Hospital and 40 MRSA isolates from St. James' Hospital, Dublin.

The MSSA, MRSA and CNS isolates were selected without

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prior knowledge of mupirocin susceptibility. Isolates were identified by conventional methods, including DNase and slide agglutination test (Slidex Staph-kit, bioMérieux, Hazelwood, MO). Methicillin susceptibility was also ascertained according to CLSI guidelines.[10]

All organisms were stored on nutrient agar slopes and were subcultured on Columbia blood agar (Oxoid, Basingstoke, Hampshire, UK) before testing.

Two American Type Culture Collection (ATCC) strains, *S. aureus* ATCC 25923 and *S. aureus* ATCC 29213, were included as controls.

Methods were carried out following CLSI guidelines.[10] Broth microdilutions were performed using Mueller-Hinton broth (Oxoid) and lithium mupirocin reference powder of known potency (kindly supplied by GlaxoSmithKline, Worthington, West Sussex, England).

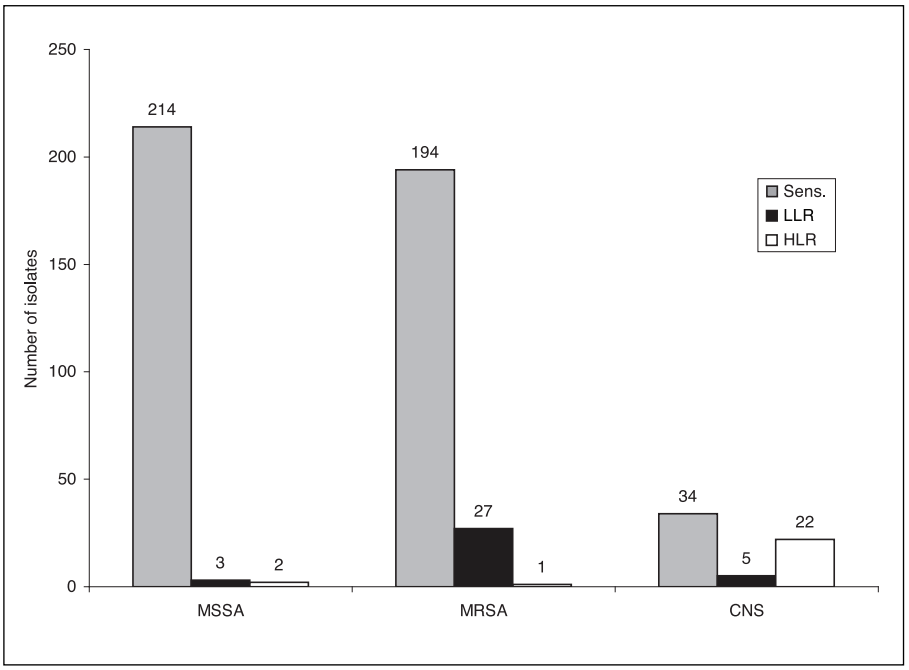

Fig. 1. Distribution of mupirocin susceptibility in the 502 staphylococcal isolates tested.

A 1–2 x 10^6 colony-forming unit (cfu)/mL inoculum was prepared and incubated with broth containing mupirocin in two-fold dilutions ranging from 0.004 µg/mL to 512 µg/mL on a microtitre plate.[10] The MIC was recorded as the lowest concentration of mupirocin that inhibited visible bacterial growth.[10]

Disc-diffusion tests were performed using Mueller-Hinton agar (Cruinn Diagnostics, Dublin). Susceptibility to mupirocin and vancomycin was assessed using two mupirocin 5 µg discs (different lot numbers) and one 30 µg vancomycin disc (Oxoid), which acted as an internal control. The surface of a Mueller-Hinton agar plate was inoculated with a 1–2 x 10^8 cfu/mL suspension, allowed to dry and then the discs were placed on the surface of the plate.

