

Detection of Relatively Penicillin G-Resistant *Neisseria meningitidis* by Disk Susceptibility Testing

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Beginning in 1985, relatively penicillin G-resistant (Pen^r) meningococci which did not produce β -lactamase were isolated from the blood and cerebrospinal fluid of patients in Spain. We identified 16 Pen^r (mean MIC, 0.3 μ g/ml; range, 0.1 to 0.7 μ g/ml) and 12 penicillin-susceptible (Pen^s; mean MIC, \leq 0.06 μ g/ml) strains of *Neisseria meningitidis* by the agar dilution technique using an inoculum of 10⁴ CFU and questioned which disk susceptibility test would best differentiate these two populations. We compared the disk susceptibility of strains using disks containing 2 (P2) and 10 (P10) U of penicillin G, 2 (Am2) and 10 (Am10) μ g of ampicillin, and 1 μ g of oxacillin (OX1). We also investigated susceptibility with disks containing 30 μ g of each of cephalothin (CF30), cefoxitin (FOX30), cefuroxime (CXM30), and cefotaxime (CTX30) and 75 μ g of cefoperazone (CFP75) and determined by cluster analysis any correlation with the zone diameters obtained with P2 disks. Using the P2 and AM2 disks (in contrast to the P10 and AM10 disks), we correctly differentiated all the Pen^r from Pen^s isolates. In addition, the zone diameters with the P2 disk gave the best correlation with the penicillin G MIC determinations. All 16 Pen^r strains and 3 of 12 Pen^s strains showed zone diameters of 6 mm around OX1 disks, limiting the usefulness of OX1 disks. The zone diameters obtained with CF30, CXM30, and OX1 disks correlated with those obtained with the P2 disk, which suggests that these antibiotics have similar effects on these strains. In contrast, the data obtained with FOX30, CTX30, and CFP75 disks did not cluster with those obtained with the P2 disk, which suggests that there was a difference in the bacterial target or reflects their greater activity. We conclude that the P2 disk tests more readily identify Pen^r meningococci than do the standard P10 disk tests.

In the clinical laboratory, routine antimicrobial susceptibility testing of meningococci has not been required since these organisms have remained highly susceptible to penicillin (4). Thus, National Committee for Clinical Laboratory Standards guidelines for susceptibility testing of *Neisseria meningitidis* are not available (5).

Meningococci that were relatively resistant to penicillin G and that did not have detectable β -lactamase activity were isolated from the blood and cerebrospinal fluid of patients in Spain (J. A. Sáez-Nieto, D. Fontanals, J. García de Jalón, V. Martínez de Artola, P. Peña, M. A. Morera, R. Verdager, I. Sanfeliú, C. Belio-Blasco, J. L. Pérez-Sáenz, and J. Casal, *J. Infect.*, in press). The emergence of these relatively penicillin G-resistant isolates indicates that susceptibility testing needs to be performed. We sought to identify a disk susceptibility test which would accurately predict the resistance phenotype. We compared 16 penicillin-resistant (Pen^r) and 12 penicillin-susceptible (Pen^s) isolates using standard 10-U penicillin G and 10- μ g ampicillin disks. In addition, we tested the isolates with a 2-U penicillin G disk and one containing 2 μ g of ampicillin. We also examined the utility of disks containing oxacillin, cephalothin, cefuroxime, cefoxitin, cefoperazone, and cefotaxime to differentiate these strains.

MATERIALS AND METHODS

Bacterial strains. All strains were identified as *N. meningitidis* by using standard bacteriological methods, including

Gram stain, oxidase and aminopeptidase activity, and carbohydrate degradation tests (4). Serogrouping was performed by agglutination by using commercial antisera (Difco Laboratories, Detroit, Mich.). β -Lactamase activity was assayed with the chromogenic cephalosporin nitrocefin (7). All strains were isolated from cerebrospinal fluid or blood from patients in Spain during 1985 or 1986. All 28 isolates (16 Pen^r and 12 Pen^s) were initially characterized by a reference laboratory in Madrid, Spain, during a retrospective surveillance. These strains were isolated in different geographical areas of Spain, but 75% of them came from urban hospitals in Barcelona or nearby cities. Of 16 of the relatively penicillin-resistant isolates included in this study, 10 have been described previously (9; Sáez-Nieto et al., in press). Although 2 of the 10 strains were isolated from two patients who died, their clinical course could not be correlated with antibiotic resistance. The susceptible strains were isolated from the same geographic areas and hospitals.

