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Emerging Antimicrobial Resistance, Plasmid Profile and Pulsed-field Gel Electrophoresis Pattern of the Endonuclease-digested Genomic DNA of *Neisseria gonorrhoeae*

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Abstract

Resistant gonococci are very prevalent in many countries, particularly in Asia. This study was conducted to determine the trend of resistance, the effect of decreasing the ciprofloxacin susceptibilities of gonococci on the prevalence of penicillinase-producing *N. gonorrhoeae* (PPNG), and to compare the epidemiology of strains with the previous studies. A total of 602 strains of gonococci were isolated from prostitutes in 1997−1999. Antimicrobial susceptibility was tested by NCCLS disk diffusion and agar dilution methods. For epidemiologic analysis, plasmid analysis and pulsed-field gel electrophoresis (PFGE) were performed. The proportion of PPNG remained high (79%), and the strains with decreased susceptibility to ciprofloxacin increased significantly from 67% in 1997 to 84% in 1999. Compared to our previous study, the PFGE patterns were similar, while the proportion of strain with the 3.2-MDa plasmid markedly decreased. In conclusion, a rapid increase in ciprofloxacin-nonsusceptible strains may suggest difficulties in the treatment of gonococcal infections in the near future with the drug. The recent decrease of PPNG with the 3.2-MDa plasmid may suggest that there is an epidemiological change in gonococcal infections, and the prevalence of related PFGE patterns suggests the dissemination of a few clones among the high risk populations.

Key Words: *Neisseria gonorrhoeae*, β-lactamase, ciprofloxacin resistance, plasmid, pulsed-field gel electrophoresis

INTRODUCTION

Neisseria gonorrhoeae is one of the most important causative agents of sexually transmitted diseases. Although the incidence of gonococcal infection cannot be estimated accurately because only a small proportion of cases are reported, its incidence has slowly declined in developed countries.[1] On the other hand, it still prevalent in developing countries.[2]

The emergence of resistance to antimicrobial agents in *N. gonorrhoeae* had made treatment more difficult. It is well known that the proportion of PPNG is prevalent worldwide,[3,4] but it has been decreasing in several countries.[5-7] In 1989 and 1993, the CDC recommended 3rd-generation cephalosporins and fluoroquinolones for the treatment of uncomplicated gonococcal infections.[8,9] However, the decreased susceptibility of *N. gonorrhoeae* to fluoroquinolones has recently been documented by several studies.[10-14]

Therefore, antimicrobial resistance surveillance of gonococci is very important for the empirical treatment of gonococcal infections, because the antimicrobial susceptibility of gonococci is not tested routinely. Since 1992, we have been participating in a WHO Western Pacific Region Gonococcal Antimicrobial Surveillance Programme, which has showed the high prevalence of PPNG and a rapid decrease of ciprofloxacin-susceptible strains.[2,4] Our previous study showed that these resistance trends are continuing, and that the PPNG strains with the 3.2-MDa plasmid, which had not been reported previously in

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Korea, has been introduced, and that the high prevalence of PPNG is probably due to the dissemination of a few resistant clones among the high risk population.[15]

The aim of this study was to determine the trend of resistance, the effect of decreasing ciprofloxacin susceptibilities on the prevalence of PPNG, and to compare the β-lactamase plasmid profile and the PFGE pattern of endonuclease-digested genomic DNA with those of previous studies.

MATERIALS AND METHODS

Endocervical specimens were collected from Korean prostitutes in 1997−1999. The specimens were inoculated onto modified Thayer-Martin medium and incubated in a CO_2 incubator. Species identification was based on conventional cultural and biochemical tests.[16]

A total of 602 strains of *N. gonorrhoeae* were isolated. NCCLS disk diffusion susceptibility[17] was performed immediately after isolation. GC II agar base supplemented with 1% IsoVitaleX (Becton Dickinson, Cockeyville, MD, USA) was used for susceptibility testing. Antimicrobial disks were obtained from Becton Dickinson (Cockeyville, MD, USA). β-lactamase production was determined with a Cefinase disk (Becton Dickinson, Cockeyville, MD, USA).

The strains were kept frozen at −70°C until used for agar dilution susceptibility testing[18] and other studies. Antimicrobial powders used were penicillin G (Sigma Chemical Co., Saint Louis, MO, USA), ceftriaxone (Hanmi Pharmaceutical, Seoul, Korea), tetracycline (Pfizer Korea, Seoul, Korea) and ciprofloxacin (Miles Pharmaceutical, West Point, CT, USA). An inoculum of 10^4 CFU was applied with a Steers replicator (Craft Machine, Chester, PA, USA) onto chocolate agar containing antimicrobial agents. Plates were incubated in a 5% CO_2 incubator at 35°C for 24 h before the results were read. *N. gonorrhoeae* ATCC 49226 was used as a control strain.

