# Genomic Epidemiology of Azithromycin-Nonsusceptible *Neisseria gonorrhoeae*, Argentina, 2005–2019

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Azithromycin-nonsusceptible *Neisseria gonorrhoeae* strains are an emerging global public health threat. During 2015–2018, the prevalence of azithromycin-nonsusceptible gonococcal infection increased significantly in Argentina. To investigate the genomic epidemiology and resistance mechanisms of these strains, we sequenced 96 nonsusceptible isolates collected in Argentina during 2005–2019. Phylogenomic analysis revealed 2 main clades, which were characterized by a limited geographic distribution, circulating during January 2015–November 2019. These clades included the internationally spreading multilocus sequence types (STs) 1580 and 9363. The ST1580 isolates, which had MICs of 2–4 µg/mL, had mutations in the 23S rRNA. The ST9363 isolates, which had MICs of 2–4 or ≥256 µg/mL, had mutations in the 23S rRNA, a mosaic *mtr* locus, or both. Identifying the geographic dissemination and characteristics of these predominant clones will guide public health policies to control the spread of azithromycin-nonsusceptible *N. gonorrhoeae* in Argentina.

Gonorrhea, caused by infection with the bacterium *Neisseria gonorrhoeae*, is the second most prevalent bacterial sexually transmitted infection (STI) globally (*1*,*2*). The World Health Organization (WHO) estimated that in 2016, a total of 86.9 million incident gonorrhea cases occurred among persons 15–49 years of age, including 13.8 million cases in the WHO Region of the Americas (*1*). Researchers have documented antimicrobial resistance (AMR) to all drugs used to treat gonorrhea (*2*,*3*). Ceftriaxone, an extended-spectrum cephalosporin, is the last option for first-line empirical treatment, but the emergence of ceftriaxone resistance has raised concerns about future treatments (*2*,*4*). Consequently, WHO guidelines and national guidelines of many countries now recommend a combination of ceftriaxone (250 mg–1 g) and azithromycin (1–2 g) as first-line treatment for uncomplicated gonorrhea (*5*,*6*). However, in 2016 Fifer et al. (*7*) reported the failure of dual therapy. Two years later, a gonococcal strain with ceftriaxone resistance and high-level azithromycin resistance was isolated in Australia and England (*8*–*10*). In recent years, international reports of azithromycin-resistant *N. gonorrhoeae* have substantially increased (*2*,*3*,*11*,*12*). The WHO Global Gonococcal Antimicrobial Surveillance Program showed that in 2016, a total of 48.4% of reporting countries had an >5% increase in rates of azithromycin resistance (*3*).

Argentina has reported low azithromycin resistance levels since the early 2000s (*13*). In Argentina, the proportion of azithromycin-nonsusceptible isolates (i.e., requiring MICs >1 µg/mL) increased from 0.1% in 2015 to 4.3% in 2018 ($p<0.01$) (*14*). The Clinical and Laboratory Standards Institute currently states a susceptible-only breakpoint for azithromycin (*15*); for simplicity, we refer to these isolates as resistant. High-level azithromycin-resistant isolates requiring MICs ≥256 µg/mL have emerged in several countries, including Argentina (*16*–*20*). Azithromycin resistance threatens the effectiveness of dual antimicrobial gonorrhea treatment.

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[1]Members of this group are listed at the end of the article.

Whole-genome sequencing (WGS) provides higher resolution and accuracy than other typing methods, making it an ideal method to study the dissemination and transmission dynamics of *N. gonorrhoeae* strains on a national and international level (*21*,*22*). Furthermore, WGS data offer insights into AMR determinants, thereby enabling prediction, enhanced detection, and characterization of high-risk clones (*22*,*23*). Several studies have found *N. gonorrhoeae* lineages and clones driving AMR transmission among *N. gonorrhoeae* strains within local, national, and international networks (*16*,*17*,*24*–*26*). Genomic surveillance provides information on current and emerging trends of circulating strains. Phenotypic, epidemiologic, and genomic surveillance data are critical for designing public health interventions and treatment strategies. Genomic approaches, including molecular epidemiology and detection of AMR determinants, are crucial for monitoring resistance to first-line drugs. We examined the genomic background of azithromycin-resistant *N. gonorrhoeae* isolates with MICs ≥2 µg/mL collected throughout Argentina during 2005–2019.

## Materials and Methods

We examined 96 azithromycin-resistant *N. gonorrhoeae* isolates (MICs ≥2 µg/mL) from male and female patients treated at STI hospitals throughout Argentina. We selected 95 isolates from 8,002 consecutive isolates collected through the Gonococcal Antimicrobial Susceptibility Surveillance Programme—Argentina during January 2005–November 2019; we also included an isolate with high-level azithromycin resistance cultured in 2001 (*20*). We confirmed the *N. gonorrhoeae* species by culture on selective agar media, microscopic analysis using Gram staining, rapid oxidase positivity, superoxol test, carbohydrate utilization test, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (microflex LT/SH; Bruker Daltonik, https://www.bruker.com) (*27*). The study was approved by the Research Ethics Committee of the Hospital General de Agudos "Bernardino Rivadavia" (Buenos Aires, Argentina). MIC determinations and whole-genome sequencing for all isolates were conducted using methods previously described (Appendix, https://wwwnc.cdc.gov/EID/article/27/9/20-4843-App1.pdf).

### WGS Analysis

We identified AMR determinants (i.e., the *mtrR*-35A, $mtr_{120}$, and mosaic *N. meningitidis*–like *mtrR* mutations) in addition to the MtrR A39T and G45D amino acid mutations in silico from WGS data, as described (*26*,*28*). We aligned and compared the *mtr* locus and *rplD*, *rplV*, and *macAB* sequences with the *N. gonorrhoeae* FA1090 reference genome (GenBank accession no. AE004969). To identify the frequency of 23S rRNA A2059G and C2611T mutations (named using *Escherichia coli* numbering), we mapped sequence reads against a single copy of the FA1090 23S rRNA gene using Burrow-Wheeler Aligner version 0.7.17 (http://bio-bwa.sourceforge.net) with the default settings. We determined base counts using a custom script, enabling the estimation of the proportion of copies with the A2059G, C2611T, or both mutations. We examined additional macrolide resistance genes (e.g., *ereA*, *ereB*, *ermA*, *ermB*, *mefA*, *mefB*, *msrA*, and *msrC*) using ARIBA version 2.14.4 and the ResFinder (https://cge.cbs.dtu.dk/services/ResFinder) and CARD (https://card.mcmaster.ca) databases (*29*). We identified alleles in silico from WGS data using *N. gonorrhoeae* multiantigen sequence typing (NG-MAST), multilocus sequence typing (MLST), and *N. gonorrhoeae* sequence typing for antimicrobial resistance (NG-STAR). We used the MLST (https://pubmlst.org/neisseria), NG-MAST (http://www.ng-mast.net), and NG-STAR (https://ngstar.canada.ca) databases to assign allele numbers and sequence types (ST)s (*30*,*31*). We grouped closely related NG-MAST STs using a previously described genogroup definition (*28*).

