Short Communication

Correspondence

Marcia Giambiagi-deMarval
marciagm@micro.ufrj.br

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Improvement of mupirocin E-test for susceptibility testing of *Staphylococcus aureus*

Pedro Juan José Mondino,^{1,2} Kátia Regina Netto dos Santos,¹
Maria do Carmo de Freire Bastos¹ and Marcia Giambiagi-deMarval¹

¹Instituto de Microbiologia Prof. Paulo de Góes, Universidade Federal do Rio de Janeiro, Brazil

²Departamento de Patologia, Universidade Federal Fluminense, Rio de Janeiro, RJ, Brazil

Interpretation of the mupirocin E-test for low-level mupirocin-resistant *Staphylococcus aureus* strains has been improved by adding the indicator dye tetrazolium. E-tests were compared with agar dilution methods for assessing mupirocin susceptibility. MICs obtained by the agar dilution method and E-tests showed 89.3 % agreement within 2 log₂ dilution criteria. The agreement between MICs increased to 100 % in the 1 log₂ dilution definition when the indicator dye tetrazolium was added to the E-test. The use of the E-test with tetrazolium reduction is more accurate for determining mupirocin MICs for *S. aureus* strains.

Introduction

Mupirocin is an effective antibiotic for elimination of methicillin-resistant *Staphylococcus aureus* (MRSA) from nasal colonization and has been used to control the spread of these organisms among patients during outbreaks (Eltringham, 1997). Mupirocin-resistant strains can be found, and they are grouped in two distinct categories: low-level or intermediate resistance (Mup^I), with MICs of 4–256 µg ml⁻¹, and high-level resistance (Mup^R), with MICs ≥512 µg ml⁻¹ (Eltringham, 1997). Mup^R has been associated with the failure to clear the organism in patients. However, it has been suggested that Mup^I nasal isolates may still be controlled with mupirocin therapy (Hudson, 1994), since the ointment used contains a much higher mupirocin concentration (20 000 µg ml⁻¹).

We have previously shown that the acquisition of mupirocin resistance in the Hospital of the Federal University of Rio de Janeiro was associated with the prior use of mupirocin in the nosocomial environment, indicating that mupirocin should be used solely for the treatment and prevention of serious MRSA infections (Santos *et al.*, 1996).

In this context, the E-test appears to be an excellent tool for determining the level of mupirocin resistance in *S. aureus*. However, *Staphylococcus* strains tend to give lower MICs in the mupirocin E-test than in the corresponding mupirocin agar dilution (Simpson *et al.*, 1995). It has been shown that the disc diffusion method can be improved by adding the indicator dye tetrazolium–phenazine methosulfate (Bartlett & Mazens, 1979). Furthermore, the E-test for antimicrobial susceptibility of *Helicobacter pylori* was more accurate when tetrazolium/egg-yolk agar was employed (Valdez *et al.*, 1998).

In the present paper, we investigate the possibility of improving the *S. aureus* mupirocin E-test by adding the tetrazolium indicator dye.

Methods

Strains and cultivation. MICs were evaluated for 42 MRSA isolates from patients from the Teaching Hospital of the Federal University of Rio de Janeiro, Brazil. Fourteen strains (Table 1) were selected on the basis of their low-level mupirocin resistance, as characterized by the agar dilution method (Santos *et al.*, 1999). *S. aureus* strains HU1A (Mup^R; Bastos *et al.*, 1999) and ATCC 2913 [mupirocin-susceptible (Mup^S)] were used as controls. Mueller–Hinton agar (Difco) was used throughout all assays.

Susceptibility agar dilution method. Agar plates containing double-dilution series of mupirocin (512–1 µg ml⁻¹) were inoculated with 2 µl (~10⁴ c.f.u. per spot) of each strain using a Steers replicator and incubated for 18 h at 35 °C (NCCLS, 2001). MICs were determined as the lowest concentration of antibiotic that inhibited bacterial growth completely.

Susceptibility E-test method. Mupirocin susceptibility was determined by the E-test according to the manufacturer's guidelines (AB Biodisk). Plates were swabbed in three directions with 0.5 McFarland inocula. The E-test strip was applied to each plate with sterile forceps. Following incubation at 35 °C for 18 h, E-test MIC values were read by two independent operators, as the point of intersection of the zone of inhibition with the E-test strip. Immediately, 100 µl of a dye-indicator solution containing 1 mg 2(*p*-iodophenyl)-3(*p*-nitrophenyl)-5-phenyl-tetrazolium chloride and 60 µg phenazine methosulfate ml⁻¹ (Sigma) (Bartlett & Mazens, 1979) was added on the inhibition ellipse edge along each E-test strip. After 30 min, each plate was examined for colour development and the intersection-point of the zone of inhibition was again read by two independent operators. A magnifying glass was used to confirm the absence of isolated colonies or hazes.

Comparison of MICs determined by the agar dilution method and E-test. Since E-test MICs are determined on continuous antibiotic

concentration gradients, E-test MIC values that fell between two-fold dilutions were rounded up to the standard $\log_2$ scale of MICs from agar dilution. Hence, E-test MIC values of 3–4 $\mu\text{g ml}^{-1}$ were rounded up to 4 $\mu\text{g ml}^{-1}$, those of 6–8 $\mu\text{g ml}^{-1}$ to 8 $\mu\text{g ml}^{-1}$, 12–16 $\mu\text{g ml}^{-1}$ to 16 $\mu\text{g ml}^{-1}$ and 24–32 $\mu\text{g ml}^{-1}$ to 32 $\mu\text{g ml}^{-1}$.

