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Neisseria gonorrhoeae Sequence Typing for Antimicrobial Resistance, a Novel Antimicrobial Resistance Multilocus Typing Scheme for Tracking Global Dissemination of N. gonorrhoeae Strains

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ABSTRACT A curated Web-based user-friendly sequence typing tool based on antimicrobial resistance determinants in *Neisseria gonorrhoeae* was developed and is publicly accessible (https://ngstar.canada.ca). The *N. gonorrhoeae* Sequence Typing for Antimicrobial Resistance (NG-STAR) molecular typing scheme uses the DNA sequences of 7 genes (*penA*, *mtrR*, *porB*, *ponA*, *gyrA*, *parC*, and 23S rRNA) associated with resistance to β-lactam antimicrobials, macrolides, or fluoroquinolones. NG-STAR uses the entire *penA* sequence, combining the historical nomenclature for *penA* types I to XXXVIII with novel nucleotide sequence designations; the full *mtrR* sequence and a portion of its promoter region; portions of *ponA*, *porB*, *gyrA*, and *parC*; and 23S rRNA sequences. NG-STAR grouped 768 isolates into 139 sequence types (STs) (*n* = 660) consisting of 29 clonal complexes (CCs) having a maximum of a single-locus variation, and 76 NG-STAR STs (*n* = 109) were identified as unrelated singletons. NG-STAR had a high Simpson's diversity index value of 96.5% (95% confidence interval [CI] = 0.959 to 0.969). The most common STs were NG-STAR ST-90 (*n* = 100; 13.0%), ST-42 and ST-91 (*n* = 45; 5.9%), ST-64 (*n* = 44; 5.72%), and ST-139 (*n* = 42; 5.5%). Decreased susceptibility to azithromycin was associated with NG-STAR ST-58, ST-61, ST-64, ST-79, ST-91, and ST-139 (*n* = 156; 92.3%); decreased susceptibility to cephalosporins was associated with NG-STAR ST-90, ST-91, and ST-97 (*n* = 162; 94.2%); and ciprofloxacin resistance was associated with NG-STAR ST-26, ST-90, ST-91, ST-97, ST-150, and ST-158 (*n* = 196; 98.0%). All isolates of NG-STAR ST-42, ST-43, ST-63, ST-81, and ST-160 (*n* = 106) were susceptible to all four antimicrobials. The standardization of nomenclature associated with antimicrobial resistance determinants through an internationally available database will facilitate the monitoring of the global dissemination of antimicrobial-resistant *N. gonorrhoeae* strains.

KEYWORDS *Neisseria gonorrhoeae*, antimicrobial resistance, molecular epidemiology, sequence typing

Neisseria gonorrhoeae, the causative agent of gonorrhea, is a global public health concern causing an estimated 78 million new cases of gonorrhea among adults every year (1). *N. gonorrhoeae* has developed resistance to almost all of the antimicrobials previously used for the treatment of gonorrhea, including penicillins, tetracyclines, and fluoroquinolones (2, 3); the remaining treatment options are now threatened by the emergence of resistance to third-generation cephalosporins and azithromycin (4–13).

The genes attributed to cephalosporin, fluoroquinolone, and azithromycin resistances have been well characterized (3); however, the lack of an internationally standardized classification nomenclature makes it difficult to compare the mutations implicated in resistance in individual gonococcal isolates. Agreement on the nomenclature of antimicrobial resistance (AMR) determinants through an internationally available, curated, user-friendly database containing sequence information and appropriate microbiological, genetic, clinical, and epidemiological data is of the utmost importance (14–16).

Variations in nucleotide sequences of *penA* (encoding penicillin binding protein 2 [PBP2]) are associated with resistance to cephalosporin and penicillin (2, 14, 17) and include the acquisition of *penA* mosaic sequences through recombination with *penA* genes from other *Neisseria* species as well as various other amino acid substitutions. Historically, the nomenclature used for the *penA* gene has been the assignment of PBP2 types I to XXXVIII based on amino acid substitution profiles at 82 selected amino acid positions (9) rather than nucleotide sequence differences. *N. gonorrhoeae* Sequence Typing for Antimicrobial Resistance (NG-STAR) uses this historical amino-acid-based nomenclature as a basis for allele numbering while creating a more convenient and easier-to-maintain naming system. Mutations in the promoter and/or coding region of the *mtrR* repressor may result in the overexpression of the MtrCDE efflux pump. Known *mtrR* mutations associated with resistance to macrolides, cephalosporins, tetracycline, and penicillin include a −35A deletion in the promoter sequence, an A39T or G45D mutation in the coding region, or the presence of *N. meningitidis*-like sequences (2, 14, 18–21). Variations in *porB1b*, encoding an outer membrane porin protein, causing amino acid substitutions at G120 and A121 (or G101 and A102, respectively, if the 19-amino-acid signal sequence is not included), also historically referred to as the *penB* resistance determinant, can decrease membrane permeability and the uptake of cephalosporins, tetracycline, and penicillin (2, 14, 22–25). The L421P variation caused by a mutation in *ponA* (PBP1) is involved in chromosomally mediated resistance to penicillins. The L421P mutation is present in many circulating *N. gonorrhoeae* strains, including those with elevated cephalosporin MICs; however, this mutation has not caused significantly increased cephalosporin MICs in transformation experiments (2, 3, 26). Fluoroquinolone resistance in *N. gonorrhoeae* is conferred via variations in *gyrA* (subunit A of DNA gyrase) that induce changes at amino acid positions S91 and/or D95 and those in *parC* (topoisomerase IV subunit C) that alter amino acids at positions D86, S87, and/or S88 (2, 3, 27). Point mutations in the peptidyl-transferase loop region in domain V of 23S rRNA confer resistance to azithromycin (2, 3, 28, 29). By convention, the locations of the 23S rRNA nucleotide mutations are based on the *Escherichia coli* coordinates of A2059G and C2611T, which correspond to A2045G and C2597T, respectively, of *N. gonorrhoeae* NCCP11945 (GenBank accession number NC_011035.1). The *N. gonorrhoeae* genome carries 4 gene alleles encoding 23S rRNA, and although the number of mutated alleles has been correlated with increasing resistance (28), a strain with one mutated allele can rapidly acquire mutations in all 4 alleles (29).

In the present study, a novel sequence-based molecular antimicrobial resistance typing scheme and Web-based analysis software, NG-STAR, were developed through an international collaboration. NG-STAR addresses the complex nomenclature applied to variations in multiple antimicrobial resistance genes of *N. gonorrhoeae* and can be used to monitor the global dissemination of antimicrobial-resistant *N. gonorrhoeae* strains.

