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Rapid Sulfonamide Disc Sensitivity Test for Meningococci

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Minimal inhibitory concentrations (MIC) of 90 strains of *Neisseria meningitidis* were determined by a plate dilution technique that employed twofold changes in concentrations of sulfadiazine. The geometric mean of three MIC determinations on each strain was correlated with inhibition zones produced by a 300-μg sulfathiazole disc. The linear relationship between the logarithm of the geometric mean MIC values and the zone diameters was highly significant. Strains were separated into sensitive and resistant populations by both test procedures. Quantitative criteria for interpreting the sensitivity of a strain by the disc test were established.

Sulfonamide prophylaxis has been remarkably effective in terminating epidemics of meningococcal meningitis and in eradicating nasopharyngeal carriage of meningococcal strains. However, the proportion of meningococci resistant to 1.0 mg/100 ml or more of sulfadiazine has been increasing during the past several years. In 1967, 43% of the isolates submitted to the National Communicable Disease Center were not inhibited by this concentration (7). Because of the increasing resistance of meningococci, the usefulness of sulfonamides for prophylaxis is decreasing. Thus far, it has been found that other antimicrobial agents are unable to eradicate sulfonamide-resistant strains of meningococci from the nasopharynx (3, 4). If resident strains of meningococci in a given population could rapidly be shown to be sulfonamide-sensitive, sulfadiazine could be used with confidence as a prophylactic agent in situations in which prophylaxis is indicated.

The current methods (tube dilution and agar dilution) used to establish sulfonamide sensitivities require considerable time, equipment, and experience, and are performed in relatively few laboratories in the United States. Often, the results of such studies are not available soon enough to guide physicians in selecting appropriate prophylactic therapy, or to allow the use of sulfonamide susceptibility as an epidemiologic marker in ongoing investigations of meningococcal outbreaks.

Interpretations based on standardized disc tests are available for a wide variety of antimicrobial agents against most bacterial pathogens, but such interpretive data have not been published for disc susceptibility testing of *Neisseria meningitidis* against sulfonamides (1).

Therefore, the present study was undertaken to determine whether a reliable, simple, and rapid disc test for sulfonamide susceptibility testing of meningococci could be developed, with materials readily available to most laboratories.

MATERIALS AND METHODS

Ninety strains of *N. meningitidis*, consisting of 69 isolates from blood or cerebrospinal fluid of persons with meningococcal infections and 21 isolates from nasopharyngeal cultures, were studied. The strains had been isolated from 1964 to 1966 and had been stored in defibrinated sheep or rabbit blood at −40 or −70 C. An attempt was made to obtain a wide variety of serogroups. Sulfonamide sensitivities had been performed by different laboratories on 81 of these isolates, using different techniques and dilution schemes. Approximately equal numbers of strains with previously recorded minimal inhibitory concentrations (MIC) of 1.0 mg/100 ml or more and strains with recorded MIC values of less than 1.0 mg/100 ml were selected. Six strains had recorded MIC values of 1.0 mg/100 ml. After subculture of frozen stock cultures on blood-agar, single colonies of each strain were transferred and maintained on cystine tryptic agar by weekly transfer.

All strains showed typical gram-negative diplococci on Gram stain, utilized maltose and glucose, failed to grow on nutrient agar at 22 C, and gave positive oxidase reactions. None of the 90 strains fermented sucrose or lactose, but 7 strains initially selected from stock cultures fermented lactose and they were excluded from analysis with the 90 typical strains of meningococci (6). The following serogroups were represented by these 90 strains of *N. meningitidis*: group

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A, 0; group B, 53; group C, 12; group D, 2; group X, 1; group Y, 7; group Z, 8; untypable, 6; and rough, 1. [Serogroups A, B, C, and D pertain to Branham's classification (2), whereas X, Y, and Z are those of Slaterus (9).]

