Association of Phylogenomic Relatedness among *Neisseria gonorrhoeae* Strains with Antimicrobial Resistance, Austria, 2016–2020

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We investigated genomic determinants of antimicrobial resistance in 1,318 Neisseria gonorrhoeae strains isolated in Austria during 2016–2020. Sequence type (ST) 9363 and ST11422 isolates had high rates of azithromycin resistance, and ST7363 isolates correlated with cephalosporin resistance. These results underline the benefit of genomic surveillance for antimicrobial resistance monitoring.

Gonorrhea, a sexually transmissible infection (STI) caused by *Neisseria gonorrhoeae*, is the second most common bacterial STI (*1*). Most gonorrhea cases are mild, but serious complications can occur. Gonorrhea is treated with antibiotics, and the recommended treatment is dual extended-spectrum cephalosporin (ESC)/azithromycin therapy or ceftriaxone monotherapy (*2*).

One of the main characteristics of *N. gonorrhoeae* is the plasticity of its genome, favoring the acquisition and dispersion of antimicrobial resistance (AMR). AMR is an increasing issue for gonorrhea treatment, and untreatable gonorrhea represents an imminent global health threat (*3*).

Whole-genome sequencing (WGS) provides high-resolution data that can support AMR surveillance. We combined phenotypic AMR testing with WGS to investigate 1,318 *N. gonorrhoeae* strains isolated in Austria during 2016–2020 and identify genetic risk factors associated with AMR.

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The Study

This study encompassed 1,318 *N. gonorrhoeae* isolates collected in Austria during 2016–2020; isolates were available at the National Reference Centre for Gonococci. We tested all isolates for phenotypic resistance to azithromycin, cefixime, ceftriaxone, ciprofloxacin, tetracycline, and benzylpenicillin, as well as production of β-lactamase (i.e., cefinase positive) (Appendix, https://wwwnc.cdc.gov/EID/article/28/8/22-0071-App1.pdf). We followed European Committee on Antimicrobial Susceptibility Testing guidelines (*4*) to determine MIC thresholds used in this study.

We performed genomic DNA isolation, WGS, assembly, and contig filtering as described previously (*5*) (Appendix). We deposited raw reads in the National Center for Biotechnology Information Sequence Read Archive (project no. PRJNA771206). We obtained sequences types (STs) from WGS data by using the PubMLST schemes (*6*,*7*). We generated a local *N. gonorrhoeae* core-genome multilocus sequence typing (cgMLST) scheme with SeqSphere+ target definer tool version 6.0.0 (Ridom, ttps://www.ridom.de) (*7*) (Appendix). We investigated AMR genes by using allele libraries based on PathogenWatch in TOML format version 0.0.14 (*8*).

We performed time series analysis, linear regression, univariate analysis, multivariate analysis (logistic regression), and data visualization by using R version 4.0.4 (Appendix). We defined statistical significance as $p<0.05$. We computed neighbor-joining

trees in SeqSphere+ by using the number of cgMLST allelic differences and exported the trees into R.

We classified isolates according to AMR (Figure 1, panel A; Table) and determined MIC distributions (Figure 1, panel B). We observed high levels of resistance to ciprofloxacin (60%) and tetracycline (46%) (Figure 1, panel A), which increased 5% per year for ciprofloxacin (p<0.0001) and 6% per year for tetracycline (p<0.0001). The percentage of penicillin-resistant isolates was 16% and decreased over the study period (2% per year; p<0.0001) (Figure 1, panel C); 14% of isolates were cefinase-positive, which increased by 2.7% per year (p<0.0001).

We detected azithromycin resistance in 9% of the isolates, which increased by 5% per year (p<0.0001) (Figure 1). Two isolates from 2020 exhibited high levels of azithromycin resistance (MIC ≥256 μg/mL) but no other AMR. Resistance to ESC was rare; only 3% of isolates were resistant to cefixime, none were resistant to ceftriaxone, and 2.5% had reduced susceptibility to ceftriaxone (MIC >0.032 μg/mL). Cefixime resistance decreased by 0.9% per year (p<0.0001). Among cefixime-resistant isolates, 23/35 were resistant to ciprofloxacin and penicillin, qualifying as multidrug resistant.

The isolates belonged to 119 different STs in mutlilocus sequence typing, including 23 newly defined (STs 15803–15825). The most prevalent STs were ST7363 (170 isolates), ST9363 (151 isolates), and ST8156 (113 isolates), which comprised 33% of the isolates. We identified 215 NG-MAST types for 873/1,318 isolates; the most prevalent STs were 12302 (73 isolates), 5441 (59 isolates), and 387 (50 isolates).