Zone diameters of inhibition were measured using Mitutoyo digital callipers (Giles Scientific, Santa Barbara CA). This study compared the zone diameters obtained with the callipers to those obtained with a semi-automated system (BIOMIC, Giles Scientific).

E-tests were performed on Mueller-Hinton agar using mupirocin E-test strips (AB Biodisk, Solna, Sweden). Determination of MIC using E-test strips was performed following the manufacturer's instructions. As mupirocin is a bacteriostatic agent the MIC was recorded at 80% inhibition, and MIC determination at 100% was used for comparative analysis only.

Comparative analyses of the methods were performed using Pearson's product moment coefficient and the paired *t*-test. Interpretive breakpoint criteria for mupirocin were established by the error-rate bounded method of Metzler and DeHann.[11]

Results

Fifty replicate tests of the control strain *S. aureus* ATCC 29213, measured by broth microdilution, showed 50 identical mupirocin MIC values of 0.016 µg/mL. Zone diameters from replicate testing of the control strain *S. aureus* ATCC 25923 ranged from 21 to 24 mm when measured by callipers and the BIOMIC system.

Table 1. An estimation of the degree of correlation between methods used to determine zone diameter and MIC.

Method 1	Method 2	r value
Calliper zone determination	BIOMIC zone determination	0.980
Broth MIC determination	E-test (80%) MIC determination	0.999
E-test (80%) MIC determination	E-test (100%) MIC determination	0.999

Table 2. Evaluation of statistical differences between methods.

Method 1	Method 2	*t* value	Critical value	*P* value
Broth MIC determination	E-test (80%) MIC determination	–1.692	1.964	0.1
Calliper zone determination	BIOMIC zone determination	–7.329	1.964	<0.001

