# E Test as Susceptibility Test and Epidemiologic Tool for Evaluation of *Neisseria meningitidis* Isolates

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The E test (AB Biodisk, Solna, Sweden), a new approach developed to test antimicrobial susceptibility, was compared with the agar dilution method for seven-drug antibiogram analysis of *Neisseria meningitidis* isolates. The overall E-test quantitative accuracy ($\pm 1$ $\log_2$ dilution) was 93% compared with that of agar dilution testing. The E test was then used to perform the susceptibility tests on a 10-year sample of 102 *N. meningitidis* isolates, including 5 from a recent epidemic outbreak in the University of Iowa (Iowa City) community. The E test proved to be an efficient methodology for identifying common source clusters of meningococcal disease having resistance to rifampin or sulfonamides. Moreover, the data demonstrated a recent increase in penicillin MICs (MIC for 90% of strains, 0.094 $\mu$g/ml) and an escalation of high-level resistance to trimethoprim-sulfamethoxazole (33%) and rifampin (14%). The E test should be considered a simple and accurate susceptibility method for the emerging need to test meningococci and other pathogenic neisserias. Chocolate Mueller-Hinton agar was observed to provide the best support of growth and E-test MIC results that correlated well with results of the reference agar dilution method previously used for neisserias.

*Neisseria meningitidis* is a gram-negative diplococcus that produces a broad spectrum of human diseases, ranging from transient fever and bacteremia to fulminant meningococcemia and meningitis (11, 17). In cases of severe meningococcal infection, the onset and progression of symptoms are rapid, and the end result is frequently death or a significant morbidity (11). Moreover, meningococcal isolates have the capacity to spread rapidly from person to person, usually in relatively confined populations of young people such as college students or military recruits (11).

Meningococci have long been considered uniformly susceptible to penicillin (11, 17). For that reason, susceptibility testing was seldom undertaken by clinical laboratories. Recently, however, penicillin-resistant strains of *N. meningitidis* have begun to emerge (3–5, 7, 12–14, 16, 17). Given the fulminant course of *N. meningitidis* infections and the organism's potential to cause community outbreaks of disease, rapid and accurate antimicrobial susceptibility testing plays an increasingly important role in identifying penicillin-resistant isolates for introducing appropriate antimicrobial chemotherapy and prophylaxis. Moreover, antibiogram analysis using very precise quantitative methods could also be useful as an epidemiologic tool for comparing isolates from different patients in suspected outbreaks (2).

The E test (AB Biodisk, Solna, Sweden) is a relatively new susceptibility testing method based on the diffusion of a continuous concentration gradient of an antimicrobial agent from a plastic carrier strip into an agar medium (1, 2, 8, 15). There have been several studies evaluating the E test as a routine susceptibility testing method and comparing it with reference methods such as broth microdilution, agar dilution, and disk diffusion (1, 15). In general, the E-test-determined MICs have compared favorably to those from the reference methods, some investigations using fastidious species or pathogenic neisserias (8, 15). Moreover, the E test

has the added advantage of being easy to perform and interpret. The purpose of this study was to correlate the E-test MICs with the National Committee for Clinical Laboratory Standards (NCCLS) standardized GC agar dilution MICs (10) by using *N. meningitidis* and also to evaluate the utility of the E test as an epidemiologic tool (2) for antibiogram analysis of isolates from two recent community outbreaks of meningococcal disease.

## MATERIALS AND METHODS

**Antimicrobial agents.** The E-test strips were prepared with appropriate antimicrobial agents and provided by AB Biodisk. Antimicrobial powders for comparison MIC tests were obtained from their domestic manufacturers. The following antibiotics were tested by both methods: penicillin, rifampin, ceftriaxone, cefotaxime, trimethoprim-sulfamethoxazole, tetracycline, erythromycin, and ciprofloxacin.

