EPIDEMIOLOGY OF INFECTION

Analysis of *Neisseria gonorrhoeae* in Ontario, Canada, with decreased susceptibility to quinolones by pulsed-field gel electrophoresis, auxotyping, serotyping and plasmid content

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The incidence of *Neisseria gonorrhoeae* with reduced susceptibility to quinolones increased from 0.18% (63 of 3285) in 1992 to 0.56% (15 of 2663) in 1993 and 0.62% (46 of 2846) in 1994. In all, 65 of the 67 isolates of *Neisseria gonorrhoeae* with decreased susceptibility to quinolones were characterised by pulsed-field gel electrophoresis (PFGE), auxotyping, serotyping and plasmid content. The strains were distributed among 14 auxotype/serovar (A/S) classes. Thirty isolates (46.2%) which were penicillin-susceptible with ciprofloxacin MIC₉₀ of 0.12 mg/L and norfloxacin MIC₉₀ of 1.0 mg/L belonged to a single A/S class, OUHL/IA-2. All but two of the 30 isolates had identical PFGE restriction profiles with *NheI* restriction endonuclease. Fifteen isolates (23.1%) with MICs in the intermediate (or resistant) categories for penicillin and with ciprofloxacin and norfloxacin MIC₉₀ of 0.25 and 4.0 mg/L and (0.5 and 4.0 mg/L) respectively, belonged to A/S class P/IB-1. The 15 isolates showed nine different patterns with *NheI* and eight patterns with *SpeI* restriction endonucleases. Two of three β -lactamase-producing (PPNG) isolates belonged to A/S class P/IB-5 and had a dissimilar PFGE restriction profile with *NheI* endonuclease; the other isolate belonged to A/S class P/IB-8. The remaining 17 isolates were distributed among 11 A/S classes. Three isolates within the common A/S class NR/IB-1 were subdivided into two types by PFGE as were three isolates belonging to A/S class NR/IB-2. Overall the 65 isolates of *N. gonorrhoeae* were distributed into 30 *NheI* and 26 *SpeI* macrorestriction profiles. All but one isolate harboured the 2.6-MDa cryptic plasmid and 18 isolates carried the 24.5-MDa transferable plasmid. The three PPNG isolates carried the 4.5-MDa Asian β -lactamase-producing plasmid and a 25.2-MDa conjugative plasmid was found in the two TRNG isolates.

Introduction

Fluoroquinolones such as ciprofloxacin have excellent penetration into the genitourinary tract and have been shown to be highly active against *Neisseria gonorrhoeae*, including penicillinase-producing strains (PPNG) [1, 2]. In several countries, quinolone agents have been used as first line therapy for uncomplicated gonorrhoeal infections [3]; however, strains with

decreased susceptibility to fluoroquinolones have been reported in various parts of the world [3–6].

In a recent report, Harnett *et al.* [7] describes the emergence of isolates of *N. gonorrhoeae* in Ontario, Canada, with decreased susceptibility to quinolones. In previous studies, several typing systems have been employed to characterise strains of *N. gonorrhoeae* [8, 9]. The purpose of the present study was to carry out a more detailed epidemiological study of the Ontario isolates to determine whether decreased susceptibility to quinolone agents was caused by the dissemination of a single resistant strain or of multiple strains. This report describes the analysis of 65

isolates by pulsed-field gel electrophoresis (PFGE), auxotyping, serotyping and plasmid content.

Materials and methods

Bacterial isolates and culture conditions

Sixty-five isolates of *Neisseria gonorrhoeae* obtained between Jan. 1992 and Dec. 1994, with MICs of ciprofloxacin ≥ 0.12 mg/L, were included in this study. In 1992, a total of 3285 isolates were tested for susceptibility patterns, and in 1993 and 1994, 2663 and 2846 isolates were examined, respectively. All isolates were grown on GC Agar Base (Difco) with lysed horse blood 1.5% and Kellogg's supplement 1% and incubated at 35°C in humidified air with CO₂ 5% for 18 h.