Medium. The medium used for growth and determination of susceptibility studies was Mueller-Hinton agar (Difco) supplemented with 5% sheep blood (MHBd). Plate cultures were incubated at 36.5°C in ambient air (without increased CO₂).

Antibiotics and chemicals. Penicillin G was obtained from Sigma Chemical Co., St. Louis, Mo. Antibiotic susceptibility disks were obtained from BBL Microbiology Systems, Cockeysville, Md. The β -lactamase substrate nitrocefin was obtained from Glaxo Research, Ltd., Greenford, Middlesex, England.

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Determination of MIC. Penicillin G susceptibility testing was performed by the agar dilution method with MHBd (6). The strains were streaked onto fresh MHBd plates from vials of 50% tryptic (Difco) soy broth–10% glycerol stored at -70°C and incubated overnight. The next day the strains were suspended in phosphate-buffered saline, and the density was adjusted to a 0.5 McFarland standard. Colony counts were performed in duplicate on antibiotic-free media. The inocula that were tested contained 10^5 , 10^4 , and 10^3 CFU per spot and were plated with a Steers replicator. The concentrations of penicillin tested were 0.01, 0.03, 0.06, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1 $\mu\text{g}/\text{ml}$. The plates were prepared by mixing 1 volume of the antibiotic solution with 9 volumes of MHBd at 55°C . The MIC was defined as the lowest concentration of antibiotic which inhibited visible growth of the inoculum in comparison with growth on antibiotic-free media. Plates were examined after 18 to 24 h of incubation in ambient air at 36.5°C .

Disk susceptibility testing. After overnight growth on MHBd plates, whole-cell suspensions in phosphate-buffered saline were adjusted to a density equal to a 0.5 McFarland standard and were swabbed onto the surface of MHBd plates (diameter, 150 mm). The following disks were placed on the surfaces of two plates (five disks per plate): 2 and 10 U of penicillin G, 2 and 10 μg of ampicillin, 1 μg of oxacillin, 30 μg of cephalothin, 30 μg of cefuroxime, 30 μg of cefoxitin, 30 μg of cefotaxime, and 75 μg of cefoperazone. After overnight incubation in 5% CO_2 , the zone diameters were measured independently by four observers who had no knowledge of the suspected antibiotic susceptibility pattern. The mean values (rounded off to the nearest whole number) of the four observations are reported.

Statistics. (i) **Linear regression analysis.** The log of the penicillin MIC for the 10^4 inoculum for all 28 isolates was plotted against the zone diameters obtained with penicillin and ampicillin disks. Using the method of least squares, we determined the line of best fit and calculated the correlation coefficients.

(ii) **Cluster analysis.** Hierarchical cluster analysis (1) was used to investigate possible grouping of zone diameter values obtained with the 2-U penicillin G disk to determine if the Pen^r isolates expressed two levels of resistance and could be further subdivided. Computations were performed with S, a datum analysis, and a graphics system (2). The distance between individual pairs of points or between clusters of points was determined by hierarchical cluster analysis. The simple arithmetic difference between zone diameter values of two points was used in the calculations. Cluster analysis was also used to compare the performance of the various antibiotic disks (zone diameter values for each antibiotic were standardized to have a mean of 0.0 and a variance of 1.0) with all 28 strains. For the comparison of disks, the distance measure used was the Euclidean distance between the vectors of zone diameter values for individual disks. Clusters based on complete linkage (greatest distance between two clusters) were examined. The complete linkage method compared with the single linkage (nearest neighbor or closest distance between two clusters) and average linkage (average distance) methods was the most powerful in detecting individual clusters of multiple points.

