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Supplementary appendix 1

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Supplement to: Gernert K M, Seby S, Schmerer M W, et al. Azithromycin susceptibility of *Neisseria gonorrhoeae* in the USA in 2017: a genomic analysis of surveillance data. *Lancet Microbe* 2020; **1:** e154–64.

Supplemental Materials:

Azithromycin susceptibility of *Neisseria gonorrhoeae* in the USA in 2017: a genomic analysis of surveillance data

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Running title: Reduced azithromycin susceptible *Neisseria gonorrhoeae* with a mosaic *mtr* efflux pump locus

Supplemental Materials

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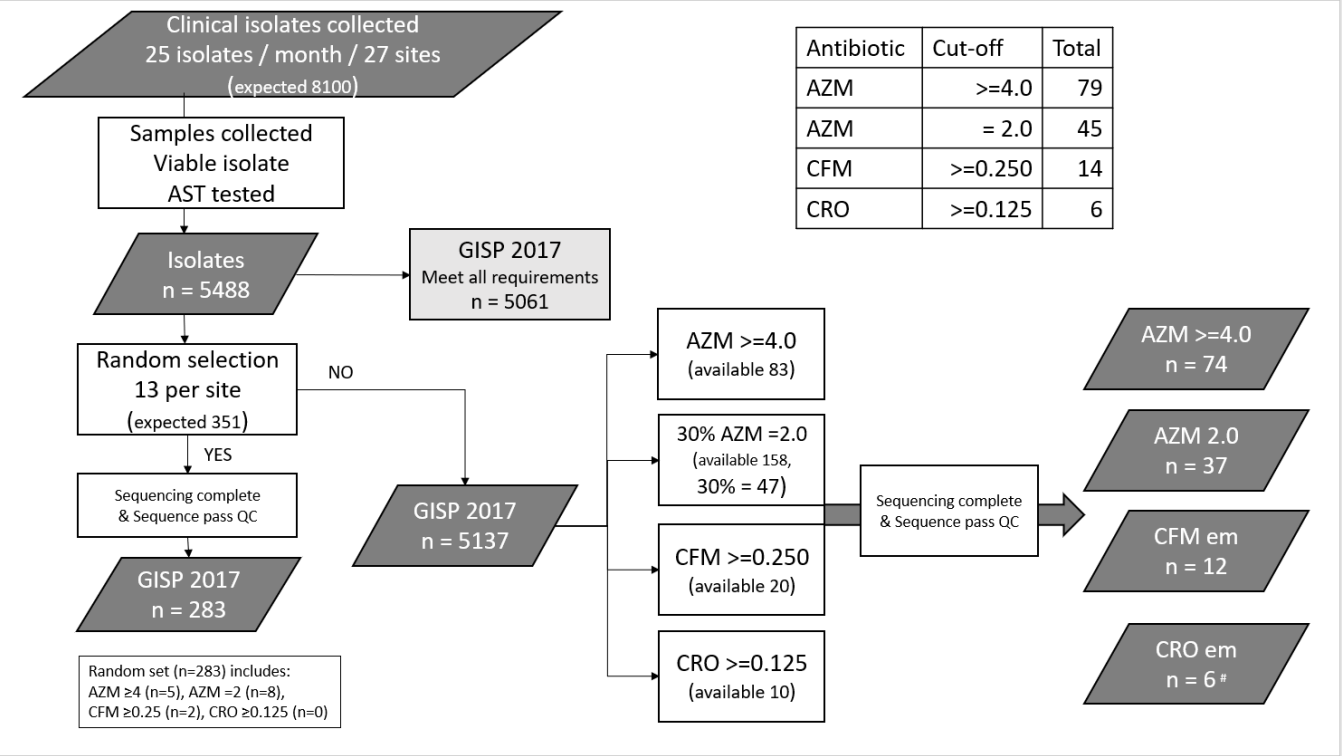
Supplemental Figure 1.



Supplemental Figure 1. Schematic representation of sample selection.

