Autobac Susceptibility Testing of Methicillin-Resistant *Staphylococcus aureus* Isolated in an Australian Hospital

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Semiautomated rapid broth elution (Autobac Multi-Test System; General Diagnostics, Div. Warner-Lambert Co., Morris Plains, N.J.) and disk diffusion tests were compared with an agar dilution breakpoint method to determine the antibiotic susceptibility of 147 methicillin-resistant *Staphylococcus aureus* isolates from our hospital. Although the disk diffusion method, in general, correlated well with the agar dilution tests, the overall agreement of the Autobac tests with agar dilution tests was only 79%, with many very major discrepancies occurring with clindamycin (88%), gentamicin (33%), and methicillin (15%). When we used a 10-fold higher inoculum for the Autobac tests, all isolates were shown to be resistant to methicillin, but significant numbers of major and minor discrepancies occurred with chloramphenicol, fusidic acid, and neomycin. The majority of isolates were shown to belong to three biotypes, distinguishable by lactose fermentation, lipolysis, hemolysis, and pigment production. The antibiotic susceptibility profile of one biotype was found to be markedly different from those of the other biotypes and contained a high incidence of clindamycin susceptibility and neomycin, gentamicin, and kanamycin resistance. In contrast, the other two biotypes had a high incidence of clindamycin, gentamicin, and kanamycin resistance and neomycin susceptibility and accounted for most of the very major discrepancies in the clindamycin and aminoglycoside tests. In these methicillin-resistant *S. aureus* strains, discrepancies possibly may arise from partial expression of methicillin resistance, dissociated or inducible clindamycin resistance, and instability of gentamicin resistance.

In 1961, methicillin-resistant *Staphylococcus aureus* was found in Britain, only 2 years after the introduction of methicillin to treat infections due to β -lactamase-producing staphylococci (21). Further methicillin-resistant *S. aureus* outbreaks were reported throughout Europe (5, 8), the United States (27), and Australia (28). From 1970 to 1980, methicillin-resistant *S. aureus* strains were a major cause of nosocomial infections in many countries (19, 22, 27, 29) and led to many severe infections (11, 12).

The management of severe staphylococcal infection depends on rapid determination of antibiotic susceptibility. Methicillin-resistant *S. aureus* is reportedly somewhat difficult to recognize in the laboratory, and is often identified retrospectively. Many strains of methicillin-resistant *S. aureus* show heterogeneous resistance to methicillin and require increased incubation times, reduced temperature (30°C), or increased osmolality for full expression of methicillin resistance (32). Although the Autobac Multi-Test System (MTS; General Diagnostics, Div. Warner-Lambert Co., Morris Plains, N.J.) can determine the antibiotic susceptibility of many important bacterial species within 3 to 5 h (33), several reports suggest that discrepant results occur with some methicillin-resistant *S. aureus* strains against antibiotics such as methicillin, gentamicin, clindamycin, and cephalothin (1, 9, 16).

Various methicillin-resistant *S. aureus* strains may be differentiated by their biochemical characteristics (19, 23), antibiotic susceptibility, plasmids (2, 10, 18, 25), and phage typing patterns (35). Vickery et al. (35) have described a biotyping scheme based on antibiotic susceptibility pattern, tellurite production, lipolysis, lactose fermentation, and phage typing using both the basic international set, and new experimental phages. J. Andrew, P. Carson, V. Moritz, and

D. Olden (personal communication) developed a biotyping system based on lactose fermentation, lipolysis, hemolysis, and pigment production combined with susceptibility to chloramphenicol, gentamicin, methicillin, fusidic acid, neomycin, and clindamycin.

In 1981, an outbreak of nosocomial infections due to methicillin-resistant *S. aureus* occurred at The Queen Elizabeth Hospital (20). In this study we set out to (1) investigate whether Autobac could be used to test the antibiotic susceptibility of methicillin-resistant *S. aureus* isolates; (2) detect any discrepancies between Autobac MTS, disk diffusion, and agar dilution tests; and (3) determine whether any discrepant results correlate to particular biotypes present in our methicillin-resistant *S. aureus* population.

