Agar Dilution Method For Susceptibility Testing of *Neisseria gonorrhoeae*

Marta C de Castillo+, Olga A de Saab, Norma P de Fernandez, Olga M de Nader, Aída P de Ruiz Holgado

Instituto de Microbiología, Facultad de Bioquímica y Farmacia, Universidad Nacional de Tucumán, Av. J. B. Justo 1192, (4000) S. M. de Tucumán, Argentina

The antibiotic susceptibilities of Neisseria gonorrhoeae *isolates obtained from patients attending a clinic for sexually transmitted diseases in Tucumán, Argentina, were determined by the agar dilution method (MIC). 3.5% of the isolates produced ß-lactamase. A total of 96.5% of ß-lactamase negative isolates tested were susceptible to penicillin (MIC £ 2 mgml^{-1}); 14.03% of the tested isolates were resistant to tetracycline (MIC £ 2 mgml^{-1}), and 98% of the tested isolates were susceptible to spectinomycin (MIC £ 64 mgml^{-1}). The MICs for 95% of the isolates, tested for other drugs were: £ 2 mgml^{-1} for cefoxitin, £ 0.06 mgml^{-1} for cefotaxime, £ 0.25 mgml^{-1} for norfloxacin, £ 10 mgml^{-1} for cephaloridine, £ 10 mgml^{-1} for cephalexin, and £ 50 mgml^{-1} for kanamycin.*

Antibiotic resistance among N. gonorrhoeae *isolates from Tucumán, Argentina, appeared to be primarily limited to penicillin and tetracycline, which has been a general use against gonorrhoeae in Tucumán since 1960.*

Periodic monitoring of the underlying susceptibility profiles of the N. gonorrhoea*e strains prevalent in areas of frequent transmission may provide clues regarding treatment options and emerging of drug resistance.*

Key words: *Neisseria gonorrhoeae* - antimicrobial susceptibility - agar dilution method

In 1976, strains of *Neisseria gonorrhoeae* able to synthesize ß-lactamase, codified by plasmids, were described for the first time more or less simultaneously in the United Kingdom and in the USA (Philips 1976, Ashford et al. 1980, Seth & Johnston 1980, Jaffe et al. 1981).

In 1983 the high prevalence of tetracycline-resistant, ß-lactamase producing *N. gonorrhoeae* (PPNG) strains led to the adoption of spectinomycin as recommended therapy for gonorrhoea in Thailand (Traisupa et al. 1990). While spectinomycin-resistant strains developed rapidly in both the Republic of Korea and the Philippines, spectinomycin has retained its effectiveness in Thailand and remains the drug of choice for the treatment of gonorrhoea (Jones et al. 1983, Berliner & No 1986, Joyce et al. 1988, 1989, Traisupa et al. 1990).

MATERIALS AND METHODS

Gonococcal isolates - 57 isolates of *N. gonorrhoeae* were obtained from patients with symptomatic sexually transmitted diseases during 1990-1991. There was no selection of the isolates; all isolates growing on subcultures were tested. No information was available on the incidence of treatment failures or repeated isolates from the same patient. Initial isolations were made on modified Thayer-Martin agar (BBL Microbiology Systems, Cockeysville, Md., USA). Suspected colonies were identified by colony morphology, Gram staining, and oxidase activity (Spot Test Oxidase reagent; Difco Laboratories, Detroit, Mich., USA). Overnight subcultures were transferred into a cryoprotective medium (Trypticase soy broth [BBL] with 25% glycerol [Mallinckrodt, Inc., Paris, Ky., USA]) and were frozen at -70°C until they were tested. Thawed specimens were placed onto chocolate agar prepared from CG agar base (BBL), 1% bovine hemoglobin (BBL), and 1% Iso VitaleX (BBL). Pure colonies re-isolated on chocolate agar were tested after 18 hr for growth in second subcultures.

ß-lactamase testing - ß-lactamase production was tested by the cephalosporin chromogenic method, using Nitrocefin (Shoid-Glaxo) with *Haemophylus influenzae* ATCC 10211 used as a negative control (Kammer et al. 1975); *Escherichia coli* ATCC 35218 was used as a positive control.

Antimicrobial agents - The following standard antimicrobial reference powders were provided as dry experimental substances with known capacity by the Microbiological Institute "Carlos G. Malbran": penicillin, ampicillin, tetracycline,

+Corresponding author: Fax: +54-81-248025
Received 19 March 1996
Accepted 6 August 1996

cefotaxime, norfloxacin, cefoxitin, spectinomycin, cephaloridine, cephalexin and kanamycin.