RESULTS

NG-STAR currently contains 215 sequence types (STs) corresponding to unique allelic profiles consisting of concatenated allele identifications for *penA*, *mtrR*, *porB*, *ponA*, *gyrA*, *parC*, and 23S rRNA loci.

penA alleles. NG-STAR currently includes 49 *penA* types, including 21 historical and 28 novel amino acid profiles, with 80 *penA* gene alleles (Table 1). The most allelic diversity was seen for *penA* type II (*penA* alleles 2.001 to 2.006), *penA* type XXII (alleles 22.001 to 22.006), and *penA* type XXXIV (alleles 34.001 to 34.006). There were 16 mosaic ($n = 228$) and 32 nonmosaic ($n = 541$) *penA* types among the 769 isolates analyzed. The most common mosaic type was *penA* type XXXIV ($n = 197$), with 79.7% ($n = 157$) of isolates carrying *penA* allele 34.001, and the most common nonmosaic type was *penA* type II ($n = 169$), where allele 2.001 accounted for 88% of the isolates ($n = 149$).

mtrR alleles. There are currently 51 *mtrR* allele types in the database (Table 2), with 6 alleles having the −35A deletion in the promoter region, 19 having either the A39T or G45D substitution in MtrR, 6 having an *N. meningitidis*-like mosaic sequence, and 20 alleles having none of these mutations. The *mtrR1* allele, which has −35A promoter deletion only, accounted for 43.2% ($n = 332$) of the isolates included in this study.

porB alleles. There are currently 16 *porB* allele types in the database, 14 of which are *porB1b* and 2 of which correspond to *porB1a* sequences. Of the *porB1b* sequences, 13 have the corresponding G120 and/or A121 amino acid substitution in PorB1b. The *porB11* allele (with G120K/A121N) was most common among the isolates tested, representing 30.8% ($n = 237$) of the *porB* alleles, followed by *porB1* (wild type at G120/A121), with 16.8% ($n = 129$), and *porB8* (G120K/A121D), with 16.6% ($n = 128$).

ponA alleles. Six *ponA* alleles are currently present in NG-STAR, four of which have the PBP1 L421P substitution present. The *ponA1* allele (L421P) was predominant among the strains tested, representing 52.8% ($n = 406$) of the isolates.

gyrA alleles. There are 10 *gyrA* alleles listed in NG-STAR so far, 5 of which possess a S91 and/or a D95 substitution in GyrA. Among the strains analyzed, 47.7% ($n = 367$) harbored *gyrA0* (wild type with no mutations), and 39.5% ($n = 304$) harbored *gyrA1* (S91F/D95G).

parC alleles. NG-STAR currently includes 21 *parC* alleles, and 13 have substitutions at amino acid positions D86, S87, and/or S88 in ParC. The most predominant allele among the strains in this study was *parC3* (S87R), in 37.3% ($n = 287$) of the isolates, followed by *parC0* and *parC1* (both wild type at D86, S87, and S88), in 16.8% ($n = 129$) and 15.0% ($n = 115$) of the strains, respectively.

23S rRNA alleles. There are currently 10 23S rRNA alleles in the NG-STAR database, with 2 having a C2611T mutation that is associated with low to moderate resistance (MIC = 2 to 32 μg/ml depending upon the number of alleles mutated) and 1 having an A2059G mutation that causes high-level resistance (MIC ≥ 256 μg/ml with more than 2 mutated alleles). The 23S rRNA wild-type allele 23S-0 accounted for 64.6% ($n = 497$), 23S-2 (with C2611T) accounted for 21.7% ($n = 167$), and 23S-1 (A2059G) accounted for 1.2% ($n = 9$) of the isolates tested.

Sequence types. Analysis of 768 *N. gonorrhoeae* isolates identified 215 NG-STAR STs (see Table S1 in the supplemental material). goeBURST analysis of the NG-STAR allelic profiles (Fig. S1) grouped 660 isolates into 28 clonal complexes (CCs) of 215 NG-STAR STs having single-locus variations, and 109 isolates were identified as 76 singleton NG-STAR STs having ≥2 locus variations, which generated a Simpson's diversity index value of 96.5% (95% confidence interval [CI] = 0.959 to 0.969). Sixteen NG-STAR STs were composed of 10 or more isolates (Table S2) ($n = 439$; 57.1%), and 134 NG-STAR STs consisted of a single isolate ($n = 134$; 17.4%). Multilocus sequence typing (MLST) analysis produced 86 MLST STs, with 435 isolates grouping into 6 CCs, and 39 MLST STs were singletons ($n = 222$), producing a Simpson's diversity index value of 86.6% (95% CI = 0.846 to 0.886). Analysis by *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) produced 209 NG-MAST STs, grouping 537 isolates into 20 CCs and 181 isolates into 103 singleton NG-MAST STs, resulting in a Simpson's diversity index value of 97.0%

(95% CI = 0.964 to 0.975). The congruence of the three typing methods was determined with the adjusted Wallace coefficient (AW), with $AW_{NG-STAR \to MLST}$ equaling 0.780 (95% CI = 0.712 to 0.848) and $AW_{NG-STAR \to NG-MAST}$ equaling 0.368 (95% CI = 0.321 to 0.416), meaning that isolates grouped by NG-STAR have a 78% chance of grouping by MLST and a 37% chance of grouping by NG-MAST.

The most common NG-STAR ST was ST-90 ($n = 100$; 13.0%), followed by ST-42 and ST-91 (each $n = 45$; 5.9%), ST-64 ($n = 44$; 5.72%), and ST-139 ($n = 42$; 5.5%). NG-STAR ST-90 was also the most diverse ST (see Table S2 in the supplemental material) and consisted of 7 MLST and 24 NG-MAST STs ($n = 96$). NG-STAR ST-91 had 2 MLST and 7 NG-MAST STs ($n = 42$), NG-STAR ST-42 had 1 MLST and 2 NG-MAST STs ($n = 45$), NG-STAR ST-64 had 3 MLST and 2 NG-MAST STs ($n = 42$), and ST-139 had 1 MLST and 10 NG-MAST STs ($n = 42$).

Some NG-STAR STs were correlated geographically with Brazilian isolates grouping into 8 NG-STAR STs, 5 of which were unique to Brazil ($n = 5$), whereas 22 NG-STAR STs were observed among isolates from the Netherlands having 15 unique NG-STAR STs ($n = 23$), 20 NG-STAR STs ($n = 31$) were unique to the United States out of a total of 28, and all Chinese isolates ($n = 10$) were associated with 7 unique NG-STAR STs (Table 3).

NG-STAR STs, allelic profiles, and antimicrobial MICs for a selection of internationally available reference strains are presented in Table 4, and the characteristics of prevalent NG-STAR STs are presented in Table 5. Specific NG-STAR STs were associated with characteristic antimicrobial resistance levels. Among the common NG-STAR STs consisting of 10 or more isolates, 92.3% of isolates with NG-STAR ST-61, ST-64, ST-79, ST-91, or ST-139 ($n = 156$) had an azithromycin MIC of ≥ 2.0 μg/ml, compared to 6.2% ($n = 17$) of isolates of other prevalent STs ($P < 0.001$) (Table 5). Similarly, 86.1% ($n = 142$) of NG-STAR ST-90, ST-91, or ST-97 isolates had a ceftriaxone MIC of ≥ 0.06 μg/ml ($P < 0.001$), and 92.1% ($n = 165$) had a cefixime MIC of ≥ 0.125 μg/ml ($P < 0.001$). Concurrently reduced susceptibilities to azithromycin and cephalosporins were present among NG-STAR ST-91 strains, where 86.7% ($n = 39$) had an azithromycin MIC of ≥ 2.0 μg/ml and a ceftriaxone MIC of ≥ 0.06 μg/ml ($P < 0.001$). NG-STAR ST-26, ST-90, ST-91, ST-97, ST-150, and ST-158 were associated with ciprofloxacin resistance, with an MIC of ≥ 1.0 μg/ml (98.0%; $n = 196$; $P < 0.001$), whereas NG-STAR ST-42, ST-43, ST-63, ST-81, and ST-160 ($n = 106$) were susceptible to all four antimicrobials. Minimum spanning tree analysis using goeBURST further illustrated the association of NG-STAR STs with antimicrobial susceptibilities (Fig. 1). NG-STAR STs with reduced susceptibilities to ciprofloxacin (Fig. 1A), ceftriaxone (Fig. 1C), and cefixime (Fig. 1D) clustered together, and highly susceptible NG-STAR STs clustered oppositely in the minimum spanning tree, while those with intermediate MIC ranges were located in between. This pattern was not as evident for azithromycin susceptibility, where clusters of resistant NG-STAR STs were scattered throughout the tree (Fig. 1B).

