DISC AGAR DIFFUSION ANTIMICROBIAL SUSCEPTIBILITY TESTS WITH β-LACTAMASE PRODUCING *NEISSERIA GONORRHOEAE*

J. W. BIDDLE, J. M. SWENSON and C. THORNSBERRY*

Bacteriology Division, Bureau of Laboratories Center for Disease Control,
Public Health Service
U.S. Department of Health, Education, and Welfare
Atlanta, Georgia 30333, U.S.A.

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The emergence of β-lactamase-producing strains of *Neisseria gonorrhoeae* has led to a reexamination of the role of the disc agar diffusion method in susceptibility testing of gonococci. Our data show that the disc agar diffusion test can be used to screen for β-lactamase production by these organisms. The disc tests were done on GC Agar Base supplemented with 1% IsoVitaleX. An inoculum of 10^8 colony forming units/ml and either a 10-unit-penicillin or a 10-μg-ampicillin disc were used. A zone diameter of ≤19 mm was indicative of β-lactamase production. These results were compared with results of chemical tests for β-lactamase and with minimal inhibitory concentrations. Recommendations were also made for a disc test with tetracycline and spectinomycin, but these methods must remain tentative because of the lack of resistant strains.

For the 20 to 25 years preceding 1972, many *Neisseria gonorrhoeae* isolates became progressively more resistant to penicillin[1,2] but remained sensitive to treatment with this antibiotic when it was used in the dosage recommended by the U.S. Public Health Service[3]. Because the isolates were still susceptible to penicillin, the drug of choice, and because of the nutritional and atmospheric requirements and the occasional fastidiousness of the organism, at that time we recommended that routine antimicrobial susceptibility testing be not performed on these organisms.

Our laboratory has monitored the susceptibility of gonococci from various areas of the U.S.A. and the rest of the world since 1972. The discovery of β-lactamase-producing gonococci in some patients with gonorrhea[1,4,8,9] has caused us to reexamine the role of disc agar diffusion tests in susceptibility testing of *N. gonorrhoeae*. This report contains a description of a disc agar diffusion test which is used primarily to screen for β-lactamase production (hence penicillin resistance) in gonococci, but which also determines relative susceptibility to other drugs.

Materials and Methods

Cultures

The β-lactamase-producing strains of *N. gonorrhoeae* were submitted to us from various states in the U.S.A. and from the Far East. The β-lactamase-negative strains, representing the full range of minimum inhibitory concentrations (MICs), 0.008 to 2 μg/ml, were selected from cultures collected in the Therapy Monitor Study[5,6].

Antibiotics

The antibiotics used in the MIC tests were supplied by the manufacturers in powder form suitable for susceptibility tests. The 10-unit-penicillin, 10-μg-ampicillin, 30-μg-tetracycline, and 30-μg-cephalothin discs were selected from the supply of discs used in the BAUER-KIRBY test[2]. Discs containing 10 μg, 25 μg, 50 μg and 100 μg of spectinomycin were prepared in our laboratory. All discs were

stored with a desiccant at −70°C until they were used.

Media

The general *N. gonorrhoeae* growth medium was Proteose No. 3 Agar supplemented with 1% hemoglobin and 1% IsoVitaleX*. In order to determine the MICs, we supplemented the above medium with various concentrations of antibiotics. For the disc agar diffusion tests, we used a clear medium, GC Agar Base supplemented with 1% IsoVitaleX. MUELLER-HINTON broth was used as a suspension medium for preparing the inocula for both tests.

Inoculum Preparation

Organisms to be tested were inoculated onto the supplemented Proteose No. 3 Agar and incubated overnight in a candle extinction jar at 35°C. Some of the resulting growth was suspended in MUELLER-HINTON broth and adjusted to a turbidity equivalent to a 0.5 MCFARLAND standard. This adjusted inoculum was used for the disc agar diffusion tests; it was also used in the agar dilution tests for MIC determination after further dilution of 1: 10.

