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Limited detectability of linezolid-resistant *Staphylococcus aureus* by the Etest method and its improvement using enriched media

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The aim of this study was to evaluate Etest for detectability of linezolid-resistant meticillin-resistant *Staphylococcus aureus* (MRSA). The MIC of linezolid obtained by the Etest method in 18 linezolid-resistant strains of MRSA was compared with that obtained using standard agar and broth dilution methods according to Clinical and Laboratory Standards Institute guidelines. The mean linezolid MIC obtained by Etest in 18 linezolid-resistant strains of MRSA using Mueller–Hinton (MH) agar was 12.6-fold lower than that obtained by the agar dilution method, with the result that 78 % of the linezolid-resistant strains were incorrectly classified as linezolid-susceptible. The MIC of linezolid by Etest on brain–heart infusion (BHI) agar had a mean value 2.5-fold lower than that obtained by the agar dilution method, suggesting that replacing MH agar with BHI agar considerably improved the detectability of linezolid-resistant MRSA. Use of blood agar (MH agar supplemented with 5 % sheep blood) and 48 h of incubation resulted in 100 % agreement with the agar and broth dilution methods. Thus, this study revealed that the Etest on MH agar and BHI agar yielded false-negative results in a significant fraction of the linezolid-resistant MRSA. Hence, the use of blood agar and prolonged incubation is highly recommended for the accurate detection of linezolid-resistant MRSA using Etest.

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Abbreviations: CLSI, Clinical and Laboratory Standards Institute; MRSA, meticillin-resistant *Staphylococcus aureus*.

INTRODUCTION

Linezolid is the first oxazolidinone antimicrobial agent used for treatment of meticillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* infections. Linezolid inhibits the growth of susceptible cells by arresting protein synthesis by binding to the domain V region of the 23S rRNA (Bozdogan & Appelbaum, 2004). The MIC of linezolid in unexposed *S. aureus* has been found to be <4 mg l^{-1}, which is considered a break point (Bozdogan & Appelbaum, 2004; Shinabarger, 1999). Linezolid-resistant mutants have been reported sporadically worldwide (Ikeda-Dantsuji *et al.*, 2011b; Meka & Gold, 2004; Meka *et al.*, 2004; Tsiodras *et al.*, 2001). The most common mechanism of linezolid resistance involves a single-nucleotide substitution, G2576T, in the gene encoding the domain V region of the 23S rRNA (Meka & Gold, 2004). Other reported mutations are a T2500A substitution in the 23S rRNA gene (Meka *et al.*, 2004), chloramphenicol/florfenicol resistance (*cfr*)-mediated modification of the 23S rRNA (Arias *et al.*, 2008) and mutations in the genes encoding the ribosomal proteins L3 and L7 (Locke *et al.*, 2009).

Antibiotic susceptibility tests may be carried out following the Clinical and Laboratory Standards Institute (CLSI) protocol (CLSI, 2007) or routinely in clinical laboratories by a disc diffusion method or Etest. A study reported that a *cfr* mutant (i.e. linezolid-resistant) was found to be linezolid-susceptible by Etest (Arias *et al.*, 2008). In contrast, Etest was found to be a reliable method for linezolid susceptibility testing compared with the Wider and standard methods (Gómez-Garcés *et al.*, 2010). Thus, the reliability of Etest for linezolid susceptibility testing is confusing. These observations prompted us to refine the linezolid susceptibility test using the Etest method.

METHODS

The linezolid-susceptible strains used were the reference strain ATCC 29213, four pre-linezolid-treatment clinical isolates and 13 randomly selected clinical strains from several regionally distant hospitals. These strains showed linezolid MICs of 2–4 and 1–4 mg l^{-1} by the agar and broth dilution methods, respectively (CLSI, 2007). Eighteen linezolid-resistant strains were collected from 13 patients in seven hospitals all over Japan and their linezolid MICs varied from 8 to 64 mg l^{-1} by the agar and broth dilution methods. All strains had at least one G2576T mutation in the chromosomal gene encoding domain V of the 23S rRNA, confirmed by PCR and subsequent sequencing analyses (Ikeda-Dantsuji *et al.*, 2011b). The MIC of linezolid was also determined by Etest (AB Biodisk) using Mueller–Hinton (MH) agar, brain–heart infusion (BHI) agar and blood agar (MH agar with 5% sheep blood; Becton Dickinson). Briefly, cells were grown in MH broth overnight, the cell density was adjusted to 0.5 McFarland Units and a 0.2 ml sample was streaked on an appropriate agar plate. An Etest strip was placed onto the agar surface and the plate was incubated at 35 °C for 24 and 48 h.

