Genomic and Phenotypic Variability in *Neisseria gonorrhoeae* Antimicrobial Susceptibility, England

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Antimicrobial resistance (AMR) in *Neisseria gonorrhoeae* is a global concern. Phylogenetic analyses resolve uncertainties regarding genetic relatedness of isolates with identical phenotypes and inform whether AMR is due to new mutations and clonal expansion or separate introductions by importation. We sequenced 1,277 isolates with associated epidemiologic and antimicrobial susceptibility data collected during 2013–2016 to investigate *N. gonorrhoeae* genomic variability in England. Comparing genetic markers and phenotypes for AMR, we identified 2 *N. gonorrhoeae* lineages with different antimicrobial susceptibility profiles and 3 clusters with elevated MICs for ceftriaxone, varying mutations in the *penA* allele, and different epidemiologic characteristics. Our results indicate *N. gonorrhoeae* with reduced antimicrobial susceptibility emerged independently and multiple times in different sexual networks in England, through new mutation or recombination events and by importation. Monitoring and control for AMR in *N. gonorrhoeae* should cover the entire population affected, rather than focusing on specific risk groups or locations.

Antimicrobial resistance (AMR) in *Neisseria gonorrhoeae* is a global concern and affects all classes of antimicrobial drugs used for treatment. Penicillin, ciprofloxacin, and cefixime were the recommended first-line antimicrobial drug therapies until AMR prevalence breached the World Health Organization (WHO) recommended threshold of ≥5% of local isolates demonstrating resistance; at that point, ceftriaxone became the preferred antimicrobial drug treatment (*1*). However, ceftriaxone resistance has been reported in many countries and frequently in East and Southeast Asia, probably because of poor antimicrobial stewardship (*2*). Ceftriaxone resistance has been linked to mutations in the *penA* gene, which has been reported in several continents, including North America and Europe (*3*–*5*).

To clarify the spread of AMR in *N. gonorrhoeae* and the population groups most at risk, surveillance programs and research studies often link phenotypic susceptibility data with data on the epidemiologic characteristics of cases (*6*,*7*). However, these analyses are limited because isolates with identical phenotypes might not be genetically related. Consequently, determining the extent to which AMR transmission is due to clonal dissemination or separate introductions is challenging and these data are essential to guide the public health response.

Combining phenotypic and genomic data can help resolve uncertainties. Whole-genome sequencing (WGS) enables investigation of genetic determinants for AMR and how these are distributed in the pathogen population (*4*,*5*). WGS also can contribute evidence toward the development of rapid antimicrobial susceptibility tests to improve treatment decisions (*8*,*9*). However, few *N. gonorrhoeae* WGS studies have been conducted in England, and none include representative geographic coverage over time (*10*–*13*).

We investigated the genomic and phenotypic variability in *N. gonorrhoeae* antimicrobial susceptibility in England. We described the epidemiologic characteristics of genetically distinct clusters of infection with reduced susceptibility to cefixime, ceftriaxone, and azithromycin and resistance to ciprofloxacin and penicillin. We focused on *N. gonorrhoeae* with mutations in the *penA* allele, which contribute to reduced susceptibility to ceftriaxone. In addition, we assessed the genetic similarity of *N. gonorrhoeae* in England, Europe, and the United States to determine the

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extent to which international travel might influence the spread of AMR in *N. gonorrhoeae*.

Methods

Isolate Selection

We selected *N. gonorrhoeae* isolates from the archives of the Gonococcal Resistance to Antimicrobials Surveillance Programme (GRASP), a sentinel program implemented by Public Health England (PHE) in 2000. GRASP is designed to represent the gonococcal population in England (*14*) and includes clinical, sociodemographic, and behavioral data collected through the GUMCAD STI Surveillance System (https://www.gov.uk/guidance/gumcad-sti-surveillance-system) and directly from clinics. During a 3-month period each year, GRASP tests for antimicrobial susceptibility in consecutive isolates from all culture-positive *N. gonorrhoeae* cases identified in 26 sexual health clinics in England and Wales (*15*). GRASP collects ≈1,200–2,500 isolates annually for antimicrobial susceptibility testing (*16*).

We selected isolates collected from 5 GRASP clinics, 2 in London and 3 in other geographically distinct areas in England: Birmingham, Bristol, and Liverpool. We chose these locations to obtain isolates from cases representing a broad range of sociodemographic and behavioral characteristics, including sex, sexual orientation, age, ethnicity, and HIV status (Appendix Table 1, https://wwwnc.cdc.gov/EID/article/26/3/19-0732-App1.pdf). We sequenced all isolates collected during 2013–2016 by the 5 clinics and stored in the GRASP archive. We chose the most recent years of GRASP data to investigate prevailing trends and patterns. We did not include isolates from a 2015 outbreak of high-level azithromycin-resistant *N. gonorrhoeae* in the United Kingdom in this sampling frame because the isolates did not meet the eligibility criteria of our study.

Ethics Considerations

PHE has permission to process confidential patient data obtained by GRASP under Regulation 3 (Control of Patient Information) of the Health Service Regulations 2002. Information governance advice and ethics approval for this study were granted by the PHE Research Ethics and Governance Group.

Antimicrobial Susceptibility Testing

GRASP tests isolates for antimicrobial susceptibility by using agar dilution methods, records MICs for antimicrobial drugs, and defines AMR by using European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints. For our study, we used data on MICs for ceftriaxone, azithromycin, cefixime, penicillin, and ciprofloxacin (*17*). In GRASP, epidemiologic data were linked to MICs for antimicrobial drugs for each isolate phenotype.

Isolation and WGS

We retrieved selected isolates from the GRASP archive by culturing on nonselective gonococcus agar (Difco BBL GC II Agar Base [Becton, Dickinson and Company, https://www.bd.com] plus 1% Vitox [Oxoid, http://www.oxoid.com]). We extracted DNA from a subculture of a single colony of each isolate by using the automated QIAsymphony DNA Mini Kit (QIAGEN, https://www.qiagen.com). WGS was conducted at the Wellcome Sanger Institute (Cambridge, UK) by using the HiSeq X Ten system (Illumina, https://www.illumina.com) and processed in the routine Sanger WGS data management pipeline (Appendix).

Data Sources from Europe and the United States

We compared the study sample with published WGS and associated metadata for *N. gonorrhoeae* isolates from international studies. The collection from Europe (European Nucleotide Archive (ENA; accession no. PRJEB9227) contained 1,054 isolates from the European Gonococcal Antimicrobial Surveillance Programme (Euro-GASP) (*5*). Isolates were collected in 2013 from 20 countries and included 106 isolates from England. We excluded 21% (22/106) that were duplicates of isolates in the study sample, leaving 948 isolates from Europe. The metadata for isolates from Europe included reporting country, antimicrobial susceptibility profile, MICs for ceftriaxone and cefixime, and the presence of the *penA*-34 allele. We grouped MICs to match the categories used in GRASP.

Isolates from the United States were from 2 previous studies investigating the association between phenotype and genotype for AMR in *N. gonorrhoeae* (*4*,*9*). The collection from the United States contained 1,114 isolates collected during 2000–2013 (ENA accession nos. PRJEB2999 and PRJEB7904). The metadata for the isolates from the United States included antimicrobial susceptibility profiles; 270 had reduced susceptibility to cephalosporin (MIC ≥0.25 mg/L for cefixime or MIC ≥0.125 mg/L for ceftriaxone); 294 had reduced susceptibility to azithromycin (MIC ≥2 mg/L); and 594 were ciprofloxacin-resistant (MIC ≥1 mg/L). Metadata also included sexual orientation of case-patients and the presence of the *penA*-34 allele. We grouped MICs to match the categories used in GRASP.

Phylogenetic Analysis

We created phylogenetic trees and removed genetic recombination events by using default settings in Gubbins version 2.4.0 (*18*), including 5 iterations and ≥3 base substitutions to identify a recombination event, and the RAxML (Geneious, https://www.geneious.com) or FastTree (*19*) tree building option (Appendix). We created 3 phylogenetic trees: isolates from England only, isolates from England and other countries in Europe, and isolates from England and the United States.

We identified known genetic markers of AMR, including mutations in the *penA* allele, by using ARIBA (*20*). We compared MICs to the genetic markers by using the ARIBA micplot module.

We identified the genotype of isolates in large and distinct clusters of *N. gonorrhoeae* with elevated MICs for ceftriaxone (MIC ≥0.015 mg/L) and cefixime (MIC ≥0.03 mg/L) from the phylogenetic trees. We compared the epidemiologic characteristics of cases in the clusters by using the χ^2 or Fisher exact test.

Statistical Analysis

We used univariate and multivariable analyses to assess differences in the epidemiologic characteristics and antimicrobial susceptibility of isolates between lineages identified in the phylogenetic tree. We analyzed the following explanatory variables: year and location the isolate was collected; case-patient information, including gender, sexual orientation, age, ethnicity, country of birth, whether they had a symptomatic *N. gonorrhoeae* infection or previous sexually transmitted infection (STI), HIV status, and the number of sexual partners they had in the United Kingdom or through travel-associated sexual partnerships ≤3 months before diagnosis; and isolate susceptibility data, including reduced susceptibility to ceftriaxone (MIC ≥0.015 mg/L), cefixime (MIC ≥0.03 mg/L), or azithromycin (MIC ≥0.25 mg/L); or resistance to penicillin (MIC >1 mg/L or β-lactamase positive) or ciprofloxacin (MIC >0.06 mg/L). We used elevated MIC thresholds for ceftriaxone, cefixime, and azithromycin to provide a robust sample size for regression analysis.