Specific NG-STAR alleles were also associated with levels of resistance. Isolates with the 23S rRNA allele 23S-1 (with an A2059T mutation) were the only isolates that also had azithromycin MICs of ≥ 256 μg/ml ($n = 8$). Allele 23S-2 with a C2611T mutation as the sole known azithromycin resistance determinant (not having an *mtrR* −35A deletion or A39T or G45D mutation, an *N. meningitidis*-like promoter, or a 23S rRNA A2059T mutation) was identified in 33 of 34 azithromycin-resistant isolates, whereas only 2 of the 70 isolates with no known determinants were resistant ($P < 0.001$). The *mtrR27*, *mtrR28*, *mtrR30*, *mtrR39*, *mtrR44*, and *mtrR46* alleles contain an *N. meningitidis*-like *mtrR* promoter sequence, which was the sole determinant in 17 azithromycin-resistant isolates and only 2 susceptible isolates ($P < 0.001$). Similarly, 24 of 27 isolates with only the *gyrA* S91 and D95 ciprofloxacin resistance determinants, corresponding to the *gyrA1* and *gyrA7* alleles, respectively, were ciprofloxacin resistant (MIC ≥ 1 μg/ml), whereas only 4 of 399 isolates with no resistance determinants (both wild-type *gyrA* S91/D95 and wild type *parC* D86/S87/S88) were resistant ($P < 0.001$).

TABLE 1 Amino acid profiles of previously described and novel *penA* types[a]

penA type by NG-STAR	*penA* type by Onishi et al.[c]	Mosaic[b]	Amino acid profile
0	Wild type	No	MCAKDDVVNYGEDQQAADRRAIVAGTDLNERLQPSPR.SRGAEFEITLNRRPAVLQIFESRENPTTAFANVAAHGAPPKII.A
1	I	No	....
2	II	No	....D.........................
3	III	No	....D.........LV..G.........VNV
4	IV	No	....D.........LV..G..S........
5	V	No	....D.........LV..G..S......VNV
6[d]	VI	No	....D.........LV..............
7	VII	No	....D.........LV......L.......
8[d]	VIII	No	....D.........VLV...G..S......
9	IX	No	....D.........VLV...S.L.......
10	X	Yes	...E.ASHAGEE..VEKQ.MTS.V.ATDTFLSATQ.TMTPK.DV..S.QKVEVKVIA.KKEA..LVY....N.ST.VQVVNV
11[d]	XI	No	....D.........VLV...G.....L...
12	XII	No	....D.........LV...G.....S....
13	XIII	No	....D.........VLV...G.....S....
14	XIV	No	....D.........LV...G.N........
15	XV	No	....D...............N.........
16[d]	XVI	No	....D.........VLV...G........NV
17	XVII	No	....D.........VLV...G..S.....VNV
18	XVIII	No	....D.........TLV...G..S.....VNV
19	XIX	No	....D.........LV..G.N........VNV
20[d]	XX	No	....D.........LV..G.N........VNV
21	XXI	No	....L.................VLV..G.N...VQVVNV
22	XXII	No	....D.........LV.G.N....VQVVNV
23[d]	XXIII	Semi	....VEVKVIA.KKE............LVY....N..T.VQVVNV
24	XXIV	No	....D.........LV...G..S..S...VNV
25[d]	XXIV	Yes	...E.ASHAGEE..VEKQ.MTS.V.ATDTFLSATQ.TMTPK.DV..S.QKVEVKVIA.KKEA..LVY....N.ST.VQVVNV
26[d]	XXVI	Yes	..NE..H...K..N..MTS.V.ATDTFLSATQ.TMTPK.DV..S.QKVEVKVIA.KKEA.VLV..N..T.VQVVNV
27	XXVII	Yes	...ASHAGEE..VEKQ.MTS.V.ATDTFLSATQ.TMTPK.DV..S.QKVEVKVIA.KKEA..LVY....N.ST.VQVVNV
28[d]	XXVIII	Yes	.EAASHAGEE..VEKQ.MTS.V.ATDTFLSATQ.TMTPK.DV..S.QKVEVKVIA.KKEA..LVY....N.ST.VQVVNV
29[d]	XXIX	Yes	...E.A.HAGEE..VEKQ.MTS.V.ATDTFLSATQ.TMTPK.DV..S.QKVEVKVIA.KKEA..LVY....N.ST.VQVVNV
30[d]	XXX	Yes	...E.ASHAGEE..VEKQ.MTS.V.ATDTFLSATQ.TMTPK.DV..S.QKVEVKVIA.KKEA..VLVY...N.ST.VQVVNV
31[d]	XXXI	Yes	...E.ASHAGEE..VEKQ.MTS.V.ATDTFLSATQ.TMTPK.DV..S.QKVEVKVIA.KKEA..LVY.VN.ST.VQVVNV
32	XXXII	Yes	...E.ASHAGEE..VEKQ.MTS.V.ATDTFLSATQ.TMTPK.DV..S.QKVEVKVIA.KKEA..LVY....N.S.L.....
33[d]	XXXIII	No	............................T...............................D.........VLV...G..S....
34	XXXIV	Yes	...E.ASHAGEE..VEKQ.MTS.V.ATDTFLSATQ.TMTPK.DV..S.QKVEVKVIA.KKEA..LVY....N.S.......
35	XXXV	Semi	IGT.E...HAGE.K....MTS.V.ATDTFLSATQ.TMTPK.DV.................................I.....
36[d]	XXXVI	Yes	...VEKQ....E.N....AQ...SSK.S....K..V.VKVIA.KKEA.....LV....N..T.VQVVNV
37		Yes	...E.ASHAGEE..VEKQVMPS.V.TTDTFL.ATQ.TMTPK.DVSV.K..VEVKVIA.KKEASI.LVY...N.ST.VQVVNV
38		Semi	IGT.E...H....K.......E.N.....AQ....SSKL..SA.K..VEVKVIA.KKEA..LV....N..T...V......
39		Semi	..............................D.MTPK.DV..S.QKVEVKVIA.KKEA..LV....N..T...V......
40		No	....S...............................
41		No	.......HAGEE...D.........LV..G..S.......
42		Yes	...E.ASHAGEE..VEKQ.MTS.V.ATDTFLSATQ.TMTPK.DV..S.QKVEVKVIA.KKEA.PLVY..N.S........VNV
43		No	....D.........VLV..G..........
44		No	....TLV..G.....L.......
45		No	....D.........LV..G.N...VQVVNV
46		No	....D.........LV..G...V........