For phylogenetic analysis, we identified single-nucleotide polymorphisms (SNPs) in sequence reads mapped against the WHO P reference genome using the variant calling tool Snippy version 4.4.5 (https://github.com/tseemann/snippy). We identified and filtered recombinant regions using Gubbins version 2.1.0 (Sanger, https://sanger-pathogens.github.io/gubbins); the resulting core SNP alignment consisted of 9,415 sites. We used IQ-tree version 1.6.1 (http://www.iqtree.org) to infer a maximum-likelihood tree from the whole-genome SNP alignment with a generalized time-reversible model of evolution using gamma correction for among-site rate variation with 4 rate categories; branch support was estimated by bootstrap analysis of 10,000 replicates (*32*). We visualized the resulting phylogeny with Figtree version 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree) and phandango (*33*). We clustered sequences using RAMI with a branch length threshold of 0.01 (*34*). For comparison, we selected international isolates and publicly available genomic data on the basis of MICs, MLST STs (i.e., ST9363 and ST1580), and NG-MAST genogroups (i.e., G470 and G12302) from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov), European Molecular Biology

Laboratory (https://www.embl.org), and the DNA Data Bank of Japan (https://www.ddbj.nig.ac.jp). We found 17 genomes from the United Kingdom, 3 from Canada, 3 from Scotland, 17 from Australia, 28 from the United States, 7 from Brazil, and 11 from Norway (*16*,*17*,*24*,*25*,*35*–*37*). We generated a phylogenetic tree of 86 international and 96 isolates from Argentina as described for domestic isolates and visualized the tree in Figtree version 1.4.4. Sequence reads are available from the European Nucleotide Archive (accession no. PRJEB41007).

## Results

### Patient Data

The 96 *N. gonorrhoeae* isolates were collected from male (90.6%) and female (6.3%) patients; sex was unreported for 3.1% of patients. Patient age was reported for 88 (91.7%) isolates. Patients were 4–47 years of age (mean 24.3 years of age); 79.5% were <30 years of age. In total, 72 isolates were cultured from the urethra, 11 from urine, 3 from the cervix, 2 from the vagina (in children 4 and 6 years of age), 1 from the pharynx, and 7 from an unreported site.

The isolates were collected in 7/24 provinces. Among these, Córdoba and Ciudad Autónoma de Buenos Aires (CABA), 2 of the most populated provinces in Argentina, had the highest percentage of isolates (Córdoba had 47.9%; CABA had 39.6%) (Figure 1). We observed a lower percentage of isolates from the provinces of Buenos Aires (5.2%), Rio Negro (3.1%), Neuquén (2.1%), La Pampa (1.0%), and Santa Fe (1.0%).

### Antimicrobial Susceptibility of *N. gonorrhoeae* Isolates

Overall, 78 (81.3%) isolates had azithromycin MICs of 2–4 µg/mL, 13 (13.5%) had MICs of 8–16 µg/mL, and 5 (5.2%) had MICs of ≥256 µg/mL (Table 1). Among 5 isolates with MICs ≥256 µg/mL, 3 were collected in CABA in 2001 (n = 1) and 2019 (n = 2); the other 2 isolates were collected in Buenos Aires in 2018 and Córdoba in 2019. All 96 azithromycin-resistant isolates were susceptible to ceftriaxone, cefixime, and spectinomycin. However, 2 isolates collected in 2016 from Córdoba, each had a MIC of 4 µg/mL, showed decreased susceptibility to ceftriaxone (MIC = 0.06 µg/mL) and cefixime (MIC = 0.125 µg/mL) (Table 2).

### Molecular AMR Determinants

All 5 isolates with MICs of ≥256 µg/mL had the A2059G mutation in all 4 23S rRNA alleles, whereas none of the 91 isolates with MICs of 2–16 µg/mL had this SNP (Table 1). Most (72; 75%) isolates with MICs of 2–16 µg/mL had the 23S rRNA C2611T mutation. Nearly all (70; 97.2%) of these isolates had the C2611T mutation in all 4 23S rRNA alleles, except for 2 isolates: 1 with a single mutated allele that had a MIC of 4 µg/mL and 1 with 3 mutated alleles that had a MIC of 8 µg/mL. Interspecies mosaics in the *mtr* locus (which encodes the tripartite MtrCDE efflux pump), as well as mutations in the *mtrR* promoter, coding region, or both, have been associated with increased azithromycin MICs (*38*–*40*). Among the 80 (83.3%) isolates with *mtrR* mutations, 17 (17.7%) had an *mtrR*-35A promoter deletion, 44 (45.8%) had an MtrR G45D amino acid mutation, 1 (1.0%) had an *mtrR*-35A deletion and MtrR G45D substitution, and 18 (18.8%) had a mosaic *N. meningitidis*–like *mtrR* promoter. We did not identify any isolates with the $mtr_{120}$ mutation. Eighteen isolates, all of which had MICs of 2–4 µg/mL, had no 23S rRNA mutations; however, 13 contained a mosaic *mtrR* promoter and 5 had a *mtrR*-35A deletion. Among 18 isolates with mosaic *mtrR* promoters, 100% also had mosaic sequences in the *mtrD*, 100% in *mtrC*, and 94.4% in *mtrE* loci. Fifteen isolates with a mosaic *mtrD* allele had sequences identical to the *N. meningitidis*–like mosaic previously described (*39*,*40*); 2 isolates had sequences sharing 97.8% identity and 1 had a sequence sharing 97.3% identity with the *N. meningitidis*–like mosaic (Appendix Figure 1). Isolates containing a mosaic-like *mtr* locus had MICs of ≥2 to ≥256 µg/mL. Isolates with MICs of ≥256 µg/mL also contained the 23S rRNA A2059G mutation.

We did not find any mutations associated with macrolide resistance in the *rplD* gene, which encodes ribosomal protein L4, or the *rplV* gene, which encodes ribosomal protein L22 (*23*). In addition, we did not find AMR mutations in *macAB*, which encodes the MacA-MacB efflux pump, or the acquired macrolide resistance genes, *ere*, *mef*, *erm*, *mph*, and *msr* (*38*). An isolate that had a MIC of 2 µg/mL had an unclear resistance mechanism.