We also explored the relationship between travel-associated sexual partnerships and reduced susceptibility to antimicrobial drugs by conducting univariate and multivariable analyses with reduced susceptibility or resistance as the outcome and travel-associated sexual partnerships as the primary explanatory variable. We considered CI of the odds ratio (OR) >1.0 and $p<0.05$ by χ^2 test as statistically significant.

We developed multivariable logistic regression models by using a forward approach and including only statistically significant variables associated with the outcome in the univariate model to control for possible confounding between variables. We used the likelihood ratio test to determine which explanatory variables should remain in the multivariable model by using $p<0.05$ as the threshold of statistical significance.

Results

Sample Description

Of the eligible isolates, we successfully sequenced 91% (1,277/1,407); the bacteria of the remaining 130 isolates were no longer viable for DNA extraction. For all antimicrobial drugs tested, the MIC distributions of the study isolates were similar to those of all GRASP isolates (Appendix Table 2). We found that 3.6% of isolates were resistant to azithromycin (MIC >0.5 mg/L) and 2 isolates were highly resistant (MIC ≥256 mg/L); 0.6% were resistant to cefixime (MIC >0.125 mg/L), 36.3% to ciprofloxacin (MIC >0.06 mg/L), 16.6% to penicillin (MIC >1 mg/L), and none to ceftriaxone (MIC >0.125 mg/L). The MIC distribution of the isolates not sequenced was similar to the distribution of the sequenced isolates. Most (69%; 881/1,277) isolates were from genital infections, 23.4% (299/1,277) were from rectal infections, and 6.3% (80/1,277) were from pharyngeal infections. Overall, we identified 226 different sequence types (STs) by using *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) (Appendix). We deposited novel sequences extracted for this study into ENA (accession no. ERP022090) and provide metadata (Appendix).

N. gonorrhoeae Lineages Circulating in England

We noted 2 distinct lineages in the phylogenetic tree (Figure 1). Compared with lineage B, lineage A was more likely to contain isolates from clinics in London than those outside of London (outside London:in London adjusted odds ratio [aOR] 1.74, 95% CI 1.27–2.67; p = 0.001). Lineage A also contained more isolates from persons ≥35 years of age than persons ≤24 years of age (aOR 1.68, 95% CI 1.16–2.40; p = 0.006). Asian ethnicity also was associated more frequently with isolates from lineage A compared with white ethnicity (aOR 1.86, 95% CI 1.01–3.45; p = 0.048) (Table 1). Lineage A was less likely to contain isolates from women (aOR 0.14, 95% CI 0.09–0.22; p<0.001) or men who reported having sex with women exclusively (MSW; aOR 0.33, 95% CI 0.23–0.47; p<0.001) compared with MSM. This lineage also was less likely to contain isolates from persons reporting black Caribbean ethnicity compared with persons reporting

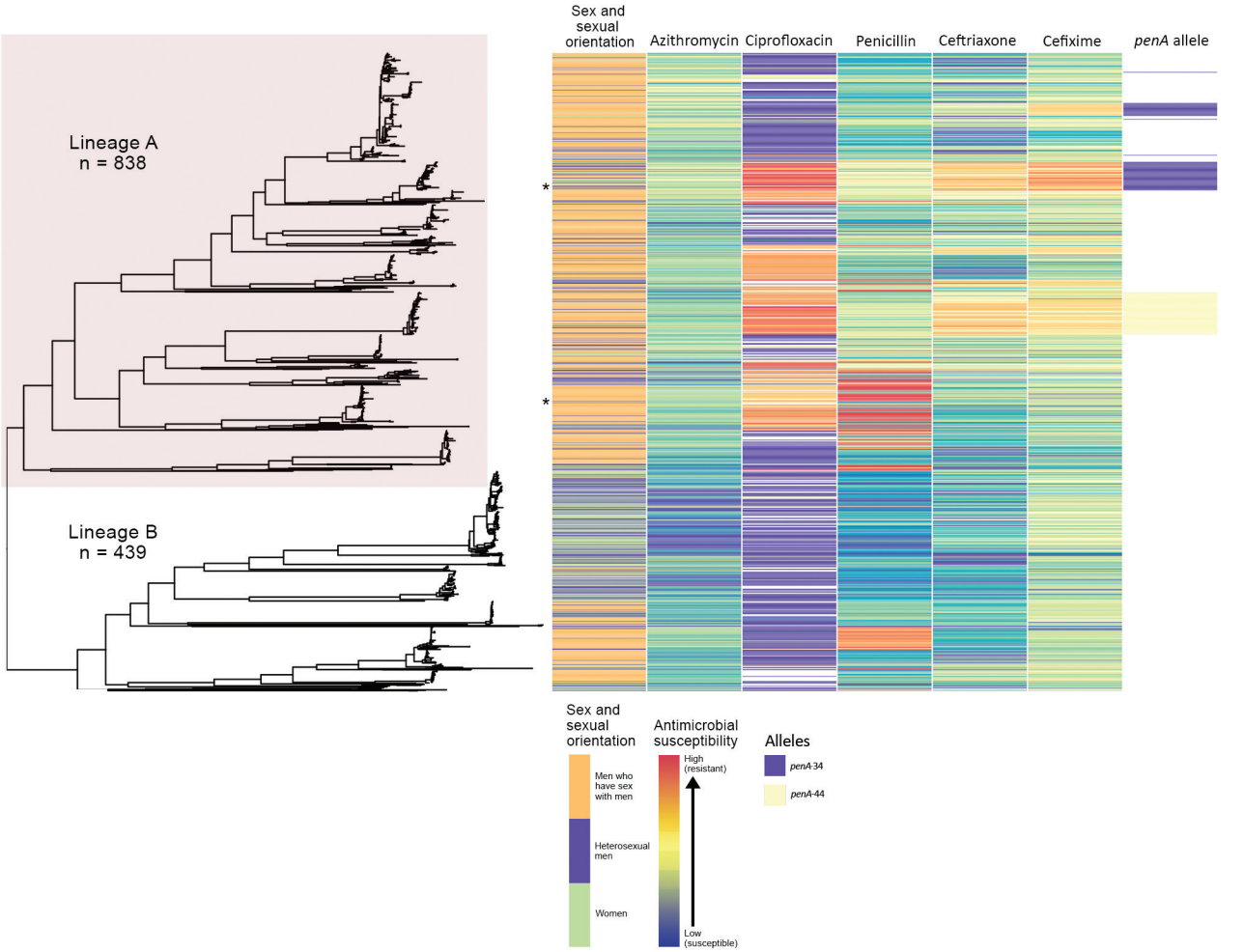

Figure 1. Phylogeny and antimicrobial susceptibility of *Neisseria gonorrhoeae* isolates from England, 2013–2016. Maximum-likelihood phylogeny with recombination events removed of all *N. gonorrhoeae* isolates annotated with gender and sexual orientation, antimicrobial susceptibility phenotype, and *penA* genotype. Asterisks represent location in tree of isolates with high-level azithromycin resistance (MIC ≥256 mg/L). Heterosexual men were those who reported sex with women exclusively.

white ethnicity (aOR 0.49, 95% CI 0.32–0.76; p = 0.001). Lineage A was more likely to contain isolates from persons who reported a travel-associated sexual partnership compared with isolates from lineage B (crude odds ratio [cOR] 1.96, 95% CI 1.20–3.21; p = 0.006), but this association did not persist in the multivariable model (aOR 1.66, 95% CI 0.94–2.91; p = 0.078 adjusting for location, age, sex, sexual orientation, and ethnicity). However, isolates from persons who had a recent travel-associated sexual partnership were more likely to be resistant to ciprofloxacin (aOR 1.83, 95% CI 1.13–2.96; p = 0.015, adjusting for location, age, sex, sexual orientation, and ethnicity). We saw no statistically significant association between recent travel-associated sexual partnerships and *N. gonorrhoeae* with reduced susceptibility to ceftriaxone, cefixime, or azithromycin, or resistance to penicillin.

Isolates with reduced susceptibility to ceftriaxone, cefixime, or azithromycin or resistance to ciprofloxacin and penicillin were dispersed throughout the phylogenetic tree (Figure 1). However, compared with lineage B, isolates in lineage A were more likely to have higher MICs for ceftriaxone (aOR 15.4, 95% CI 8.50–27.8; p<0.001), cefixime (aOR 3.97, 95% CI 2.76–5.76; p<0.001), azithromycin (aOR 7.5, 95% CI 5.37–10.5; p<0.001), and penicillin (aOR 18.2, 95% CI 11.4–29.2; p<0.001) (Table 2).

