

Longitudinal Analysis of the Evolution and Dissemination of *Neisseria gonorrhoeae* Strains (Saskatchewan, Canada, 2005 to 2008) Reveals Three Major Circulating Strains and Convergent Evolution of Ciprofloxacin and Azithromycin Resistance

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A longitudinal study combining multilocus sequence typing with molecular evolutionary analysis determined the distribution, population structure, and evolution of antibiotic resistance in *Neisseria gonorrhoeae* isolates in Saskatchewan that were collected between 2005 and 2008. Of 195 gonococcal isolates examined, 29 sequence types (STs) were identified with 3 major circulating strains (ST-1 through ST-3) comprising 52% of all gonococcal isolates studied. The prevalences, persistence, distribution patterns, and clonalities of these isolates strongly suggest that gonorrhea endemicity within this broad geographic region was driven by these 3 circulating strains. ST-1 exhibited a significantly ($P = 0.001$) higher prevalence throughout the study than did the others, accounting for ~25% of the tested isolates each year. The spatial distributions of the gonococcal strains indicated that ST-1 in 2007 entered a linear component of the sexual network, reaching the remote north and resulting in the further spread and maintenance of infection. Ciprofloxacin and azithromycin resistances were observed in distantly related gonococcal lineages, clearly indicating the convergent acquisition of these antibiotic-resistant phenotypes. In addition, all ciprofloxacin- and azithromycin-resistant lineages were found at the edges of the minimum spanning tree, far from the major lineages, suggesting that these antibiotic phenotypes were most likely introduced into the province. In contrast, resistance to penicillin was found mostly in the endemic gonococcal lineages, suggesting that penicillin resistance was probably acquired in Saskatchewan as a result of spontaneous mutations in already-established lineages. Tetracycline resistance was present in all STs except one, indicating its ubiquitous nature in the gonococcal population studied.

Neisseria gonorrhoeae, a fastidious Gram-negative diplococcus, is an obligate human pathogen that causes the disease gonorrhea. Complications from the spread of the organism along the mucosal surfaces may result in endometritis, salpingitis, and peritonitis in females (3), which can lead to infertility or ectopic pregnancy (27). In Canada, between 1999 and 2008, the overall rate of gonococcal infection increased by 116.5%, from 17.6 to 38 cases per 100,000 (20). Saskatchewan has had the highest infection rate of all the Canadian provinces, with an increase from 45.2 cases per 100,000 in 2000 to 131.3 cases per 100,000 in 2008, resulting in a 290.4% increase (173.4% above the national average) (20). Antimicrobial resistance in *N. gonorrhoeae* is a major public health problem, as treatment options are becoming increasingly limited. Recently, *N. gonorrhoeae* isolates with decreased susceptibilities ($MIC \geq 0.25$) to third-generation cephalosporins (cefixime and ceftriaxone) have been identified in Japan, Europe, the United States, and Canada (4, 15, 16, 19, 29, 30). These antibiotics are the last remaining options for treating gonorrhea in a single-dose fashion, a preferred mode of treatment for gonococcal infections.

Strain typing can be successfully used to identify specific strains that are spreading globally or to identify temporal and geographic changes in strain types and the emergence and transmission of individual strain phenotypes (31). The multilocus sequence typing (MLST) genotyping method is based on obtaining the DNA sequences of various housekeeping genes that are under stabilizing selection (32) (i.e., nonsynonymous substitutions are likely to be purged relatively rarely). As these genes evolve slowly, the genetic relatedness of a bacterial population can be ascertained over

the long term more confidently than with genotyping methods based on antigen-encoding genes that are under positive selection pressure and that change rapidly over the short term (32, 34). A combination of MLST with molecular evolutionary analysis, such as eBURST (12) and the minimum spanning tree, provides reliable insight into the evolutionary pathways and transmission routes of gonococcal isolates. In addition, the emergence and evolution of antibiotic-resistant *N. gonorrhoeae* strains can be better resolved using MLST with the analytical methods discussed above to reveal their evolutionary paths and clonal complexes (CCs).

The aim of this research was to characterize and analyze gonococcal isolates from the province of Saskatchewan that were collected between 2005 and 2008 to uncover the distribution patterns and population structures of these strains and to gain insight into the evolution of antibiotic-resistant phenotypes. This investigation is a unique longitudinal population-based study in which 195 consecutively obtained *N. gonorrhoeae* isolates over a 4-year period were examined by MLST and analyzed by eBURST and the

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minimum spanning tree methods to investigate gonococcal strain relationships and the evolution of antibiotic-resistant phenotypes.