Agar Dilution Method for MICs

The supplemented Proteose No. 3 Agar and the inoculum described above were used for these tests. Antibiotics to be tested were dissolved in the proper solvent[10] and added to the agar medium at 50°C in various amounts to yield the desired concentrations. Penicillin was tested in $\log_2$ dilution steps ranging from 16 μg/ml to 0.008 μg/ml. Other drugs were tested in ranges from 32 to 0.03 μg/ml (ampicillin), 16 to 4 μg/ml (spectinomycin), and 4 to 0.25 μg/ml (tetracycline and cephalothin). Approximately 30 ml of the media were poured into square Petri plates (90 mm). Plates not used immediately were sealed in plastic bags and stored at 4~10°C. The plate surfaces were dried before tests were performed. The organisms were adjusted to a turbidity equivalent to a 0.5 MCFARLAND standard {approximately 10^8 colony forming units (CFU) per ml}, then diluted 1: 10 and added to the media with a Steers replicator[11]. The replicator delivers 0.001~0.002 ml of inoculum, resulting in approximately 10^3 CFU of organisms per inoculum "spot". Previous counts of these inocula have shown the actual number is always between 10^3 and 10^4 CFU, with a mean of approximately 5×10^3 CFU. After the inocula dried, the plates were inverted and incubated in a candle jar for 24 hours at 35°C. The MIC was defined as the lowest concentration of drug that permitted the growth of no more than one colony. Reference strains and a plate without antibiotics were included as controls in each test.

Disc Agar Diffusion Method

Sixty milliliters of the GC Agar Base −1% IsoVitaleX medium was poured into each 140 mm plate. If the plates were not used the same day, they were sealed in plastic bags and stored at 4~10°C. The inoculum was prepared as described above to a turbidity equivalent to a 0.5 MCFARLAND standard (approximately 10^8 CFU/ml). The adjusted inoculum was spread on the dry agar surface in three planes as described for the BAUER-KIRBY test[2]. The discs were pressed onto the surface of the agar with sterile forceps. The plates were inverted and incubated in a candle jar for 24 hours at 35°C. The zones of inhibition were measured with a ruler placed on the back of the plate. Because the contrast between growth and agar was often poor, we placed a light behind the plate while measuring the zones.

β-Lactamase Tests

Some of these cultures were tested for β-lactamase activity by the rapid acidometric, iodometric, and chromogenic cephalosporin tests[13], but most were tested only by the latter method.

Results

The MICs of the five antibiotics for the 82 *N. gonorrhoeae* isolates which we used are shown in Table 1. Of those strains listed as 0.06 or less for penicillin, one was 0.008 μg/ml and another

* Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health, Education and Welfare.

Table 1. Minimum inhibitory concentrations (MICs) of β-lactamase positive and negative strains of *N. gonorrhoeae*

Antibiotic	β-Lactamase	% of strains with MIC (μg/ml) of										
		≤0.06	0.12	0.25	0.5	1	2	4	8	12	16	32
Penicillin	−	28	16	18	32	4	2			—		
	+			3	3	13	22	31	25	—	3	
Ampicillin	−	14	40	38	6	2				—		
	+						6	16	63	—	12	3
Cephalothin	−	—[a]	—	26[b]	12	24	34	4[c]		—		
	+	—	—	16[b]	26	45	10	3		—		
Tetracycline	−	—	—	8[b]	28	28	16	20		—		
	+	—	—	12[b]	22	41	22	3		—		
Spectinomycin	−	—	—	—	—	—	—	2[b]	48	46	4	
	+	—	—	—	—	—	—	3[b]	59	38		

a) Not tested at these concentrations
b) Equal to or less than these MICs
c) One of these strains (2%) was >4 μg/ml

Fig. 1. Relationship of penicillin zone diameters and MICs obtained with β-lactamase negative and β-lactamase positive strains of *N. gonorrhoeae*.

