Whole-Genome Phylogenomic Heterogeneity of Neisseria gonorrhoeae Isolates with Decreased Cephalosporin Susceptibility Collected in Canada between 1989 and 2013

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A large-scale, whole-genome comparison of Canadian Neisseria gonorrhoeae isolates with high-level cephalosporin MICs was used to demonstrate a genomic epidemiology approach to investigate strain relatedness and dynamics. Although current typing methods have been very successful in tracking short-chain transmission of gonorrheal disease, investigating the temporal evolutionary relationships and geographical dissemination of highly clonal lineages requires enhanced resolution only available through whole-genome sequencing (WGS). Phylogenomic cluster analysis grouped 169 Canadian strains into 12 distinct clades. While some N. gonorrhoeae multiantigen sequence types (NG-MAST) agreed with specific phylogenomic clades or subclades, other sequence types (ST) and closely related groups of ST were widely distributed among clades. Decreased susceptibility to extended-spectrum cephalosporins (ESC-DS) emerged among a group of diverse strains in Canada during the 1990s with a variety of nonmosaic penA alleles, followed in 2000/2001 with the penA mosaic X allele and then in 2007 with ST1407 strains with the penA mosaic XXXIV allele. Five genetically distinct ESC-DS lineages were associated with penA mosaic XXXIV alleles and nonmosaic XII and XIII alleles. ESC-DS with coresistance to azithromycin was observed in 5 strains with 23S rRNA C2599T or A2143G mutations. As the costs associated with WGS decline and analysis tools are streamlined, WGS can provide a more thorough understanding of strain dynamics, facilitate epidemiological studies to better resolve social networks, and improve surveillance to optimize treatment for gonorrhoeal infections.

Neisseria gonorrhoeae is a Gram-negative diplococcus bacterium that causes gonorrhea infections. Gonorrhea is the second most reported bacterial sexually transmitted infection (STI) in Canada, with reported cases increasing from 15.5 per 100,000 in 1997 to 36.2 per 100,000 in 2012 (1), and approximately 106 million cases are estimated annually worldwide (2). N. gonorrhoeae bacteria have developed resistance against sulfonamides, penicillins, tetracyclines, and fluoroquinolones (3, 4), and current treatment options now include third-generation extended-spectrum cephalosporins (ESC), namely, cefixime (CFM) and ceftriaxone (CRO) (5). MIC creep has seen the modal MIC values rise between 2001 and 2010 in Canada from 0.016 μg/ml to 0.125 μg/ml and 0.063 μg/ml for CFM and CRO, respectively (6). These results coincide with recent clinical reports of treatment failures to primarily CFM monotherapy in Canada (7, 8) and additional global reports of high-level CRO MICs in isolates from Japan and Europe (9–13). Furthermore, isolates with decreased susceptibility to cephalosporins and coresistance to azithromycin (AZM), a recommended cotherapy (5), have recently been identified in Canada (14).

Decreased susceptibility to extended-spectrum cephalosporins (ESC-DS) and resistance to AZM have been attributed to several molecular mechanisms. The primary mechanism for ESC-DS is modification of the penA gene (penicillin binding protein 2 [PBP2]) including various mutations of the wild-type gene and a recombinant mosaic allele containing portions of genetic sequences from commensal Neisseria (3, 4, 9, 15–18). Mutations in the promoter and/or the coding region of the repressor gene mtrR cause overexpression of the MtrCDE efflux pump (3, 4, 15, 19, 20) and porB1b gene mutations (penB G120 and/or A121 mutation) have been shown to cause decreased membrane permeability and contribute to decreased susceptibility toward cephalosporins (3, 4, 15, 19–22). Other genes associated with penicillin resistance

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include pilQ and porA; however, the role in ESC-DS remains unclear (3, 9, 10, 20, 23–27). The currently identified resistance determinants do not fully account for the observed ESC MICs, and it is hypothesized that other factors may be involved (4, 9, 10, 15, 20, 24, 25). AZM resistance has been associated with mutations in 23S rRNA alleles (4, 28) as well as mtrR (29–31).