**Bacterial strains.** A total of 102 *N. meningitidis* isolates were studied; 85 of the isolates were from Iowa, and 17 were from Illinois. The 85 Iowan isolates included 5 from an outbreak of meningococcemia and septic arthritis that occurred in the University of Iowa (Iowa City) community between November 1992 and January 1993, with 6 strains from other cases distributed throughout the state during the same time period. The remaining Iowan isolates were subcultured from the stock culture collection of the Anti-Infectives Research Center and the laboratory of Peter Densen (University of Iowa College of Medicine). The latter isolates had been collected over a 9-year period (June 1984 to January 1993) and included isolates from 55 cases of documented invasive *N. meningitidis* infection and 18 cases of asymptomatic colonization. The 17 Illinoisan isolates included 12 from an outbreak of meningococcal disease that occurred on the University of Illinois Champaign-Urbana campus. The remaining five Illinoisan isolates were from sporadic cases that occurred throughout that state over the same interval of time.

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**Susceptibility methods.** The agar dilution MICs were determined by using GC agar and Mueller Hinton (MH) agar according to the NCCLS method (10). Briefly, agar plates were inoculated with $10^4$ CFU per spot and incubated at 35°C under an atmosphere of 5 to 7% $CO_2$. The MICs were interpreted following 24 h of incubation. Because of poor and inconsistent growth support with approximately 20% of strains, the MH agar data were deleted. However, the 80% of strains that displayed adequate growth on MH agar gave MIC results that were nearly identical ($\pm 1$ $\log_2$ dilution) to those obtained with GC agar (data not shown). Components (supplements) of the GC agar standardized test inhibited trimethoprim-sulfamethoxazole dilution tests, precluding direct comparisons to E-test results. However, two populations of MICs that indicated resistance or susceptibility to sulfonamide-like drugs were easily separated (see below).

The E test was performed according to the manufacturer's package insert. In brief, the inoculum concentration was adjusted to the turbidity of a 0.5 McFarland standard (9), a cotton swab was used to apply the inoculum, resulting in a confluent growth of bacteria, and no more than four E-test strips were placed onto each 150-mm-diameter plate agar surface. All plates were incubated at 35°C in conditions identical to those for the reference method. The plates were examined for formation of an elliptical zone of inhibited growth. The value printed on the strip edge at the intersection between the growth inhibition zones was recorded as the MIC for the organism. The E-test MICs were easy to interpret, particularly on chocolate MH agar plates, and there were generally clear, sharp end points for all of the antimicrobial agents tested. Growth problems as described for the dilution tests also occurred on MH agar with the disk test inoculation method (9) used for the E test. In MIC comparisons between MH agar alone and MH with chocolate, only organisms growing well on both media were utilized.

Definitions of susceptibility were as follows: for penicillin, $\leq 0.06$ $\mu g/ml$ indicated susceptibility, $>0.06$ to 1 $\mu g/ml$ indicated relative resistance, and $>1$ $\mu g/ml$ indicated resistance (4, 17); for all other antimicrobial agents, published NCCLS breakpoint definitions were utilized (9, 10). The breakpoint MIC for penicillin-susceptible strains is approximately 10-fold less than the expected peak levels in cerebrospinal fluid in patients with meningitis (6). Statistical analyses were performed on a computer to compare results of methods, media, and supplements. The method of least squares was utilized, and data were presented as regression equations and correlation coefficients ($r$).

## RESULTS

Table 1 summarizes the comparative results of the E test and agar dilution against 20 isolates of *N. meningitidis* that were randomly selected from the available pool of 102 clinical isolates, and Fig. 1 shows the erythromycin scattergram comparing E-test and agar dilution MICs. The total quantitative accuracy ($\pm 1$ $\log_2$ dilution step) obtained between the E-test and agar dilution results was 93% for both MH agar and chocolate MH agar. Rifampin and ceftriaxone had the highest E-test quantitative accuracy (100% for both agents on both media). The poorest correlation was obtained with ciprofloxacin, as the E-test MIC for 40% of isolates tested was 2 $\log_2$ dilutions greater than the GC agar dilution MIC. There was a trend for E-test penicillin and rifampin MICs on MH agar to be 1 $\log_2$ dilution lower than the MIC determined with reference agar. But the E-test results on