RESULTS AND DISCUSSION

First, we determined the linezolid MIC using the agar dilution method in reference strain ATCC 29213 and the linezolid-susceptible isolates. The MIC values were 2 mg l^{-1} in 16 of the strains and 4 mg l^{-1} in two of the strains (Table 1). KY1, KT4, TK429 and SN1 were the strains isolated from the linezolid pre-treatment patients, and these strains were found to be resistant after linezolid treatment. The strain initials indicate the hospital codes. The linezolid MICs in these strains obtained by Etest on MH agar after 24 h incubation were 0.19, 0.5, 0.75 and 1 mg l^{-1} in two, five, ten and one strains, respectively. These values were on average 3.5-fold lower than the mean value obtained by the agar dilution method, whilst the value at 48 h by Etest was on average 2.6-fold lower. The MIC of linezolid in BHI agar at 24 and 48 h incubation by Etest was on average 2.6-fold and 2.2-fold lower, respectively, than the values obtained by the agar dilution method. Similarly, the values obtained by Etest in blood agar at 24 and 48 h were on average 2.2-fold and 1.5-fold lower, respectively, than the values obtained by the agar dilution method. None of the strains showed a linezolid MIC of $\geqslant 4$ mg l^{-1} in any assay medium as tested by Etest. These results revealed that the Etest significantly underestimated the linezolid MIC on MH agar and BHI agar in all the linezolid-susceptible strains except for one strain, KU2. Although the values obtained on blood agar appeared to be close to those obtained by the CLSI method, there were still small differences. As all the strains described above were linezolid-susceptible MRSA, the impact of this inconsistency might be clinically marginal. However, the impact might be unpredictable if the same tendency was applicable to linezolid-resistant MRSA.

Accordingly, we determined the MIC of linezolid in 18 linezolid-resistant strains available for this experiment by Etest using the three different media, and the MIC values were compared with those obtained by the agar dilution method (Table 2). KY5, KT1–KT3, KT6, KT7, KS510, KS227, KS310, TK471, TK487 and SN10 were the linezolid-resistant strains. The MIC values obtained by the agar dilution method varied from 8 to 64 mg l^{-1} among the strains, whilst that obtained by Etest on MH agar ranged from 0.5 to 8 mg l^{-1} at 24 h incubation. As the direct comparison of MIC values in individual strains obtained using different media did not yield much information, we calculated the relative MIC values of linezolid by dividing the MIC value obtained by the agar dilution method by the respective values in MH agar, or vice versa. The ratios in MH agar at 24 h incubation varied from 4 to 42.6 and those at 48 h varied from -1.5 to 10.6. The mean ratios at 24 and 48 h incubation were 12.6 and 4.0, respectively, suggesting that the MIC of linezolid obtained by Etest on MH agar was significantly underestimated. The Etest method only detected four and 11 strains from a total of 18 as linezolid-resistant (MIC >4 mg l^{-1}) at 24 and 48 h, respectively. Thus, it should be noted that use of the Etest on MH agar may not be reliable for determining linezolid MICs.