GISP guidelines include collection of isolates from the first 25 males with gonococcal urethritis each month or up to 300 isolates per year at 27 clinical sites across the U.S. in 2017. The targeted number of isolates collected per year is 8100 (expected 8100). 5488 bacterial cultures were collected; 5061 were successfully isolated, AST tested, and met GISP criteria. Isolates (n=3039) were not available or were excluded based on (1) not all sites were active for 12 months in 2017 and samples were not collected, (2) not all sites collected 25 specimens per month, (3) not all clinical specimens were able to provide viable, purified isolates, (4) >25 isolates were collected within a single month. From the annual isolate pool, 13 isolates were randomly selected for sequencing, per clinical site, without replacement. From the 5137 isolates not selected from the random process (5488 – 351), isolates were selected, exclusively, without replacement, based on elevated MIC to AZM (100% MIC >=4·0 µg/mL or 30% MIC =2·0), CFM (>=0·25) or CRO (>=0·125). Lists were compared to assure no isolate duplication.

(NOTE [#]Two isolates carried elevated MIC to CFM and CRO; each provided only a single sequenced sample.) A small subset of isolates (n=17, 4%) were part of the original clinical isolates collected but were later found to not meet all GISP criteria. These isolates remained part of the dataset. Isolates were excluded from the process based on inability to obtain high quality WGS sequence, even after requests for re-sequencing. Ultimately, not all isolates selected were sequenced, 2 out of 27 sites showed low sequence numbers (less than 50% of the random selection), 17/27 were missing 1-4 sequences. These omissions, since distributed, were expected to result in a low bias. The sequencing resulted in 410 isolates, 283 random selection, 74 with AZM MIC >=4·0 µg/mL, 37 with AZM MIC=2·0, 10 with CFM MIC >=0·25, 4 with CRO MIC >=0·125, 2 with elevated MIC to both CFM and CRO. Additional limitations on GISP sample collection exist; for instance, GISP collection does not discriminate from repeat individuals or networked partners (whether presenting at the same clinic or at different clinics). Both conditions may introduce bias, although the occurrence is expected to be very low. As noted by, out of the 70 specimens within this dataset with anonymized patient identifiers, no two isolates were from the same patient.

Supplemental Table 1 (Appendix 2) provides the following information on the 410 GISP 2017 isolates included in this study: NCBI SRA accession numbers, HHS region, MLST and NG-MAST sequence types, MICs for AZM, CFM, CRO, antimicrobial resistance determinant calls, sexual orientation and HIV status.

Supplemental Table 2.

Comparison of GISP isolates and sequenced isolates of different AZM MIC values with respect to HHS region.

HHS region	ALL GISP (all processed) (n=5488)		Official GISP data (n=5061)	
HHS:1	31	1%	31	1%
HHS:2	339	6%	339	7%
HHS:3	283	5%	283	6%
HHS:4	533	10%	531	10%
HHS:5	1371	25%	1126	22%
HHS:6	557	10%	557	11%
HHS:7	361	7%	300	6%
HHS:9	1648	30%	1529	30%
HHS:10	365	7%	365	7%
TOTAL	**5488**	**100%**	**5061**	**100%**

HHS region	ALL GISP (n=5061)		Sequenced samples	
HHS:1	31	1%	14	3%
HHS:2	339	7%	24	6%
HHS:3	283	6%	31	8%
HHS:4	531	10%	32	8%
HHS:5	1126	22%	101	25%
HHS:6	557	11%	45	11%
HHS:7	300	6%	15	4%
HHS:9	1529	30%	131	32%
HHS:10	365	7%	17	4%
TOTAL	**5061**	**100%**	**410**	**100%**

AZM MIC =2 μg/mL

30% MIC=2 μg/ml

HHS region	ALL GISP		Sequenced samples	extrapolated to 100%	
HHS:1	0	0%	0	0	0%
HHS:2	9	7%	3	10	7%
HHS:3	11	8%	0	0	0%
HHS:4	1	1%	1	3	2%
HHS:5	54	39%	25	83	56%
HHS:6	10	7%	3	10	7%
HHS:7	3	2%	2	7	4%
HHS:9	35	26%	10	33	22%
HHS:10	14	10%	1	3	2%
TOTAL	**137**	**100%**	**45**	**150**	**100%**

