Reevaluation of the Disk Diffusion Method for Sulfonamide Susceptibility Testing of Neisseria meningitidis

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Lack of correlation between quantitative minimal inhibitory concentration (MIC) determinations and disk diffusion susceptibility tests in our laboratory prompted a study to reevaluate the use of the disk diffusion test for sulfonamide susceptibility testing of Neisseria meningitidis. One hundred and sixty-three recent clinical isolates of N. meningitidis were examined for sulfonamide susceptibility by the agar dilution and disk diffusion methods. Optimal inocula for each of the tests were determined, and thereafter all disk diffusion tests were compared with quantitative MICs as determined by the agar dilution method using sulfadiazine and an inoculum of 10^6 colony-forming units (CFU)/ml. The clearest and most reproducible zone diameters were obtained with a 10^3-CFU/ml inoculum in the disk diffusion test. There was complete correlation between the disk zone diameters for 300-μg disks of sulfadiazine and sulfathiazole and the agar dilution test MICs. All isolates with a zone diameter of <20 mm were resistant to sulfadiazine, whereas those with zone diameters of ≥30 mm were susceptible. False susceptible and false resistant readings were obtained with 300-μg sulfisoxazole disks. These data suggest that inocula and type of sulfonamide are critical factors in the disk diffusion test for meningococcal susceptibility testing. Sulfonamide disks are not interchangeable for susceptibility testing of meningococci.

Disagreements between quantitative minimal inhibitory concentrations (MICs) and disk diffusion susceptibilities for sulfonamide testing of Neisseria meningitidis were noted on several occasions in our laboratory. This prompted the present study to reevaluate selected technical aspects of the disk diffusion method for estimation of sulfonamide susceptibility of N. meningitidis. Inaccuracies in these results may be reflected clinically by inappropriate use of chemoprophylactic agents in meningococcal carriers and occasionally by therapeutic errors, and may influence certain epidemiological observations.

MATERIALS AND METHODS

Bacteria. One hundred and sixty-three recent clinical isolates of N. meningitidis were tested for sulfonamide susceptibility by agar dilution and disk diffusion methods. These bacteria were isolated from contacts and index cases of meningococcal meningitis and/or bacteremia and stored in sheep blood at −70°C. Serogroups were as follows: group A, 25; group B, 58; group C, 42; group Y, 9; group 29e, 6; group X, 3; and 20 were not typed.

Media. Mueller-Hinton agar (Difco) (Ca++, 7.0 mg/100 ml; Mg++, 3.6 mg/100 ml) and Mueller-Hinton broth (Difco) were used for all agar dilution and disk diffusion tests. Sheep blood agar plates were used for subculture of isolates from frozen sheep blood.

Antimicrobial agents. Disks (300 μg) of sulfadiazine, sulfathiazole, and sulfisoxazole (Difco) were tested. Sulfadiazine was provided in powder form by May and Baker Canada Ltd. A stock solution of 800 mg/100 ml was prepared and used immediately for preparation of the agar dilution plates.

Agar dilution tests. Square petri dishes (100 by 15 mm; Falcon) were prepared within 48 h of testing by combining twofold dilutions of sulfadiazine (0.5 ml) with 9.5 ml of Mueller-Hinton agar, yielding test combinations of 40 to 0.009 mg of sulfadiazine per 100 ml. The inoculum was prepared by swabbing sufficient growth from a pure blood agar culture into 10 ml of Mueller-Hinton broth to produce an optical density of 0.13 to 0.15 at 625 nm in a Coleman Junior spectrophotometer. This standard, and suspensions prepared by eye comparison, yielded viable bacterial counts of approximately 10^6 colony-forming units (CFU)/ml (MacFarland no. 1 turbidity standard can be used as an alternative to the spectrophotometric standard providing it is prepared on a biyearly basis or when clumping is noted). A 10^{-2} dilution of this inoculum was placed in a Steers replicator, and the plates were inoculated and dried at room temperature (3). Plates were read after incubation in 5% CO₂ at 37°C for 16 to 20 h. MIC was defined as the lowest concentration of drug giving an 80% (or sudden) reduction in surface growth of bacteria.

Disk diffusion test. Round petri dishes (100 by 15
mm; Fisher) were prepared with a uniform agar depth of 4 mm and placed in the incubator for 30 min before inoculation. The plates were inoculated with a swab dipped into a 10⁻¹ dilution of the standard inoculum (described above), giving a colony count of approximately 10⁶ CFU/ml. The swab was squeezed on the side of the tube to remove excess fluid and then streaked on the plate in four directions. After drying for 3 to 5 min, disks were applied and the plates were inverted and incubated in 5% CO₂ at 37°C for 16 to 20 h. The zone diameter was measured with a ruler from the back of the plate, ignoring any haze within the definite margin of inhibition.