MIC values were determined by a plate dilution method. Twofold increments in concentrations of sulfadiazine from 0.01 to 41.0 mg/100 ml were prepared in plastic petri dishes (100 mm in diameter) containing 9.5 ml of 1.5% Mueller-Hinton agar and 0.5 ml of sulfadiazine solution in sterile distilled water. Growth from the surface of the 24-hr culture of test strains on Mueller-Hinton agar was suspended in 10 ml of Mueller-Hinton broth by transferring with a loop. A sufficient number of organisms was suspended to obtain an optical density of 0.13 to 0.15 at 625 mμ. Plate counts established that these suspensions contained approximately 5×10^7 colony-forming units per ml. The suspended bacteria were inserted, undiluted, into a Steers-Foltz replicator, and the strains were then applied to freshly prepared Mueller-Hinton agar plates containing sulfadiazine and a control plate without sulfonamide. After drying for 10 min under a drying lamp, the inoculated plates were incubated in a candle jar overnight at 37 C. The MIC was defined as the plate concentration on which a substantial and sudden reduction from uninhibited or moderate growth to slight or no growth occurred. To improve the accuracy of this means of estimating the susceptibility of a strain, the geometric mean of three MIC determinations that were done on different days was used.

Disc tests were performed with 300-μg sulfathiazole discs (Difco). Overnight growths of test strains on Mueller-Hinton agar were transferred with a bacteriological loop and were suspended in 10 ml of Mueller-Hinton broth. A sufficient number of organisms was transferred to obtain *minimally visible turbidity*. No attempt was made to standardize these suspensions to a given optical density, but spot checks usually revealed optical density readings of 0.04 to 0.06 at 625 mμ. A sterile cotton swab was dipped into the suspension, and excess fluid was removed from the swab by gentle pressure against the inside of the test tube. Organisms were then immediately inoculated onto the surface of a plastic petri dish (100 mm in diameter) containing 19 ml of Mueller-Hinton agar. To ensure an even inoculum, the surface of the agar was thoroughly streaked with a swab in four different axes. After the agar had dried for 3 to 5 min, a single 300-μg sulfathiazole disc was applied to the center of the plate with a sterile forceps and gently pressed onto the agar surface to ensure good contact. Uninverted plates were incubated for 18 hr at 37 C in a candle jar, and inhibition zones were then read against a dark background. All measurements were made to the nearest millimeter and included the disc diameter. A measurement of 6 mm represented the diameter of the disc alone. Slightly oval zones were sometimes encountered and, in these instances, the average of the maximal and minimal diameters was used; the difference between maximal and minimal readings was never more than 3 mm. The disc test was considered reliable only if confluent growth was observed on the plates outside the inhibition zones. Occasionally, isolated colonies rather than confluent growth was observed, and repeat tests were performed since the lesser inoculum resulted in significantly larger zones. The outer margins of inhibition zones were always distinct, and the zones were usually completely clear. However, a slight film of growth occasionally occurred within small inhibition zones. In these instances, the measurement was made to the clearly distinguishable outer margin of the inhibition zone. Disc tests on each strain were performed on 3 different days to assess the reproducibility of the test.

RESULTS

A significant linear relationship ($P<0.001$) was observed between the logarithm of the geometric mean MIC values and the arithmetic average zones resulting from disc tests performed on these strains (Fig. 1). All strains with geometric mean MIC values at or above 1.3 mg/100 ml had average inhibition zones of 31 mm or less, whereas all strains with MIC values of 0.78 mg/100 ml or less had inhibition zones greater than 44 mm. None of the tested strains had a geometric mean MIC of 0.79 to 1.2 mg/100 ml, and no inhibition zone of 33 to 39 mm was observed among 270 individual disc tests on these 90 strains.