### Molecular Epidemiology and Phylogenomic Analysis

Among the 96 *N. gonorrhoeae* isolates, we observed 42 NG-MAST STs, including 21 new STs and 25 STs represented by single isolates. We found 24 isolates belonging to ST470, 7 belonging to ST20102, 6 belonging to ST696, 4 belonging to ST12302, and 4 belonging to ST20104. We found 3 NG-MAST genogroups comprising ≥3 isolates: 33 belonged to G470, 10 belonged to G12302, and 10 belonged to G20102. We also documented 14 MLST STs, including 2 new STs and 8 STs represented by single isolates. We found 43 isolates belonging to ST1580, 14 belonging to ST1901, 14 belonging to ST9363, and 10 belonging to ST1584. NG-STAR showed 32 types, of which 11 were new and 20 were represented by single isolates.

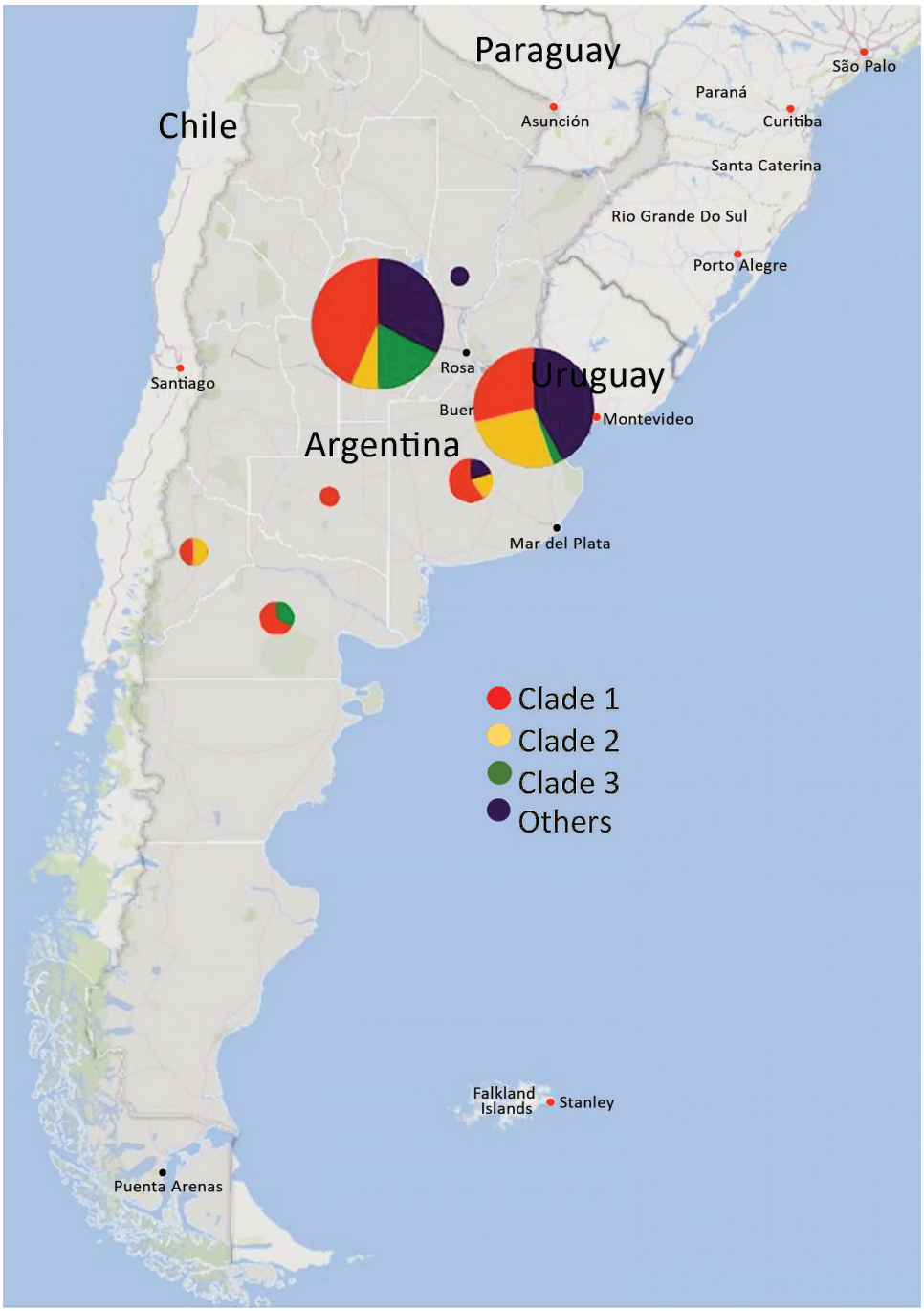


**Figure 1.** Geographic distribution of *Neisseria gonorrhoeae* isolates with azithromycin MICs of ≥2 µg/mL, Argentina, January 2005–November 2019. Circle size corresponds to the number of isolates in each location. Circle colors indicate the proportion of isolates belonging to the 3 main genomic clades compared with other clades.

We found 32 isolates belonging to NG-STAR type 1038, 10 belonging to type 179, 5 belonging to type 168, and 5 belonging to type 3200.

Analysis of the phylogenomic tree revealed 14 clades. In total, 63 (65.6%) isolates were grouped into 3 clades, each containing 10–38 isolates (Figure 2) (https://microreact.org/project/AZM_Project/006b822d). The remaining 33 isolates were singletons or belonged to smaller clonal groups of 2–6 isolates each.

Clade 1 comprised 38 isolates, most of which belonged to NG-MAST G470 (86.8%), MLST ST1580 (97.4%), or NG-STAR ST1038 (84.2%). Clade 1 isolates had mean SNP difference of 8.5 (range 0–39). The isolates required MICs of 2–16 µg/mL; most (76.3%; 29/38) required an MIC of 4 µg/mL. The oldest isolate in clade 1 was identified in CABA in 2013. The proportion of clade 1 isolates increased significantly from 1.0% (1/96) in 2013 to 11.4% (11/96) in 2019 ($p<0.05$). Clade 1 was

**Table 1.** Characteristics of 96 azithromycin-resistant *Neisseria gonorrhoeae* isolates, Argentina, January 2005–November 2019*

