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Detection of Oxacillin-Resistant Staphylococci by the AutoMicrobic System

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The AutoMicrobic system (Vitek Systems, Inc., Hazelwood, Mo.) is an automated instrument designed for rapid microbiological identification and susceptibility reporting in the clinical laboratory. The reliability of a rapid, automated approach to testing methicillin-resistant staphylococci was evaluated. To determine the accuracy in detecting oxacillin-methicillin resistance by the AutoMicrobic system, 746 staphylococci from seven different geographical areas were tested. Results were compared with the Bauer-Kirby agar disk diffusion technique as the reference method. Of the 304 staphylococci, 209 coagulase-positive and 95 coagulase-negative strains were resistant to oxacillin-methicillin. These organisms fell into three categories of resistance detection. The first category had resistance levels high enough for initial detection, the second category had low resistance levels requiring modified data analysis techniques for detection, and the third category had resistance levels too low for detection. Of the resistant strains tested, 21% showed a category two resistant growth pattern. Major errors, as a result of hetero-resistant growth patterns of the tested strains, were resolved by computer analysis of growth curves. These data analysis applications enabled detection of 96% of the oxacillin-methicillin-resistant organisms. Results for all resistant staphylococci tested were available in an average time of 5.5 h.

The appearance of methicillin-resistant strains of *Staphylococcus aureus* has been increasing since the late 1970s. Recent reports indicate that the methicillin-resistant (MR) *S. aureus* strains have caused nosocomial outbreaks in many U.S. hospitals and have become endemic in other hospitals (10). The resistance mechanism in *S. aureus* is not fully understood at this time; however, it is intrinsic, and within the parent population, there is a low frequency of mutants which are significantly more resistant to methicillin (hetero-resistance) (11). In the past, doubts have been raised as to the reliability of standard methods to accurately detect MR *S. aureus* isolates (1, 4, 5). Several factors influence and enhance the in vitro detection of methicillin resistance. These factors are increased salt concentration, increased inoculum size, increased incubation time, and decreased incubation temperature. These factors have also contributed to the problems automated systems have had with rapid, accurate detection of methicillin-resistant staphylococci. The shorter incubation period of automated systems is an area of particular concern since the methicillin hetero-resistant population of these strains has a much slower rate of growth (3, 6, 7).

Recent reports concerning the reliability of automated instrumentation to detect MR *S. aureus* organisms have raised questions as to the capability of the AutoMicrobic System (AMS; Vitek Systems, Inc., Hazelwood, Mo.) gram-positive susceptibility test kit to detect these strains. A study was therefore conducted with several hundred oxacillin-methicillin-resistant staphylococci from seven different U.S. geographical areas to determine whether the AMS would have similar difficulties in detecting MR *S. aureus* hetero-resistance and correct any weaknesses observed in the system. The AMS results were compared with the disk diffusion method as the standard method.

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MATERIALS AND METHODS

Organisms. Strains of staphylococci were collected from seven different geographical areas in the United States (Table 1). All *S. aureus* isolates were from clinical sources. A number of coagulase-negative staphylococci were obtained from W. E. Kloos, North Carolina State University, Raleigh, N.C. Organisms were confirmed as *S. aureus* by their ability to coagulate rabbit plasma. Coagulase-negative strains were identified by the Kloos and Smith scheme of identifica-

TABLE 1. Categories of resistance patterns for staphylococci tested by geographical site

Organisms	Boston, Mass.				Chapel Hill, N.C.				Chicago, Ill.			
	No. of strains tested	Category			No. of strains tested	Category			No. of strains tested	Category		
		1[a]	2[b]	3[c]		1[a]	2[b]	3[c]		1[a]	2[b]	3[c]
S. aureus	44	9	1	1	19	13	5	1	122	3	2	0
Coagulase-negative staphylococci	34	26	7	1	1	1	0	0	126	24	0	0
Staphylococcus saprophyticus	2	2	0	0	10[d]	0	1	0	9	0	0	0
Staphylococcus hominis					16[d]	2	2	0				
S. cohnii					10[d]	1	1	1	1	0	0	0
S. simulans					8[d]	0	0	0				
Staphylococcus haemolyticus					5[d]	1	0	1				
S. warneri					11[d]	0	0	0				

[a] Resistance levels high enough for initial detection.
[b] Low resistance levels requiring modified analysis for detection.
[c] Low resistance levels not detected.
[d] Organisms from collection of W. E. Kloos.

tion (9). All strains received were maintained at −70°C in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) with 20% glycerol.