AZM MIC ≥4 μg/mL

HHS region	ALL GISP		Sequenced samples	
HHS:1	1	1%	1	1%
HHS:2	2	2%	1	1%
HHS:3	24	29%	20	27%
HHS:4	1	1%	1	1%
HHS:5	16	19%	14	19%
HHS:6	5	6%	5	7%
HHS:7	0	0%	0	0%
HHS:9	32	38%	30	40%
HHS:10	3	4%	3	4%
TOTAL	**84**	**100%**	**75**	**100%**

Supplemental Table 2

Supplemental Table 2 provides comparisons of the isolate counts and percentages between all 2017 GISP isolates (n=5488) and sequenced isolates included in this dataset (n=410) to identify representation by category (HHS region, AZM MIC 2 μg/mL, and AZM MIC ≥4 μg/mL). Column 1 provides the HHS region and in parentheses the number of sites within that HHS region. In 2017, no clinical collections were obtained in HHS region 8. Some sites contribute the full complement of specimens (25 per month per site); other sites, for instance HHS region 1, contribute fewer due to a lower burden of infection. HHS region 5 and HHS region 9 have 6 and 8 sites per region, respectively, and thus account for the highest number of specimens. These HHS regions provide the highest number of isolates with elevated MIC to AZM. HHS region 3 shows a high burden of AZM MIC ≥4 μg/mL with respect to its overall numbers. Since AZM^{ds} MIC =2 μg/mL were selected at 30% ("30% MIC =2 μg/mL, Sequenced Samples"), the count of isolates sequenced for AZM^{ds} MIC =2 μg/mL was extrapolated to 100% and adjusted to the sum of collected GISP isolates ("Extrapolated to 100%"). For AZM MIC=2 μg/mL, HHS3 shows underrepresented and HHS5 shows overrepresentation.

Methods:

PulseNet protocols

Supplemental Table 3A: PulseNet Protocol Document Control Numbers

Title	**Document Control Number**
PulseNet SOP for Requesting Anonymized WGS Identification Numbers for Clinical Isolates to be Sequenced	PND17
Laboratory SOP for PulseNet Nextera XT Library Prep and Run Setup for the Illumina MiSeq	PNL32 v7
PulseNet SOP for Illumina MiSeq Data Quality Control	PNQ07 v6
PulseNet SOP for Submitting a Biosample to National Center for Biotechnology Information (NCBI)	PND18
PulseNet SOP for Transferring Data from the Illumina MiSeq	PND19
Agar Dilution Susceptibility Testing of *Neisseria gonorrhoeae*	

WGS was performed at the Antibiotic Resistance Laboratory Network and Hawaii Department of Health following PulseNet protocols as listed in Supplemental Table 3A. These PulseNet protocols have been revised and replaced by the following (PNL33, PNL34, PNL35, PNL38, PNQ07 (Supplemental Table 3B)) and are available online: https://www.cdc.gov/pulsenet/pathogens/protocols.html.

Supplemental Table 3B.

Title	PNL32	Document Control Number
DNA Extraction and Quality	Step 4.5	PNL33
PN Nextera XT Library Prep	Step 4.6 – 4.6.2.3	PNL34
DNA Flex Protocol Library Prep	Step 4.6 – 4.6.2.3	PNL35
WGS on MiSeq	Step 4.6.2.4 – 4.6.3.6	PNL38 v1.0

Bioinformatic analyses

The WGS analysis pipeline as previously published [1] included the following steps.