Antimicrobial susceptibility testing

The MIC for all isolates was determined by the agar dilution method on GC agar base with Kellogg's supplement 1%. The inoculum was prepared and plates were inoculated as recommended by the National Committee of Clinical Laboratory Standards (NCCLS) [10]. The concentrations (mg/L) of antibiotics tested were as follows: two-fold dilutions of penicillin 0.015–256; tetracycline 0.25–32.0; erythromycin 0.12–16.0; ciprofloxacin 0.001–2.0; norfloxacin 0.5–32.0; cefixime 0.25; ceftriaxone 0.000062–0.25 and spectinomycin 16.0 and 32.0. MIC values were determined after incubation for 24 h at 35°C in CO₂ 5% atmosphere. MIC values were defined as the lowest concentration of an antimicrobial agent that completely inhibited visible growth. A control strain of *N. gonorrhoeae* (ATCC 49226) recommended by NCCLS and WHO reference strains were included with each test run.

DNA preparations and restriction endonuclease digestion of agarose plugs

Genomic DNA from the isolates of *N. gonorrhoeae* was prepared by a modification of the method of Smith and Cantor [11]. Fresh overnight cultures were harvested, washed twice with 1.0 ml (per 5-cm plate culture) of 1 M NaCl, 10 mM EDTA, 10 mM Tris buffer (pH 7.6) and resuspended in the same buffer. Agarose plugs were prepared by adding 200 μ l of cell suspension to 1.0 ml of low melting agarose (BioRad Laboratories, Mississauga, Ontario, Canada) 1.0% at 50°C and c. 200 μ l of this mixture were distributed into the slots of an acrylic mould (BioRad) and allowed to solidify. The agarose plugs were transferred into 2.0 ml of EC lysis buffer (6 mM Tris-HCl, pH 7.6, 1 M NaCl, 100 mM EDTA) Brij 58 0.5%, sodium deoxycholate 0.2%, lysozyme 1 mg/ml, RNAase 20 μ g/ml and incubated at 37°C for 24 h with gentle shaking. After further incubation in ESP buffer (0.5 M EDTA, pH 9.3, sodium lauroyl sarcosine 1.0%, proteinase K 1 mg/ml) for 48 h at 50°C with gentle shaking, the plugs were

placed in TE buffer (10 mM Tris (pH 7.5), 0.1 mM EDTA) containing phenylmethylsulphonyl fluoride (PMSF) 174 μ g/ml for 6 h. The plugs were then washed in TE buffer and stored at 4°C in the same buffer. Slices 1 cm length from these plugs were equilibrated for 30 min on ice in 100 μ l of the appropriate 1 $\times$ restriction endonuclease buffer, 10–20 units of endonuclease were added and equilibrated on ice for a further 30 min. Digestion was carried out overnight at 37°C. The restriction endonucleases used were *Nhe*I (BRL, Mississauga, Ontario) and *Spe*I (Promega Labs). Restriction endonuclease buffers for *Nhe*I (React 4) and *Spe*I (Buffer B) were provided with the enzymes by the suppliers.

PFGE

Digested slices were sealed into slots in an agarose (Pulsed field certified agarose, BioRad) 1.0% gel and electrophoresed in a CHEF DR II apparatus (BioRad) for 24 h at 200 V with pulse times of 5–15 s. Gels were stained with ethidium bromide solution, destained in distilled water and photographed under UV illumination. Phage lambda concatamers were used as mol. standards.

Determination of auxotype and serotype

Nutritional growth requirements (auxotype) were assessed by the method of Hendry and Stewart [12] as modified by Hendry [13]. Serological classification was by co-agglutination with monoclonal antibodies to protein I [14]. Serovars were designated following Knapp's nomenclature [15].

Plasmid analysis

Plasmid DNA was isolated as described earlier [16] and analysed on agarose 0.7% gels. *N. gonorrhoeae* isolates from the collection at the Central Public Health Laboratory and *Escherichia coli* strains containing plasmids RSF 1030 (23 MDa) and S-a (5.5 MDa) were included as size markers.

Results

This study characterised 65 isolates of *N. gonorrhoeae* with reduced susceptibility to quinolone agents that were isolated between Jan. 1992 and Dec. 1994. Six reduced susceptibility strains, out of a total of 3285 isolates (0.18%), were identified in 1992 and 15 (0.56%) of 2663 in 1993. In 1994, 46 (1.62%) of 2846 isolates showed decreased susceptibility to fluoroquinolones. Two isolates with decreased susceptibility to quinolones failed to grow on subculture and were not included in this study.