Gram-Positive Susceptibility Card. The AMS Gram-Positive Susceptibility Card (GPS) incorporates 11 antimicrobial agents and β-lactamase detection for testing group B and group D streptococci and staphylococci (Fig. 1). The modified Mueller-Hinton broth in the GPS contains the following (in grams per liter): Mueller-Hinton (Difco Laboratories, Detroit, Mich.), 21.0 g; Dextrose (Sigma Chemical Co., St. Louis, Mo.), 3.0 g; yeast extract (Difco), 3.0 g; total calcium ($CaCl_2$, anhydrous; Sigma), 0.06 g; total magnesium ($MgCl_2 \cdot 6H_2O$; Sigma), 0.03 g; XV factor (Difco), 0.7 g; 0.4% Aniline Blue (Manufacturing Chemists, Norwood, Ohio), 40.0 ml. The final pH was 7.2 ± 0.5 after drying and rehydration.

Susceptibility testing methods. The AMS testing was performed according to the GPS test kit package insert. Organisms 18 to 24 h old were taken from blood agar plates and suspended in sterile 0.45% saline to a turbidity equal to a McFarland standard of 0.5. Of this suspension, 200 μl was then diluted with 1.8 ml of 0.45% sterile saline and inoculated into the test kit. Resistance or susceptibility was determined by the amount of growth achieved in the 2-μg/ml oxacillin well.

The Bauer-Kirby disk diffusion procedure was used as the reference method (2). Mueller-Hinton agar plates (diameter, 100 mm) supplemented with 5% sheep blood (Remel, Lenena, Kans.) were inoculated and incubated at 35°C. Zones of inhibition for a 5-μg methicillin disk and a 1-μg oxacillin disk (Baltimore Biological Laboratories, Baltimore, Md.) were recorded after 18 to 24 h of incubation. Organisms were considered methicillin resistant when the zone of inhibition was ≤9 mm in diameter for methicillin and ≤10 mm in diameter for oxacillin. Susceptible strains gave zones of inhibition ≥14 mm in diameter for methicillin and ≥13 mm in diameter for oxacillin. When major discrepancies occurred between the AMS and reference method, The Bauer-Kirby plate was incubated at 30°C for an additional 18 to 24 h.

AMS data analysis. The AMS contains diode emitters which emit light through each of the growth wells in the test kit at a wavelength of 665 nm. Growth wells were scanned every hour, the data were collected by sensors, and then the data were stored in the computer. The sensors detected increases in turbidity and color change due to acid production from glucose. The first recorded reading was considered to be the base line from which any decrease in optical density was detected as percent change. Once growth in the positive control (base broth without antimicrobial agents) achieved a preset threshold of 30%, determination of oxacillin resistance was calculated. An organism was determined resistant when the percent change in the oxacillin well was equal to 65% of the recorded value for the positive control well; otherwise it was considered to be susceptible. The susceptibility results were printed automatically by the AMS printer.

RESULTS

Initial test results demonstrated several different MR *S. aureus* growth patterns of resistance. In analyzing the optical density values from the instrument, we observed that different growth patterns appeared, depending on the hetero-resistant frequency within a given strain population. These patterns fell into three categories. Category one had a pattern of resistance levels high enough for initial detection by the AMS. Category two had low resistance levels requiring modified analysis for detection. Category two strains also showed increased levels of resistance when successively transferred onto blood agar plates. Strains with category three patterns had resistance levels too low for detection by the system and showed no increased levels of resistance when successively transferred onto blood agar plates. Of the 307 resistant staphylococci tested, 232 (75%) could be described in a category one resistance pattern (Table 1). The growth characteristics for these strains showed detectable hetero-resistance expression in their cell population. From the remaining 75 strains, 64

TABLE 1—*Continued*

St. Louis, Mo.				Houston, Tex.				Los Angeles, Calif.				San Francisco, Calif.			
No. of strains tested	Category			No. of strains tested	Category			No. of strains tested	Category			No. of strains tested	Category		
	1[a]	2[b]	3[c]		1[a]	2[b]	3[c]		1[a]	2[b]	3[c]		1[a]	2[b]	3[c]
81	9	0	2	104	75	28	1	45	34	11	0	17	11	6	0
78	21	0	3												