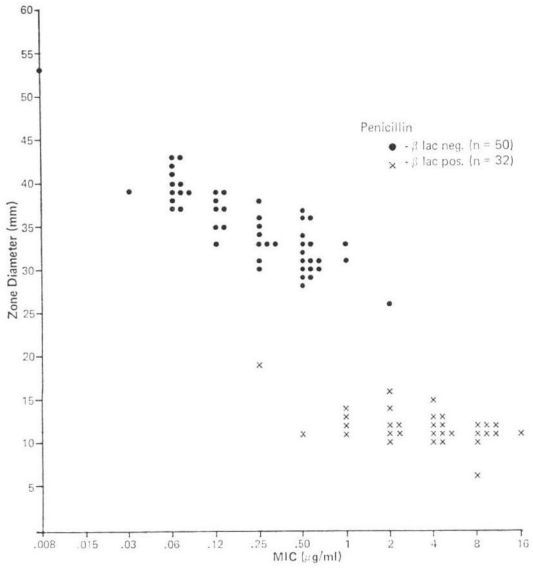
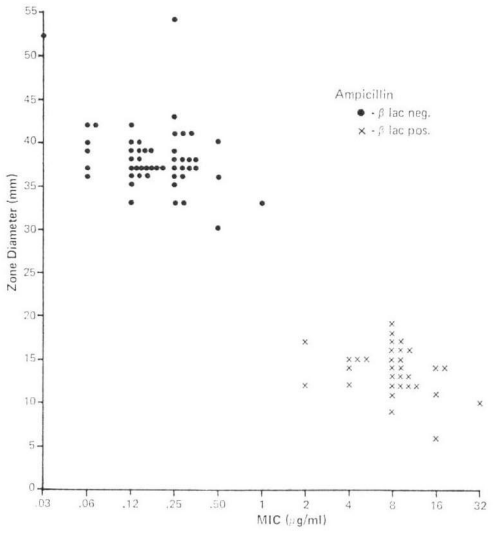


Fig. 2. Relationship of ampicillin zone diameters and MICs obtained with β-lactamase negative and β-lactamase positive strains of *N. gonorrhoeae*.



was 0.03 μg/ml; for ampicillin, one strain listed in the 0.06 μg/ml group was actually 0.03 μg/ml (see Figs. 1 and 2). Although there was some overlap of penicillin MICs, those of penicillin and ampicillin for the β-lactamase-positive and negative strains showed a bimodal distribution. Such a distribution was not seen with another β-lactam antibiotic, cephalothin, or with tetracycline and spectinomycin.

The distribution of zone sizes for these organisms and antibiotics is shown in Table 2. The bimodal distribution was complete for penicillin and ampicillin, but was absent for the other three antibiotics.

Discs with spectinomycin concentrations of 10, 25, 50, and 100 μg were tested with these strains.

Table 2. Range of zone diameters obtained with β-lactamase negative and positive strains of *N. gonorrhoeae*

Antibiotic	β-Lactamase	% of strains with zone diameters (mm) of					
		6~12	13~19	20~30	31~40	41~50	51~60
Penicillin	−			16	74	8	2
	+	75	25				
Ampicillin	−			2	80	14	4
	+	34	66				
Cephalothin	−			26	66	6	2
	+			48	52		
Tetracycline	−				58	42	
	+				47	53	
Spectinomycin[a]	−			80	20		
(100 μg disc)	+			91	9		

a) One strain of *N. gonorrhoeae* not shown in the table was resistant to spectinomycin with an MIC of >2,048 μg/ml and no zone (*i.e.*, 6 mm) around a 100 μg disc.

Fig. 3. Relationship of cephalothin zone diameters and MICs obtained with β-lactamase-negative and β-lactamase-positive strains.

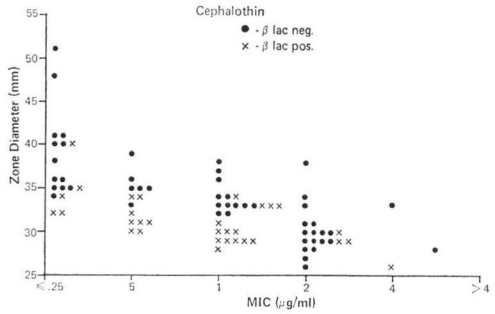


Fig. 4. Relationship of tetracycline zone diameters and MICs obtained with β-lactamase-negative and β-lactamase-positive strains of *N. gonorrhoeae*.

