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Screening Pneumococci for Penicillin Resistance

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Eighty-four pneumococci with various MICs of penicillin (38 with MICs of ≤0.06 μg/ml [susceptible], 35 with MICs of 0.12 to 1.0 μg/ml [relatively resistant], and 11 with MICs of >1.0 μg/ml [resistant]) were screened by a disk diffusion test using oxacillin and methicillin to see how well they distinguished penicillin-susceptible strains from those with decreased susceptibility to penicillin. The effects of Mueller-Hinton agar plus 5% sheep blood and Trypticase soy agar plus 5% sheep blood and two atmospheres, ambient air and a candle extinction jar (increased CO_2), were compared. There were no obvious differences between the effects of the two media, but zones were generally larger in ambient air than in increased CO_2. Although the oxacillin test can separate penicillin-susceptible and -resistant strains, it cannot separate penicillin-resistant from relatively penicillin-resistant strains by using the breakpoint of <20 mm recommended by the National Committee for Clinical Laboratory Standards. When the 20-mm breakpoint was applied to methicillin, 12% of the relatively resistant strains tested were erroneously classified as susceptible. When different breakpoints were used for methicillin, there was better separation of the two classes of penicillin-resistant isolates, but a few relatively resistant strains were still classified as susceptible. We recommend that oxacillin, not methicillin, be used as the screening agent with Mueller-Hinton sheep blood agar and ambient air incubation and that the breakpoint be <20 mm to indicate resistance or relative resistance.

The use of disk susceptibility tests with oxacillin as a screen to detect penicillin resistance in *Streptococcus pneumoniae* has been recommended for several years (3, 8, 11). Dixon et al. were the first to report the usefulness of a diffusion test with a 1-μg oxacillin disk for distinguishing penicillin-resistant from penicillin-susceptible pneumococci (3). The National Committee for Clinical Laboratory Standards (NCCLS) recommends the test in their disk diffusion standard M2-A3, stating that both oxacillin and methicillin can be used (8). However, only breakpoints for oxacillin are included in the table of interpretive breakpoints, the assumption being that oxacillin breakpoints can also be used for methicillin.

Jacobs et al. (5) studied the disk diffusion test as a method for detecting penicillin resistance in pneumococci, using oxacillin (1-μg), methicillin (5-μg), and penicillin (0.15- and 0.018-μg) disks. They reported that all disks except the 0.018-μg penicillin disk were useful in separating susceptible pneumococci (MICs, ≤0.06 μg/ml) from relatively resistant pneumococci (MICs, 0.12 to 1.0 μg/ml) and from resistant pneumococci (MICs, >1.0 μg/ml); the 0.018-μg disk could not distinguish resistant from relatively resistant strains. Jacobs et al. also recommended breakpoints other than those given by the NCCLS for the 5-μg methicillin disk.

In several College of American Pathologists surveys, pneumococci that were relatively resistant and resistant to penicillin were sent to participants to determine whether they could detect decreased susceptibility to penicillin (6, 7). Although many laboratory personnel were aware that an oxacillin disk diffusion test should be performed against pneumococci, many of the same personnel also performed a penicillin 10-U disk test and reported that the strains were penicillin susceptible. Since pneumococci with penicillin MICs of ≥0.12 μg/ml frequently have penicillin zones of >30 mm and occasionally even >35 mm, the penicillin disk test cannot be used to screen for decreased susceptibility to penicillin.

We have found that oxacillin works well in distinguishing susceptible strains from those with decreased susceptibility (11). However, we could not determine whether a strain is highly resistant or only relatively resistant to penicillin by using the oxacillin screen test. We undertook this study to see whether diffusion tests with methicillin could be used as a screen for penicillin resistance, whether the tests would separate resistant from relatively resistant strains, and whether methodological variables such as increased CO_2 or use of Trypticase soy agar-blood (TSAB) plates would affect results with either oxacillin or methicillin.

MATERIALS AND METHODS

Organisms. The isolates were received in our laboratory for diagnositc purposes or as a part of two surveillance studies (4, 10). Eighty-four isolates were tested: 38 were penicillin susceptible, 35 were relatively penicillin resistant, and 11 were penicillin resistant. The strains had been kept frozen at −70°C or colder and before use had been subcultured onto TSAB plates (BBL Microbiology Systems, Cockeysville, Md.).

MICs. The MICs for these isolates had been determined in previous studies and were not retested. All strains had been tested by broth microdilution with plates containing cation-supplemented Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) and 5% lysed and centrifuged horse blood (11).