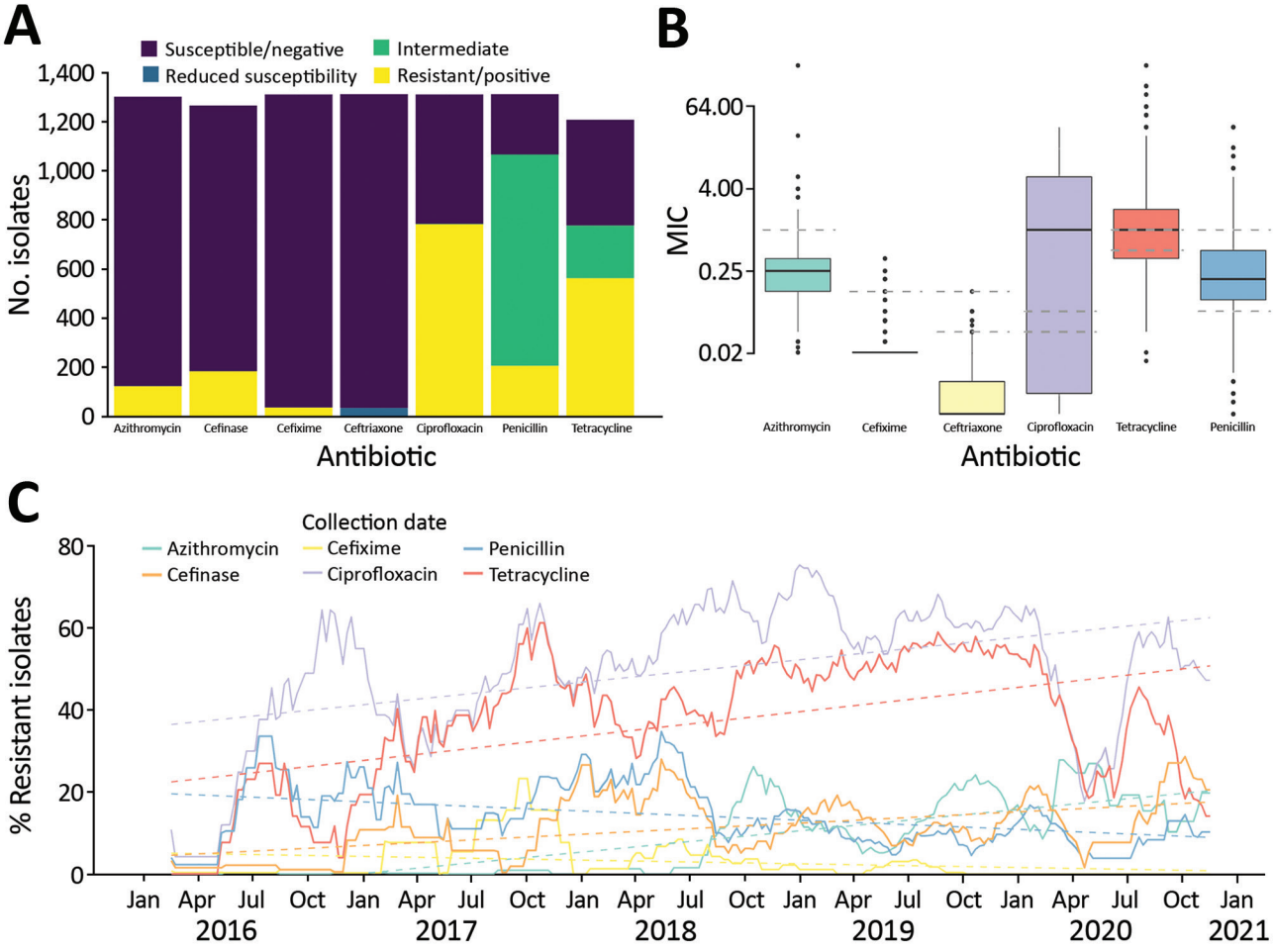

Figure 1. Antimicrobial resistance in 1,318 *Neisseria gonorrhoeae* isolates, Austria, 2016–2020. A) Number of isolates classified as susceptible, intermediate, or resistant. For ceftriaxone, isolates with reduced susceptibility are indicated in blue. For cefinase, β-lactamase producing isolates are indicated as positive (yellow). B) Boxplots of MIC obtained by Etest. Dashed lines indicate the thresholds used to classify the isolates as susceptible, intermediate, or resistant for ciprofloxacin, tetracycline, and penicillin, as susceptible or resistant for azithromycin, cefixime, and as susceptible, reduced susceptibility, or resistant for ceftriaxone. Horizontal lines within boxes indicate median, box tops and bottoms indicate quartiles 1 and 3, and dots indicate potential outliers. C) Evolution of the frequency of resistant isolates over time. Plain lines indicate the 13-week moving average of the percentage of isolates classified as resistant. Trends over time (obtained by linear regression) are represented by the dashed lines.

Table. Antimicrobial resistance classification and mean MIC of 1,318 *Neisseria gonorrhoeae* isolates, Austria, 2016–2020

Antibiotic	Antimicrobial resistance	No. isolates	Total no. isolates*	Frequency, %
Azithromycin	Susceptible (≤1)	1,180	1,302	90.6
	Resistant (>1)	122	1,302	9.4
	MIC, µg/mL		0.8432 (0.2937–1.3927)	
Cefixime	Susceptible (≤0.125)	1,276	1,311	97.3
	Resistant (>0.125)	35	1,311	2.7
	MIC, µg/mL		0.0289 (0.0266–0.0311)	
Ceftriaxone	Susceptible (≤0.032)	1,279	1,312	97.5
	Reduced Sensitivity (>0.032)	33	1,312	2.5
	Resistant (>0.125)	0	1,312	
	MIC, µg/mL		0.007 (0.0064–0.0076)	
Ciprofloxacin	Susceptible (≤0.032)	528	1,311	40.3
	Intermediate	1	1,311	0.1
	Resistant (>0.064)	782	1,311	59.6
	MIC, µg/mL		6.4455 (5.8446–7.0463)	
Tetracycline	Susceptible (≤0.5)	431	1,208	35.7
	Intermediate	215	1,208	17.8
	Resistant (>1)	562	1,208	46.5
	MIC, µg/mL		7.0349 (5.9602–8.1096)	
Penicillin	Susceptible (≤0.064)	246	1,312	18.8
	Intermediate	861	1,312	65.6
	Resistant (>1)	205	1,312	15.6
	MIC, µg/mL		2.2397 (1.8598–2.6196)	
Cefinase	Negative	1,083	1,266	85.5
	Positive	183	1,266	14.5
All			1,318	100