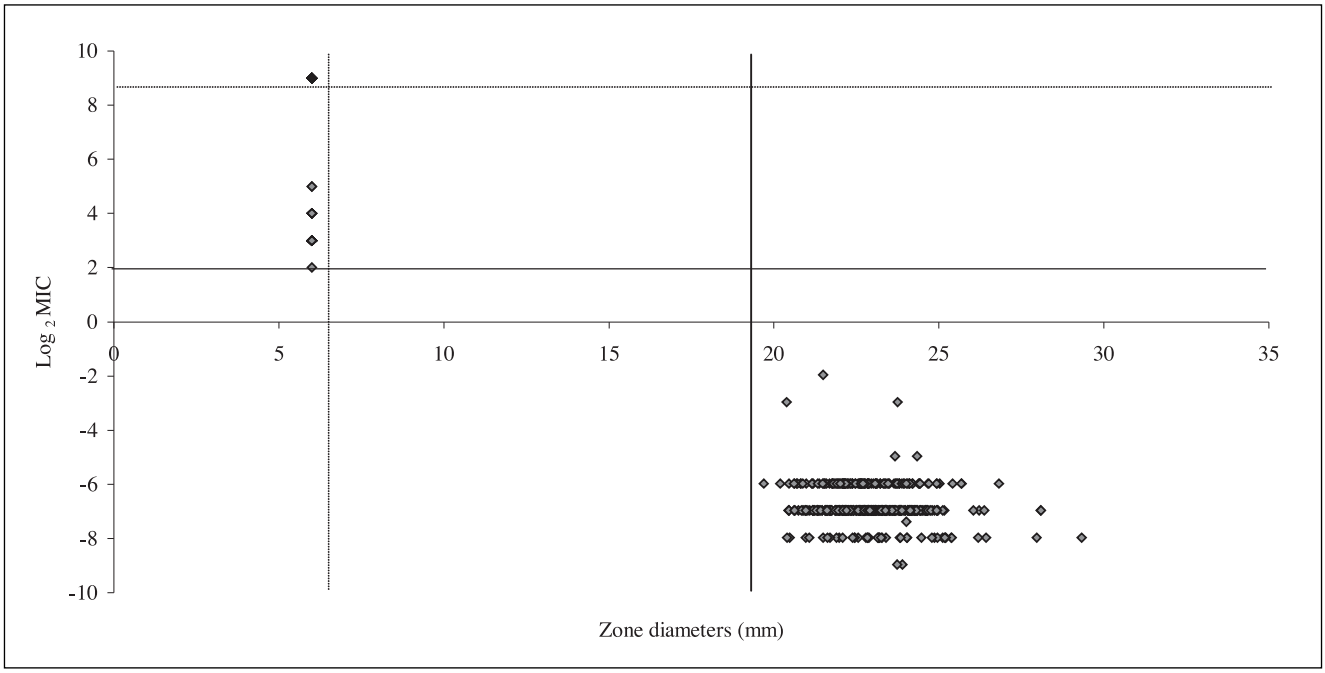

Fig. 2. Scattergram of mupirocin $\log_2$ MICs versus 5 µg disc inhibitory zone diameters.

When 95% confidence limits of two standard deviations were applied to the data for calliper measurements, 95.92% of sensitive isolates (424/442) fell within the limits. Zone diameters for the vancomycin 30 µg disc ranged from 17 to 19 mm, which is within current CLSI limits of >15 mm as the breakpoint for susceptibility.

Pearson's product moment coefficient (r) was used to analyse the level of correlation between comparable methods, and r values are presented in Table 1. Pearson's correlation reflects the degree of linearity between two variables. It ranges from +1 (a perfect positive relationship) to –1 (a perfect negative relationship). Disc-diffusion zone sizes (mm) were compared with $\log_2$ MIC (µg/mL), and an r value of –0.941 was derived. A paired *t*-test was used to compare two sets of related quantitative data. Results are presented in Table 2.

Following analysis by broth microdilution and disc diffusion, the distribution of susceptibility to mupirocin for the 502 staphylococcal isolates is illustrated in Figure 1. In this study, neither organisms showing low-level resistance nor high-level resistance to mupirocin (5 µg) exhibited a zone of inhibition.

Low-level resistance (LLR) isolates showed MIC values in the range 8–256 µg/mL. High-level resistance (HLR) isolates showed MIC values >512 µg/mL, as determined by broth microdilution.[6]

In Figure 2, a scattergram represents the MIC results (in logarithmic format) plotted against zone diameters. Interpretive criteria were established using the error rate bounded method of Metzler and DeHann, based on MIC breakpoints of <4 µg/mL for sensitive, 8–256 µg/mL for LLR and >512 µg/mL for HLR isolates. Corresponding zone diameter breakpoints for sensitive and resistant isolates were >19 mm and 6 mm, respectively.

The full horizontal line in Figure 2 represents the cut-off MIC value of 4 µg/mL ($\log_2$ 2) for resistant isolates. The full vertical line represents the smallest zone diameter (19 mm) obtained from the sensitive population. Neither HLR nor LLR populations demonstrated zone diameters of inhibition. The broken vertical line represents a zone size of 6 mm, which corresponds to the diameter of the disc. The broken horizontal line represents the MIC that defines HLR (i.e., >512 µg/mL).

All isolates fell within the proposed parameters for susceptibility or for LLR or HLR.

Discussion

In antimicrobial susceptibility testing, the MIC susceptibility breakpoints are defined by the correlation of MIC data for the infecting organisms with disc-diffusion zone diameters. The breakpoint established should predict organism susceptibility while ensuring misclassification errors are kept to a minimum.[12]

Determination of such breakpoints through an error-rate bounded classification scheme was first proposed by Metzler and DeHann in 1974.[11] This method involves one MIC breakpoint that separates susceptible and resistant strains. More recently, researchers, including CLSI, have preferred to use two MIC breakpoints that separate susceptible, intermediate and resistant strains.[12]

In the present study, using the error-rate bounded method, the proposed parameters for definition of susceptibility were <4 µg/mL (MIC) and >19 mm (zone diameter). All resistant organisms had a zone diameter of inhibition of 6 mm.