MATERIALS AND METHODS

Bacterial isolates. The Saskatchewan Disease Control Laboratory (SDCL) serves as the microbiological reference center for the province of Saskatchewan. Clinics and hospitals across the province submit *N. gonorrhoeae* specimens to the SDCL for identification and confirmation. From 2005 to 2008, the SDCL received 195 consecutive gonococcal isolates for culture, representing 195 unique patients. Over 98% of the isolates were urogenital in origin. Primary identification was undertaken at the SDCL using standard microbiological methods (9, 41). Information, such as city of isolation and the age and sex of the patients, was available for each isolate. Isolates were stored at -80°C in brain heart infusion medium (Difco BD Biosciences, Oakville, ON, Canada) containing 20% glycerol and were shipped on dry ice to Saskatoon for molecular typing. They were cultured on Difco GC medium base (Becton, Dickinson and Company, Sparks, MD) supplemented with 1% modified Kellogg's supplement GC medium base (GCMB) followed by incubation at 35°C with 5 to 7% CO_2 in a humid environment for 18 to 24 h (9, 41). Isolates were reconfirmed as *N. gonorrhoeae* using the oxidase test and Gram staining (37).

Antimicrobial susceptibility testing. The MICs of the *N. gonorrhoeae* isolates to penicillin, tetracycline, ciprofloxacin, spectinomycin, cefixime, and ceftriaxone were assessed, in duplicate, by the agar dilution method as described previously (9, 41). All antimicrobial agents were purchased from Sigma-Aldrich (Oakville, ON, Canada). WHO strains B, C, and F and strain ATCC 49226 were used as reference strains for antimicrobial susceptibility testing (9, 28, 35). Antimicrobial susceptibility criteria followed CLSI recommendations (9); interpretative criteria for azithromycin resistance have been described previously (7). β -Lactamase production was determined using nitrocefin (Calbiochem, EMD Chemicals Inc., Darmstadt, Germany). *N. gonorrhoeae* isolates identified as having plasmid-mediated tetracycline resistance (i.e., TRNG) based on MICs of ≥ 16 mg/liter were confirmed by *tetM* analysis with WHO strain M as a positive control (40).

Multilocus sequencing typing. Growth from an overnight culture on GC medium base was collected using a sterile cotton swab and resuspended in 1 ml of 0.9% saline. After centrifugation at $10,000 \times g$ for 1 min, the supernatant was removed, and genomic DNA was extracted using the Qiagen DNeasy tissue kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions.

All gonococcal isolates were typed using the MLST method described by Tazi et al. (26), and sequence types (STs) were randomly assigned. The following seven markers were used: *fumC*, *gdh*, *glnA*, *gnd*, *pilA*, *pyrD*, and *serC*. The oligonucleotide primers used in this study were purchased from Life Technologies–Invitrogen (Burlington, ON, Canada). Amplicons were purified using a PCR purification kit (Qiagen, Mississauga, ON, Canada), and their nucleotide sequences on both strands were determined using an Applied Biosystems 3730X1 DNA analyzer (Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, SK, Canada). DNA sequences were analyzed using DNASTAR Lasergene 7 (DNASTAR Inc., USA). DNA sequences for each locus were compared, and every unique sequence was assigned as a distinct allele using Sequence Output (www.mlst.net). Each unique combination of allele numbers was assigned as a different sequence type (ST) which unambiguously defined strains of *N. gonorrhoeae*.

Phylogenetic and eBURST analysis. MEGA4 (25) was used to construct phylogenetic trees from concatenated sequences of the housekeeping genes. Evolutionary distances were computed using the maximum composite likelihood method (24). The neighbor-joining algorithm (22) was used to generate the initial phylogenetic tree. To predict ancestral profiles and clonal complexes, each genotype identified in this study was analyzed by eBURST (12). For identification of the clonal complexes, the most stringent default group definition was used, where all STs must be single-locus variants of at least one ST in the clonal complex.

Minimum spanning tree. The minimum sum of the weights of the edges (genetic distances) was used to create a minimum spanning tree from the MLST profile data. Prim's algorithm was implemented to determine the links between different STs. The highest priority was given to STs with the most numerous single-locus variants. The two STs with the greatest number of single-locus and then double-locus variants were linked first, preferably using intermediate STs.

