Emergence and Characterization of *Neisseria gonorrhoeae* Isolates With Decreased Susceptibilities to Ceftriaxone and Cefixime in Canada: 2001–2010

Irene Martin, BSc, Pam Sawatzky, BSc,* Vanessa Allen, MD,† Linda Hoang, MD,‡§ Brigitte Lefebvre, PhD,¶ Neil Mina, MD,‖ Tom Wong, MD, MPH,** and Matthew Gilmour, PhD**

Background: Globally, *Neisseria gonorrhoeae* antimicrobial resistance has been increasing, and in particular, reports of isolates with reduced susceptibility to third-generation cephalosporins have surfaced. We examined the phenotypic and genetic characteristics of 155 *N. gonorrhoeae* isolates with decreased susceptibilities to third-generation cephalosporins isolated in Canada between 2001 and mid-2010.

Methods: Minimum inhibitory concentrations (MICs) were determined by agar dilution on *N. gonorrhoeae* isolates, and those displaying elevated MICs to cefixime (MIC = 0.25 μg/mL and 0.5 μg/mL) and ceftriaxone (MIC = 0.125 μg/mL and 0.25 μg/mL) were examined using *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) and sequencing of resistance determinants associated with decreased cephalosporin susceptibilities (*penA, mtrR, ponA, porB1b* (*penB* alteration).

Results: Between 2001 and 2010, there has been a shift in the modal MICs from 0.016 to 0.125 μg/mL for cefixime and from 0.016 to 0.063 μg/mL for ceftriaxone. Thirty-seven different sequence types (STs) were identified among the isolates using *N. gonorrhoeae* multiantigen sequence typing; ST3158, ST225, and ST1407 were most prevalent at 25.9%, 19.4%, and 14.8%, respectively. The *penA* mosaic was present in 60% of the isolates, with the most common *penA* mosaic types XXXII and X identified at 51.0% and 7.7%, respectively, whereas the nonmosaic *penA* type XII was identified in 36.8% of the isolates.

Conclusions: In Canada, *N. gonorrhoeae* isolates with decreased susceptibilities to third-generation cephalosporins have increased over the years. The alterations in *penA*, *mtrR*, and *porB1b* (*penB* alteration) are important determinants identified in these isolates. The most common STs identified among these Canadian isolates have also been reported worldwide.

From the *Bacteriology and Enteric Diseases Program, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Manitoba, Canada; †Public Health Ontario Laboratories, Toronto, Ontario, Canada; ‡British Columbia Centres for Disease Control Public Health Microbiology & Reference Laboratory, Vancouver, British Columbia, Canada; §Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, British Columbia, Canada; ¶Laboratoire de santé publique du Québec, Ste-Anne-de-Bellevue, Québec, Canada; ‖Department of Medical Microbiology, University of Alberta, Edmonton, Alberta, Canada; and **Centre for Communicable Diseases and Infection Control, Public Health Agency of Canada, Ottawa, Ontario, Canada

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Correspondence: Irene Martin, BSc, Bacteriology and Enteric Diseases Program, National Microbiology Laboratory, Public Health Agency of Canada, 1015 Arlington St., Winnipeg, Manitoba R3E 3R2, Canada. E-mail: irene.martin@phac-aspc.gc.ca.

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Neisseria gonorrhoeae, the causative agent of gonorrhea remains a global public health issue, and the World Health Organization (WHO) recently reported that approximately 88 million people yearly are affected by gonococcal infections worldwide.[1] In Canada, gonorrhea is the second most commonly reported bacterial sexually transmitted infection with over 11,000 cases reported in 2009.[2] The Canadian reported rate of gonorrhea is on the rise and has increased by 122% from 4.9 per 100,000 in 1997 to 33.1 per 100,000 in 2009.[2]

N. gonorrhoeae has evolved over the years and developed resistance to many of the antibiotics used to treat it including the penicillins (PENs), tetracyclines (TETs), macrolides, and quinolones.[3] Reduced susceptibility to third-generation cephalosporins has begun to develop in Asia with possible importation to Australia and Europe.[3] The earliest case reports of treatment failures with the use of third-generation cephalosporins were from Japan, as early as 2000.[4] More recently, a cefixime (CFM) treatment failure for urogenital gonorrhea has been described from Norway[5] and England,[6] as well as the first reports of ceftriaxone (CRO) treatment failure of pharyngeal gonorrhea in Australia,[7] Sweden,[8] and Japan.[9]

Several molecular mechanisms for decreased in vitro susceptibility and resistance to cephalosporins have been described. Two classes of *penA* gene alterations are possible: the first is the acquisition of a *penA* mosaic allele that encodes an altered PEN-binding protein 2 (PBP2); the second is an alteration of amino acids (A501, G542, P551) of PBP2 in nonmosaic *penA* alleles.[3,10,11] Mutations in the promoter and/or coding sequence of the repressor gene *mtrR*, which cause overexpression of the MtrCDE efflux pump system, have also been associated with a decrease in cephalosporin susceptibility.[3,12] Finally, *porB1b* gene mutations (the *penB* resistance determinant) that alter amino acid G120 and A121 in the outer membrane PorB1b porin result in decreased permeability and, thus, further decreased susceptibility to cephalosporins.[3,12]

In this report, we present the phenotypic and genetic characteristics of *N. gonorrhoeae* with decreased susceptibilities to third-generation cephalosporins isolated between 2001 and 2010, results from a Canadian laboratory based antimicrobial susceptibility program coordinated by the National Microbiology Laboratory (NML), Public Health Agency of Canada.

