

Comparison of Broth Microdilution and E-test for Susceptibility Testing of *Neisseria meningitidis*

ALVARO PASCUAL,^{1,2*} PROVIDENCIA JOYANES,² LUIS MARTÍNEZ-MARTÍNEZ,^{1,2}
ANA ISABEL SUÁREZ,² AND EVELIO J. PEREA^{1,2}

Department of Microbiology, School of Medicine, University of Seville,¹
and University Hospital V. Macarena,² Seville, Spain

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The susceptibilities of 54 clinical isolates of *Neisseria meningitidis* to penicillin, cefotaxime, ceftriaxone, cefepime, imipenem, ciprofloxacin, chloramphenicol, and rifampin were determined by the microdilution method in both cation-adjusted Mueller-Hinton broth (CAMHB) and *Haemophilus* test medium (HTM). Poor growth was observed in 16.6 and 9% of the strains in CAMHB and HTM, respectively. As a result, the growth of the 54 *N. meningitidis* strains was evaluated in three other commercially available batches of CAMHB and in one in-house batch of HTM. Poor growth was observed for 9.3 to 16.6% of the strains in all four batches. More important, three of the CAMHB batches failed to support growth for 3.7 to 33.3% of the strains; 3.7% of the strains did not grow in the in-house-prepared HTM. Ten (18.7%) strains were relatively resistant to penicillin (RRP; MIC, >0.125 µg/ml) in CAMHB and 13 (24%) strains were RRP in HTM. The percentages of agreement obtained by using CAMHB as the reference ranged from 78% for cefepime to 100% for ceftriaxone. Seven minor errors were observed for penicillin; five of them were for strains susceptible to penicillin in CAMHB and RRP in HTM. All strains were susceptible to the other antimicrobial agents evaluated. The growth of *N. meningitidis* was also evaluated in four batches of Mueller-Hinton agar (MHA). In two of them, 3.7 and 44.4% of the strains did not grow, and considering all four batches, 5.5 to 11.1% grew poorly. All strains grew adequately in MHA supplemented with blood (MHA-b). The activities of penicillin and cefotaxime were also evaluated by the E-test in MHA and MHA-b. The proportion of RRP strains were 24% in MHA and 59% in MHA-b. For penicillin, the percentages of agreement of the E-test with the microdilution method in CAMHB (reference) were 64.8 and 70.3% in MHA and MHA-b, respectively. For cefotaxime, the agreement was 98.1%. Minor errors for the penicillin MIC were detected for 38% of the strains tested. Further studies are needed to define adequate culture media for reference methods to evaluate the susceptibility of *N. meningitidis* to antimicrobial agents.

Neisseria meningitidis is an important cause of meningitis and community-acquired septicemia in children (23). Penicillin G has been considered the treatment of choice for infections caused by this organism. Over the past decade, *N. meningitidis* strains with decreased susceptibility to penicillin (MICs of penicillin G, ≥ 0.125 µg/ml) have been described in Europe (22, 25–27, 30), South Africa (3), South America (13), and North America (11, 24). The incidence in Spain of meningococci with decreased susceptibility to penicillin increased from 0.4% in 1985 (25) to 67% in 1994 (9). Resistance in these strains is related to altered forms of PBP 2 (14). A few β -lactamase-producing strains have also been described in South Africa, Spain, and Canada (3, 7, 8, 20).

The National Committee for Clinical Laboratory Standards (NCCLS) has recommended microdilution (with cation-adjusted Mueller-Hinton broth [CAMHB]) or agar dilution (with Mueller-Hinton agar [MHA]) for susceptibility testing of *N. meningitidis* (17), but several investigators have observed that some strains grow poorly in Mueller-Hinton media (13). For this reason, other media, including *Haemophilus* test medium (HTM) (13), GC agar (29), and MHA supplemented with blood (MHA-b) (4, 5), have also been used. There are no specific guidelines from NCCLS regarding the susceptibility of meningococci by disk diffusion, but the use of 1-µg oxacillin

and 2-U penicillin G disks has been proposed for the screening of *N. meningitidis* moderately susceptible to penicillin (and/or ampicillin) (4, 5). A more recent possibility for the quantitative determination of antimicrobial activity is the E-test. This system can be used to study the susceptibilities of microorganisms, including fastidious bacteria such as *Haemophilus influenzae* or *Streptococcus pneumoniae*, and is particularly useful when limited numbers of antimicrobial agents are tested (15). When compared with the agar dilution method, the E-test has been shown to be a reliable method of evaluating the antimicrobial susceptibility of *N. meningitidis* (10, 12).