MATERIALS AND METHODS

Organisms. All strains used in this study were clinical isolates from 147 patients at The Queen Elizabeth Hospital and were confirmed as methicillin-resistant *S. aureus* organisms by Gram stain, catalase, DNase, and disk diffusion susceptibility tests (4). They were stored at ambient temperature in maintenance medium described by Crowder (13).

Susceptibility tests. All susceptibility tests were carried out on fresh overnight cultures obtained by streaking stock cultures on Columbia agar containing 5% defibrinated horse blood. Four to five morphologically similar colonies were suspended in 0.1 M phosphate-buffered saline (pH 7.2). Turbidity of each suspension was adjusted in the Autobac nephelometer to ca. 10^8 CFU/ml. When diluted 10-fold in 0.1 M phosphate-buffered saline, the turbidity of the suspension was equivalent to that of standardized inoculum (ca. 10^7 CFU/ml). Both inocula were used for Autobac tests, while the latter was used for both agar dilution and disk diffusion tests.

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TABLE 1. Percentage antibiotic resistance of 147 methicillin-resistant *S. aureus* isolates tested by agar dilution, disk diffusion, and Autobac methods

Antimicrobial agent	% Antibiotic resistance by the following methods:					
	Agar dilution	Disk diffusion	Autobac (AIS)		Autobac (AISH)	
			Resistance	Intermediate resistance	Resistance	Intermediate resistance
Chloramphenicol	0.7	0.7	0.7	0.0	52.4	33.3
Clindamycin	89.1	72.8	0.7	0.0	61.2	20.4
Erythromycin	NT ^a	100.0	85.7	4.8	99.3	0.7
Fusidic acid	0.7	0.7	0.0	0.0	0.7	26.5
Gentamicin	75.5	74.8	34.0	8.2	74.1	12.2
Kanamycin	76.2	76.2	70.8	3.4	80.3	8.2
Methicillin	100.0	100.0	79.6	6.1	100.0	0.0
Neomycin	15.6	14.9	4.1	2.0	21.1	36.1
Penicillin	NT	100.0	100.0	0.0	100.0	0.0
Tetracycline	NT	100.0	97.3	2.0	100.0	0.0
Vancomycin	0.0	0.0	0.0	0.0	0.0	2.7

^a NT, Not tested.

An agar dilution breakpoint test modified from the method described previously by Ericsson and Sherris (15) was performed on Iso-Sensitest agar (Oxoid Ltd., London, England) with the following antibiotics at the indicated final concentrations: chloramphenicol (8 µg/ml; Sigma Chemical Co., St. Louis, Mo.); clindamycin (8 µg/ml; The Upjohn Co., Kalamazoo, Mich.); fusidic acid (1 µg/ml; Leo Pharmaceuticals); gentamicin (4 µg/ml; Schering Corp., Bloomfield, N.J.); kanamycin (4 µg/ml; Bristol Laboratories, Syracuse, N.Y.); methicillin (8 µg/ml; Beecham, Australia); and vancomycin (4 µg/ml; Eli Lilly & Co., Indianapolis, Ind.). A Steers replicator (30) delivering ca. 5×10^4 CFU was used to inoculate the antibiotic plates.

Disk diffusion tests were carried out by a modified Kirby-Bauer method (4) on Iso-Sensitest agar. After preliminary experiments with methicillin-resistant *S. aureus* and those antibiotics used in this report, results with Iso-Sensitest agar were comparable to those with Mueller-Hinton agar (unpublished data) such that interpretation of the zone sizes on Iso-Sensitest agar followed the recommendations of the National Committee for Clinical Laboratory Standards (26).

Autobac Interpretive Susceptibility (AIS) tests were performed in sterile eugonic broth (Pfizer Inc., New York, N.Y.) according to the manufacturer's instructions with two inocula for each strain. All antibiotic elution disks were obtained from Pfizer except for fusidic acid (30 µg; Oxoid).

All susceptibility tests were incubated at 35°C. During this study, it was found to be unnecessary to incubate methicillin susceptibility tests at 30°C because all isolates were resistant to methicillin at 35°C.

