ANTIMICROBIAL RESISTANCE

Discrimination of methicillin-resistant *Staphylococcus aureus* from borderline-resistant and susceptible isolates by different methods

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Methicillin-resistant *Staphylococcus aureus* (MRSA) are important nosocomial pathogens. Diseases caused by these resistant bacteria frequently are serious and there is a need to control the spread of epidemic MRSA clones in hospitals. However, detection is complicated by the fact that expression of the resistance is variable and, commonly, heterogeneous within strains. The reliability of several tests recommended to discriminate heterogeneous MRSA isolates from borderline-resistant and susceptible strains was evaluated. Screening for growth on agar with methicillin 25 mg/L was the only method that detected all MRSA strains tested, but screening on agar with methicillin 10 mg/L or oxacillin 6 mg/L detected all but one of 10 heterogeneously resistant strains tested. None of the borderline-resistant nor any truly susceptible staphylococci tested grew on any of these screening plates.

Introduction

Methicillin was the first β -lactamase-stable β -lactam antibiotic described and overcame resistance caused by staphylococcal penicillinase. Subsequently, however, methicillin-resistant *Staphylococcus aureus* (MRSA) strains have emerged worldwide as major causes of hospital infections [1–4]. The determinant of methicillin resistance is the *mecA* gene, which encodes penicillin-binding protein 2a (2'), a component that is not present in methicillin-susceptible or borderline-resistant strains [5, 6]. This 78-kDa membrane protein has low affinity for β -lactam antibiotics [7]. Although *mecA* has been detected in all MRSA so far tested, there is enormous variation in the phenotypic expression of methicillin resistance, with many strains showing heterogeneous resistance [8]. Several auxiliary genes [9, 10] seem to be involved in determining the heterogeneous phenotype.

MRSA isolates can be divided into four phenotypic classes by population analysis profile [11]. These profiles are stable *in vitro* [11, 12] and *in vivo* [13]. For 'Class 1' heterogeneous strains the MICs for most cells (*c.* 99%) are low (1.5–3.0 mg/L) and only a small sub-population (10^{-8} – 10^{-6}) is highly resistant.

In 'Class 2' heterogeneous MRSA, the MIC values for the majority of cells are higher (6–25 mg/L) and the highly resistant sub-population comprises a larger fraction (10^{-5} – 10^{-4}) of the total. MICs for Class 3 strains typically exceed 50 mg/L and the proportion of highly resistant cells is increased to 10^{-3} – 10^{-1} . The least frequent class of MRSA – Class 4 – comprises strains with homogeneous resistance. MIC values for these organisms exceed 400 mg/L [12].

These complex phenotypes complicate the detection of resistance: in the Class 1 phenotype, methicillin MICs for most cells may be as low as 1.5 mg/L, compared to 0.5–1.0 mg/L for truly susceptible *S. aureus* [14]. MICs as high as those for the majority of cells in the class 1 phenotype are also seen for some 'borderline-resistant' *S. aureus* strains that lack the *mecA* gene [14, 15] and which may hyperproduce β -lactamase or have reduced penicillin affinity for both PBPs 1 and 2, coupled to overproduction of PBP 4 [15].

Earlier studies showed that MRSA Classes 1 and 2 could hardly be discriminated from borderline-resistant strains by disk diffusion tests [14]. Other studies [16–18] have compared different methods – including screening for growth on methicillin-containing agar – to detect MRSA strains, but did not consider the phenotypic variability among heterogeneous MRSA.

This study examined whether heterogeneous MRSA

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and borderline-resistant *S. aureus* strains could be discriminated from the truly susceptible strains by any recommended test.

Materials and methods

Isolates

Ninety-nine *Staphylococcus aureus* clinical isolates from our collection were used, and were from various hospitals in Brazil. *S. aureus* ATCC 25923 was used as the control organism in disk diffusion and broth macrodilution tests.

For preparation of stock cultures, clinical isolates from agar slants were plated on to trypticase soy agar (TSA) to confirm purity. One isolated colony was then grown to the stationary phase in trypticase soy broth (TSB) and stored at -70°C with sterile glycerol 10% v/v. *S. aureus* strains representing each class of methicillin heteroresistance were obtained from Dr A. Tomasz.

Methicillin resistance was confirmed with a DNA probe comprising a *Pst*I–*Xba*I internal fragment of the *mecA* gene cloned in pTZ219 [14].

Population analysis profile (PAP)

Overnight cultures (10^9 – 10^{10} cfu/ml) were plated at different dilutions on TSA containing serial two-fold dilutions of methicillin at concentrations of 0 and 0.75–800 mg/L. Colonies were counted after incubation at 37°C for 48 h [13].