The present study was undertaken to evaluate the capabilities of different culture media (and batches) commonly used in susceptibility testing assays to support the growth of *N. meningitidis*. Moreover, the susceptibility patterns of *N. meningitidis* organisms isolated in Seville, in southern Spain, and the usefulness of the E-test as a method of recognizing *N. meningitidis* isolates relatively resistant to penicillin were also evaluated.

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MATERIALS AND METHODS

Bacteria. Fifty-four strains of *N. meningitidis* isolated between 1992 and 1994 from cerebrospinal fluid (24), blood (8), joint fluid (1), and pharyngeal exudates (21) at the Microbiology Laboratories of both the University Hospital Virgen Macarena and the University Hospital Virgen de Valme in Seville (Spain) were used. The strains were identified by standard bacteriological methods, including

* Corresponding author. Mailing address: Department of Microbiology, School of Medicine, Apdo. 914, 41080 Seville, Spain. Phone: 34-5-4557448. Fax: 34-5-4377413.

Gram stain, oxidase, aminopeptidase activity, and carbohydrate degradation tests (16). Serogrouping was performed by agglutination with commercial antisera (Difco Laboratories, Detroit, Mich.). β -Lactamase activity was assayed with the chromogenic cephalosporin nitrocefin. After identification, the bacteria were maintained in 10% glycerol in tryptic soy broth at -70°C until they were ready for use. Before they were used, the bacteria were subcultured twice on Columbia agar plates supplemented with 5% sheep blood agar at 35°C in 5% CO_2 . *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25299, and *Pseudomonas aeruginosa* ATCC 27853 were used as control strains in microdilution assays, and *S. aureus* ATCC 25923 was also studied for the E-test.

Media. The growth of the 54 strains of *N. meningitidis* was evaluated in several media. Four batches of Mueller-Hinton broth (MHB, three from Difco [11497, 59462 JG, and 69437 JC], and one from Oxoid [040-46950; Oxoid, Basingstoke, England]), two batches of HTM (one from BBL [I5EPVT; Becton Dickinson, Cockeysville, Md.] and one prepared in house according to NCCLS guidelines), and four batches of MHA (three from Difco [34970 JD, 63154 JC, and 65892 JA] and one from Oxoid [060-48017]) were evaluated.

MHA (Difco) from batch 34970 JD was used to prepared MHA supplemented with 5% sheep blood (MHA-b).

The culture media were inoculated and incubated according to NCCLS guidelines for susceptibility testing of *N. meningitidis*. The growth was evaluated independently by three different observers.

According to the results obtained from these experiments (see below), batch 34970 JD of MHA, batch 11497 of MHB, and batch I5EPVT of HTM were used for susceptibility testing assays.

Microdilution assay. An in-house microdilution method was used. We followed the guidelines described by NCCLS (17) using either CAMHB or HTM. The following antimicrobial agents were used as powders of known potency: penicillin G (Sigma, St. Louis, Mo.), cefotaxime (Hoechst, Barcelona, Spain), ceftriaxone (Sigma), cefepime (Bristol Myers Squibb, Madrid, Spain), imipenem (Merck Sharp & Dohme, Madrid, Spain), ciprofloxacin (Bayer AG, Barcelona, Spain), chloramphenicol (Sigma), and rifampin (Sigma). Twofold dilutions across a range of 0.008 to 16 $\mu\text{g/ml}$ were used. The inoculum (5×10^4 CFU per well) was prepared in CAMHB from cultures grown (for 20 to 24 h) on Columbia agar with 5% sheep blood. The plates were incubated at 35°C in a 5% CO_2 atmosphere, and the MIC results were read after 24 h.