Statistical analyses. Data were analyzed using SAS (SAS Institute Inc., Cary, NC) by applying the Marascuilo procedure test to determine if there was a significant difference between the ratios for every pair of STs. Also, a trend in changes in the prevalence of gonococcal strains over the 4-year period was evaluated using the Chi-square multiple-hypothesis test.

Nucleotide sequence accession numbers. Unique DNA sequences were deposited in GenBank with accession numbers HM359031 through HM359049 and HM359053 through HM359071.

RESULTS

Identified sequence types and clonal complexes of *N. gonorrhoeae*. In total, 2,730 DNA sequences were processed. The 195 *N. gonorrhoeae* isolates were resolved by MLST into 29 different STs. Most notably, 3 distinct STs were responsible for 52% ($n = 106$) of all the *N. gonorrhoeae* isolates we examined. ST-1 accounted for 23.6% ($n = 46$) of the isolates, followed by ST-3 (15.9%, $n = 31$) and ST-2 (12.3%, $n = 24$). Seventy-four isolates were resolved into 11 STs (ST-4, ST-5, ST-6, ST-8, ST-11, ST-14, ST-15, ST-16, ST-20, ST-21, and ST-25); these STs accounted for 1.5% ($n = 3$) to 7.2% ($n = 14$) of the isolates examined. Lastly, 10 STs (ST-7, ST-12, ST-13, ST-17, ST-18, ST-22, ST-23, ST-26, ST-27, and ST-28) were represented by a single isolate, and five STs (ST-9, ST-10, ST-19, ST-24, and ST-29) were represented by two isolates each, indicating that these STs were sporadic and minor.

The genetic relatedness between the 29 STs is presented in a minimum evolution tree (Fig. 1) that also indicates the major clonal complex. The population snapshot of all 195 *N. gonorrhoeae* isolates generated by the eBURST analysis revealed the existence of one major and eight minor clonal complexes (CCs). ST-1 was identified as the ancestral strain for the major CC that also involved ST-8, ST-17, ST-23, and ST-28, as also indicated in the minimum evolution tree. The eight minor CCs comprised one triplet (STs 27, 28, and 6) and seven doublets (STs 2 and 5, 18 and 25, 13 and 12, 24 and 29, 21 and 19, 3 and 22, and 7 and 10). Seven different STs (ST-14, ST-15, ST-11, ST-20, ST-16, ST-4, and ST-9) were identified as singletons and could not be assigned to any existing CC.

Distribution of sequence types. Longitudinal data collected from 2005 to 2008 clearly showed the existence of two different classes of gonococcal strains. One class, comprising ST-1, ST-2, ST-3, ST-5, and ST-11, was characterized by the constant circulation of the STs throughout the period of testing (Fig. 2A), while the other class of strains was characterized by the STs' sporadic occurrence (Fig. 2A). Circulating sequence types (i.e., ST-1, ST-2, and ST-3) showed significantly higher prevalences ($P \leq 0.05$) than any sporadic strain identified in this study (Fig. 2A and B). In examining trends in the prevalences of gonococcal strains, separately for each year, only ST-1 showed continuously higher significance ($P \leq 0.05$) than any other ST (Fig. 2B). The strains associated with the sporadic sequence types rapidly disappeared (Fig. 2A), and the sporadic sequence types from 2005 and 2006 were completely replaced in 2008 with new sequence types.

The geographic distribution of the gonococcal sequence types across the province during the study period indicated a distinct