RESULTS

Strains. Of the 16 relatively penicillin G-resistant strains, 11 were serogroup B, 4 were serogroup C, and 1 was autoagglutinable. Of the 12 susceptible strains, 8 were

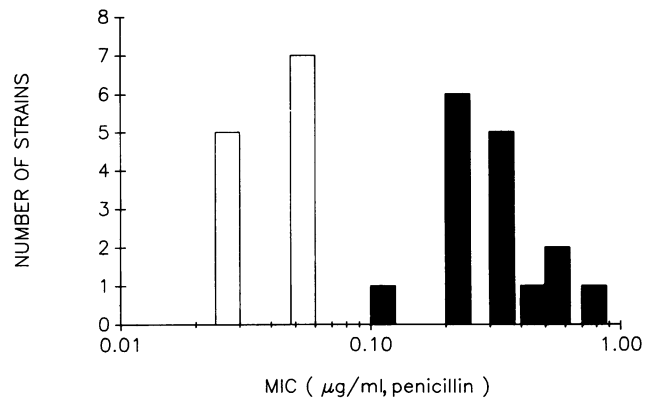


FIG. 1. Histogram of the penicillin G MIC at an inoculum of 10^4 CFU for 28 isolates of *N. meningitidis* obtained from cerebrospinal fluid or blood identified in Spain during 1985 and 1986. Symbols: □, susceptible isolates; ■, relatively resistant strains.

serogroup B, and 4 were serogroup C. None of the strains produced detectable β -lactamase.

MIC determinations. For 16 of 28 strains, penicillin G MICs were identical with the three inocula that were used (10^3 , 10^4 , and 10^5 CFU); for the remainder, a 1 log unit increase in inoculum increased the MIC by one dilution. At 10^3 CFU, the MIC for 21 of 28 strains was the same as that obtained with the 10^4 inoculum; the MIC for 16 of 28 strains was the same as that obtained with the 10^5 inoculum. For 24 of 28 strains, penicillin G MICs were the same when 10^4 or 10^5 CFU was tested. Twelve strains were inhibited by penicillin G at ≤ 0.06 $\mu\text{g}/\text{ml}$ (Fig. 1). Sixteen strains were inhibited by penicillin G concentrations of ≥ 0.1 $\mu\text{g}/\text{ml}$ (Fig. 1). Strains inhibited by penicillin G at ≤ 0.06 $\mu\text{g}/\text{ml}$ were defined as susceptible, while isolates growing in penicillin G concentrations of ≥ 0.1 and ≤ 1 $\mu\text{g}/\text{ml}$ were defined as relatively resistant.

Disk susceptibility. The relationship between the zone diameter obtained with disks containing 2 or 10 U of penicillin G and 2 or 10 μg of ampicillin and the penicillin G MIC are shown in Fig. 2. The best correlation was found with disks containing 2 U of penicillin G ($r = -0.93$) and 2 μg of ampicillin ($r = -0.89$).

The 2-U penicillin G disk differentiated all 12 susceptible strains from the 16 relatively resistant strains (Fig. 2A). Zone diameters of ≥ 28 mm correlated with susceptibility to penicillin G (MIC, ≤ 0.06 $\mu\text{g}/\text{ml}$), while zone diameters of ≤ 26 mm occurred with all relatively resistant strains. Of the Pen^r isolates, nine and seven strains, respectively, showed zone diameters between 22 and 26 mm and between 13 and 21 mm (Fig. 2A). With the standard 10-U penicillin G disk, no clear-cut zone diameter separated susceptible from resistant strains. With zone diameters of ≥ 38 mm, 10 of 12 susceptible isolates were detected (Fig. 2A). Two susceptible and two resistant strains, however, showed zone diameters of between 35 and 37 mm. The other 14 resistant strains showed zone diameters of between 27 and 34 mm (Fig. 2A).