Antibiotic stock solutions were prepared in reagent-grade water and were frozen at -70°C. Serial twofold dilutions of the antibiotics were prepared in reagent-grade water on the day of use.

Antimicrobial susceptibility testing - Antibiotic susceptibility testing was conducted as described previously (Schwarcz et al. 1990). The quality control organisms inoculated onto each plate were *Staphylococcus aureus* ATCC 29213 and ATCC 25923, *Enterococcus faecalis* ATCC 29212 and *E. coli* ATCC 25922. Subcultures were incubated in a humidified atmosphere of 5% CO_2 for 24 hr at 35°C. Agar dilution methods (MICs) were read as the lowest concentration of antibiotic that inhibited growth (Knapp 1988). Antimicrobial susceptibility was judged according to breakpoints previously defined in the literature (Jones et al. 1989,1991, NCCLS 1990).

RESULTS

Fifty-seven isolates of *N. gonorrhoeae* were examined to determine their antibiotic susceptibilities. The MICs of the tested isolates and the range of MICs for each tested antibiotic are given in Table. ß-lactamase was produced by 2 of the 57 isolates (3.5%).

The distribution of the susceptibilities to each of these antimicrobial agents are shown in Figs a, b, c, d, e, f, g, h, i, j. These isolates were categorized as susceptible or moderately susceptible to each antibiotic (Jones et al. 1989, 1991, NCCLS 1990).

Fifty-five of the 57 examined specimens (96.5%) were susceptible to penicillin, ampicillin, cephalexin and cephaloridine (Figs a, b, c, d).

Distribution of penicillin susceptibility in all 55 isolates was similar for all strains.

All (100%) were susceptible to cefoxitin, cefotaxime and norfloxacin (Figs e, f, j).

Ninety-eight percent of the strains was susceptible to kanamycin and spectinomycin (Figs h, i), and 86% was susceptible to tetracycline (Fig. g).

TABLE

Antimicrobial susceptibility testing of *Neisseria gonorrhoeae*[a]

Antibiotic	MIC limit (μgml^{-1})	
	Susceptible ≤	Resistance ≥
Penicillin	0.06	2
Ampicillin	1	4
Cephaloridine	4	16
Cephalexin	4	16
Cefoxitin	2	8
Cefotaxime	0.5	2
Kanamycin	4	64
Spectinomycin	32	128
Tetracycline	0.25	2
Norfloxacin	4	10

a: the MIC limits are according to the NCCLS (1990).

DISCUSSION

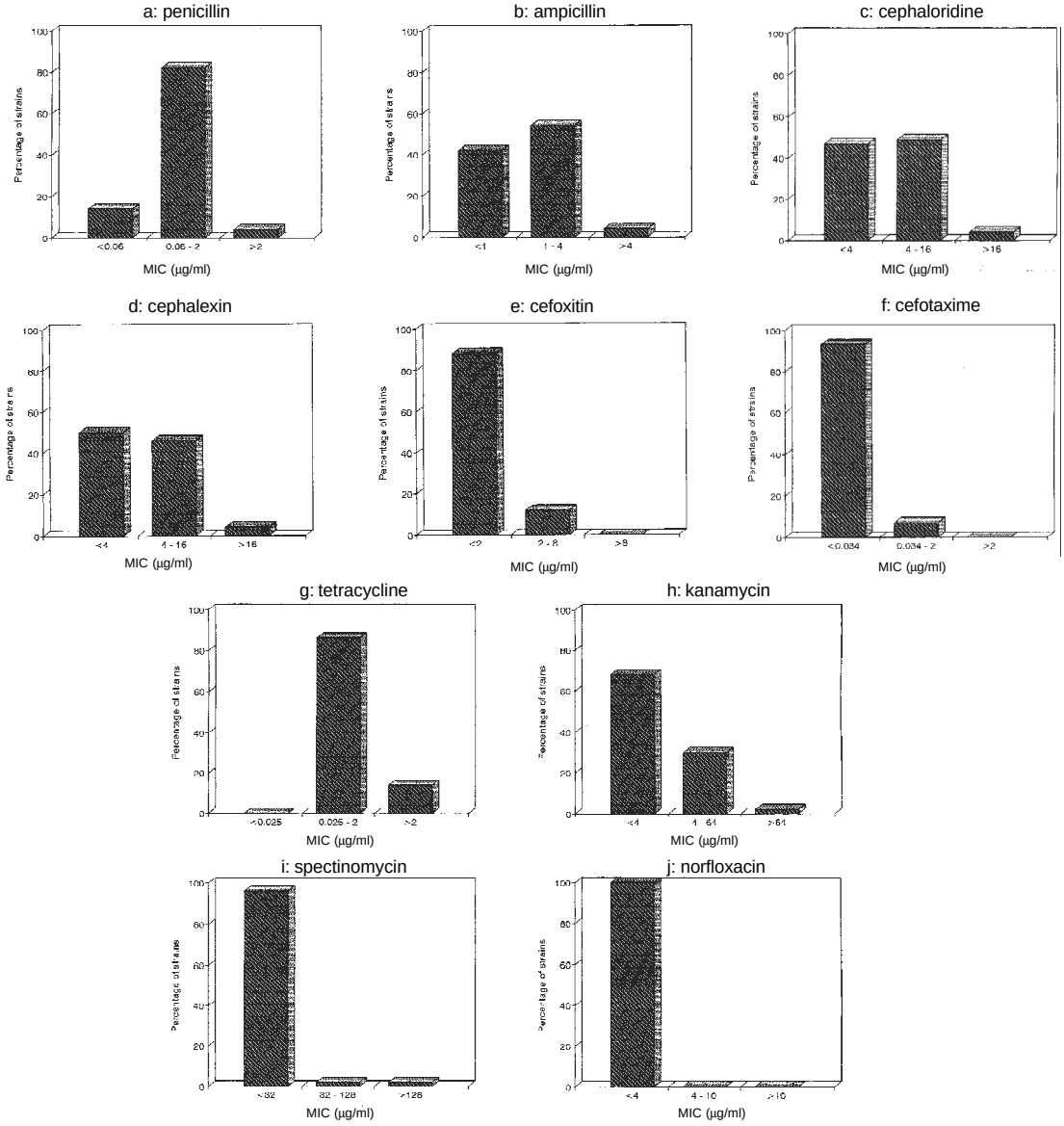
Some researchers have previous documented the resistance of *N. gonorrhoeae* to both penicillin and tetracycline in Thailand, Taiwan, The Philippines and Singapore (Sparling 1977, Sparling et al. 1997, WHO 1978, Khoo 1979, Brown et al. 1982, Chu et al. 1992).