Raw whole genome sequences (*.fastq) which passed the basic quality MiSeq metrics (Q30 and cluster density based on MiSeq cycle kit), minimal estimated chromosomal coverage based on read count, per-base and per-sequence quality scores following PulseNet criteria[2] were transferred to CDC. FastQC 0.10.1 [3] was used to verify quality of the read data set before and after trimming. Trimming via Cutadapt 1.8.3 [4] (parameters including minimum quality cutoff of 30, minimum length cutoff of 19) removed low-quality reads and sequencing adapters. Kraken 0.10.5 [5] identified contaminant reads; a quality cutoff of >90% of the reads mapped to Neisseria order was required. *De novo* assembly of the paired-end, trimmed reads was performed using SPAdes Genome Assembler 3.9.0 [6] (parameters for automatic selection of kmers, each kmer contributes to assembly, and reduce number of mismatches and short indels for bacterial genomes). The quality of the assembled genomes was assessed using Quast 4.3 [7] based on the reference genome FA19 (GenBank accession CP012026 [8]) and other reporting measures. Assessment included: number of contigs with length >500 nucleotides (<150), % genome coverage (>85%), N50 value (20000), total length of the assembled genome (1.8 – 2.5 Mb), and average chromosomal coverage (>10x). ParSNP Harvest 1.2 [9] was applied to generate core-genome, single nucleotide polymorphism (SNP) alignment with reference genome FA19 (GenBank accession CP012026 [8]). Gubbins 2.3.1 [10] removed recombinant blocks from ParSNP alignment, using hybrid alignment method. RaxML 8.2.9 [11] generated maximum likelihood phylogenetic tree (1000 bootstrap, GTRCAT substitution model). ParSNP alignment length covered 81.3% average genome, 39771 SNPs; Gubbins' alignment length covered 1785354 nucleotides, 16396 SNPs (Gubbins n=411).

Phylogenetic clades which were defined by predominant MLST, with greater than 5 isolates (Figure 1), were labeled A – S. Clades which included isolates with elevated MIC to AZM, or which were present in 2014-2016 with AZM^{ds}, were also defined and are summarized in Table 1. Clades without labels (white) either had less than 5 isolates or did not contain isolates with AZM^{ds}.

Pairwise SNP distance matrix was computed using SNP-dist [12], and custom script to calculate average SNPs distance and standard deviation (avgSNP-dist) between isolates within the clusters. StringMLST 0.3.6 [13] (https://pubmlst.org/neisseria/) was used to determine MLST types from raw reads; NGMASTER 0.4 [14] (http://www.ng-mast.net/) was used to determine ngMAST from assembled contigs. *Neisseria gonorrhoeae* AMR Profiler and Typing Tool 2.8.3 and 2.9.2 [1] is a custom python script used for identifying unique loci or SNP positions for previously identified antimicrobial resistant determinants. Phylogenetics and associated metadata were visualized using ITOL [15] (https://itol.embl.de/).

Supplemental Tables 4 A-E (Contingency tables)

Phylogenetically-related isolates with decreased susceptibility to azithromycin

Fisher's exact test were used to determine associations between pair-wise categorical variables (clade, elevated MIC, resistance determinants, sequence type). The contingency tables are summarized.

Supplemental Table 4A

One predominant cluster (ST9363; n=97), which was persistent throughout the year, included a majority of isolates with azithromycin MICs of ≥2 μg/mL (63%, n=61/97 versus 59/311*, P<0·0001, Fisher's exact test) and carried a mosaic *mtr* locus (70%, 68/97 versus 2/313 P<0·0001).

	AZM^{ds} MIC ≥2 μg/mL	AZM susceptible	***Marginal Row Totals***
Clade ST9363	61	36	97
Not in clade ST9363	59	252*	311*
Marginal Column Totals	120	288	408 (Grand Total)

* Two isolates (GCWGS_1162, GCWGS_4189) for which MIC was indeterminate were not counted in Tables 4A and 4E.

Supplemental Table 4B

The mosaic *mtr* locus as represented by a A to C nucleotide change in the *mtr* promoter (70%, 68/97 versus 2/313, P<0·0001) or as represented by a mosaic *mtrR* locus (67%, 65/97 versus 0/313, P<0·0001).

