Emergence of *Neisseria gonorrhoeae* Strains Harboring a Novel Combination of Azithromycin-Attenuating Mutations

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**ABSTRACT** The nimbleness of *Neisseria gonorrhoeae* to evade the effect of antibiotics has perpetuated the fight against antibiotic-resistant gonorrhea for more than 80 years. The ability to develop resistance to antibiotics is attributable to its indiscriminate nature in accepting and integrating exogenous DNA into its genome. Here, we provide data demonstrating a novel combination of the 23S rRNA A2059G mutation with a mosaic-multiple transferable resistance (mosaic-mtr) locus haplotype in 14 *N. gonorrhoeae* isolates with high-level azithromycin MICs (≥256 μg/ml), a combination that may confer more fitness than in previously identified isolates with high-level azithromycin resistance. To our knowledge, this is the first description of *N. gonorrhoeae* strains harboring this novel combination of resistance determinants. These strains were isolated at two independent jurisdictions participating in the Gono-coccal Isolate Surveillance Project (GISP) and in the Strengthening the U.S. Response to Resistant Gonorrhea (SURRG) project. The data suggest that the genome of *N. gonorrhoeae* continues to shuffle its genetic material. These findings further illuminate the genomic plasticity of *N. gonorrhoeae*, which allows this pathogen to develop mutations to escape the inhibitory effects of antibiotics.

**KEYWORDS** 23S rRNA mutation, azithromycin resistance, MTR mutations, *Neisseria gonorrhoeae*

Antimicrobial agents play a critical role in the fight against gonorrhea, a sexually transmitted disease caused by *Neisseria gonorrhoeae*. The use of antibiotics for gonorrhea management began in the mid-1930s (1–3). This date also marked the dawn of a perpetual battle against antibiotic-resistant *N. gonorrhoeae* (AR-Ng). Over the last 80+ years, *N. gonorrhoeae* has progressively developed resistance to all first-line antibiotics following their introduction (1). The therapeutic longevity of antibiotics is inversely dependent to the malleability of the genetics of the pathogen (3). *Neisseria gonorrhoeae* demonstrates a high degree of genetic plasticity and uses this plasticity as a mechanism to develop antimicrobial resistance.

In an effort to restrain the emergence and spread of AR-Ng, the United States and other countries adopted a combination therapy strategy consisting of ceftriaxone as a primary therapy and azithromycin as a secondary agent (4). In spite of this effort, some *N. gonorrhoeae* isolates continue to defy therapeutic intervention and evade the effect of antibiotics (5). Gonococcal isolates displaying reduced susceptibility to ceftriaxone and/or azithromycin have emerged around the globe (3, 6–10). In the United States, the percentage of isolates with reduced cephalosporin susceptibility declined after 2012;
however, the number and percentage of gonococcal isolates with reduced susceptibility to azithromycin have steadily increased since then (11). The increase in the number of *N. gonorrhoeae* isolates displaying elevated MICs to azithromycin is typically associated with the accumulation of genetic mutations in the multiple transferable resistance (*mtr*) locus or in the 23S rRNA gene (10, 12–14).

The substitution of a nongonococcal *mtr* locus (mosaic-*mtr*) of a closely related *Neisseria* species into a *N. gonorrhoeae* strain can increase its azithromycin MIC to up to 2 μg/ml (13). The decrease in azithromycin sensitivity is more pronounced in *N. gonorrhoeae* isolates harboring 23S rRNA gene mutations. An A-to-G substitution at nucleotide position 2059 (*Escherichia coli* designation) and a C-to-T substitution at position 2611 (*E. coli* designation) in the *N. gonorrhoeae* 23S rRNA gene confer reduced susceptibility to azithromycin, with in vitro MICs up to ≥256 μg/ml and 16 μg/ml, respectively (15).

Mosaic-*mtr* substitutions and 23S rRNA mutation-mediated azithromycin resistance mechanisms rarely occur simultaneously in the same *N. gonorrhoeae* isolates. So far, only five *N. gonorrhoeae* isolates in the United States have been reported to contain the mosaic-*mtr* and C2611T haplotype (13). Also, to our knowledge, the combination of a mosaic-*mtr* and A2059G 23S rRNA in 14 *N. gonorrhoeae* isolates recovered recently by two independent jurisdictions participating in the Gonococcal Isolate Surveillance Project (GISP) and the Strengthening the U.S. Response to Resistant Gonorrhea (SURRG).