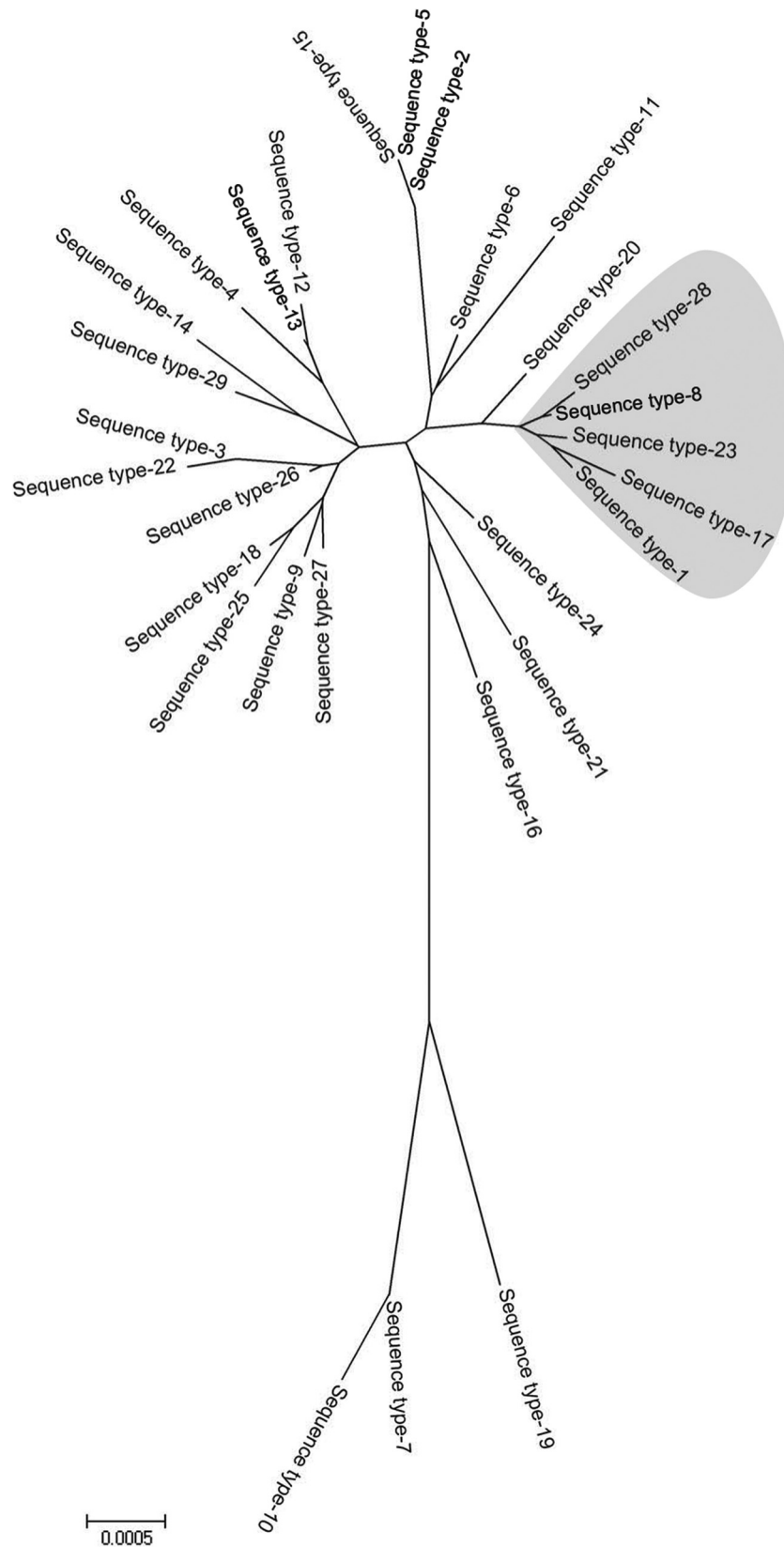


FIG 1 Minimum evolution tree of the concatenated sequences from the 29 sequence types (STs) comprising 195 *N. gonorrhoeae* isolates. The 5 lineages shaded in gray represent the major clonal complex identified in the population of *N. gonorrhoeae* isolates from Saskatchewan over the study period. The evolutionary history was inferred using the minimum evolution method (21). 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 minimum evolution tree was searched using the close-neighbor-interchange algorithm (18) at a search level of 1.