A nonrandom distribution of zones around the regression line was apparent. Within the sensitive population, the more highly sensitive strains by MIC appeared to the left of the line and the less sensitive strains to the right. A similar bias was observed in the resistant population. Valid extrapolations from regression lines require the assumption that the variables are normally distributed (5). In our experiments, the test strains represented two populations of strains and, in addition, zones within each population were not randomly dispersed around the regression line. Thus, interpretive schemes for this disc test should be based on the characteristics of the two populations rather than on estimations from the regression line on the basis of the standard error of estimate.

Strains were separated into resistant and sensitive populations by both MIC and disc tests, with complete correspondence between them. Bimodal populations were clearly more apparent in the distribution of average zone diameters than in the distribution of MIC values, and a 14-mm separation between populations was observed (Fig. 2).

The mean zone of 135 determinations of the 45 strains in the sensitive population was 50.6 mm, with a standard deviation of 4.96 mm. The 45 resistant strains had an average zone of 20.7 mm, with a standard deviation of 5.65 mm. Assuming a normal distribution within each population, the expected frequency of a given zone measurement was calculated for each population. To establish

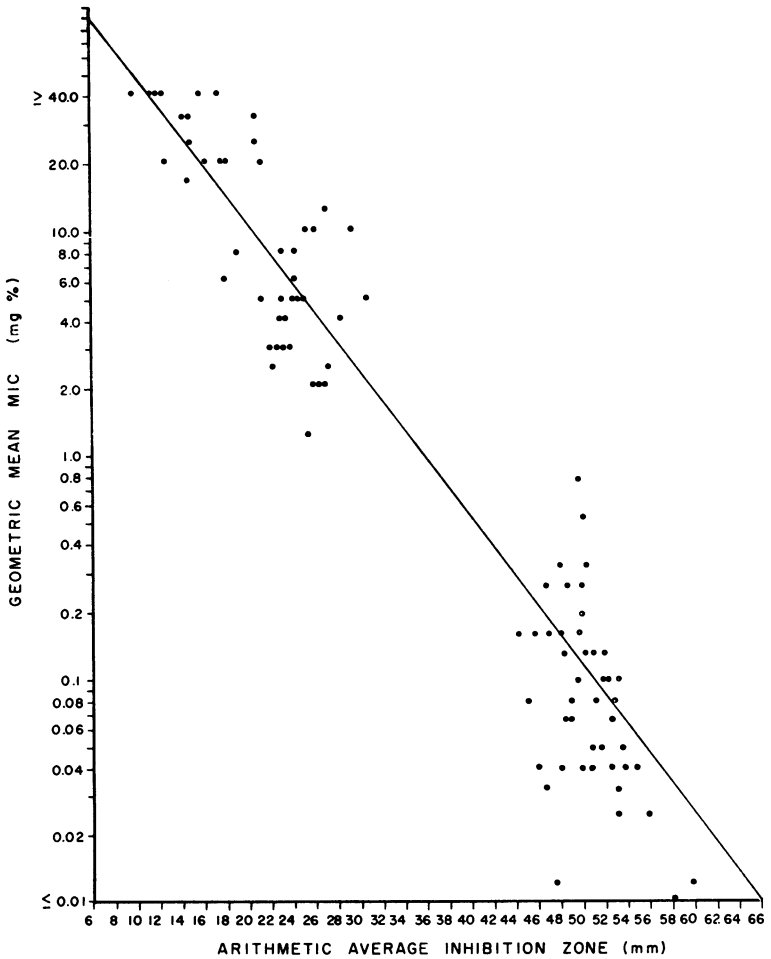

FIG. 1. *Relationship between minimal inhibitory concentration (MIC) and inhibition zone around 300-μg sulfathiazole discs.*

interpretive criteria, exact probabilities were calculated for outcomes in the critical area between populations (Table 1). Theoretically, correct decisions would be made about 91% of the time if all strains with single zone measurements of 36 mm were considered members of the resistant population, and correct decisions would be made about 95% of the time if strains with a single zone determination of 40 mm were said to be members of the sensitive population. As zones increase and decrease from these cutoff points, it becomes increasingly probable that a strain belongs to either the sensitive or resistant population, respectively. The odds are approximately equal for a strain with an inhibition zone of 38 mm to belong to either population. Thus, it is suggested that strains with zones of 36 mm or less should be considered resistant, zones of 37 to 39 mm intermediate, and zones of 40 mm or more sensitive. Assuming equal proportions of sensitive and resistant strains, only five of 1,000 disc tests would result in zones of 37 to 39 mm. Only one or two of the 270 zone determinations in this study would be expected to fall in this range, though no such results were actually observed.

