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Genomic Characterization of *Neisseria gonorrhoeae* Strains from 2016 U.S. Sentinel Surveillance Displaying Reduced Susceptibility to Azithromycin

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ABSTRACT In 2016, the proportion of *Neisseria gonorrhoeae* isolates with reduced susceptibility to azithromycin rose to 3.6%. A phylogenetic analysis of 334 *N. gonorrhoeae* isolates collected in 2016 revealed a single, geographically diverse lineage of isolates with MICs of 2 to 16 μg/ml that carried a mosaic-like *mtr* locus, whereas the majority of isolates with MICs of $\geq$16 μg/ml appeared sporadically and carried 23S rRNA mutations. Continued molecular surveillance of *N. gonorrhoeae* isolates will identify new resistance mechanisms.

KEYWORDS 23S rRNA, *Neisseria gonorrhoeae*, antimicrobial resistance, azithromycin, mosaic-like *mtrR*

Neisseria gonorrhoeae, the causative agent of the sexually transmitted disease gonorrhea, has consistently developed resistance to each recommended antibiotic, resulting in its designation as an urgent threat by the Centers for Disease Control and Prevention (CDC) and as a high-priority antibiotic-resistant pathogen by the World Health Organization (1, 2). Increasing numbers of *N. gonorrhoeae* isolates with reduced susceptibility to azithromycin (AZM) are reported in the United States and internationally, including in China (3), Canada (4), and Europe (5). In the United States, surveillance efforts through the Gonococcal Isolate Surveillance Project (GISP) indicated an increased incidence of reduced susceptibility to AZM from 0.2% in 2012 to 3.6% in 2016 (6). Given this sharp increase, we focused on understanding the genetics of all the isolates from 2016 with reduced susceptibility to AZM, defined as an MIC of $\geq$2 μg/ml. In a previous report, 117 isolates from 2016 with an AZM MIC of $\geq$2 μg/ml were genetically analyzed (7). In this report, we expand this analysis to 177 isolates, which represents 95% of all such isolates from 2016.

Isolates with an AZM MIC of <16 μg/ml cluster into a single, diverse clade sharing a common genetic mechanism. In 2016, 95% (177/186) of all gonococcal isolates collected by GISP with an AZM MIC of $\geq$2 μg/ml as determined by agar dilution were available for whole-genome sequencing (WGS) on an Illumina MiSeq (8). We investigated the relatedness of the strains by performing sequence typing using MLST and NG-MAST and a core genome single nucleotide polymorphism (SNP) analysis with recombination filtering followed by generation of a maximum-likelihood phylogenetic tree (Fig. 1). We also selected a stratified random sample of 10 isolates from each GISP sentinel site from 2016, of which 157 isolates were available for sequencing and contained mostly susceptible isolates. The distribution of AZM MICs in the sampled susceptible isolates was similar to that of all susceptible isolates in GISP, suggesting that