Disk diffusion susceptibility tests

Disk diffusion tests were carried out as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) [19]. The bacterial inoculum, (5×10^7)–(9×10^7) cfu/ml, was plated on to Müller-Hinton agar (MHA), and disks containing oxacillin (Cecon, São Paulo, Brazil) 1 μg were applied. The plates were examined after incubation at 35°C for 24 h [19].

Broth macrodilution

Broth macrodilution susceptibility tests were performed in Müller-Hinton broth according to the NCCLS guidelines [20]. Oxacillin was tested at final concentrations from 0.25 to 32 mg/L and with NaCl 2% w/v added to the broth. Incubation was at 35°C for 24 h.

Oxacillin and methicillin screening agars

MHA was supplemented with oxacillin 6 mg/L or methicillin 10 mg/L and NaCl 4% w/v. The inoculum was prepared in two ways: either (i) as recommended by NCCLS guidelines [19], giving 10^7 cfu/plate or (ii)

as suggested by Kloos and Jorgensen, giving 10^4 cfu/plate [21]. The plates were incubated at 35°C for 24 h.

Methicillin agar screen

Fifty μl of the frozen stock culture were inoculated into 2 ml of TSB and incubated at 37°C in a shaking device that ensured vigorous aeration and a high bacterial concentration (c. 10^9 – 10^{10} cfu/ml). Portions of these cultures (100 μl) were then plated on to TSA plates containing methicillin 25 mg/L. Methicillin resistance was confirmed by surface growth after incubation for 24–48 h at 37°C [14].

Results

Population analysis profiles of MRSA isolates

By comparison with the controls the clinical *S. aureus* isolates were grouped into truly susceptible isolates, borderline-resistant and MRSA heterogeneous Classes 1, 2 or 3 (Fig. 1). Class 3 MRSA isolates were the most frequent phenotype (28%); as Classes 1 and 2 MRSA isolates represented 9% and 4% of the total, respectively; whereas borderline-resistant isolates (MIC 2–4 mg/L) comprised 44% and truly susceptible *S. aureus* (MIC ≤ 1 mg/L) 14% (Fig. 2). No homogeneous (Class 4) MRSA were isolated.

Detection of MRSA by recommended methods

Three (7%) of the MRSA isolates were not identified reliably by disk diffusion tests, giving zones larger than 10 mm, whereas 20% of borderline-resistant isolates gave zones ≤ 10 mm in diameter (Table 1). Only MRSA from Class 3 and some heterogeneous Class 2 isolates were reliably identified as resistant by disk diffusion tests. All Class 3 isolates grew to the disk edge without halo formation (Table 1). Inhibition zones for borderline-resistant isolates ranged from 19 mm to 8 mm, and a similar range of zone diameters (17–8 mm) was observed for Class 1 MRSA and some Class 2 isolates (Table 1). When broth macrodilution tests with NaCl 2% w/v were used, two of six Class 1 and 2 MRSA isolates were erroneously classified as susceptible, with MICs of 4 mg/L being observed.

Screening tests with agar containing methicillin 10 mg/L and oxacillin 6 mg/L have also been advocated to discriminate Class 1 and 2 MRSA from borderline isolates [19, 21]. However, one Class 1 MRSA failed to grow on these media when the inoculum was prepared as recommended by NCCLS [19] (i.e. with c. 10^7 – 10^6 cfu/plate) and only two Class 1 and 2 isolates grew when an inoculum of 10^4 cfu/plate was used, as suggested by Kloos and Jorgensen (Table 2) [21]. The screening plate with methicillin 25 mg/L was the only method that detected all MRSA isolates tested. None of the borderline-

Fig. 1. Population analysis profiles of representative strains. a, borderline-resistant strains; b, MRSA Class 1; c, MRSA Class 2; d, MRSA Class 3.

Fig. 2. Percentages of methicillin susceptible, borderline and resistant isolates among the *S. aureus* isolates studied.

resistant nor truly susceptible staphylococci tested grew on any of the screening media.

Discussion

Staphylococci are still the most common agents of hospital infections [1] and the emergence of MRSA has

led to increased use of expensive and potentially toxic antibiotics. Infections caused by MRSA are often serious, even life-threatening. Thus there is a need to invest in control measures to prevent transmission of MRSA in hospitals [22], and this demands reliable techniques to discriminate these organisms from other staphylococci. DNA probes for *mecA* allow accurate and rapid detection of MRSA [14, 16], but the methodology needs to be made less expensive and simpler if it is to be used routinely in clinical laboratories. Accordingly, simpler microbiological tests for the detection of MRSA were recommended by the NCCLS [19, 20] and were thought to be reliable [21]. In general, addition of NaCl [23], a cooler incubation temperature (30–35°C), neutral pH and prolonged (48 h) incubation favour detection of MRSA [21]. Several studies have examined the reliability of tests incorporating these factors for the detection of MRSA [16–18], but only one of these discriminated MRSA by class of resistance [14].