(Continued on next page)

TABLE 1 (Continued)

penA type by NG-STAR	penA type by Onishi et al.	Mosaic[b]	Amino acid profile
47		Yes	................MTS.V.ATDTFL.ATQ.TMTPK.DV..S.QKVEVKVIA.KKEA..LV....N.T....V..
48		No	..................................D...........................LV..G.N...GQVVNV
49		No	..................................D..V...............................TLV..G...
50		No	..................................D..................................LV..G...A..
51		Yes	...E.ASHAGEE..VEKQ.MTS.V.ATDTFLSATQ.TMTPK.DV..S.QKVEVKVIA.KKEA..LVY....N.S...NF.P
52		Yes	...E.ASHAGEE..VEKQ.MTS.V.ATDTFLSATQ.TMTPK.DV..S.QKVEVKVIA.KKEA..LVY....N.S....F.P
53		Yes	...E.ASHAGEE..VEKQ.MTS.V.ATDTFLSATQ.TMTPK.DV..S.QKVEVKVIA.KKEA..LVY....N.S.A....
54		No	...................................D...................VLV..G....A....
55		Yes	...E.ASHAGEE..VEKQ.MTS.V.ATDTFLSATQ.TMTPK.DV..S.QKVEVKVIA.KKEA..LVY....N.S....N...
56		No	...................................D...........................VLV..G.P.GG...F.P
57		No	...................................D...........................VLV..G....A..F.
58		Yes	...E.ASHAGEE..VEKQ.MTS.V.ATDTFLSATQ.TMTPK.DV..S.QKVEVKVIA.KKEA..LVY....N.S.A...P
59		Yes	...E.A.R...KK.V.QQVMTS.V.PTDTFL.ATQ.TMTPK.DV..S.QKVEVKVIA.KKEASI.LVY....N.ST.VQVVNV
60		Yes	...............VMTS.V.PTDTFL.ATQ.TMTPK.DV..S.QKVEVKVIA.KKEASI.LVY....N.ST.VQVVNV
61		No	..E...................D...............................LV..G....L...
62		Yes	IGT.E...H....K.........AQ....SSKL..SA.K...V..A..QE....LVY....N.T....V..
63		Yes	IGT...H...K.....MTS.V.ATDTFL.ATQ.TMTPK.DV..S.QKVEVKVIA.KKEA..LV....N.T....V..
64		Yes	...E.ASHAGEE......VMTS.V.PTDTFL.ATQ.TMTPK.DV..S.QKVEVKVIA.KKEASI.LVY....N.ST.VQVVNV

[a] *penA* types correspond to historical amino acid profiles that consisted of 85 of 576 total protein amino acid residues and include *penA* types I to XXXVI (9) and novel *penA* types 37 to 59.

[b] Semimosaic (Semi) structures correspond to *penA* sequences with alterations of either the first or second half of the gene only.

[c] The wild-type PBP2 profile from *N. gonorrhoeae* strain M32091 (9).

[d] PBP2 amino acid profiles awaiting submission to NG-STAR.

TABLE 2 Molecular antimicrobial resistance determinants present in NG-STAR *mtrR*, *porB*, *ponA*, *gyrA*, *parC*, and 23S rRNA alleles

Allele	Resistance determinant(s) for NG-STAR allele[a]					
	mtrR	*porB*	*ponA*	*gyrA*	*parC*	23S rRNA
0	WT	WT	WT	WT	WT	WT
1	−35A deletion	None	L421P	S91F, D95G	None	A2059G
2	−35A deletion	A121D	WT	S91F, D95N	None	C2611T
3	None	A121S	L421P	S91Y	S87R	None
4	None	G120D	L421P	None	D86N	None
5	None	G120D, A121D	L421P	S91F	S87R, S88P	None
6	None	G120D, A121N		None	None	None
7	None	G120K, A121D		S91F, D95A	None	C2611T
8	None	G120K, A121D		WT	S87N	None
9	A39T	G120R, A121D		S91F	D86N	None
10	A39T	G120K, A121D		None	None	None
11	A39T	G120K, A121N			D86N, S88P	
12	None	G120K, A121G			S87I	
13	None	*porB1a*			S87N	
14	None	*porB1a*			S87R	
15	None	A120K, A121V[b]			S87N	
16	−35A deletion	G120N			S87R	
17	None				None	
18	None				D86N	
19	−35A deletion, G45D				None	
20	A39T				S87N	
21	None				None	
22	−35A deletion					
23	A39T					
24	A39T					
25	G45D					
26	A39T					
27	*N. meningitidis*-like					
28	*N. meningitidis* -like					
29	None					
30	*N. meningitidis* -like					
31	None					
32	WHO-P-like					
33	None					
34	−35A deletion					
35	A39T					
36	−35A deletion					
37	−35A deletion, A39T,G45D					
38	A39T					
39	*N. meningitidis* -like					
40	A39T					
41	A39T					
42	A39T					
43	A39T					
44	*N. meningitidis* -like					
45	None					
46	*N. meningitidis* -like					
47	A39T					
48	None					
49	A39T					
50	None					
51	None					

[a]WT signifies the allelic wild-type DNA sequence lacking known antimicrobial resistance molecular markers.
[b]A 2-codon deletion results in a 2-amino-acid frameshift.

DISCUSSION

The emergence of extensively antimicrobial-resistant gonococcal infections together with the increased use of nucleic acid amplification tests for diagnosis have highlighted the need for rapid and standardized molecular-based methods to enhance surveillance of this disease. NG-STAR is a novel molecular typing scheme developed to facilitate the standardization and organization of the nomenclature of *N. gonorrhoeae* loci commonly associated with resistance to macrolides, cephalosporins, penicillins,

TABLE 3 Geographical distribution of NG-STAR sequence types

NG-STAR sequence type(s) (total no. of isolates)	No. of isolates				
	Brazil	Canada	China	Netherlands	USA
26 (15)		14			1
46 (2)		1			1
56 (2)	1	1			
57 (8)		7			1
58 (10)		9		1	
63 (16)		9		6	1
64 (44)		34		4	6
90 (100)	1	59		5	35
91 (44)	2	40			2
127 (3)		2		1	
139 (42)		36		3	3
158 (10)		6		4	
209 (1), 210 (1), 214 (1), 216 (1), 217 (1)	5				
199 (1), 200 (2), 201 (1), 202 (1), 203 (2), 204 (1), 221 (2)			10		
177 (2), 178 (2), 179 (1), 180 (2), 181 (1), 182 (3), 183 (1), 184 (1), 185 (3), 186 (1), 187 (1), 188 (1), 189 (1), 190 (2), 191 (1)				23	
192 (1), 193 (6), 194 (1), 195 (1), 196 (4), 197 (1), 198 (3), 205 (1), 206 (1), 207 (1), 208 (1), 211 (2), 212 (1), 213 (1), 215 (1), 218 (1), 219 (1), 220 (1), 222 (1), 223 (1)					31
Other (382)[a]		382			
Total[b]	9	600	10	47	81

[a]Including 138 sequence types and a total of 382 isolates.
[b]Excludes 21 NCBI reference strains.

and fluoroquinolones. NG-STAR is intended as an adjunct typing scheme to integrate antimicrobial resistance information with existing successful molecular epidemiological typing schemes such as MLST and NG-MAST. Although AMR profiles have been indirectly associated with particular NG-MAST genotypes, several problems exist, including the fact that AMR associations with NG-MAST genotypes are not fully definitive, rare or infrequent types may have little associated interpretative data available, and the approach requires continual data updating (16). The association of AMR and NG-MAST genotypes may also differ regionally, and it is important for each region to test the hypothesis that NG-MAST genotypes are predictive of AMR (30). Furthermore, susceptibility of *N. gonorrhoeae* to third-generation cephalosporins has been shown to be associated with specific combined *penA-mtrR-porB* mutation patterns (31). NG-STAR has the flexibility to directly associate resistance-determining mutations of the major genes with AMR profiles and to capture novel resistance mutations within these genes.

