In vitro Comparison of Disk Diffusion and Agar Dilution Antibiotic Susceptibility Test Methods for *Neisseria gonorrhoeae*

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At present, most Neisseria gonorrhoeae *testing is done with ß-lactamase and agar dilution tests with common therapeutic agents. Generally, in bacteriological diagnosis laboratories in Argentina, study of antibiotic susceptibility of* N.gonorrhoeae *is based on ß-lactamase determination and agar dilution method with common therapeutic agents. The National Committee for Clinical Laboratory Standards (NCCLS) has recently described a disk diffusion test that produces results comparable to the reference agar dilution method for antibiotic susceptibility of* N.gonorrhoeae, *using a dispersion diagram for analyzing the correlation between both techniques. We obtained 57 gonococcal isolates from patients attending a clinic for sexually transmitted diseases in Tucumán, Argentina. Antibiotic susceptibility tests using agar dilution and disk diffusion techniques were compared. The established NCCLS interpretive criteria for both susceptibility methods appeared to be applicable to domestic gonococcal strains. The correlation between the MIC's and the zones of inhibition was studied for penicillin, ampicillin, cefoxitin, spectinomycin, cefotaxime, cephaloridine, cephalexin, tetracycline, norfloxacin and kanamycin. Dispersion diagrams showed a high correlation between both methods.*

Key words: *Neisseria gonorrhoeae* - antimicrobial susceptibility - disk diffusion - agar dilution

The ability of bacteria to develop resistance to antimicrobial agents has become a significant problem in the treatment and control of communicable sexually transmitted diseases (Perine et al. 1977, Thornsberry et al. 1977, Ashford et al. 1981, Knapp et al. 1987, CDC 1987, Heritage & Hawkey 1988). This is particularly true for *Neisseria gonorrhoeae*, which has evolved a number of different mechanisms to resist antimicrobial agents. These mechanisms are plasmid or chromosome encoded. The emergence of penicillinase-producing *N. gonorrhoeae* (PPNG) in 1976 has led to widespread high-level penicillin resistance (Ashford et al. 1976, CDC 1976, Phillips 1976, Siegel et al. 1978). High-level tetracycline resistance (MIC ³ 16 mg/ml) was reported for *N. gonorrhoeae* in 1985 by the Centers for Disease Control (CDC 1985). Spectinomycin resistance has also been reported for many strains of *N. gonorrhoeae* (Thornsberry et al. 1977, Ashford et al. 1981). Gonococcal resistances have commonly been reported to originate in southeast Asia and Western Africa (Phillips 1976).

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There is an immediate need to simplify and standardize the *in vitro* antimicrobial susceptibility testing of *N. gonorrhoeae*, because of the frequency of isolation and the increased levels of antimicrobial resistance seen with these strains. Antimicrobial susceptibility testing of *N. gonorrhoeae* in most clinical microbiology laboratories in Argentina consists of the ß-lactamase test and a 10-U penicillin disk diffusion test, both used for the detection of penicillinase-producing *N. gonorrhoeae* (Phillips 1976, NCCLS 1990a). Alternative methods, such as agar dilution antimicrobial testing (NCCLS 1990b), are generally laborious and not practical for routine use. The development of a standardized agar diffusion method for these tests is practical and allows fast and reproducible results for clinical microbiology laboratories if standards are observed (Jones et al. 1990a,b). The purpose of this study was to evaluate the agar disk diffusion technique, comparing it with the agar dilution method proposed by the National Committee for Clinical Laboratory Standard (NCCLS), using strains of *N. gonorrhoeae*, isolated in Tucumán, Argentina.

MATERIALS AND METHODS

Bacterial strains - Two gonococcal control strains (ATCC 49226: WHO A and WHO C) were selected for susceptibility to penicillin (WHO A: MIC 0.5 mg/ml and zone diameter 31.1 mm; WHO C: MIC 25 mg/ml and zone diameter 10.6 mm).

Besides, 57 *N.gonorrhoeae* strains, isolated from samples from patients attending a clinic in Tucumán between 1990 and 1991, were studied.

Antimicrobial agents - Antimicrobial agents used for the agar dilution studies were penicillin, ampicillin, tetracycline, cefotaxime, norfloxacin, cefoxitin, spectinomycin, cephaloridine, cephalexin and kanamycin, which were provided by the Microbiology Institute "Carlos G. Malbran", as dry experimental substances with known capacity.