| Characteristics | MICs for azithromycin, μg/mL | | |
|---|---|---|---|
| | 2–4 | 8–16 | ≥256 |
| Total | 78 | 13 | 5 |
| Province | Buenos Aires, CABA, Córdoba, Neuquén, La Pampa, Río Negro, Santa Fe | CABA, Córdoba | Buenos Aires, CABA, Córdoba |
| Resistance determinants | | | |
| 23S rRNA (no. mutated alleles; total no. isolates) | C2611T (4; 58); C2611T (1; 1) | C2611T (4; 12); C2611T (3; 1) | A2059G (4; 5) |
| MtrR protein (no. isolates) | A-deletion (12)†; *N. meningitidis*–like (14); G45D (41); $mtr_{120}$ (0)‡ | A-deletion (6); G45D (3); $mtr_{120}$ (0) | *N. meningitidis*–like (4); G45D (1); $mtr_{120}$ (0) |
| Mosaic *mtr* locus (no. isolates) | *mtrC* (14); *mtrD* (14); *mtrE* (13) | *mtrC* (0); *mtrD* (0); *mtrE* (0) | *mtrC* (4); *mtrD* (4); *mtrE* (4) |
| ST | | | |
| *N. gonorrhoeae* multiantigen sequence typing (no. isolates) | ST470 (23); ST20102 (7); ST696 (4); ST12302 (4); ST11062 (3); other STs (37) | ST18761 (3); ST20104 (3); singleton STs (7) | ST3935 (2); ST20106 (2); ST696 (1) |
| Multilocus sequence typing (no. isolates) | ST1580 (39); ST1584 (10); ST9363 (10); ST1901 (8); other STs (11) | ST1901 (6); ST1580 (3); ST13844 (3); ST13594 (1) | ST9363 (4); ST1580 (1) |
| *N. gonorrhoeae* sequence typing for antimicrobial resistance (no. isolates) | ST1038 (30); ST179 (10); ST168 (5); ST3200 (4); other STs (29) | ST27 (4); ST2728 (3); ST1038 (2); singleton STs (4) | ST1993 (2); ST2906 (1); ST3194 (1); ST3199 (1) |

*CABA, Ciudad Autónoma de Buenos Aires; ST, sequence type.
†Deletion of A in 13-bp inverted repeat sequence of the *mtrR* gene.
‡C-to-T transition mutation 120 bp upstream of the *mtrC* start codon.

dominated by isolates from Córdoba (52.6%; 20/38) and CABA (28.9%; 11/38) but also included isolates obtained in 4 additional provinces. In total, 92.1% of the clade 1 isolates were from male patients and 7.9% were from female patients. Clade 1 isolates were characterized by the 23S rRNA C2611T mutation in all 4 alleles and the MtrR G45D amino acid mutation.

Clade 2 comprised 15 isolates that mainly belonged to NG-MAST G12302 (66.7%) and MLST ST9363 (93.3%). Clade 2 isolates had a mean SNP difference of 13.1 (range 0–33). All clade 2 isolates were cultured from men. Most (73.3%; 11/15) required an MIC of 2 μg/mL, and 26.7% (4/15) required MICs of ≥256 μg/mL. The first clade 2 isolate was detected in Córdoba in 2016; during 2017–2019, isolates were mainly detected in CABA (71.4%; 10/14), except for 2 isolates detected in Córdoba, 1 in Neuquén, and 1 in Buenos Aires. Clade 2 isolates did not have the 23S rRNA C2611T mutation but possessed the mosaic *mtrR* promoter and *mtrCDE* locus. In addition, isolates requiring MICs of ≥256 μg/mL had the 23S rRNA A2059G mutation in all 4 alleles.

Clade 3 was composed of 10 isolates belonging to NG-MAST G20102 and MLST ST1584. Clade 3 isolates had a mean SNP difference of 1.1 (range 0–2). Eight isolates were collected in Córdoba, 1 in CABA, and 1 in Río Negro during 2017–2019; of these, 8 were from men. All isolates required an MIC of 4 μg/mL and possessed the 23S rRNA C2611T mutation in all 4 alleles.

To investigate the international context of the 2 major MLST STs in Argentina, including azithromycin-resistant ST1580 and ST9363, we conducted a phylogenomic analysis using SNPs (Figure 3) (https://microreact.org/project/AZM_Project_2/7a2032e2). The ST1580 isolates from Argentina clustered with isolates from the United States, the United Kingdom (particularly Scotland), Australia, and Brazil. The mean pairwise SNP differences between ST1580

**Table 2.** Antimicrobial susceptibility of 96 azithromycin-resistant *Neisseria gonorrhoeae* isolates, Argentina, January 2005–November 2019*

| Antimicrobial drug | Azithromycin MICs, μg/mL (no. isolates) | | | | | |
|---|---|---|---|---|---|---|
| | 2–16 (91) | | | | ≥256 (5) | |
| | $MIC_{50}$ | $MIC_{90}$ | Range | Resistance, % | MIC | Resistance, % |
| Ciprofloxacin | 0.004 | 16 | 0.001–32 | 28.6 | 0.002–4 | 20 |
| Tetracycline | 1 | 2 | 0.125–4 | 25.3 | 0.5–2 | 20 |
| Benzylpenicillin | 1 | 2 | 0.25–8 | 14.3 | 0.5–1 | 0 |
| Ceftriaxone | 0.004 | 0.03 | 0.002–0.06 | 0 | 0.004–0.016 | 0 |
| Cefixime | 0.016 | 0.03 | 0.004–0.125 | 0 | 0.008–0.03 | 0 |
| Spectinomycin | 32 | 32 | 16–32 | 0 | 32 | 0 |
| Gentamicin | 8 | 8 | 4–16 | 0 | 8–16 | 0 |

*$MIC_{50}$, MIC for 50% of isolates; $MIC_{90}$, MIC for 90% of isolates.