Peeters et al. (1987) studied in three different periods (1981-1984-1985) the susceptibility to penicillin, tetracycline and spectinomycin in 302 clinic isolations of ß-lactamase producing and ß-lactamase negative *gonococci* and compared the susceptibility variations of the strains. In 1981, 7% of the strains resulted susceptible to a penicillin MIC higher than 32 μgml^{-1}. In 1984 and 1985 this percentage was 48 and 23 respectively. A similar behaviour was presented by *gonococci* to tetracycline, in which case 50% of the strains showed a MIC higher than 1 μgml^{-1} in 1984, and only 6% reached this value in 1985. Spectinomycin showed another behaviour; the increase was gradual through the years, reaching a MIC value of 32 μgml^{-1} for 60% of the strains in 1985.

Unfortunately, the intense use of spectinomycin in the treatment of patients infected with PPNG strains has determined that nowadays PPNG strains resistant to this antibiotic are found with a certain frequency in the USA and England (CDC 1981, Ison et al. 1983). Our results indicate that only 1.75% was resistant to spectinomycin, but on the other hand they showed a MIC value higher than 25 μgml^{-1} in 7% of the *gonococci* strains, which makes an epidemiologic control necessary.

Khan et al. (1981) made a study to measure the susceptibility (*in vitro*) to ß-lactamase negative and positive strains of *N. gonorrhoeae* and observed that 90% of these strains resulted highly sensitive to cefotaxime with a MIC value of ≤ 0.015 μgml^{-1}. Cefoxitin resulted less active against both groups (ß-lactamase negative and positive strains); 28% showed a MIC value from 0.5 to 5.0 μgml^{-1}. In Tucumán, 93% of the *N. gonorrhoeae* strains presented a MIC value of ≤ 0.02 μgml^{-1} to cefotaxime.

Kerbs et al. (1983) measured the *in vitro* antimicrobial activity of 92 penicillinase producing *gonococci* strains and 88 strains susceptible to penicillin, determining the same as we did: (a) cefotaxime resulted highly effective, and, to a lesser extent, cefoxitin, and (b) the behaviour against these antimicrobials is independent to the production of ß-lactamase.



Distribution of the susceptibility to some antibiotics in 57 isolates of *Neisseria gonorrhoeae*, isolated from patients in Tucumán, Argentina: a: penicillin, b: ampicillin, c: cephaloridine, d: cephalexin, e: cefoxitin, f: cefotaxime, g: tetracycline, h: kanamycin, i: spectinomycin, j: norfloxacin.