It is evident from Figure 2 that two distinct groups fall into this category. Low-level resistant isolates had MIC values of 8–256 µg/mL and HLR isolates had MICs >512 µg/mL, demonstrated by the broken horizontal line. There were no errors when these interpretation criteria were applied to the data, most probably due to the absence of zones of inhibition for LLR isolates.

Correlation was calculated by Pearson's product moment coefficient, and a value of –0.941 demonstrates a strong

inverse relationship between the methods. Finlay *et al*.[13] suggest susceptibility parameters of <4 µg/mL and >14 mm for MIC and zone diameter, respectively. When interpretive criteria of <4 µg/mL and >18 mm were applied, five major errors occurred.

The differentiation of susceptible and resistant isolates using 5 µg discs in the present study makes disc-diffusion testing a reliable and cost-effective routine method. Of the 502 isolates studied, 442 (88%) showed zone diameters of inhibition >19 mm. However, it was not possible to distinguish between HLR and LLR in the 60 (12%) isolates exhibiting resistance.

E-test MICs showed a high degree of correlation with broth microdilution MICs over the range 4–512 µg/mL for MSSA, MRSA and CNS. E-tests were performed on the 60 resistant isolates and 58 (96.7%) of the results were within a single dilution of the corresponding broth microdilution MIC. All results were within two dilutions. This correlates with the performance of mupirocin E-test in a study by Simpson *et al*.[14]

As 5 µg discs fail to distinguish between LLR and HLR, a study by Palepou *et al*.[5] endeavoured to determine an optimum disc concentration to enable this distinction to be made. It was concluded that 25 µg mupirocin discs were most useful. However, CLSI-recommended media were not used in the study.

In the present study, however, LLR isolates showed MIC values of 8–32 µg/mL. Therefore, use of a 25 µg disc could not be recommended here. However, a 50 µg disc used in conjunction with a 5 µg disc may distinguish between LLR and HLR.

Zone sizes obtained from discs with high mupirocin concentrations may be too large to be of practical use in a clinical laboratory setting and this method requires evaluation. It would be important to monitor the MIC values of LLR strains concurrently with screening for HLR with 50 µg discs.

The effect of increasing therapeutic concentrations may be to increase MICs in LLR strains, so careful monitoring would be essential. However, the versatility and ease of use of the E-test provides an attractive alternative to dilution susceptibility tests in distinguishing between LLR and HLR in the absence of validated two-disc breakpoint data.

Pearson's coefficient was used to determine the degree of correlation between methods (Table 1). In order to determine if there was a statistically significant difference between methods, a paired *t*-test was performed (Table 2). Statistical analysis of the relationship between MIC values obtained by broth microdilution and E-test at 80% inhibition showed a strong correlation between the methods (r=0.999); however, using the paired *t*-test; no statistically significant difference was found between the methods (t=–1.692, P=0.1).

Correlation between zone diameter readings using callipers and the BIOMIC system was 0.980. However, when analysed using the paired *t*-test, it was determined that the methods were statistically different (t=–7.329, P<0.001).

Previous studies suggest that the BIOMIC system is an acceptable alternative to callipers for the determination of interpretative categories based on disc-diffusion susceptibility tests.[15] Korgenski and Daly, in a study of more than 3000 isolates, determined that discrepancies appeared to be random, with no particular organism/antimicrobial agent combination being noted as a problem.[15]

In the present study, there was a significant statistical difference between the methods to determine zone size for mupirocin, but not for the internal control vancomycin disc (data not shown). However, misclassification errors did not occur as a consequence of these events.

In an evaluation of a similar semi-automated zone reading system by Medeiros and Crellin, it was found that the reader often failed to detect light growth at the margins of the zone.[16] Consequently, the zone diameters were significantly larger for bacteriostatic antimicrobial agents, and therefore were inaccurate. However, these differences rarely affected the classification of the isolate as susceptible or resistant.

Unlike high-level mupirocin resistance, LLR is not associated with treatment failure, and can be overcome by increasing the concentration of mupirocin at the site of infection.[17] Therefore, it is important to obtain the MIC value of resistant isolates by E-test in order to determine the efficacy of mupirocin therapy in a clinical setting.