Biotyping. A biotyping scheme developed by J. Andrew, P. Carson, V. Moritz, and D. Olden was used in this study (personal communication). The biochemical characteristics selected for this biotyping scheme were lactose fermentation on MacConkey agar (Oxoid), lipolysis on lactose-egg yolk-milk agar, hemolysis on Iso-Sensitest agar containing 7% defibrinated sheep blood cells, β-hemolysin production on a sheep blood agar lawn inoculated previously with *Streptococcus agalactiae*, and pigmentation on homogenized milk agar. The biotyping plates were also inoculated with a Steers replicator with the same inocula source as that used in the agar dilution tests. The plates were incubated for 18 h at 35°C.

A two-digit numerical code system was used to score the biochemical reactions: lactose fermentation, 4; lipolysis, 2;

hemolysis, 1; β-hemolysin, 4; gold pigment, 2; cream pigment, 1; white pigment, 0.

RESULTS

Susceptibility testing of methicillin-resistant *S. aureus* isolates. The antibiotic susceptibility of 147 methicillin-resistant *S. aureus* isolates was determined by agar dilution, disk diffusion, and Autobac (with two inocula levels) methods and is shown in Table 1.

Agar dilution methods. All isolates were found to be resistant to methicillin and susceptible to vancomycin; most isolates were resistant to clindamycin (89.1%), gentamicin (75.5%), and kanamycin (76.2%); and a few isolates were resistant to neomycin (15.6%), chloramphenicol (0.7%), and fusidic acid (0.7%). Susceptibility to penicillin, tetracycline, and erythromycin was not tested by this method.

Disk diffusion method. The disk diffusion method showed that all methicillin-resistant *S. aureus* isolates were reproducibly resistant to penicillin, ampicillin, tetracycline, erythromycin, and methicillin. All methicillin-resistant *S. aureus* isolates produced only small zones of inhibition (<10 mm in diameter) around the methicillin disk at 35°C, and hence it was unnecessary to incubate these tests at 30°C to obtain full expression of methicillin resistance. Unlike clindamycin-sensitive methicillin-resistant *S. aureus*, which produced a completely clear zone (>20 mm in diameter), clindamycin-resistant methicillin-resistant *S. aureus* isolates produced light to medium background growth around the clindamycin disk, with a halo of complete inhibition at the periphery of the zone. Gentamicin-resistant isolates often produced small colonies which grew within zones (usually <16 mm in diameter). When subcultured, these gentamicin-resistant colonies demonstrated the same behavior. Thus, the innermost colonies were used to calculate the gentamicin zone of inhibition. The gentamicin-susceptible methicillin-resistant *S. aureus* isolates usually produced >25-mm-diameter zones. Clear zones of inhibition were seen in isolates sensitive to all other tested antibiotics.

Autobac tests. Using the lower standardized inocula (AIS), all isolates were resistant to penicillin and susceptible to fusidic acid and vancomycin. However, only 85.7% of isolates were found to be methicillin resistant. Most isolates were also resistant to tetracycline (99.3%), erythromycin (90.5%), and kanamycin (74.2%); some strains were resistant

TABLE 2. Summary of study with disk diffusion, Autobac, and agar dilution tests^a

Antimicrobial agents	% Discrepancies by ^b :							
	Disk diffusion		Autobac					
			Very major		Major		Minor ^c	
	Very major ^c	Major ^d	AIS	AISH	AIS	AISH	AIS	AISH
Chloramphenicol	0.0	0.0	0.0	0.0	0.7	51.7	0.0	33.3
Clindamycin	16.3	0.0	88.4	12.9	0.0	0.0	0.0	20.4
Fusidic acid	0.0	0.0	0.7	0.7	0.0	0.7	0.0	26.5
Gentamicin	0.7	0.0	33.3	1.4	0.0	2.7	8.2	12.2
Kanamycin	0.0	0.0	2.7	0.0	0.7	4.8	3.4	8.2
Methicillin	0.0	0.0	14.3	0.0	0.0	0.0	6.1	0.0
Neomycin	0.7	0.0	10.2	0.7	0.7	6.1	2.0	36.1
Vancomycin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.7

^a A total of 147 methicillin-resistant *S. aureus* isolates were studied.^b Percent average: Disk diffusion, very major, 2.2; major, 0.0. Autobac (AIS), very major, 18.7; major, 0.3; minor, 2. Autobac (AISH), very major, 2.0; major, 8.25; minor, 17.^c Very major, susceptible by disk diffusion or Autobac; resistant by agar dilution.^d Major, resistant by disk diffusion of Autobac; susceptible by agar dilution.^e Minor, intermediately resistant by Autobac.

to gentamicin (42.2%), neomycin (6.1%), clindamycin (0.7%), and chloramphenicol (0.7%).