TABLE 1. Distribution of differences in E-test and GC reference agar dilution MICs of seven antimicrobial agents for 20 *N. meningitidis* isolates

| Antimicrobial agent | E-test agar | No. of isolates with E-test difference[a] ($\log_2$ dilution[s]) in MIC | | | | |
| | | Lower | | Same (0) | Higher | |
| | | ≥2 | 1 | | 1 | ≥2 |
|---|---|---|---|---|---|---|
| Penicillin | MH | 1 | 8 | 9 | 2 | 0 |
| | Chocolate | 0 | 3 | 15 | 2 | 0 |
| Rifampin | MH | 0 | 15 | 5 | 0 | 0 |
| | Chocolate | 0 | 11 | 6 | 3 | 0 |
| Cefotaxime | MH | 1 | 5 | 12 | 2 | 0 |
| | Chocolate | 0 | 5 | 12 | 3 | 0 |
| Ceftriaxone | MH | 0 | 0 | 20 | 0 | 0 |
| | Chocolate | 0 | 0 | 20 | 0 | 0 |
| Erythromycin | MH | 0 | 4 | 14 | 2 | 0 |
| | Chocolate | 1 | 9 | 10 | 0 | 0 |
| Tetracycline | MH | 0 | 5 | 8 | 7 | 0 |
| | Chocolate | 0 | 0 | 7 | 12 | 1 |
| Ciprofloxacin | MH | 0 | 0 | 2 | 10 | 8 |
| | Chocolate | 0 | 0 | 2 | 10 | 8 |
| Total | MH | 2 | 37 | 70 | 23 | 8 |
| | Chocolate | 1 | 28 | 72 | 30 | 9 |

[a] Compared with GC reference agar dilution MFC.

chocolate MH agar were more consistent with the reference value. Moreover, the overall quantitative accuracy for each of these clinically important agents was at least 95%.

Figure 1 presents correlation data for erythromycin, an antimicrobial agent with a broad range of MICs for *N. meningitidis*. E-test and reference erythromycin MICs correlated highly ($r = 0.89$) over the 0.06- to 2-$\mu g/ml$ MIC range. Rifampin also had a high correlation ($r = 0.88$). All other drugs had narrow MIC ranges (2 to 4 $\log_2$ dilutions) and lower correlation statistics ($r = 0.23$ to 0.64). Overall, the results indicate that the E test compares quite favorably with the agar dilution method for determining MICs of antimicro-
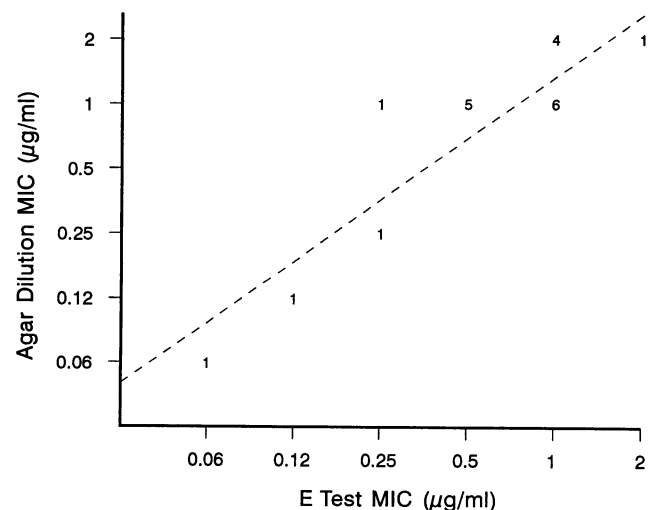


FIG. 1. Scattergram demonstrating the correlation ($r = 0.89$) between GC agar dilution reference erythromycin MICs and E-test erythromycin MICs determined on chocolate MH agar. A total of 20 strains that produced a regression equation of $y = 0.8 + 0.97x$ were tested.