	A>C in *mtrR* promoter	Other promoter	***Marginal Row Totals***
Clade ST9363	68	29	97
Not in clade ST9363	2	311	313
Marginal Column Totals	70	340	410 (Grand Total)

	Mosaic *mtrR*	wildtype *mtrR*	***Marginal Row Totals***
Clade ST9363	65	32	97
Not in clade ST9363	0	313	313
Marginal Column Totals	65	345	410 (Grand Total)

	MtrD Lys823Glu	MtrD Lys823	***Marginal Row Totals***
Clade ST9363	97	0	97
Not in clade ST9363	3	310	313
Marginal Column Totals	100	310	410 (Grand Total)

Supplemental Table 4C

Clade A represented predominantly by MLST ST9363 (84%, 81/97 isolates versus 0/313, P<0·0001).

	ST9363	Not ST9363	***Marginal Row Totals***
Clade ST9363	81	16	97
Not in clade ST9363	0	313	313
Marginal Column Totals	81	329	410 (Grand Total)

Supplemental Table 4D

61/97 isolates in Clade sc9363.1 have AZM^{ds} (>=2 μg/mL), and AZM^{ds} (>=2 μg/mL) are predominantly clustered in sc9363.1 (57/65 versus 4/32, P<0·0001).

	AZM^{ds} MIC ≥2 μg/mL	AZM susceptible	**Marginal Row Totals**
Clade sc9363.1	57	8	65
Remainder Clade ST9363.a	4	28	32
Marginal Column Totals	61	36	97 (Grand Total)