Disk diffusion. The inoculum was prepared by suspending overnight growth from a TSAB plate in 5 ml of Mueller-Hinton broth and adjusting the suspension to equal the turbidity of a 0.5 McFarland standard. Commercially prepared Mueller-Hinton agar-sheep blood (MHAB) plates (BBL) and TSAB plates were then inoculated by the NCCLS standard procedure (8). Two plates of each medium were inoculated, one for incubation in a forced ambient air incu-

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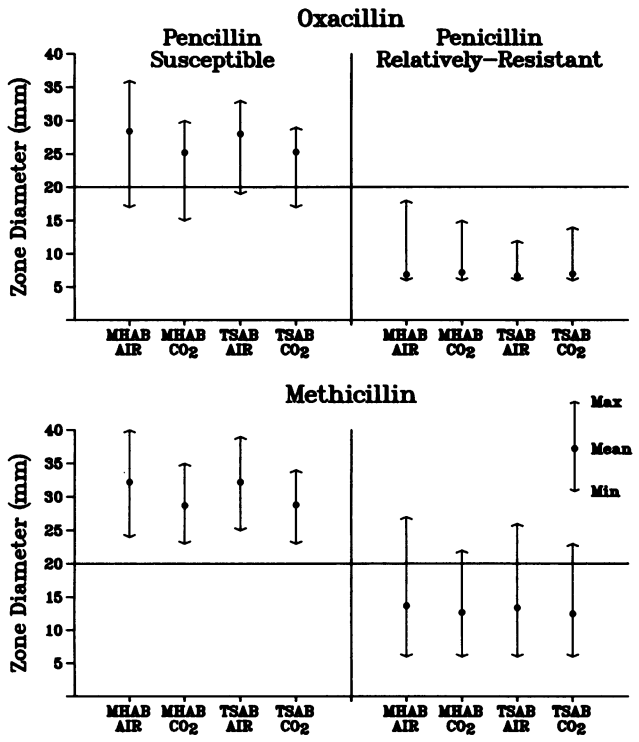

FIG. 1. Ranges (↔) and means (●) of oxacillin and methicillin zone diameters for penicillin-susceptible and -relatively resistant pneumococci tested with two media (MHAB or TSAB) and two atmospheres (AIR, forced ambient air; CO_2, candle extinction jar).

bator and one for incubation in increased CO_2, achieved by using a candle extinction jar, hereafter referred to as CO_2 incubation. All plates were incubated at 35°C for 20 to 24 h. Zone diameters were read using a small millimeter ruler specially designed for reading from the top surface of the plate. Interpretations of the zone diameters were made according to recommendations of the NCCLS (8) and of Jacobs et al. (5). We also interpreted the methicillin zones by using breakpoints different from those recommended by the NCCLS to see whether the use of different breakpoints increased the discriminatory powers of the test.

RESULTS

Effect of CO_2 and medium. A total of 5 of the 84 strains tested (6.0%) did not grow enough without increased CO_2 to yield measurable zone diameters. All strains grew well with increased CO_2.

Ranges and means of zone diameters for each of the conditions tested are given in Fig. 1. All resistant strains had zones of 6 mm for both oxacillin and methicillin and are not included. In general, zone diameters were smaller when the plates were incubated in CO_2, with the exception of the oxacillin zones for the relatively resistant strains. Type of medium had little effect on zone diameter. Although there were some miscategorizations, there were no major susceptibility category changes (i.e., susceptible to resistant or vice versa) as a result of either medium or atmosphere variations.

Effect of drugs. The distribution of penicillin MICs by zone diameters for methicillin and oxacillin is given in Table 1 for the recommended method using MHAB and incubation in air. The zone diameters are separated into groups that allow comparison with past recommendations from our laboratory (11), with those of Jacobs et al. (5), and with NCCLS recommendations (8). Percentages of strains with correct categories using NCCLS, Jacobs et al., and other breakpoints are given in Table 2.

NCCLS zone diameter breakpoints for oxacillin or methicillin are ≥20 mm for susceptible and <20 mm for resistant. With oxacillin, 2 of 34 (5.9%) susceptible strains (penicillin MIC, 0.06 μg/ml) were falsely categorized as resistant; none were falsely categorized as resistant with methicillin. However, 4 of 34 (11.8%) relatively resistant strains (penicillin MIC, 0.12 to 0.25 μg/ml) were incorrectly categorized as susceptible with methicillin.