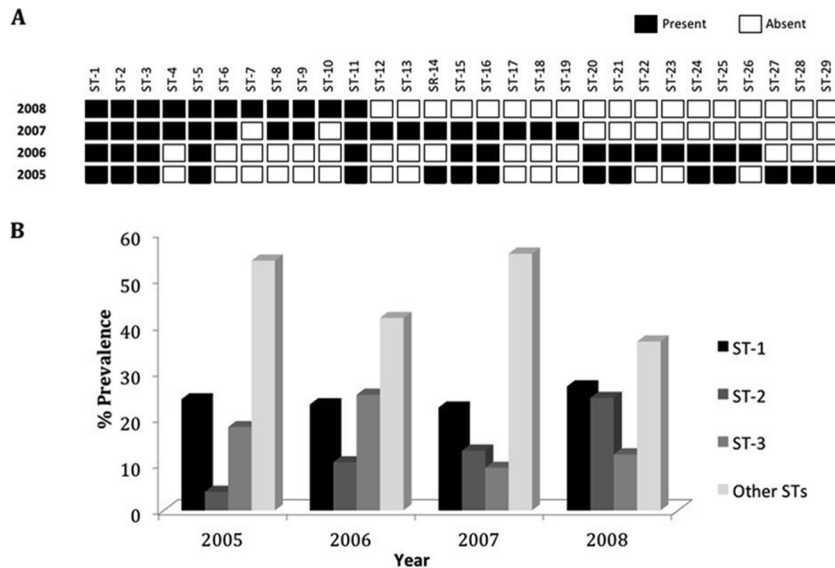


FIG 2 Longitudinal distribution of *Neisseria gonorrhoeae* strains. (A) Occurrence of the STs over a 4-year period. Solid squares indicate the presence of certain STs, while open squares indicate their absence. (B) Prevalence rates of the most predominant circulating strains compared to the prevalence rates of all other STs identified during the 2005–2008 study period in Saskatchewan.

migration route for ST-1 (Fig. 3). Isolates belonging to ST-1 were first identified in the southern part of the province (Fig. 3) and migrated toward the north over the study period. In 2008, the last year of this study, ST-1 isolates were predominantly identified in the northern part of the province (Fig. 3). Other STs showed more nondistinct migration.

Sequence types and antibiotic resistance. During the period of this study, all gonococcal isolates were found to be susceptible to cefixime, ceftriaxone, and spectinomycin. The majority of isolates (77.9%, $n = 152$) were resistant to tetracycline, with 94.1% ($n = 143/152$) of these isolates being chromosomally resistant and 5.9% ($n = 9$) carrying plasmid-mediated tetracycline resistance (TRNG). Tetracycline resistance was identified in all STs except ST-29 (Fig. 4), while TRNG isolates were identified in ST-20 (7/7), followed by ST-25 (1/3) and ST-26 (1/1). Resistance to penicillin (3.0%, 6/195), ciprofloxacin (2.6%, 5/195), and azithromycin (0.5%, 1/195) was low, and no penicillinase-producing *N. gonorrhoeae* (PPNG) isolates were detected. The majority of isolates resistant to penicillin (5/6) were found in the circulating strains ST-1 ($n = 3$) and ST-3 ($n = 2$), whereas one penicillin-resistant isolate was identified in sporadic strain ST-25. All ciprofloxacin-resistant isolates ($n = 5$) were found in sporadic sequence types, including ST-6, ST-9, ST-26, and ST-5. Also, the single azithromycin-resistant isolate ($n = 1$) was found in sporadic sequence type ST-12. All of these sporadic isolates were positioned on the edges of the minimum spanning tree (Fig. 4).

DISCUSSION

To reveal the distribution patterns and population structures of *N. gonorrhoeae* isolates from the province of Saskatchewan, we performed a longitudinal study over a 4-year period using MLST, a neutral typing method (14, 32, 34) (i.e., a method capable of buffering frequent mutations), and molecular evolutionary analyses. By using MLST, we were able to minimize some limitations associated with typing methods, such as *porB* and *N. gonorrhoeae* multiantigen sequence typing (NG-MAST), that may overdiscrimi-

nate related isolates. For example, in a recent study (34), we showed that the concatenated sequences of the housekeeping genes (MLST) retain the phylogenetic signals of the circulating gonococcal strains and can identify them over a long period of time. On the other hand, the hypervariable *porB* DNA sequence is subject to frequent mutation and recombination, further producing a distorting effect among genetically related isolates that may lead to a failure to identify the predominant related strains of *N. gonorrhoeae* that circulate within the community over an extended period of time (34). A unique characteristic of using MLST analysis is its ability to provide a buffer against the distorting effects of mutations and recombinations, further resulting in the preservation of clonal stability of *N. gonorrhoeae* isolates. Therefore, MLST is a suitable method for fine-scaled epidemiological investigations designed to monitor the spread of gonococcal isolates over an extended period of time.