E-test. Plates containing either MHA or MHA-b were used for the E-test. The inoculum was prepared by suspending bacteria grown on Columbia agar with 5% sheep blood for 24 h in CAMHB to a concentration of about 10^8 CFU/ml. The plates were inoculated with a cotton swab according to NCCLS guidelines for the disk diffusion assay (18). E-test strips (Biodisk, Solna, Sweden) of penicillin G and cefotaxime were applied to the surfaces of the plates. For each microorganism, one 150-mm-diameter plate of each medium was used, and two strips were applied to each agar surface. The plates were incubated at 35°C in a 5% CO_2 atmosphere, and the MIC results were read after 24 h.

Evaluation criteria. Because of the absence of accepted breakpoints for the assignment of *N. meningitidis* to interpretive categories, definitions of susceptibility were as follows: for penicillin an MIC of ≤ 0.06 $\mu\text{g/ml}$ indicated susceptibility, an MIC of 0.125 to 1 $\mu\text{g/ml}$ indicated relative resistance to penicillin (RRP), and an MIC of ≥ 2 $\mu\text{g/ml}$ indicated resistance. For all other antimicrobial agents, published NCCLS breakpoint definitions were used (17). For comparison of the results determined by the E-test, the MICs obtained by this method were rounded up to the next higher twofold dilution.

RESULTS

Thirty-four strains were serogroup B, 18 were serogroup C, and 2 were nonagglutinable. None of the strains produced β -lactamase.

The percentage of strains that either grew poorly or that did not grow at all in the different media and batches is presented in Table 1. Remarkable differences in the percentages of strains which did not grow in the four media evaluated in the study were observed. These ranged from 0 to 44.4% in MHA, from 0 to 33.3% in CAMHB, and from 0 to 3.7% in HTM. All the strains grew properly on MHA-b.

Data on the antimicrobial susceptibility of *N. meningitidis*, as determined in CAMHB, are presented in Table 2. When considering all 54 *N. meningitidis* strains, the MIC at which 90% of isolates are inhibited (MIC_{90}) for penicillin was 0.125 $\mu\text{g/ml}$. Ten (18.7%) strains were RRP. Eight of these strains were serogroup B (24% of serogroup B strains) and two were serogroup C (11% of serogroup C strains). All strains were susceptible to the other antimicrobial agents evaluated. The activity of imipenem was lower than that of the broad-spectrum cephalosporins.

TABLE 1. *N. meningitidis* strains that grew poorly or that did not grow in different culture media

Medium	Percent	
	Poor growth	No growth
CAMHB		
Difco batch 11497	16.6	0
Difco batch 59462 JG	13.0	20.4
Difco batch 69437 JC	9.3	33.3
Oxoid batch 040-46950	14.8	3.7
HTM		
BBL batch 15EPVT	9.0	0
In-house batch	16.6	3.7
MHA		
Difco batch 34970 JD	5.5	0
Difco batch 63154 JC	11.1	0
Difco batch 65892 JA	9.3	3.7
Oxoid batch 060-48017	11.1	44.4
MHA-b		
	0	0

When comparing the activities of the antimicrobial agents evaluated against penicillin-susceptible strains and RRP strains, it was found that the MIC_{90} s of rifampin and imipenem for the latter group were four times or greater and two times higher, respectively, than those for penicillin-susceptible strains. Moreover, the MIC_{90} s of rifampin for pharyngeal isolates were three dilution steps or more compared with those obtained for invasive strains (0.06 versus ≤ 0.008 $\mu\text{g/ml}$).

Nine of the 54 strains (16.6%) grew poorly in CAMHB, which made the reading of the endpoints for MIC determinations difficult. Therefore, we also evaluated HTM as an alternative medium for the microdilution assay. Even in this medium, poor growth of 5 (9%) strains was observed, and two of these strains also grew poorly in CAMHB. Thirteen (24%) strains were RRP in HTM, with five of them corresponding to strains which were susceptible in CAMHB.

