Antimicrobial Susceptibility Testing of *Streptococcus pneumoniae* by Micro-Broth Dilution

MARTHA M. TARPAY,* DAVID F. WELCH, AND MELVIN I. MARKS

Department of Pediatrics, Infectious Diseases Division, University of Oklahoma, Health Sciences Center, Oklahoma City, Oklahoma 73190

Thirty-three clinical isolates of *Streptococcus pneumoniae* were tested for susceptibility to penicillin and ampicillin by a standard agar dilution method. Results were compared to those obtained using a micro-broth dilution technique in which Mueller-Hinton broth was supplemented with 5% defibrinated whole sheep blood. Among the 33 strains, 2 were resistant (minimal inhibitory concentration, 8 µg/ml), 10 were relatively resistant (minimal inhibitory concentration, 0.12 to 0.5 µg/ml), and 20 were susceptible (minimal inhibitory concentration, ≤0.06 µg/ml) to penicillin by both methods. Only one strain showed a two-dilutional-step difference by micro-broth and agar dilution testing resulting in categorization as relatively resistant by the former method but susceptible by the latter. A 1-µg oxacillin disk correctly identified 11 of the 12 resistant strains. The micro-broth dilution technique is a reliable, simple method for penicillin or ampicillin susceptibility testing of pneumococci and economically feasible to perform manually or with a semiautomated system.

Penicillin-resistant isolates of *Streptococcus pneumoniae* have been noted with increasing frequency (1, 11) and have been clinically significant in patients with meningitis (7, 10). Because of this, disk diffusion susceptibility testing with a 1-µg oxacillin disk is recommended as a screening test on all *S. pneumoniae* isolates from cerebrospinal fluid. Although quantitative susceptibility testing would be desirable for strains apparently resistant by this method, the recommended technique is agar dilution, which is cumbersome and expensive when a small number of isolates require testing (15). Our objective was to evaluate a micro-broth dilution technique for the antimicrobial susceptibility testing of *S. pneumoniae*. We report this method and compare the results with those obtained with the agar dilution technique and oxacillin disk diffusion test.

MATERIALS AND METHODS

Organisms. Thirty-three strains of *S. pneumoniae* were studied. Thirty-one strains were isolated from patients in the Oklahoma area. The remaining two strains were resistant South African isolates. Identifications were based on colony morphology, Gram stain, bile solubility, and optochin susceptibility (2). All strains were initially screened for susceptibility to penicillin by using a 1-µg oxacillin disk, and those found to be resistant were tested for β-lactamase production by the chromogenic cephalosporin method (9). All were β-lactamase negative. Laboratory reference strains of *Escherichia coli* and *Staphylococcus aureus* were used as controls throughout the experiments.

Disk diffusion testing. Oxacillin disks (1 µg; Pfizer, Inc.) were tested against all strains using the methodology described by the National Committee for Clinical Laboratory Standards (8). Interpretation of zone sizes with oxacillin was as follows: ≤12 mm, resistant; 13 to 19 mm, indeterminate; ≥20 mm, susceptible (14).

Agar dilution testing. The methodology employed was essentially that described by an international collaborative study (4). The inoculum was prepared by suspending several colonies from an overnight culture of *S. pneumoniae* from 5% sheep blood agar media in Mueller-Hinton broth and adjusting to a turbidity of a 0.5 McFarland standard (approximately 1.5 x 10⁶ colony-forming units per ml). A further dilution of 1:20 was made by placing 0.5 ml of the adjusted suspension into 9.5 ml of Mueller-Hinton broth. Inocula containing approximately 10⁶ organisms were delivered by using the Steers replicator (12) onto Mueller-Hinton agar plates supplemented with 5% defibrinated sheep blood and the appropriate antibiotic concentrations. The plates were incubated at 35°C for 18 h. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of antimicrobial agent which completely inhibited bacterial growth.

Micro-broth dilution testing. The inoculum was prepared in the manner described for the agar dilution method. A further 1:10 dilution was made in Mueller-Hinton broth. Stock solutions of penicillin and ampicillin in sterile water were prepared in concentrations of 1,000 µg/ml and used immediately or stored in working samples at -20°C until used. Doubling dilutions of the antibiotics were prepared in Mueller-Hinton broth (Difco) supplemented with 5% defibrinated sheep blood. All antimicrobial solutions were prepared in large volumes (20 ml); 0.1-ml samples of the antibiotic solutions containing sheep cell-supplemented media were delivered to wells of a 96-micro well plate and inoculated by Dynatech MIC-2000 instrumenta-
tion (Dynatech Laboratories, Alexandria, Va.). The final concentration of inoculum in each well ranged from $2 \times 10^7$ to $7 \times 10^5$ colony-forming units per ml (verified by colony counting). MIC endpoints were read after 18 h of incubation at 35°C and were defined as the lowest concentration of antibiotic that resulted in no bacterial growth as indicated by absence of hemolysis of erythrocytes. The cell buttons in wells without growth were bright red, whereas those in wells containing subinhibitory antibiotic concentrations showed a greenish-black discoloration. Minimal bactericidal concentrations (MBCs) were defined as the lowest concentrations of drug that yielded no growth after subculture of each well by use of the inoculator pin assembly (Dynatech) onto 150-mm blood agar plates and overnight incubation. Organisms were considered resistant with an MIC of $>1 \mu g/ml$, relatively resistant with an MIC of 0.1 to 1 \mu g/ml, and susceptible with an MIC of $\leq 0.06$ \mu g/ml.