In contrast, by using the 10-fold-higher standardized inocula (AISH) for the Autobac tests, all isolates were found to be resistant to methicillin, tetracycline, and erythromycin. Most isolates were resistant to clindamycin (81.6%), kanamycin (88.5%), gentamicin (86.3%), chloramphenicol (85.7%), and neomycin (57.2%). Intermediate resistance was detected against fusidic acid (26.5%) and vancomycin (2.7%).

Comparison of susceptibility tests. When disk diffusion and agar dilution tests were compared, the overall agreement was 97.8%, ranging from 83.7 to 100%, with over 90% agreement for chloramphenicol, fusidic acid, gentamicin, kanamycin, methicillin, neomycin, and vancomycin (Table 2). Very major discrepancies (i.e., resistance by agar dilution, susceptibility by disk diffusion) were seen in 16.3% of isolates tested against clindamycin.

When Autobac tests using the lower standardized inocula (AIS) and agar dilution tests were compared, the overall agreement was 78.6%, with a range of 11.6 to 100% and over 90% agreement for chloramphenicol, fusidic acid, kanamycin, and vancomycin (Table 2). Very major discrepancies occurred with clindamycin (88.4%), gentamicin (33.3%), and methicillin (14.3%).

When the Autobac tests using the high inocula (AISH) and agar dilution tests were compared, the overall agreement was reduced to 72.4%, ranging from 15 to 100%, with over 90% agreement for only methicillin and vancomycin (Table 2). In contrast to the Autobac AIS tests, most discrepancies were of the minor type (17.4%), involving fusidic acid (26.5%), neomycin (36.1%), and chloramphenicol (33.3%).

Several major discrepancies also occurred with chloramphenicol (51.7%).

Biotyping. Three major biotypes were found in the isolates used in this study. First, 67% of the isolates belonged to biotype 72, which was characterized by the fermentation of lactose and the production of β -hemolysis (sheep blood agar), lipolysis (lactose-egg yolk-milk agar), and gold pigment (milk agar). Second, 18% of all isolates belonged to biotype 41/51, which characteristically were nonlipolytic, fermented lactose, produced cream pigment, and may also produce β -hemolysin. Third, 12% of all isolates belonged to biotype 35 and produced β -hemolysin, lipolysis, and cream pigment but did not ferment lactose. The remaining isolates were considered as a single biotype for convenience.

While antibiotic susceptibility patterns of isolates varied within each biotype, only biotype 35 isolates showed markedly different overall susceptibility patterns, particularly for clindamycin and the aminoglycosides (Table 3). Most biotype 35 isolates were sensitive to clindamycin, while all other isolates were clindamycin resistant by agar dilution tests. Furthermore, all the biotype 35 isolates were resistant to neomycin, gentamicin, and kanamycin by agar dilution tests, and in disk diffusion tests they did not produce zones around the gentamicin, kanamycin, and neomycin disks in contrast to other gentamicin-resistant, neomycin-sensitive isolates which produced zones of inhibition of <16 mm in diameter, with isolated single colonies growing within zones of gentamicin (unpublished data).