Jacobs et al. (5) expanded the breakpoint scheme to allow separation of resistant from relatively resistant strains. They recommended oxacillin breakpoints of >20 mm for susceptible, 7 to 20 mm for intermediate, and 6 mm for resistant and methicillin breakpoints of >25 mm for susceptible, 17 to 25 mm for intermediate, and <16 mm for resistant. Using these breakpoints, very few relatively resistant strains were correctly classified in our study (Table 2) with either methicillin or oxacillin.

To further investigate whether a resistant strain could be distinguished from a relatively resistant strain, we also considered breakpoints for methicillin other than those rec-

TABLE 1. Distribution of MICs by zone diameter for methicillin and oxacillin using MHAB incubated in ambient air and category of susceptibility determined by three different recommendations

Screening drug	Zone diam (mm)	Disk category[a]			No. of strains with penicillin MICs (μg/ml)						
					Susceptible		Relatively resistant				Resistant
		1	2	3	≤0.03	0.06	0.12	0.25	0.5	1	>1
Oxacillin	6	R	R				3	16	10	1	11
	7–12	R	I				1	1			
	13–19	R	I			2	2				
	20–25	S	S		3						
	>25	S	S		29						
Methicillin	6	R	R	R				1	1	1	11
	7–12	R	R	I				8	5		
	13–16	R	R	I			2	7	4		
	17–19	R	I	I			1				
	20–25	S	I	I		2		1			
	>25	S	S	S	32		3				

[a] R, Resistant; I, intermediate; S, susceptible. Category 1 (8): R, <20 mm; S, ≥20 mm. Category 2 (5): for oxacillin, R, 6 mm; I, 7 to 20 mm; S, ≥20 mm; for methicillin, R, <17 mm; I, 17 to 25 mm; S, >25 mm. Category 3 (other breakpoints): for methicillin, R, 6 mm; I, 7 to 25 mm; S, >25 mm.

TABLE 2. Percentage of strains with the correct category of penicillin susceptibility using MHAB incubated in ambient air

Guideline	Screening drug	No. of strains with correct category[a]/total no. of strains (%)[b]		
		Susceptible[c]	Intermediate[d]	Resistant[e]
NCCLS	Oxacillin	32/34 (94.1)		45/45 (100)
	Methicillin	34/34 (100)		41/45 (91.1)
Jacobs	Oxacillin	32/34 (94.1)	4/34 (11.8)	11/11 (100)
	Methicillin	32/34 (94.1)	3/34 (8.8)	11/11 (100)
Other	Methicillin	32/34 (94.1)	28/34 (82.4)	11/11 (100)

[a] NCCLS breakpoints for oxacillin and methicillin are: susceptible, ≥20 mm; resistant, <20 mm (8). Jacobs et al. breakpoints are: oxacillin, susceptible, >20 mm; intermediate, 7 to 20 mm; resistant, 6 mm; methicillin, susceptible, >25 mm; intermediate, 17 to 25 mm; resistant, <16 mm (5). Other breakpoints are: methicillin, susceptible, >25 mm; intermediate, 7 to 25 mm; resistant, 6 mm.

[b] Total number of strains is 79 since 5 did not grow in air.

[c] Penicillin MIC, ≤0.06 μg/ml.

[d] Penicillin MIC, 0.12 to 1.0 μg/ml (also called relatively resistant).

[e] Penicillin MIC, >1.0 μg/ml. With NCCLS recommendations, resistant and relatively resistant categories are not separated.

ommended by Jacobs et al. (5) (in Table 1, disk category 3 defines >25 mm as susceptible, 7 to 25 mm as relatively resistant, and 6 mm as resistant). Using these breakpoints, 28 or 34 (82.4%) relatively resistant strains were correctly classified, as were 32 of 34 (94.1%) susceptible strains. However, 3 of 34 (8.8%) relatively resistant strains were still falsely categorized as susceptible (Table 2). Substituting TSAB for MHAB or incubating the strains in increased CO_2 did not greatly improve the accuracy of the categorization.

When we used recommended NCCLS breakpoints with methicillin, four (8.9%) of the resistant strains were missed entirely; extending the breakpoint for resistance to >25 mm still failed to distinguish three (6.7%) of the resistant strains.