The MIC₉₀ and MIC ranges of the 65 strains for each antimicrobial agent tested are shown in Table 1. The

Table 1. In-vitro activity of selected antimicrobial agents against 65 isolates of *N. gonorrhoeae* with decreased susceptibility to fluoroquinolones

Antimicrobial agent	MIC (mg/L) for					
	Penicillin-susceptible strains (n = 30)		Penicillin-intermediate strains (n = 11)		*Penicillin-resistant strains (n = 24)	
	MIC ₉₀	Range	MIC ₉₀	Range	MIC ₉₀	Range
Penicillin	0.03	0.015–0.03	1.0	0.25–1.0	8.0	2.0–128
Ciprofloxacin	0.12	0.12	0.25	0.12–0.25	0.5	0.25–1.0
Norfloxacin	1.0	0.5–1.0	4.0	1.0–4.0	4.0	2.0–16.0
Erythromycin	0.25	0.12–0.5	4.0	0.12–4.0	4.0	1.0–8.0
Tetracycline	1.0	0.5–16.0	4.0	0.5–4.0	8.0	2.0–256
Ceftriaxone	0.00025	0.000063–0.00025	0.032	0.002–0.032	0.032	0.004–0.063

All isolates were susceptible to cefixime and spectinomycin (MICs $\leq$ 0.25 mg/L and $\leq$ 32/L respectively).

*Three isolates were penicillinase-producing *N. gonorrhoeae* (PPNG); one PPNG isolate was also TRNG with tetracycline MIC of 256 mg/L.

Table 2. Phenotypic profile and plasmid content of 65 isolates of *N. gonorrhoeae* with decreased susceptibility to quinolones

A/S class	Number of isolates tested	Number of isolates with plasmids having mol. wt (MDa) of *					
		2.6	2.6	2.6	2.6	2.6	10.5 [†]
			+	+	+	+	+
			25.2	4.5	4.5	24.5	24.5
					+		
					25.2		
OUHL/IA-2	30	29	1				
O/IB-6	1	1					
O/IB-8	2	1				1	
P/IB-1	15	2				12	1
P/IB-2	2	2					
P/IB-3	1					1	
P/IB-5	2		2				
P/IB-6	1	1					
P/IB-7	1	1					
P/IB-8	2	1			1		
P/IB-50 [‡]	1	1					
NR/IB-1	3	3					
NR/IB-2	3	1				2	
NR/IB-15	1					1	

OUHL, ornithine-; uracil-; hypoxanthine- and leucine-requiring; O, ornithine-requiring; P, proline-requiring; NR, non-requiring or wild-type.

*Mol. wt in megadaltons as determined by agarose gel electrophoresis.

[†]Probably directly repeated copies of the 2.6-MDa cryptic plasmid.

[‡]IB-50 is not an accepted Knapp designation but arbitrarily given by the National Laboratory for Sexually Transmitted Diseases as no code number is available for the agglutination pattern obtained. The pattern in terms of specific monoclonal antibodies was 3C8, 2D6, 2G2, 2H1 (A, C, G, K).

MIC₉₀ values of ciprofloxacin and norfloxacin against penicillin-resistant, penicillin-sensitive and penicillin-intermediate isolates were 0.5 and 4.0 mg/L, 0.12 and 1.0 mg/L and 0.25 and 4.0 mg/L, respectively. Three isolates of the total examined over the 3 years (8794) had ciprofloxacin MICs of 0.06 mg/L; the MIC values for the remaining isolates ranged from 0.002 to 0.016 mg/L (MIC₉₀ of 0.002 mg/L).

The 65 isolates of *N. gonorrhoeae* were distributed into 14 A/S classes (Table 2), and 30 *NheI* and 26 *SpeI* PFGE restriction patterns (Table 3). PFGE analysis of the 30 isolates belonging to A/S class OUHL/IA-2 showed identical *SpeI* restriction endonuclease profiles and all but two isolates had identical *NheI* patterns. The two isolates, 1017 and 1870, differed from the others by a single fragment with extra fragment, no.3 in isolate 1017 and no.7 in isolate 1870. Representative patterns from A/S class

Table 3. Numbers of PFGE patterns among different A/S classes of *N. gonorrhoeae* after digestion with *NheI* and *SpeI* restriction endonuclease