All our strains resulted highly sensible to norfloxacin, which coincides with the results obtained by King et al. (1982), who assayed 48 *N. gonorrhoeae* strains, all showing sensibility to norfloxacin, and 13 being PPNG; in Tucumán, all of the *N. gonorroeae* strains (100%) presented a MIC value of ≤ 0.25 µgml^{-1}. 98% of the *N. gonorrhoeae* strains was sensible to kanamycin and 68% of these 98% showed a MIC value of ≤ 0.2 µgml^{-1} (Fig. h). These data are different from those obtained from studies made in Hong Kong, where 76% of the strains presented a MIC value of ≤ 16 µgml^{-1}; this is probably due to the fact that this drug was used there as alternative against the penicillinase producing *N. gonorrhoeae* strains (PPNG). Rajan et al. (1979) recommend this antibiotic for the treatment of gonorrhoea in regions dominated by ß-lactamase producing strains.

The CDC established in 1985 (CDC 1985), that strains resistant to tetracycline, located on plasmids, must show MIC levels higher than 10 µgml^{-1}; none of our isolations exceeded this value, which could indicate the absence of a mediator plasmid with the mentioned resistance.

The results with respect to cefoxitin, cefotaxime and norfloxacin are similar as those obtained by Clendennen et al. (1992) in Thailand and Coovadia et al. (1987) in South Africa.

Fourteen percent of the isolated strains showed resistance to tetracycline; this percentage is very low compared with the resistance to this antibiotic obtained in the southwest of Africa and in Hong Kong (WHO 1977).

The norms for the gonorrhoea treatment without complications in Tucumán include the recommendation of penicillin use as preferable antibiotic. Due to the low incidence of PPNG in Tucumán, it is recommended to continue with this antimicrobial, provided that this is always done under strict vigilance of the isolations, to avoid posterior failures and proliferation of resistant strains, obtained by the presence of plasmids or by the increase of their MIC due to chromosomal mutations.

ACKNOWLEDGEMENTS

To Eng. Eric Fengler for language advice and drawing of the graphics.

REFERENCES

Ashford W, Golash R, Hemming G 1980. Penicillinase-producing *Neisseria gonorrhoeae*. *The Lancet 5:* 39.

Berliner DS, No PU, 1986. Prevalence of penicillinase-producing *Neisseria gonorrhoeae* in Korea. *Aviat Space Environ Med 57* (12 Pt 1): 1170-1175.

Brown S, Warnnissorn T, Biddle J, Panikabutra K, Traisupa A 1982. Antimicrobial resistance of *Neisseria gonorrhoeae* in Bangkok: is single-drug treatment passé? *The Lancet ii:* 1366-1368.

CDC - Centers for Disease Control 1981. Spectinomycin-resistant penicillinase-producing *Neisseria gonorrhoeae*. *MMWR 30*: 221-222.

CDC - Centers for Disease Control 1985. Tetracycline-resistant *Neisseria gonorrhoeae*-Georgia, Pennsylvania, New Hampshire. *Morbid Mortal Weekly Rep 34*: 563-564.

Chu Mong-Ling, Ho Ling-Jun, Lin Hwa-Chen, Wu Ying-Chang 1992. Epidemiology of penicillin-resistant *Neisseria gonorrhoeae* isolated in Taiwan, 1960-1990. *Clin Infect Dis 14*: 450-457.

Clendennen TE, Echeverria P, Saengeur S, Kees ES, Boslego JW, Wignall FS 1992. Antibiotic susceptibility survey of *Neisseria gonorrhoeae* in Thailand. *Antimicrob Agents Chemother 36*: 1682-1687.

Coovadia YM, Van Den Ende J, Hoosen AA, Kharsany A 1987. Susceptibility of penicillinase-producing and non-penicillinase-producing strains of *Neisseria gonorrhoeae* isolated in Durban, South Africa, to 15 ß-lactam antibiotics. *Sex Transm Dis 15*: 30-36.

Ison CA, Littleton K, Shannon KP et al. 1983. Spectinomycin resistant gonococci. *Br Med J* : 1827-1829.

Jaffe H, Biddle JW, Johnson SP, Wiesner PJ 1981. Infections due to penicillinase-producing *Neisseria gonorrhoeae* in the United States 1976-1980. *J Infect Dis 144*: 191-197.

Jones O, Strohmeyer G, Brockett J, Wright J, Grundy P, Lathrop G, Wolfe W, Herbole J 1983. Spectinomycin-resistant penicillinase-producing *Neisseria gonorrhoeae*. *Morbid Mortal Weekly Rep 32*: 51.