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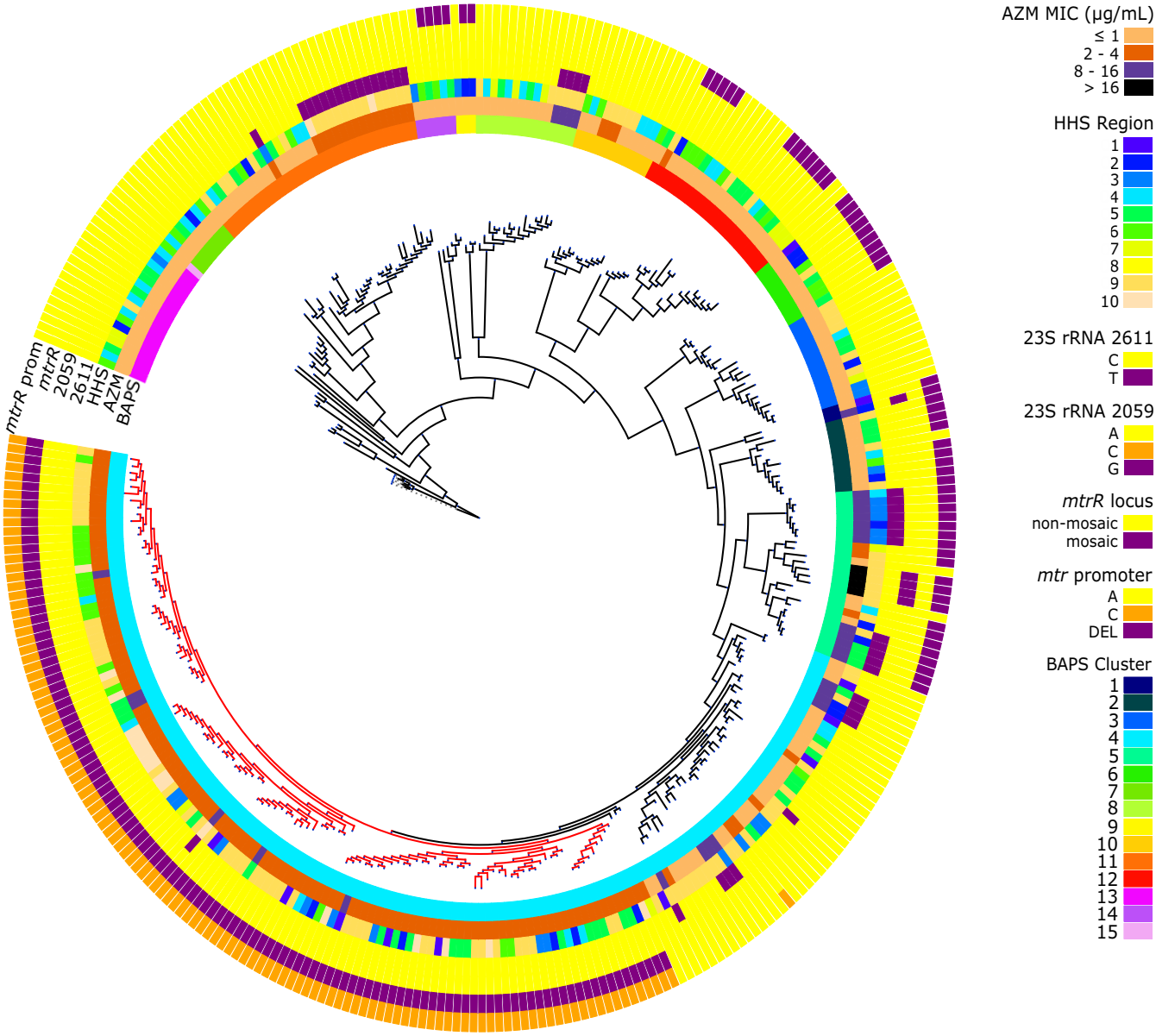

FIG 1 Maximum-likelihood phylogeny of *Neisseria gonorrhoeae* isolates collected in 2016, including all available isolates exhibiting AZM MICs of ≥2 μg/ml in GISP ($n = 117$). Lineage characterized by reduced susceptibility to AZM (2 to16 μg/ml) is highlighted in red (clade A). Rings from inside to outside are BAPS clusters, AZM MIC values, HHS regions, 23S rRNA C2611T, 23S rRNA A2059G, mosaic-like *mtrR* presence, and *mtrR* promoter mutations.

these isolates reflect the circulating U.S. AZM-susceptible gonorrhea population (compare reference 6 and Fig. S1 in the supplemental material). We also investigated alterations in the 23S rRNA alleles or the multiple transferable resistance regulator (*mtrR*) locus, which contribute to reduced susceptibility to AZM (Tables 1 and 2) using a custom pipeline written in Python (9, 10). A total of 13 clades containing three or more isolates were identified using Bayesian analysis of population structure (BAPS) (11). We identified one large clade of highly related isolates with high nodal support (clade A [$n = 119$]; within-clade difference, 87 ± 58 SNPs) (Fig. 1), which is a subset of BAPS cluster 4. Whereas the most common sequence type (ST) in clade A was ST9363 (100/119), others, including ST11422 and ST8134, were present. Clade A contained 39 total NG-MAST STs, with ST3935 (21/119) being the most common, followed by ST8241 (19/119) and ST12302 (12/119). Clade A is distinguished from the rest of the cluster by the presence of a mosaic-like *mtrR* locus, which contributes to elevated macrolide MICs by increasing expression of the MtrCDE efflux pump (12). Chi-square analysis showed