DISCUSSION

The results of a national surveillance study of serotypes and resistance patterns of 3,400 strains of *S. pneumoniae* that was performed at the Centers for Disease Control indicate that, in the United States, the overall incidence of pneumococci with decreased susceptibility to penicillin is rather low (3.7%) (2). However, some areas in the United States have reported a higher incidence of penicillin resistance. In Denver, 6.9% relative resistance was found in 101 patients screened (4), and in Oklahoma City, 15.5% relative resistance was found in 103 patients screened (10). It is important to recognize those strains that are not fully susceptible to penicillin, especially since there are reports of pneumococci in the United States that are not only fully resistant to penicillin but also resistant to other antimicrobial agents (4; M. Lukaszewski, M. Simberkoff, A. Cross, M. Al-Ibrahim, A. Baltch, P. J. Geiseler, J. Nadler, and A. Richmond, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, C300, p. 350).

In this study, we detected all strains with decreased susceptibility to penicillin. Using either oxacillin or methicillin, we were unable, however, to adequately distinguish strains resistant to penicillin (MIC, >1.0 μg/ml) from those relatively resistant to penicillin (MIC, 0.12 to 1.0 μg/ml). Varying breakpoints and using methicillin resulted in better separation of resistant and relatively resistant isolates, but the incidence of false susceptibility (11.8%) makes the use of methicillin undesirable. The breakpoints recommended by Jacobs et al. were also inadequate for our purposes.

However, the need to separate the two types of resistance at all is debatable. Because levels of penicillin in the spinal fluid may be inadequate to eradicate pneumococci that are relatively resistant (1, 9), it is only necessary to know that the strain is not susceptible in isolates from meningitis. For infections in other areas of the body, the significance of relative resistance is less clear, and the need for categorization of penicillin resistance is questionable. Response to penicillin therapy is better when infection occurs in areas other than the central nervous system, but significant numbers of failures have been reported for infection caused by both resistant and relatively resistant pneumococci (12). Therefore, the best approach may be to consider any resistance significant.

The oxacillin screening method was first described in 1977 (3) and has performed well in our laboratory as in other laboratories. Unfortunately, however, there is some question about whether the test is being interpreted correctly in some clinical laboratories. In 1981, the College of American Pathologists used a strain of *S. pneumoniae* with a penicillin MIC of 0.12 μg/ml (relatively resistant to penicillin) to test their subscribers' ability to detect relative resistance. Although many participants using the NCCLS method tested oxacillin and reported resistance to it, they also tested penicillin, and 86.2% of participants reported the strain to be susceptible to penicillin (6). In a later survey, the College sent out a strain with a penicillin MIC of >1.0 μg/ml, and 22.4% of the participants still reported the strain to be penicillin susceptible, even though all zone diameters reported for oxacillin were 6 mm (7). In a more recent survey using the same strain, 16.3% of laboratories tested penicillin and reported that the strain was susceptible even though they had also tested oxacillin and found no zone (College of American Pathologists, Critique of Infectious Disease Survey ID-D, Specimen ID-16, 1984). We emphasize that the oxacillin disk test should be used instead of the penicillin disk test to detect penicillin resistance or relative resistance and that the oxacillin disk results should not be reported as such; i.e., they should be used only to report penicillin susceptibility.

We also recommend that oxacillin rather than methicillin be used as the screening agent for detecting penicillin resistance or relative resistance in pneumococci because the oxacillin disk test as recommended by the NCCLS (8) identified all the resistant or relatively resistant isolates and methicillin did not. We further recommend that if the oxacillin zone diameter is ≤19 mm, the pneumococcus be interpreted as resistant or relatively resistant to penicillin; if the zone diameter is ≥20 mm, the isolate should be interpreted as susceptible to penicillin. We previously recommended (11) a resistant or relatively resistant breakpoint of ≤12 mm because it was more consistent with the published breakpoints in the standard disk diffusion method and because only occasional strains yielded zone diameters of 13 to 19 mm (with corresponding borderline MICs of 0.06 or 0.12 μg/ml), but the use of the ≤19 mm breakpoint will simplify the routine interpretation of this test.

The test worked well when performed as described in the NCCLS standard M2-A3 (8). Incubation in CO_2 can be done if it is necessary for growth. Although using TSAB did not substantially alter the results, we recommend MHAB because it is the standard medium (8). We do not recommend that this diffusion test be used to separate resistant from relatively resistant strains but rather that this separation be

done with an MIC test (9) with breakpoints of ≤ 0.06 μg/ml for susceptible, 0.12 to 1.0 μg/ml for relatively resistant, and >1.0 μg/ml for resistant.

In summary, we recommend that the oxacillin screen test be used to detect penicillin resistance in pneumococci but that it not be used to differentiate between resistant and relatively resistant isolates. Furthermore, we recommend that the methicillin screen test not be used.

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