TABLE 1. Primers Used for PCR Amplification and Sequencing of the *penA, mtrR, ponA, porB1b* (*penB* alteration) Genes

Primer	Nucleotide Sequence (5′ to 3′)	Nucleotide Positions	Reference
penA-A3	GCCGTAACCGATATGATCGA	1003–1022	Ito et al[17]
penA-B3	CGTTGATACTCGGATTAAGACG	1844–1865	
penA-F	TCGAACAGAAAGGCAAGTC	386–404	Whiley et al[21]
penA-R1	CTGATGGTATTCGACCGC	777–760	
penA-R2	GTAAACGGCTTCATGGCA	947–930	
penA-R3	TAGGATAAACGTGGGTATCTTGT	1054–1032	
penA-R4	CGCCCACACCTAAATCG	1168–1152	
penA-R5	GCACTTTTTTGGCAGTAGAG	1411–1392	
*mtrR*1	AACAGGCATTCTTATTTCAG	860–879	Mavroidi et al[18]
*mtrR*2	TTAGAAGAATGCTTTGTGTC	1756–1775	
*ponA*F	CGCGGTGCGGAAAACTGATATCGAT	955–978	Ropp et al[19]
*ponA*R	AGCCCGGATCGGTTACCATACGTT	2218–2195	
*porB*F	CCGGCCTGCTTAAATTTCTTA	Entire *porB*	Liao et al[20]
*porB*R	TATTAGAATTTGTGGCGCAG		

MATERIALS AND METHODS

N. gonorrhoeae Isolates

Between January 2001 and July 2010, a total of 10,301 *N. gonorrhoeae* isolates were submitted from sexually transmitted infection clinics and provincial public health laboratories to the NML for antimicrobial susceptibility testing as part of the National *Neisseria gonorrhoeae* Surveillance Program. Isolates are submitted to the NML only when the provincial laboratories identify resistance to at least 1 antibiotic or if the provincial laboratories do not conduct any antimicrobial susceptibility testing. The results presented in this report represent *N. gonorrhoeae* isolates received by NML from 3 provincial public health laboratories in Canada. In addition to the isolates, information on age and sex of the patient, province of residence, date of isolation, and anatomical site of infection were also submitted to NML. Isolates in the study included those obtained from both males and females of all ages. Sexual orientation of patient was not available.

A total of 155 random isolates obtained from urethral, rectal, cervical, and pharyngeal sites displaying increased minimum inhibitory concentrations (MICs) to CFM (MIC = 0.25 μg/mL and 0.5 μg/mL) and CRO (MIC = 0.125 μg/mL and 0.25 μg/mL) were selected for further characterization. *N. gonorrhoeae* ATCC 49226, ATCC BAA-1837 (F62) and the WHO isolates WHO B, WHO C, and WHO D reference cultures were used as controls because reference cultures that exhibit decreased susceptibility to CFM and CRO were not available.

Isolate Characterization and Antimicrobial Susceptibility Testing

Antimicrobial susceptibilities of *N. gonorrhoeae* to azithromycin (AZM; compliments of Pfizer, Pointe-Claire/Dorval, Québec, Canada), CFM (compliments of Wyeth-Ayerst Laboratories, Mason, MI), ciprofloxacin (CIP; compliments of Bayer, Etobicoke, Ontario, Canada), spectinomycin (SPT; compliments of Pharmacia & Upjohn, Kalamazoo, MI), CRO, erythromycin (ERY), PEN, and TET (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) were determined using the agar dilution method with a GC medium base containing 1% Kellogg's-defined supplement and 2-fold dilutions of antibiotic. MIC (the minimum concentration of antibiotic that will inhibit the growth of the organism) interpretations were based on the criteria of the Clinical Laboratory Standards Institute: PEN resistance (R) MIC ≥2.0 μg/mL; TET resistance MIC ≥2.0 μg/mL; CIP resistance MIC ≥1.0 μg/mL; SPT resistance MIC ≥128.0 μg/mL; CRO susceptibility ≤0.25 μg/mL; CFM susceptibility ≤0.25 μg/mL.[13] The breakpoints for ERY and AZM resistance were both MIC ≥2.0 μg/mL. β-lactamase production was analyzed using nitrocefin. Auxotyping and plasmid profiles were determined as previously described.[14,15] Isolates were subcultured on GC medium base (Difco Laboratories, Detroit, MI) containing 0.2% BioX and incubated for 24 hours at 35°C in a 5% CO_2 atmosphere with or without antibiotics and maintained in brain heart infusion broth containing 20% glycerol and stored at −80°C.