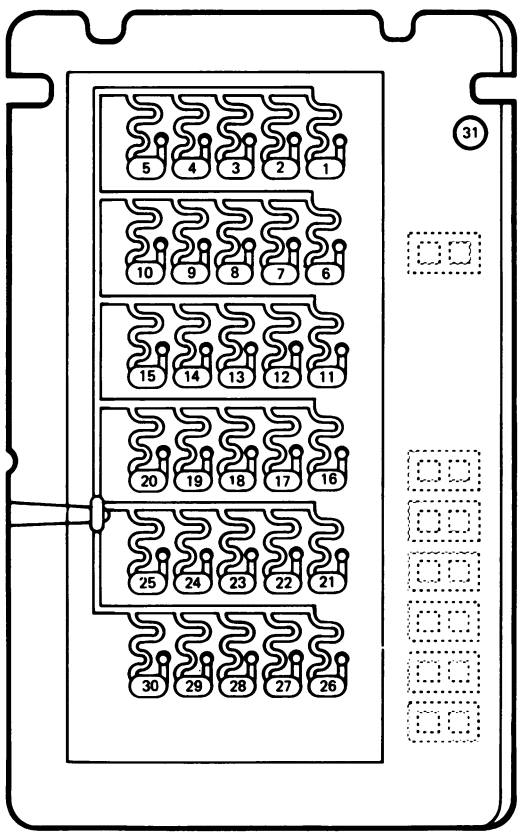

FIG. 1. Gram-Positive Susceptibility Card antimicrobial locations and concentrations (μg/ml): 1, positive control; 2, ampicillin, 0.25; 3, penicillin G, 0.03; 4, vancomycin, 1; 5, erythromycin, 0.5; 6, gentamicin, 0.5; 7, ampicillin, 8; 8, penicillin G, 8; 9, vancomycin, 8; 10, erythromycin, 4; 11, gentamicin, 4; 12, ampicillin, 32; 13, penicillin G, 128; 14, vancomycin, 64; 15, erythromycin, 32; 16, gentamicin, 16; 17, tetracycline, 1; 18, cephalothin, 2; 19, clindamycin, 0.5; 20, chloramphenicol, 1; 21, oxacillin, 2; 22, tetracycline, 8; 23, cephalothin, 16; 24, clindamycin, 4; 25, chloramphenicol, 8; 26, nitrofurantoin, 128; 27, tetracycline, 64; 28, cephalothin, 128; 29, clindamycin, 32; 30, β-lactamase; 31, catalase reaction code.

(21%) had category two and 11 (4%) had category three resistance patterns, and the frequency of strains showing these two categories of resistance varied, depending upon the geographical areas of isolation. As can be seen, the coagulase-negative staphylococci also expressed all three category patterns of hetero-resistance. The only species not illustrating these patterns from iso-

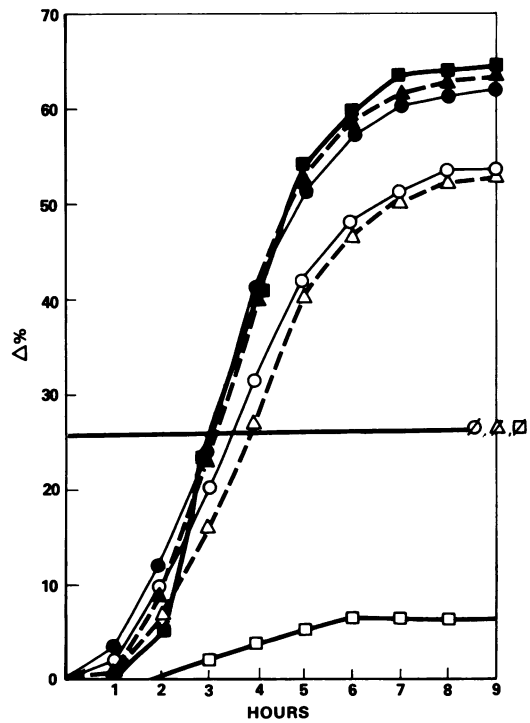

FIG. 2. MR *S. aureus* strains 1022 (-----), 1020 (-----), and 722 (——) which were tested in the GPS comparing growth patterns in the positive control with the 2-μg/ml oxacillin well. For the strains to be resistant, the oxacillin well (○, △, □) must achieve a threshold (Ø, Δ, Ø) when the positive control (●, ▲, ■) is ≥30%.