A/S class	Number of isolates tested	Number of PFGE patterns with	
		<i>NheI</i>	<i>SpeI</i>
OUHL/IA-2	30	3	1
O/IB-8	2	2	2
P/IB-1	15	9	8
P/IB-2	2	2	2
P/IB-5	2	2	1
P/IB-8	2	2	2
NR/IB-1	3	2	2
NR/IB-2	3	2	2
O/IB-6	1	1	1
P/IB-3	1	1	1
P/IB-6	1	1	1
P/IB-7	1	1	1
P/IB-50	1	1	1
NR/IB-15	1	1	1

OUHL, ornithine-; uracil-; hypoxanthine- and leucine-requiring; O, ornithine-requiring; P, proline-requiring; NR, non-requiring or wild type. IA serovar, serogroup WI; IB serovar, serogroup WII/III.

OUHL/IA-2 are shown in Figs. 1a and 1b. In contrast, PFGE demonstrated diversity within the P/IB-1, P/IB-2, P/IB-5, P/IB-8, NR/IB-1, NR/IB-2 and O/IB-8 classes. The 15 isolates belonging to A/S class P/IB-1 showed nine different profiles with *NheI* and eight different patterns with *SpeI*. The patterns after digestion with *NheI* are shown in Fig. 2, c. 12–15 fragments were identified. The three NR/IB-1 isolates displayed two PFGE profiles with each of the two endonucleases as did the three belonging to A/S class NR/IB-2. The *SpeI* restriction profiles of the three NR/IB-1 and three NR/IB-2 isolates are shown in Fig. 3, the isolates which differed were readily distinguishable from the others. These differences are also evident in the *NheI* pattern of NR/IB-2 (Fig. 1a). The two O/IB-8 isolates and two P/IB-8 exhibited different profiles with each endonuclease. The *SpeI* restriction endonuclease patterns of the O/IB-8 isolates are shown in Fig. 3 (lanes 8 and 9) and the *NheI* patterns of isolates belonging to A/S class P/IB-8 are shown in Fig. 4 (lanes 2 and 3). Two PPNG isolates belonging to A/S class P/IB-5 had an identical profile with *SpeI*, but small differences were detected with *NheI* (Fig. 4, lanes 4 and 5). The differences were in a single extra band, (band 6, lane 5) and an extra band 15 (lane 4).

Two restriction patterns were observed among isolates belonging to A/S class P/IB-2 with both *NheI* and *SpeI* restriction endonucleases. The profiles of isolates belonging to A/S class P/IB-2 after digestion with *NheI* are shown in Fig. 4 (lanes 6 and 7). The unique profiles of isolates belonging to eight different A/S classes are displayed in Fig. 5, while the distinct patterns from seven different A/S classes are shown in Fig. 6. A comparison of restriction endonucleases

Fig. 2. PFGE analysis of genomic DNA from *N. gonorrhoeae* isolates with reduced susceptibility to quinolones after digestion with *NheI*. Lane 1, λ DNA; 2, isolate 463; 3, 638; 4, 642; 5, 852; 6, 959; 7, 1380; 8, 1606; 9, 1919; 10, 2455. All strains belong to A/S class P/IB-1 and show dissimilar profiles.

NheI and *SpeI* in differentiating isolates of four A/S types is shown in Fig. 7. Isolates 1248 (P/IB-2) and 890 (P/IB-6) which appear similar with *SpeI* (lanes 4 and 5) can be distinguished with *NheI* (lanes 2 and 3). Similarly, isolates 861 (NR/IB-1) and 1381 (O/IB-8) which appear similar with *NheI* (lanes 6 and 7) are differentiated with *SpeI* (lanes 8 and 9).