Distribution of *penA* Alleles across the Phylogeny

Overall, we identified 32 different known mutations in 8 genes associated with resistance to ceftriaxone, cefixime, azithromycin, ciprofloxacin, or penicillin. For all antimicrobial drugs, we noted isolates with the same combination of genotypic markers of

Table 1. Univariate and multivariable analyses comparing the epidemiologic characteristics of cases of *Neisseria gonorrhoeae* between 2 phylogenetic lineages, England*

	Lineage, no.		Lineage A outcomes					
			Univariate			Multivariable		
Characteristics	A	B	cOR	95% CI	p value	aOR	95% CI	p value
Total	838	439						
Year								
2013	220	106	Ref					
2014	210	123	0.82	0.60–1.13	0.234			
2015	260	107	1.17	0.85–1.62	0.339			
2016	148	103	**0.69**	**0.49–0.98**	**0.035**			
Clinic location								
Outside London	630	136	Ref			Ref		
London	463	109	**3.73**	**2.86–4.88**	**<0.001**	**1.74**	**1.27–2.67**	**0.001**
Sex and sexual orientation								
MSM	630	136	Ref			Ref		
MSW	150	154	**0.21**	**0.15–0.29**	**<0.001**	**0.33**	**0.23–0.47**	**<0.001**
F	57	149	**0.08**	**0.05–0.12**	**<0.001**	**0.14**	**0.09–0.22**	**<0.001**
Age, y								
≤24	188	196	Ref			Ref		
25–34	342	161	**2.21**	**1.67–2.93**	**<0.001**	1.14	0.83–1.59	0.413
≥35	308	82	**3.92**	**2.81–5.46**	**<0.001**	**1.68**	**1.16–2.40**	**0.006**
Ethnicity								
White	586	238	Ref			Ref		
Black Caribbean	51	81	**0.26**	**0.17–0.38**	**<0.001**	**0.49**	**0.32–0.76**	**0.001**
Black African	27	20	**0.55**	**0.30–1.00**	**0.046**	0.84	0.43–1.64	0.607
Black Other	6	4	0.61	0.17–2.18	0.442	0.57	0.14–2.37	0.441
Asian	57	17	1.36	0.78–2.39	0.280	**1.86**	**1.01–3.45**	**0.048**
Other	24	8	1.22	0.54–2.75	0.634	0.99	0.41–2.44	0.999
Mixed	62	43	**0.59**	**0.39–0.89**	**0.011**	0.82	0.51–1.32	0.413
Place of birth								
United Kingdom	473	309	Ref					
Not United Kingdom	305	102	**1.95**	**1.49–2.56**	**<0.001**			
Symptomatic infection								
No	219	119	Ref					
Yes	526	277	1.03	0.79–1.35	0.818			
New STI diagnosis ≤1 year, excluding HIV								
No or unknown	615	363	Ref					
Yes	223	75	**1.75**	**1.31–2.35**	**<0.001**			
HIV status								
Negative or unknown	653	398	Ref					
Positive	185	41	**2.75**	**1.91–3.96**	**<0.001**			
Number of partners in the United Kingdom ≤3 months of diagnosis								
0	27	20	Ref					
1	175	162	0.80	0.43–1.48	0.478			
≥2	304	167	1.35	0.73–2.48	0.335			
Travel-associated sexual partnerships ≤3 months of diagnosis								
No	442	325	Ref					
Yes	64	24	**1.96**	**1.20–3.21**	**0.006**			

*The multivariable model was adjusted for location, gender and sexual orientation, age, and ethnicity. Bold text indicates statistical significance (p<0.05 and 95% CI does not cross 1.0). aOR, adjusted odds ratio; cOR, crude odds ratio; MSM, men who have sex with men; MSW, men who reported sexual activity exclusively with women; Ref, referent; STI, sexually transmitted infection.

resistance but differing phenotypic MICs (Appendix Figures 1–5).

The larger, distinct clusters with elevated MICs for ceftriaxone and cefixime contained the *penA*-34 allele and the *penA*-44 allele (Figure 1). All isolates with the *penA*-34 allele (n = 86) had a MIC of ≥0.015 mg/L for cefixime and 67 had a MIC of ≥0.015 mg/L for ceftriaxone. Most (81/84; 96%) isolates with the *penA*-44 allele had a MIC of ≥0.015 mg/L for cefixime, a MIC of ≥0.015 mg/L for ceftriaxone (83/84; 98%), or both (81/84; 96%).

The 2 largest clusters with the *penA*-34 allele (cluster 1, n = 57; cluster 2, n = 26) were genetically distinct from each other and isolates in the 2 groups had statistically significant differences by year, clinic, sexual orientation, and HIV status (Table 3). Most (81%; 21/26) isolates in cluster 2 were from London in 2014–2015, and most (67%; 38/57) in cluster 1 were from outside London but distributed across all 4 years of the study. Most (96%; 25/26) isolates in cluster 2 were from MSM; whereas cluster 1 was more mixed and composed of isolates from women (21%; 12/57), MSW (37%; 21/57), and MSM (42%; 24/57). Cluster 2 had a higher percentage of persons living with HIV (35%; 9/26) than did cluster 1 (7%; 4/57). Most (82%;

69/84) isolates with the *penA*-44 allele were from MSM, persisted over all 4 years of the study, and were found both inside and outside of London. The characteristics of isolates with the *penA*-44 allele were more similar to the characteristics of isolates in cluster 2 of the *penA*-34 group than to isolates in cluster 1 (Appendix Table 3).

Comparison of Isolates from England, Europe, and the United States

Isolates from England were genetically interspersed with isolates from other countries in Europe (Figure 2) or the United States (Figure 3), although some large clades of isolates came only from England or the United States. Isolates in cluster 1 with the *penA*-34 allele in England were clustered with isolates from Europe and the United States (Figures 2–3). Isolates with the *penA*-34 allele in cluster 2 from England that were only found after 2013 were not related genetically to isolates from the United States or from other countries in Europe.

Discussion

We conducted a large study on genomic variability of antimicrobial susceptibility in *N. gonorrhoeae* in England. We sampled isolates from geographically dispersed clinics in England, and our data likely represent patterns at a national level. Our data suggest that *N. gonorrhoeae* with reduced susceptibility to antimicrobial drugs, including ceftriaxone, has emerged in England through novel mutation and recombination events, repeated introduction from overseas, clonal expansion, or a combination of these.

We observed 3 distinct clusters with 2 different *penA* alleles and reduced susceptibility to ceftriaxone and cefixime and found patients in each cluster with differing epidemiologic characteristics. The genetic similarity of isolates from England, Europe, and the United States is consistent with global dissemination of *N. gonorrhoeae* concerning genotypic and phenotypic features. Our data highlight the potential influence of travel-associated sexual partnerships in AMR transmission.

As seen in other *N. gonorrhoeae* studies, the high frequency of DNA recombination requires computational strategies to use single-nucleotide polymorphism differences arising through mutation rather than recombination. We identified and removed recombinant DNA, but some likely remained, which might lead to incorrect inferences about relatedness for some isolates.

We found 2 distinct lineages of *N. gonorrhoeae* with different antimicrobial susceptibility profiles circulating in England. The larger lineage contained isolates with elevated MICs or resistance to all 5 antimicrobial drugs tested, consistent with findings from recent studies in Europe and globally (*5*,*21*). The authors of those studies hypothesized that differing susceptibility profiles of the 2 lineages were associated with different sexual orientation networks, but neither study had complete data on sexual orientation to support the hypothesis. Our study includes sexual orientation data for 99% of cases. Our findings strongly support the hypothesis that MSM are more frequently infected with *N. gonorrhoeae* strains with reduced susceptibility to antimicrobials, whereas MSW and women are more frequently infected with the more susceptible lineage. Nevertheless, many MSW were infected with *N. gonorrhoeae* with reduced susceptibility to antimicrobial drugs and MSM were

Table 2. Association between antimicrobial susceptibility of *Neisseria gonorrhoeae* isolates and presence in lineage A of the phylogeny, England*

Susceptibility	Lineage A, no. isolates	Lineage B, no. isolates	aOR	95% CI	p value
Reduced					
Ceftriaxone, MIC ≥0.015 mg/L					
No	572	418	Referent	–	–
Yes	263	15	**15.4**	**8.50–27.8**	**<0.001**
Cefixime, MIC ≥0.03 mg/L					
No	544	370	Referent	–	–
Yes	291	63	**3.97**	**2.76–5.76**	**<0.001**
Azithromycin, MIC ≥0.25 mg/L					
No	328	367	Referent	–	–
Yes	507	66	**7.50**	**5.37–10.5**	**<0.001**
Resistant					
Penicillin, MIC >1 mg/L or β-lactamase positive					
No	671	378	Referent	–	–
Yes	164	55	1.33	0.92–1.93	0.134
Ciprofloxacin, MIC >0.06 mg/L					
No	400	408	Referent	–	–
Yes	435	25	**18.2**	**11.4–29.2**	**<0.001**

*Each model adjusted for location inside or outside of London, and patient age, sexual orientation, and ethnicity. Nine isolates did not have MIC data. Bold text indicates statistical significance, i.e., p<0.05 and 95% CI does not cross 1. aOR, adjusted odds ratio.

infected by antimicrobial-susceptible *N. gonorrhoeae*, and we noted intralineage variation by sexual orientation. MSM have more bacterial STI diagnoses and greater exposure to antimicrobial drugs, thereby increasing selection pressures for AMR, a hypothesis supported by mathematical models (*21*–*24*). Resistant strains also might persist in the absence of selective pressure because the organism's biologic fitness is unaffected or compensatory mutations mitigate a deleterious effect (*25*–*27*).

By combining WGS, epidemiologic, and phenotypic data, we found that reduced susceptibility to ceftriaxone and cefixime emerged repeatedly in separate sexual networks in England. Without WGS data, we would have grouped all *penA*-34 samples from MSM together. Likewise, if we restricted sequencing to the *penA* gene, we would not have identified unique clusters with the same *penA*-34 allele.