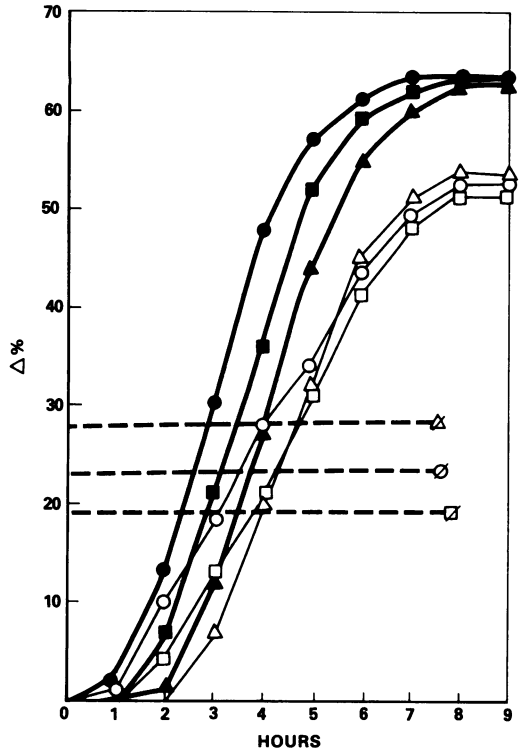

FIG. 3. MR *S. aureus* strain 1021 tested in the GPS comparing growth patterns in the positive control well (——) with the 2-μg/ml oxacillin well (-----) after successive blood agar plate transfers (transfer 1, ●, ○; transfer 2, ▲, △; transfer 3, ■, □). Thresholds (-----) are given which strain 1021 must achieve in the oxacillin well to be resistant for transfer 1, ∅; transfer 2, ⍲; and transfer 3, ⍁, when the positive control is ≥30Δ%.

lated strains tested were *Staphylococcus simulans* and *Staphylococcus warneri*. Figure 2 compares results of two different MR *S. aureus* strains with a category one resistance to the results of strain 722, which is susceptible to oxacillin. Resistance in these two strains was detected by the AMS in 4 h. In strains which expressed a category two pattern, oxacillin resistance could not be detected at the time the positive control achieved sufficient growth for susceptibility determination (≥30% change). Figure 3 illustrates MR *S. aureus* strain 1021, which has this category two pattern. After this strain was subcultured successively, however, a growth rate increase was seen in the oxacillin well with the second and third transfer with respect to the positive control. Strains with this pattern also had a tendency to have different growth characteristics in the positive control well, reflecting different resistant thresholds for each transfer. Figure 4 represents MR *S. aureus* strain 1025 with growth patterns which show a category three resistance pattern. Even when subcultured through a series of successive transfers, the oxacillin resistance could not be detected when the positive control was ≥30%. Growth patterns from three different coagulase-negative staphylococci are presented in Fig. 5. Strain 2296 represents a category one oxacillin-resistant organism, whereas strain 2589 has a similar hetero-resistant pattern exhibited by some category two MR *S. aureus* organisms. Strain 2587 demonstrates the growth pattern of an oxacillin-susceptible organism. What emerged from the data were varying patterns of hetero-resistance. The levels were such that the AMS was unable to detect sufficient growth for a resistance determination to be made at the time results were reported. A modification of the analysis technique in the software enabled the recognition of the hetero-resistant growth pattern of MR *S. aureus* strains. When the percent change recorded in the 2-μg/ml oxacillin well is <10% and the