The aim of the present study was to investigate whether these tests could reliably discriminate heterogeneous MRSA. Both oxacillin 1-μg disks and MIC determinations by broth macrodilution failed to detect

Table 1. Behaviour of representative *S. aureus* clinical isolates with different classes of methicillin resistance

Isolate no.	Inhibition zone diameter (mm)*	Growth on methicillin 25 mg/L plate†	MIC (mg/L)‡	Class§
54	8 (R)	48	1.5–3	1
69	17 (S)	400	1.5–3	1
79	11 (R)	1000	1.5–3	1
95	8 (R)	108	1.5–3	1
97	13 (I)	60	1.5–3	1
96	9 (R)	62	1.5–3	1
56	None (R)	C	12–25	2
73	None (R)	C	12–25	2
92	8 (R)	C	12–25	2
99	15 (S)	300	12–25	2
47	None (R)	C	400	3
52	None (R)	C	400	3
55	None (R)	C	200	3
56	None (R)	C	200	3
58	None (R)	C	800	3
61	None (R)	C	800	3
49	12 (I)	None	2.0	B
50	15 (S)	None	2.0	B
60	19 (S)	None	2.0	B
62	12 (I)	None	2.0	B
63	13 (S)	None	2.0	B
77	15 (S)	None	2.0	B
12	10 (S)	None	2.0	B

*The disk diffusion test was according to the NCCLS protocol [19].

†See *Materials and methods*. Numbers indicate cfu; C, confluent growth on plates after incubation for 18 h at 37°C.

‡MIC values and grouping were based upon population analysis profile experiments (Fig. 1) [13].

§1, 2 and 3, class of methicillin resistance; B, borderline resistance not associated with *mecA*.**Table 2.** Comparison of different screening tests for the detection of MRSA

Isolate	Class	Growth on agar with methicillin 25 mg/L	Growth on screening plates with			
			methicillin 10 mg/L		oxacillin 6 mg/L	
			10 ⁴ *	10 ⁷ †	10 ⁴ *	10 ⁷ †
54	1	+	–	–	–	–
69	1	+	–	+	–	+
79	1	+	–	+	–	+
95	1	+	–	+	–	+
96	1	+	–	+	–	+
97	1	+	–	+	–	+
56	2	+	+	+	+	+
73	2	+	+	+	+	+
92	2	+	–	+	–	+
99	2	+	–	+	–	+
49	B	–	–	–	–	–
50	B	–	–	–	–	–
60	B	–	–	–	–	–
62	B	–	–	–	–	–
63	B	–	–	–	–	–
70	B	–	–	–	–	–
77	B	–	–	–	–	–

*Inoculum of 10⁴ cfu/plate, as recommended by Kloos and Jorgensen [21].†Inoculum of 10⁷ cfu/plate, as recommended by the NCCLS [19].

c. 30% of Class 1 and 2 MRSA tested. Moreover, the ranges of inhibition zone diameters of oxacillin 1- μ g disks for Class 1 and some Class 2 MRSA overlapped those for borderline-resistant strains, confirming previous data [14]. It is unclear why some *mecA* negative borderline-resistant *S. aureus* gave zones as small as those for MRSA in diffusion tests. Most of these organisms were β -lactamase positive and may have hyperproduced this enzyme, especially if it was

strongly induced by oxacillin, but this aspect was not investigated.

When broth macrodilution tests in the presence of NaCl 2% w/v were used, two of six Class 1 MRSA were not recognised. More reliable results were obtained with screening plates containing oxacillin 6 mg/L or methicillin 10 mg/L in the presence of NaCl 4% w/v if the NCCLS-recommended inoculum

of $c. 10^7$ cfu/ml was employed. With these systems, all but one Class 1 MRSA were detected; however, when a less dense inoculum (10^4 cfu) was employed, as recommended by Kloos and Jorgensen [21], only two of 10 Class 1 and 2 isolates were detected as MRSA. Methicillin 25 mg/L screening plates, employing a very dense inoculum (10^9 – 10^{10} cfu), allowed detection of all Class 1 and 2 MRSA. These results demonstrated that inoculum size is critical when screening agar tests are used and that excessively dilute inocula produce false-negative results.

It may seem surprising that the failure of MRSA detection tests to identify heterogeneous Class 1 and 2 isolates has not been remarked upon previously [14]. However, it is possible that few such isolates have been tested; at least in Brazil, recent clinical isolates with Class 1 or 2 behaviour seem to be sporadic [24].

In conclusion, the general reliability of methicillin and oxacillin screening plates for the detection of heterogeneous MRSA clinical isolates was confirmed, although failure of detection still appears to be a risk when testing Class 1 MRSA. For the longer term we recommend that accurate and rapid molecular tests should be developed, but until then, screening plates should be the methods of choice for recognition of MRSA in clinical laboratories.

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