

ANTIBIOTIC SENSITIVITIES OF *Neisseria gonorrhoeae* IN
THE FAR EAST: COMPARISON OF PLASMID SPECIES IN
ISOLATES FROM SIX COUNTRIES

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OBJECTIVES : To determine the antibiotic susceptibilities of 36 *Neisseria gonorrhoeae* isolated in the Philippines, Thailand, Indonesia, Malaysia, Singapore, and Hong Kong in 1979 and 1980 and to compare the plasmids encoding for antibiotic resistance in these isolates.

BACKGROUND : Strains of *Neisseria gonorrhoeae* containing β -lactamase producing plasmids were isolated for the first time in 1976 in England and the United States (1-3). Since then these resistant pathogens have been isolated throughout the world and most cases of infections with β -lactamase positive *N. gonorrhoeae* (PPNG) infections have been epidemiologically linked with either the Far East or West Africa. Strains originating in Asia have been reported to contain 4.4 megadalton (Md) plasmids while those from West Africa contain 3.2 megadalton plasmids encoding for β -lactamase (1). These plasmids have been shown to be readily lost in the absence of selective antibiotic pressure and their emergence and prevalence throughout Asia is assumed to be due to the widespread prophylactic use of oral penicillin especially by prostitutes.

Spectinomycin and kanamycin have been used for treatment of PPNG infections. Recently, however, strains resistant to these antibiotics have been isolated in Asia. An individual with a spectinomycin resistant PPNG acquired his infection in the Philippines (4). Two refugees in Thailand who had received intramuscular kanamycin prophylactically were infected with kanamycin resistant gonococci two weeks later. *N. gonorrhoeae* isolated in Hong Kong, Indonesia, Malaysia, the Philippines, Singapore, and Thailand during 1979 and 1980 were, therefore, examined to determine antibiotic sensitivities of isolates and whether strains from diverse sources in Asia contained similar plasmids.

METHODS :

Source of isolates : The gonococcal strains examined were isolated from men and women attending public health clinics, private clinics, or during surveys of hospitality girls in the six Southeast Asian countries studied during 1979 and 1980. *N. gonorrhoeae* were identified by their morphology, cultural characteristics, and by fermentation of glucose but not sucrose,

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maltose, or lactose. These strains were lyophilized until ready for susceptibility testing and plasmid analysis. *Escherichia coli* 711 containing plasmids of known molecular weight RI (62×10^6 daltons), RP4 (36×10^6 daltons), Sa (24×10^6 daltons), RSF 1010 and RSF 1030 (5.5×10^6 daltons) were used as standards to determine the molecular weight of *N. gonorrhoeae* plasmids. Each isolate of PPNG was grown on Mueller-Hinton agar containing 100 μ g of ampicillin and retested for β -lactamase production before being examined (5).

Antibiotic susceptibility tests : Antibiotics were obtained from the following manufacturers: benzylpenicillin, cefuroxime and cephalexin from Glaxo Laboratory; chloramphenicol, thiamphenicol, ampicillin and tetracycline from Sigma Chemical Co.; streptomycin from Meiji Seika Kaisha Ltd., ceftriaxone (R013-9904), sulfamethoxazole and trimethoprim from Hoffman La Roche Inc., erythromycin from Abbott Laboratories, kanamycin from Pierrel s.p.a., spectinomycin from Upjohn Co., cefamandole, cephalothin and moxalactam from Eli Lilly & Co., cefoxitin from Merck Sharp and Dohme International Co., cephradine from E.R. Squibb & Sons, cefotaxime from Hoechst Roussel Pharmaceuticals Inc., cefoperazone from Pfizer Pharmaceuticals Inc.

The susceptibility of *N. gonorrhoeae* strains were tested by the agar dilution method, in which doubling dilutions of the antibiotics were incorporated into the test medium, which was GC (gonococcus) base (Difco) supplemented with 1% hemoglobin and 1% GC supplement without antibiotics (Oxoid Ltd., London, England). This medium was used for all antibiotics except sulfamethoxazole-trimethoprim; these two drugs were combined in a ratio of 19:1 and tested on diagnostic sensitivity test agar (Oxoid) with 5% lysed horse blood and 1% Defined Supplement (Oxoid).