N. gonorrhoeae Multiantigen Sequence Typing

The molecular genotyping of the *N. gonorrhoeae* isolates using the *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) method incorporated the amplification of the porin gene (*por*) and the transferrin-binding protein gene (*tbpB*) as previously described.[16] The resulting polymerase chain reaction products were purified (QIA Quick PCR Purification Kit, Qiagen, Mississauga, Ontario or Agencourt AMPure, Beckman Coulter, Beverly, MA), and the DNA sequences of both strands obtained by the DNA Analyzer 3730xl (Applied Biosystems, Foster City, CA) were edited, assembled, and compared using software from DNAStar, Inc. (Madison, WI). The resulting sequences were submitted to the NG-MAST Web site (http://www.ng-mast.net/) to determine the sequence types (STs).

Nucleotide Sequencing of Resistance Genes *penA, mtrR, ponA, and porB1b* (*penB* Alteration)

The *penA*, *mtrR*, *ponA*, *and porB1b* (*penB* alteration) nucleotide sequences of the isolates were amplified as previously reported using the primers listed in Table 1.[17–21] The resulting sequences were aligned with the respective gene sequences of *N. gonorrhoeae* wild-type strain F62 using MegAlign to identify patterns and mutations. The *penA* amino acid sequence patterns were classified consistent with types found by Ito et al,[17] Whiley et al,[10] and Allen et al.[22]

Genetic Analysis

Concatenated sequences of the *por* and *tbpB* alleles were created for all STs identified in this study. The evolutionary history was inferred using the Neighbor-Joining method to create a phylogenetic tree.[23] The evolutionary distances were computed using the p-distance method and are in the units of

the number of base differences per site.[24] Evolutionary analyses were conducted in MAEGA5.05.[25]

Statistical Analysis

A 2 × 2 χ^2 test was used to compare 2 proportions to detect significant differences between groups of isolates (*P* values were calculated with a 95% confidence interval). Statistical analysis was carried out using EpiCalc 2000 version 1.02.

RESULTS

Isolate Information

A total of 155 *N. gonorrhoeae* isolates with increased MICs to CRO (MIC = 0.125 μg/mL and 0.25 μg/mL) and CFM (MIC = 0.25 μg/mL and 0.5 μg/mL) were identified from the following provinces: Ontario (53.5%, N = 83); British Columbia (34.2%, N = 53); Québec (12.3%, N = 19). Of these selected isolates observed with reduced susceptibility, 23 were identified between 2001 and 2007 (14.8%), which increased to 84 isolates (54.2%) by 2008. By 2009 and 2010, 23 isolates (14.8%) and 25 isolates (16.1%) were observed, respectively. Limited demographic data were available for the isolates submitted for testing. Of those isolates with information on gender, 87.2% (130/149) were from males with a mean age of 33.6 years (age range of 16–61 years). A total of 12.8% (19/149) of isolates were from females with a mean age of 26.9 years (age range of 16–58 years). Sources of the isolates included urethral (n = 26), rectal (n = 18), pharyngeal (n = 5), cervical (n = 5), and the source for the remaining 101 isolates was unknown.

Antimicrobial Susceptibilities

The MICs of CFM and CRO as well as the antimicrobial resistance profile of all of the isolates are described in Table 2. The majority of isolates, 95.3% (143/155) were found to be chromosomally resistant to PEN, TET, and ERY (CMRNG) or probable CMRNG (one of the MIC values of either PEN, TET or ERY = 1 μg/mL, the other two ≥2.0 μg/mL). Based on the CLSI guidelines, 3 isolates (1.9%, 3/155) were identified as nonsusceptible (NS) to CFM (CFM-NS) (MIC = 0.5 μg/mL). Each of these 3 isolates was also resistant to other antimicrobials: one CFM-NS/CIP-R; one CFM-NS/CIP-R/ERY-R; one CFM-NS/PEN-R/TET-R/CIP-R. A total of 3.8% of isolates (6/155) were found to be resistant to one antimicrobial, CIP. Two isolates were identified as CIP-R/TET-R, and the remaining isolate was found to have high-level plasmid-mediated TET resistance (TRNG)/CIP-R/ERY-R, harboring the 25.2-Mda plasmid along with the 2.6-Mda cryptic plasmid. All but one of the isolates were found to harbor the 2.6-Mda cryptic plasmid. Auxotyping revealed that 61.9% (96/155) were proline-requiring, 37.4% (58/155) were nonrequiring isolates, and 1 (0.6%) was identified as a hypoxanthine-requiring isolate. All 155 isolates were susceptible to SPT, AZM, and CRO.

Based on all *N. gonorrhoeae* isolates submitted to NML, a right shift in the modal MICs of both CRO and CFM has been observed between 2001 and 2010, from 0.016 to 0.125 μg/mL for CFM and from 0.016 to 0.063 μg/mL for CRO during this time period. In 2001, 27.8% and 32.0% of all isolates tested (N = 1058) had MICs equal to 0.016 μg/mL for CRO and CFM, respectively. By 2010, 29.8% of all isolates tested (N = 503) had a CFM MICs equal to 0.125 μg/mL and 35.8% had a CRO MIC equal to 0.063 μg/mL (Fig. 1).