*Total number of isolates for which variable data were available.

cgMLST showed a branch including isolates with no or little AMR (Figure 2). We found no clear correlation with the cgMLST classification for penicillin, cefinase, tetracycline, and ciprofloxacin resistance. All cefixime-resistant isolates belonged to a single branch of ST7363 isolates, which also contained 24/32 isolates with reduced susceptibility to ceftriaxone. This branch had above average rates of ciprofloxacin, tetracycline, and penicillin resistance. A branch containing ST9363 and ST11422 isolates had a high rate of azithromycin resistance.

We searched isolate sequences for genes and point mutations associated with AMR (Appendix Table 3). For ciprofloxacin resistance, *gyrA* D95 substitutions were the main risk factor (adjusted odds ratio [aOR] 7.56 [95% CI 2.33–33.1]) and explained >99% of ciprofloxacin resistance. Tetracycline resistance was strongly associated with *tetM* carriage (aOR 157 [95% CI 48–965]), which we found in 33% of tetracycline-resistant isolates. For β-lactams, the main risk factor was bla_{TEM} carriage (aOR 67.9 [95% CI 35.2–139] for penicillin and aOR 234 [95% CI 93.3–683] for cefinase). Mutations in *penA* were also associated with cefinase positivity (aOR 35.6 [95% CI 14–97.4]).

We found mutations in the *macAB* promoter or mosaic *mtr* genes in 138/149 azithromycin-resistant isolates (93%). All cefixime-resistant isolates carried *penA* G545S substitution. The major risk factor for reduced susceptibility to ceftriaxone was *penA* A501T/V (aOR 73.9 [95% CI 6.9–3,170]).

Conclusions

This study combined phenotypic AMR and genomic data to analyze *N. gonorrhoeae* strains isolated in Austria during 2016–2020. We used a convenience sample (National Reference Centre for Gonococci collection) and results should be interpreted in light of this limitation. The percentage of *N. gonorrhoeae* strains resistant to azithromycin, ciprofloxacin, and tetracycline, or producing β-lactamase was increasing during the study period. The rate of azithromycin resistance rate was >13% during 2019–2020, which was high considering that an azithromycin/cefixime combination is a standard treatment for gonorrhea (*2*). We found no ceftriaxone-resistant isolates, and cefixime resistance rate was low.

We performed isolate typing by using multilocus sequence typing, *N. gonorrhoeae* multiantigen sequence typing (NG-MAST), and cgMLST. Only 37 isolates belonged to ST1901, which was predominant in isolates from Austria in a European study in 2013, highlighting the fast diversification of *N. gonorrhoeae* (*9*). The most common NG-MAST type was 12302; all isolates belonged to ST9363 and 71% were resistant to azithromycin. NG-MAST type 12302 and ST9363 have been associated with azithromycin resistance in other studies (*10*,*11*). cgMLST classification highlighted 3 branches with specific AMR patterns: 1 with low rates of AMR, 1 including azithromycin-resistant isolates, and 1 including ESC-resistant isolates. Previous studies comparing AMR and phylogenomic distributions in

different countries showed either that azithromycin/ESC resistance emerged repeatedly in different networks or that their spread was largely clonal (*12*,*13*). In Austria, azithromycin and ESC resistance clustering was in favor of single introductions. The use of cgMLST among available classification methods has limitations (i.e., no counting of mutations within 1 gene, exclusion of intergenic regions, and resolution) but also advantages (i.e., no correction of recombination events necessary and one scheme fitting all isolates). This tool corresponds to the need for surveillance, where its lower resolution does not have a major effect.