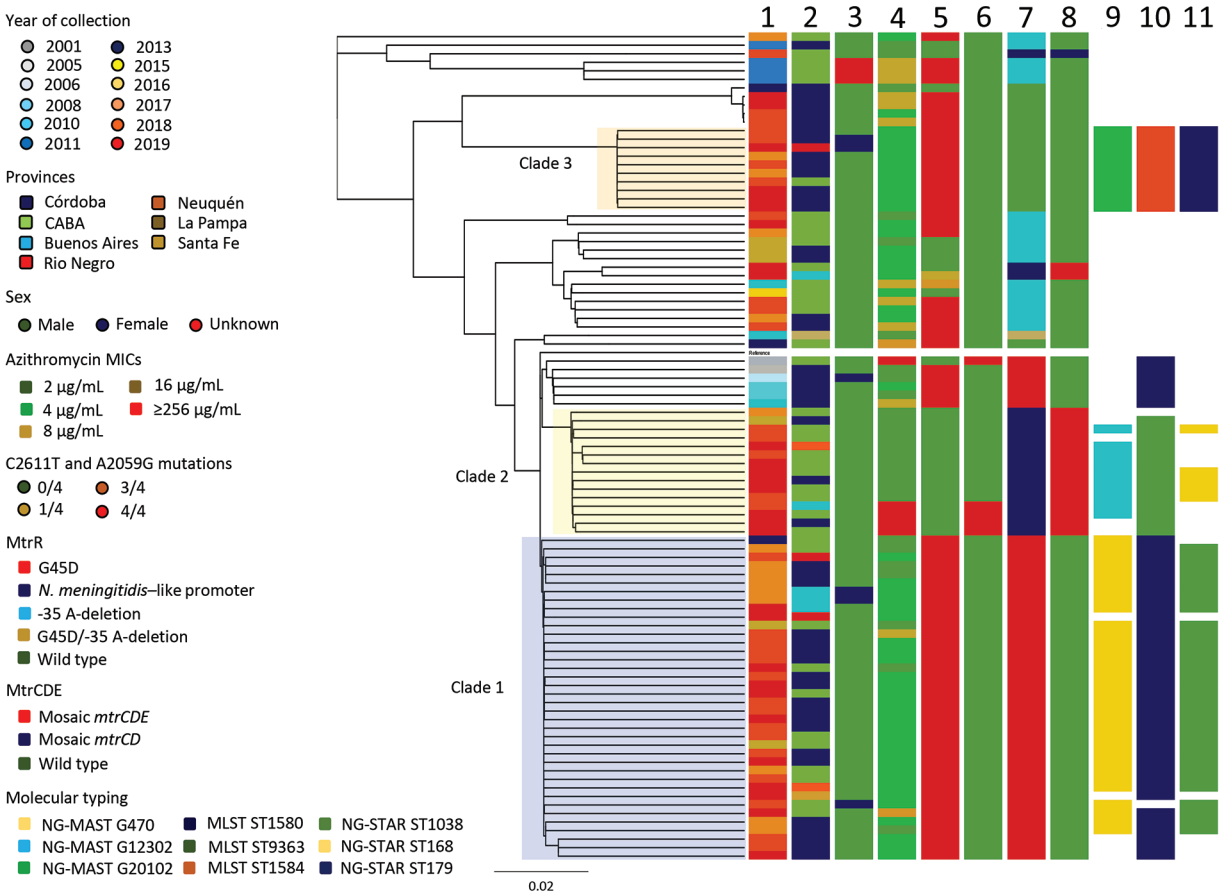


**Figure 2.** Phylogenomic tree of 96 *Neisseria gonorrhoeae* isolates with azithromycin MICs of ≥2 µg/mL, Argentina, January 2005–November 2019. Lane 1, year; lane 2, province; lane 3, sex; lane 4, azithromycin MICs; lane 5, 23S C2611T; lane 6, 23S A25059G; lane 7, MtrR; lane 8, MtrCDE; lane 9, NG-MAST; lane 10, MLST; lane 11, NG-STAR. Scale bar indicates substitutions per site. CABA, Ciudad Autónoma de Buenos Aires; MLST, multilocus sequence typing; NG-MAST, *N. gonorrhoeae* multiantigen sequence typing; NG-STAR, *N. gonorrhoeae* sequence typing for antimicrobial resistance; ST, sequence type.

isolates from Argentina and other countries were 6.8 (range 1–23) for the isolates from the United States, 6.9 (range 1–22) for isolates from Australia, 7.9 (range 4–22) for isolates from Scotland, 11.4 (range 4–28) for isolates from Brazil, and 16.8 (range 13–31) for isolates from the United Kingdom (excluding Scotland). Isolates from Scotland and the United Kingdom had MICs of ≥256 µg/mL whereas isolates from the United States, Australia, and Brazil had MICs of 2–8 µg/mL. All isolates with MICs of 2–8 µg/mL had the 23S rRNA C2611T mutation and all isolates with MICs of ≥256 µg/mL had the A2059G mutation. In addition, 2 isolates from Brazil had mosaic *mtrD* alleles, but no mutations in the 23S rRNA gene; these isolates had MICs of 2 µg/mL. The ST9363 isolates from Argentina clustered with other ST9363 isolates from the United States, Australia, Canada, Brazil, and Norway. ST9363 isolates from Argentina had a mean pairwise SNP difference of 7.7 (range 0–20) with isolates from Brazil, 10.1 (range 1–23) with isolates from Norway, 12.5 (range 5–25) with isolates from Canada, 13.1 ( range 2–42) with isolates from the United States, and 14.8 (range 2–35) with isolates from Australia. All isolates had mosaic *mtrR* promoters and *mtrD* alleles. All isolates with MICs of ≥256 µg/mL had the 23S rRNA A2059G mutation and 4 isolates with MICs of 8–16 µg/mL had the 23S rRNA C2611T mutation.

## Conclusion

We characterized the genomes of azithromycin-resistant *N. gonorrhoeae* isolates collected in Argentina during 2005–2019. Phylogenomic analysis showed that isolates from Argentina clustered into distinct clades, including 3 clades comprising 63 (65.6%) isolates collected during 2016–2019. All isolates also were

resistant to benzylpenicillin, tetracycline, ciprofloxacin or some combination, but susceptible to ceftriaxone and cefixime.

In Argentina, dual therapy is recommended as first-line treatment for uncomplicated gonorrhea, according to the WHO guidelines (*6*). The Argentine Ministry of Health and the Sociedad Argentina de Infectología recommend a single 1-g dose of azithromycin monotherapy for the treatment of *Chlamydia trachomatis* and *Mycoplasma genitalium* infections (*41*,*42*). These guidelines also recommend antimicrobial treatment for suspected infections. Azithromycin has a long half-life, resulting in detectable drug concentrations in human plasma for up to 14 days (*43*). Undiagnosed *N. gonorrhoeae* infections concurrent with the treatment of *C. trachomatis* and *M. genitalium* infections might lead to prolonged exposure to subinhibitory concentrations of azithromycin, thereby prompting the induction of or selection for resistance genes. In the United States and United Kingdom, dual therapy is no longer the first-line treatment. Instead, high-dose ceftriaxone monotherapy (500 mg in the United States or 1 g in the United Kingdom) is now recommended for treatment of uncomplicated gonorrhea (*44*,*45*). Moreover, additional treatment with doxycycline (100 mg 2×/d for 7 d) is recommended if chlamydial infection has not been excluded (*44*). Similar empirical antimicrobial therapies for gonorrhea and chlamydial infections might be of benefit in Argentina to reduce patient exposure to azithromycin and avoid the emergence of resistant gonococcal strains.