Monitoring the spread of resistant N. gonorrhoeae involves phenotypic and molecular biology-based typing methodologies (32, 33) that have been used in combination with antimicrobial susceptibility data for epidemiological studies. The internationally recognized N. gonorrhoeae multiantigen sequence typing (NG-MAST) system has been effective in tracking short-chain transmission of infections (33) (www.ng-mast.net). However, application of whole-genome sequencing (WGS) for comparative studies provides higher resolution through a genomic epidemiology approach to investigate strain relatedness and dynamics (34–36).

In this report, we have applied the enhanced discriminatory power of WGS to describe the dissemination, relatedness, and emergence of Canadian N. gonorrhoeae isolates with elevated ESC MICs.

**MATERIALS AND METHODS**

*N. gonorrhoeae isolates*. This study included 169 N. gonorrhoeae isolates collected between 1989 and 2013 from across Canada and 10 international reference strains. Canadian isolates were primarily selected for decreased susceptibility to CRO (*n* = 65) consisting of 19 CRO-DS isolates that were submitted to the National Microbiology Laboratory (NML), Public Health Agency of Canada, between 1989 and 2006 (83%), 6/18 from 2007, 5/83 from 2008, 7/96 from 2009, 12/217 from 2010, 5/207 from 2011, and 11/168 from 2012. Additional isolates (*n* = 105) were included to provide a broad range of NG-MAST sequence types (ST), geographical distributions, and antimicrobial susceptibilities.

Isolate characterization and antimicrobial susceptibility testing. Antimicrobial susceptibilities of *N. gonorrhoeae* to spectinomycin, ceftriaxone, erythromycin, penicillin, tetracycline, azithromycin, cefixime (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada), and ciprofloxacin (generously provided by Bayer, Etobicoke, Ontario, Canada) were determined using the agar dilution method as previously described (37). Interpretation of the MIC was based on established criteria (38, 39); cefoxime decreased susceptibility MIC of ≥0.25 μg/ml, ceftriaxone decreased susceptibility MIC of ≥0.125 μg/ml (2), and azithromycin resistance MIC of ≥2.0 μg/ml (40). *N. gonorrhoeae* ATCC 49226, WHO-B, WHO-C, WHO-D, WHO-F, WHO-G, WHO-K, and WHO-P reference cultures were used as controls (41). Susceptibility categories were assigned as very susceptible, susceptible, moderate susceptibility, decreased susceptibility or resistant, and high-level resistance for drugs as follows: for CRO, 0.00025 to 0.004 μg/ml, 0.008 to 0.016 μg/ml, 0.032 to 0.063 μg/ml, 0.125 to 0.5 μg/ml, and 1 to 2 μg/ml, respectively; for CEF, 0.00025 to 0.004 μg/ml, 0.008 to 0.016 μg/ml, 0.032 to 0.125 μg/ml, 0.25 to 0.5 μg/ml, and 1 to 4 μg/ml, respectively; and for AZM, 0.032 to 0.063 μg/ml, 0.125 to 0.25 μg/ml, 0.5 to 1 μg/ml, 2 to 16 μg/ml, and 32 to 256 μg/ml, respectively.

**Statistical comparisons.** To determine the magnitude of the contributions of the known antimicrobial resistance markers to ESC-DS, isolates were divided into four categories based on CRO MIC values and the presence or absence of the molecular marker of interest (including *penA* genotype, *mtrR* −35 A deletion, the G-to-D change at position 39 encoded by *mtrR* [mtrR A39T], *mtrR* G45D, *porA* L421P, *porB* G120, and *porB* A121); true positive (TP) having moderate to very high MICs (0.032 to 2 μg/ml) and the presence of the genetic marker of interest; false positive (FP) having very low to low MICs (≤0.016 μg/ml) and the marker; false negative (FN) having moderate to very high MICs and no marker; and true negative (TN) having very low to low MICs and no marker. Calculations were performed as follows: sensitivity (SENS) = TP/(TP + FN) × 100 and specificity (SPEC) = TN/(FP + TN) × 100 (42).

The measure of association was determined using χ² or Fisher exact test using OpenEpi version 3.01 (43). Two-tailed differences of P < 0.05 at 95% confidence were considered statistically significant.