Mutations in the *penA* Gene

Sequencing of the *penA* gene revealed that 60% (93/155) of the isolates contained the *penA* mosaic allele. The sequence pattern types observed in these isolates included pattern XXXII (84.9%, 79/93), X (12.9%, 12/93), and XXXVIII (2.2%, 2/93). The remaining 40% (62/155) non-*penA* mosaic allele isolates had sequence pattern type XII (91.9%, 57/62), V (4.8%, 3/62), XIII (1.6%, 1/62), and a pattern type previously not identified (1.6%, 1/62). This new pattern designated XXXIX was deposited into GenBank under the accession number JF893455. It displayed the D345A insertion typical of non-*penA* mosaic types; however, it lacked any of the other amino acid mutations associated with these types. It also has combinations of the mutations identified in mosaic patterns X and XXXII as shown in Figure 2. Over time, the proportion of *penA* pattern X dropped significantly from 100% in 2001, 2004, and 66.7% in 2006 to 1.2% and 0% in 2008 and 2009, respectively ($P < 0.001$). *PenA* pattern XXXII was not identified in the early years of the study period but the proportion of XXXII increased significantly from 21.4% in 2007 to 95.7% in 2009 ($P < 0.001$). Geographically, *penA* pattern XII was identified at significantly higher rates in Ontario (51.8%) and Québec (63.2%) as compared with British Columbia (3.8%, $P < 0.001$). However, British Columbia had significantly more *penA* pattern XXXII isolates (86.8%) than Ontario (32.5%) or Québec (31.6%, $P < 0.001$).

Of the 62 isolates with nonmosaic *penA* alleles, 93.5% (58/62) and 4.8% (3/62) had the amino acid substitution P551S and G542S, respectively. One isolate (1.6%) was identified as having the A501V mutation of the *penA* gene. This isolate, which also had the P551S mutation, was found to be a *penA* type XIII. Other mutations identified in this isolate include the *mtrR* deletion of A, the *ponA* L421P and the *porB1b* (*penB* alterations) G120K and A121G.

Mutations in the *mtrR* Gene

A single nucleotide (A) deletion in the 13-bp inverted repeat located between the −10 and −35 sequence of the *mtrR* promoter region that causes overexpression of the MtrCDE efflux pump was identified in 95.5% (148/155) of the isolates. Four of these isolates (2.6%) also displayed a G45D alteration in addition to the promoter region A deletion. Three isolates (1.9%) did not have the well-described A deletion in the promoter region but 2 of these displayed a G45D alteration, and 1 had an A39T alteration. A total of 2.6% (4/155) isolates did not have the A deletion in the promoter region or any other alteration in *mtrR*.

Mutations in the *porB1b* and *ponA* Genes

All 155 isolates displayed alterations in *porB1b* (*penB* alteration), including G120K, A121N (49.7%, 77/155); G120K, A121D (45.2%, 70/155); G120K, A121G (2.6%, 4/155); and G120R, A121D (0.6%, 1/155). Three isolates (1.9%) did not have alterations in amino acid 120 but did have the A121S alteration. The *ponA* alteration L421P was detected in all of the isolates. Alterations in *penA*, *mtrR*, *ponA*, *porB1b* (*penB* alteration) for all the isolates are displayed in Table 2.

Comparison of NG-MAST Data

Thirty-seven different NG-MAST STs were identified among the isolates; ST3158, ST225, and ST1407 were the most prevalent at 25.8%, 19.4%, and 14.8%, respectively. There were no statistically significant differences in distribution of most STs over time except for an increase in proportion of ST1407 from 1.2% in 2008 to 28.0% in 2010 ($P < 0.001$). Geographically, significantly more ST225 were found in Ontario (27.7%, $P < 0.001$) and Québec (31.6%, $P < 0.001$) than

TABLE 2. Minimum Inhibitory Concentrations (MICs), NG-MAST Sequence Types (STs) and Mutations of *penA*, *mtrR*, *porB1b* of 155 *Neisseria gonorrhoeae* Isolates With Increased MICs to Cefixime (0.25 μg/ml and 0.5 μg/ml) and Ceftriaxone (0.125 μg/ml and 0.25 μg/ml)