The present population-based study shows that the majority of gonococcal infections in Saskatchewan between 2005 and 2008, a region with one of the highest gonorrhea infection rates worldwide (36), were mainly caused by three distinct circulating gonococcal strains. Another study examining the population genetics of *N. gonorrhoeae* isolates in Liverpool, United Kingdom, also identified a couple of gonococcal strains, ST-1596 and ST-1583, with profound geographic persistence, indicating that these gonococcal strains may have a fitness advantage which enables them to persist over a long period of time (2). However, these persistent gonococcal strains found in Liverpool did not show the high prevalences that the three major circulating strains showed in our study. In comparing the gonococcal population from Saskatchewan to one from Shanghai (26), we found that the main characteristic of gonococcal isolates from Saskatchewan is a high degree of genetic homogeneity. For instance, 195 gonococcal isolates from this study were resolved into 29 different STs, while the population of 96 isolates from Shanghai was resolved into 76 STs. In addition, the gonococcal population from Saskatchewan showed

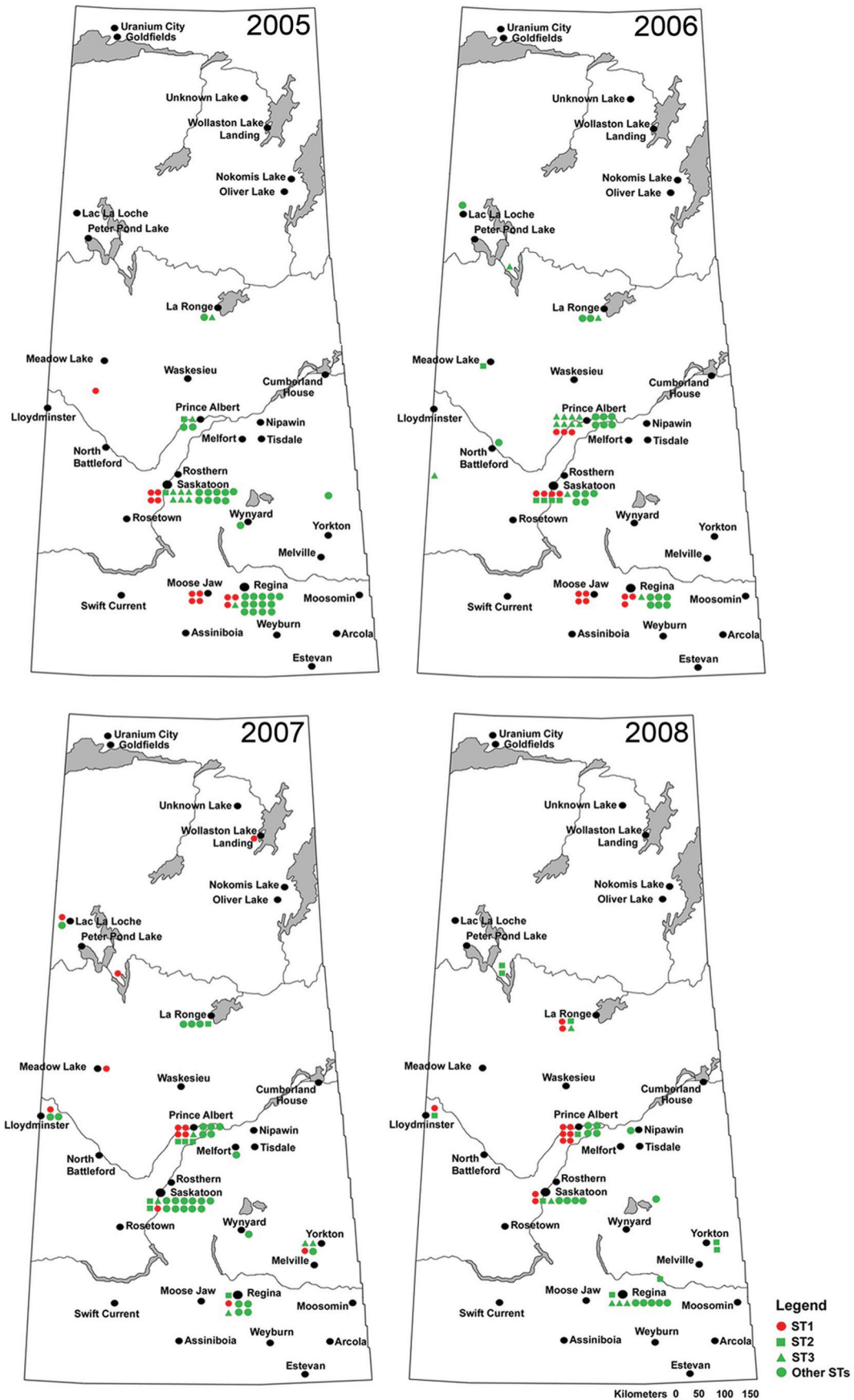


FIG 3 Spatial distribution of *Neisseria gonorrhoeae* strains in Saskatchewan during the 2005–2008 study period. The most predominant ST-1 strain is shown in red to emphasize its importance. The other two circulating strains, ST-2 and ST-3, are shown in green squares and triangles, respectively. All of the other 26 sequence types identified in this study are indicated by green circles. (Reprinted from reference 33.)