Biotype-specific discrepancies between Autobac and agar dilution tests. The discrepancies between Autobac and agar dilution tests were analyzed to determine whether any

TABLE 3. Percentage of antibiotic resistance within each methicillin-resistant *S. aureus* biotype

Biotype	No. of strains	% Resistance of the following antibiotics:						
		Chloramphenicol	Clindamycin	Fusidic acid	Gentamicin	Kanamycin	Methicillin	Neomycin
72	98	0	100	1	76	77	100	3
41/51	26	3	100	0	62	62	100	0
35	17	0	6	0	100	100	100	100
Other	6	0	100	0	67	67	100	0
All strains	147	0.7	89.1	0.7	75.5	76.2	100	15.6

TABLE 4. Susceptibility of different biotypes in 147 methicillin-resistant *S. aureus* isolates by Autobac and agar dilution tests

Biotype	No. of strains	% Discrepancies with the following antibiotics ^a :														
		Methicillin			Clindamycin						Gentamicin					
		Very major	Major	Minor	Very major		Major		Minor		Very major		Major		Minor	
		AIS	AIS	AIS	AIS	AISH	AIS	AISH	AIS	AISH	AIS	AISH	AIS	AISH	AIS	AISH
72	98	16	0	2	100	16	0	0	0	13	37	2	0	2	11	16
41/51	26	19	0	15	96	8	0	0	0	23	39	0	0	4	4	8
35	17	0	0	12	6	0	0	0	0	53	0	0	0	0	0	0
Others	6	0	0	17	100	17	0	0	0	33	50	0	0	0	0	0
All strains	147	14.2	0	6.1	88.4	12.9	0	0	0	20.4	33.3	1.4	0	2.7	8.2	12.2

^a For explanation of very major, major, and minor, see footnotes c, d, and e in Table 2.

biotype-specific patterns emerged. Only methicillin, clindamycin, and gentamicin tests were considered.

When Autobac AIS and agar dilution tests for methicillin were compared, the overall agreement was 80%, with a range of 65 to 88% for the different types (Table 4). Autobac did not detect methicillin resistance in 16% of biotype 72 and 19% of biotype 41/51 isolates. Minor discrepancies occurred in 12 to 17% of isolates (excluding biotype 72). Major discrepancies were not seen. The Autobac AISH tests detected methicillin resistance in all isolates.

When Autobac AIS and agar dilution tests for clindamycin were compared, the overall agreement was 11.6%, ranging from 0 to 94.1% for the different biotypes, with over 90% agreement for biotype 35 isolates (Table 4). The agar dilution tests showed that 89% of all isolates were resistant to clindamycin, yet the Autobac AIS tests detected clindamycin resistance in only one isolate. The good agreement between Autobac and agar dilution tests for biotype 35 isolates correlated with their low incidence of clindamycin resistance. Excluding biotype 35 isolates, 99% of the very major discrepancies were observed in the methicillin-resistant *S. aureus* isolates for clindamycin.

However, when Autobac AISH and agar dilution tests for clindamycin were compared, the overall agreement increased to 66.7% for all isolates (Table 4). Very major discrepancies decreased to 13%, while minor discrepancies increased to 20% of all isolates. By using the high inocula in these Autobac tests, 53% of biotype 35 isolates (clindamycin sensitive) were interpreted as having intermediate resistance, yet clindamycin resistance was still not detected in 8 to 17% of isolates from other biotypes.

When the Autobac AIS and agar dilution tests for gentamicin were compared, the overall agreement was 58.5%, ranging from 50 to 100% for the different biotypes (Table 4). All biotype 35 isolates were resistant to gentamicin, neomycin, and kanamycin by both test methods. In the remaining isolates, very major (38%) and minor (9%) discrepancies occurred overall in each biotype. In contrast, when the higher inoculum was used in the Autobac tests, the overall agreement between Autobac and agar dilution tests for gentamicin increased to 84%, and a few minor discrepancies were observed in biotypes 72 and 41/51 (Table 4).

DISCUSSION

The need for rapid and accurate detection of methicillin-resistant organisms is becoming more important as the incidence of these organisms increases in clinical laboratories. Several problems have been reported in the literature concerning the ability of standard methods (3, 14, 32) and automated systems (1, 6, 7, 9, 16, 31, 33) to consistently

detect methicillin-resistant *S. aureus* strains. We undertook this comparative study to evaluate the Autobac's ability to detect methicillin resistance in the methicillin-resistant *S. aureus* isolates endemic in our hospital and to determine whether any discrepant results occurred within the specific methicillin-resistant *S. aureus* biotypes.