No. Isolates	Antimicrobial Characterization	Ceftriaxone MICs	Cefixime MICs	NG-MAST ST*	*penA* Type†	*mtrR* Mutations	*porB1b* (*penB* Alteration)
40	CMRNG/CIP-R, ProbableCMRNG/CIP-R	0.063–0.25	0.063–0.25	ST3158	(M) XXXII	Deletion of A	G120K, A121N
30	CMRNG/CIP-R, ProbableCMRNG/CIP-R, CIP-R	0.125, 0.25	0.016–0.25	ST225	(NM) XII	Deletion of A	G120K, A121D
23	CMRNG/CIP-R, ProbableCMRNG/CIP-R	0.125	0.063–0.25	ST1407	(M) XXXII (22), (M) XXXVIII (1)	Deletion of A	G120K, A121N
10	CMRNG/CIP-R, ProbableCMRNG/CIP-R	0.125	0.032–0.125	ST3550	(NM) XII, P551S	Deletion of A	G120K, A121D
6	CMRNG/CIP-R, CIP-R	0.125, 0.25	0.063, 0.125	ST3132	(NM) XII, P551S	Deletion of A	G120K, A121D
4	CMRNG/CIP-R	0.063, 0.125	0.25	ST2569	(M) XXXII	Deletion of A	G120K, A121D
3	CMRNG/CIP-R	0.125	0.25, 0.125	ST3149	(M) XXXII	Deletion of A	G120K, A121N
3	CFM-RS/CIP-R, CIP-R/TET-R	0.063	0.25, 0.5	ST4427*	(M) X	Wild type	G120K, A121G
3	CMRNG/CIP-R	0.125	0.25	ST4771	(M) XXXII	Deletion of A	G120K, A121N
2	CMRNG/CIP-R, CFM-RS/CIP-R/ERY-R	0.125	0.032, 0.5	ST3108	(NM) V, G542S	Deletion of A	A121S
2	ProbableCMRNG/CIP-R	0.125	0.25	ST4014	(M) X	No deletion, G45D	G120K, A121D
2	CMRNG/CIP-R	0.125	0.25	ST4383*	(M) X	Deletion of A	G120K, A121D
2	CMRNG/CIP-R	0.125	0.032, 0.63	ST4385*	(NM) XII, P551S	Deletion of A	G120K, A121D
2	CMRNG/CIP-R	0.125	0.25	ST4462	(M) XXXII	Deletion of A	G120K, A121N
1	CMRNG/CIP-R	0.125	0.063	ST3123	(NM) XII, P551S	Deletion of A	G120K, A121D
1	CMRNG/CIP-R	0.125	0.25	ST1424	(M) X	Deletion of A, G45D	G120K, A121D
1	TRNG/CIP-R/ERY-R	0.125	0.125	ST1612	(NM) XIII, A501V, P551S	Deletion of A	G120K, A121G
1	CMRNG/CIP-R	0.125	0.25	ST3505	(M) X	Deletion of A, G45D	G120K, A121D
1	CMRNG/CIP-R	0.125	0.063	ST3599	(NM) XII, P551S	Deletion of A	G120K, A121D
1	ProbableCMRNG/CIP-R	0.125	0.25	ST3779	(M) XXXVIII	Deletion of A	G120K, A121N
1	CMRNG/CIP-R	0.125	0.25	ST3935	(M) XXXII	Deletion of A	G120K, A121N
1	CMRNG/CIP-R	0.125	0.063	ST437	(NM) V, G542S	Deletion of A	G120K, A121D
1	CMRNG/CIP-R	0.125	0.25	ST4386*	(NM) XII, P551S	Deletion of A	G120K, A121D
1	CMRNG/CIP-R	0.125	0.125	ST4428*	(NM) XII, P551S	Deletion of A	G120K, A121D
1	CMRNG/CIP-R	0.125	0.032	ST4429*	(NM) XII, P551S	Deletion of A	G120K, A121D
1	CMRNG/CIP-R	0.032	0.25	ST4430*	(M) XXXII	Deletion of A	G120K, A121N
1	CMRNG/CIP-R	0.125	0.125	ST4431*	(M) XXXII	Deletion of A	G120K, A121N
1	CIP-R	0.125	0.032	ST4432*	(NM) XXXIX	Deletion of A	G120K, A121D
1	CMRNG/CIP-R	0.125	0.032	ST4433*	(NM) XII, P551S	Deletion of A	G120K, A121D
1	CMRNG/CIP-R	0.125	0.063	ST4434*	(NM) XII, P551S	Deletion of A	G120K, A121D
1	CIP-R	0.125	0.125	ST4435*	(NM) XII, P551S	No deletion, A39T	A121S
1	ProbableCMRNG/CIP-R	0.063	0.25	ST4436*	(M) X	Wild type	G120K, A121S
1	PEN-R/TET-R/CIP-R/CFM-RS	0.063	0.5	ST4437*	(M) X	Deletion of A, G45D	G120K, A121S
1	CMRNG/CIP-R	0.063	0.25	ST4438*	(M) X	Deletion of A, G45D	G120R, A121S
1	CMRNG/CIP-R	0.125	0.032	ST4439*	(NM) XII, P551S	Deletion of A	G120K, A121S
1	CMRNG/CIP-R	0.25	0.25	ST4797	(M) XXXII	Deletion of A	G120K, A121S
1	CMRNG/CIP-R	0.25	0.25	ST4811	(M) XXXII	Deletion of A	G120K, A121S

*These sequence types (STs) were newly identified in this study.
†*pen*A type, mosaic (M), non-mosaic (NM), sequence pattern type, significant mutations of P551, G542, and P501.