**Whole-genome sequencing and assembly.** DNA samples were extracted from cultures following standard protocol with Epicentre MasterPure complete DNA and RNA extraction kit (Mandal Scientific, Guelph, Ontario, Canada). Multiplexed libraries were created with Nextera XT sample preparation kits (Illumina, San Diego, CA). Paired-end, 250-bp indexed reads were generated on the Illumina MiSeq platform (Illumina, San Diego, CA) yielding an average of 1,167,540 reads/genome and an average genome coverage of 127.

**De novo assembly.** The quality of the reads was assessed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), merged using FLASH (44), and assembled with SPAdes (45) and annotation was accomplished with Prokka (46). The average number of contigs was 147, the average contig length was 16,506, and the average N₅₀ contig length was 62,730.

**Phylogenomic analysis based on core single nucleotide polymorphisms.** FASTQ files for forward and reverse reads were concatenated into one fastq FASTQ file per isolate and were used for further analysis. Read ends were trimmed, and poor-quality reads were filtered to improve assembly quality using the script run_assembly_clean.pl from CG-Pipeline (47) with the following options: “–min-quality 25 –bases_to_trim 10 –min_avg_quality 25 –min_length 36 -p 1”, and read qualities were assessed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

The high-quality reads were then mapped to the publicly available reference genome, *N. gonorrhoeae* NCCP11945 (NCBI accession no. NC_011035) (G. T. Chung et al. PubMed identifier [PMID] 18586945) with SMALT version 0.7.4 (http://www.sanger.ac.uk/resources/software/smal/) with the following options: smalt_index “–k 13 -s 6” and smaltmap “–s samsoft -r -t 1”.

Single nucleotide variants were called using FreeBayes (Erik Garrison, Garbor Marth [2012] arXiv:1207.3907[q-bio.GN]) using the following parameters: “–pvar 0 –ploidy 1 –left-align-inds –min-mapping-quality 30 –min-base-quality 30 –min-alternate-fraction 0.75 –min-coverage 15” with additional variant confirmation using SAMTools mpileup (48). The following parameters were used to run SAMtools: “samtools mpileup -BQO -d100000000” and “bcftools view –cq”. Positions where variant calls were not in agreement between both variant callers were excluded. Variant calls within potential problematic regions including repetitive regions (MUMmer v.3.23), predicted phages (PHAST), genomic islands (IslandViewer), and 10 suspected highly recombinant regions were excluded from the analysis containing >90 single nucleotide polymorphisms (SNPs) per 10,000 bp (see Fig S1 and S1 Table in the supplemental material). All remaining variant calls were merged into a single meta-alignment file. There were a total of 6,509 core SNP positions for the population (Table S2). The meta-alignment of informative core SNP positions was used to create a maximum likelihood phylogenetic tree using PhyML with generalized time reversible model (49) using parameters “–quiet -b -4 -m GTR -s BEST.” Phylogenomic clades were assigned by cluster analysis using PhyloPart (http://sourceforge.net/projects/phylopart/files) with a percentile distance threshold value of 0.10 for all clades except clade F that clustered at a threshold value of 0.105.

*N. gonorrhoeae* multiantigen sequence types (NG-MAST) and antimicrobial resistance molecular markers. NG-MAST types were determined by the NG-MAST PCR method as previously described (33) and in *silico* using WGS data. The sequences were submitted to the NG-MAST website (http://www.ng-mast.net/) to determine the sequence type. NG-MAST groups were determined by the trimming concatenated *porB* and *rpbB* NG-MAST gene sequences. Sequences were aligned, and a neighbor-joining phylogenetic tree was generated using ClustalX (50) and visualized radially using FigTree (http://tree.bio.ed.ac.uk/software).
RESULTS

Strain distribution. Core SNP phylogenetic analysis was performed on 169 Canadian isolates of *N. gonorrhoeae* isolated between 1989 and 2013 from Ontario (*n* = 82), British Columbia (*n* = 23), Quebec (*n* = 30), Saskatchewan (*n* = 13), Nova Scotia (*n* = 12), New Brunswick (*n* = 4), and Manitoba (*n* = 1) and 10 international reference isolates including France F89 (sample 34842) (10), ATCC 49226, WHO-F, WHO-L, WHO-O, WHO-K, WHO-G, WHO-N, WHO-M, and WHO-P (41). The Canadian isolates included 41 (24%) from female patients, 126 (75%) from male patients, and 2 (1%) with no gender provided. Patient ages ranged from 2 months to 64 years with a median age of 27 years for the 137 isolates for which an age was available (see Table S3 in the supplemental material). Enhanced epidemiological data such as patient sexual orientation, clinical isolation site, and treatment history were not available.