The distribution of the differences in the MICs of the antimicrobial agents evaluated in CAMHB or HTM are presented in Table 3. The percentages of agreement within ± 1 twofold dilution step by using CAMHB as the reference ranged from 78% for cefepime to 100% for ceftriaxone. For penicillin the percentage of agreement was 81%. In this medium, 13 (24%) strains were RRP. Seven (13%) minor errors were observed for penicillin. No discrepancies were observed for the other antimicrobial agents.

With the E-test, the MIC_{90} s of penicillin in MHA and MHA-b were 0.25 and 0.38 $\mu\text{g/ml}$, respectively. By this

TABLE 2. Antimicrobial susceptibilities of *N. meningitidis* to eight antimicrobial agents in CAMHB

Antimicrobial agent	MIC ($\mu\text{g/ml}$)		
	50%	90%	Range
Penicillin	0.03	0.125	≤ 0.008 –0.25
Cefotaxime	≤ 0.008	≤ 0.008	≤ 0.008 –0.03
Ceftriaxone	≤ 0.008	≤ 0.008	≤ 0.008 –0.015
Cefepime	≤ 0.008	0.06	≤ 0.008 –0.06
Imipenem	0.125	0.25	0.015–0.25
Ciprofloxacin	≤ 0.008	≤ 0.008	≤ 0.008 –0.06
Chloramphenicol	0.5	1	0.125–2
Rifampin	≤ 0.008	0.015	≤ 0.008 –0.125

TABLE 3. Distribution in differences of MICs of eight antimicrobial agents for 54 strains of *N. meningitidis* determined in CAMHB (reference) and HTM

Antimicrobial agent	% Isolates with the following differences in MIC:							% Agreement
	≤-3	-2	-1	0	+1	+2	≥+3	
Penicillin	5.6	3.7	31.5	38.8	11.1	9.23	0	81.4
Cefotaxime	0	1.8	0	94.5	3.7	0	0	98.2
Ceftriaxone	0	0	3.7	94.5	1.8	0	0	100
Cefepime	5.6	13.0	18.5	55.5	7.4	0	0	81.4
Imipenem	11.1	9.3	40.8	25.9	11.1	1.8	0	77.8
Ciprofloxacin	1.8	1.8	1.8	90.9	3.7	0	0	96.5
Chloramphenicol	3.7	0	7.4	46.3	29.6	13.0	0	83.3
Rifampin	0	0	5.6	88.9	1.8	3.7	0	96.3

method, the percentages of RRP strains were 24% in MHA and 59% in MHA-b. Sixteen of the 54 strains (29%) including 11 from cerebrospinal fluid, 3 from pharyngeal exudates, and 2 from blood cultures were susceptible to penicillin in this medium but were RRP when they were tested on MHA-b. When comparing the results obtained in both media, no differences in the MICs (within the ranges that we studied) of cefotaxime determined by the E-test were observed.

The distribution of differences in the MICs of penicillin determined by the reference microdilution method in CAMHB and the E-test in either MHA or MHA-b are presented in Table 4. The percentages of agreement were 65 and 70% in MHA and MHA-b, respectively. When cefotaxime was considered, the agreement of the E-test with the reference microdilution method was 98.1% in both media.

DISCUSSION

The emergence of resistance to penicillin in a few β -lactamase-producing strains of *N. meningitidis*, and, in particular, the appearance of RRP strains in different countries (3, 11, 13, 22, 24–27), could throw open the question of the empirical treatment of meningococcal diseases with penicillin.

The correct evaluation of the resistance of *N. meningitidis* to penicillin and other antimicrobial agents should be based on a standardized methodology that allows comparison of results from different laboratories. At the moment NCCLS recommends the use of either an agar dilution method with MHA or a broth microdilution assay in CAMHB (17).

Nevertheless, the results from the present study show the great variability in the growth of *N. meningitidis* in different MHA and CAMHB culture media and in different batches of the same medium. The only medium allowing sufficient growth of *N. meningitidis* was MHA-b. These results question the usefulness of the culture media currently recommended by NCCLS and suggest that further studies are needed in order to establish the medium of choice for susceptibility testing of *N. meningitidis*.