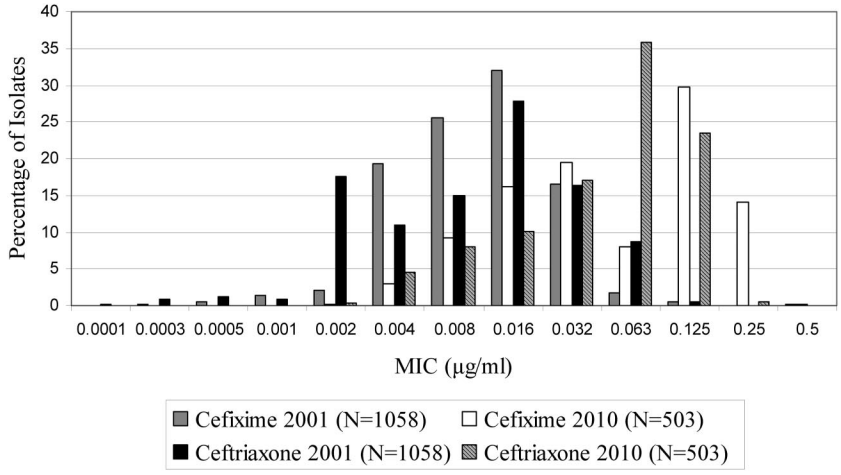

Figure 1. Ceftriaxone and cefixime trends of minimum inhibitory concentrations (MICs) for *Neisseria gonorrhoeae* isolates tested between 2001 and mid-2010.

in British Columbia (1.9%). Of the 37 STs, 16 STs had not been identified in the NG-MAST database and were newly identified in this study. Twenty-three (14.8%) STs were represented by only 1 isolate. Of the 37 different STs, 11 STs all contained the *tbpB*-110 allele which represented 51.6% (80/155) isolates.

Figure 3 demonstrates the evolutionary relationships between the STs found in this study. It reveals how ST225 is very closely related to 6 other STs including ST3132, ST4428, ST3599, ST4433, ST4434, and ST3123 (Subtree A). Subtree A represents 26.4% (41/155) of the isolates in this study, and all isolates in Subtree A differ by 1 nucleotide. All of the isolates in Subtree A are the nonmosaic *penA* pattern type XII, contain the *mtrR* A deletion, the *ponA* L421P mutation, and the *porB1b* (*penB* alteration) G120K and A121D mutations. An additional 4 STs (ST437, ST4429, ST4435, and ST3108) differ by up to 32 nucleotides when compared with Subtree A. In total, Subtree A and its closely related STs represent 29.7% (46/155) of all isolates in this study.

Subtree B includes ST3158 and 5 other STs (ST1407, ST3779, ST3149, ST4462, and ST4771) which differ by 1 to 4 nucleotides when compared with each other. Isolates of this subtree represent 46.5% (72/155) of all isolates in this study. Subtree B *penA* alleles are all mosaic and 70 (97.2%) of them are *penA* type XXXII. The other 2 (2.8%) are type XXXVIII