Phylogenomic tree. Maximum likelihood phylogenetic core SNPs and cluster analysis grouped 143 of the 179 isolates into 12 major clades (Fig. 1). The reference strains WHO-G, WHO-N, and WHO-M appeared independently between clades E and F, and reference strain NCCP11945 appeared between clades D and E. Reference strains ATCC 49226, WHO-F, WHO-L, and WHO-O appeared between clades H and I, while ESC-resistant France F89 reference strain (sample 34842) clustered in clade A, reference strain WHO-K (Japan) clustered in clade F; and reference strain WHO-P (United States) clustered in clade E.

Isolates 35593, 34772, 33967, 27777, and 22890 were the most genetically distant averaging 1,039, 1,022, 1,017, 1,013, and 1,004 SNPs from the rest of the 175 strains, respectively. These isolates were also members of the most distinctly related NG-MAST group 1 (Fig. 2) and were the only strains in the data set with the wild-type penA allele (GenBank accession no. M32091 [Table S4 in the supplemental material]) (9). The greatest pairwise distances observed were 1,183 SNPs between isolates 35593 and 23836 and 1,181 SNPs between isolate 35593 and clade E isolates (Table S2). Clade G (*n* = 6) had the most closely related isolates with a maximum SNP difference of 3 and an average of 1.6 SNPs between each isolate, whereas clade J (*n* = 8) had the highest strain diversity with a maximum of 865 SNPs and an average of 245 SNPs between isolates (Table 1).

In our data set, 75% of the isolates were from male patients. Clades A, C, D, E, F, G, H, and I had a higher proportion of isolates from males (88% male [93/106]) compared to the isolates that did not cluster into clades and those of clade I (63% male [27/43]; *P* < 0.002). In clades J, K, and B, isolates from females predominated (33% male [6/18]; *P* < 0.001).

Antimicrobial resistance. Of the 169 Canadian isolates analyzed, 67 (40%) exhibited decreased susceptibility to extended-spectrum cephalosporins (ESC-DS), 29 exhibited CRO-DS/CFM-DS, 36 exhibited CRO-DS/CFM-S, and 2 exhibited CRO-S/CFM-DS. ESC-DS strains were present in 5 genetically distinct lineages corresponding to clades A (35 ESC-DS/63 isolates in clade), E (1/8), F (4/4), G (4/6), and H (10/16) and some that did not cluster into distinct clades (13 ESC-DS/28 nonclade isolates) (Fig. 1). All isolates of clades B, C, D, I, J, K, and L were ESC-S.

ESC-DS was observed in 60.4% (55/91) of the isolates that clustered into clades A, F, G, and H, significantly greater than isolates of clades B, C, D, E, I, J, K, and L where only 1.9% (1/53) exhibited ESC-DS (*P* < 0.001). Among the isolates that did not group into clades, ESC-DS (40% [14/35]) was significantly greater than those of clades B, C, D, E, I, J, K, and L (*P* < 0.001) isolates, but not significantly different from the proportion of ESC-DS seen in the clade A, F, G, and H isolates (*P* = 0.06).

The earliest ESC-DS isolates were observed among miscellaneous isolates located between clades I and H on the phylogenomic tree (Fig. 1). The first CRO-DS strain isolated in Canada in 1989 from Saskatchewan (isolate 883218) was ST919 (NG-MAST group 3 in Fig. 2) and possessed a novel penA nonmosaic allele most closely resembling type II with an additional A550V substitution (see Table S4 in the supplemental material). Other early CRO-DS strains clustering early in the phylogeny included strains from 1994 to 1998 isolated in Quebec, Alberta, and Ontario in Canada with a variety of ST types, NG-MAST groups, and penA alleles (Fig. 1 and 2; see Table S3 in the supplemental material). During 2000 to 2001, ESC-DS strains with the penA mosaic X allele were observed in clade F from Quebec (NG-MAST group 4) as well as in clade A from Ontario (NG-MAST group 9). Then in 2006, ESC-DS strains were observed to form another lineage, clade H, with the penA nonmosaic XII allele and NG-MAST groups 2 and 9.