Inocula were prepared from overnight growth on GC medium. Several colonies of each test and control strains (*N. gonorrhoeae* 76-073389 and 77-083718 from Centers for Disease Control, Atlanta, GA.) were suspended in tryptic soy broth until the turbidity matched that of a 0.5 McFarland standard. The suspension was further diluted 1:50 in the same broth and approximately 10^3 CFU/ml were spot inoculated onto the test plates with a Dynatech multipoint inoculator. After the inocula had dried, the plates were inverted and incubated in a 5% CO₂ incubator (National Weinicke Co., Oregon, U.S.A.) for 24 hrs. Minimal inhibitory concentration (MIC) was the lowest concentration of antibiotic that inhibited growth. The presence of a single colony was ignored.

Isolation and characterization of plasmid DNA : Plasmid deoxyribonucleic acid (DNA) of *N. gonorrhoeae* were examined by a method developed by Portnoy et al (6). Briefly isolates were grown on Mueller-Hinton agar in 5% CO₂ at 37°C for 24 hours. PPNG isolates were grown on Mueller-Hinton agar containing 100 μ g/ml of ampicillin. Cells from a plate with confluent growth were suspended in 2 ml of TE buffer (50mM Tris-hydrochloride, pH=8.0, 10mM EDTA) (optical density of approximately 1.0 at 650nm), washed, and resuspended in 40 μ l of TE. Cells were then transferred to a 1.5 ml polypropylene microcentrifuge tube (Brinkman 22-26-411-1) containing 0.6 ml of lysis buffer (TE + 4% SDS, pH=12.42) and mixed immediately by inversion. The sample was then incubated at 37°C for 20 minutes and then neutralized to pH 8.0 by adding 30 μ l of 2 M Tris-hydrochloride pH 7.0 and mixed by gentle inversion. 0.16 ml

of 5 M NaCl was added, the tubes held in an ice bath for at least 60 minutes and then centrifuged at 15,600 g in a microcentrifuge (Eppendorf model 5412) for 5 minutes. The supernatant was decanted immediately into another tube and the plasmid DNA was precipitated by adding 0.55 ml of isopropanol and submerging the sealed tube in a bath of dry-ice-95% ethanol for 5 minutes. The specimen was then centrifuged at 15,600 g for 3 minutes, the supernatant discarded, and the pellet dried in a vacuum jar. The pellet was then resuspended in 30 μ l of TES (30mM Tris-hydrochloride pH 8.0, 5mM EDTA, 50mM NaCl) and 15 μ l of the sample was subjected to electrophoresis in Tris-borate buffer 0.7% vertical agarose gel at 120 volts (constant voltage) and photographed under long wave ultraviolet light.

RESULTS :

Antibiotic sensitivities : The MICs of 27 PPNG and 9 NPPNG strains from the different S.E. Asian countries tested were summarized in Table 1. It is clear that there is notable difference between the MICs of penicillin and ampicillin for these two types of strains. In no instance were the MICs of NPPNG strains for these antibiotics greater than 1 μ g/ml. Both PPNG and NPPNG were susceptible to chloramphenicol and thiamphenicol requiring not more than 4 μ g/ml to inhibit 90% of all strains. In comparison, 50% of the strains required tetracycline MICs of >2 μ g/ml and 75% required streptomycin MICs of >128 μ g/ml. The MICs of erythromycin required to inhibit 90% of the isolates were 7.3 and 6.0 μ g/ml for the PPNG and NPPNG respectively. Eighty-two percent of the PPNG and 100% of NPPNG strains examined required kanamycin MICs of >32 μ g/ml. On the other hand, spectinomycin and sulphamethoxazole-trimethoprim (19:1) were effective against these strains and all were inhibited by these drugs at 32 μ g/ml.

Among the ten cephalosporins tested, the third generation compounds: cefoperazone, moxalactam, cefotaxime, and ceftriaxone were the most effective. The latter two compounds were 10-fold more active (on an MIC basis) than cefoperazone and moxalactam with MICs required to inhibit all strains not greater than 0.06 μ g/ml. The order of *in vitro* activities of the second generation cephalosporins was cefuroxime > cefoxitin > cefamandole. Cephalothin was as effective as cefamandole and 90% of the strains were inhibited at a concentration of 1.2 μ g/ml. Cephadrine and cephalixin were the least active compounds: 58 and 33% of the strains, respectively, required MICs of > 8 μ g/ml.