Similar to the data with the 2-U penicillin G disk, the 2- μg ampicillin disk differentiated all the susceptible and relatively resistant strains (Fig. 2B). A zone diameter of ≥ 26 mm correlated with susceptibility to penicillin G, and one of ≤ 24 mm correlated with resistance. With the 10- μg ampicillin disk (Fig. 2B), a zone diameter of ≥ 36 mm was found with 11 of 12 susceptible strains. A susceptible strain (penicillin G MIC, 0.06 $\mu\text{g}/\text{ml}$) and a resistant strain with the lowest

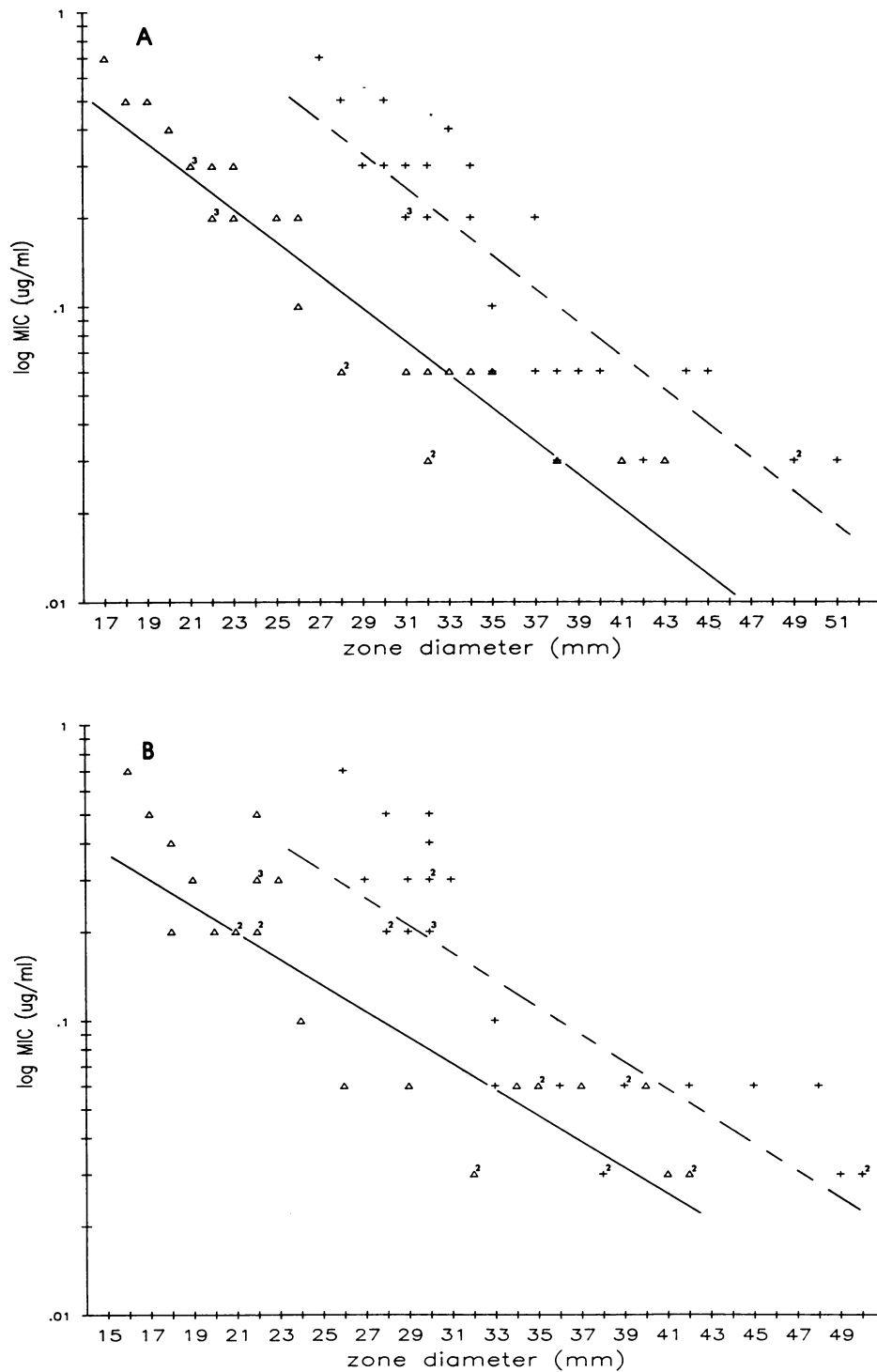


FIG. 2. Regression analysis of the MIC of penicillin G, with *N. meningitidis* strains plotted against zone diameters obtained with disks containing 2 U (Δ ; $r = -0.9334$) or 10 U (+; $r = -0.8723$) of penicillin G (A) or disks containing 2 μg (Δ ; $r = -0.8915$) or 10 μg (+; $r = -0.8523$) of ampicillin (B). The solid line and the dashed line represent the regression lines obtained with disks containing 2 U or 2 μg (—) or 10 U or 10 μg (---) of β -lactam antibiotic. Each symbol represents a single isolate unless otherwise noted by a superscript.

penicillin G MIC (0.1 $\mu\text{g}/\text{ml}$) each showed a zone diameter of 33 mm. The other relatively resistant strains showed zone diameters of ≤ 31 mm (Fig. 2B). With the 1- μg oxacillin disk (Fig. 3), all relatively resistant strains showed zone diameters of 6 mm, whereas three susceptible strains showed zone diameters of 6 (two strains) or 7 (one strain) mm.

Statistics. The results of cluster analysis applied to the zone diameter with disks containing 2 U of penicillin G indicated that with the exception of three lower-level-resistance strains (MIC, 0.1 or 0.2 $\mu\text{g}/\text{ml}$) in the susceptible cluster, all of the remaining relatively resistant strains appeared as one cluster, while all the susceptible strains

TABLE 1. Penicillin MIC and mean zone diameters for 10 β -lactam antibiotics with 28 *N. meningitidis* isolates

Strain	n ^a	MIC (μ g/ml) ^b		Zone diam (mm) for the following antibiotic disk ^c :									
		50%	90%	P2	P10	AM2	AM10	OX1	CF30	CXM30	CTX30	FOX30	CFP75
Susceptible	12	0.06	0.06	34	42	35	42	11	41	48	54	41	48