The plasmid content of the 65 isolates is shown in Table 2. All but one isolate carried the 2.6-MDa cryptic plasmid. Thirteen (86.7%) of 15 isolates belonging to A/S class P/IB-1 harboured the 24.5-MDa plasmid and the one isolate without a 2.6-MDa plasmid carried a 10.5-MDa plasmid. Two of three

Fig. 1. PFGE analysis of genomic DNA from *N. gonorrhoeae* isolates with reduced susceptibility to quinolones after digestion with *NheI*. (a) Lane 1, isolate 1870 (OUHL/IA-2); 2, 2199 (OUHL/IA-2); 3, 2287 (OUHL/IA-2); 4, 2222 (NR/IB-2); 5, 2379 (NR/IB-2); 6, 2380 (NR/IB-2); 7, Megabase II DNA Standard (Gibco-BRL). Arrow indicates extra band in isolate 1870 (lane 1). (b) Lane 1, 315 (P/IB-8); 2, 104–95 (P/IB-8); 3, 2473 (OUHL/IA-2); 4, 2424 (OUHL/IA-2); 5, 2294 (OUHL/IA-2); 6, 1042 (OUHL/IA-2); 7, 1017 (OUHL/IA-2). Arrow indicates extra band in isolate 1017 (lane 7).

Fig. 3. PFGE analysis of genomic DNA from *N. gonorrhoeae* isolates with reduced susceptibility to quinolones after digestion with *Spe*I. Lane 1, λ DNA; 2, isolate 861 (NR/IB-1); 3, 1153 (NR/IB-1); 4, 582 (NR/IB-1); 5, 2222 (NR/IB-2); 6, 2379 (NR/IB-2); 7, 2380-94 (NR/IB-2); 8, 1381 (O/IB-8); 9, 2919 (O/IB-8); 10, λ DNA (New England BioLabs).

Fig. 4. PFGE analysis of genomic DNA from *N. gonorrhoeae* isolates with reduced susceptibility to quinolones after digestion with *Nhe*I. Lane 1, λ DNA; 2, isolate 104-95 (P/IB-8); 3, 315 (P/IB-8); 4, 1108 (P/IB-5); 5, 1144 (P/IB-5); 6, 1172 (P/IB-2); 7, 1248 (P/IB-2); 8, 2797 (P/IB-2); 9, 749 (P/IA-2); 10, 2380-92 (P/IA-6); lanes 8-10 contain isolates with ciprofloxacin MICs of 0.06 mg/L.

isolates belonging to A/S class NR/IB-2, one isolate of A/S class O/IB-8 and the single isolates of A/S class NR/IB-15 and P/IB-3 also harboured the 24.5-MDa plasmid. The two PPNG isolates belonging to A/S class P/IB-5 carried the 4.5-MDa Asian β -lactamase plasmid in addition to the cryptic 2.6-MDa plasmid and the remaining PPNG isolate, which was also tetracycline-resistant (PP/TRNG), harboured a 25.2-MDa plasmid as well as a 4.5-MDa Asian β -lactamase producing and a 2.6-MDa plasmid. This PP/TRNG isolate belonged to A/S class P/IB-8. One isolate of A/S class OUHL/IA-2, which was resistant to tetracycline, harboured a 25.2-MDa conjugative plasmid in addition to the 2.6-MDa plasmid.

Fig. 5. PFGE analysis of *Nhe*I-cleaved genomic DNA from *N. gonorrhoeae* isolates with reduced susceptibility to quinolones showing dissimilar profiles. Strains belong to eight different A/S classes. Lane 1, isolate 2222 (NR/IB-2); 2, 861 (NR/IB-1); 3, 1248 (P/IB-2); 4, 53 (P/IB-3); 5, 890 (P/IB-6); 6, 1236 (P/IB-7); 7, 1381 (O/IB-8); 8, 315 (P/IB-8); 9, 2919 (O/IB-8); 10, Megabase II DNA Standard (Gibco-BRL).

Fig. 6. PFGE analysis of *Nhe*I cleaved genomic DNA from *N. gonorrhoeae* isolates with reduced susceptibility to quinolones showing dissimilar profiles. Strains belong to seven different A/S classes. Lane 1, λ DNA; 2, isolate 582 (NR/IB-1); 3, 966 (NR/IB-15); 4, 1144 (P/IB-5); 5, 104-95 (P/IB-8); 6, 315 (P/IB-8); 7, 1021 (P/IB-50); 8, 1868 (O/IB-6); 9, 461 (OUHL/IA-2).