NG-STAR was validated with a wide selection of reference strains and internationally collected clinical isolates and produced a desirable high Simpson's diversity index value (32) comparable to that of NG-MAST and better than that of MLST. Specific NG-STAR STs are defined by mutations within antimicrobial resistance-determining alleles; therefore, each distinctive allelic profile is directly associated with a characteristic antimicrobial resistance profile. Each allele number relates to specific molecular antimicrobial resistance determinants, and consequently, each allelic profile will represent a composite of such determinants, creating an NG-STAR ST that should have a characteristic antimicrobial resistance phenotype. NG-STAR ST-90, ST-91, and ST-97 isolates possess mosaic

TABLE 4 NG-STAR sequence types of select *Neisseria gonorrhoeae* reference strains with MICs and molecular antimicrobial resistance determinant profiles

Strain[a]	NG-STAR type	Allelic profile (penA, mtrR, porB, ponA, gyrA, parC, 23S rRNA)[b]	MIC (μg/ml)[c] PEN	CRO	CIP	CFM	AZM	penA Type	Mosaic	mtrR −35A	A39	G45	MEN	porB G120	A121	ponA L421P	gyrA S91	D95	parC D86	S87	S88	23S rRNA A2059	C2611
ATCC 49226	1	22.001, 10, 13, 0, 0, 2, 0	1	0.016	0.004	0.016	0.5	XXII	No	WT	A39T	WT	WT	NA	NA	WT	WT	WT	WT	WT	WT	WT	WT
WHO-F	2	15.001, 14, 14, 0, 0, 7, 0	0.032	0.002	0.004	0.016	0.125	XV	No	WT	WT	WT	WT	NA	NA	WT	WT	WT	WT	WT	WT	WT	WT
WHO-G	3	2.001, 1, 13, 1, 5, 1, 0	0.5	0.008	0.125	0.016	0.25	II	No	Del	WT	WT	WT	NA	NA	L421P	S91F	WT	WT	S87R	WT	WT	WT
WHO-K	4	10.001, 19, 8, 1, 2, 5, 0	2	0.064	>32	0.5	0.25	X	Yes	Del	WT	G45D	WT	G120K	A121D	L421P	S91F	D95N	WT	S87R	S88P	WT	WT
WHO-L	5	7.001, 25, 8, 1, 2, 11, 0	2	0.125	>32	0.25	0.5	VII	No	WT	WT	G45D	WT	G120K	A121D	L421P	S91F	D95N	D86N	WT	S88P	WT	WT
WHO-M	6	2.001, 19, 8, 1, 1, 1, 0	8	0.012	2	0.016	0.25	II	No	Del	WT	G45D	WT	G120K	A121D	L421P	S91F	D95G	WT	WT	WT	WT	WT
WHO-N	7	2.001, 26, 13, 1, 1, 12, 0	8	0.004	4	0.016	0.125	II	No	WT	A39T	WT	WT	NA	NA	L421P	S91F	D95G	WT	S87I	WT	WT	WT
WHO-O	8	12.001, 1, 8, 1, 0, 7, 0	>32	0.032	0.008	0.016	0.25	XII	No	Del	WT	WT	WT	G120K	A121D	L421P	WT	WT	WT	WT	WT	WT	WT
WHO-P	9	2.001, 32, 2, 0, 0, 0, 0	0.25	0.004	0.004	0.016	2	II	No	WT	WT	WT	WT	WT	A121D	WT	WT	WT	WT	WT	WT	WT	WT
NCCP11945	10	5.003, 1, 12, 1, 1, 3, 0	ND	ND	ND	ND	ND	V	No	Del	WT	WT	WT	G120K	A121G	L421P	S91F	D95G	WT	S87R	WT	WT	WT
FA1090	11	1.002, 5, 0, 0, 0, 0, 0	ND	ND	ND	ND	ND	I	No	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
MS11	12	22.001, 10, 5, 1, 0, 1, 0	ND	ND	ND	ND	ND	XXII	No	WT	A39T	WT	WT	G120D	A121D	L421P	WT	WT	WT	WT	WT	WT	WT
FA19	13	15.001, 5, 14, 0, 0, 2, 0	ND	ND	ND	ND	ND	XV	No	WT	WT	WT	WT	NA	NA	WT	WT	WT	WT	WT	WT	WT	WT
FA6140	14	12.001, 22, 8, 1, 0, 1, 0	ND	ND	ND	ND	ND	XII	No	Del	WT	WT	WT	G120K	A121D	L421P	WT	WT	WT	WT	WT	WT	WT
35/02	15	10.001, 19, 8, 1, 5, 2, 0	ND	ND	ND	ND	ND	X	Yes	Del	WT	G45D	WT	G120K	A121D	L421P	S91F	D95G	WT	S87R	WT	WT	W
32867	30	12.001, 1, 8, 3, 1, 3, 1	4	0.125	32	0.063	≥512	XII	No	Del	WT	WT	WT	G120K	A121D	L421P	S91F	D95G	WT	S87R	WT	A2059G	WT
38202	64	2.001, 9, 1, 0, 0, 0, 2	0.25	0.008	0.004	0.016	8	II	No	WT	A39T	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	C2611T
WHO-U	224	2.001, 50, 1, 1, 0, 1, 2	0.125	0.002	0.004	<0.016	4	II	No	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	C2611T
WHO-V	225	5.002, 1, 8, 1, 1, 3, 1	>32	0.064	>32	<0.016	>256	V	No	Del	WT	WT	WT	G120K	A121D	L421P	S91F	D95G	WT	S87R	WT	A2059G	WT
WHO-W	4	10.001, 19, 8, 1, 2, 5, 0	4	0.064	>32	0.25	0.5	X	Yes	Del	WT	G45D	WT	G120K	A121D	L421P	S91F	D95N	WT	S87R	S88P	WT	WT
WHO-X	226	37.001, 1, 8, 1, 2, 5, 0	4	2	>32	4	0.5	37	Yes	Del	WT	WT	WT	G120K	A121D	L421P	S91F	D95N	WT	S87R	S88P	WT	WT
WHO-Y	16	42.001, 1, 11, 1, 1, 3, 0	1	1	>32	2	1	42	Yes	Del	WT	WT	WT	G120K	A121N	L421P	S91F	D95G	WT	S87R	WT	WT	WT
WHO-Z	227	64.001, 51, 8, 1, 2, 5, 0	2	0.5	>32	2	1	64	Yes	WT	WT	WT	WT	G120K	A121D	L421P	S91F	D95N	WT	S87R	S88P	WT	WT

[a] Strains corresponding to WHO-F to -Z were reported previously by Unemo et al. (38); strains NCCP11945 (GenBank accession number NC_011035.1), FA1090 (accession number NC_002946.2), MS11 (accession number NC_022240.1), FA19 (accession number NZ_CP012026.1), FA6140 (accession number NZ_CP012027.1), and 35/02 (accession number NZ_CP012028.1) were obtained from the NCBI; and strains 32867 and 38202 were clinical strains submitted to the National Microbiology Laboratory (NML), Winnipeg, Canada, for routine reference testing purposes.