The large group of isolates in England with the *penA*-34 allele clustered with isolates from Europe and the United States that had the same allele. Some of the *penA*-34 allele isolates belonged to the NG-MAST 1407 lineage, a widely disseminated clone associated with elevated MICs for ceftriaxone and cefixime and the catalyst for changing national treatment guidelines in the United Kingdom from cefixime as

Table 3. Epidemiologic characteristics of patients from whom *Neisseria gonorrhoeae* isolates were collected in the 2 largest *penA*-34 clusters, England*

Characteristics	Total	Cluster 1, n = 57, no. (%)	Cluster 2, n = 26, no. (%)	p value†
Year				
2013	26	26 (45.6)	0	**<0.001**‡
2014	29	14 (24.6)	15 (57.7)	
2015	20	10 (17.5)	10 (38.5)	
2016	8	7 (12.3)	1 (3.8)	
Sex and sexual orientation				
MSM	49	24 (42.1)	25 (96.2)	**<0.001**‡
MSW	21	21 (36.8)	0	
F	13	12 (21.1)	1 (3.8)	
Clinic location				
Outside London	43	38 (66.7)	5 (19.2)	**<0.001**‡
London	40	19 (33.3)	21 (80.8)	
Age, y				
≤24	28	23 (40.4)	5 (19.2)	0.081
25–34	29	20 (35.1)	9 (34.6)	
≥35	26	14 (24.6)	12 (46.2)	
Ethnicity				
White	59	37 (68.5)	22 (84.6)	0.408‡
Black Caribbean	6	6 (11.1)	0	
Black, Other	2	2 (3.7)	0	
Asian	5	4 (7.4)	1 (3.8)	
Other	4	2 (3.7)	2 (7.7)	
Mixed	4	3 (5.6)	1 (3.8)	
Place of birth				
United Kingdom	40	30 (52.6)	10 (38.5)	0.262‡
Not United Kingdom	40	26 (45.6)	14 (53.9)	
Unknown	3	1 (1.7)	2 (7.7)	
Symptomatic infection				
No	28	13 (24.5)	15 (57.7)	**0.004**
Yes	51	40 (75.5)	11 (42.3)	
New STI diagnosed ≤1 year, excluding HIV				
No	68	51 (89.5)	17 (65.4)	**0.013**‡
Yes	15	6 (10.5)	9 (34.6)	
HIV status				
Negative or unknown	70	53 (93.0)	17 (65.4)	**0.003**‡
Positive	13	4 (7.0)	9 (34.6)	
Number of sexual partners in the United Kingdom ≤3 mo of *N. gonorrhea* diagnosis				
0	6	6 (13.3)	0	0.675‡
1	19	16 (35.6)	3 (33.3)	
≥2	29	23 (51.1)	6 (66.7)	
Travel-associated sexual partnership				
No	43	34 (75.6)	9 (100)	0.178‡
Yes	11	11 (24.4)	0	

*Bold text indicates statistical significance (i.e., p<0.05 and 95% CI does not cross 1). MSM, men who have sex with men; MSW, men who reported sexual activity exclusively with women.
†Calculated using χ^2 test, except where noted.
‡Calculated using Fisher exact test.

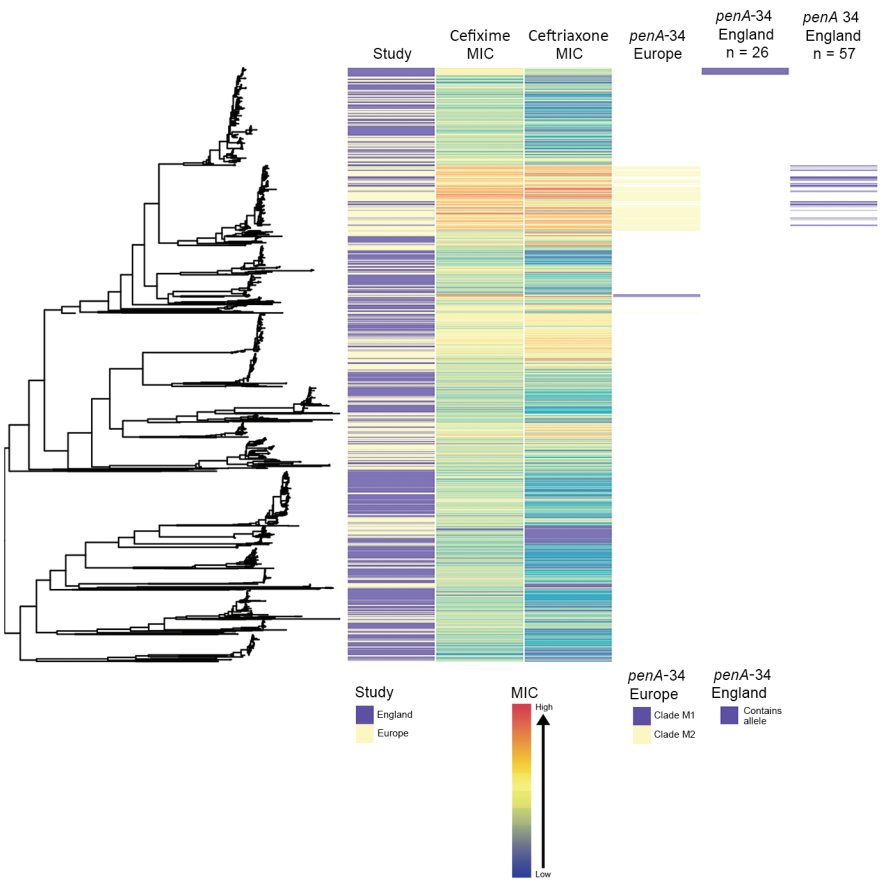

Figure 2. Phylogenetic tree of *Neisseria gonorrhoeae* isolates from England and other countries in Europe in a study of antimicrobial susceptibility, 2013–2016, including metadata for study type, MICs for ceftriaxone and cefixime, and presence of *penA*-34 alleles. We sequenced 1,277 isolates; 948 isolates were from other countries in Europe. The *penA*-34 clades from Europe are labeled M1 and M2, as noted by Harris et al. (*5*).

first-line therapy to ceftriaxone in 2011 (*6*,*22*). Therefore, the larger *penA*-34 group probably represents clonal spread of a previously identified endemic strain of *N. gonorrhoeae*; the smaller *penA*-34 group represents a new strain emerging in a different sexual network largely comprising MSM in London with a history of STIs, including HIV. Consequently, restricting public health resources that measure, prevent, and control AMR in *N. gonorrhoeae* to specific risk groups or geographic locations could be ineffective because AMR appears to emerge independently in different sexual networks and locations.

We found some evidence for importation of AMR. Isolates from persons who recently had a travel-associated sexual partnership were more likely to be infected with *N. gonorrhoeae* that was resistant to ciprofloxacin. Although de novo development of high-level resistance to azithromycin in the United Kingdom has been described, some studies have concluded that importation events probably initiate AMR spread in countries with low population prevalence, such as England (*11*,*27*,*28*). The success of antimicrobial stewardship policies and compliance with treatment guidelines that aim to curtail AMR in the endemic gonococcal population in England could be undermined by the importation and subsequent spread of resistant isolates. These data support the importance of promoting STI prevention messages and testing to international travelers, particularly those visiting countries where AMR *N. gonorrhoeae* is endemic. Quantifying the relationship between *N. gonorrhoeae* circulating in England and internationally also could help parameterize mathematical models exploring the relative contribution of importation and de novo development on AMR prevalence and distribution.

Rapid molecular tests for genetic markers that are highly predictive of an antimicrobial susceptibility phenotype could lead to more effective use of antimicrobials. Tests detecting markers of ciprofloxacin and cephalosporin antimicrobial susceptibility are already in development (*8*,*29*,*30*). However, as found in our study and elsewhere, the association between genotype and phenotype is much stronger for ciprofloxacin resistance than for cephalosporin resistance (*4*,*31*,*32*). Most rapid tests for cephalosporin resistance focus on detecting mutations in the *penA* allele, but reduced susceptibility to cephalosporins also can