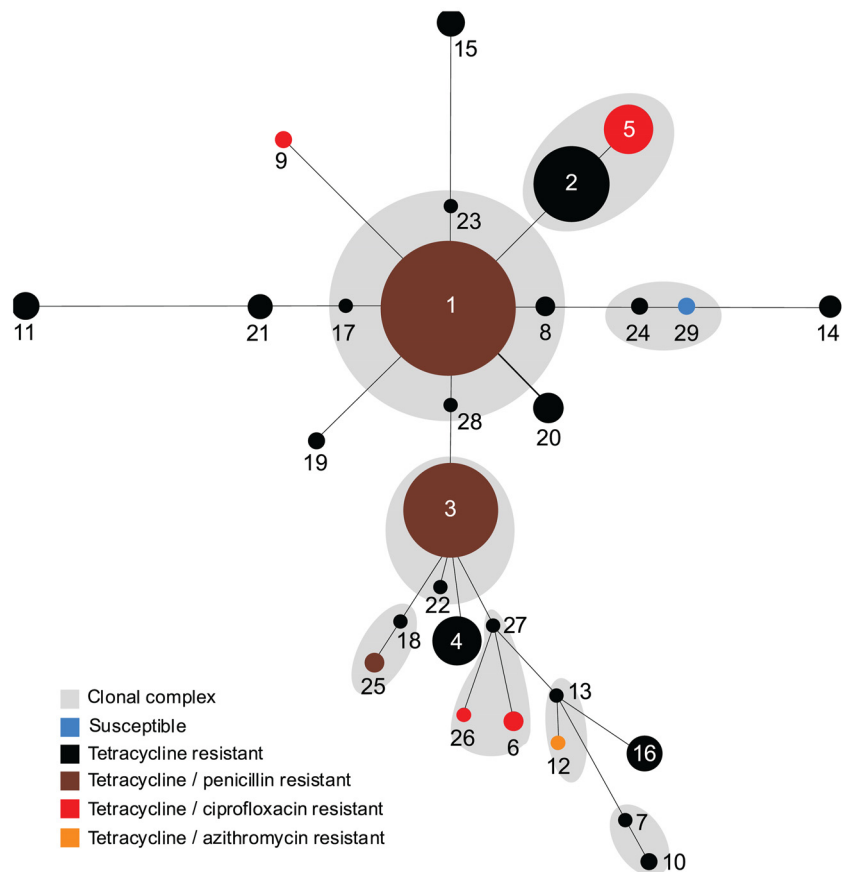


FIG 4 Minimum spanning tree based on the multilocus sequence typing allelic profiles depicting the antibiotic-resistant phenotype distribution across *Neisseria gonorrhoeae* lineages and their clonal complexes. Each circle represents a unique ST. The number of isolates of each ST is directly proportional to the area of the circle. The blue circle represents the ST susceptible to all tested antibiotics, while black indicates resistance to tetracycline alone, brown indicates resistance to penicillin and tetracycline, red indicates resistance to ciprofloxacin and tetracycline, and orange indicates resistance to azithromycin and tetracycline. Gray shadings represent the clonal complexes which consist of single-locus variants.

temporal clustering, whereas no temporal clustering was observed among the isolates from Shanghai. These studies suggest the existence of two distinct gonococcal populations. The gonococcal population from Shanghai was very diverse, with no predominant strains, whereas the gonococcal population from Saskatchewan, Canada, was homogenous, with the existence of several predominant strains that spread throughout the province.