[b] Alleles in the profile correspond to penA, mtrR, porB, ponA, gyrA, parC, and 23S rRNA sequences, respectively.

[c] PEN, penicillin; CRO, ceftriaxone; CIP, ciprofloxacin; CFM, cefixime; AZM, azithromycin. MIC values for NCCP11945, FA1090, MS11, FA19, FA6140, and 35/02 were not determined (ND) in this study.

[d] Presence of antimicrobial molecular resistance markers including the mtrR −35A promoter nucleotide deletion (Del) and *Neisseria meningitidis*-like mosaic sequences (MEN); molecular antimicrobial resistance determinants of mtrR, porB, ponA, gyrA, and parC; and nucleotide point mutations of 23S rRNA. WT signifies the wild-type DNA sequence lacking currently recognized antimicrobial resistance molecular markers. NA for porB molecular markers indicates the presence of porB1a alleles for which porB1b amino acid substitutions are not applicable.

**TABLE 5** Characterization of predominant NG-STAR sequence types

NG-STAR sequence type (no. of isolates)	% of isolates with MIC(μg/ml)[a]				Allelic profile (penA, mtrR, porB, ponA, gyrA, parC)[b]	penA type[c]	Resistance marker[d]													
							mtrR				porB		ponA	gyrA		parC			23S rRNA	
	CRO ≥0.63	CFM ≥0.125	AZM ≥2	CIP ≥1			−35A	A39	G45	MEN	G120	A121	L421	S91	D95	D86	S87	S88	A2059	C2611
26 (15)	57	29	21	100	12.001, 1, 8, 1, 1, 3, 0	XII	Del	WT	WT	WT	G120K	A121D	L421P	S91F	D95G	WT	S87R	WT	WT	WT
42 (45)	2	0	0	0	14.001, 10, 3, 0, 0, 1, 0	XIV	Del	WT	WT	WT	WT	A121S	WT	WT	WT	WT	WT	WT	WT	WT
43 (14)	0	0	0	0	14.001, 10, 3, 0, 0, 1, 6	XIV	Del	A39T	WT	WT	WT	A121S	WT	WT	WT	WT	WT	WT	WT	C2611T
58 (10)	0	0	90	0	2.001, 27, 1, 0, 0, 0, 0	II	WT	A39T	WT	MEN	WT	A121S	WT	WT	WT	WT	WT	WT	WT	WT
61 (17)	0	0	100	0	2.001, 5, 3, 0, 0, 0, 2	II	WT	A39T	WT	MEN	WT	A121S	WT	WT	WT	WT	WT	WT	WT	C2611T
63 (16)	0	0	0	0	2.001, 9, 1, 0, 0, 0, 0	II	WT	A39T	WT	WT	WT	A121S	WT	WT	WT	WT	WT	WT	WT	WT
64 (44)	2	2	98	0	2.001, 9, 1, 0, 0, 0, 2	II	WT	A39T	WT	WT	WT	A121S	WT	WT	WT	WT	WT	WT	WT	C2611T
79 (11)	0	2	91	0	2.001, 0, 3, 0, 0, 1, 2	XXII	WT	WT	WT	WT	WT	A121S	WT	WT	WT	WT	WT	WT	WT	C2611T
81 (10)	0	0	0	0	22.001, 0, 0, 0, 0, 2, 0	XXII	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	S87R	WT	WT	WT
90 (100)	84	94	4	97	22.002, 11, 0, 0, 0, 2, 0	XXII	Del	WT	WT	WT	G120K	A121N	WT	S91F	D95G	WT	S87R	WT	WT	WT
91 (45)	88	88	98	100	34.001, 1, 11, 1, 1, 3, 2	**XXXIV**	Del	WT	WT	WT	G120K	A121N	L421P	S91F	D95G	WT	S87R	WT	WT	C2611T
97 (27)	89	93	0	100	34.002, 1, 11, 1, 1, 3, 0	**XXXIV**	Del	WT	WT	WT	G120K	A121N	L421P	S91F	D95G	WT	S87R	WT	WT	WT
139 (42)	21	7	79	7	9.001, 1, 8, 1, 0, 2, 0	IX	Del	WT	WT	WT	G120K	A121D	L421P	WT	WT	WT	WT	WT	WT	WT
150 (11)	36	0	9	91	41.001, 1, 11, 1, 1, 3, 0	41	Del	WT	WT	WT	G120K	A121D	L421P	S91F	D95G	WT	S87R	WT	WT	WT
158 (10)	10	0	0	100	44.001, 16, 10, 1, 1, 10, 0	44	Del	WT	WT	WT	G120K	A121D	L421P	S91F	D95G	WT	S87R	WT	WT	WT
160 (22)	0	0	0	0	14.002, 41, 1, 0, 0, 7, 6	XIV	WT	WT	WT	WT	G120K	A121D	WT	WT	WT	WT	WT	WT	WT	WT

[a] Percentage of isolates with the indicated MICs for ceftriaxone (CRO), cefixime (CFM), azithromycin (AZM), and ciprofloxacin (CIP).

[b] Alleles in the profile correspond to penA, mtrR, porB, ponA, gyrA, parC, and 23S rRNA sequences, respectively.

[c] penA sequence types correspond to amino acid profiles as described previously by Ohnishi et al. (9). penA type XXXIV, in boldface type, is a mosaic allele.

[d] Presence of antimicrobial molecular resistance markers including the mtrR −35A promoter nucleotide deletion (Del) and Neisseria meningitidis-like mosaic sequences (MEN); molecular antimicrobial resistance determinants of mtrR, porB, ponA, gyrA, and parC; and nucleotide point mutations of 23S rRNA. WT signifies the wild-type DNA sequence lacking currently recognized antimicrobial resistance molecular markers.
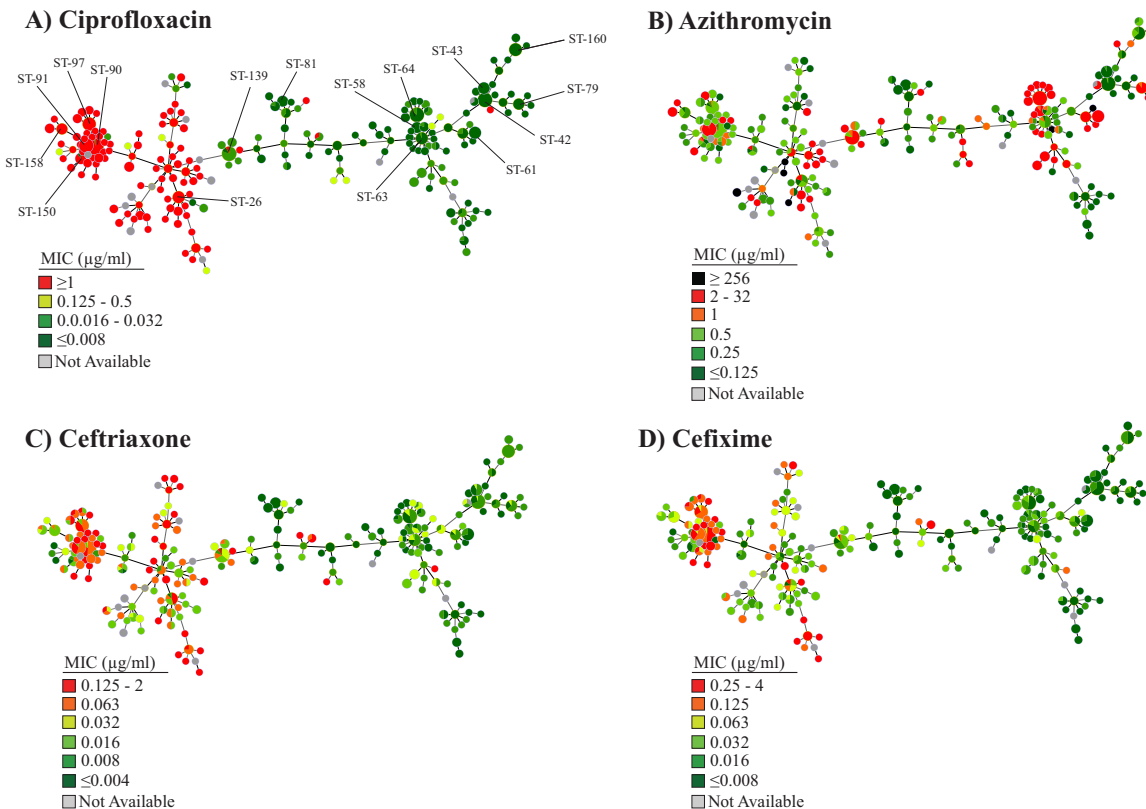
FIG 1 Antimicrobial resistance and genetic relatedness of 768 *Neisseria gonorrhoeae* isolates determined by goeBURST minimum spanning tree analysis of NG-STAR allelic profiles of seven antimicrobial resistance-related genes. Colored nodes indicate MICs of ciprofloxacin, azithromycin, ceftriaxone, and cefixime. The size of a node is proportional to the number of isolates, and branches are not to scale.