Plasmid species of *Neisseria gonorrhoeae* : β -lactamase production was associated with a 4.4 megadalton plasmid in all 27 PPNGs examined as exemplified in Fig. 1. Ninety-three percent (25/27) of PPNG and 22 percent (2/9) NPPNG strains contained a 24 megadalton plasmid (Table 2). Single strains isolated in Malaysia, Indonesia, Singapore, and the Philippines contained 12.5 and/or 7.5 megadalton plasmids (Table 2) which could not be associated with resistance to any specific antibiotics. Two NPPNG from Thailand contained a 3.2 megadalton plasmic which was similar in size to the plasmids encoding for β -lactamase in strains traceable to West African sources (1). All strains of *N. gonorrhoeae* carried a previously described 2.5 megadalton cryptic plasmid.

PLAN :

1. *N. gonorrhoeae* isolated at the Cholburi Venereal Disease clinic in 1981 will be screened for resistance to antibiotics specifically kanamycin and spectinomycin.

Table 1. Susceptibility of penicillinase-producing (PPNG) and non penicillinase-producing (NPPNG) *N. gonorrhoeae* strains isolated in S.E. Asian countries to 20 antibiotics.

Antimicrobial agent	PPNG (n = 27 ^a)			NPPNG (n = 9) ^a		
	MIC ($\mu\text{g/ml}$)					
	Range	For % of isolates 50%	90%	Range	For % of isolates 50%	90%
Penicillin	1-32	3.5	17.0	0.06-0.5	0.213	1.0
Ampicillin	2-32	11.5	25.0	0.125-1	0.225	0.57
Chloramphenicol	1-8	1.6	3.9	0.25-4	0.86	2.9
Thiamphenicol	0.5-4	0.75	1.5	0.25-1	0.48	0.8
Tetracycline	0.5-4	1.17	2.75	0.5-4	0.71	2.25
Erythromycin	0.06-16	2.8	7.3	0.5-8	1.73	6.0
Streptomycin	8->128	44.0	119.0	16->128	94.0	122.5
Kanamycin	0.5-64	25.0	43.8	32-64	27.0	53.0
Spectinomycin	2-32	6.05	14.8	2-16	4.2	11.6
Sulfamethoxazole- trimethoprim ^b	2-32	2.7	9.1	2-16	3.25	11.5
Cephradine	2-32	4.15	7.8	0.5-8	5.1	7.2
Cephalexin	2-16	3.2	6.9	2-8	5.3	18.0
Cephalothin	0.06-4	0.58	1.2	0.25-2	0.62	1.08
Cefamandole	0.125-2	0.42	1.27	0.125-2	0.33	1.17
Cefoxitin	0.125-1	0.34	0.57	0.5-1	0.37	0.53
Cefuroxime	0.015-0.125	0.04	0.06	0.015-1	0.03	0.138
Cefoperazone	0.0018-0.25	0.036	0.08	0.0075-0.125	0.025	0.09
Moxalactam	0.015-0.25	0.023	0.04	0.03-0.125	0.033	0.05
Cefotaxime	0.0018-0.06	0.004	0.008	0.0018-0.06	0.012	0.035
Ceftriaxone	0.0018-0.015	0.002	0.004	0.0018-0.015	0.004	0.0086

a = Number of strains tested

b = Ratio of sulfamethoxazole-trimethoprim at 19:1

Table 2. Plasmid species of 29 PPNG and 9 NPPNG isolated in the Far East.