Azithromycin resistance (i.e., MICs of ≥2 µg/mL) in *N. gonorrhoeae* has been mainly associated with mutations in the 23S rRNA target (*38*). The 23S rRNA A2059G mutation causes high-level resistance (i.e., MICs of ≥256 µg/mL) and the C2611T mutation causes low-level resistance (i.e., MICs of 2–16 µg/mL) (*38*). We found that 75% of isolates had the C2611T mutation. These isolates were phylogenetically diverse; however, clade 1, which was predominated by MLST ST1580 and NG-MAST G470, comprised 38 (52.8%) isolates. NG-MAST ST470 has been associated with high-level resistance to azithromycin in Scotland (*18*). In addition, NG-MAST ST470 has >99% similarity to ST9768, which caused an outbreak of high-level azithromycin-resistant *N. gonorrhoeae* in the United Kingdom (*16*). Previous gonococcal evolution studies have estimated that ≈4 (range 0–14) SNPs occur per year per genome, enabling phylogenetic analysis (*21*). Isolates from Argentina differed from isolates from Scotland by ≥4 (mean 7.9) SNPs and the United Kingdom by 13 (mean 16.8) SNPs. In addition,

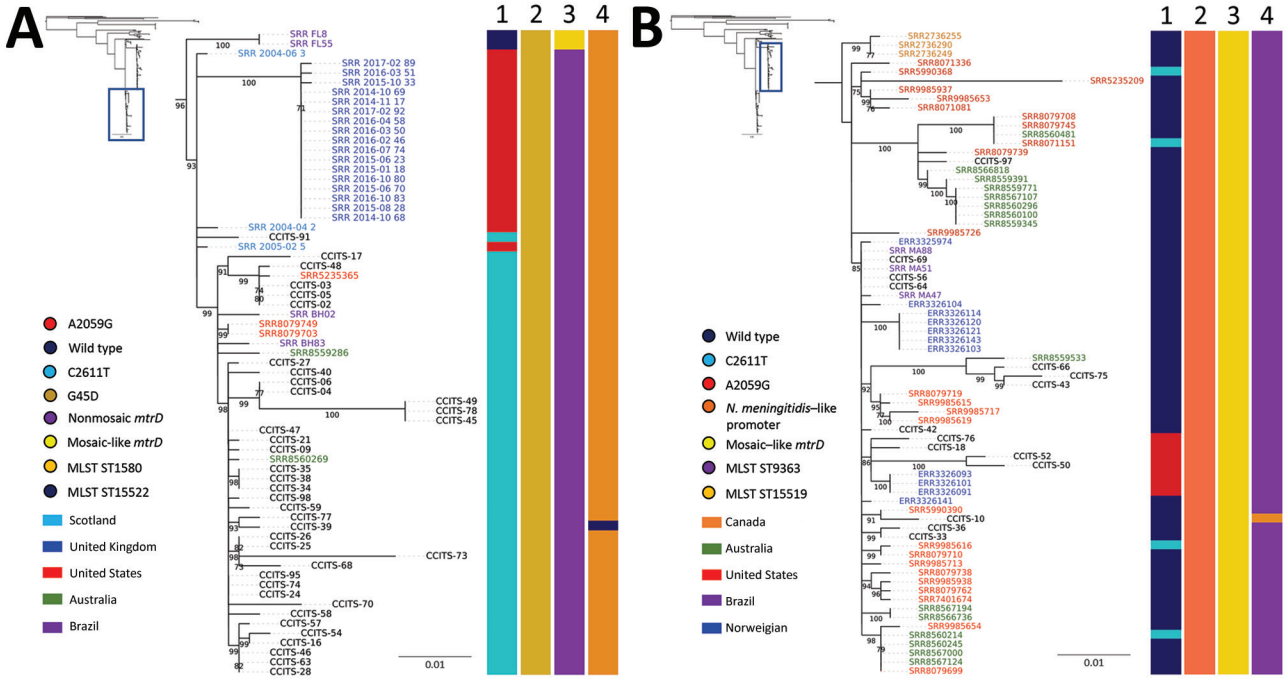


**Figure 3.** Phylogenomic tree of *Neisseria gonorrhoeae* isolates with azithromycin MICs of ≥2 µg/mL, 2004–2017. A) MLST ST1580 and NG-MAST genogroup 470 isolates from Argentina in the context of selected isolates from Scotland (2004–2005), the United States (2016), Australia (2017), Brazil (2015–2016), and the United Kingdom (2014–2017). B) MLST ST9363 and NG-MAST genogroup G12302 isolates from Argentina in the context of selected isolates from Australia (2017), the United States (2014–2017), Brazil (2015–2016), Norway (2017), and Canada (2013–2014). Lane 1, 23S rRNA; lane 2, *mtrR*; lane 3, *mtrD*; lane 4, MLST. Labels indicate isolate identity; font colors indicate country of isolation. Bar colors indicate distribution of mutations. Insets indicate relationship of sequences to larger phylogenetic tree. Scale bar indicates substitutions per site. MLST, multilocus sequence typing; NG-MAST, *N. gonorrhoeae* multiantigen sequence typing.

NG-MAST ST470 isolates from the United States, Brazil, and Australia, all of which showed low-level resistance to azithromycin, were closely related to isolates from Argentina (mean 7–11 SNPs). These findings support the hypothesis that NG-MAST G470 strains from Argentina might be descended from 1 lineage of the ST470 clone, which has spread internationally and can develop high-level and low-level resistance to azithromycin. Previous research, especially that of Unemo et al. (*38*), hypothesized that gonococcal antimicrobial-resistant strains emerge through genetic events, such as horizontal gene transfer or spontaneous mutations; these strains can spread quickly within a geographic region through sexual networks. Furthermore, compensatory mutations or gene exchange might have preserved this lineage in Argentina. The presence of additional STs, such as the co-circulation of MLST ST1584 and MLST ST1580 (NG-MAST G470), suggests that novel introductions also have occurred.

The *mtr* locus recently has been described as a hotspot for genetic recombination; mosaic-like *mtr* loci are associated with decreased susceptibility to azithromycin (i.e., MICs of 1–4 µg/mL) and contribute to the survival and transmission of *N. gonorrhoeae* (*39*,*40*, *46*). Most clade 2 isolates were associated with MLST ST9363 and had a mosaic-like *mtr* locus. MLST ST9363 was the predominant strain type of isolates with MICs of 2–4 µg/mL identified in Australia during 2017 and the United States during 2014–2017 (*24*,*25*,*35*). We found that MLST ST9363 isolates from Argentina shared a high level of genomic similarity with the ST9363 clones reported in Australia, the United States, Canada, Norway, and Brazil, indicating that importation and dissemination has occurred. Those data further support the hypothesis that *N. gonorrhoeae* isolates carrying a mosaic-like *mtr* locus contribute to the emergence of isolates with low-level resistance to azithromycin in many countries (*24*,*25*). Isolates with MICs of ≥256 µg/mL have recently reemerged in Argentina (*20*). Those isolates belonged to clade 2 and were distinguished by the mosaic-like *mtr* locus and the A2059G mutation in all 4 23S rRNA gene alleles. The phylogenetic tree showed that these isolates were closely related to isolates from Norway (mean 10.2 SNPs) that also had MICs of ≥256 µg/mL, suggesting that strains carrying a mosaic-like *mtr* locus and 23S rRNA A2059G mutation can disseminate internationally. Previous studies have suggested that isolates carrying the A2059G mutation or mosaic *mtr* locus have enhanced fitness; elucidating the effects of both mechanisms on *N. gonorrhoeae* evolution might help predict the emergence and spread of azithromycin resistance (*39*,*46*,*47*).