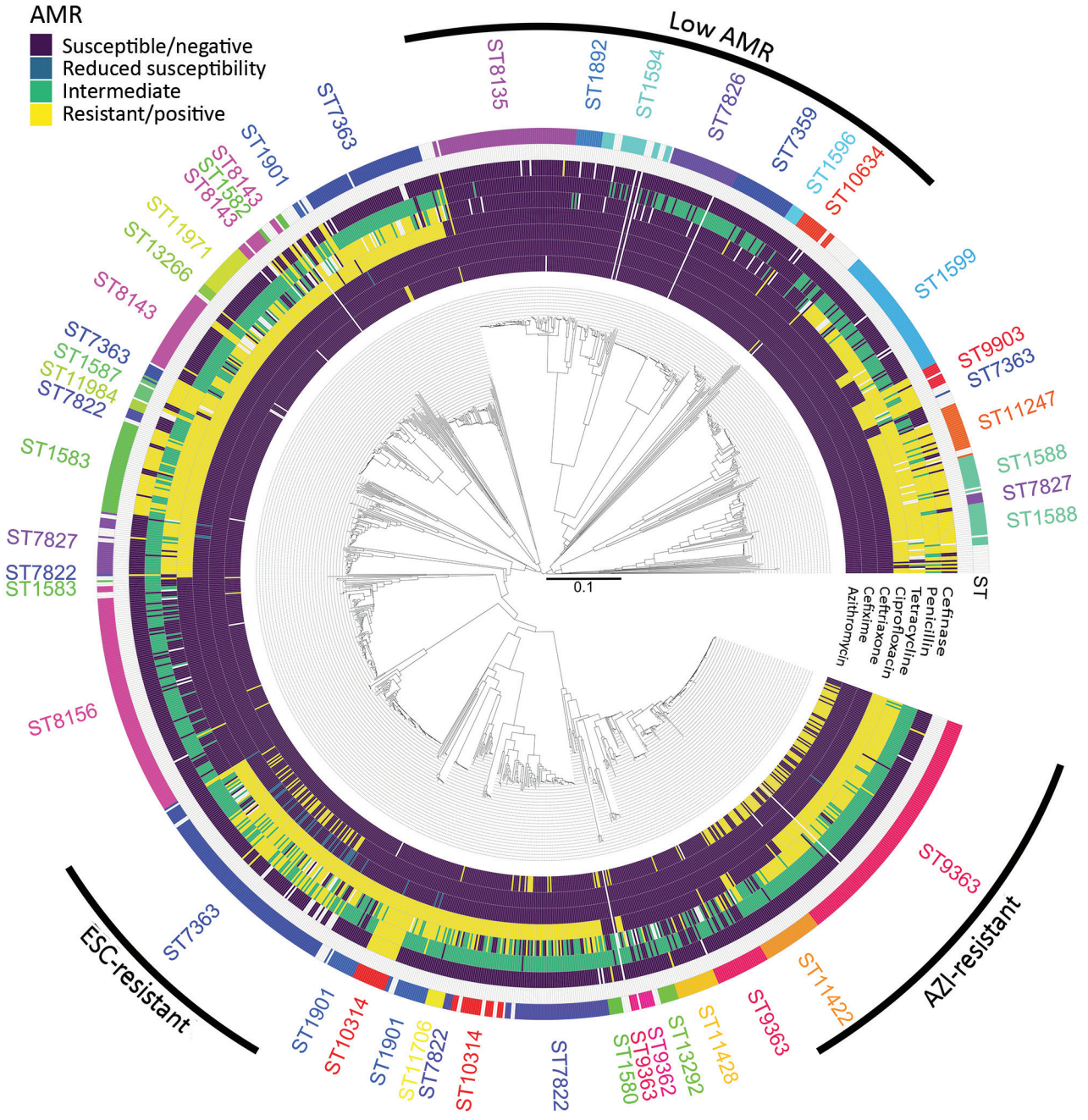

Figure 2. Correlation between population structure and antimicrobial resistance in *Neisseria gonorrhoeae* isolates, Austria, 2016–2020. Dendrogram was computed from the distance matrix of the core-genome multilocus sequence typing analysis (N = 1,304). Rims indicate the isolate classification as susceptible, intermediate, or resistant. For ceftriaxone, isolates with reduced susceptibility are indicated in blue. For cefinase, β-lactamase producing isolates are indicated as positive (yellow). The outer rim indicates sequence types corresponding to ≥2 consecutive isolates. Three branches with specific antimicrobial resistance patterns are indicated. AMR, antimicrobial resistance; AZI, azithromycin; ESC, extended-spectrum cephalosporin.

We used our WGS data to search for genetic determinants of AMR (*8*,*14*). Ciprofloxacin resistance matched well with *gyrA* mutations (*9*,*12*). Tetracycline resistance correlated with *tetM*, and penicillin resistance correlated bla_{TEM}. Mutations in *penA* and *mtrR* were associated with ESC resistance. Neither substitution C1192U in *16S rDNA* nor *rpsE* V25 mutations, associated with spectinomycin resistance, were found, suggesting a low prevalence of spectinomycin resistance.

Our study provides an overview of the *N. gonorrhoeae* strains circulating in Austria and their evolution over the past 5 years, both at the phenotypic and genomic level. It also underlines the benefits of genomic surveillance of *N. gonorrhoeae*, which can support epidemiologic investigations and provide information on specific genes and alleles thought to confer AMR (*14*).

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Appendix

Methods

Whole-Genome Sequencing

Genomic DNA isolation, WGS, assembly and contig filtering were performed as described previously (*1*). High-molecular-weight DNA was isolated from cultures using the MagAttract HMW DNA Kit (QIAGEN, Hilden, Germany), following the manufacturer's protocol for Gram-negative bacteria. Ready-to-sequence libraries were obtained with NexteraXT kit following the manufacturer's protocol (Illumina, CA, United States). Paired-end sequencing (2 × 300 bp) was performed on a MiSeq instrument as recommended by the manufacturer (Illumina). Raw reads were de novo assembled into a draft genome using SPAdes (version 3.11.1) (*2*). Contigs were filtered for a minimum coverage of 5 and minimum length of 200 bp. Sequencing quality was checked with FastQC. Sequencing generated 106,428 to 2,927,502 reads, a coverage of 12- to 272-fold (mean 76, 95% confidence interval [74.3–77.6]), a mean N50 of 38,513 (95% confidence interval [174–153,250]) and a mean contig length of 8,395 (95% confidence interval [208–23,184]).