The antimicrobial agent-containing disks included penicillin (10 U), tetracycline (30 mg), cefotaxime (30 mg) norfloxacin (10 mg), spectinomycin (100 mg), cefoxitin (30 mg), ampicillin (10 mg), cephaloridine (30 mg), cephalexin (30 mg) and kanamycin (30 mg). All drug solutions were prepared and stored according to the manufacturers' instructions. All disks were provided by the Microbiology Institute "Carlos G. Malbran" stored in desiccated storage units at 4°C.

The antimicrobial agents tested, their agar diffusion breakpoints and their MIC limits are shown in Tables I and II. Values are according to the NCCLS (NCCLS 1990a,b).

Laboratory identification of N. gonorrhoeae - All specimens were obtained with Dacron swabs from male urethral samples. The specimens were immediately plated onto GC-agar (Difco Laboratories, Detroit, Mi) plates and incubated at 35°C in a candle jar. The isolates were classified as *Neisseria* spp. by the identification of gram-negative diplococci from the urethral swab, characteristic colony morphology, and an oxidase-positive test. All presumptive gonococcal isolates were frozen at -70°C in tryptic soy broth with 25% glycerol. The isolates were later plated onto GC-agar plates supplemented with 1% Iso Vitalex (Becton Dickinson) and incubated for 24 hr at 37°C under an atmosphere of 5 to 8% CO_2.

The organisms were harvested and retested for diplococcal morphology and the production of oxidase. All strains were confirmed with a *N. gonorrhoeae*-specific monoclonal antibody assay (Phadebact Monoclonal GC OMNI Test 50; Remel, Lenexa, Ka). All confirmed organisms were tested for the presence of ß-lactamase production with nitrocefin (Cefinase, Glaxo Research Ltd, Greenford, Middlesex, England).

Antimicrobial susceptibility testing - The agar dilution method was performed according to the method established by the NCCLS with GC-agar with 1% of a defined supplement (NCCLS 1990b). The disk diffusion method was performed with GC-agar using 1% GC supplement (Prepared Media Laboratories), also according to the recommendations of the NCCLS (NCCLS 1990a). The contents of the GC-agar and the supplement have been described previously by Jones et al. (1989). All agar plates (agar dilution and disk diffusion) were incubated at 35°C under a 5 to 8% CO_2 atmosphere, and results were read after 24 hr.

Data analysis - A dispersion diagram was carried out for each of the antibiotics as to determine the correlation between the MIC and the disk zone diameter susceptibility tests. The correlation coefficient according to Pearson (r) is used for measuring the linear correlation between $\log_2$ MIC x diameter (Lorian 1986).

RESULTS AND DISCUSSION

Tables I and II show the distribution of the susceptibility of the studied strains. The isolated strains are highly susceptible to the examined antibiotics except for tetracycline (12.5% of the strains showed resistance to this drug).

Two strains out of 57 (3.5%) were resistant to penicillin, ampicillin and cephalosporin of the first generation (cephaloridine-cephalexin).

The distribution of the MIC's and the inhibition halos of the other antibiotics assayed, was similar for both ß-lactamase producing PPNG (plasmid encoded) and non-producing gonococci. Therefore these drugs (tetracycline, spectinomycin, norfloxacin and cephalosporins of the 2nd and 3rd generation) were considered as alternatives for the treatment of infections, which could not be cured with penicillin. The results agreed with those obtained by Mc Cormack (1982).

Jones et al. (1989) determined that 90% of the laboratories routinely used the ß-lactamase test on all gonococcal strains or selected isolates. The most commonly used methods (93%) were commercially prepared chromogenic cephalosporin (nitrocefin or PADAC) and acidimetric reagents. PPNG strains were rarely found. Few laboratories performed other susceptibility tests on *N. gonorrhoeae* strains. When another method was used, the disk diffusion procedure was preferred in 86% of the laboratories. The most-tested anti-gonococcal drugs were penicillin, tetracycline, ß-lactamase-stable cephalosporins and spectinomycin.

The standard disk diffusion test allowed to obtain reproducible results, when compared with the MIC method. Gonococci showed 1.7% more resistance against penicillin, cephalexin and kanamycin with the disk diffusion test than using the MIC method. For tetracycline the difference between both tests was 3.5% (the disk diffusion test showing more resistance again). Similar results were obtained in assays carried out by Stratton (1984) and by Fekete (1993).