Plasmids were isolated by the alkaline lysis method[19] and their size estimated by comparing their electrophoretic mobilities with those of *E. coli* V517. The method of Birren and Lai[20] was used for PFGE with some modifications. Briefly, to prepare the plugs, one loopful of the cells grown overnight on chocolate agar was suspended in 1 ml of saline EDTA solution. The genomic DNA was digested with *Nhe*I (Takara, Tokyo, Japan) for about 18 h at 35°C. A CHEF DR II instrument (Bio-Rad, Hercules, CA, USA) was used to separate the fragments, with switch times of 0.5 s (initial) and 54 s (final) and a running time of 20 h at 6 V/cm. The band patterns were compared according to the recommendations of Tenover et al.[21]

RESULTS

Among the 602 strains of gonococci isolated in 1997−1999, none showed susceptibility to penicillin G disk. The rate of β-lactamase-producing strains was 74−84% (mean 79%) depending on the year of isolation (Table 1). All of the isolates were susceptible to ceftriaxone and spectinomycin, but none were susceptible to low-level tetracycline. The proportion of ciprofloxacin susceptible isolates decreased from 33%

Table 1. Antimicrobial Susceptibility of *N. gonorrhoeae* Tested by the Disk Diffusion Test

Year	No. of isolates tested	% of isolates with:								
		Penicillin			Tetracycline			Ciprofloxacin		
		I	R	PPNG	I	R	TRNG	S	I	R
1997	382	8	92	79	0	100	1	33	47	20
1998	134	10	90	74	0	100	0	37	52	11
1999	86	4	96	84	0	100	0	16	71	13

PPNG, penicillinase-producing *N. gonorrhoeae*; TRNG, high-level tetracycline resistant *N. gonorrhoeae*; S, susceptible; I, intermediate; R, resistant.

Table 2. Antimicrobial Susceptibility of *N. gonorrhoeae* Tested by the Agar Dilution Test

Antimicrobial agents	No. of isolates tested	MIC (μg/ml)			% of isolates with		
		Range	50%	90%	S	I	R
Penicillin G	Non-PPNG, 29	0.12−1	0.25	1	0	100	0
	PPNG, 144	2−>128	8	128	0	0	100
Ceftriaxone	173	≤0.008−0.06	0.015	0.03	100	−	0
Tetracycline	173	1−8	4	4	0	13	87
Ciprofloxacin	Non-PPNG, 29	≤0.008−0.5	0.015	0.5	59	41	0
	PPNG, 144	≤0.008−1	0.25	0.5	35	64	1

Table 3. Plasmid Profile of Penicillinase-producing *N. gonorrhoeae* Isolates

Pattern	Plasmid (MDa)				No (%) of strains by year		
	24.5	4.4	3.2	2.6	1993	1997	1998
I	+	+	−	+	28 (24.6)	8 (50)	44 (77.2)
II	+	−	+	+	58 (50.8)	4 (25)	3 (5.3)
III	−	+	−	+	28 (24.6)	4 (25)	10 (17.5)
Total					114 (100)	16 (100)	57 (100)

Table 4. PFGE Patterns of *Nhe*I-digested Genomic DNA of *N. gonorrhoeae*

Year	Organism (No.of isolates tested)	No. of isolates with PFGE pattern:																												
		A2	B	C1	C2	C3	C4	C5	C6	D1	D2	E	F1	F2	G1	G2	G3	G4	G5	G6	G7	H1	H2	H3	H4	I1	I2	I3	J	K	L
1995	PPNG (44)	0	1	4	1	1	1	0	0	4	3	0	3	0	10	4	5	2	0	0	0	1	1	1	1	0	0	0	0	0	0
	Non-PPNG (13)	0	0	5	1	0	0	0	0	0	0	1	1	1	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1998	PPNG (40)	2	0	1	3	0	0	3	6	2	2	0	0	0	0	0	2	0	10	1	1	0	0	0	0	2	0	1	1	1	1
	Non-PPNG (10)	1	0	2	0	0	0	1	0	0	2	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	1	0	0	0	0

in 1997 to 16% in 1999.

The MIC range of pencillin G for non-PPNG was 0.12−1 μg/ml, while that for PPNG was 2−>128 μg/ml (Table 2). MIC ranges and MIC_{90s} of ciprofloxacin for non-PPNG and PPNG were similar, but MIC_{50s} were 0.015 μg/ml and 0.25 μg/ml, respectively (Table 2).

Among the PPNG strains, the proportion of strains with 24.5-MDa, 4.4-MDa and 2.6-MDa plasmids increased, 24.6% in 1993, 50% in 1997, and 77% in 1999. However, the proportion of the strains with 3.2-MDa plasmid markedly decreased from 50.8% in 1993 to 5.3% in 1998 (Table 3, Fig. 1).