The reproducibility of the two susceptibility testing techniques was studied by examining the differences between the arithmetic mean zone or MIC of the strain and the three separate results contributing to each of these averages. The coefficients of variation for the disc test results on sensitive and resistant strains were substantially less than those for the MIC test (Table 2). The coefficient of variation for the MIC test on resistant strains was substantially less than the coefficient of variation for this test on sensitive strains; this largely resulted from the assumption that no variation occurred between tests on several strains

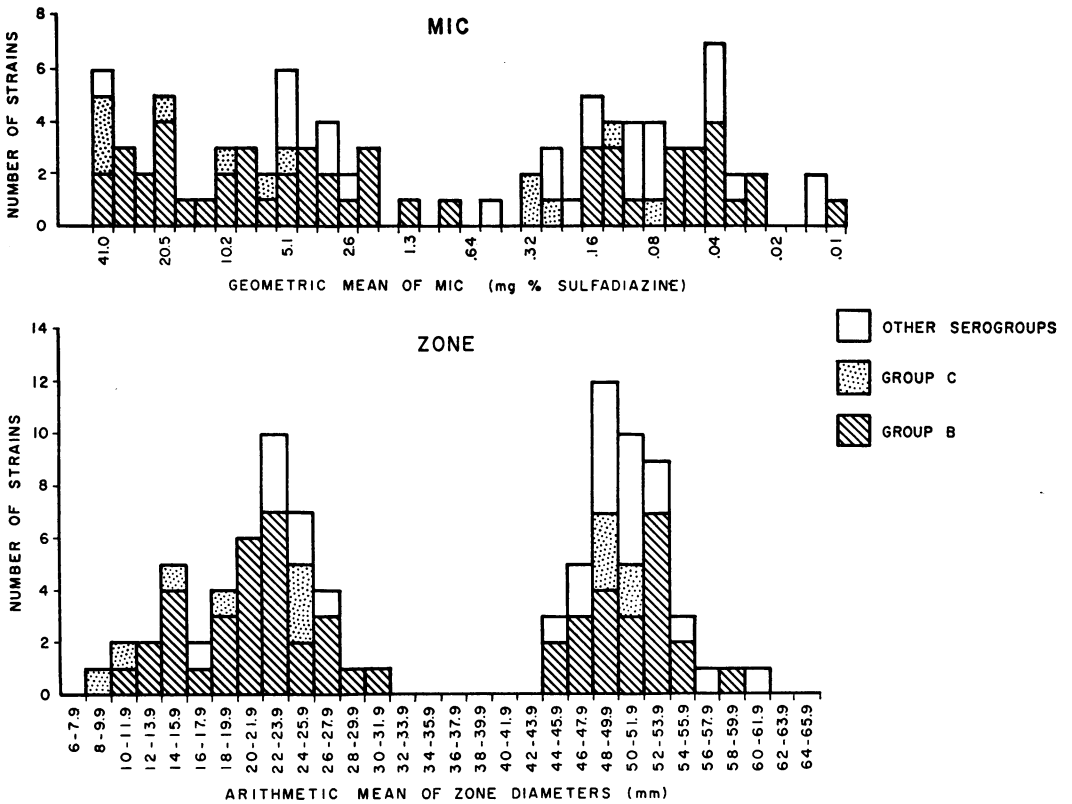

FIG. 2. *Distribution of average minimal inhibitory concentration (MIC) and zone in 90 meningococcal strains.*