Core-Genome MLST (cgMLST)

A local *N. gonorrhoeae* cgMLST scheme was generated with SeqSphere+ target definer tool (version 6.0.0, Ridom, Münster, Germany) (*3*). Strain MS11 was used as a seed genome (NCBI accession number NC_022240.1) and 47 complete *N. gonorrhoeae* genomes were used as query sequences (accession numbers NC_002946.2, NC_011035.1, NZ_CP012026.1, NZ_CP012027.1, NZ_CP012028.1, NZ_CP016015.1, NZ_CP016016.1, NZ_CP016017.1, ABZF00000000.1, ABZG00000000.1, ABZH00000000.1, ACIG00000000.1,

ADAA00000000.1, ABZJ00000000.2, ABZI00000000.1, ABZM00000000.1, ABZL00000000.1, ABZN00000000.1, ABZO00000000.1, ABZP00000000.1, ABZQ00000000.1, CQLK00000000.1, CQJM00000000.1, CQME00000000.1, CQJI00000000.1, CQIM00000000.1, CQHK00000000.1, CQLD00000000.1, CQNW00000000.1, CQKW00000000.1, CQJY00000000.1, CQIY00000000.1, CQJB00000000.1, CQKU00000000.1, CQOV00000000.1, CQIR00000000.1, CQJZ00000000.1, CQKM00000000.1, CQMI00000000.1, CQMT00000000.1, CQKB00000000.1, CQOT00000000.1, CQJD00000000.1, CHZN00000000.1, CFRU00000000.1, AKCG00000000.1, AKCH00000000.1), with default software parameters. A 1,524 loci cgMLST scheme and a 463 loci accessory target scheme were obtained, which were used in a previous publication (*4*).

Antimicrobial Resistance Genes Identifier, Adapted from PathogenWatch

Genotypic antibiotic resistance was investigated using allele libraries of *16S rDNA* (coding for 16S ribosomal RNA), *23S rDNA* (coding for 23S ribosomal RNA), *blaTEM*, *ereA*, *ereB*, *ermA*, *ermB*, *ermC*, *ermF*, *folP*, *gyrA*, *macAB promoter*, *mefA*, *mtrC*, *mtrR*, *mtrR promoter*, *mtr mosaic*, *norM promoter*, *parC*, *parE*, *penA*, *ponA1*, *porB1b*, *rplD*, *rplV*, *rpoB*, *rpoD*, *rpsE*, *rpsJ* and *tetM*, based on the library of PathogenWatch in TOML format (version 0.0.14) (*5*). Each allele library was implemented in SeqSphere+ (Ridom) and used to search assembled genomes. Alleles were matched if they reached 99% alignment to reference sequences. Alleles with >90% identity to reference sequences but no match were defined as “new allele” and aligned with reference sequences to identify mutations. All 1,318 study isolates were searched for genetic AMR using this tool.

Data Analysis

Statistical analysis was performed using R version 4.0.4. A positive outcome was defined as resistance to azithromycin, cefixime, ciprofloxacin, tetracycline, or penicillin, reduced susceptibility to ceftriaxone, or positivity for cefinase. For time series analysis, thirteen-weeks moving averages of collection dates were calculated (R packages ISOweek (*6*), zoo (*7*)). The percentage of resistant isolates (or with reduced susceptibility to ceftriaxone/positive for cefinase) over time was plotted, and trends were calculated by linear regression.

For risk factor identification, odds ratios (OR) were calculated for each outcome using univariate analysis (package epitools (*8*)). Multivariate analysis consisted in logistic regression including several explanatory variables (function glm and package broom (*9*)). Only genes or mutations reported to induce AMR to a given antibiotic by the PathogenWatch tool (*5*) were considered as potential explanatory variables. Explanatory variables were progressively included in the model until the lowest Akaike information criterion was reached. Adjusted odds ratio (aOR) were calculated

Data Visualization

Isolates were characterized by seven loci MLST scheme (*10*), NG-MAST (*11*) and by an in-house cgMLST scheme using SeqSphere+ (Ridom). Minimum spanning trees (MST) were computed using the number of cgMLST allelic differences between 1,304 isolates (14 were excluded due to <90% cgMLST good targets). Neighbor-joining tree (NJT) of the cgMLST analysis was exported from SeqSphere+ (Ridom, Münster, Germany) and loaded into R to compute dendrograms (packages ggplot2 (*12*), ggpubr (*13*), ape (*14*), ggtree (*15*)). Histograms and boxplots were created with R packages ggplot2 (*12*), viridis (*16*), RColorBrewer (*17*) and scales (*18*).

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Appendix Table 1. Measures of association between the different classes of antimicrobial resistance (N = 1,318)*