Similarly, the linezolid MIC obtained by Etest on BHI agar was compared with that obtained by the agar dilution

Table 1. MICs (μg ml^{-1}) of linezolid determined by the agar and broth dilution methods and Etest in linezolid-susceptible strains

Strain	CLSI method		Etest					
	Agar dilution	Broth dilution	MH agar		BHI agar		Blood agar	
			24 h	48 h	24 h	48 h	24 h	48 h
ATCC 29213	2	2	0.75	1	1	1	1	1.5
KY1	2	2	0.19	0.5	0.25	0.5	0.75	1.5
KT4	2	2	0.19	0.5	0.38	0.5	0.5	1
TK429	4	4	0.5	1	1	1	1.5	3
SN1	2	2	0.75	1	0.75	0.75	0.75	1
NG2	2	2	0.5	0.75	0.75	0.75	1	1
NG3	2	2	0.5	0.75	0.75	0.75	0.75	1
HG1	2	2	0.75	0.75	1	1	1	1
KU1	2	2	0.75	1	0.75	0.75	0.75	1
KU2	2	1	1	1	1	1	0.75	0.75
KU3	2	1	0.5	0.75	0.75	0.75	0.75	1
TM17	2	2	0.75	1.5	1	1.5	1.5	2
TM19	2	2	0.75	1.5	1	1.5	1	2
SW1	2	2	0.75	0.75	0.75	1	1	1.5
SW2	4	2	0.75	2	2	3	2	3
SW3	2	1	0.75	1.5	0.75	0.75	0.75	1
SW24	2	1	0.5	1	0.75	0.75	1	1
HG2	2	2	0.75	1	0.75	1	1	1.5

method. The MIC of linezolid by the Etest appeared to be on average 8.1-fold and 2.5-fold lower than that obtained by the agar dilution method at 24 and 48 h incubation, respectively. Among 18 strains tested, only six and 15 strains were classified as resistant on BHI agar at 24 and 48 h incubation, respectively. These results suggested that

Table 2. MICs (μg ml^{-1}) of linezolid determined by the CLSI method and Etest in the linezolid-resistant strains

Strain	CLSI method		Etest					
	Agar dilution	Broth dilution	MH agar		BHI agar		Blood agar	
			24 h	48 h	24 h	48 h	24 h	48 h
KY5	16	32	4	24	6	24	8	64
KT1	16	32	1.5	8	2	8	4	32
KT2	16	32	1.5	8	2	8	6	64
KT3	16	32	3	12	4	16	8	32
KT6	32	64	4	12	8	48	16	64
KT7	32	64	6	24	8	32	16	64
KS510	64	64	6	12	6	24	12	64
KS227	32	16	0.75	2	1	4	6	32
KS310	16	16	0.5	3	1	6	3	16
TH1	32	64	8	16	12	16	16	64
TH2	16	64	3	12	4	8	6	32
TH3	8	32	0.75	1.5	1	1.5	1.5	6
TH4	8	8	0.5	1.5	1	6	2	12
TH5	8	8	0.75	1.5	1	1.5	2	8
TK471	16	32	2	4	3	6	6	24
TK487	16	32	3	8	3	16	2	16
YM103	32	64	1	3	3	6	6	16
SN10	32	64	6	24	8	32	16	64
No. resistant strains	18	18	4	11	6	15	12	18

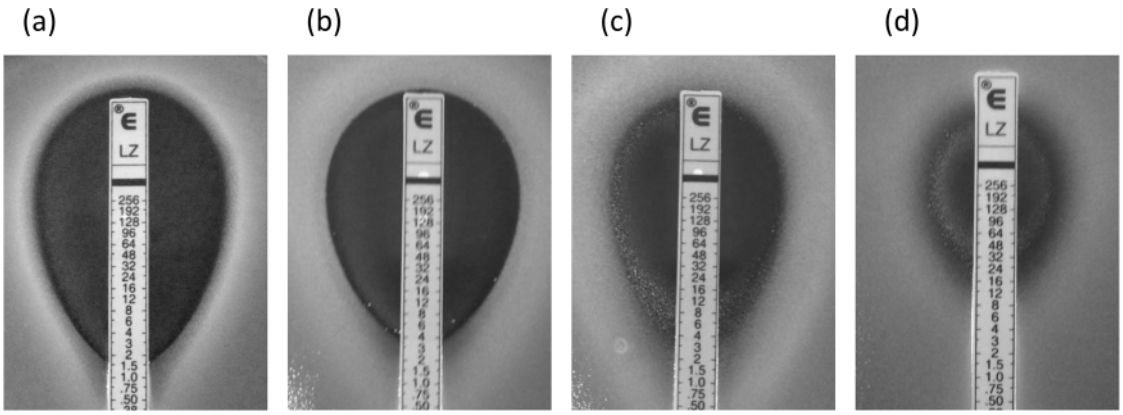

Fig. 1. Typical growth inhibitory profiles determined by Etest. (a) Linezolid-susceptible reference strain ATCC 29213; (b) linezolid-resistant strain TH3 with microcolonies in the clear zone; (c) linezolid-resistant strain KS310 with a blurred gradient growth inhibitory zone; (d) linezolid-resistant strain SN10 with double ring of the growth inhibitory zone.