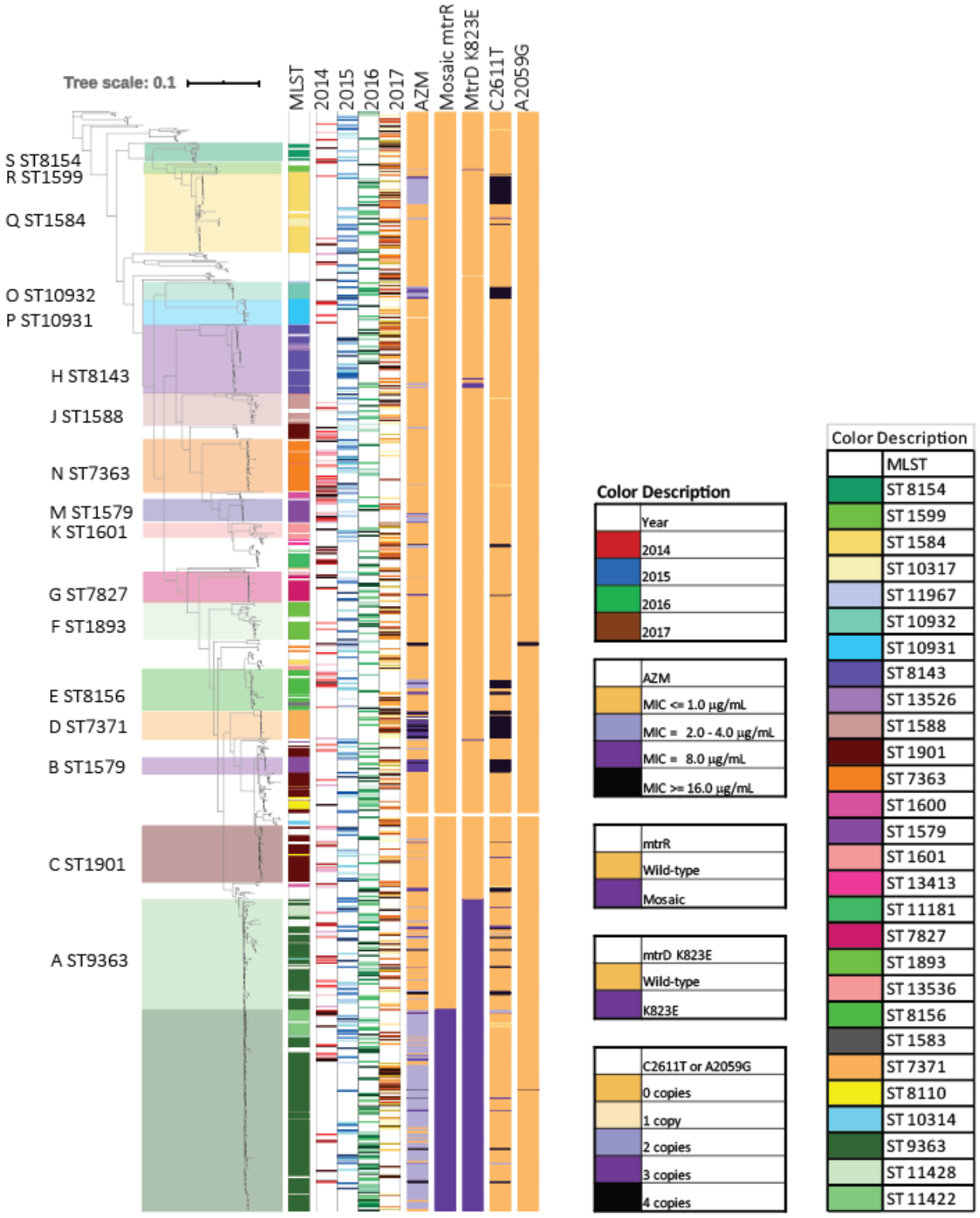
Supplemental Table 4E

Majority of remaining isolates not in Clade ST9363 (n=313) with elevated azithromycin MIC (>=4 μg/mL; n=57) were attributed to 23S rRNA variants (98%, 56/57).

	AZM^{ds} MIC ≥4 μg/mL	AZM MIC <=2 μg/mL	***Marginal Row Totals***
23S rRNA variants (C2611T, A2059G)	56	1	57
wildtype 23S rRNA	1	253	254
Marginal Column Totals	57	254	311* (Grand Total)

* Two isolates (GCWGS_1162, GCWGS_4189) for which MIC was indeterminate were not counted in Tables 4A and 4E.

Supplemental Figure 2



Supplemental Figure 2. Maximum likelihood core-genome SNP phylogeny of 1056 GISP *Neisseria gonorrhoeae* isolates from U.S. in 2014 – 2017 (2014-2016 (n=646), 2017 (n=410)) was generated (with FA19 reference genome, and recombination removed (based on 1583698 nucleotides, 20758 SNPs)).

MLST, date of specimen collection (2014, 2015, 2016, 2017), AZM MIC, mosaic *mtrR*, *mtrR* promoter variant, MtrD Lys823Glu and 23S rRNA (C2611T, A2059G) are represented in columns 1-10. Isolates from all four years are present in Clade ST9363, clade ST1584. Isolates from years 2015 – 2017 are shown in Clades ST1584.1, ST10932.1, and from years 2016 – 2017 in clades ST7371, ST1579. Color scheme: MIC susceptible (orange (≤1·0 μg/mL), increasing MIC (light purple (2-4 μg/mL), purple (8 μg/mL), dark purple (16 μg/mL)). The year and month of specimen collection date are represented in columns 2-5 (2014 (red), 2015 (blue), 2016 (green), 2017 (brown)), respectively, with each month represented from light (January) to dark (December). *mtrR* locus is described by *mtrR* mosaic (wild-type, non-mosaic *mtrR* (light orange) or mosaic *mtrR* (dark purple)); *mtrR* promoter (C-substitution (dark purple) or an A deletion (light purple) in the 13-bp inverted repeat); MtrD K823E (MtrD Lys823Glu) substitution (wild-type orange or mutation dark purple)) representing *mtrD* mosaicity. AMR 23S rRNA C2611T and A2059G are colored by cumulative number of variant rRNA copies present (1 to 4 copies, light orange, light purple, purple, dark purple). MLSTs with low representation are uncolored (white). Unknown values are colored white.

Supplemental Table 5.

Percentage of isolates with 23S rRNA variants or mosaic *mtr* locus in datasets 2012 – 2017.