Fifty isolates in 1998 were tested for PFGE patterns of *Nhe*I-digested genomic DNA, and compared to those of previous studies. The most common pattern in 1998 was type G5 (10 PPNG and 1 non-PPNG), while in 1995 it was type G1. When the subtypes were combined, type G was most prevalent, 44% in 1995 and 32% in 1998 (Table 4, Fig. 2).

DISCUSSION

The resistance of gonococci to some antimicrobial agents is now widespread, and is either chromosomally mediated or plasmid-mediated. Resistance to

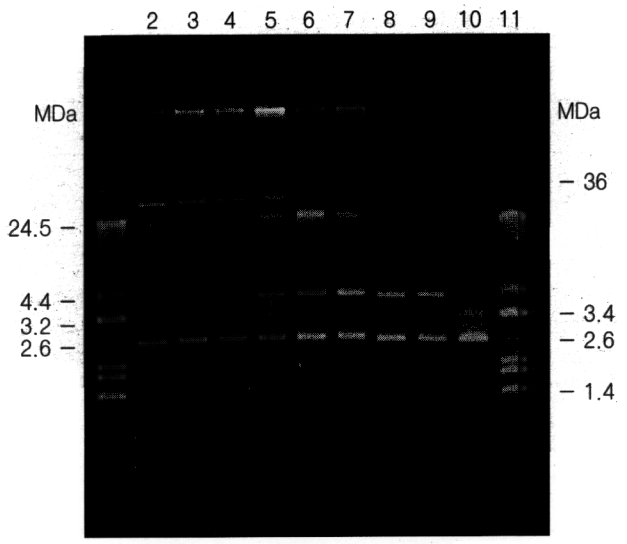

Fig. 1. Plasmid profile of PPNG strains showing 4.4-MDa (lanes 2-9) and 3.2-MDa (lane 10) β-lactamase plasmids and 24.5-MDa conjugative plasmid (lanes 2-7, 10). Lanes 1 and 11 are Escherichia coli *V517 plasmid size markers. The sizes of plasmids of gonococci and* E. coli *V517 are shown on the left and right of the photograph, respectively.*

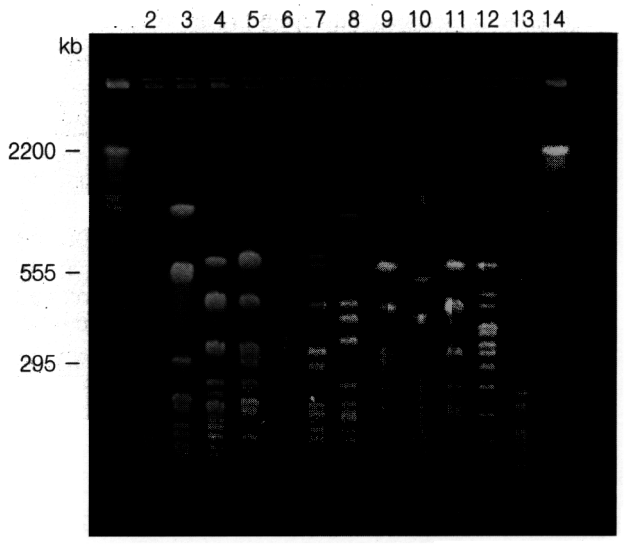

Fig. 2. PFGE separation of NheI-digested chromosomal DNA of N. gonorrhoeae. *Prevalent patterns were C6 (lane 5; 6 isolates) and G5 (lane 9; 11 isolates). Molecular markers are chromosomes of* Saccharomyces cerevisiae *(lanes 1 and 14).*

high levels of penicillins and tetracyclines are plasmid-mediated and have the potential to spread horizontally.[6] Since the first isolation of PPNG in 1976,[22] its proportion has steadily increased worldwide, particularly in Korea.[15]

The proportion of PPNG is high in many Asian countries compared to that in other developed countries.[3-7] Surveillance by the WHO Western Pacific Region in 1997 showed that PPNG was widely distributed throughout the region.[2] PPNG is particularly prevalent in the Philippines (81.8%), Korea (79.3%), Singapore (61.3%), Vietnam (64.1%), and Malaysia (41.0%). Interestingly, the rates are very low in other countries, such as Japan (2.3%), Australia (6.4%), and New Zealand (7.4%). In the study by Tanaka et al.,[23] plasmid-mediated penicillin resistance (PPNG) decreased significantly from 7.9% in 1993–1994 to 2.0% in 1997–1998, and chromosomally mediated penicillin resistance decreased from 12.6% in 1993–1994 to 1.9% in 1995–1996 and subsequently increased in 1997–1998 (10.7%). However, in our study, none of the Korean isolates were susceptible to penicillin G. The proportion of PPNG was about 80%, which was slightly higher than that of our previous study.[15] The proportion of PPNG was high in this study probably because the isolates were from prostitutes.