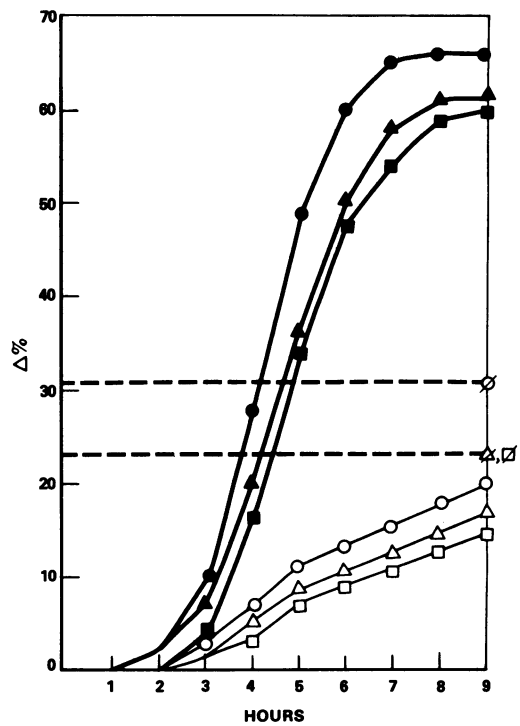

FIG. 4. MR *S. aureus* strain 1025 tested in the GPS comparing growth patterns in the positive control well (——) with the 2-μg/ml oxacillin well (-----) after successive blood agar plate transfers (transfer 1, ●, ○; transfer 2, ▲, △; transfer 3, ■, □). Thresholds (-----) are given which strain 1025 must achieve in the oxacillin well to be resistant for transfer 1, ∅; transfer 2, ⍲; and transfer 3, ⍁, when the positive control is ≥30Δ%.

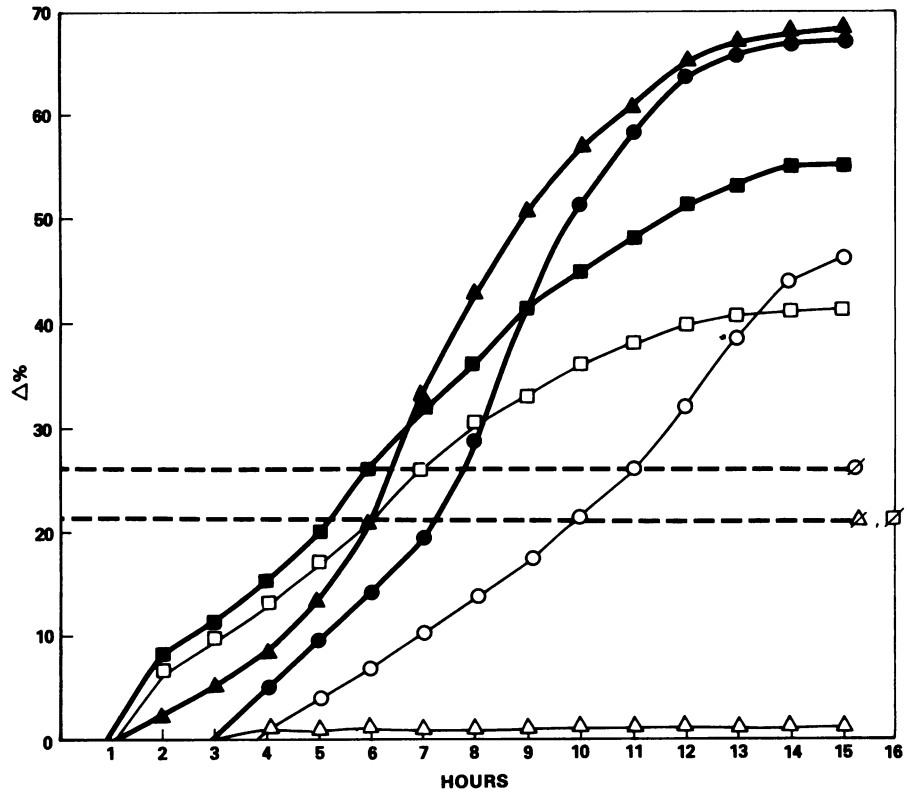

FIG. 5. Three coagulase-negative staphylococci: strain 2296 (■, □), strain 2587 (▲, △), and strain 2589 (●, ○). The positive control (——) for each strain was compared with the 2-μg/ml oxacillin well (——). Resistance to oxacillin is determined by the threshold (------) which has to be achieved by strains 2296 (∅), 2587 (⍙), and 2589 (⍁) when the positive control is ≥30Δ%.