Relatively resistant	16	0.3	0.5	22	32	21	29	6	30	35	47	38	43

^a n, Number of isolates tested.

^b The determination of MIC and disk susceptibility testing were performed by standard technique (see the text). 50% and 90%, MIC for 50 and 90% of strains, respectively.

^c P, Penicillin G; AM, ampicillin; CTX, cefotaxime; CF, cephalothin; OX, oxacillin; CXM, cefuroxime; FOX, cefoxitin; CFP, cefoperazone. The numbers after the abbreviations indicate the concentration of antibiotic contained in each disk (in units for penicillin or micrograms for the other β -lactam agents).

appeared as a separate cluster (data not shown). No obvious clustering of the seven lower-level-resistance strains (penicillin MIC, 0.1 or 0.2 μ g/ml) was seen. Thus, no natural grouping exists for these lower-level-resistance strains by using the level of resolution provided by the number of strains tested. The susceptible strains showed a large range of zone diameters and a narrow range of penicillin G MICs (Table 1). All of the relatively resistant strains showed a wide range of zone diameters and a wide range of penicillin G MICs (Table 1; Fig. 1). Based on the results of cluster analysis, the lower-level-resistance strains which grew in penicillin G concentrations of 0.1 or 0.2 μ g/ml behaved more like resistant strains in terms of their zone diameters.

Comparison of the zones of inhibition of the other antibiotic disks with that of the 2-U penicillin G disk revealed that six antibiotic disks clustered together with the 2-U penicillin G disk; these were the 10-U penicillin G disk, the 2- μ g ampicillin disk, the 10- μ g ampicillin disk, the 30- μ g cephalothin disk, the 30- μ g cefuroxime disk, and the 1- μ g oxacillin disk. These antibiotics had zone diameter values which correlated with those of the 2-U penicillin G disks and, therefore, with the penicillin G MIC. This clustering suggests that the antibiotics have similar effects on the strains and may reflect an underlying similarity in their mechanisms of action. The other three disks, which contained 30 μ g of cefoxitin, 30 μ g of cefotaxime, and 75 μ g of cefoperazone, did not cluster with the 2-U penicillin G disk.