TABLE 1 Distribution of *mtrR* mutations

	Distribution (no. isolates) of:														
	mtrR locus		*mtrR* promoter[c]			MtrR									
AZM MIC (μg/ml)	Wild type	Mosaic	Wild type	Del A	C SNP	39A	39T	44H	44R	45D	45G	47L	47A	Full length	Premature stop
<2.0	157[a]	0	119[a]	38	0	100[a]	57	26	131[b]	48	109[a]	157	0	28	129
2.0–4.0	29	113[a]	24	4	114[a]	134[a]	8	6	136[b]	0	142[a]	142	0	19	123
8.0–16.0	25	6	12	13	6	20	11	4	27	8	23	31	0	0	31
>16	4	0	0	4	0	4	0	0	4	0	4	4	0	0	4

[a]$P < 0.0001$.
[b]$P < 0.005$.
[c]Del A, deletion of an adenine in the inverted repeat of the *mtrR* promoter; C SNP, A-to-C transversion in the inverted repeat of the *mtrR* promoter.

that a mosaic-like *mtrR* locus or an A-to-C transversion was highly associated with an MIC range of 2 to 4 μg/ml ($\chi^2 = 204.71$, $P < 0.0001$; or $\chi^2 = 174.98$, $P < 0.0001$, respectively) (Table 1). The success of the organisms in clade A, as suggested by the presence of similar isolates in a genomic analysis of GISP isolates from 2000 to 2013 (13) and whose presence has been continuous since 2014 (7), suggests that the mosaic-like *mtrR* locus contributes to their ability to survive and be transmitted. In support of this hypothesis, the transformation of a susceptible strain with a mosaic-like *mtr* locus is sufficient to increase the AZM MIC of the recipient strain (12, 14). Across the data set, the most predominant MLST type was ST9363 ($n = 130$) (Fig. 1), followed by ST1584 ($n = 23$), ST11422 ($n = 12$), ST1893 ($n = 11$), and ST1579 ($n = 10$). Some STs, e.g., ST9363, were found across the country, whereas others were more restricted (e.g., ST7371 or ST8154 on the East Coast). For NG-MAST, ST3935 was the most common ($n = 23$), followed by ST8241 ($n = 19$), ST7638 ($n = 15$), and ST12302 ($n = 12$) (for new NG-MAST STs, see Fig. S3 and Supplementary Methods in the supplemental material).

Isolates with an AZM MIC of ≥16 μg/ml appear sporadically and contain mutations in the 23S rRNA genes. Twenty isolates had an AZM MIC of ≥16 μg/ml as determined by agar dilution antimicrobial susceptibility testing. Those were further tested by Etest (bioMérieux), and 4 had an AZM MIC of ≥256 μg/ml. Those isolates were represented by ST1579 ($n = 4$), ST1901 ($n = 4$), ST7371 ($n = 6$), ST9363 ($n = 5$), and ST7822 ($n = 1$). The four isolates with MICs of ≥256 μg/ml were part of a previously described cluster of isolates with high-level AZM resistance and reduced susceptibility to ceftriaxone, which was successfully contained (15). Isolates with AZM MICs of ≥16 μg/ml were found throughout the tree and grouped into small subclusters (e.g., BAPS cluster 5 contained three subclusters) (Fig. 1). These isolates all contained mutations in either position 2611 or 2059 (*E. coli* numbering) of the 23S rRNA, which is the target of macrolides (Fig. 1 and Table 2). In *N. gonorrhoeae*, there are four copies of the 23S rRNA gene in the genome. Four copies of the 23S rRNA allele carrying the C2611T alteration were associated with an MIC range of 8 to 16 μg/ml ($\chi^2 = 129.65$; $P < 0.0001$) (Table 2). Isolates with four alleles carrying the A2059G alteration were found to have an AZM MIC of ≥256 μg/ml (Fig. 1 and Table 2). When found in clinical isolates or generated by site-directed mutagenesis in a susceptible strain, the A2059G alteration leads to an AZM MIC of ≥256 μg/ml and, potentially, treatment failure (9, 16, 17).