Discussion

The present study has shown that, based on the distribution of MICs, reduced susceptibility to fluoroquinolone agents was associated with three populations of *N. gonorrhoeae* in Ontario. One population demonstrated marginally increased MICs of fluoroquinolones and all but one isolate in this population showed susceptibility to penicillin (pen^s) and other antimicrobial agents. The single exception showed high level resistance to tetracycline (TRNG). Twenty-eight of the 30 isolates in this group, belonging to A/S class OUHL/IA-2, had identical macrorestriction profiles. The remaining two isolates differed by only a single fragment, indicating a close similarity among the

Fig. 7. PFGE analysis of genomic DNA from *N. gonorrhoeae* isolates with reduced susceptibility to quinolones after digestion with *NheI* and *SpeI*. Lane 1, λ DNA; 2, isolate 1248 (P/IB-2); 3, 890 (P/IB-6) (digested with *NheI*); 4 and 5, 1248 (P/IB-2) and 890 (P/IB-6) (digested with *SpeI*); 6 and 7, 861 (NR/IB-1) and 1381 (O/IB-8) (digested with *NheI*); 8 and 9, 861 (NR/IB-1) and 1381 (O/IB-8) (digested with *SpeI*).

isolates. Goering [17] has proposed that a single genetic event can cause as many as three differences among PFGE patterns; therefore, isolates differing by fewer than four bands should be considered subtypes of the same strain. On this basis, all 30 isolates that belong to A/S class OUHL/IA-2 are clonally related. Isolates of this A/S class are generally susceptible to all antimicrobial agents, so it is interesting that not only are isolates now showing decreased susceptibility to fluoroquinolones, but one strain has acquired the 25.2-MDa *Tet M*-containing plasmid which confers high-level resistance to tetracycline. The antimicrobial susceptibility patterns seen in this group of isolates are similar to those reportedly acquired in the UK [18] which showed penicillin-susceptibility and reduced susceptibility to ciprofloxacin with MICs of 0.12 mg/L. It is noteworthy that, in the present study, all 30 isolates belonged to A/S class OUHL/IA-2 while those in the UK belonged to serovar IB-6.

It is also important to note that pen^s-quinolone-susceptible strains in Ontario were more heterogeneous when examined by A/S class and PFGE (data not shown). These observations would suggest that a single strain with reduced quinolone susceptibility is disseminating rather than the occurrence of independent selections for reduced quinolone susceptibility among patients infected with a common fully susceptible clone.

The second population consists of a heterogeneous group showing resistance to penicillin (pen^R) tetracycline and erythromycin. This group included three PPNG isolates, one of which demonstrated high-level tetracycline resistance (PP/TRNG). The third group of organisms consisted of penicillin-intermediate isolates

(pen^I), exhibiting a more diverse pattern of resistance to other antimicrobial agents and MICs to fluoroquinolones which ranged from those associated with the pen^s isolates to those associated with the pen^R isolates. The results in the present study differ from those of a number of studies which reported only PPNG isolates with quinolone insensitivity [5, 19]. The MIC₉₀ values of fluoroquinolones against *N. gonorrhoeae* which were pen^R or pen^I were more closely comparable to those reported by Tanaka *et al.* [20] and by Clendennen *et al.* [21]. The MIC₉₀ values of ciprofloxacin and norfloxacin for the Japanese isolates [20] were 0.5 mg/L and 2.0 mg/L, respectively, while for those from the Philippines [21] MIC₉₀ values of ciprofloxacin and norfloxacin were 0.25 mg/L and 2.0 mg/L. In this study, pen^R and pen^I isolates had MIC₉₀ values of 0.5 mg/L and 4.0 mg/L, and 0.25 mg/L and 4.0 mg/L, respectively, to ciprofloxacin and norfloxacin; resistance to norfloxacin was slightly higher than that observed in the previous studies [20, 21]. The 35 isolates of the pen^R and pen^I categories in the present study were distributed amongst 13 A/S classes. Fifteen (42.9%) of these isolates belonged to A/S class P/IB-1. PFGE analysis after digestion of the isolates with *NheI* further subdivided the 15 isolates of the identical class P/IB-1 into nine distinct patterns. PFGE was also more discriminatory than A/S classification for differentiating the two isolates belonging to A/S class O/IB-8 which had distinct PFGE patterns, the three NR/IB-1 strain types which displayed two PFGE restriction profiles, and three NR/IB-2 strains which were also subdivided into two restriction patterns. Overall, for the majority of the A/S classes studied, it was possible to differentiate subtypes within each class and the patterns obtained were stable over repeated subcultures.