RESULTS

Preliminary experiments were carried out to determine suitable inocula for the disk diffusion and agar dilution tests. An inoculum of 10⁶ CFU/ml gave the clearest results for the agar dilution test. When an inoculum of 10⁶ CFU/ml was used for both the agar dilution and disk diffusion tests, trailing end points occurred, making the interpretation of the exact MIC difficult; several false susceptible disk readings resulted. When 10⁵ CFU/ml was used for the disk diffusion test, however, false susceptible results were also encountered. An inoculum of 10⁵ CFU/ml produced a preconfluent growth on the surface of the agar as well as two clearly distinguishable populations. Therefore, an inoculum of 10⁴ CFU/ml was used for the agar dilution studies, and 10⁴ CFU/ml was used for disk diffusion.

Several other parameters were also examined. Incubation of plates in 9% CO₂, 5% CO₂, and a candle jar (approximately 2% CO₂) did not alter the disk or agar dilution test results. Similarly, agar depth (10 ml versus 25 ml) had no effect on agar dilution MICs, and each isolate had the same MIC to sulfadiazine, sulfathiazole, and sulfoxazole.

A total of 163 recent clinical isolates of *N. meningitidis* were tested for susceptibility with sulfathiazole disks, and the results were correlated with the MIC to sulfadiazine determined by the agar dilution method (Fig. 1). All 45 isolates with a disk zone diameter of <20 mm were resistant (MIC ≥ 1 mg/100 ml), whereas all 118 isolates with a zone diameter of ≥30 mm were susceptible.

Sixty-nine of these isolates were also tested with 300-μg disks of sulfadiazine and sulfoxazole, and the arithmetic mean of three determinations was correlated with the sulfadiazine agar dilution MICs (Fig. 2 and 3). Although <20- and >30-mm zone diameters with sulfathiazole and sulfadiazine disks clearly separated the populations into resistant and susceptible, respectively, comparable criteria for sulfoxazole were <33 and >40 (Fig. 3).

Although zone diameter variations for a single isolate as great as 12 mm were recorded when tests were repeated in triplicate, there was no cross-over from resistant to susceptible populations, or vice versa, with sulfathiazole.
and sulfadiazine disks. However, several false susceptible and resistant results were noted with repeated sulfisoxazole disk diffusion tests. This is not reflected in Fig. 3 since only the mean zone diameters of three separate experiments are illustrated. Zone diameter variations increased in direct proportion to the size of the zone of inhibition.

DISCUSSION

Our studies confirm the accuracy and reproducibility of the disk diffusion test in predicting resistance and susceptibility of *N. meningitidis* to sulfonamides. The method we used for disk diffusion testing is the same as described by Bennett et al. (1) without the modifications subsequently used by Wiggins et al. (4). A critical difference in our results, however, is the different disk zone criteria for susceptibility and resistance. The disk zone diameters that we found useful in predicting agar dilution MICs for *N. meningitidis* are ≥30 mm = susceptible and <20 mm = resistant, using sulfisoxazole and sulfathiazole 300-µg disks and the inocula and other test characteristics described above. Previous publications recommended disk zone diameters of >40 and <30 mm, respectively (1, 4).

The inoculum used in our agar dilution studies was smaller than that described by Bennett et al. (1). We were only able to accurately reproduce clear end points for MIC determinations of *N. meningitidis* with an inoculum of 10⁶ CFU/ml. Bennett et al. (1), however, were able to do this with an inoculum of 5 × 10⁶ CFU/ml, whereas Wiggins et al. (4) used a different method of standardizing their inocula to a McFarland no. 5 tube. These differences are small ones and do not appear to account for the difference in disk zone diameter readings mentioned above. Nevertheless, these results, and those obtained in the disk diffusion studies, illustrate the importance of carefully standardizing the inoculum for use in determining susceptibilities of *N. meningitidis* to sulfonamides.

It has been stated that different sulfonamide preparations are interchangeable for disk susceptibility testing, although this was not specifically tested for *N. meningitidis* (2). In our studies, sulfathiazole and sulfadiazine gave almost identical results; however, sulfisoxazole was less useful, and its use was associated with false readings. There is no ready explanation for this difference in disk activity. Differences in antibacterial activity of the three sulfonamide preparations were ruled out, but diffusion was not evaluated.

As stated by Bennett et al. (1) and Wiggins et al. (4), *N. meningitidis* with zone diameters in the intermediate zone of susceptibility (20 to 30 mm in the test described above) should have their susceptibilities to sulfonamide confirmed by a quantitative method, if clinically indicated. It is clear that certain technical aspects of meningococcal susceptibility testing are critical, and any changes in inocula, type of sulfonamide, disk concentration, media, etc., should be evaluated in a careful comparative manner with a quantitative test before introduction into the clinical diagnostic laboratory.

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LITERATURE CITED