TABLE 2. Summary of oxacillin-methacillin susceptibility with modified analysis

Organisms (no. tested)	Method of analysis				% Total agreement with strains tested[e]	Average time to call susceptible (h)[f]	Average time to call resistant (h)[g]
	Standard[a]		AMS[b]				
	R[c]	S[d]	R[c]	S[d]			
S. aureus (435)	212	223	207	228	98 (98)[h]	4.4	4.5
Coagulase-negative staphylococci (239)	83	156	79	160	95 (98)	5.9	5.8
S. saprophyticus (21)	3	18	2	19	66 (95)	6.8	8.5
S. hominis (16)	4	12	4	12	100 (100)	7.2	7.2
S. warneri (11)	0	11	0	11	(100)	5.8	NA[i]
S. cohnii (11)	3	8	2	9	66 (91)	8.1	8.5
S. simulans (8)	0	8	0	8	(100)	4.6	NA
S. haemolyticus (5)	2	3	1	4	50 (80)	7.2	8

[a] Bauer-Kirby disk diffusion technique.
[b] Oxacillin well in the GPS.
[c] R, Resistant.
[d] S, Susceptible.
[e] Of 746 organisms tested, the average percent agreement with resistant strains was 96.
[f] Of 746 organisms tested, the average time to call the organisms susceptible was 5.0 h.
[g] Of 746 organisms tested, the average time to call the organisms resistant was 5.5 h.
[h] Of 746 organisms tested, the average percent agreement was 98.
[i] NA, Not applicable.

positive control achieves ≥30% change, a susceptible result is reported. If the oxacillin value is ≥10%, the incubation period is extended by 1 to 2 h, at which time the analysis technique calculates whether the organism is susceptible or resistant to oxacillin.

After the data analysis technique was incorporated, all strains with major errors were retested. Strains not showing problems with oxacillin were not affected by the data analysis modification. The summary of all the organisms tested is given in Table 2. Approximately 70% of all resistant staphylococci tested were MR *S. aureus* strains. The MR *S. aureus* correlation was 98%, with an average detection time of 4.5 h. Of the oxacillin-methicillin-resistant, coagulase-negative staphylococci, 93% were detected with an average time ranging from 5.8 h for *Staphylococcus epidermidis* to 8.5 h for *Staphylococcus cohnii*. The overall correlation for the 307 methicillin-resistant organisms was 96%.

DISCUSSION

The need for rapid and accurate detection of methicillin-resistant organisms is becoming more important as the frequency of occurrence of these isolates is increasing in clinical laboratories. Several problems have been cited in the literature concerning the ability of standard methods (1, 8, 12) and the automated systems (3, 7, 10, 13) to consistently detect MR *S. aureus* isolates. During this study, it was noted that for some strains tested, the oxacillin and methicillin disk diffusion results did not always show agreement in the 18- to 24-h incubation period. In these cases, methicillin was resistant and the oxacillin result was susceptible; however, when incubation was continued at 30°C for an additional 18 to 24 h, the strain was resistant to both antibiotics. Various attempts were made to increase the detection rate of hetero-resistant strains of *S. aureus*, including decreased incubation temperature, addition of NaCl to the oxacillin broth, and increased inoculum size. Although many of these variations affected the growth observed, optimizing the software appeared to be the best solution. Once these different hetero-resistant patterns were recognized, data analysis modifications were made within the computer system. With these modifications, the AMS was able to detect 96% of the 307 oxacillin-methicillin-resistant staphylococci.

From the three category resistance patterns, it was of interest to find that resistance patterns for categories one and two could be found within isolates of a single parent colony. However, a parent colony with a category three resistance pattern did not have isolates of any other category (data not shown). Also, when a strain had a category three resistance pattern, it could not be detected by the system. Of the MR *S. aureus* strains tested, 25% (53) had a category two resistance pattern, and 2% (5) were category three strains, confirming the variable levels of resistance expressed by this phenotype and the difficulty in their detection.

The ability of the AMS to accurately detect oxacillin-methicillin resistance lies in the flexible software of the system and the micro-aerophilic environment in the test kit. The constant environment is created by the polyester tape, allowing the system to detect any small changes which may occur. It has recently been reported that with a particular methicillin-resistant strain of *S. aureus*, methicillin interfered with normal cell division but did not appear to inhibit septal peptidoglycan synthesis (14). This would suggest that the rapid detection time is related to cell viability and is not dependent on the slower process of cell division for the expression of the resistance.

From the results presented here, staphylococci resistant to oxacillin-methicillin can be accurately detected by means of automated and computer-assisted methodology. The AMS had an overall correlation of 98% from the 746 organisms tested and had a detection range of 4.4 to 8.5 h with an average time of 5.0 h.

ACKNOWLEDGMENTS

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