Three major biotypes were found in these methicillin-resistant *S. aureus* isolates, demonstrating that a number of different strains of methicillin-resistant *S. aureus* had spread throughout our hospital. The predominant biotype 72 strains, which account for 67% of all strains, differed from the other strains by pigment and either lactose fermentation (biotype 35) or lipolysis (biotype 41/51). Biotype 35 strains, in contrast to the other strains, were largely susceptible to clindamycin and resistant to neomycin, gentamicin, and kanamycin. Within the other biotypes 60 to 75% of isolates were resistant to gentamicin and kanamycin, while only 2% were resistant to neomycin.

Autobac AIS produced reliable results with chloramphenicol, fusidic acid, kanamycin, penicillin, tetracycline, and vancomycin but failed to detect resistance to clindamycin (88%), erythromycin (14%), methicillin (14%), and neomycin (10%). When the higher inocula were used in the Autobac test, erythromycin and methicillin tests were in agreement with conventional methods, but many tests, including those with chloramphenicol, clindamycin, fusidic acid, gentamicin, kanamycin, and neomycin, produced many discrepancies in comparison with conventional methods.

In earlier reports (1, 9, 16, 33), Autobac produced only 20 to 75% agreement with conventional tests for methicillin, compared with the 80% agreement reported here. Methicillin resistance in *S. aureus* has been characterized by heterogeneity, in which only a few cells are resistant to methicillin and grow better at 30°C or in higher concentrations of salt (32). However, all isolates in this study showed high resistance to methicillin, being fully expressed at 35°C, and therefore Autobac seemed to perform better by detecting 86% of methicillin-resistant strains in this study. Although discrepancies were more common in biotype 72 and 41/51 strains, the reasons remain unclear.

Two classes of gentamicin resistance have been reported in Australian methicillin-resistant *S. aureus* strains (34), and this became apparent in the isolates used in this study. Biotype 35 isolates were highly resistant to neomycin, gentamicin, and kanamycin, and consequently the Autobac and conventional methods agreed when these isolates were tested with all three antibiotics. Autobac failed to detect gentamicin resistance in 58% of the remaining gentamicin-resistant isolates. A representative, neomycin-susceptible, gentamicin-resistant strain was shown by Lyon et al. (24) to

contain the pSK3 plasmid which carries gentamicin resistance genes. These isolates demonstrate low-level resistance to gentamicin, perhaps in part due to the relative instability of the resistance plasmid (unpublished data). Hence, Autobac probably failed to detect gentamicin resistance due to the combination of the instability of the gentamicin resistance plasmid and the low level of resistance that these plasmids encode. However, these gentamicin-resistant isolates were detected by the Autobac tests when the inocula were increased 10-fold. Alternatively, kanamycin susceptibility remains a good indicator of gentamicin susceptibility in our isolates.

The clindamycin-resistant methicillin-resistant *S. aureus* isolates grew well on plates containing 8 µg of clindamycin per ml, produced background growth around clindamycin disks in the disk diffusion tests, and showed antagonism between erythromycin and clindamycin disks (unpublished data). Garrod (17) first described dissociated resistance, whereby erythromycin-resistant staphylococci were susceptible to related antibiotics, such as clindamycin, yet became resistant to clindamycin in the presence of erythromycin. Erythromycin is the specific inducer of this type of resistance. Observations from this study suggest that the methicillin-resistant *S. aureus* isolates possess inducible resistance to clindamycin and that Autobac fails to detect such inducible resistance. The biotypes did not appear to affect the performance of Autobac in tests with clindamycin. Rather, the good agreement found in biotype 35 strains was due to the low incidence of clindamycin resistance in these isolates.

Autobac fails to detect resistance to methicillin, gentamicin, and clindamycin in methicillin-resistant *S. aureus* isolates, probably due to test conditions that might not fully express methicillin resistance, the relative instability of plasmids encoding low-level resistance to gentamicin, and the inducible resistance to clindamycin.

Currently, in our laboratory methicillin-resistant *S. aureus* isolates are detected by the Autobac tests by virtue of the multiple resistance patterns within our hospital and are confirmed by biotyping and agar dilution tests.

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