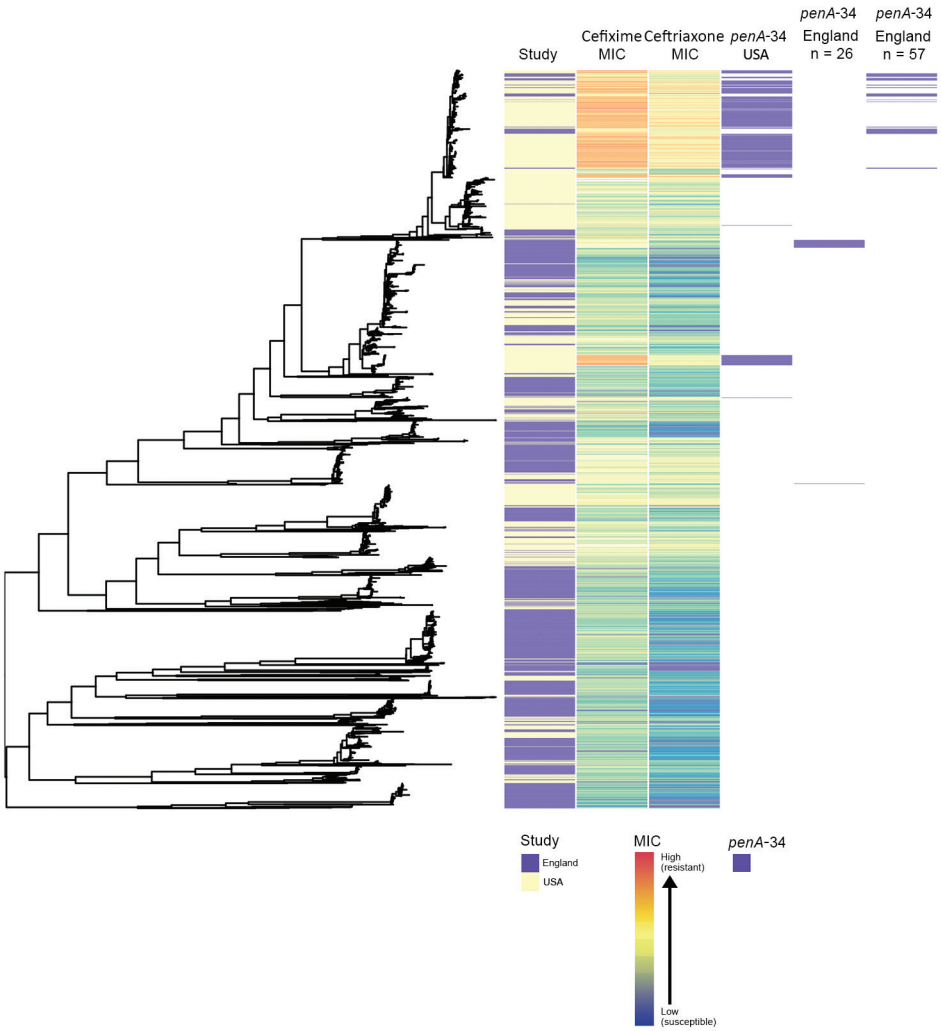

Figure 3. Phylogenetic tree of *Neisseria gonorrhoeae* isolates from England and the United States in a study of antimicrobial susceptibility, 2013–2016, including metadata for study type, MICs for ceftriaxone and cefixime, and presence of *penA*-34 alleles. We sequenced 1,277 isolates; 1,114 isolates were from the United States.

be caused by mutations in the semimosaic *penA* allele, *penA*-35, the nonmosaic *penA* allele, *penA*-44, or even in the *mtrR* and *penB* genes (*30*,*33*).

Molecular tests that focus on the presence or absence of 1 mutation without considering the additive effect of multiple mutations could be insufficient for detecting resistance and predicting treatment failure. Epistasis, in which phenotypic resistance is dependent on complex interactions of multiple mutant genes, is known to occur in *N. gonorrhoeae* (*22*,*31*,*34*). In our study, the antimicrobial susceptibility of isolates with identical genetic markers of resistance varied by >2 doubling dilutions, and most isolates with resistance markers were sensitive. Nonetheless, the presence of 1 mutation that belongs to a complex of mutations required for resistance indicates the potential for phenotypic resistance to develop. Clinicians could prioritize patients infected with these strains for a test of cure or consider use of alternative antimicrobial drugs unaffected by the resistance marker. In any event, mathematical modeling studies have shown that molecular tests should only be implemented if they are highly sensitive; otherwise, they could accelerate the spread of AMR (*23*). Elucidation of the mechanisms and genomic markers of cephalosporin resistance is needed and can be achieved through a combination of microbiologic and genomic studies, including genomewide association studies. WGS cannot replace phenotypic testing for all antimicrobial susceptibility because it can only detect known mutations associated with resistance, and novel mutations associated with resistance develop constantly in *N. gonorrhoeae* (*31*).

In conclusion, phylogenetic analyses with WGS data revealed transmission patterns of *N. gonorrhoeae* with reduced susceptibility in England that would not have been identified by using only epidemiologic and phenotypic data. Reduced susceptibility to

antimicrobial drugs likely has emerged and spread independently in different sexual networks in England through multiple de novo mutation and recombination events and through some repeated importation by persons who have travel-associated sexual partnerships. Consequently, public health actions to limit dissemination of AMR in England should aim to reduce risk behaviors that support *N. gonorrhoeae* transmission and encompass the diffuse distribution and epidemiologic diversity of the population groups affected.

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Dr. Town was the senior surveillance scientist on the Gonococcal Resistance to Antimicrobials Surveillance Programme (GRASP) at Public Health England and graduated from University College London with a PhD in epidemiology. Dr. Town's research focuses on investigating new methods for understanding, monitoring, and preventing the spread of *Neisseria gonorrhoeae* and antimicrobial resistance.

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Genomic and Phenotypic Variability in *Neisseria gonorrhoeae* Antimicrobial Susceptibility, England

Appendix

This research was undertaken as part of the National Institute for Health Research Health Protection Research Unit (NIHR HPRU) in Blood-Borne and Sexually Transmitted Infections at University College London, England, UK.

Methods

Antimicrobial Susceptibility Testing External Quality Assurance

Quality assurance of antimicrobial susceptibility testing was performed by including 12 control strains, WHO A, WHO D, WHO E, WHO J, WHO K, WHO L, TR01, 1336, 1339, A02, A24, and QA07–09. In addition, we participated in external quality assurance (EQA) exchanges with the national reference laboratories of Scotland and Belgium, and in the Euro-GASP EQA.

Antimicrobial Resistance Definitions

The Gonococcal Resistance to Antimicrobials Surveillance Programme (GRASP) uses European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints to define antimicrobial resistance as follows: ceftriaxone, MIC >0.125 mg/L; cefixime, MIC >0.125 mg/L; azithromycin, MIC >0.5 mg/L; ciprofloxacin, MIC >0.06 mg/L; penicillin, MIC >1 mg/L.

Whole-Genome Sequencing Methods

Whole-genome sequencing (WGS) was conducted at the Wellcome Sanger Institute by using the HiSeq X Ten system (Illumina, http://www.illumina.com), and put through the routine Sanger WGS data management pipeline (*1*). The following measures were used to assess the quality of the WGS data for each isolate included in the phylogenetic analyses: a quality score >30 for the nucleotides called during the sequencing process, the majority of raw reads identified as *N. gonorrhoeae* when cross-referenced to a public database of pathogen genomes by using Kraken (*2*), the assembly length similar to the *N. gonorrhoeae* reference genome FA1090 (*3*),

2,153,922 nt, the assembly guanine and cytosine content (53%) similar to the *N. gonorrhoeae* reference genome FA1090, and >90% of the reference genome covered by reads.

After passing quality control, the raw reads were aligned to the reference genome FA1090 to create a consensus whole-genome sequence for each isolate. We used the Burrows-Wheeler Aligner Maximal Exact Match (BWA-MEM) algorithm with the option to flag duplicate shorter reads that match as secondary for removal (option M) (*4*). The Sequence Alignment/Map (SAM) (*5*) file output was converted into a Binary Alignment/Map (BAM) file using SAMTools to reduce the size of the file for faster computer processing (*5*). The Genome Analysis Toolkit (GATK) was used to realign indels, which helps the process of identifying single-nucleotide polymorphisms (SNPs) (*6*). SAMTools mpileup was used to identify the variant nucleotides identified in each read and the haploid option of Binary Call Format (BCF) tools from SAMTools filtered this information to select the variant nucleotides based on the following conditions: the minimum base call quality was ≥50 (quality of the base was previously determined using the Phred score system in SAMTools); the minimum mapping quality score by BWA-MEM was 20; ≥8 reads have the same variant and ≥3 are from each strand direction, forward and back; and the specific variant called is the same in 80% of the reads used. The consensus sequence for each isolate was compiled into 1 multiple FASTA file and used for the analyses.

Phylogenetic Tree Construction for Isolates from England

Gubbins version 2.4.0 (*7*) was used with the default settings (5 iterations and ≥3 base substitutions to identify a recombination event) and the tree building option Randomized Axelerated Maximum Likelihood (RAxML) version 8.2.8 (*8*) to create the phylogenetic tree with recombination events removed. Prior to this, the *opa* and *pil* genes, phages (*9*), and the Gonoccocal Genetic Island (GGI) (*10*) were manually removed from the alignment. The output phylogenetic tree was midpoint rooted, i.e., the root of the tree was placed half-way between the 2 isolates with the largest SNP difference, using Figtree version 1.4.3 (hhtp://tree.bio.ad.uk/software/figtree), and the branches were ladderized, i.e., branches were rotated so that they were ordered by increasing clade size at each node to aid visualization. Statistical support for the structure of the phylogenetic tree was assessed by using the Booster program (*11*). First, the phylogenetic tree was recreated by using Gubbins and RAxML with the bootstrap option to create 100 trees and input into Booster along with the reference tree used for

analysis. Booster was used to calculate the transfer bootstrap expectation (TBE), a value that quantifies the presence of each branch at a particular position in the bootstrap trees. A value of 1 indicates that the branch is in all bootstrap trees and a value of 0 indicates that the bootstrap trees are random. Statistical support for the phylogenetic tree was high: 79% (1,014/1,276) of nodes had a TBE value >70%.

Phylogenetic Tree Construction for Isolates from England, Europe, and the United States

For each international dataset, the consensus sequences were combined with the study consensus sequences and the mobile and repetitive elements removed. Phylogenetic trees with genetic recombination events removed were created by using Gubbins version 2.4.0 (*9*) with the default settings (5 iterations and ≥3 base substitutions to identify a recombination event) and the tree building option FastTree version 2.1.4 (*12*), which also uses a heuristic approach to find the tree with the maximum likelihood of producing the data given the model. FastTree has been shown to produce similar phylogenetic trees as RAxML methods but can analyze larger datasets within 1 day (*13*).