The distribution of susceptibility for the 502 isolates of staphylococci in the present study is illustrated in Figure 1. The majority of LLR isolates (27/35) were found in MRSA strains. Conversely, the majority of HLR strains (22/25) were found in CNS. Of the 22 HLR CNS, 21 were isolated from known MRSA-positive patients. These findings may suggest that there was a high level of resistance in CNS from patients likely to have been treated with mupirocin.

The resistance profiles of staphylococcal isolates to mupirocin in a previous study show that the overwhelming majority of these were resistant to methicillin.[18] Mupirocin is used principally for treatment of nosocomial infections caused by methicillin-resistant organisms, even though it is also used to treat *S. aureus* carriage in healthcare personnel, and so the selective pressure of mupirocin use is focused mainly on such strains.

In a study by Leski *et al*., mechanisms for the spread of mupirocin resistance were investigated in a large hospital.[18] Using MIC values, it was determined that the prevalence of mupirocin resistance in the staphylococcal population was almost 20%. The high proportion of resistant CNS compared to that of MSSA and MRSA indicates that CNS may constitute a significant reservoir of resistant staphylococcal isolates.

Morton *et al*. demonstrated the horizontal transfer of a conjugative mupirocin plasmid in *S. aureus* isolates between patients in different areas of the hospital.[19] Furthermore, Udo *et al*. showed that unrelated strains could harbour *mup*A plasmids of the same phenotype, and that conjugative plasmids move readily between CNS and *S. aureus*.[3] There is evidence to suggest that mupirocin-resistant CNS can act as a reservoir for *mup*A plasmid transfer to MSSA and MRSA. Further studies are needed to investigate this potential plasmid transfer among the study population.

After initial reports of low-level mupirocin resistance, it was believed that its unique mode of action, combined with the scarcity of such resistance, would have made high prevalence of LLR a rather remote possibility.[17] It is now evident that given the ease and rapidity with which the mupirocin resistance gene can be mobilised to a conjugative plasmid, and the large potential reservoir for these plasmids in CNS, this is not the case.

A study by Petinaki *et al*. showed that the rate of HLR CNS increased dramatically from 9% in 1999 to 33% in 2002.[20] This would suggest that careful monitoring of mupirocin usage

and more definitive routine testing of staphylococci for mupirocin resistance is necessary.

Estimating the incidence of mupirocin resistance is difficult for a number of reasons. Most laboratories test mupirocin susceptibility of a subset of staphylococcal isolates only (i.e., those where mupirocin may be used). In a study by Cookson, it was found that less than half of laboratories surveyed used a breakpoint method to determine mupirocin MIC.[17] Therefore, the remaining laboratories did not distinguish between HLR and LLR. Until such time as there are interpretive criteria established for mupirocin, difficulties associated with determining resistant strains will remain.

The emergence of mupirocin resistance and the potential loss of one of the most important MRSA control strategies emphasises the significance of using the agent judiciously. Eradication strategies for MRSA should be designed carefully with reliable laboratory screening for resistance. Periodic antibiotic prescribing and infection control audits should be performed. This will ensure that emerging resistance may be detected and control measures implemented to minimise further spread.

It can be concluded from the findings of the present study that the MIC value for susceptible strains should be set at <4 μg/mL. In tests with the 5 μg disc, the zone diameter of inhibition for sensitive isolates should be >19 mm. Further determination of the type of resistance present requires MIC investigation, and it has been shown that E-test determination is an acceptable alternative to standard broth methods.

Additional studies are necessary to evaluate the usefulness of a higher-concentration mupirocin disc to distinguish between LLR and HLR. If validated, this method would be of greater value than E-test MIC determination in a clinical setting, as definitive results would be available earlier. Also, with the advent of interpretive criteria, the true parameters of mupirocin susceptibility or resistance may be ascertained and inter-country comparisons made, in order to achieve a global perspective on MRSA treatment strategies. □

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