<u>Masses of plasmids (Md)</u>	<u>Number of isolates containing different species of plasmids</u>						
	<u>Thailand</u>	<u>Malaysia</u>	<u>Singapore</u>	<u>Indonesia</u>	<u>Hong Kong</u>	<u>Philippines</u>	
<u>PPNG</u>							
24, 12.5, 7.5, 4.4, 2.5	0	1	0	0	0	1	
24, 12.5, 4.4, 2.5	0	0	1	0	0	0	
24, 4.4, 2.5	4	7	4	4	3	0	
7.5, 4.4, 2.5	0	0	0	0	0	1	
4.4, 2.5	0	0	0	1	0	0	
<u>NPPNG</u>							
24, 2.5	1	0	0	1	0	0	
12, 2.5	0	0	0	1	0	0	
3.2, 2.5	2	0	0	0	0	0	
2.5	0	0	0	2	0	2	

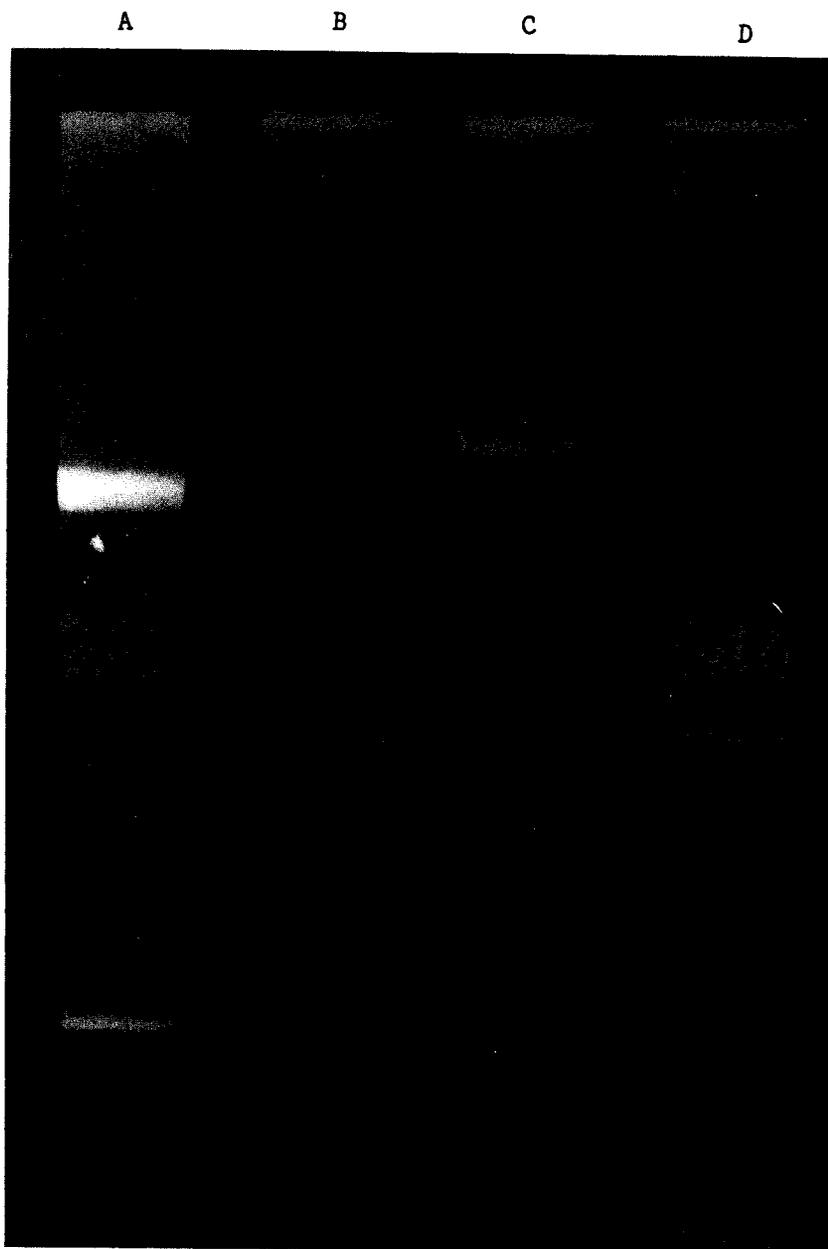


Figure 1. Plasmid profiles of a β -lactamase positive *Neisseria gonorrhoeae* and R1, RP4, and RSF 1030

- (A) A β -lactamase positive *N. gonorrhoeae* from Thailand containing 24, 12.5, 7.5, 4.4, and 2.5 Md plasmids.
- (B) *E. coli* 711 containing R1, ^a62 Md plasmid.
- (C) *E. coli* 711 containing RP4, ^a36 Md plasmid and
- (D) *E. coli* 711 containing RSF 1030, ^a5.5 Md plasmid.

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