**RESULTS**

All 14 *N. gonorrhoeae* isolates shared a similar antibiogram and a high-level resistance to azithromycin. The *N. gonorrhoeae* isolates were collected from patients who presented at health clinics participating in the national gonococcal surveillance program established by the Centers for Disease Control and Prevention (CDC). Clinics participating in the gonococcal surveillance program routinely culture *N. gonorrhoeae* for antimicrobial susceptibility testing (AST). Between August 2017 and August 2018, 14 isolates from urethral (n = 10), pharyngeal (n = 2), and rectal (n = 2) samples were determined to display high-level resistance to azithromycin through routine AST. These isolates were cultured from 12 patients at two clinics in two different cities. Two urethral and pharyngeal isolate pairs were recovered from two patients. Initial MICs obtained using Etest by the local public health labs (SURRG) on all 14 isolates were ≥256 μg/ml for azithromycin and 0.016 μg/ml for both cefixime and ceftriaxone (Table 1). The MICs were later confirmed at the AR Lab Network using agar dilution and at the CDC using both AST methods. The CDC and AR Lab Network labs also assessed the susceptibilities of these isolates to ciprofloxacin, gentamicin, penicillin, and tetracycline using agar dilution (Table 1). The MICs for these antibiotics were within one doubling dilution from each other by either AST method.

All 14 *N. gonorrhoeae* isolates harbored a novel combination of azithromycin resistance markers. The DNA sequences of the 23S rRNA genes and the *mtr* locus were analyzed to determine whether these resistance mechanisms are present in these isolates and may explain the observed high level of azithromycin resistance. Across these loci, all 14 isolates had identical sequences. Analysis of the 23S rRNA gene revealed a single-nucleotide aberration, an A-to-G transversion mutation across all four alleles, at position 2059. No mutations were found at the 2611 position of the 23S rRNA gene (Table 1).

DNA analysis of the *mtr* locus revealed evidence of exogenous DNA integration at this site (Table 1). In all 14 isolates, the partially analyzed *mtrC* and *mtrR* genes and their associated promoter regions were replaced with nongonococcal counterparts (mosaic-*mtr*). The DNA sequence of this fragment (706 bp) showed high identity to *N. meningitidis* and *Neisseria lactamica*, at 97% and 96%, respectively (Fig. 1).

Whole-genome sequencing (WGS) analysis confirmed the results of 23S rRNA (A2059G) mutation and a mosaic-*mtr* locus obtained by PCR and Sanger sequencing, and it revealed that the mosaicism seen in the *mtr* locus extended through a large part of the genome.
### TABLE 1 Modal MICs were determined using both Etest and agar dilution MICs

<table>
<thead>
<tr>
<th>Isolate ID</th>
<th>WGS ID</th>
<th>Isolate source</th>
<th>Antibiogram modal MIC (µg/ml) by drug</th>
<th>23S rRNA mutation</th>
<th>mtr locus</th>
<th>Ribosomal gene present</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>AZI CRO CFX CIP PEN GEN TET</td>
<td>A2059G C2611T</td>
<td>mtr120</td>
<td>G45D H105Y rplD rplV</td>
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<tr>
<td>3001042919</td>
<td>LRRBGS_0305</td>
<td>Rectum</td>
<td>&gt;256 0.016 0.016 0.015 1</td>
<td>4/4</td>
<td>No</td>
<td>Yes C No No No No No</td>
</tr>
<tr>
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<td>Urethra</td>
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<td>2</td>
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<tr>
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</tr>
<tr>
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<td>4</td>
<td>Yes C</td>
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</tr>
</tbody>
</table>

**Notes:**
- Azithromycin Etest MICs are reported; the maximum concentration for an azithromycin Etest strip is 256 µg/ml. Mutational analyses were achieved through Sanger sequencing and WGS.
- ID, identifier.
- AZI, azithromycin; CRO, ceftriaxone; CFX, cefixime; CIP, ciprofloxacin; PEN, penicillin; GEN, gentamicin; TET, tetracycline.
of the entire mtrCDE operon (data not shown). WGS analysis, however, did not reveal a −35 delA in the mtr promoter (ΔAmtrR-p) nor any additional nonsynonymous amino acid mutations in the mtrR gene (G45D and H105Y) or in the rplV and rplD ribosomal genes, mutations previously reported to have an influence on azithromycin MICs (Table 1) (10).