Results

N. gonorrhoeae Multiantigen Sequence Typing (NG-MAST) Data

The most common sequence types (STs) were ST51 (8.8%; 113/1,277), ST2992 (5%; 67/1,277), ST292 (3%; 45/1,277). By NG-MAST, the 2 isolates highly resistant to azithromycin had ST649 and ST13124, which are not the same STs seen in the United Kingdom during a 2015 outbreak of high-level azithromycin–resistant *N. gonorrhoeae*.

The largest *penA*-34 cluster contained 22 different previously reported STs and 4 novel STs. The 3 most frequently found STs were ST1407 (14/57; 25%), ST3169 (6/57; 10%), and ST8953 (5/57; 9%). The smaller *penA*-34 cluster primarily contained isolates with ST4244 (21/26; 81%). The remaining isolates were ST11084 (n = 2), ST3808 (n = 1), ST9897 (n = 1), and 1 isolate had a previously unidentified ST by NG-MAST. The *penA*-44 cluster contained 16 different STs, 30% (25/84) were ST2400, 21% (18/84) were ST10149, 17% (14/84) were ST6360, and 5 isolates had an ST that had not been identified previously.

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Appendix Table 1. Epidemiologic characteristics of the study sample compared to all gonorrhea cases diagnosed in England, 2013–2016

Characteristics	Study sample, no. (%)	England, no. (%)	p value*
Total	1,267 (100.0)	146,369 (100.0)	
Year			
2013	326 (25.5)	31,213 (21.3)	**<0.001**
2014	333 (26.1)	37,178 (25.4)	0.580
2015	367 (28.7)	41,396 (28.3)	0.718
2016	251 (19.7)	36,582 (25.0)	**<0.001**
Clinic location			
London	527 (44.8)	72,809 (49.7)	**<0.001**
Outside London	705 (55.2)	73,560 (50.2)	**<0.001**
Gender and sexual orientation†			
MSM	766 (60.0)	72,660 (49.6)	**<0.001**
MSW	304 (23.8)	34,330 (23.5)	0.768
F	206 (16.1)	36,178 (24.7)	**<0.001**
Missing	1 (<0.1)	3,201 (2.2)	N/A
Age, y			
≤24	384 (30.1)	55,029 (37.6)	**<0.001**
25-34	503 (39.4)	54,143 (37.0)	0.077
≥35	390 (30.5)	37,197 (25.4)	**<0.001**
Ethnicity			
White	824 (64.5)	104,028 (71.1)	**<0.001**
Black Caribbean	132 (10.3)	8,280 (5.7)	**<0.001**
Black African	47 (3.7)	5,858 (4.0)	0.559
Black, other	10 (0.8)	3,238 (2.2)	**<0.001**
Asian	74 (5.8)	5,750 (3.9)	**<0.026**
Other	32 2.5)	4,747 (3.2)	0.138
Mixed	105 (8.2)	8,614 (5.9)	**<0.001**
Missing	53 (4.2)	5,815 (4.0)	0.747
Place of birth			
United Kingdom	782 (61.2)	96,189 (65.7)	**<0.001**
Not United Kingdom	407 (31.9)	38,334 (26.2)	**<0.001**
Missing	88 (6.8)	11,846 (8.1)	0.117
New STI diagnosed ≤1 year, excluding HIV			
N	1,015 (79.5)	117,493 (80.3)	0.481
Y	262 (20.5)	28,876 (19.7)	0.481
HIV status			
Negative or unknown	1,051 (82.3)	130,198 (89.0)	**<0.001**
Positive	226 (17.7)	16,171 (11.0)	**<0.001**

*****p value calculated by using 2 sample proportions z-test. Bold text indicates statistical significance.
†MSM, men who report having sex with men; MSW, men who report having sex with women exclusively.

Appendix Table 2. Phenotypic antimicrobial susceptibility profile of study sample and all GRASP isolates, 2013–2016*

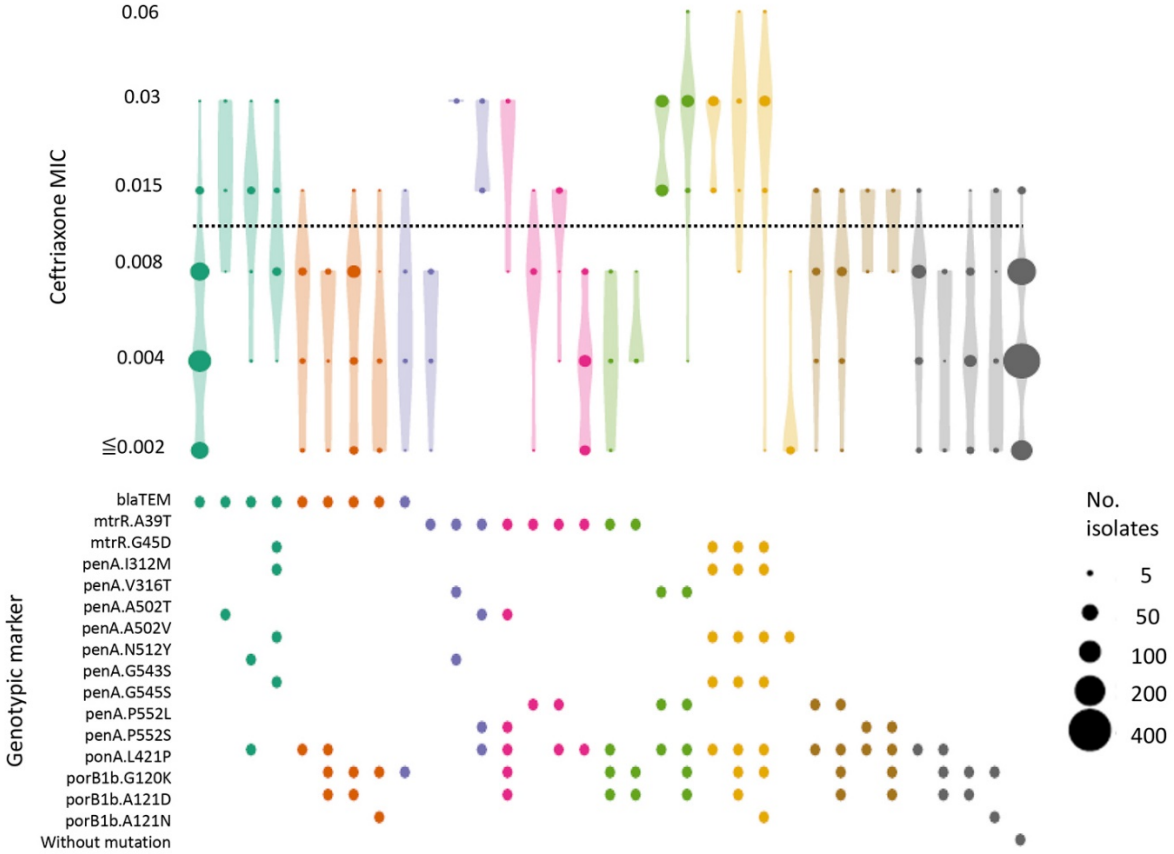
MIC	Study sample, no. (%)†	GRASP, no. (%)	p value‡	Study sample as a % of GRASP
Total	1,267 (100.0)	6,184 (100.0)		20.5
Ceftriaxone (threshold for resistance, MIC >0.125 mg/L)				
<0.002	207 (16.3)	938 (15.2)	0.569	22.1
0.004	409 (32.3)	1,999 (32.3)		20.5
0.008	374 (29.5)	1,760 (28.5)		21.3
0.015	150 (11.8)	833 (13.5)		18.0
0.03	121 (9.6)	601 (9.7)		20.1
0.06	7 (0.6)	52 (0.8)		13.5
0.125	0	1 (<0.1)		0
Azithromycin (threshold for resistance, MIC >0.5 mg/L)				
<0.03	127 (10.0)	598 (9.7)	0.847	21.2
0.06	186 (14.7)	924 (14.9)		20.1
0.125	382 (30.1)	1,873 (30.3)		20.4
0.25	402 (31.7)	1,913 (30.9)		21.0
0.50	125 (9.9)	622 (10.1)		20.1
1.00	39 (3.1)	192 (3.1)		20.3
2.00	1 (0.1)	31 (0.5)		3.2
4.00	3 (0.2)	15 (0.2)		20.0
8.00	1 (0.1)	3 (0)		33.3
16.0	0	3 (0)		0
≥256	2 (0.2)	10 (0.2)		20.0
Cefixime (threshold for resistance, MIC >0.125 mg/L)				
0.002	37 (2.9)	200 (3.2)	0.795	18.5
0.004	62 (4.9)	305 (4.9)		20.3
0.008	331 (26.1)	1,691 (27.3)		19.6
0.015	484 (38.2)	2,187 (35.4)		22.1
0.03	174 (13.7)	882 (14.3)		19.7
0.06	144 (11.4)	729 (11.8)		19.8
0.125	28 (2.2)	140 (2.3)		20.0
0.25	8 (0.6)	46 (0.7)		17.4
0.50	0	4 (0.1)		0
Ciprofloxacin (2013–2015; threshold for resistance, MIC >0.06 mg/L)				
0.03	632 (49.9)	3,009 (48.7)	0.952	21.0
0.06	12 (0.9)	65 (1.1)		18.5
0.125	6 (0.5)	28 (0.5)		21.4
0.25	2 (0.2)	14 (0.2)		14.3
0.50	3 (0.2)	28 (0.5)		10.7
1.00	18 (1.4)	84 (1.4)		21.4
2.00	13 (1.0)	78 (1.3)		16.7
4.00	68 (5.4)	329 (5.3)		20.7
8.00	145 (11.4)	698 (11.3)		20.8
16.0	90 (7.1)	427 (6.9)		21.1
32.0	37 (2.9)	140 (2.3)		26.4
Ciprofloxacin (2016 breakpoint plates; threshold for resistance, MIC >0.06 mg/L)				
≤0.06	164 (12.9)	851 (13.8)	0.302	19.3
>0.06 and <0.50	5 (0.4)	13 (0.2)		38.5
≥0.50	73 (5.8)	420 (6.8)		17.4
Penicillin (threshold for resistance, MIC >1.0 mg/L)				
0.06	92 (7.3)	386 (6.2)	0.066	23.8
0.125	298 (23.5)	1,400 (22.6)		21.3
0.25	399 (31.5)	2,112 (34.2)		18.9
0.50	186 (14.7)	1,009 (16.3)		18.4
1.0	82 (6.5)	413 (6.7)		19.9
2.0	34 (2.7)	168 (2.7)		20.2
4.0	44 (3.5)	156 (2.5)		28.2
8.0	133 (10.5)	540 (8.7)		24.6