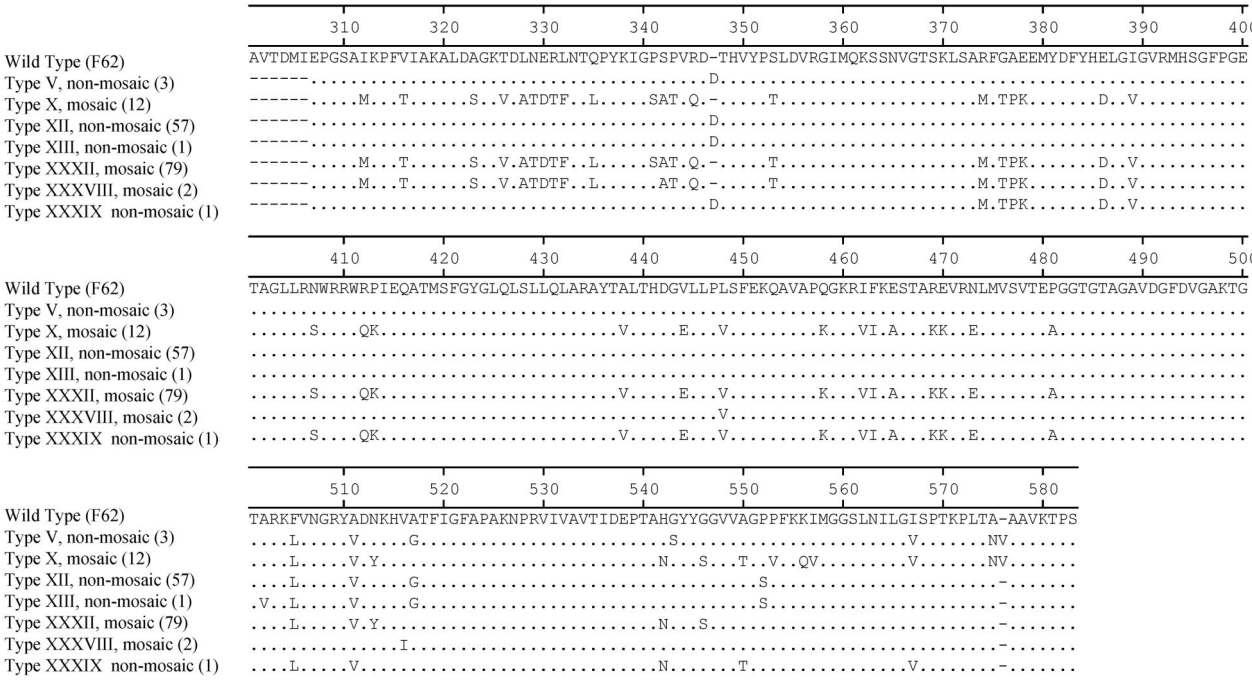

Figure 2. Amino Acid sequences of PBP 2 from *Neisseria gonorrhoeae* isolates with reduced susceptibility to cefixime and ceftriaxone. A comparison of types V, X, XII, XIII, XXXII, and XXXVIII with a novel sequence pattern (XXXIX) identified in this study and deposited into GenBank under the accession number JF893455. The numbers of isolates within each pattern are indicated in brackets. Periods indicate amino acid residues identical to those of wild-type ATCC BAA-1837(F62) and dashes are blanks.

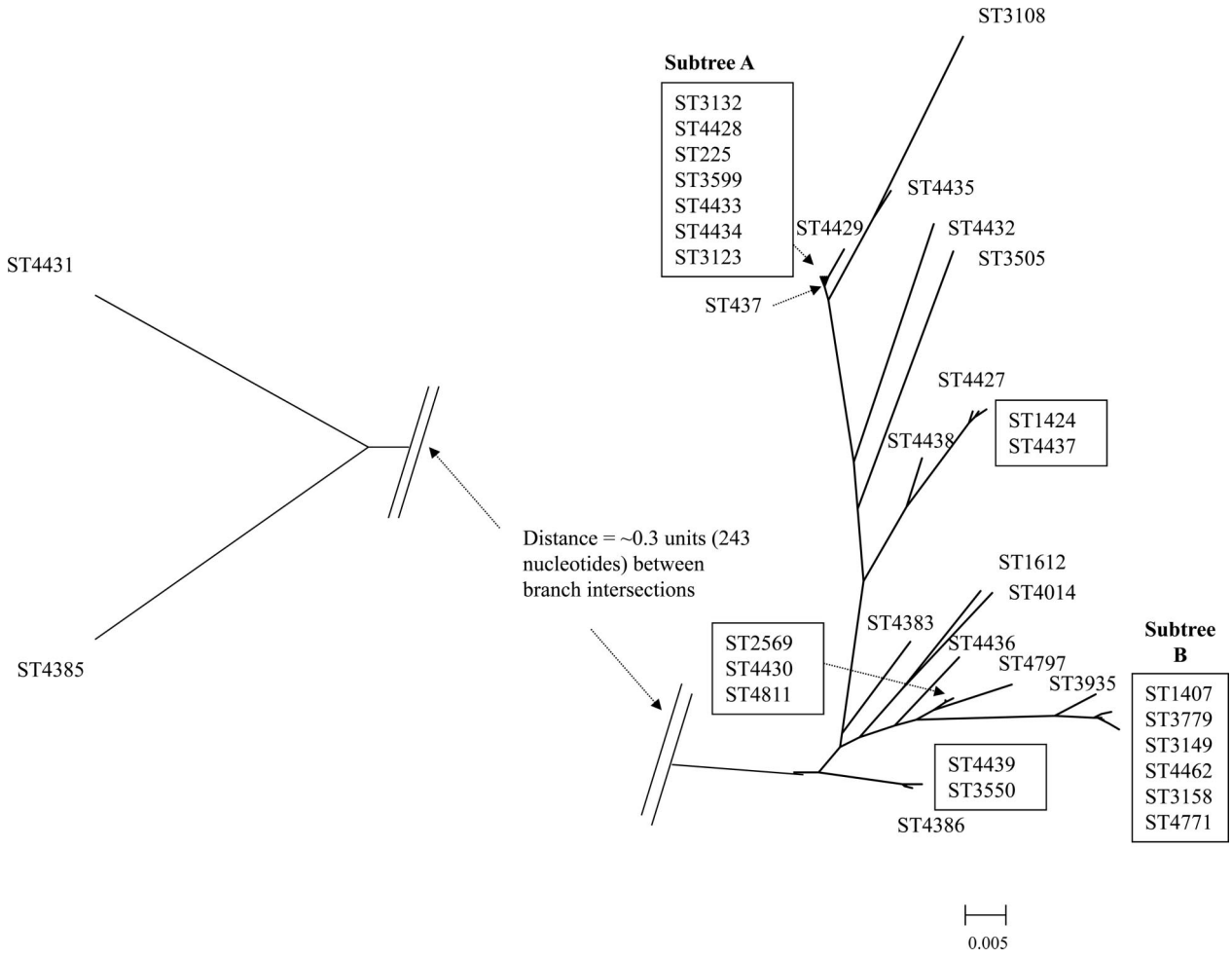

Figure 3. Phylogenetic reconstruction of concatenated sequences of *por* and *tbpB* alleles for all sequence types (STs) in this study. Two major subtrees were found surrounding the most prevalent STs (ST225, ST1407, and ST3158). The 2 subtrees are genetically separated by approximately 74 nucleotides. The evolutionary history was inferred using the neighbor-joining method.[24] The optimal tree with the sum of branch length = 0.67455331 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method[23] and are in the units of the number of base differences per site. The analysis involved 37 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 810 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.05.[25]

which is a slightly modified version of type XXXII. All Subtree B isolates have the *mtrR* A deletion, the *ponA* L421P and *porB1b* (*penB* alteration) G120K and A121N mutations. An additional 9 different STs differing from Subtree B by up to 45 nucleotides can be included in this cluster for a total of 55.5% (86/155) of all isolates in this study.