TABLE 2. Susceptibility testing results for 102 *N. meningitidis* isolates by the E test[a] with eight drugs

| Antimicrobial agent | MIC (μg/ml)[b] | | | % Susceptible (μg/ml[c]) |
|---|---|---|---|---|
| | 50% | 90% | Range | |
| Penicillin | 0.064 | 0.094 | 0.016–0.25 | 84.3 (≤0.06) |
| Cefotaxime | 0.006 | 0.012 | ≤0.002–0.023 | 100.0 (≤0.25) |
| Ceftriaxone | ≤0.002 | ≤0.002 | ≤0.002–0.003 | 100.0 (≤0.25) |
| Rifampin | 0.032 | 0.19 | ≤0.016–>256 | 97.1 (≤1) |
| Trimethoprim-sulfamethoxazole[d] | 0.5 | >32 | 0.012->32 | 70.6 (≤2) |
| Ciprofloxacin | 0.006 | 0.008 | ≤0.002–0.012 | 100.0 (≤1) |
| Erythromycin | 0.75 | 1.5 | 0.047–2.0 | 42.2 (≤0.5) |
| Tetracycline | 0.75 | 1.5 | 0.25–2.0 | 100.0 (≤4) |

[a] Results were obtained with chocolate MH agar.
[b] 50% and 90%, $MIC_{50}$ and $MIC_{90}$.
[c] Breakpoint MIC in for susceptibility (10).
[d] Listed concentrations are of the trimethoprim component of a 1:19 ratio test.

bial agents against *N. meningitidis* and that chocolate MH agar would support the growth of the greatest number of clinical strains compared with MH agar alone.

Table 2 shows the susceptibility testing results for 102 *N. meningitidis* isolates and eight antimicrobial agents. A high potency was observed for cefotaxime, ceftriaxone, and ciprofloxacin (MICs for 90% of strains [$MIC_{90}$s], ≤0.012 μg/ml). Drugs of choice such as rifampin were 98.0% effective against the isolates, and 84.3% of strains tested were susceptible (≤0.06 μg/ml) to penicillin. Penicillin MICs for all remaining isolates were between 0.094 and 0.25 μg/ml, i.e., isolates were relatively resistant. The penicillin $MIC_{50}$ was at the widely used susceptibility breakpoint concentration of 0.06 μg/ml (4, 17). Greater resistance was identified with erythromycin and trimethoprim-sulfamethoxazole, with overall susceptibilities of only 42.2 and 70.6%, respectively.

An extreme polarity of MICs was observed for trimethoprim-sulfamethoxazole by the E test. MICs for all trimethoprim-sulfamethoxazole-susceptible meningococci were ≤1.5 μg/ml (70.6%), in contrast to the MICs of >32 μg/ml for the resistant strains. MICs for all rifampin-resistant isolates were >256 μg/ml.

Table 3 provides a chronologic grouping of the rifampin and trimethoprim-sulfamethoxazole susceptibility patterns of all 102 *N. meningitidis* strains according to year (1984 to 1993) of isolation. The table clearly demonstrates that trimethoprim-sulfamethoxazole resistance has emerged since 1990 (33% among 1992 to 1993 Iowan isolates). The only previous trimethoprim-sulfamethoxazole-resistant strains were in 1985 to 1986 (33 to 37% resistant). All of the rifampin-resistant strains appeared in a 1992 to 1993 Iowa City epidemic cluster of patients.

Table 4 displays the antibiogram profile of 11 *N. meningitidis* isolates collected in the state of Iowa between November 1992 and January 1993. Five of the cases (cases 1 to 5, Table 4) occurred in the University of Iowa community, while the remaining six cases were distributed sporadically throughout the state. The five University of Iowa-associated

cases occurred in two different clusters. One cluster involved two students (cases 1 and 2, Table 4). The *N. meningitidis* isolates from these two patients had identical E-test antibiograms (phenotype A) and displayed a high-level resistance to trimethoprim-sulfamethoxazole (>32 μg/ml). A similar antimicrobial susceptibility profile was seen in the isolates from the 1991 University of Illinois outbreak (Table 4), and the two Iowan patients had recently visited the University of Illinois campus for an athletic event.