		Resistant			Susceptible			Univariate analysis	
AMR		tot	#	%	tot	#	%	OR [95%CI]	p.value
Azithromycin	Cefixime	121	0	0%	1180	35	3%	0 [0-NA]	0.06878
	Ceftriaxone	122	1	0.8%	1180	32	2.7%	0.296 [0.04–2.19]	0.3581
	Ciprofloxacin	122	88	72.1%	1179	688	58.4%	1.85 [1.22–2.79]	0.0035
	Tetracycline	121	86	71.1%	1077	472	43.8%	3.15 [2.09–4.75]	0
	Penicillin	122	2	1.6%	1180	202	17.1%	**0.081 [0.02–0.329]**	0
	Cefinase	121	1	0.8%	1135	181	15.9%	**0.044 [0.006–0.316]**	0
Cefixime	Azithromycin	35	0	0%	1266	121	9.6%	0 [0-NA]	0.06878
	Ceftriaxone	35	16	45.7%	1276	17	1.3%	**62.4 [27.5–142]**	0
	Ciprofloxacin	35	35	100%	1275	746	58.5%	Inf [NA-Inf]	0
	Tetracycline	27	19	70.4%	1180	542	45.9%	2.8 [1.21–6.44]	0.01761
	Penicillin	35	23	65.7%	1276	182	14.3%	11.5 [5.63–23.6]	0
	Cefinase	35	1	2.9%	1230	182	14.8%	0.169 [0.023–1.25]	0.04921
Ceftriaxone	Azithromycin	33	1	3%	1269	121	9.5%	0.296 [0.04–2.19]	0.3581
	Cefixime	33	16	48.5%	1278	19	1.5%	**62.4 [27.5–142]**	0
	Ciprofloxacin	33	32	97%	1278	750	58.7%	22.5 [3.07–165]	0
	Tetracycline	25	19	76%	1183	543	45.9%	3.73 [1.48–9.41]	0.00375
	Penicillin	33	22	66.7%	1279	183	14.3%	12 [5.71–25.1]	0
	Cefinase	29	2	6.9%	1237	181	14.6%	0.432 [0.102–1.83]	0.41838
Ciprofloxacin	Azithromycin	776	88	11.3%	525	34	6.5%	1.85 [1.22–2.79]	0.0035
	Cefixime	781	35	4.5%	529	0	0%	Inf [NA-Inf]	0
	Ceftriaxone	782	32	4.1%	529	1	0.2%	22.5 [3.07–165]	0
	Tetracycline	714	480	67.2%	494	82	16.6%	10.3 [7.76–13.7]	0
	Penicillin	782	200	25.6%	529	4	0.8%	**45.1 [16.6–122]**	0
	Cefinase	748	177	23.7%	517	5	1%	**31.7 [12.9–77.8]**	0
Tetracycline	Azithromycin	558	86	15.4%	640	35	5.5%	3.15 [2.09–4.75]	0
	Cefixime	561	19	3.4%	646	8	1.2%	2.8 [1.21–6.44]	0.01761
	Ceftriaxone	562	19	3.4%	646	6	0.9%	3.73 [1.48–9.41]	0.00375
	Ciprofloxacin	562	480	85.4%	646	234	36.2%	**10.3 [7.76–13.7]**	0
	Penicillin	562	141	25.1%	646	33	5.1%	6.22 [4.17–9.27]	0
	Cefinase	545	143	26.2%	617	19	3.1%	11.2 [6.83–18.4]	0
Penicillin	**Azithromycin**	204	2	1%	1098	120	10.9%	**0.081 [0.02–0.329]**	0
	Cefixime	205	23	11.2%	1106	12	1.1%	11.5 [5.63–23.6]	0
	Ceftriaxone	205	22	10.7%	1107	11	1%	12 [5.71–25.1]	0
	Ciprofloxacin	204	200	98%	1107	582	52.6%	**45.1 [16.6–122]**	0
	Tetracycline	174	141	81%	1034	421	40.7%	6.22 [4.17–9.27]	0
	Cefinase	194	150	77.3%	1072	33	3.1%	**107 [66.2–174]**	0
Cefinase	**Azithromycin**	182	1	0.5%	1074	120	11.2%	**0.044 [0.006–0.316]**	0
	Cefixime	183	1	0.5%	1082	34	3.1%	0.169 [0.023–1.25]	0.04921
	Ceftriaxone	183	2	1.1%	1083	27	2.5%	0.432 [0.102–1.83]	0.41838
	Ciprofloxacin	182	177	97.3%	1083	571	52.7%	**31.7 [12.9–77.8]**	0
	Tetracycline	162	143	88.3%	1000	402	40.2%	11.2 [6.83–18.4]	0
	Penicillin	183	150	82%	1083	44	4.1%	**107 [66.2–174]**	0

*A positive outcome was defined as resistance to azithromycin, cefixime, tetracycline and penicillin, reduced susceptibility to ceftriaxone and positivity for cefinase. For each variable, number of isolates (#), total number of isolates and frequency (%) are indicated for resistant (or with reduced susceptibility/positive) and susceptible (or negative) isolates. Odds ratio (OR) and 95% confidence interval were calculated by univariate analysis and association was tested with Fisher exact test.

Appendix Table 2. Genes and point mutations associated with antimicrobial resistance in *N. gonorrhoeae* isolates (N = 1,318). For each gene, number of isolates (#), total number of isolates for which the gene was found (tot) and frequency (%) are indicated

Gene	Variant	#	%
16S_rDNA	C1450	639	48.5%
	none	545	41.4%
	NA	134	10.2%
23S_rDNA	C2597	3	0.2%
	C2597.C265	1	0.1%
	C265	123	9.3%
	none	1124	85.3%
	NA	67	5.1%
blaTEM	not found	1185	89.9%
	found	133	10.1%
ereA	not found	1318	100%
	found	0	
ereB	not found	1318	100%
	found	0	
ermA	not found	1318	100%
	found	0	
ermB	not found	1318	100%
	found	0	
ermC	not found	1318	100%
	found	0	
ermF	not found	1314	99.7%
	found	4	0.3%
folP	R228	1081	0.82
	none	229	17.4%
	NA	8	0.6%
gyrA	D95	484	36.7%
	D95.S91	291	22.1%
	none	529	40.1%
	NA	14	1.1%
macAB_promotor	mut-10	129	9.8%
	none	1182	89.7%
	NA	7	0.5%
mefA	not found	1318	100%
	found	0	
mtr_mosaic	not found	1146	86.9%
	found	172	13.1%
mtrC	frameshift	23	1.7%
	none	1282	97.3%
	NA	13	1.0%
mtrR	found	1258	95.4%
	A39	337	25.6%
	A39.G45	48	3.6%
	frameshift	126	9.6%
	G45	160	12.1%
	none	637	48.3%
	NA	10	0.8%
mtrR_promoter	C187G	7	0.5%
	del-35	302	22.9%
	ins266A+ins253G	88	6.7%
	none	731	55.5%
	NA	190	14.4%
norM_promoter	ins211	62	4.7%
	ins211.ins250	124	9.4%
	ins250	5	0.4%
	none	1117	84.7%
	NA	10	0.8%
parC	D86N	284	21.5%
	E91G/Q	80	6.1%
	E91G/Q.S87I	12	0.9%
	E91G/Q.S87N	20	1.5%
	E91K.S87N	25	1.9%
	S87I	1	0.1%
	S87N	29	2.2%
	S87N.S88P	2	0.2%
	S87R	198	0.15
	S87R.S88P	103	7.8%
	S88P	21	1.6%