The first ST1407 strains of clade A (NG-MAST group 5) were isolated in 2005 from Quebec, Canada, and had moderate cephalosporin susceptibilities (CRO MIC of 0.032 μg/ml and CFM MICs of 0.63 and 0.125 μg/ml) and a penA mosaic XXXIV allele, and in 2007 the first ESC-DS ST1407 strains were identified in Ontario, Canada. A subclade of isolates in clade A with penA mosaic XXX allele included two isolates from 2008, with ST1407 and ST3779, each having moderate ESC susceptibilities (MICs of 0.032 μg/ml), and one ST1407 isolate from 2010 that was highly susceptible to ESC (MIC of 0.008 μg/ml). The ESC-resistant European strain F89 (isolate number 34842) was also part of clade A and had penA mosaic XXXIV allele with an A501P mutation (see Table S4 in the supplemental material). Most recently in 2012, ESC-DS was seen in a distinct lineage of clade G consisting of isolates that were ST7989 (NG-MAST group 2) and had penA nonmosaic XIII allele.

Five ESC-DS strains were also resistant to AZM. These five strains included one isolate clustering between clades H and I with an MIC of 2 μg/ml and no 23S rRNA mutations but with the mtrR –35 A deletion present, two isolates of clade A with MICs of 2 and 8 μg/ml and C2599T mutations in 2/4 and 4/4 of the 23S rRNA alleles, and two isolates with extremely high MICs of ≥64 μg/ml and ≥256 μg/ml, both with A2143G mutations in all alleles, clustering between clades F and G, and clustering in clade H, respectively.

*N. gonorrhoeae* multiantigen sequence typing (NG-MAST). All 79 NG-MAST types determined by the NG-MAST PCR method (including 3 novel types, ST10443, ST10469, and ST10476) matched those determined *in silico* from the WGS data and clustered into 10 closely related NG-MAST groups (Fig. 2). Clade A (Fig. 1) consisted almost entirely of ST1407-like strains (NG-MAST group 5; blue in Fig. 2) with a single non-ST1407-like
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**Chart NG**

**Chart MIC Interpretation**

**Colour (Figure 2)**

<table>
<thead>
<tr>
<th>Group 1</th>
<th>ST</th>
<th>Other ST</th>
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<td>Group 7</td>
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</table>

**Antimicrobial Susceptibility**

1. **High Level Resistance**
2. **Very Susceptible**

**Clade**

- **Clade A**
- **Clade B**
- **Clade C**
- **Clade D**
- **Clade E**
- **Clade F**
- **Clade G**
- **Clade H**
- **Clade I**
- **Clade J**
- **Clade K**
- **Clade L**

**Isolate**

**CROCFM pen R A Del R A39T por B 120**
NG-MAST type ST4383 (NG-MAST group 9; light green in Fig. 2) present. The 35 ST1407 strains were distributed throughout the clade, and other closely related NG-MAST types such as ST4985, ST3158, and ST5643 formed subclades. Isolates of clades B, C, D, G, H, and L were mainly of NG-MAST group 2 (red in Fig. 2), with specific NG-MAST types correlating into individual clades, with other NG-MAST group 2 isolates falling into clade I and independently between clades. Similarly, clade E consisted largely of ST2992 isolates (NG-MAST group 10; gray in Fig. 2), with other NG-MAST group 10 strains clustered independently and in clade I. Clade F was composed of a variety of NG-MAST types, all of which were members of NG-MAST group 4 (purple in Fig. 2); however, NG-MAST group 4 strains also clustered distantly throughout the phylogenomic tree.

**FIG 1** Whole-genome-based core SNP phylogenomic tree of *N. gonorrhoeae* strains based on maximum likelihood. NG-MAST types, antimicrobial susceptibility, and antimicrobial resistance molecular markers are indicated. Antimicrobial susceptibility to ceftriaxone (CRO), cefixime (CFM), and azithromycin (AZM) is shown. Red in the molecular marker columns (rightmost columns) represents presence of the marker. For *penA*, red represents a mosaic allele. In the *penA* column, WT, wild type; I*, nonmosaic I allele with a P413S substitution; II*, nonmosaic II allele with A505V; II**, nonmosaic II allele with A502V; V*, nonmosaic V allele with Y201H, G202A, E203G, D204E, and Q214E; XXXIV*, mosaic XXXIV allele with A502P.