**DISCUSSION**

The sustained increase in the number of *N. gonorrhoeae* isolates with elevated MICs for azithromycin around the world in recent years threatens the effectiveness of...
Azithromycin as part of the current treatment regimen (3, 6). In recent years, many countries have observed an increase in the prevalence of reduced susceptibility to azithromycin in *N. gonorrhoeae*. The percentage of isolates displaying an elevated MIC to azithromycin ranged from 4.7% to 32% in 2014 to 2017 (3, 6–10). Most of the isolates with elevated azithromycin MICs can be associated with mutations in either the 23S rRNA gene or the *mtr* locus (15). Moreover, an A-to-G transversion mutation at nucleotide position 2059 in the 23S rRNA can lead to high-level azithromycin resistance (i.e., MIC, ≥256 μg/ml).

In our study, we investigated 14 *N. gonorrhoeae* isolates displaying an MIC of ≥256 μg/ml for azithromycin. These 14 isolates were susceptible to ceftriaxone, cefixime, and ciprofloxacin but demonstrated intermediate resistance to penicillin and resistance to tetracycline. DNA sequence analysis revealed that all 14 isolates harbored the mosaic-*mtr* and the A2059G mutation in all four copies of their 23S rRNA gene. While investigators from many countries have reported on the A2059G mutation or the mosaic-*mtr*, none described these two haplotypes together (5, 10, 12–14, 16–25). The 14 isolates in this study are the first known isolates to have possessed both the A2059G mutation and the mosaic-*mtr*.

The dual azithromycin-attenuating mutations found in these 14 isolates were likely acquired through spontaneous and intragenus recombination. Genetic modification to gain antimicrobial resistance is a common phenomenon in *N. gonorrhoeae* (13, 15, 24). The A2059G point mutation in the 23S rRNA was likely acquired through spontaneous mutation. Chisholm et al. demonstrated that a single-allelic mutation at this residue could further transfer to the wild-type 23S rRNA alleles via endogenous homologous recombination under selection pressure (15). Conversely, the “mosaic” allele was likely acquired from other neisserial species through intragenus recombination (13, 24, 25). DNA sequences of *mtr* found in these 14 isolates shared high genetic identity to those of *N. meningitidis* and *N. lactamica* (Fig. 1). Furthermore, Wadsworth et al. demonstrated that the *mtr* from these two neisserial species, when transformed into *N. gonorrhoeae*, could recapitulate the azithromycin susceptibility pattern elicited by mosaic-*mtr* (13).

Although the A2059A mutation confers resistance to azithromycin, it has been posited that this same mutation exerts a fitness cost on *N. gonorrhoeae* isolates (14). This may explain why isolates with this genotype failed to transmit and persist in the population. In contrast, it has been speculated that mutations in the *mtr* locus may result in overexpression of the *mtrCDE* efflux pump and may increase fitness in *N. gonorrhoeae* (26, 27). Interestingly, the mosaic-*mtr* has been shown to increase MtrCDE protein expression in *N. gonorrhoeae* (13, 28). Thus, the A2059G-mosaic-*mtr* genotype might be more fit than the A2059G-alone genotype and perhaps is better able to sustain transmission of a gonococcal strain that confers high-level azithromycin resistance. Future exploration is needed to investigate this hypothesis.

The treatment and control strategies for *N. gonorrhoeae* infections have constantly evolved because of the organism’s ability to overcome the growth-inhibitory effect of first-line antibiotics. This bacterium develops resistance to antimicrobials at a high rate (1). As a result, the only recommended treatment against uncomplicated gonorrhea is dual therapy with an extended-spectrum cephalosporin (ceftriaxone) plus azithromycin. The ability to evade the effects of antibiotics displayed by *N. gonorrhoeae* is an attribute of the promiscuity in DNA uptake and the pliable nature of this bacterium’s genome to integrate exogenous DNA and/or its ability to develop mutations *de novo*. With the emergence of isolates possessing the dual azithromycin-attenuating genotype, A2059G-mosaic-*mtr*, *N. gonorrhoeae* isolates might have a mechanism to both harbor resistance and sustain transmission. More research on the fitness cost of these strains could further elucidate these hypotheses.