The importance of examining more than one restriction endonuclease digest pattern for the macrorestriction analysis of isolates was also evident in this study. Although *NheI* provided data which enabled better discrimination of subtypes, in at least two instances isolates of different A/S types, which had a similar restriction profile with one endonuclease could easily be differentiated with a second endonuclease, in this case it was *SpeI*. Three or more band differences were present among PFGE types within a common serovar, e.g., P/IB-1. These differences may result from genetic events such as insertions, deletions, mutations or inversions of DNA. Differences among different A/S types also showed three or more fragment differences from a conserved core pattern.

In the present study, the cryptic 2.6-MDa plasmid was present in all but one isolate. These results are similar to others which have shown that the 2.6-MDa plasmid is common among isolates of *N. gonorrhoeae* [22, 23]. Strains lacking the 2.6-MDa plasmid have been found among isolates requiring proline, citrulline and uracil

for growth [22, 24]. While screening the 65 isolates for plasmid content one strain was encountered which contained a plasmid of *c.* 10.5 MDa, as determined by agarose gel electrophoresis. This strain also possessed a 24.5-MDa conjugative plasmid but lacked the 2.6-MDa plasmid. The 10.5-MDa plasmid is of an unusual size which has not been described previously for a plasmid in *N. gonorrhoeae*. Atypical plasmids of 7.5 and 7.8 MDa have been shown to occur in strains of *N. gonorrhoeae* [25, 26]. Johnson *et al.* [25] have demonstrated that these plasmids are composed of directly repeated copies of the 2.6-MDa cryptic plasmids which can be highly unstable [27].

It is noteworthy that three isolates (Fig. 4) had ciprofloxacin MICs of 0.06 mg/L which is interpreted as susceptible by NCCLS. Two of these isolates were obtained in 1992 and one in 1994. These isolates are interesting because of reports of clinical failure with fluoroquinolones in patients with *N. gonorrhoeae* isolates with MIC of 0.06 mg/L [4]. This suggests that infections caused by strains with ciprofloxacin MICs of 0.06 mg/L should be closely monitored as far as treatment with quinolones is concerned. In Canada, fluoroquinolones were introduced as first line therapy for the treatment of uncomplicated gonorrhoea in 1992 [28]. The recommended dosage was 500 mg of ciprofloxacin orally in a single dose. The emergence of isolates of *N. gonorrhoeae* in Ontario with reduced susceptibility to fluoroquinolones is probably a result of a combination of treatment failures after use of the agent, spread of a particular clone, and introduction of isolates from outside Canada.

Resistance to quinolone agents in gram-negative bacteria involves alterations in DNA gyrase and decreased permeability of bacterial membranes [2]. The mechanism by which isolates of *N. gonorrhoeae* become resistant to these antimicrobial agents is not known with certainty. A study by Stein *et al.* [29] has suggested that low level resistance was associated with the *gyrB* gene while high level resistance probably represents a mutation in *gyrA*. A recent study [30] also found mutations in the *gyrA* gene associated with quinolone resistance in clinical isolates of *N. gonorrhoeae* and Belland *et al.* [31] have indicated that alterations in topoisomerase IV, an enzyme related to DNA gyrase, have also been associated with quinolone resistance in gonococci.

In this report PFGE analysis proved to be more discriminatory than auxotyping or serotyping in the epidemiological investigation of *N. gonorrhoeae* isolates with reduced susceptibility to quinolones. Subtle differences between phenotypically similar but epidemiologically unrelated clones were detected by PFGE, providing further evidence for the genetic diversity of *N. gonorrhoeae* [32]. Plasmid profile analysis also provided some degree of discrimination

for these isolates, particularly distinguishing the PPNG isolates as carrying the Asian type plasmid but, in general, did not add significant information to the study. An examination of the overall picture suggests that a combination of antimicrobial susceptibility, PFGE and A/S type may be more suitable for improved epidemiological investigation of quinolone-insensitive gonococci.

The data presented in this report also show that the incidence of *N. gonorrhoeae* isolates with reduced susceptibility to quinolones is increasing steadily in Ontario, and suggest that continued monitoring of the susceptibility of *N. gonorrhoeae* isolates to fluoroquinolones is necessary.

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