*GRASP, Gonococcal Resistance to Antimicrobials Surveillance Programme.
†Denominator is less than study sample because 10 samples from 2016 failed routine phenotypic testing so AMR data are unavailable.
‡p value from χ^2 test comparing distribution of MICs in the study sample to the GRASP sample.

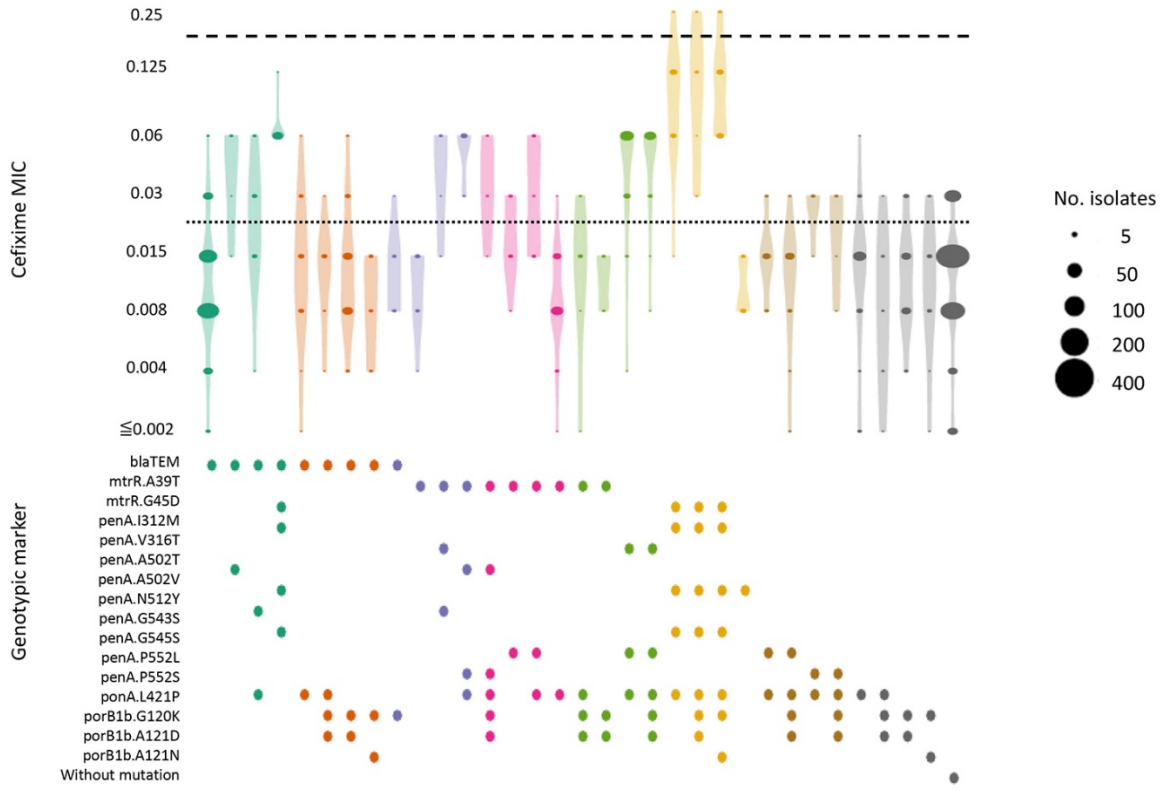
Appendix Table 3. Epidemiologic characteristics of cases in *penA*-44 group compared to 2 *penA*-34 groups*

	penA-44 (n = 84)	*penA*-34 comparison, p value†	
Characteristics	No. (%)	Cluster 1 (n = 57)	Cluster 2 (n = 26)
Year			
2013	39 (46.4)	0.952	**<0.001**‡
2014	23 (27.4)		
2015	12 (14.3)		
2016	10 (11.9)		
Gender and sexual orientation§			
MSM	69 (82.1)	**<0.001**	0.116‡
MSW	11 (13.1)		
F	4 (4.8)		
Clinic location			
Outside London	38 (45.2)	**0.012**	**0.021**‡
London	46 (54.8)		
Age, y			
≤24	16 (19.0)	**0.021**	0.508
25–34	39 (46.4)		
≥35	29 (34.5)		
Ethnicity			
White	60 (72.3)	0.867‡	0.836‡
Black Caribbean	5 (6.0)		
Black African	1 (1.2)		
Black, other	1 (1.2)		
Asian	6 (7.2)		
Other	4 (4.8)		
Mixed	6 (7.2)		
Place of birth			
United Kingdom	56 (66.7)	0.201‡	**0.019**‡
Not United Kingdom	26 (31.0)		
Unknown	2 (2.4)		
Symptomatic infection			
N	19 (25.3)	0.917	**0.003**
Y	56 (74.7)		
New STI diagnosed <1 year, excluding HIV			
N	60 (71.4)	**0.01**	0.557
Y	24 (28.6)		
HIV status			
Negative or unknown	61 (72.6)	**0.003**	0.478
Positive	23 (27.4)		
Number of sexual partners in the United Kingdom ≤3 mo before diagnosis			
0	1 (1.9)	0.082	0.915
1	18 (34.0)		
≥2	34 (64.2)		
Travel-associated sexual partnership			
N	51 (96.2)	**0.003**	1.00‡
Y	2 (3.8)		

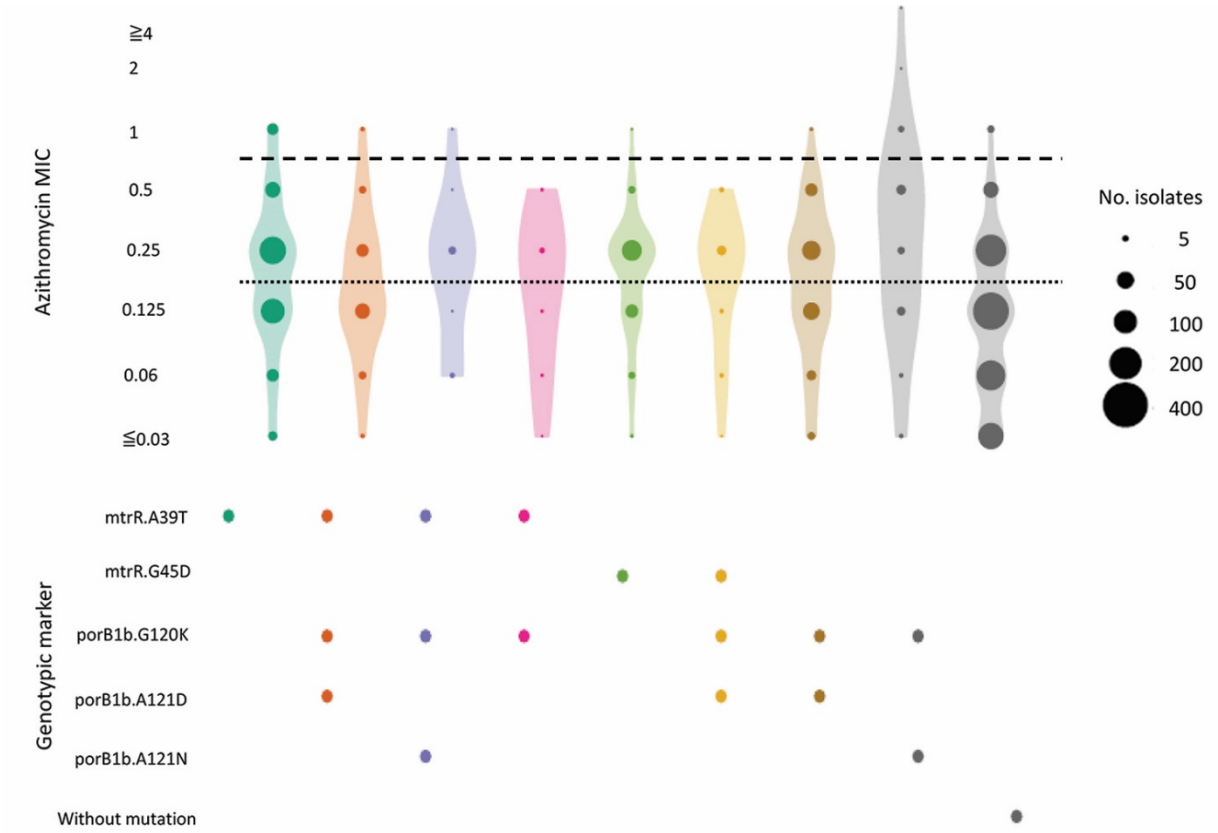
*Bold text indicates a statistically significant result, p<0.05 and CI does not cross 1. See Table 2 in the main manuscript for epidemiologic characteristics of isolates with *penA*-34 allele.
†p value calculated by using χ^2 test, except where indicated.
‡p value calculated by using Fisher exact test.
§MSM, men who reported sex with men; MSW, men who reported sex with women exclusively.