**MATERIALS AND METHODS**

*Neisseria gonorrhoeae* culture. Gonococcal isolates were obtained through the national surveillance programs GISP ([http://www.cdc.gov/std/gisp/](http://www.cdc.gov/std/gisp/)) and SURRG. All isolates were propagated on GC...
medium base agar supplemented with 1% hemoglobin and/or 1% IsoVitaleX (BD, Franklin Lakes, NJ, USA), and were incubated at 36 ± 1°C and 5% CO2. Species identification of the gonococcal isolates was confirmed using matrix-assisted laser desorption ionization–time of flight mass spectrometry (Bruker Daltonics, Boston, MA, USA).

Antimicrobial susceptibility testing. The MICs of all 14 N. gonorrhoeae isolates were assessed using the agar dilution and the Etest antimicrobial susceptibility testing (AST) methods, as described previously (29). MICs for ceftoxime, ceftriaxone, azithromycin, ciprofloxacin, gentamicin, penicillin, and tetracycline were determined for all isolates. Etest strips were purchased from bioMérieux (Durham, NC, USA), and antibiotic powders were purchased from Sigma-Aldrich (St. Louis, MO, USA). The susceptibility interpretations were based on CLSI interpretation guidelines (30) when available.

Molecular analysis of the 23S rRNA genes and the mtr locus. (i) Sanger DNA sequencing. PCR and Sanger sequencing were employed to analyze the DNA sequence of the 23S rRNA genes as well as the promoter and coding regions of the mtrR and mtrC genes. Total genomic DNA was purified using the QIAamp DNA minikit, as described by the manufacturer (Qiagen, Valencia, CA, USA). Amplification and sequencing of the four 23S rRNA alleles were achieved using the primers previously described by Ng et al. (31). In brief, an allele-specific primer was paired with the gonrRNA-F primer to amplify each allele. Then, each allele-amplicon was sequenced bidirectionally using the gonfrRNA-F and gonfrRNA-R2 primers. The amplification of the partial mtrR and mtrC genes and their promoters was carried out using the forward primer NGmtrC-F (5′-TGT CGA TCT GAT ACA GCG GC-3′) and the previously described reverse primer MTR2 (32). The mtrR amplicon was pair-end sequenced with the NGmtrC-F and NGmtrC-R (5′-CCA AGA ACC TCC TTC GGC GC-3′) and the MTR1339 (33) and MTR2 primer pairs. The conditions for PCR and DNA sequencing were previously described (34).

(ii) Whole-genome sequencing. Whole-genome sequencing of N. gonorrhoeae isolates was performed either at the Tennessee Department of Health Laboratory or at the CDC. DNA extraction, library preparation, and Illumina-based sequencing were performed according to a standardized protocol (https://www.cdc.gov/pulseNET/pdf/PNL32-MiSeq-Nextera-XT.pdf).

Bioinformatics analysis. DNA sequences acquired through Sanger sequencing were visualized and processed using the Geneious program (Biomatters Ltd., San Francisco, CA, USA). The DNA sequences of the 23S rRNA gene and the mtr region from the N. gonorrhoeae strain ATCC 49226 were used as references. The mtr sequence identity was determined using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with the sequence identity was determined using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The mtr sequences of Neisseria meningitides (GenBank accession no. CP002422.1), Neisseria lactamica (GenBank accession no. FN995097.1), N. gonorrhoeae (ATCC 49226), and the gonococcal mosaic-mtr were also aligned using CLUSTALW (https://www.genome.jp/tools-bin/clustalw).

WGS data were analyzed using a custom script written in Python version 3.6. Raw reads in FASTQ format were used as input for analysis on a per-isolate basis. The raw reads were first analyzed by FastQC version 0.11.5 (35) and then filtered and trimmed using TrimGalore version 0.3.7 (36) with the default settings and for reference mapping using breseq version 0.30 (37) with N. gonorrhoeae FA19 (GenBank accession no. CP012026) as the reference genome. In order to extract variants in the Neisseria gonorrhoeae 23S rRNA and count the number of copies in the genome, breseq was run a second time using the sequence for a single allele of the 23S rRNA gene from strain MS11 as the mapping reference (GenBank accession no. X67293) with the polymorphism-prediction option turned on. The de novo genome assembly and the output from breseq were used for the next steps in the analysis pipeline, which uses the PANDAS version 0.22 and Biopython (38) packages to extract variants and format the data.

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