Because we received a small number of isolates from some provinces, our dataset might have been limited by selection bias. In addition, we did not have access to therapy strategies and treatment success rates, which might have provided insight into the generation of resistance or the selection of azithromycin-resistant isolates. Finally, we obtained limited data regarding patients' sexual orientation and HIV status, but found that clade 2 strains were slightly more associated with male patients, including men who have sex with men, than clade 1 strains (100.0% vs. 92.1%). In addition, 3 patients who had infections caused by clade 2 strains were HIV-positive (data not shown). Increased awareness of the transmission dynamics of azithromycin-resistant gonococcal strains within sexual networks is crucial to confirming these observations. Continuing surveillance of the prevalence and distribution of azithromycin-resistant strains in addition to genomic monitoring using individual-level epidemiologic data should provide a more complete picture of azithromycin-resistant gonococcal strains. These data will inform public health strategies to control azithromycin-resistant *N. gonorrhoeae*.

In conclusion, the recent increase in the prevalence of azithromycin-resistant *N. gonorrhoeae* isolates in Argentina was mainly the result of the introduction and expansion of 2 clones belonging to MLST ST1580 and ST9363. The integration of appropriate STI diagnosis and antimicrobial prescription into health services combined with genomic, phenotypic, and epidemiologic gonococcal surveillance data will be critical in preventing the dissemination of gonococcal clones resistant to azithromycin, ceftriaxone, or both, and preserving the current available therapeutic option for gonorrhea.

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# Genomic Epidemiology of Azithromycin-Nonsusceptible *Neisseria gonorrhoeae*, Argentina, 2005–2019

## Appendix

## Appendix Methods

### Antimicrobial Susceptibility Testing

We subcultured N. gonorrhoeae isolates on Difco GC medium agar base (Becton, Dickinson and Company, https://www.bd.com) supplemented with 1% Britalex enrichment supplement (Laboratorios Britania S.A., https://www.britanialab.com) for 18–24 h at 35°C in a humid 5% carbon dioxide–enriched atmosphere before conducting antimicrobial susceptibility testing. We determined the MICs of azithromycin, ceftriaxone, cefixime, ciprofloxacin, spectinomycin, benzylpenicillin, tetracycline, and gentamicin (MilliporeSigma, https://www.sigmaaldrich.com) using the agar dilution method (Reference *1* in Appendix). We interpreted the MICs using CLSI breakpoints (*15*), except for gentamicin, for which we used previously published interpretive criteria (Reference *2* in Appendix). We used the N. gonorrhoeae strain ATCC 49226 and 8 WHO reference strains documented in 2008 for quality control (*15*, Reference *3* in Appendix).

### Whole-Genome Sequencing

We extracted genomic DNA using QIAamp DNA Mini Kit (QIAGEN, https://www.qiagen.com) according to the manufacturer's instructions. We determined DNA concentration using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, https://www.thermofisher.com) and stored samples at −20°C. We conducted WGS on all isolates using the Nextera XT DNA library preparation kit and the MiSeq Platform (Illumina, https://www.illumina.com) according to the manufacturer's instructions. We assessed the quality of the sequences using FastQC version 0.11.9 (Babraham Institute,

http://www.bioinformatics.babraham.ac.uk) and identified contaminants using Kraken 2 version 2.08 (Johns Hopkins University, http://ccb.jhu.edu). We assembled de novo reads using Unicycler version 0.4.8, which is based on SPAdes version 3.13.0 (Reference *4* in Appendix), and assessed assembly quality using Quast version 5.0.2 (Reference *5* in Appendix). On average, the numbers of contigs was 103 and the N50 contig length (i.e., the length for which half of the bases of a draft genome are situated in contigs of that length or longer) was 48,458 bp.

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N. gonorrhoeae FA1090  MAKFFIDRPI FAWVISIFII AAGIFGIKSL PVSQYPSVAA PTITLHAIYP GASAQVMEGS VLSVIERNMN GVEGLDYMST SADSSGSGSV SLTFTPDTDE NLAQVEVQNK LSEVLSTLPA  120
CDC2                   .......... .......... .......... .......... .N...R.T.. ........D. .......... .......... .......... ......E... D......... ......M...  120
CCITS-50 (Clade 2)     .......... .......... .......... .......... .N...R.T.. ........D. .......... .......... .......... ......E... D......... ......M...  120
GCGS0402               .......... .......... .......... .......... .N...R.T.. ........D. .......... .......... .......... ......E... D......... ......M...  120
CCITS-20 (n=1)         .......... .......... .......... .......... .N...R.T.. ........D. .......... .......... .......... ......E... D......... ......M...  120
CCITS-60 (n=2)         .......... .......... .......... .......... .N...R.T.. ........D. .......... .......... .......... ......E... D......... ......M...  120

N. gonorrhoeae FA1090  TVQQYGVTVS KARSNFLMIV MLSSDVQSTE EMNDYAQRNV VPELQRIEGV GQVRLFGAQR AMRIWVDPKK LQNYNLSFAD VGSALSAQNI QISAGSIGSL PAVRGQTVTA TVTAQGQLGT  240
CDC2                   .......... .......... .......... .........I .......... .......... .......... .......... I......... .......... ...Q...... ........S.  240
CCITS-50 (Clade 2)     .......... .......... .......... .........I .......... .......... .......... .......... I......... .......... ...Q...... ........S.  240
GCGS0402               .......... .......... .......... .........I .......... .......... .......... .......... I......... .......... ...Q...... ........S.  240
CCITS-20 (n=1)         .......... .......... .......... .........I .......D.. .K.Q...... .......... .......... .......... .......... ...Q...... ........S.  240
CCITS-60 (n=2)         .......... .......... .......... .........I .......D.. .K.Q...... .......... .......... .......... .......... ...Q...... ........S.  240

N. gonorrhoeae FA1090  AEEFGNVILR ANTDGSNIYL KDVAKVGLGM EDYSSSTRLN GVNTTGMAVM LSNSGNAMAT AKAVKERLAV LEKYFPQGMS WKTPYDTSKF VEISIEKVIH TLIEAMVLVF VVMYLFLQNI  360
CDC2                   .......... .......V.. .......... .......... .......... .......... .......M.T .......... .......... .......... ..L....... ..........  360
CCITS-50 (Clade 2)     .......... .......V.. .......... .......... .......... .......... .......M.T .......... .......... .......... ..L....... ..........  360
GCGS0402               .......... .......V.. .......... .......... .......... .......... .......M.T .......... .......... .......... ..L....... ..........  360
CCITS-20 (n=1)         .......... .......V.. .......... .......... .......... .......... .......M.T .......... .......... .......... .......... ..........  360
CCITS-60 (n=2)         .......... .......V.. .......... .......... .......... .......... .......M.T .......... .......... .......... .......... ..........  360