Results and Discussion

We observed that the reading of MIC values determined by the E-test could be improved by the addition of the indicator dye tetrazolium. For one *S. aureus* isolate (HU2D), the MIC was determined as either 8 $\mu\text{g ml}^{-1}$ by standard E-test or 16 $\mu\text{g ml}^{-1}$ after adding tetrazolium to the E-test plate (Fig. 1).

Therefore, we decided to analyse the MICs of 14 nosocomial strains of *S. aureus* exhibiting low-level mupirocin resistance by agar dilution, E-test and E-test with tetrazolium (Table 1). The intercepts of the E-test scale were read by two different operators and end-points were determined. For the MICs obtained in the E-test, when seven assays were read independently by two operators, five of the resulting 14 readings (35.7%) were in agreement. After tetrazolium reduction, determination of the interception point was much clearer and easier. The readings of MICs from the E-test with tetrazolium were more similar than those observed for the conventional E-test. The operators agreed on 13 of 14 readings, thus increasing the agreement from 35.7 to 92.8% (Table 1).

Fig. 1. E-test MICs of mupirocin for a clinical isolate of *S. aureus* Mup^I, strain HU2D, without (a) or with (b) tetrazolium reduction.

Table 1. Mupirocin MICs of *S. aureus* strains from the agar dilution test, E-test and E-test after tetrazolium reduction

Values are $\mu\text{g ml}^{-1}$. E-test values were rounded up to the standard $\log_2$ scale of MICs from agar dilution. Op, Operator.

Strain	Agar dilution	E-test		E-test+tetrazolium	
		Op I	Op II	Op I	Op II
HU1a	32	4	8	16	16
HU1B	32	8	8	16	16
HU2D	32	8	8	16	16
HU4A	32	32	32	16	32
HU4B	32	8	16	16	16
HU5A	32	16	16	16	16
HU5B	32	8	16	16	16
HU6B	16	8	16	16	16
HU20A	32	4	4	16	16
HU21A	32	8	16	32	32
HU26A	32	16	16	16	16
HU27A	32	8	12	16	16
HU30A	32	32	32	32	32
HU30B	32	16	16	32	32
Controls					
ATCC 29213	1	0.062	0.062	0.125	0.125
HU1A	≥ 512	256	256	> 1024	> 1024

The coefficients of agreement of MICs between E-test and agar dilution were 89.3% within 2 $\log_2$ dilutions, 57.2% within 1 $\log_2$ dilution and 17.9% at identical dilutions (Table 2). Whenever the MICs did not agree exactly, we observed a clear bias towards E-test values being lower than those obtained by agar dilution. These differences were reduced by the addition of the indicator tetrazolium. There was 100% agreement between the MICs of E-test with tetrazolium and the agar dilution method by the 1 $\log_2$ dilution definition and

Table 2. Differences between MICs to mupirocin from the agar dilution method and E-test with or without tetrazolium

Values are numbers (percentages) of strains showing the difference indicated.

Operator	Log ₂ difference between MICs from agar dilution and E-test			
	0	1	2	>2
Without tetrazolium				
I	2 (14.3)	4 (28.6)	6 (42.9)	2 (14.3)
II	3 (21.4)	7 (50.0)	3 (21.4)	1 (7.1)
Total	5 (17.9)	11 (39.3)	9 (32.1)	3 (10.7)
With tetrazolium				
I	4 (28.6)	10 (71.4)	0 (0)	0 (0)
II	5 (35.7)	9 (64.3)	0 (0)	0 (0)
Total	9 (32.2)	19 (67.8)	0 (0)	0 (0)

32.2 % of MICs were identical (Table 2). No strain showed differences in the MICs obtained by agar dilution and E-test with tetrazolium, regardless of operators, within the criterion limits of 2 or $>2 \log_2$ dilutions. It should be noted that control strains, exhibiting high-level resistance or sensitivity to mupirocin, also presented lower values of MICs when comparing standard E-test with agar dilution. These results reinforce the advantage of adding the tetrazolium dye for the E-test method.

The need to perform antimicrobial susceptibility tests on *Staphylococcus* isolates is becoming more pressing. The versatility and feasibility of the E-test make the method an attractive alternative to conventional diffusion and dilution susceptibility tests. Therefore, the purpose of this study was to test the validity of tetrazolium reduction for improving mupirocin E-testing. It was shown that the mupirocin E-test is a reliable method for determining the susceptibility of staphylococci to mupirocin, although there was a tendency for the E-tests to give lower MICs when they did not correspond to the agar MICs (Simpson *et al.*, 1995). It should be emphasized that the high-level mupirocin-resistant control strain HU1A used in this work also presented an MIC with an E-test value lower than that observed for the agar dilution method. This reinforces the advantage of using the tetrazolium dye for the E-test method.

Here, we present an improvement of the mupirocin E-test by tetrazolium reduction and show that this modified technique brings the MICs determined by the E-test closer to the 'gold standard' agar dilution method.

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