We used the broth microdilution assay to study the activities of eight antimicrobial agents against 54 isolates of *N. meningitidis* isolated in southern Spain. In our study, the prevalence

of RRP strains was 18.7% in CAMHB, but it was 24% in both HTM and MHA and 59% in MHA-b. Other investigators from Spain have reported prevalences ranging from 48% (4) to 67% (9) by disk diffusion and agar dilution methods, respectively.

Considering the results obtained in CAMHB, we have not observed a higher percentage of serogroup C strains among the RRP strains, as was previously reported in our country (1, 2). Although we evaluated only 10 RRP strains, decreased activity of rifampin (and, to a lesser extent, that of imipenem) against these strains was observed. This decreased activity of rifampin was observed in pharyngeal isolates but not in invasive strains. The explanation for this finding remains obscure.

Since 16.6% of the *N. meningitidis* strains included in the study grew poorly in CAMHB, an alternative medium, HTM, was also evaluated in the microdilution assay. Although better growth was observed in HTM, 9% of the strains nonetheless grew poorly in this medium. When the results obtained with HTM were compared with those obtained with CAMHB, the agreements for the different antimicrobial agents ranged from 78% for cefepime to 100% for ceftriaxone. Minor errors were observed for penicillin (13%) but not for the other antimicrobial agents tested, and in fact, five strains which were susceptible in CAMHB were RRP in HTM. Differences in growth in both media may explain these results. Lopardo et al. (13) also observed a better growth of *N. meningitidis* in HTM than in MHB, but the differences in the MICs of penicillin observed with both media did not differ by more than 1 dilution step.

Both the disk diffusion assay and the E-test have been proved to be cost-effective for evaluating the activity of penicillin against *N. meningitidis*. Disks of β -lactams, including oxacillin (1 μ g) (4), penicillin (2 or 10 U) (4), and amdinocillin (10 μ g) (21), have been used to separate penicillin-susceptible and RRP strains of *N. meningitidis*, but the clinical usefulness of the results obtained by these screening tests is controversial (28). With the E-test, the inclusion of a broad-spectrum cephalosporin strip together with the penicillin strip will allow us to determine the activities of the antimicrobial agents more commonly used in the treatment of meningococcal disease.

The agreement of the E-test in MHA with the standard microdilution method in CAMHB within ± 1 dilution step was

TABLE 4. Difference in MICs of penicillin determined by the reference microdilution method in CAMHB and the E-test determined in either MHA or MHA-b

Medium	% Isolates with the following differences in MICs:							% Agreement
	≤-3	-2	-1	0	+1	+2	≥+3	
MHA	1.9	0	7.4	25.9	31.5	28.8	5.5	64.8
MHA-b	1.9	7.4	5.5	18.5	46.3	13.0	7.4	70.3

only 64.8%. All strains grew on MHA-b, but the agreement of the E-test in this medium with the microdilution method in CAMHB was still only 70.3%. These disagreements are related to increased MICs determined by the E-test. In fact, 34.3 and 20.4% of the MICs determined by the E-test in MHA and MHA-b, respectively, were ≥ 2 dilution steps higher than the MICs determined by the microdilution method in CAMHB. Data were also discrepant when results from the E-test in MHA and MHA-b were compared: 38% of minor errors were caused by strains susceptible to penicillin in MHA but RRP in MHA-b. We might speculate that the poor growth observed for some strains of *N. meningitidis* in CAMHB and MHA is the cause of such low levels of agreement between the E-test and the microdilution method. Other investigators have obtained higher levels of agreement when the E-test was compared with the agar dilution method (10, 12, 21). Carroll et al. (6) have also reported a high degree of correlation of the E-test with the reference microdilution method, but only strains susceptible to penicillin (≤ 0.06 $\mu\text{g/ml}$) were evaluated by those investigators.

Our results suggest that it is very difficult to establish whether an isolate of *N. meningitidis* is RRP. Fortunately, the clinical manifestations in and outcomes for patients infected with RRP strains are similar to those for patients infected with strains that are not resistant, and it has been indicated that similar treatments may be used for both *N. meningitidis* isolates that are penicillin susceptible and RRP (1). Molecular methods based on the detection of altered PBP 2 could help to elucidate whether a particular strain should be considered RRP, but until this is done, further studies on the activity of penicillin against *N. meningitidis* are warranted.

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