penA type XXXIV and *porB* mutations that cause substitutions in amino acids G120 and A121, and these STs exhibit the expected reduced susceptibility to extended-spectrum cephalosporins (3, 33, 34). Similarly reduced cephalosporin susceptibilities were also observed among NG-STAR STs with nonmosaic *penA* alleles with an A501V substitution, such as *penA* 43.001, 43.002, and 13.001. Nonmosaic *penA* type IX, XIII, XVII, or XVIII with combinations of the A501V, P551S, G542S, and/or P551S/L mutation has been shown to be associated with *N. gonorrhoeae* isolates with cefixime and ceftriaxone MICs of ≥0.03 μg/ml (31, 35). In addition to the cephalosporin resistance determinants, NG-STAR ST-91 also had 23S rRNA allele 23S-2 with a C2611T point mutation, which confers concurrent resistance to macrolides. High-level azithromycin resistance (MIC ≥ 256 μg/ml) is associated with NG-STAR ST-30, ST-36, ST-69, ST-192, ST-202, ST-223, and ST-225, all of which possess the 23S rRNA A2059G mutation of allele 23S-1. Previous molecular studies have shown that these isolates possessed the A2059G mutation in all 4 alleles (36). Although more than a single mutated 23S rRNA gene in the genome is required to confer elevated azithromycin MICs (28, 36), the acquisition of a single mutated allele facilitates the rapid acquisition of a full complement of mutated alleles under selective pressures (3, 29); therefore, the presence of a single mutation may be considered a precursor to full resistance, especially if inappropriate treatment adds to this selective pressure. It is for this reason that NG-STAR records the presence of a mutation, even if present in a single allele, as being sufficient to identify the potential for full azithromycin resistance and to recommend an alternative treatment. Azithromycin-resistant STs did not cluster to the same extent as those with decreased susceptibility to other antimicrobials in the minimum spanning tree (Fig. 1B), potentially reflecting the more sporadic, nonclonal nature of the acquisition of azithromycin resistance among many *N. gonorrhoeae* strains (3, 29, 34, 36), although some subsequent clonal dissemination has been documented (37).

A Web-based user-friendly typing tool based on the well-characterized antimicrobial resistance genes of *N. gonorrhoeae* was developed, where nucleotide sequences of *penA*, *mtrR*, *porB*, *ponA*, *gyrA*, *parC*, and 23S rRNA may be queried against curated allelic libraries employing a standardized nomenclature. Although NG-STAR provides a satisfactory discriminatory molecular typing scheme for *N. gonorrhoeae*, it would be more useful in combination with other epidemiological typing schemes such as NG-MAST to enhance the molecular surveillance of antimicrobial resistance. Future planned enhancements of NG-STAR include interrogation of whole-genome sequence data and improved analytical functionality such as built-in sequence alignment and protein translation tools and bulk querying mechanisms. NG-STAR will enable public health practitioners and researchers around the world to communicate in a common "molecular language" to facilitate improved responses and monitoring of emerging drug-resistant clones of *N. gonorrhoeae* and to provide an enhanced understanding of the dissemination, transmission, and dynamics of gonorrhea, which will inform public health interventions and reduce the burden of this disease.

MATERIALS AND METHODS

***N. gonorrhoeae* isolates and antimicrobial susceptibility testing.** This study examined 768 *N. gonorrhoeae* isolates, which included 600 isolates from Canada, 47 from the Netherlands, 31 from the United States, 10 from China, and 9 from Brazil. Additional international reference strains WHO-F, WHO-G, WHO-K, WHO-L, WHO-M, WHO-N, WHO-O, WHO-P, WHO-U, WHO-V, WHO-W, WHO-X, WHO-Y, and WHO-Z (38) and strains ATCC 49226, NCCP11945 (GenBank accession number NC_011035.1), FA1090 (accession number NC_002946.2), MS11 (accession number NC_022240.1), FA19 (accession number NZ_CP012026.1), FA6140 (accession number NZ_CP012027.1), and 35/02 (accession number NZ_CP012028.1) from the NCBI were also included. Fifty genomes downloaded from the CDC *Neisseria gonorrhoeae* Antimicrobial Resistance Bank (available at http://www.cdc.gov/drugresistance/resistance-bank/currently-available.html [accessed December 2016]) were included to provide a broader range of strains. The WHO and CDC *Neisseria gonorrhoeae* Antimicrobial Resistance Bank strains are representative of the known antimicrobial resistance determinants for *N. gonorrhoeae*.

Susceptibilities to azithromycin, ciprofloxacin, ceftriaxone, and cefixime (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) were determined by using the agar dilution method and quality control strains as previously described (39, 40).

NG-MAST, MLST, and NG-STAR typing. NG-MAST, MLST, and NG-STAR sequence typing was performed by using gene sequences extracted *in silico* from whole-genome sequencing (WGS) data and submitted to the NG-MAST (http://www.ng-mast.net/), *Neisseria* MLST (http://pubmlst.org/neisseria/), and NG-STAR (https://ngstar.canada.ca) websites to determine the respective STs. The relatedness of allelic profiles was visualized by using PHYLOViZ 2.0 (41, 42) to produce a full goeBURST minimum spanning tree. MLST and NG-STAR CCs were defined by a single-locus variation in allelic profiles from a founding ST, while an NG-MAST CC was determined by 5 or more single-nucleotide polymorphisms of concatenated and aligned *porB* and *tbpB* sequences.