N. gonorrhoeae FA1090  RYTLIPTIVV PISLLGGFAF ISYMGMSINV LTMFAMILVI GIVVDDAIVV VENVERIMAG EGLPPKEATK KAMGQISGAV IGITAVLISV FVPLAMFSGA AGNIYKQFAL TMASSIAFSA  480
CDC2                   .......... .......... .......... ......V... .......... .......... ......A... .......... .......M.. .......... T......... ...A......  480
CCITS-50 (Clade 2)     .......... .......... .......... ......V... .......... .......... ......A... .......... .......M.. .......... T......... ...A......  480
GCGS0402               .......... .......... .......... ......V... .......... .......... ......A... .......... .......M.. .......... T......... ...A......  480
CCITS-20 (n=1)         .......... .......... .......... ......V... .......... .......... ......A... .......... .......M.. .......... T......... ...A......  480
CCITS-60 (n=2)         .......... .......... .......... ......V... .......... .......... ......A... .......... .......M.. .......... T......... ...A......  480

N. gonorrhoeae FA1090  FLALTLTPAL CATMLKTIPK GHHEEKKGFF GWFNKKFDSW THGYEGRVAK VLRKTFRMMV VYIGLAVVGV FLFMRLPTSF LPTEDQGFVM VSVQLPAGAT KERTDATLAQ VTQLAKSIPE  600
CDC2                   .......... .......... .......... .......N.. ......W... .....L.... ..V....... .......... .......... .......... .......... ..........  600
CCITS-50 (Clade 2)     .......... .......... .......... .......N.. ......W... .....L.... ..V....... .......... .......... .......... .......... ..........  600
GCGS0402               .......... .......... .......... .......N.. ......W... .....L.... ..V....... .......... .......... .......... .......... ..........  600
CCITS-20 (n=1)         .......... .......... .......... .......N.. ......W... .....L.... ..V....... ...T...... .......... .......... .......... ..........  600
CCITS-60 (n=2)         .......... .......... .......... .......N.. ......W... .....L.... ..V....... ...T...... .......... .......... .......... ..........  600

N. gonorrhoeae FA1090  IENIITVSGF SFSGSGQNMA MGFAILKDWN ERTASGSDAV AVAGKLTGMM MGTLKDGFGI SVVPPPILEL GNGSGLSINL QDRNNTGHTA LLAKRNELIQ KMRASGLFDP STVRAGGLED  720
CDC2                   .......... .......... ....M..... ..KTP....E S......... .......... AI.....M.. .......... ........A. .......... ...T....N. ..........  720
CCITS-50 (Clade 2)     .......... .......... ....M..... ..KTP....E S......... .......... AI.....M.. .......... ........A. .......... ...T....N. ..........  720
GCGS0402               .......... .......... ....M..... ..KTP....E S......... .......... AI.....M.. .......... ........A. .......... ...T....N. ..........  720
CCITS-20 (n=1)         .......... .......... ....M..... ..KTP....E S......... .......... AI.....M.. .......... ........A. ..V....... ...T....N. ..........  720
CCITS-60 (n=2)         .......... .......... ....M..... ..KTP....E S......... .......... AI.....M.. .......... ........A. ..V....... ...T....N. ..........  720

N. gonorrhoeae FA1090  SPQLKIDINR AAAAAQGISF ADIRTALASA LSSSYVSDFP NQGRLQRVMV QADEDARMQP ADILNLTVPN KSGVAVPLST IATVSWENGT EQSVRFNGYP SMKLSASPAT GVSTGQAMAA  840
CDC2                   A......... .......V.. .......... .......... .......... ...G...... .......... S..I.....S ......QM.. .......... A.E..G.... ........E.  840
CCITS-50 (Clade 2)     A......... .......V.. .......... .......... .......... ...G...... .......... S..I.....S ......QM.. .......... A.E..G.... ........E.  840
GCGS0402               A......... .......V.. .......... .......... .......... ...G...... .......... S..I.....S ......QM.. .......... A.E..G.... ........E.  840
CCITS-20 (n=1)         A......... S..S...... .S......N. .G....N... .......... ...AS..... .......... S..I.....S ......QM.. .......... A.E..G.... ........E.  840
CCITS-60 (n=2)         A......... S..S...... .S......N. .G....N... .......... ...AS..... .......... S..I.....S ......QM.. .......... A.E..G.... ........E.  840

N. gonorrhoeae FA1090  VQKMVDELGG GYSFEWGGQS REEAKGGSQT LILYGLAVAA VFLVLAALYE SWSIPLAVIL VIPLGLIGAA AGVTGRNLFE GLLGSVPSFA NDIYFQVGFV TVMGLSAKNA ILIIEFAKDL  960
CDC2                   .........S ...L...... .......... IA..A..AV. .......... ........L. .M....A... .......... .......... .......... .......... ..........  960
CCITS-50 (Clade 2)     .........S ...L...... .......... IA..A..AV. .......... ........L. .M....A... .......... .......... .......... .......... ..........  960
GCGS0402               .........S ...L...... .......... IA..A..AV. .......... ........L. .M....A... .......... .......... .......... .......... ..........  960
CCITS-20 (n=1)         .......... ...L...... .......... IA..A..AV. .......... ........L. .M....A... ........T. LM..GI.A.. .......... .......... ..........  960
CCITS-60 (n=2)         .......... ...L...... .......... IA..A..AV. .......... ........L. .M....A... ........T. LM..GI.A.. .......... .......... ..........  960

N. gonorrhoeae FA1090  QAQGKSAVEA ALEAARLRFR PIIMTSFAFI LGVVPLYIAA GASSASQRAI GTTVFWGMLV GTLLSVFLVP LFYVVVRKFF KETAHEHEMA VRHASKAGIT GSDDKQY* 1068
CDC2                   .......... .......... .......... .........G .......... .........I .......... .......... .......... .K..AE.... ....S.H* 1068
CCITS-50 (Clade 2)     .......... .......... .......... .........G .......... .........I .......... .......... .......... .K..AE.... ....S.H* 1068
GCGS0402               .......... .......... .......... .........G .......... .........I .......... .......... .......... .K..AE.... ....S.H* 1068
CCITS-20 (n=1)         .......... .......... .......... .......... .......... .........I .......... .......... .......... .......... .......* 1068
CCITS-60 (n=2)         ......V... .......... .......... .........G .......... .........I .......... .......... .......... .K..AE.... ....S.H* 1068
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**Appendix Figure.** Amino acid sequences of mosaic MtrD loci in *N. gonorrhoeae* isolates, Argentina, 2005–2019. Amino acid sequences are aligned to the wildtype sequence of *N. gonorrhoeae* FA1090 (GenBank accession no. AE004969) and previously described mosaic MtrD sequences from CDC-2 and GCGS0834 strains (*44*,*45*). Dashes indicate amino acid residues identical to those of FA1090.