**FIG 2** Genetic relatedness of NG-MAST sequence types. Genetic relationships were inferred using a neighbor-joining method of trimmed, aligned, and concatenated NG-MAST internal *porB* and *tbpB* gene sequences with ClustalX and visualized using FigTree software. NG-MAST types clustering on each branch were assigned to the same group.

NG-MAST type ST4383 (NG-MAST group 9; light green in Fig. 2) present. The 35 ST1407 strains were distributed throughout the clade, and other closely related NG-MAST types such as ST4985, ST3158, and ST5643 formed subclades. Isolates of clades B, C, D, G, H, and L were mainly of NG-MAST group 2 (red in Fig. 2), with specific NG-MAST types correlating into individual clades, with other NG-MAST group 2 isolates falling into clade I and independently between clades. Similarly, clade E consisted largely of ST2992 isolates (NG-MAST group 10; gray in Fig. 2), with other NG-MAST group 10 strains clustered independently and in clade I. Clade F was composed of a variety of NG-MAST types, all of which were members of NG-MAST group 4 (purple in Fig. 2); however, NG-MAST group 4 strains also clustered distantly throughout the phylogenomic tree.
Antimicrobial resistance molecular markers. The known ESC-DS molecular determinants that have been reported to influence cephalosporin susceptibility, including penA, mtrR, and porB mutations, do not fully account for the ESC susceptibilities observed in this study (Table 2). Of the 114 isolates that had CRO MICs of 0.032 to 2 μg/ml, the factors having the greatest influence on susceptibility were the penA mosaic allele (SENS/SPEC = 61%/98%), the porB G120K mutation (SENS/SPEC = 93%/94%), and the mtrR −35 A deletion (SENS/SPEC = 91%/86%). A combination of markers such as the penA mosaic allele and/or porB G120K

### TABLE 1 Number of single nucleotide polymorphisms in the core genome within and between the major phylogenomic clades of N. gonorrhoeae strains

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<tr>
<th>Clade</th>
<th>No. of isolates</th>
<th>Avg no. of SNPs within clade</th>
<th>Maximum no. of SNPs within clade</th>
<th>Avg no. of SNPs from previous clade</th>
<th>Minimum no. of SNPs from previous clade</th>
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* Number of single nucleotide polymorphisms (SNPs) compared to the previous closest common ancestral clade.

### TABLE 2 Molecular profiles associated with decreased susceptibility to ceftriaxone

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* Symbols: +, present; −, absent.

* MIC range categories for ceftriaxone include the following: very low, 0.00025 to 0.004 μg/ml; low, 0.008 to 0.016 μg/ml; moderate, 0.032 to 0.063 μg/ml; high, 0.125 to 0.5 μg/ml; very high, 1 to 2 μg/ml.

* porB A121 substitutions include A121D, A121F, A121G, A121N, and A121G.
and nonmosaic $A121D$ mutations but had a variety of mosaic patterns that could be investigated.

Isolates that were ESC-DS generally also had the $mtrR - 35A$ deletion and the $penA$ L421P and $porB$ G120K and A121 (includes A121D, -F, -G, -N, and -S) mutations but had a variety of mosaic and nonmosaic $penA$ alleles (Fig. 1). Clade A strains predominantly had the $penA$ mosaic XXXIV allele ($n = 59$), with a subcluster of mosaic type XXXV allele ($n = 3$), and one strain with a mosaic type $X$ ($n = 1$ allele), whereas clade A was associated with the $penA$ mosaic type $X$ allele, clade $G$ was associated with the nonmosaic type XIII allele, and clade $H$ was associated with the nonmosaic type XII alleles. Novel $penA$ genotypes were identified in 2 CRO-DS nonclade strains corresponding to a nonmosaic type II allele with an A502V substitution, and the third moderately CFM-susceptible strain had an A550V substitution (see Table S4 in the supplemental material). Other novel $penA$ genotypes observed included the following: a $penA$ nonmosaic I allele with a P413S mutation in an ESC-S isolate of clade $K$; a $penA$ mosaic XXXIV allele with an A502P mutation in strain F89 of clade $A$; and a $penA$ nonmosaic V allele with Y201H, G202A, E203G, D204E, and Q214E substitutions seen in 3 isolates of clade B with CRO MICs of 0.032 to 0.063 μg/ml.