TABLE 2 Distribution of 23S rRNA mutant alleles

	Distribution (no. isolates) of:					
	C2611T				A2059G	
AZM MIC (μg/ml)	Wild type	2 mutated loci	3 mutated loci	4 mutated loci	Wild type	4 mutated loci
<2.0	156[a]	1	0	0	157	0
2.0–4.0	126[a]	0	1	15[b]	142	0
8.0–16.0	6	0	1	24[a]	30	1
>16.0	4	0	0	0	0	4

[a]$P < 0.0001$.
[b]Not significant.

In contrast to the widespread presence of isolates containing the mosaic-like *mtrR* locus, isolates with the C2611T alteration appear more sporadically. This is consistent with a hypothesis that the 23S rRNA mutations occur spontaneously and carry a fitness cost. The largest clade of these contains 15 isolates (MLST ST1584 and NG-MAST ST7638) and is found only in HHS regions 9 and 10. Many of these smaller clades were geographically isolated (Fig. 1), further suggesting spontaneous mutation followed by clonal expansion.

WGS for *N. gonorrhoeae* surveillance in 2016 did not include routine selection of enough susceptible isolates to assess when the 23S rRNA mutations arose in the U.S. *N. gonorrhoeae* population and whether certain strain types are more prone to developing these mutations. Defined selection criteria for routine WGS of GISP isolates were established in late 2017 and include subsets of both susceptible and resistant isolates. These sequences will provide a valuable resource for tracking of known strains with reduced susceptibility to AZM and detection of emerging strains that appear in the United States.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.05 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.4 MB.

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REFERENCES

1. World Health Organization. 2017. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. World Health Organization, Geneva.
2. Centers for Disease Control and Prevention. 2019. Antibiotic resistance threats in the United States, 2019. U.S. Department of Health and Human Services, Atlanta, GA. https://www.cdc.gov/drugresistance/pdf/threats-report/2019-ar-threats-report-508.pdf.
3. Wan C, Li Y, Le WJ, Liu YR, Li S, Wang BX, Rice PA, Su XH. 2018. Increasing resistance to azithromycin in *Neisseria gonorrhoeae* in eastern Chinese cities: resistance mechanisms and genetic diversity among isolates from Nanjing. Antimicrob Agents Chemother 62:e02499-17. https://doi.org/10.1128/AAC.02499-17.
4. Martin I, Sawatzky P, Liu G, Allen V, Lefebvre B, Hoang L, Drews S, Horsman G, Wylie J, Haldane D, Garceau R, Ratnam S, Wong T, Archibald C, Mulvey MR. 2016. Decline in decreased cephalosporin susceptibility and increase in azithromycin resistance in Neisseria gonorrhoeae, Canada. Emerg Infect Dis 22:65–67. https://doi.org/10.3201/eid2201.151247.
5. Cole MJ, Spiteri G, Jacobsson S, Woodford N, Tripodo F, Amato-Gauci AJ, Unemo M, Euro-GASP Network. 2017. Overall low extended-spectrum cephalosporin resistance but high azithromycin resistance in Neisseria gonorrhoeae in 24 European countries, 2015. BMC Infect Dis 17:617. https://doi.org/10.1186/s12879-017-2707-z.
6. Centers for Disease Control and Prevention. 2017. Sexually transmitted disease surveillance 2016. U.S. Department of Health and Human Services, Atlanta, GA. https://www.cdc.gov/std/stats16/default.htm.
7. Thomas JC, Seby S, Abrams AJ, Cartee J, Lucking S, Vidyaprakash E, Schmerer M, Pham CD, Hong J, Torrone E, Cyr SS, Shafer WM, Bernstein K, Kersh EN, Gernert KM, Antimicrobial-Resistant Neisseria gonorrhoeae Working Group. 2019. Evidence of recent genomic evolution in gonococcal strains with decreased susceptibility to cephalosporins or azithro-