With each type of antibiotic a dispersion diagram was made to analyze the correlation between the susceptibility tests of the diffusion in agar and the MIC (Fig.). The index used for measuring the

TABLE I

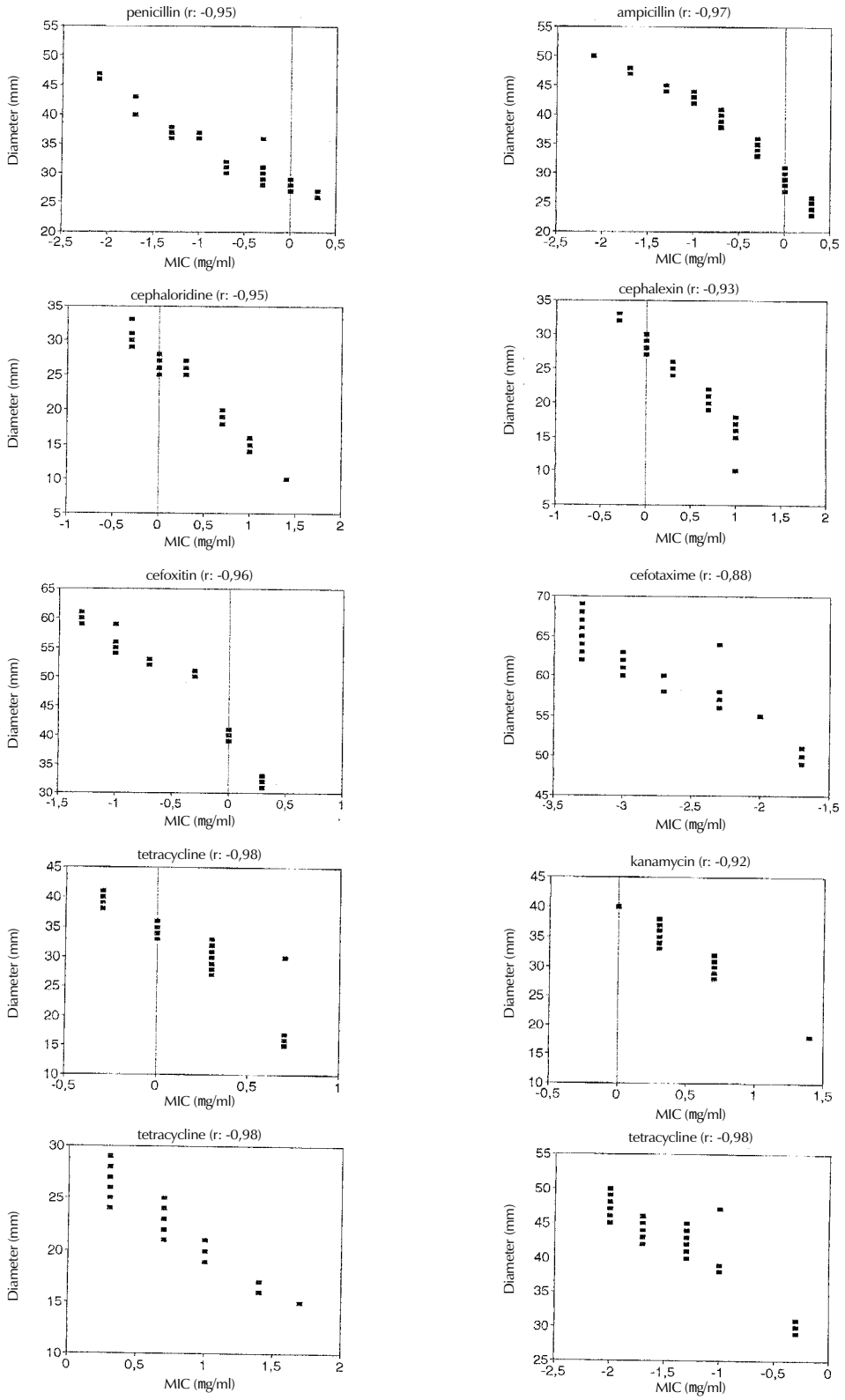
Diffusion method: comparison of the inhibition diameters (zone diameter breakpoint) of 57 strains of *Neisseria gonorrhoeae* from Tucumán, Argentina; for the tested antibiotics; susceptible strains are in bold