Fluoroquinolones, such as ciprofloxacin and ofloxacin, are highly effective as a single dose regimen for uncomplicated gonococcal infections and is one of the recommended drugs.[9] However, the emergence of gonococci exhibiting intermediate resistance or resistance to fluoroquinolones have been reported in many countries since 1992.[24] The proportion of intermediate resistant strains by disk diffusion test remained particularly high in China (51.5%) and Hong Kong (42.1%) in 1997.[2] During the same period, fluoroquinolone-resistant strains accounted for approximately 50.0%, 38.6%, and 28.5% of all strains in the Philippines, Hong Kong, and China, respectively. Moreover, strains with high ciprofloxacin resistance, i.e., MICs of $\geq 8\ \mu g/ml$, have been reported in several countries.[25] However, all of our isolates were inhibited by $\leq 1\ \mu g/ml$ of ciprofloxacin. With increasing fluoroquinolone resistance, treatment failure of gonococcal infections with fluoroquinolones has also been reported.[13,24]

In our previous study, a rapid decrease of ciprofloxacin-susceptible isolates was noted, from 91% in 1992 to 46% in 1996.[15] However, most of the non-susceptible strains were intermediate, and less than 5% of the isolates were resistant. In this study, 33% of the isolates in 1997 and 16% in 1999 were susceptible by the disk diffusion test. The resistance rate

of all of the isolates was 1% by agar dilution test, but the MIC of ciprofloxacin for the resistant isolates was only 1 μg/ml. The MIC ranges and MIC_{90s} of ciprofloxacin for both of strains in 1993 and 1998 were equal, $\leq 0.008-1$ μg/ml and 0.25 μg/ml, but the MIC_{50s} were 0.015 μg/ml and 0.25 μg/ml, respectively, showing gradual increase of resistance.

With the increase of fluoroquinolone-resistant gonococci, a decrease of PPNG has been reported, from 71% in 1983 to 28% in 1990 in Bangkok, Thailand,[5] from 25.5% in 1993 to 4.3% in 1994 in Hong Kong[6] and from 7.9% in 1993-1994 to 2.0% in 1997-1998 in Japan.[23] It has been suggested that the decrease of PPNG is due to the therapeutic use of active drugs other than penicillins and use of fluoroquinolone, which possibly cured β-lactamase plasmids.[6] In this study, the MIC ranges and MIC_{90s} of ciprofloxacin for non-PPNG and PPNG were similar, but the MIC_{50s} were 0.015 μg/ml and 0.25 μg/ml, respectively. It has been suggested that the decrease in PPNG may be associated with full resistance to fluoroquinolones, but the decline of PPNG and emergence of fluoroquinolone resistance may be coincidental.

The isolation trend of high level tetracycline-resistant *N. gonorrhoeae* (TRNG) may be useful for the epidemiologic study of gonococcal infections. The presence of TRNG has been documented worldwide,[1] but the prevalence varies and is country dependent: 82% in Singapore, 55% in Malaysia, 1-2% in Japan and China.[2] In Korea, TRNG was about 3% in 1992-1996[15] and less than 1% in 1997-1999.

Spectinomycin and ceftriaxone are very active drugs for the treatment of gonococcal infections. No resistance to ceftriaxone has been evident until now.[2] A small number (0.5%) of spectinomycin-resistant strains were reported in China.[2] In our study, these two drugs were very active against gonococci. Therefore, either spectinomycin or ceftriaxone may be used effectively for empirical therapy.

Evolution of β-lactamase genes carrying plasmids is known.[26] It was considered that the 4.4-MDa plasmid is the Asian type and that the 3.2-MDa plasmid is the African type.[3] In Korea, only 4.4-MDa plasmid had been reported until 1994.[27] In our previous study, a significant proportion of PPNGs with the 3.2-MDa plasmids were noted, 51% in 1993 and 25% in 1997.[15] However, 3.2-MDa plasmids were detected in only 5% of strains in this study. The cause of this change is not known.

In this study, the PFGE pattern of the *Nhe*I-digested genomic DNA of gonococci from prostitutes showed two predominant clones. This finding was very similar to those of previous studies,[15,28] with minor differences in the subtypes. This may suggest that a few resistant clones may be spreading among the high-risk populations.

In conclusion, in Korea, the continued high prevalence of PPNG precludes the empiric use of penicillins and the gradual increase of ciprofloxacin-nonsusceptible strains may suggest difficulties in the treatment of gonococcal infections with the drug in the future. The recent decrease of PPNG with the 3.2-MDa plasmid may suggest that there is an epidemiological change in gonococcal infections, and the prevalence of related PFGE patterns suggests the dissemination of a few clones among the high risk populations.

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