NG-STAR software platform. The NG-STAR analysis platform was written in Perl with the Perl Catalyst Model-View-Controller (MVC) framework (http://www.catalystframework.org/). It uses the MariaDB database and Apache Web server software on main and backup servers. The system is available to the research community as a virtual machine image running the CentOS 7 operating system. The software is released under the Apache 2.0 open-source license (available at https://github.com/phac-nml/ngstar). The NG-STAR Web service is publicly available (https://ngstar.canada.ca).

NG-STAR takes the full set of user-submitted locus sequences (trimmed or untrimmed) and first looks for exact matches by using SQL queries. If exact matches are found for all loci, NG-STAR returns the allele type and corresponding NG-STAR type (sequence type) if it exists. If the allele type combination is not associated with an NG-STAR type, the system will report the allele types and give an option to submit the novel allele type combination for curation and possible inclusion in the NG-STAR allele schema. Submitted sequences without exact matches in the schema are aligned against its corresponding set of alleles by using nucleotide-level BLAST+ (43); high-scoring segment pairs above a similarity of 90% and any match length are reported as a partial match. If one or more of the submitted sequences have partial matches, the user is given an option to submit the sequence for curation, although the system will not report a sequence type. Novel alleles (full length) and profiles may be submitted for manual curation through a curator notification functionality, where the submitter provides the DNA sequence; trace file; contact information (e-mail); and, ideally, additional background information such as isolation date, clinical source, country of origin, and antimicrobial MICs.

NG-STAR loci. The DNA sequences of 7 genes commonly associated with resistance to β-lactam antimicrobials (e.g., cephalosporins and penicillins), macrolides, or fluoroquinolones (14) were used, including *penA*, *mtrR*, *porB*, *ponA*, *gyrA*, *parC*, and 23S rRNA. Primers recommended for PCR and conventional Sanger sequencing and amplicon sizes and fragment sizes used for typing are listed in Table 6.

NG-STAR uses the historical 82-amino-acid-based *penA* nomenclature developed by Ohnishi et al. (9) as a basis for allele numbering. This includes *penA* types I to XXXVIII (now *penA* types 1 to 38 in Arabic numerals) and sequentially adds novel types as they are submitted, beginning with *penA* type 39 (Table

TABLE 6 Gene targets and suggested primers utilized in the NG-STAR typing scheme

Target (reference)	Primer	Nucleotide sequence (5′–3′)	Size of PCR amplicon (bp)	Allele size in NG-STAR (bp)	Approximate size of full gene (bp)
penA (17)	PenA-A1 (forward)	CGGGCAATACCTTTATGGTGGAAC	669	1,746–1,752	1,746–1,752
	PenA-B1 (reverse)	AACCTTCCTGACCTTTGCCGTC			
	PenA-A2 (forward)	AAAACGCCATTACCCGATGGG	581		
	PenA-B2 (reverse)	TAATGCCGCGCACATCCAAAG			
	PenA-A3 (forward)	GCCGTAACCGATATGATCGA	863		
	PenA-B3 (reverse)	CGTTGATACTCGGATTAAGACG			
	PenA-A4 (forward)	AATTGAGCCTGCTGCAATTGGC			
mtrR (18)	MTR1 (forward)	AACAGGCATTCTTATTTCAG	916	698–708 (includes promoter region)	Gene, 633; promoter, 251
	MTR2 (reverse)	TTAGAAGAATGCTTTGTGTC			
ponA (26)	ponA1-f (forward)	CGCGGTGCGGAAAACTATATCGAT	1,240	75	2,397
	ponA1-r (reverse)	AGCCCGGATCGGTTACCATACGTT			
porB1b (22)	por-NGMAST-F (forward)	CAAGAAGACCTCGGCAA	737	30	1,047
	por-NGMAST-R (reverse)	CCGACAACCACTTGGT			
gyrA (27)	GYRA-1 (forward)	AACCCTGCCCGTCAGCCTTGA	270	264	2,751
	GYRA-2 (reverse)	GGACGAGCCGTTGACGAGCAG			
parC (47)	parC F (forward)	GTTTCAGACGGCCAAAAGCC	332	332	2,304
	parC R (reverse)	GGCATAAAATCCACCGTCCCC			
23S rRNA (28)	gonrRNAF (forward)	ACGAATGGCGTAACGATGGCCACA	712	567	2,890
	gonrRNAR2 (reverse)	TTCGTCCACTCCGGTCCTCTCGTA			

1). When a novel amino acid substitution profile is detected, a sequential whole number is assigned to the allele. Novel whole-gene DNA sequences with synonymous mutations coding for a preexisting *penA* type will be indicated by an incremental decimal number. For example, the 12th unique whole-gene DNA sequence for the historical *penA* type XXXIV amino acid profile would be assigned allele number 34.012. For the remainder of the loci, each unique sequence is assigned a sequential allele number. NG-STAR *mtrR* alleles include a portion of the promoter starting approximately 66 to 68 bp before the ATG start codon of the coding region and extending the length of *mtrR*. To limit a potentially very large number of alleles of the hypervariable *porB* gene, a small 60-bp segment is used in NG-STAR that includes the G120- and A121-encoding region associated with antimicrobial resistance. Although the G120/A121 mutations apply only to *porB1b*, due to the high level of homology of *porB1a* sequences, and for enhanced typing purposes, both genes are included in NG-STAR. NG-STAR uses a 75-bp portion of *ponA* spanning amino acid L421; a 264-bp portion of *gyrA* spanning amino acids S91 and D95; and a 332-bp portion of *parC* spanning amino acids D86, S87, and S88. 23S rRNA mutations of interest include A2059G and/or C2611T (*E. coli* coordinates, corresponding to A2045G and C2597T, respectively, of *N. gonorrhoeae* NCCP11945 [GenBank accession number NC_011035.1]). Of the four 23S rRNA alleles present in the *N. gonorrhoeae* genome, only a single 567-nucleotide sequence is analyzed by NG-STAR. If no mutated alleles are present (wild type), any one of the alleles may be submitted; however, if a mutation is present in any one of the alleles, the sequence with a mutation is submitted, even if it is in the minority. The combination of allele numbers for all 7 genes (allelic profile) corresponds to a unique NG-STAR type.

Information for a representative reference strain for each allele and NG-STAR type, such as the level of antimicrobial resistance and relevant clinical and epidemiological data, is available. Allelic queries with sequences longer than those stored in the library are automatically trimmed to give an exact allele type match if present in the database, whereas shorter sequences will return possible allele type matches (hits), and users are encouraged to submit a longer DNA sequence to accurately identify an allele. An NG-STAR type is returned only with a complete allelic profile generated from exact allelic matches. Novel alleles (full-length queries containing mismatches) and profiles may be submitted through curator notification functionality, where the submitter provides the DNA sequence(s); contact information (e-mail); and, ideally, additional background information such as isolation date, clinical source, country of origin, epidemiological background information, and antimicrobial MICs. Curator comments for each allele and NG-STAR type will be presented, which may include characteristic mutations or other items of interest for that allele.

Statistical comparisons and calculations. The measure of association was determined by using χ^2 or Fisher's exact test using OpenEpi version 3.01 (44). Two-tailed differences with *P* values of <0.05 at a 95% CI were considered statistically significant. The discriminatory power of the NG-STAR, NG-MAST, and MLST schemes was calculated by using Simpson's diversity index (32, 45), and congruence of the methods was determined by using the adjusted Wallace coefficient (46) (calculated by using the online tool available at http://comparingpartitions.info).

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