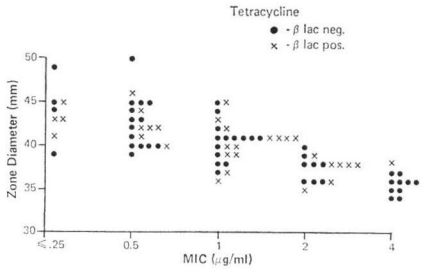
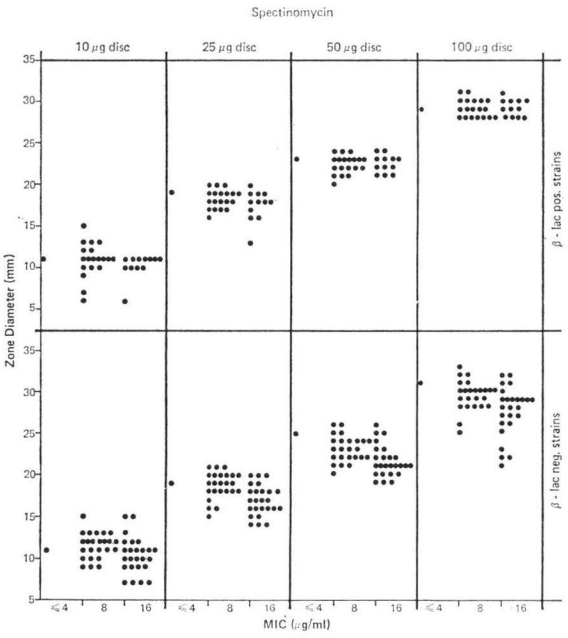


Fig. 5. Relationship of spectinomycin zone diameters (10, 25, 50 and 100 μg discs) and MICs obtained with β-lactamase negative and β-lactamase positive strains of *N. gonorrhoeae*.



These results are shown in Table 3. These discs yielded values from 6 mm (no zone) to 33 mm, with the zones becoming progressively larger with increasing concentration. One strain, not shown here, had a spectinomycin MIC of >2,048 μg/ml, and no zone with a 100-μg disc. This strain was β-lactamase negative.

Comparisons of zone sizes and MICs for each drug are shown in the scattergrams in Figs. 1~5. Figs. 1 and 2 show that β-lactamase-negative and positive strains can be separated on the basis of

penicillin and ampicillin zone size, although penicillin MICs do overlap. A breakpoint of 20 mm ($\leq$19 mm indicates β-lactamase production) is applicable for both drugs. This clear division is not evident in the results with cephalothin, the other β-lactam antibiotic (Fig. 3), although the zone sizes for the β-lactamase-producing strains are, in general, slightly smaller than those of the β-lactamase-negative strains. No division points are present in the results for tetracycline and spectinomycin.

Table 3. Zone diameters obtained with 10, 25, 50, and 100 μg spectinomycin discs and β-lactamase positive and negative *N. gonorrhoeae*

Disc (μg)	β-Lactamase	Range of zone diameters (mm)
10	−	7～15
	+	6～15
25	−	14～21
	+	13～20
50	−	19～26
	+	20～24
100	−	21～33
	+	28～31

These results were obtained with inocula adjusted to approximately 10^8 CFU/ml for the disc test and further diluted for the MIC test. With heavier concentration of inocula, the MICs for the β-lactamase strains in particular, change drastically, although there is a less marked effect on the β-lactamase negative strains than on the positive. An increase in inoculum also affects the zone sizes obtained in the disc test, in many cases rendering invalid the penicillin and ampicillin breakpoint of $\geq$20 mm for β-lactamase negative strains.

Discussion

Between 1950 and 1970 increasing numbers of gonococci became generally more resistant to penicillin[12]. However, this resistance was relative, and the organisms were still susceptible to achievable serum levels of penicillin[6]. In a study of strains from various regions of the U.S.A., we have shown that since 1972 the trend toward greater penicillin resistance has leveled off, and overall, strains have become slightly more sensitive[5], (unpublished information). Because all strains were still sensitive to penicillin, and because the overall cure rate of gonorrhea with penicillin and probenicid therapy was 97%[6], routine susceptibility tests were not recommended.