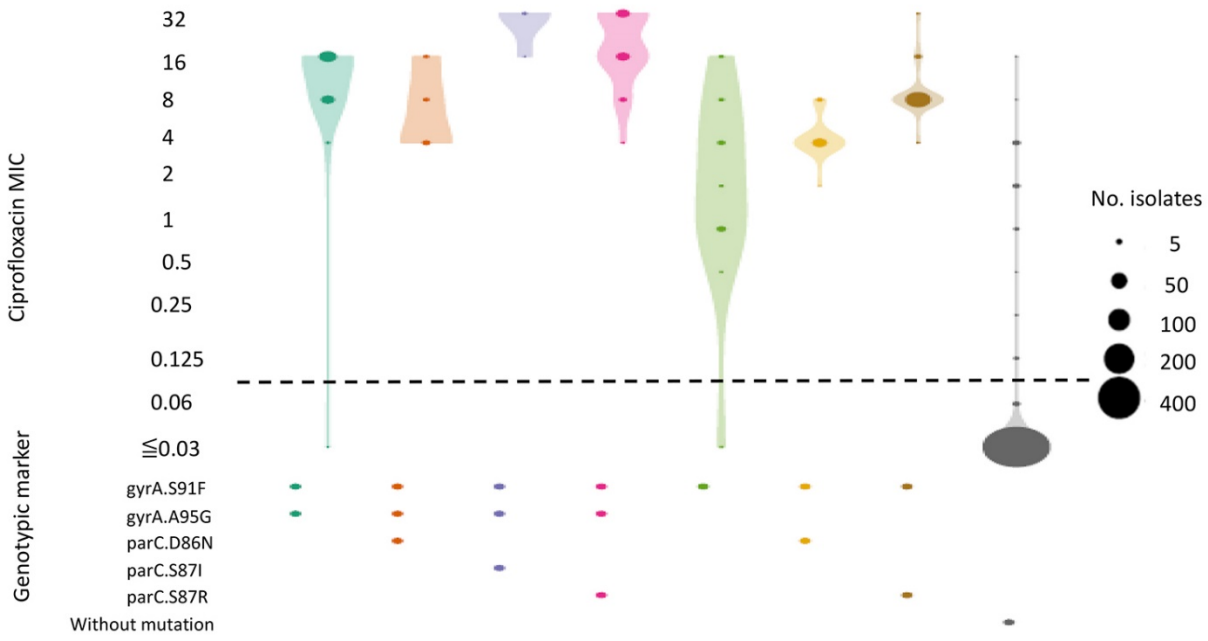
Appendix Figure 1. Genetic markers of antimicrobial resistance and MIC for ceftriaxone in a study of antimicrobial susceptibility of *Neisseria gonorrhoeae*, England, 2013–2016. Only marker combinations with ≥5 isolates included. Dotted line indicates reduced susceptibility threshold of MIC ≥0.015 mg/L. Colors are a visual aide to distinguish every 4 groups of genotypic markers and are not representative of any genotypic types. AMR, antimicrobial resistance; MIC, minimal inhibitory concentration.



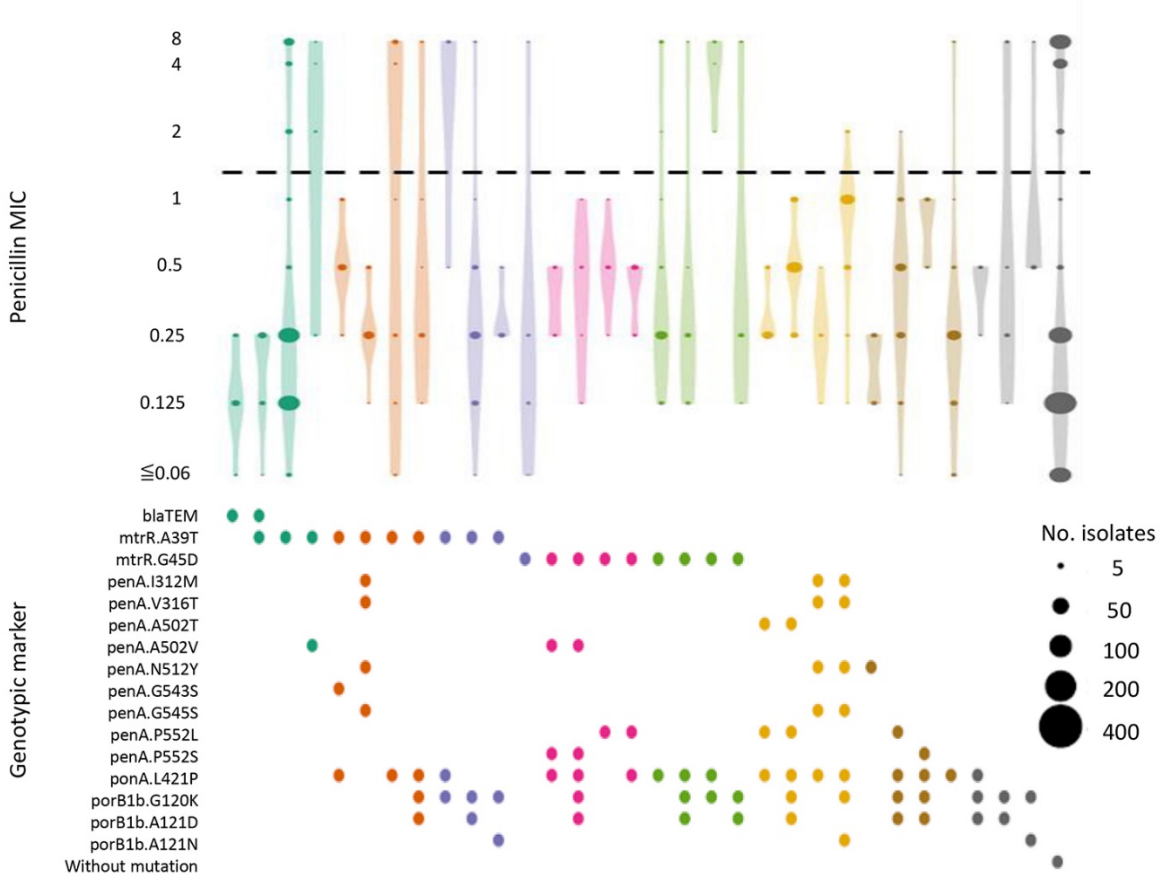
Appendix Figure 2. Genetic markers of antimicrobial resistance and MIC for cefixime in a study of antimicrobial susceptibility of *Neisseria gonorrhoeae*, England, 2013–2016. Only marker combinations with ≥5 isolates included. Dotted line indicates reduced susceptibility threshold of MIC ≥0.015 mg/L. Dashed line indicates resistance threshold of MIC >0.125 mg/L. The colors are a visual aide to distinguish every 4 groups of genotypic markers and are not representative of any genotypic types. AMR, antimicrobial resistance; MIC, minimal inhibitory concentration.



Appendix Figure 3. Genetic markers of antimicrobial resistance and MIC for azithromycin in a study of antimicrobial susceptibility of *Neisseria gonorrhoeae*, England, 2013–2016. Only marker combinations with ≥5 isolates included, dotted line indicates reduced susceptibility threshold of MIC ≥0.25 mg/L. Dashed line indicates resistance threshold of MIC >0.5 mg/L. Colors are a visual aide to distinguish every 4 groups of genotypic markers and are not representative of any genotypic types. AMR, antimicrobial resistance; MIC, minimal inhibitory concentration.



Appendix Figure 4. Genetic markers of antimicrobial resistance and MIC for ciprofloxacin in a study of antimicrobial susceptibility of *Neisseria gonorrhoeae*, England, 2013–2016. Only marker combinations with ≥5 isolates included. Dashed line indicates resistance threshold of MIC ≥0.06 mg/L. The colors are a visual aide to distinguish every 4 groups of genotypic markers and are not representative of any genotypic types. For isolates from 2016, only data on whether the isolate was susceptible or resistant to ciprofloxacin were available, so for MIC analyses all resistant isolates were allocated an MIC of 1 mg/L and all sensitive isolates were allocated an MIC of ≤0.03 mg/L. AMR, antimicrobial resistance; MIC, minimal inhibitory concentration.



Appendix Figure 5. Genetic markers of antimicrobial resistance and MIC for penicillin in a study of antimicrobial susceptibility of *Neisseria gonorrhoeae*, England, 2013–2016. Only marker combinations with ≥5 isolates included. Dashed line indicates resistance threshold of MIC ≥1 mg/L. The colors are a visual aide to distinguish every 4 groups of genotypic markers and are not representative of any genotypic types. AMR, antimicrobial resistance; MIC, minimal inhibitory concentration.