Dataset	Year	#Percentage of AZM^{ds} sequenced which carry the variant		**Adjusted percentage of GISP AZM^{ds} isolates which carry the variant	
		% 23S rRNA	**% mosaic *mtr* clade ST9363**	**% 23S RNA**	**% mosaic *mtr* clade ST9363**
Grad YH. JID 2016.[16]	**2012**	53	6	53	12
	2013	27	30	34	28
Thomas, JC. JID 2019.[1]	**2014**	38	40	32	40
	2015	43	52	21	51
	2016	28	65	29	63
This work	**2017**	55	46	30	69

Percentage of AZM^{ds} which carry the variant is determined by the number of isolates sequenced per category (eg. 23S rRNA in 2012) divided by the total AZM^{ds} sequenced per year.

**The adjusted percentage provides an estimate per category if 100% of GISP AZM^{ds} isolates were sequenced. This was calculated to account for the fact that only 30% of AZM^{ds} MIC 2 μg/mL for 2017 was selected and sequenced. The count of isolates per category (e.g. 23S rRNA, MIC 16 μg/mL in 2012) was multiplied by the reciprocal of the percent sequenced per category, and an "adjusted percent" of total was recalculated. As reported in the publications, for 2000-2013 approximately 100% of AZM^{ds} were sequenced,[16] and for 2014-2016 the percentage sequenced per year ranged from 30-60%.[1] For 2017, 94% of AZM^{ds} MIC >=4 μg/mL were sequenced and 32% of AZM^{ds} MIC =2 μg/mL.

Supplemental Table 5 provides the percentage of isolates with reduced AZM susceptibility which are associated with either 23S rRNA variants or mosaic mtr locus. The AZM^{ds} isolates from clade ST9363 with mosaic *mtr* locus were first identified in the U.S. in 2012.[16] In 2017 95% of isolate with AZM MIC 2 μg/mL carried mosaic mtr operon and 33% of isolates with AZM MIC 4 μg/mL, and together they represented a majority of the AZM^{ds}

isolates (64%). The high representation of this strain aligns with the increase seen in the percentage of U.S. GISP surveillance isolates with reduced AZM susceptibility (AZM^{ds}) from 0·27% in 2012 to 4·4% in 2017.[17]

Table 6 (A, B)

23S rRNA determinants are present in phylogenetically diverse clades

Six isolates within this dataset were determined (via AMR Profiler) to have variants in the 23S rRNA in fewer than four copies of the rRNA. Supplemental Table 6A shows that the AZM MIC for these isolates ranged from 2–16 µg/mL, which agrees with S. Johnson, [18] who showed that elevated MICs to AZM correlated with the fraction of mutated copies of 23S rRNA. 4 copies gave MIC 8–16 µg/mL; 2-3 copies MIC 2–16 µg /mL. Supplemental Table 6B provides a summary of the clades which contain isolates with four copies of 23S rRNA variants at either C2611T or A2059G, and the associated MIC per clade.

Supplemental Table 6A

Sample Identifier	MLST	23S rRNA C2611T	Number of copies	AZM MIC µg/mL
GCWGS_0674	0	T	1	4
GCWGS_2116	9363	T	1	4
GCWGS_3276	9363	T	3	8
GCWGS_3304	9363	T	3	16
GCWGS_3620	8134	T	2	2
GCWGS_3586	1901	T	3	4

Supplemental Table 6 B

MLST	Number of isolates	23S rRNA variant	Number of Copies	AZM MIC µg/mL
1584	16	C2611T	4	4
1579	9	C2611T	4	8
10932	4	C2611T	4	4 - 8
8156	2	C2611T	4	8
7827	1	C2611T	4	8
7371	21	C2611T	4	8 - 16
12093	3	C2611T	4	2 - 16
9363	1	C2611T	4	16
11417	1	C2611T	4	16
9363	2	A2059G	4	16