The spatial distribution of the gonococcal sequence types showed that the most predominant type, ST-1, was initially identified in the south-central part of the province (Fig. 3), mainly in the most populated areas of the province. Over the next 2 years (2005-2006), infections associated with ST-1 remained in the same areas, showing characteristics of endemic infection. By 2007, strain ST-1 exhibited a distinct northward motion, reaching remote places which are accessible only by airplane or boat. This type of ST-1 distribution reflects the linear component of a sexual network (38). Using routinely collected case/contact information by public health authorities, Wylie and Jolly (38) identified two types of components, radial and linear, within a large sexual network in the province of Manitoba, Canada. Linear-type components were characterized by a larger number of distinct geographic locations than the radial-type components. From a public health perspective, linear components represent an important reservoir

for gonorrhea maintenance and spread (38). Further, Wylie et al. (39) found that the linear component included extensive interconnections present within a large sexual network, resulting in frequent bridging events between different demographic groups. For instance, in a study that examined a large linear-type component in Manitoba, Canada, involving 82 people, the authors found two prominent central figures that caused the network to span significant distances, resulting in the infection of people living in various communities scattered throughout this large region of western Canada (38). In contrast to linear components, radial components were characterized by local transmissions through direct or indirect links between members of the same community. In our study, the observation that ST-1 is frequently found over a vast area of the Saskatchewan province and connects otherwise geographically disconnected areas clearly indicates its importance in spreading and maintaining gonococcal infection. We were unable to discern in this study whether ST-1 is common in other parts of Canada or globally, because the MLST scheme used, described earlier by Tazi et al. (26), is not posted online and is therefore unavailable for international comparison.

The population snapshot of 195 gonococcal isolates created by our eBURST analysis shows the existence of a single major clonal complex together with several minor clonal complexes. Most no-

tably, the highly predominant ST-1 was found as an ancestor of the major clonal complex, further suggesting that this circulating strain has been present in Saskatchewan for a long period. Unlike the gonococcal population in Shanghai (26), where all STs within the major clonal complexes did not differ in size, the founder of the major clonal complex in Saskatchewan was profoundly larger than the other STs. Our eBURST analysis suggested that, in Saskatchewan, there exists a predominant gonococcal lineage, which is well established, whereas in Shanghai the gonococcal population is more dynamic, without predominant gonococcal strains.

Emerging resistance to extended-spectrum cephalosporins (19, 29, 30), as well as resistance to fluoroquinolone (15, 41) and macrolide (8, 10, 13, 23) antibiotics, among gonococcal isolates is an ongoing challenge for the treatment and control of gonorrhea infections. In this study, we have provided insight into the antimicrobial profiles of *N. gonorrhoeae* isolates collected over a large geographic area using past and present antimicrobial agents recommended for gonococcal treatment (5, 6). The most prevalent antibiotic resistance was chromosomal resistance to tetracycline. The high rate of resistance to this antibiotic may be attributed to the history of tetracycline use in the cotreatment of chlamydial infections, as tetracycline has not been recommended for the primary treatment of gonorrhea in Canada since the late 1980s (11). Notably, the prevalence rate of ciprofloxacin resistance (2.6%) in gonococcal isolates from this study was significantly lower than that reported in the recent study (28.2%) carried out in Ontario, Canada (1). The depiction of ciprofloxacin resistance across the gonococcal lineages on the minimum spanning tree (Fig. 4) clearly suggests the convergent acquisition (genetically distant lineages acquiring the same antimicrobial phenotype) of drug resistance by these isolates rather than the clonal expansion. Furthermore, all gonococcal lineages resistant to ciprofloxacin were positioned at the edges of the minimum spanning tree, indicating a very distant genetic relatedness to the major gonococcal lineages, and further suggesting that ciprofloxacin resistance was most likely introduced from outside the province. Mavroidi et al. (17) found that the emergence and rapid recent increase of a quinolone-resistant strain of *N. gonorrhoeae* in Greece were due to the importation of a quinolone-resistant strain, ST-1901, from other countries. Although resistance to ciprofloxacin in Saskatchewan is presently low, it may emerge and spread, as we already observed the importation of several quinolone-resistant *N. gonorrhoeae* strains within this province. Chromosomal resistance to penicillin was observed among the major gonococcal lineages (Fig. 4), further suggesting that this phenotype was probably acquired in Saskatchewan as a result of spontaneous mutations in established lineages.

In summary, this study has revealed the structure of the gonococcal population from the province of Saskatchewan and clearly indicates the high impact of several circulating strains on the overall rates and spread of *N. gonorrhoeae* isolates. In addition to providing current insight into the antibiotic susceptibilities of gonococcal isolates, this work also illustrates how combining antimicrobial susceptibility data, MLST, and evolutionary analysis (eBURST and the minimum spanning tree) provides a more comprehensive view of the evolution and dissemination of antimicrobial susceptibility traits in the gonococcal population.

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