The source of the second community outbreak was traced to a barroom in Iowa City. For these three isolates (cases 3 to 5, Table 4) E-test MICs were also nearly identical (phenotype B); however, the isolates' resistance to rifampin and pan-susceptibility to the remaining seven antimicrobial agents clearly distinguished them from the other epidemic cluster. The remaining six Iowan isolates that occurred sporadically throughout the state (two in the same town) over the same time period were all susceptible to the eight antimicrobial agents (phenotype C) tested (cases 6 to 11, Table 4). Variation of the E-test MIC antibiograms between isolates with the same phenotype was less than 1 $\log_2$ dilution step.

## DISCUSSION

Although penicillin remains the drug of choice for serious meningococcal disease, the drug appears to be less active because of emerging penicillin-binding protein-mediated resistance (3–5, 7, 12, 13, 15, 16). β-Lactamases have also been reported in meningococci, but this resistance mechanism has remained rare (3, 5). Furthermore, widely used prophylactic agents such as sulfonamides and rifampin are not applicable to many clinical settings because of organism resistance (11, 17, 18) or other pharmacokinetic factors. These problems were confirmed in this investigation that identified (i) increased resistance to penicillin among recent isolates (84.3% susceptible), (ii) reduced activity of rifampin and trimethoprim-sulfamethoxazole against epidemic isolates, and

TABLE 3. Trimethoprim-sulfamethoxazole and rifampin susceptibility patterns of 102 *N. meningitidis* isolates (1984 to 1993)

| Antimicrobial agent | % Susceptible in isolation yr (no. tested): | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1984 (8) | 1985 (12) | 1986 (8) | 1987 (9) | 1988 (4) | 1989 (7) | 1990 (14) | 1991 (19[a]) | 1992–1993 (21) |
| Trimethoprim-sulfamethoxazole | 100 | 67 | 63 | 100 | 100 | 100 | 71 | 32 | 67 |
| Rifampin | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 86 |

[a] Includes 17 strains from Illinois.

TABLE 4. E-test antimicrobial susceptibility patterns of 22 *N. meningitidis* isolates from Iowa (1992 to 1993) and Illinois (1991)

| Case no. (serogroup) | MIC (μg/ml) of: | | | | | | | | Phenotype |
|---|---|---|---|---|---|---|---|---|---|
| | Penicillin | Trimethoprim-sulfamethoxazole | Rifampin | Ciprofloxacin | Cefotaxime | Ceftriaxone | Erythromycin | Tetracycline | |
| Iowan cases | | | | | | | | | |
| 1 (C$^a$) | 0.064 | >32 | 0.032 | 0.008 | 0.012 | ≤0.002 | 0.75 | 1.5 | A |
| 2 (C$^a$) | 0.094 | >32 | ≤0.016 | 0.012 | 0.008 | ≤0.002 | 1.0 | 1.0 | A |
| 3 (C$^b$) | 0.094 | 0.38 | >256 | 0.006 | 0.012 | ≤0.002 | 0.5 | 1.0 | B |
| 4 (C$^b$) | 0.064 | 0.50 | >256 | 0.008 | 0.008 | ≤0.002 | 0.5 | 1.0 | B |
| 5 (C$^b$) | 0.064 | 0.25 | >256 | 0.006 | 0.008 | ≤0.002 | 1.0 | 0.75 | B |
| 6 (C) | 0.094 | 0.38 | 0.125 | 0.006 | 0.012 | ≤0.002 | 0.5 | 1.5 | C |
| 7 (C) | 0.064 | 0.38 | 0.064 | 0.008 | 0.008 | ≤0.002 | 0.38 | 1.5 | C |
| 8 (C) | 0.064 | 0.38 | 0.094 | 0.006 | 0.008 | ≤0.002 | 1.0 | 1.0 | C |
| 9 (B) | 0.047 | 0.19 | 0.047 | 0.006 | 0.006 | ≤0.002 | 0.5 | 0.75 | C |
| 10 (B) | 0.023 | 0.094 | 0.023 | 0.004 | 0.004 | ≤0.002 | 0.5 | 0.75 | C |
| 11 (X) | 0.047 | 0.125 | 0.19 | 0.006 | 0.006 | ≤0.002 | 1.5 | 0.5 | C |
| Illinoisan cases (range$^c$) | 0.047–0.125 | >32 | ≤0.016–0.094 | 0.006–0.023 | 0.008–0.023 | ≤0.002 | 0.75–1.5 | 0.75–1.5 | A |

$^a$ Epidemiologically related cluster also similar to that identified in Illinois.
$^b$ Second meningococcal cluster with unusual rifampin resistance.
$^c$ Range of MICs for epidemic strains (12 cases from Illinois).