TABLE 1. *Probabilities of obtaining various zone diameters from strains belonging to resistant and sensitive populations*

Recorded zone	Actual range of measurements	Probability that zone was produced by a strain of either population		Relative chance (%) that strain is	
		Resistant	Sensitive	Resistant	Sensitive
mm	*mm*				
35	34.5–35.4	.00300	.00005	98.3	1.7
36	35.5–36.4	.00180	.00017	91.3	8.7
37	36.5–37.4	.00100	.00038	74.2	25.8
38	37.5–38.4	.00064	.00085	43.0	57.0
39	38.5–39.4	.00037	.00174	17.5	82.5
40	39.5–40.4	.00020	.00341	5.5	94.5
41	40.5–41.4	.00012	.00622	1.9	98.1

TABLE 2. *Reproducibility of susceptibility tests*

Population	Standard deviation	Arithmetic avg	Coefficient of variation
			%
Sensitive population			
Disc test	2.4 mm	50.6 mm	4.8
MIC test	0.154 μg/ml	0.142 μg/ml	108.5
Resistant population			
Disc test	2.7 mm	20.7 mm	13.1
MIC test	5.36 μg/ml	14.58 μg/ml	36.8

with MIC values consistently above the highest concentration of sulfadiazine. No significant differences in the distribution of sensitivities of the major serogroups were observed (Fig. 2).

The necessity to correlate inhibition zones with MIC values is illustrated by the test results. Definite inhibition zones were observed with the most highly resistant strains (MIC values greater than 41.0 mg/100 ml of sulfadiazine), and the need to interpret disc results by quantitative measurement of inhibition zones is obvious. The perfectly bimodal population achieved with the MIC or disc test is striking, although the equal number of strains in both populations is largely the result of the initial selection procedures. Intermediate zones are definitely expected with further

use of this test, although they should occur infrequently if the quantitative susceptibilities of the 90 tested strains are representative of meningococci generally.

If only one MIC, rather than the geometric average of several MIC values, was correlated with inhibition zones, then the relationship between these tests would appear to be weak or absent. This probably reflects the variability in the MIC tests, since the bimodal population is separated by only approximately one plate (0.79 to 1.2 mg/100 ml), and it is likely that strains could be misclassified on the basis of a single plate test. Standard tests for susceptibility testing of meningococci usually use sulfadiazine, and this compound was also used in our study.

The decision to use 300-μg sulfathiazole discs was made because these discs are commonly available and because the interpretation of results from such discs is said to be interchangeable with the results with other sulfonamides (8), although we did not specifically study this issue. However, these high-content discs result in very large zones with sensitive strains, and obviate the possibility of performing more than one disc test on a single plate.

The reproducibility of test results with both discs and plates depends on performing the method exactly as described. The agar medium and thickness and many other variables may affect the test results. Indeed, it was discovered in preliminary work that freshly prepared sulfathiazole plates were essential for reproducible MIC values, since significant trends for higher MIC values were noted when plates had aged as little as 5 days, presumably due to deterioration of the drug.

Past experience has shown that meningococci with MIC values of less than 1.0 mg/100 ml of sulfathiazole can be eradicated from the nasopharynx, and 1.0 mg/100 ml by a plate test has been considered a reliable concentration for separating sensitive and resistant strains (4). Of course, the value of an MIC may vary according to the test procedure. However, the bimodal population should persist despite differences in methodology and changes in the relative size of each population. Primary emphasis in the interpretation of disc test results has been placed on the bimodal population. However, the recommended quantitative interpretations of the disc test results are consistent with the use of MIC values of 1.0 mg/100 ml or more as an indication of resistance of meningococci to sulfonamides.

The standardized disc test described here should allow rapid and reliable determinations of sulfonamide susceptibility of meningococcal strains, should make such determinations accessible to small laboratories, and should prove useful as a guide to prophylactic therapy and as an epidemiologic tool.

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