Gene	Variant	#	%
	none	529	40.1%
	NA	14	1.1%
penA	A501T/V	1	0.1%
	A501T/V.ins346D	63	4.8%
	A501T/V.ins346D.P551S/L	94	7.1%
	G545S.I312M+V316T.I312M+V316T	304	23.1%
	I312M+V316T	2	0.2%
	I312M+V316T.P551S/L	1	0.1%
	ins346D	706	53.6%
	ins346D.P551S/L	16	1.2%
	none	119	0.09
	NA	12	0.9%
ponA1	L421	561	42.6%
	none	738	0.56
	NA	19	1.4%
porB1b	A121	162	12.3%
	A121.G120	392	29.7%
	G120	33	2.5%
	none	579	43.9%
	NA	152	11.5%
rplD	G68	18	1.4%
	G70	40	0.03
	none	1252	0.95
	NA	8	0.6%
rpsE	D11	141	10.7%
	none	1170	88.8%
	NA	7	0.5%
rpsJ	V57	961	72.9%
	none	342	25.9%
	NA	15	1.1%
tetM	not found	1061	80.5%
	found	257	19.5%
All		1318	100%

Appendix Table 3. Genetic risk factors associated with resistant *N. gonorrhoeae* isolates (N = 1,318)*

	Patient data		Resistant			Susceptible			Univariate analysis		multivariate analysis	
Amr	Variable	Category	Tot	#	%	Tot	#	%	OR [95%CI]	p.value	OR [95%CI]	p.value
Azithromycin	23S_rDNA	C265T	115	3	2.6%	1122	120	10.7%	0.224 [0.07–0.715]	0.00294		
	macAB_promotor	mut-10	120	80	66.7%	1175	48	4.1%	**47 [29.1–75.7]**	**0**	**27.7 [1.3–231]**	**0.00566**
	mtrR_promoter	del-35	12	7	58.3%	1101	293	26.6%	3.86 [1.22–12.3]	0.02107	2.59 [0.594–11.2]	0.18732
	mtr_mosaic	found	122	103	84.4%	1180	68	5.8%	**88.7 [51.3–153]**	**0**		
	mtrR	A39T	120	11	9.2%	1172	370	31.6%	0.219 [0.116–0.412]	0		
		frameshift	120	1	0.8%	1172	121	10.3%	**0.073 [0.01–0.527]**	**0.00012**		
		G45D/S	120	3	2.5%	1172	204	17.4%	0.122 [0.038–0.387]	1.00E-06	1.54e-07 [NA-1.63e+37]	0.98996
Cefixime	mtrR_promoter	del-35	35	0	0%	1088	302	27.8%	0 [0-NA]	2.00E-05	3.95e-09 [2.43e-312–9.92e+39]	0.99335
	mtrR	A39T	35	0	0%	1266	383	30.3%	0 [0-NA]	6.00E-06	3.62e-09 [1.94e-281–1.05e+35]	0.99256
		G45D/S	35	29	82.9%	1266	179	14.1%	**29.4 [12–71.7]**	**0**	2.27 [0.875–6.71]	0.10868
	penA	G545S	35	35	100%	1264	268	21.2%	Inf [NA-Inf]	0	3.7e+08 [4.46e+25–1.06e+258]	0.98916
Ceftriaxone	penA	A501T/V	32	5	15.6%	1268	153	12.1%	1.35 [0.512–3.56]	0.57969	**73.9 [6.9–3.17e+03]**	**0.00421**
		G545S	32	26	81.2%	1268	277	21.8%	**15.5 [6.32–38]**	**0**	**16.2 [2.95–369]**	**0.01321**
		I312M+V316T	32	26	81.2%	1268	280	22.1%	**15.3 [6.23–37.5]**	**0**		
		ins346D	32	5	15.6%	1268	869	68.5%	**0.085 [0.033–0.222]**	**0**	**0.0723 [0.00231–2.18]**	**0.09253**
Ciprofloxacin	gyrA	D95N/G/A/Y	772	767	99.4%	525	5	1%	**1.6e+04 [4.6e+03–5.54e+04]**	**0**	**7.56e+03 [2.33e+03–3.31e+04]**	**8.66E-27**
		S91F/T	772	290	37.6%	525	1	0.2%	**315 [44.1–2.25e+03]**	**0**		
	norM_promoter	ins211A	775	161	20.8%	526	24	4.6%	5.49 [3.52–8.56]	0	8.11 [1.04–53.2]	0.03637
		ins250T	775	117	15.1%	526	11	2.1%	8.32 [4.44–15.6]	0		
	parC	D86N	774	252	32.6%	523	29	5.5%	8.22 [5.49–12.3]	0	4.5 [0.814–26.8]	0.09268
		E91G/Q	774	91	11.8%	523	22	4.2%	3.03 [1.88–4.9]	1.00E-06		
		S87N	774	75	9.7%	523	1	0.2%	**56 [7.76–404]**	**0**		
		S87R	774	299	38.6%	523	2	0.4%	**164 [40.6–662]**	**0**	**24.2 [2–463]**	**0.03343**
Tetracycline	mtrR_promoter	del-35	437	128	29.3%	584	151	25.9%	1.19 [0.9–1.57]	0.22835	5.3 [3.29–8.71]	1.74E+02
	mtrR	A39T	560	162	28.9%	639	190	29.7%	0.962 [0.75–1.23]	0.79937	0.535 [0.306–0.921]	0.02552
		G45D/S	560	110	19.6%	639	79	12.4%	1.73 [1.26–2.37]	0.00062	2.52 [1.53–4.2]	0.00033
	rpsJ	V57M	555	552	99.5%	639	323	50.5%	**180 [57.3–566]**	**0**	**113 [41.1–467]**	**3.18E-01**
	tetM	found	562	223	39.7%	646	3	0.5%	**141 [44.8–444]**	**0**	**345 [104–2.14e+03]**	**1.44E-01**
Penicillin	blaTEM	found	205	100	48.8%	1107	33	3%	**31 [19.9–48.2]**	**0**	**67.9 [35.2–139]**	**1.08E-19**
	mtrR_promoter	del-35	203	30	14.8%	920	272	29.6%	0.413 [0.273–0.624]	1.1e-05	0.257 [0.118–0.53]	0.00035
		ins266A+ins253G	203	20	9.9%	920	68	7.4%	1.37 [0.811–2.31]	0.24839	3.55 [1.42–8.45]	0.00501
	mtrR	A39T	204	47	23%	1098	336	30.6%	0.679 [0.478–0.964]	0.02974		
		frameshift	204	34	16.7%	1098	92	8.4%	2.19 [1.43–3.35]	0.00068		
		G45D/S	204	74	36.3%	1098	134	12.2%	4.09 [2.92–5.74]	0	2.29 [1.18–4.42]	0.01352
	penA	G545S	203	77	37.9%	1097	226	20.6%	2.35 [1.71–3.24]	0		
		I312M+V316T	203	77	37.9%	1097	229	20.9%	2.32 [1.68–3.19]	1.00E-06		
		P551S/L	203	9	4.4%	1097	102	9.3%	0.453 [0.225–0.91]	0.02004	0.368 [0.121–1.01]	0.06114
	ponA1	L421P	203	136	67%	1090	424	38.9%	3.19 [2.32–4.38]	0	4.5 [2.3–8.95]	1.28E+08
	porB1b	A121D/N/S/G/V	173	101	58.4%	988	451	45.6%	1.67 [1.2–2.32]	0.00222		
		G120K/N/D/Q/R	173	113	65.3%	988	311	31.5%	4.1 [2.92–5.76]	0	6.15 [3.16–12.3]	1.60E+07
Cefinase	blaTEM	found	183	118	64.5%	1083	13	1.2%	**149 [80–279]**	**0**	**234 [93.3–683]**	**2.73E-13**