replacing MH agar with BHI agar considerably improves the detectability of linezolid-resistant MRSA, although the detection rate was still unsatisfactory.

We assumed that the growth rate of the cells might affect the MIC value. Accordingly, we predicted that blood agar might be even better than BHI agar. Thus, the MH agar was supplemented with 5 % sheep blood and this was used for MIC determination. The linezolid MIC on this medium obtained by Etest after 24 h incubation was on average 3.7-fold lower than that obtained by the agar dilution method, with the result that 12 out of 18 strains were classified as linezolid-resistant MRSA. After the same plates were incubated for 48 h, there was on an average a −0.78-fold difference in the MIC ratio, showing that the MIC of linezolid obtained by Etest on blood agar after 48 h incubation was fully comparable with that obtained by the CLSI method. All 18 resistant strains were classified as linezolid-resistant, which was in 100 % agreement with the results obtained by the agar and broth dilution methods (Table 2). Thus, it was evident that the Etest underestimated the MICs of linezolid when MH agar and BHI agar were used. Therefore, the use of blood agar is highly recommended with incubation for 48 h for the determination of linezolid MIC in MRSA using the Etest method.

In addition, we encountered difficulties in reading the MIC of linezolid using the Etest method in some strains. After the linezolid-impregnated Etest strip was placed on blood agar plates smeared with bacterial cells, the majority of linezolid-susceptible and -resistant cells showed a clear zone of growth inhibition and the MIC could be read without difficulty (Fig. 1a). However, some of the resistant strains showed a markedly different profile: (i) TH3 and TH4 cells showed microcolonies in the clear zone of growth inhibition (Fig. 1b), which were more or less similar in appearance to those of the linezolid-resistant clinical strain HG503 with the T2500A mutation in the 23S rRNA gene, as reported recently (Ikeda-Dantsuji *et al.*, 2011a); (ii) KS310 and KS227 cells showed a halo zone of growth inside a hazy growth ring (Fig. 1c); and (iii) SN10 cells showed a gradient of the growth inhibitory zone, the border of which was not clear (Fig. 1d). Although hazy colonies or microcolonies were not observed on MH agar by the agar dilution method, these appeared on BHI agar in some strains. Previous studies have reported the following: (i) an MRSA strain with a *cfr* mutation with a linezolid MIC of 8 mg l^{-1} obtained by the agar dilution method showed a faint halo of growth with the Etest method and the MIC was difficult to read (Arias *et al.*, 2008); (ii) the MICs of linezolid by Etest in linezolid-susceptible clinical isolates appeared to be one to two dilution values lower than those obtained by the microdilution method (Tubau *et al.*, 2001); and (iii) the MIC reading by the disc diffusion and Etest methods in staphylococci needs to be improved (Tenover *et al.*, 2007). The results reported in these studies are consistent with the present results, although a solution for these difficulties remains to be determined.

Possible reasons for the underestimation of the linezolid MICs using the Etest method might be because: (i) the penetration of linezolid into the agar plates may be faster than the expected cell growth; or (ii) due to the ribosomal mutation, the growth rate of the resistant cells might be slower than that of the susceptible cells. In addition, it should be noted that use of the disc diffusion method on MH agar also might not be reliable for determining the linezolid susceptibility (data not shown). Therefore, the use of enriched media may be required.

In conclusion, the MIC of linezolid determined by Etest on MH agar and BHI agar in *S. aureus* appeared to be significantly lower compared with that determined by the agar and broth dilution methods. Consequently, a large fraction of the linezolid-resistant strains were falsely classified as susceptible. The use of blood agar and an incubation of 48 h was found to resolve this problem.

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