mycin in the United States, 2014–2016. J Infect Dis 220:294–305. https://doi.org/10.1093/infdis/jiz079.
8. NCBI. 2016. BioProject: GCWGS_SL Neisseria gonorrhoeae genome sequencing. https://www.ncbi.nlm.nih.gov/bioproject/PRJNA317462.
9. Chisholm SA, Dave J, Ison CA. 2010. High-level azithromycin resistance occurs in *Neisseria gonorrhoeae* as a result of a single point mutation in the 23S rRNA genes. Antimicrob Agents Chemother 54:3812–3816. https://doi.org/10.1128/AAC.00309-10.
10. Wadsworth CB, Sater MRA, Bhattacharyya RP, Grad YH. 2019. Impact of species diversity on the design of RNA-based diagnostics for antibiotic resistance in *Neisseria gonorrhoeae*. Antimicrob Agents Chemother 63:e00549-19. https://doi.org/10.1128/AAC.00549-19.
11. Cheng L, Connor TR, Siren J, Aanensen DM, Corander J. 2013. Hierarchical and spatially explicit clustering of DNA sequences with BAPS software. Mol Biol Evol 30:1224–1228. https://doi.org/10.1093/molbev/mst028.
12. Rouquette-Loughlin CE, Reimche JL, Balthazar JT, Dhulipala V, Gernert KM, Kersh EN, Pham CD, Pettus K, Abrams AJ, Trees DL, St Cyr S, Shafer WM. 2018. Mechanistic basis for decreased antimicrobial susceptibility in a clinical isolate of Neisseria gonorrhoeae possessing a mosaic-like *mtr* efflux pump locus. mBio 9:e02281-18. https://doi.org/10.1128/mBio.02281-18.
13. Grad YH, Harris SR, Kirkcaldy RD, Green AG, Marks DS, Bentley SD, Trees D, Lipsitch M. 2016. Genomic epidemiology of gonococcal resistance to extended-spectrum cephalosporins, macrolides, and fluoroquinolones in the United States, 2000–2013. J Infect Dis 214:1579–1587. https://doi.org/10.1093/infdis/jiw420.
14. Wadsworth CB, Arnold BJ, Sater MRA, Grad YH. 2018. Azithromycin resistance through interspecific acquisition of an epistasis-dependent efflux pump component and transcriptional regulator in *Neisseria gonorrhoeae*. mBio 9:e01419-18. https://doi.org/10.1128/mBio.01419-18.
15. Papp JR, Abrams AJ, Nash E, Katz AR, Kirkcaldy RD, O'Connor NP, O'Brien PS, Harauchi DH, Maningas EV, Soge OO, Kersh EN, Komeya A, Tomas JE, Wasserman GM, Kunimoto GY, Trees DL, Whelen AC. 2017. Azithromycin resistance and decreased ceftriaxone susceptibility in Neisseria gonorrhoeae, Hawaii, USA. Emerg Infect Dis 23:830–832. https://doi.org/10.3201/eid2305.170088.
16. Gose SO, Soge OO, Beebe JL, Nguyen D, Stoltey JE, Bauer HM. 2015. Failure of azithromycin 2.0 g in the treatment of gonococcal urethritis caused by high-level resistance in California. Sex Transm Dis 42:279–280. https://doi.org/10.1097/OLQ.0000000000000265.
17. Zhang J, van der Veen S. 2018. Neisseria gonorrhoeae 23S rRNA A2059G mutation is the only determinant necessary for high-level azithromycin resistance and improves in vivo biological fitness. J Antimicrob Chemother 74:407–415. https://doi.org/10.1093/jac/dky438.