RESULTS

The disk diffusion test using a 1-\mu g oxacillin disk correctly identified all but one penicillin-resistant strain. The zone of inhibition was 20 mm for this isolate and the penicillin MIC was 0.25 \mu g/ml. Repeated disk testing (10 consecutive separate determinations) with this strain yielded a mean zone diameter of $17 \pm 2.67$ mm. The zone diameter of inhibition was always larger than 20 mm for all susceptible strains.

Among the 33 S. pneumoniae strains tested, 2 were resistant (MIC, 8 \mu g/ml), 10 were relatively resistant (MIC, 0.1 to 0.5 \mu g/ml), and 20 isolates were susceptible to penicillin by both the micro-broth and agar dilution methods. One strain was relatively resistant to penicillin (MIC, 0.25 \mu g/ml) by the micromethod but susceptible by the agar dilution technique (MIC, 0.06 \mu g/ml). The ampicillin MIC for this strain was 0.12 \mu g/ml by both methods. Representative mean and standard deviation values for the micro-broth method, based on at least four independent determinations for a relatively resistant strain, were 0.1875 ± 0.072 \mu g/ml when testing ampicillin and 0.315 ± 0.125 \mu g/ml when testing penicillin. Repeated testing of single strains by agar dilution gave the same values or, rarely, a one-dilution-step difference.

The typical appearance of micro-broth dilution plates after inoculation and overnight incubation showed endpoints as being the first well in the dilution series with no darkening of the red cell button. The color change in wells containing growth was apparently secondary to the partial hemolysis of erythrocytes in the presence of pneumococci similar to the type of alpha hemolysis produced by these organisms on blood agar media.

Comparison and distribution of the MICs obtained by the agar dilution and the micro-broth techniques are shown in Fig. 1. The MIC of penicillin was the same or one dilution different for 31 strains. Good correlation between the two methods was also obtained with ampicillin. Agar dilution endpoints were the same as or one dilution different from microbroth endpoints for 32 of the strains tested against ampicillin. However, 54.5% of the isolates were one doubling dilution more susceptible to ampicillin by the micromethod as compared with the agar dilution technique. The MICs of penicillin and ampicillin were the same for 17 strains. Ten strains were found to be one dilution more susceptible to ampicillin than to penicillin, whereas two strains were two dilutions more susceptible. Four isolates were more susceptible to penicillin than to ampicillin. The penicillin and ampicillin MBCs were performed to identify possible tolerant strains and to verify the accuracy of the micro-broth dilution endpoint interpretations. The penicillin MICs and MBCs were the same for 26 strains. The penicillin MBC was one dilution higher than the MIC in six strains and three dilutions higher in one strain. The MIC of this strain was 0.03 \mu g/ml by both methods, whereas the MBC was 0.25. The ampicillin MIC and MBC were 0.03. This strain was a typical S. pneumoniae in all other respects. Ampicillin MICs and MBCs were the same in 24 strains; the MBC was one dilution higher than the MIC in 8 strains and two dilutions higher in 1 strain.

DISCUSSION

S. pneumoniae can no longer be assumed to be uniformly susceptible to penicillin. Although recent reports (3, 6) suggest that the isolation
rate of relatively resistant strains of *S. pneumoniae* is low in North America (2.4%), rates in excess of 10% have been reported in two different studies from Oklahoma (11, 13). There is a need for a simple and reliable method for quantitative susceptibility testing of these organisms.

The micromethod described herein utilizes a commercially available semiautomated microbroth dilution susceptibility testing system. Good correlation between this microtechnique and the standard agar dilution method was obtained. This method of susceptibility testing is economically feasible to perform on even a small number of isolates, and the endpoints are easily interpretable. Microplates without blood can be prepared and stored for up to 2 months at −20°C without loss of antimicrobial activity. Blood can be added to the thawed media just before testing. In laboratories where semiautomated systems are not available, the test can be performed manually. Thornsberry and Swenson (14) recently described a micromethod for the susceptibility testing of *S. pneumoniae* using Mueller-Hinton broth with lysed horse blood. However, no comparison with the standard agar dilution method was presented. The technique employed in the present study requires less preparation, is easily performed in the average clinical laboratory, and correlates well with agar dilution results.

Of particular interest in the present study was that 36.3% of the *S. pneumoniae* isolates were more susceptible to ampicillin by one to two dilutions than to penicillin and that all of the relatively resistant strains were more susceptible to ampicillin than penicillin.

The disk diffusion test using the 1-μg oxacillin disk has been recommended as a screening test for the identification of penicillin-resistant strains. In our study one of the relatively resistant isolates was initially placed in the penicillin-susceptible group because of a 20-mm zone of inhibition around the oxacillin disk. Statistical analysis verified that this must be attributed to variability of the disk test, but it may be important to recognize that strains with penicillin MICs of 0.25 μg/ml can have oxacillin zones that approach or exceed 20 mm on occasion. This variability appears to be rarely encountered (3, 5). Therefore, this does not diminish the usefulness of the oxacillin disk test, but a quantitative method as described herein could be used as a more definitive measure of susceptibility, particularly for blood, cerebrospinal fluid, and other significant isolates.

**LITERATURE CITED**