**Temporal and regional epidemiological tracking.** Clade C consisted of three ST757 strains (Fig. 1), with two isolates having no SNP differences (isolates 33997 and 32902) collected from male patients in central Canada in March and November of 2010. The third isolate (26870) collected 5 years earlier differed from the other two by 53 SNPs indicating a temporal separation of infections. Similarly, clade D consisted of four ST3108 strains, three of which differed by a maximum of 2 SNPs (strains 18361, 31284, and 28541) and were collected between June and September 2006 in central Canada, and the fourth was collected during January 2006 from western Canada and differed by 16 SNPs, demonstrating regional separation within the cluster. A third cluster of three ST3150 strains (32849, 33586, and 33756) formed clade B, differing by a maximum of three SNPs, were collected in January and February of 2010 from female patients in central Canada suggesting temporal, regional, and a possible social network commonality that could be investigated.

**DISCUSSION.**

*N. gonorrhoeae* isolates are genetically diverse in Canada with the majority of isolates distributed through 12 phylogenomic clades and 28 miscellaneous clinical isolates falling outside these groups. Along with the recent clonal spread of ST1407, isolates of *N. gonorrhoeae* with decreased ESC susceptibility have also been observed sporadically in Canada since at least 1989 among several distinct lineages of strains, indicating that the spread of ESC resistance in gonorrhea in Canada is not entirely due to clonal expansion nor restricted solely to the presence of a $penA$ mosaic allele in the genome.

The ESC resistance mechanism is a complex, multifaceted system consisting of numerous components contributing to overall levels of susceptibility. The molecular markers previously described as contributing to cephalosporin resistance, such as $penA$, $mtrR$, $porB$, $penA$, and $pilQ$ mutations, do not adequately account for the observed phenotypes (4, 9, 10, 15, 20, 24-26, 51). Of these markers identified in silico using the WGS data, the presence of a $penA$ mosaic allele, $mtrR - 35A$ deletion and/or a $porB$ G120K mutation were the best indicators of decreased susceptibility to cephalosporins (Table 2).

ESC-DS has been associated with the presence of a $penA$ mosaic allele that evolved through recombination events with other commensal *Neisseria* species; the $penA$ mosaic allele was first seen in 1998 in Japan as a $penA$ mosaic X allele (see Table S4 in the supplemental material) (9, 52). The $penA$ mosaic XXXIV allele was first reported in the United States in 2008 (52–55) and has spread worldwide. In 2009, an isolate was identified in Japan (H041) with a novel $penA$ mosaic allele, NG-MAST type ST4220, and CRO and CFM MICs of 2 and 8 μg/ml, respectively (9). A second highly ESC-resistant isolate was identified in France in 2010 (F89) with a novel $penA$ mosaic mutation that resembled the $penA$ mosaic XXXIV allele with an A502P substitution, a NG-MAST type ST1407, and CRO and CFM MICs of 2 and 4 μg/ml, respectively (10). Despite being highly ESC-DS, F89 was genetically similar to Canadian clade A strains in the phylogenomic tree (Fig. 1).