Antibiotic	Disk load	Range	Zone diameter breakpoint (mm)																														
	µg	mm	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	46	48	50	52	54	56	58	60	>60	Resistance **%**
Penicillin	10	28-47	2												1	**12**	**20**	**8**		**5**	**5**	**1**	**1**		**1**	**1**								5.26
Ampicillin	10	21-40	2											**2**	**3**	**5**	**16**	**6**	**3**	**3**	**2**	**3**	**3**	**4**	**2**	**2**	**1**							3.50
Cephaloridine	30	14-18	1				1		**3**	**16**	**5**	**4**			**12**	**9**	**4**	**1**	**1**															3.50
Cephalexin	30	15-19	2				1			**8**	**2**	**9**	**6**	**6**	**6**	**5**	**6**	**4**	**2**															5.26
Cefoxitin	30	23-28														**1**	**1**	**2**	**3**			**4**	**2**				**2**	**3**	**5**	**6**		**20**	**8**	0
Cefotaxime	30	31-35																		**1**		**1**			**1**		**2**	**2**		**3**	**8**	**4**	**37**	0
Kanamycin	30	13-18	1						**1**		**1**					**2**	**8**	**5**	**5**	**18**		**1**												3.50
Spectinomycin	100	14-20	1							**2**	**2**	**7**	**11**	**6**	**11**	**15**	**2**				**15**													1.75
Tetracycline	30	30-38						1		4	2					**3**	**18**	**10**	**3**	**7**	**1**	**4**	**2**							**2**				17.50
Norfloxacin	10	14-18															**1**	**3**			**1**	**3**	**11**	**11**	**15**	**10**	**2**							0

TABLE II

Dilution method *in vitro* susceptibility of 57 strains of *Neisseria gonorrhoeae* from Tucumán, Argentina; for the tested antibiotics susceptible strains are in bold

		MIC (mg/ml)																	
Antibiotic	Range	0,0005	0,001	0,003	0,005	0,01	0,02	0,05	0,1	0,25	0,5	1	2	5	10	25	50	100	200
Penicillin	0,06-2					**2**	**2**	**4**	**5**	**8**	**14**	**17**	**3**						2
Ampicillin	1-4					**1**	**2**	**3**	**4**	**5**	**9**	**26**	**5**						2
Cephaloridine	4-16										**6**	**10**	**11**	**9**	**9**		1	1	
Cephalexin	4-16										**6**	**11**	**12**	**15**	**11**	2			
Cefoxitin	2-8							**27**	**8**	**6**	**3**	**6**	**5**	**2**					
Cefotaxime	0,034-2	**30**		**8**		**3**		**6**		**2**	**4**		**1**	**2**	**1**				
Kanamycin	4-64											**1**	**38**	**15**		**1**	**1**	1	
Spectinomycin	32-128												**30**	**12**	**10**	**3**	**1**		1
Tetracycline	0,25-2									**2**	**7**	**9**	**31**	7	1				
Norfloxacin	4-10					**15**	**15**	**16**	**4**	**4**									



Dispersion diagram between the agar dilution and the agar diffusion methods for 57 *Neisseria gonorrhoeae* strains from Tucumán, Argentina. Correlation coefficient according to Pearson (r) (see text for details) between both methods is given in brackets for each of the antibiotics studied.

linear regression analysis grade is the Pearson coefficient (r). A strong correlation between both methods could be observed.

When estimating the sensitivity and the specificity of both methods taking the MIC as the standard value, and classifying the strains in resistant and non-resistant (sensitive) ones, the following results were obtained: a sensitivity of 89% and a specificity of 91%. Similar results have been found by Jones et al. (1988), obtaining a correlation of 98% between both methods.

Due to the frequency of gonococcal infections various methods have been developed to study their *in vitro* susceptibility (Maier et al. 1974, Putnam et al. 1992).

In 1978 the scientific group of the World Health Organization recommended a standard method, with which they achieved to compare the results of different national and international laboratories, as well as the effectiveness of the antimicrobial therapies applied in different geographic areas (WHO 1978).

The results of the disk diffusion test for this microorganism and for the tested antibiotics were perfectly reproducible and comparable with the dilution agar method, MIC, if standardized parameters are observed. Analysis with a dispersion diagram showed a high correlation between both, with a sensitivity of 89% and a specificity of 91%.

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