(iii) the need for a simple susceptibility test to direct therapy, prophylaxis, and epidemiologic studies.

Penicillin-resistant or relatively penicillin-resistant isolates have been reported essentially worldwide and in some locations represent approximately one-half of all strains (4). The mechanism of resistance to the penicillins is an altered penicillin-binding protein 2 that may have been derived from a related *Neisseria* sp. such as *N. flavescens* or *N. lactamica* (15). Although Campos (4) indicated that some cephalosporins and other penicillins (ampicillin and oxacillin) may also be less active against these strains, our experience with cefotaxime (MIC$_{90}$, 0.012 μg/ml) and ceftriaxone (MIC$_{90}$, ≤0.002 μg/ml) showed an average potency 8- to 64-fold greater than that of penicillin. Thus, the newer parenteral cephalosporins emerge as potential therapeutic agents with excellent activity and favorable pharmacokinetics.

The current prophylaxis agents such as rifampin and sulfonamide also are less usable against contemporary *N. meningitidis* isolates, and high-level resistance to these drugs has developed on chemoprophylaxis (18), resulting in clinical infections. Alternative oral agents include the fluoroquinolones (ciprofloxacin MIC$_{90}$, 0.008 μg/ml) and the newer oral cephalosporins. Ciprofloxacin was utilized for some patient contacts in the Iowa City epidemic with apparent clinical success, i.e., no development of meningococcal disease.

To identify the need for alternative therapy or prophylaxis regimens, a simple in vitro susceptibility test is a must (17). Dilution tests for these infrequently encountered meningococcal cases or epidemics are very expensive, cumbersome, and yet to be standardized (9, 10, 17). Attempts to modify the disk diffusion method (4) were promising but required special potency disks and several disk interpretations and have not been reproducible in other laboratories (17).

The Centers for Disease Control and Prevention have recommended an MH broth microdilution method for laboratories wishing to test *N. meningitidis* isolates (17). Alternatively, plain MH agar or chocolate MH agar dilution tests (10) could be used (17). However, the E-test method was proven comparable to the Centers for Disease Control and

Prevention (17) and NCCLS (10) methods for pathogenic neisserias. In the current study, nearly all (≥95%) penicillin MICs by the E test were ±1 log$_2$ dilution step compared to recommended agar dilution results (10, 17). Endpoints for the E test on chocolate MH agar were easily read, the medium supported all strains tested, and a single 150-mm-diameter plate could rapidly produce the needed information for penicillin, a newer cephalosporin (cefotaxime or ceftriaxone), rifampin, a fluoroquinolone (ciprofloxacin or ofloxacin), and trimethoprim-sulfamethoxazole. Strains relatively resistant (MICs > 0.06 to 1 μg/ml) to penicillin (4 of the 20 strains tested) were readily categorized, and the high-level resistance to rifampin and sulfonamides was clearly defined. These results, although preliminary, strongly suggest that the E test is an accurate, efficient methodology for susceptibility testing of meningococci.

The E-test method applied to epidemiology (2) and therapeutics in the cited epidemic was valuable in defining the most active treatments, prophylaxis, and epidemic patient clusters. The preliminary antibiograms were confirmed by molecular microbiology methods using restriction digests and contour-clamped homogeneous electric field procedures (6a). The antibiogram, however, could be of limited value among the pan-susceptible strains and the emerging, more highly prevalent sulfonamide-resistant strains. The use of an E-test antibiogram and serologic typing could screen meningococcal strains for subsequent testing by the more expensive, time-consuming molecular epidemiologic procedures. The cost of an E-test antibiogram (five drugs) would average $10.50 to $13.50 for reagents plus minimal labor costs.

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