Amr	Patient data		Resistant			Susceptible			Univariate analysis		multivariate analysis	
	Variable	Category	Tot	#	%	Tot	#	%	OR [95%CI]	p.value	OR [95%CI]	p.value
	mtrR_promoter	del-35	180	25	13.9%	898	267	29.7%	0.381 [0.244–0.595]	6.00E-06	0.0892 [0.0248–0.277]	8.56E+09
		ins266A+ins253G	180	21	11.7%	898	64	7.1%	1.72 [1.02–2.9]	0.04805	5.77 [2.23–14.2]	0.00017
	mtrR	A39T	182	76	41.8%	1074	300	27.9%	1.85 [1.34–2.56]	0.00023		
		frameshift	182	35	19.2%	1074	88	8.2%	2.67 [1.74–4.09]	2.00E-05		
	penA	A501T/V	181	56	30.9%	1073	97	9%	4.51 [3.09–6.58]	0	**35.6 [14–97.4]**	**3.43E+01**
		G545S	181	29	16%	1073	253	23.6%	0.618 [0.406–0.942]	0.02655		
		I312M+V316T	181	29	16%	1073	255	23.8%	0.612 [0.402–0.933]	0.02117		
		ins346D	181	150	82.9%	1073	706	65.8%	2.52 [1.68–3.78]	3.00E-06	3.49 [1.66–7.57]	0.00116
		P551S/L	181	8	4.4%	1073	97	9%	0.465 [0.222–0.974]	0.04125	0.107 [0.0287–0.378]	0.00058
	porB1b	A121D/N/S/G/V	147	52	35.4%	973	472	48.5%	0.581 [0.405–0.833]	0.00334		
		G120K/N/D/Q/R	147	60	40.8%	973	336	34.5%	1.31 [0.917–1.86]	0.13973	4.56 [2.17–9.67]	6.61E+09

*A positive outcome was defined as resistance to azithromycin, cefixime, tetracycline and penicillin, reduced susceptibility to ceftriaxone and positivity for cefinase. For each variable, number of isolates (#), total number of isolates and frequency (%) are indicated for resistant (or with reduced susceptibility/positive) and susceptible (or negative) isolates. For univariate analysis, odds ratio (OR) and 95% confidence interval were calculated and association was tested with Fisher exact test. For multivariate analysis, variables with significant association in univariate analysis were included in a logistic regression model. Adjusted odds ratio (aOR), 95% confidence interval and p_{value} were calculated for the model with the lowest Akaike information criterion.