Jones RN, Gavan TL, Thornsberry C, Fuchs PC, Gerlach EH, Knapp JS, Murray P, Washington JA 1989. Standardization of disk diffusion and agar dilution susceptibility test for *Neisseria gonorrhoeae*: interpretive criteria and quality control guidelines for ceftriaxone, penicillin, spectinomycin and tetracycline. *J Clin Microbiol 27*: 2758-2766.

Jones RN, Gerlach EH, Koontz FP, Murray PR, Pfaller MA, Washington JA, Erwin ME, Knapp CC 1991. Interpretive criteria and quality control guidelines for *Neisseria gonorrhoeae* susceptibility test standardization for cefotetan. *J Clin Microbiol 29*: 363-366.

Joyce MP, Aying BB, Vaughan GH, Herip DS, Hayes CG, Espinosa G, Andrada A, Dally OP, Laughlin LW 1988. Drug resistance patterns of gonococcal isolates in the Philippines. Abstr. C-42, p. 339 in Abstract of 88th Annual Meeting of American Society for Microbiology, Washington D.C.

Joyce MP, Aying BB, Vaughan GH, Herip DS, Muallem RM, Bernardo ST, Andrada A, Coolbaugh JC 1989. Susceptibilities of penicillinase-producing *Neisseria gonorrhoeae* in the Philippines. Program Abstract of 29th Interscience Conference Antimicrobial Agents Chemotherapy, abstr. 986.

Kammer RB, Preston DA, Turner JR, Hawlwy LC 1975. Rapid detection of ampicillin-resistant *Haemophilus influenzae* and their susceptibility to sixteen antibiotics. *Antimicrob Agents Chemother 8:* 91-94.

Kerbs SB, Stone JR, Berg SW, Harrison WO 1983. *In vitro* antimicrobial activity of eight new ß-lactam antibiotics against penicillin-resistant *Neisseria gonorrhoeae*. *Antimicrob Agents Chemother 23*: 541-544.

Khan MY, Siddiqui Y, Simpson ML, Gruninger RP 1981. Comparative *in vitro* activity of cefmenoxime, cefotaxime, cefuroxime, cefoxitin and penicillin against *Neisseria gonorrhoeae*. *Antimicrob Agents Chemother 20*: 681-682.

Khoo R 1979. Gonorrhoeae: problems in control and effective treatment. *Med Prog 6*: 20.

King A, Warren C, Shannon K, Phillips I 1982. *In vitro* antibacterial activity of norfloxacin (MK-0366). *Antimicrob Agents Chemother 21*: 604-607.

Knapp JS 1988. Laboratory methods for the detection and phenotypic characterization of *Neisseria gonorrhoeae* strains resistant to antimicrobial agents. *Sex Transm Dis 1*5: 225-233.

NCCLS - National Committee for Clinical Laboratory Standards 1990. Standard methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically. Approved standard M7-A2. National Committee for Clinical Laboratory Standards, Villanova, Pa.

Peeters M, Frost EH, Collet M, Ossari S, Yvert F, Ivanoff B 1987. Changing antibiotic susceptibility of *Neisseria gonorrhoeae* in Franceville, Gavon. *Antimicrob*

Agents Chemother 31: 1288-1290.

Philips I 1976. ß-lactamase producing penicillin-resistant gonococcus. *The Lancet ii*: 656-657.

Rajan VS, Pang R, Tan NJ, Sng EH 1979. Kanamycin in the treatment of penicillinase-producing gonococcal infections. *Asian J Infect Dis 3*: 37-39.

Schwarcz SK, Zenilman JM, Schnell D, Knapp JS, Hook III EW, Thompson S, Judson FN, Holmes KK 1990. National surveillance of antimicrobial resistance in *Neisseria gonorrhoeae*. *JAMA 264*: 1413-1417.

Seth QA, Johnston N 1980. Penicillin-resistant gonococcus. *The Lancet 2*: 531.

Sparling PF 1977. Antibiotic resistance in the gonococcus, p. 111-135. In RB Roberts. *The gonococcus*. John Wiley and Sons, Inc. New York.

Sparling PF, Biswas GD, Sox TE 1977. Transformation of the gonococcus, p. 155-176. In RB Roberts. *The gonococcus*. John Wiley and Sons, Inc. New York.

Traisupa A, Ariyarit C, Saengsur S, Theeratum C, Tharavanich S 1990. Spectinomycin-resistant gonococci in Thailand. *Clin Ther 12*: 101-104.

WHO - World Health Organization 1977. A new complication in the fight against gonorrhoeae. *WHO Chron 31*: 38-39.

WHO - World Health Organization 1978. *Neisseria gonorrhoeae* and gonococcal infection. Report of a WHO Scientific Group. *WHO Tech Rep Series 616*: 65-91.