DISCUSSION

Meningococci that are relatively resistant to penicillin G have been increasingly recognized in Spain. In the first 6 months of 1986, the National Reference Laboratory in Madrid found a 5% incidence among 168 isolates, compared with 1 of 3,264 strains tested in 1985 (Sáez-Nieto et al., in press).

The characterization of penicillin G-resistant strains of *N. meningitidis* has been limited; only a single genital isolate has been described, and that strain had plasmid-mediated β -lactamase production (3). The relatively resistant strains described in this report did not produce detectable β -lactamase when tested with nitrocefin. An alternative mechanism of resistance for these strains includes degradation of the penicillin molecule (other than by β -lactamase), decreased target susceptibility (altered penicillin-binding proteins), and impermeability. Results of our initial studies have revealed reduced penicillin binding to penicillin-binding protein 3 of a relatively resistant strain in comparison with that to penicillin-binding protein 3 of a susceptible isolate (P. M. Mendelman, J. Campos, D. O. Chaffin, A. L. Smith, and J. A. Sáez-Nieto, Abstr. Am. Soc. Microbiol. Conf. Antibiotic Inhibition of Bacterial Cell Surface Assembly and Function, Philadelphia, 17 to 20 May 1987, abstr. no. 48, p. 25).

The National Committee for Clinical Laboratory Standards has no guidelines for disk susceptibility testing of *N. meningitidis*. With the emergence of clinically significant relatively penicillin G-resistant isolates, however, susceptibility testing should be performed routinely. To do this, criteria that define resistance are needed.

Because of the low level of resistance expressed by these strains, the 2-U penicillin G disk most accurately differentiated all susceptible strains from the relatively resistant strains. The 1- μ g oxacillin disk appeared not to be acceptable because it falsely identified three susceptible isolates as resistant.

These relatively penicillin G-resistant isolates appeared to form a common group because we could not statistically subdivide these into lower- and higher-level subsets. Unfortunately, evolution of meningococci to an even higher level of resistance (MIC, ≥ 1 μ g/ml) may occur with an additional mutational event, as has been observed with gonococci (8).

At this time, clinically significant resistance to penicillin remains to be demonstrated for meningococci. Indeed, selection of other antimicrobial agents for therapy of infections caused by resistant strains may become necessary. Based on cluster analysis of our disk susceptibility tests, it appears that disks containing cephalothin, oxacillin, and cefuroxime reflect the relative penicillin G resistance and may have similar targets. In contrast, disks containing cefoxitin, cefoperazone, and cefotaxime are not reflective of penicillin G resistance and thus appear to have different targets. Alternatively, the disk test data for cefoxitin, cefoperazone, and cefotaxime may reflect their greater activity.

Until resistance becomes more common, for any meningococcal isolate identified as relatively resistant by our zone

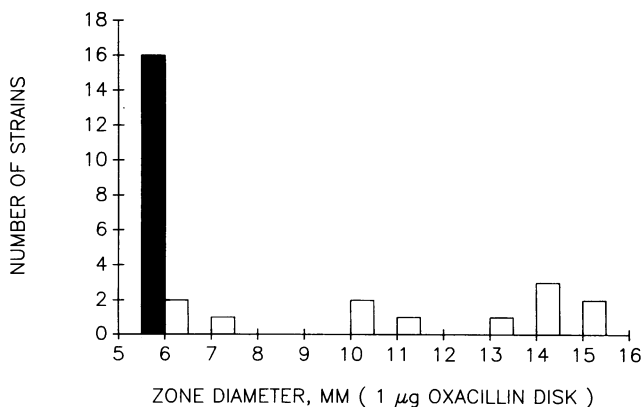


FIG. 3. Distribution of zone diameters obtained with disks containing 1 μ g of oxacillin by testing the following strains: 12 penicillin G-susceptible isolates (□; MIC 0.03 or 0.06 μ g/ml) and 16 relatively resistant isolates (■; MIC 0.1 to 0.7 μ g/ml).

diameter criteria or associated with suspected treatment failure, the penicillin G MIC should be determined and confirmed at a reference laboratory.

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