Supplemental Table 7

Genomic and protein variants

	Accession number: sequence identifier	**Accession number: sequence identifier**
Genomic variants	**Nucleotide**	**Amino Acid**
2611C→T	X67293.1: r.2599C→T	
2059A→G	X67293.1: r.2047A→G	
mtr promoter (*mtrR* and *mtrCDE*)		
delA	NZ_CP012026.1: g.1110846del	
A→C	NZ_CP012026.1: g.1110846A→C	
mtrR premature stop *	NZ_CP012026.1: g. (1110901_?_1111533)del	(AKP10809.1) p.(1_?_210)del
Protein variants		
MtrR Ala39Thr	NZ_CP012026.1: g.1111015G>A	(AKP10809.1) p.(Ala39Thr)
MtrR Gly45Asp	NZ_CP012026.1: g.1111034G>A	(AKP10809.1) p.(Gly45Asp)
MtrR His105Tyr	NZ_CP012026.1: g.1111213C>T	(AKP10809.1) p.(His105Tyr)
MtrD Ser821Ala	NZ_CP012026.1: g.1106940A>C	(AKP10807.1) p.(Ser821Ala)
MtrD Lys823Glu	NZ_CP012026.1: g.1106934T>C	(AKP10807.1) p.(Lys823Glu)
PorB Gly120Lys	NZ_CP012026.1: g.1598401GGC>AAG	(AKP11294.1) p.(Gly120Lys)
PorB Gly121Asp	NZ_CP012026.1: g.1598404GGC>GAC	(AKP11294.1) p.(Gly121Asp)
PorB Gly121Asn	NZ_CP012026.1: g.1598404GGC>AAC	(AKP11294.1) p.(Gly121Asn)
PBP1 Leu421Pro	NZ_CP012026.1: g.2080172T>C	(AKP11771.1) p.(Leu421Pro)
PBP2 Asp345 insertion	NZ_CP012026.1: g.1302135+TCG	(AKP10994.1) p.(Asp345ins)
PBP2 Ala501Thr	NZ_CP012026.1: g.1301669C>T	(AKP10994.1) p.(Ala501Thr)
PBP2 Ala501Val	NZ_CP012026.1: g.1301668G>A	(AKP10994.1) p.(Ala501Val)
GyrA Ser91Phe	NZ_CP012026.1: g.359891G>A	(AKP10068.1) p.(Ser91Phe)
GyrA Asp95Ala	NZ_CP012026.1: g.359879T>G	(AKP10068.1) p.(Asp95Ala)
GyrA Asp95Gly	NZ_CP012026.1: g.359879T>C	(AKP10068.1) p.(Asp95Gly)
GyrA Asp95Asn	NZ_CP012026.1: g.359880C>T	(AKP10068.1) p.(Asp95Asn)
ParC Ser87Arg	NZ_CP012026.1: g.993821A>C	(AKP10706.1) p.(Ser87Arg)
ParC Ser87Asn	NZ_CP012026.1: g.993822G>A	(AKP10706.1) p.(Ser87Asn)
ParC Ser87Ile	NZ_CP012026.1: g.993822G>T	(AKP10706.1) p.(Ser87Ile)
Genomic Mosaicity		
mtrR mosaic †	KT954125.1: c.(1–797)	
penA mosaic ‡	NZ_CP012026.1: c.(1301424-1303169)	

Variants described according to Human Genome Variation Society guidelines (http://varnomen.hgvs.org/bg-material/refseq/).

* MtrR premature stop (AKP10809.1) p.(1_?_210)del : The complete nucleotide sequence *mtrR* was scanned for a stop codon or deletion in any position. If a stop codon or deletion was found in any position, it was assigned as a premature stop.

† *mtrR* mosaic KT954125.1: c.1–797 : The complete nucleotide sequence of gene *mtrR* was aligned to the reference to calculate percent similarity and determine mosaicity.

‡ *penA* mosaic NZ_CP012026.1: c.1301424-1303169 : The complete nucleotide sequence of gene *penA* was blasted against the PubMLST database penA locus (NEIS1753) to identify penA Type and NG STAR penA allele. https://pubmlst.org/neisseria/

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