In the United States, two lineages of strains with ESC-DS were observed, and they had CRO and CFM MICs of 0.125 and 0.50 μg/ml (35). Strains with ESC-DS in Canada had MIC values similar to those reported in the United States; however, Canadian strains tend to be more broadly distributed and genetically heterogeneous, grouping into 5 lineages with other isolates grouping outside the defined phylogenomic clades. The emergence of CRO-DS in Canada occurred during the early 1990s among a group of sporadic isolates with nonmosaic $penA$ alleles, located between clades H and I within the phylogenomic tree (Fig. 1), followed by the $penA$ mosaic X allele during 2000 to 2004 among strains of clade F and clade A. Clade A was the largest group of isolates associated with ESC-DS consisting of NG-MAST types closely related to ST1407. The $penA$ mosaic XXXIV allele was predominant in this group, with the first isolates having MIC values of 0.032 μg/ml, which was observed in 2005, and then with decreased susceptibility (MIC = 0.125 μg/ml) in 2007. Despite a recent report of clonal expansion of strains with $penA$ nonmosaic XXII and IX alleles in Saskatchewan, Canada (56), no regional trends could be deduced among the Canadian isolates. Although only general temporal relationships could be established with the emergence of various lineages of ESC-DS strains, recent isolations indicate that these lineages arose independently—most likely from imported sources (57–59). Despite a paucity of social network information available for this study, certain lineages tended to have a greater proportion of isolates isolated from women; these lineages included clades B, J, K and to a lesser extent the nonclade miscellaneous isolates and clade I, suggesting that these lineages may be more likely to be circulating within the heterosexual population.

In the current study, using core SNP comparisons, we identified several isolate pairs exhibiting close relatedness that represent a promising avenue to determine potential targets for novel antimicrobial resistance mechanisms, including isolates of the ST1407-like clade A that had identical resistance marker profiles ($penA$ mosaic XXXIV allele, $mtrR - 35A$ deletion, and $porB$ G121K) with variable ESC MIC values.

AZM resistance is of concern, as it is the recommended cotherapy for gonorrhea (5), and AZM resistance with elevated ESC MICs has recently been described in Ontario (14), Quebec, and British Columbia in Canada (see Table S3 in the supplemental material).
material. In contrast to Grad et al. (35) who reported that AZM resistance was clonal in the United States, we observed five geographically separated AZM-resistant strains distributed widely through the phylogenomic tree, indicating that this phenotype is not clonal but arose spontaneously from independent accumulation of mtrR –35 A deletion and 23S rRNA mutations.

Of interest is the international epidemic clone ST1407 (and related NG-MAST types such as ST3150, ST3158, and ST4985) that has elevated ESC MICs and increased frequency of AZM-resistant strains (6, 52, 60) that threaten the efficacy of currently recommended treatments. The ST1407 clone is thought to have originated in Japan and spread globally, with the first report of a ST1407 strain harboring a penA mosaic XXXIV allele identified in the United States in 2008 (10). Although a precise date for the actual emergence of ST1407 is not known, our study indicates emergence of the strain in North America prior to 2005.

Core SNP phylogenomic analysis provides greater resolution when grouping strains; however, the NG-MAST typing scheme is generally concordant with the phylogenomic tree (Fig. 1). Although some NG-MAST groups were distributed among several clades, individual NG-MAST types tended to correlate with specific clades and subclades. The miscellaneous strains that did not cluster into distinct phylogenomic clades similarly were associated with a variety of NG-MAST groups and types. Although this study lacks detailed epidemiological information, the differentiation of clade B, C, and D strains beyond the level of the NG-MAST type demonstrates the utility of WGS to identify and characterize social networks and transmission of strains.

The selection of isolates for WGS was based primarily on ESC-DS, resulting in a data set that was enriched for isolates from larger population jurisdictions, recent collection dates, and to the ST1407-like strain groups that are currently the most prevalent strain circulating within Canada and most likely to be associated with antimicrobial resistance (6, 61). Although 85% of Canadian ESC-DS strains isolated between 1989 and 2006 were included in this study, it is possible that some recent lineages and clonal groups have not been detected because of fewer isolates analyzed from subsequent years. Similarly, although isolates with a variety of NG-MAST types, geographical jurisdictions, and antibiotic susceptibilities were selected, clusters of isolates within these smaller groups may also be underrepresented in the phylogeny.

WGS was used to catalog the distribution and relatedness of ESC-DS strains collected in Canada between 1989 and 2013. Analysis of the data suggested a heterogeneous landscape which is dominated by the global epidemic clone ST1407, yet ESC-DS has arisen independently in several lineages. While there is a high correlation of NG-MAST sequence types to phylogenomic lineages, the latter provides greater resolution and discrimination of isolates. As WGS becomes more widely adopted to identify the molecular relationships among isolates, only the addition of epidemiologic contact tracing can fully leverage the great resolving power of WGS to more effectively inform public health interventions, ultimately reducing the burden of disease.

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