DISCUSSION

The upward shift of MICs in third-generation cephalosporins in *N. gonorrhoeae* has been described in many countries worldwide and is particularly worrisome as these are the last remaining treatment options to cure gonorrhea. This study describes the genotypic and phenotypic characteristics of *N. gonorrhoeae*, found to be resistant to at least one antibiotic, isolated in Canada between 2001 and mid-2010 with reduced susceptibility to third-generation cephalosporins. Monitoring MICs over time, the detection of *penA* mosaic alleles as well as the identification of alterations A501, G542, P551 in PBP2 and the characterization of *mtrR* and *porB1b* (*penB* alterations) determinants become very important for surveillance purposes and to monitor the emergence and spread of isolates with decreased third-generation cephalosporins susceptibility. The relationship of these genetic determinants and the increase in MICs to cephalosporins has been well described.[3,12,26]

The majority of the isolates in this study had both the *penA* mosaic allele and nonmosaic allele sequence pattern types that have been previously reported and 1 nonmosaic allele isolate with a novel sequence pattern type. Regarding the non-*penA* mosaic allele mutations, our study identified 1 isolate with the A501V mutation of the *penA* gene, in contrast with reports from countries such as Sweden[27] and Japan.[11] However, the majority of our non-*penA* mosaic isolates did have either

P551S or G542S mutations, which were recently linked to decreased susceptibility to CRO.[26] Monitoring these *penA* alterations in combination with the *mtrR* deletion and *porB1b* mutations is important in understanding the progression of *N. gonorrhoeae* as the MICs to the third-generation cephalosporins continue to increase. Alterations L421P in *ponA* (encoding PBP1) have also been previously suggested to contribute to the decreased susceptibility to third-generation cephalosporins. All the isolates tested in this study did in fact have the L421P *ponA* alteration. However, recent studies have shown that, in contrast with the PENs, *ponA* alterations do not significantly affect susceptibility to third-generation cephalosporins in *N. gonorrhoeae*.[12]

An ST cluster analysis revealed that the majority of all the isolates studied belonged to 5 different STs and some of these STs were highly related, indicating a clonal relationship between many of these Canadian isolates. This clonal distribution of isolates is similar to a recent report from England.[6] In contrast, other studies from Sweden[27] and Australia[10] have reported a higher degree of diversity amongst their reduced susceptibility gonococci. Part of the collection of Canadian isolates did in fact have a high degree of diversity, which can be explained by the high polymorphism of the 2 alleles involved in the NG-MAST method. A large number of isolates belonged to a single unique ST, which may be due to the local emergence of new STs or the recent introduction of gonorrhea from other countries. At least two of the STs identified in these Canadian isolates have been reported in other countries including England, the United States, and Australia.[6,28,29] ST1407 is very closely related to ST3158, reported in Ontario, Canada by Allen et al[22] and also identified in Sweden.[27]

As reports of decreased susceptibility to third-generation cephalosporins in *N. gonorrhoeae* continue, gonococcal treatment guidelines are being reviewed. In a recent report, Chisholm et al[30] describe that gonococci with CRO and CFM MICs of 0.125 to 0.25 μg/mL are likely to be on the edge of responsiveness to current treatments and suggest strategies to control antimicrobial resistance in gonorrhea, including higher cephalosporin doses, multidose cephalosporin regimens, and drug cycling. Furthermore, pharmacodynamic analysis predicts that failures with the standard 400-mg CFM orally and 250-mg CRO intramuscularly treatments become possible around these MICs.[30]

A major challenge faced by the laboratories that perform surveillance of antimicrobial resistance of *N. gonorrhoeae* is the shift from the use of cultures (currently required for antimicrobial susceptibility testing) to the Nucleic Acid Amplification Test for the diagnosis of gonorrhea, explaining the decrease in the number of isolates available for susceptibility testing and interfering with clinical treatment. The current passive Canadian surveillance system for *N. gonorrhoeae* is limited by the collection of only resistant isolates, which may introduce a bias and cause a lack of representativeness.

In response, the current Canadian surveillance system for *N. gonorrhoeae* is being strengthened through a sentinel clinic-laboratory surveillance mechanism capable of integrating epidemiologic, laboratory, and treatment failure information for both drug-sensitive and drug-resistant *N. gonorrhoeae*. Such a mechanism is imperative for both patient management and surveillance purposes as multidrug resistant *N. gonorrhoeae* with decreasing susceptibilities to third-generation cephalosporins are emerging.

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