The emergence of penicillin resistance in gonococci as a result of their β-lactamase production raises the question of a need for routine susceptibility tests for gonococci. Patients infected with β-lactamase-producing gonococci are unlikely to respond to penicillin therapy. Therefore, being able to test gonococci for β-lactamase production has become important for both therapeutic and epidemiological reasons. This testing can be done by any of three rapid chemical methods[13], in addition to which our data show that a disc agar diffusion test is also effective, when either a 10-unit-penicillin disc or a 10-μg-ampicillin disc and an inoculum of approximately 10^8 CFU/ml are used. Unless there is a drastic change in chromosomal resistance to penicillin in these organisms, any strains yielding zone sizes of $\leq$19 mm are almost certain to be β-lactamase producers. One must be aware, however, that some strains of *N. gonorrhoeae* are extremely slow growers, and are thus likely to produce large zones. β-Lactamase-negative strains of the fastidious type most often yield penicillin zone diameters $\geq$50 mm; β-lactamase-positive strains of this type usually have zone sizes near 19 mm, and undoubtedly, some strains will yield zones $>$19 mm. Therefore, results with these fastidious strains should be interpreted with caution; if the zone diameter is between 20～30 mm, a chemical test for β-lactamase should be done for confirmation.

The distribution of results shown in Figs. 1～4, indicates that there is probably some inverse relationship between MIC and zone size. We did not do statistical tests because, with the penicillin and ampicillin disc test, our aim was only to screen for β-lactamase production, and with the other antibiotics we do not have resistant strains to include. For example, with the exception of the one strain very resistant to spectinomycin, the MICs for this drug were virtually the same for all strains (either 8 or 12 μg/ml) (Table 1 and Fig. 5). The same is true for over 9,000 other strains we have

tested with spectinomycin.

MAIER *et al.* have reported on the use of a disc test for *N. gonorrhoeae*[7]. Where direct comparison is possible, their results and ours are very similar.

WASHINGTON and YU reported on the activity of spectinomycin against a variety of bacteria[16]. They recommended the use of a 100-μg-spectinomycin disc, and breakpoints in which zones $\geq$18 mm indicate susceptibility, 15~17 mm indicate intermediate susceptibility, and $\leq$14 mm indicate resistance. Although they did not test gonococci, our results with both the 50 μg and 100 μg discs, show our sensitive strains conforming to their susceptibility category. The only known spectinomycin-resistant strains[10,14] are chromosomal mutants with very high levels of resistance, which yield no zones and thus present no problems in detection. If, however, strains should develop an enzymatic resistance to spectinomycin (*e.g.*, adenylase), further studies would be necessary to determine the adequacy of current zone-size breakpoints. Until enough strains with a wider range of susceptibility are available for further study, we recommend that the 100-μg-spectinomycin disc be used to test gonococci, and that the tentative zone-size breakpoint for sensitive strains be $\geq$18 mm. Strains with zones $<$18 mm should be further investigated to determine mechanism of resistance.

Gonococci isolated from patients who have not responded to penicillin therapy, from patients who have traveled in areas where the incidence of β-lactamase-positive strains is high, or from patients epidemiologically associated with patients known to have harbored the β-lactamase-positive organism should be tested for β-lactamase production. We prefer one of the rapid β-lactamase tests[13], but our results show that the disc test can also be used to screen for β-lactamase. We recommend the use of the clear medium, GC Agar Base supplemented with 1% IsoVitaleX, a 10-unit-penicillin or 10-μg-ampicillin disc, and an inoculum of approximately 10^8 CFU/ml. Our disc test results show that a zone $\leq$19 mm indicates that the strain produces β-lactamase, whereas $\geq$20 mm indicates that it does not. Generally β-lactamase-negative gonococci yield zones of $\geq$30 mm.

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