Neisseria gonorrhoeae diagnostic escape from a *gyrA*-based test for ciprofloxacin susceptibility can lead to increased zoliflodacin resistance

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Abstract

The etiologic agent of gonorrhea, *Neisseria gonorrhoeae*, has become resistant to each of the first line antibiotics used to treat it. However, most isolates in the US remain susceptible to ciprofloxacin, leading to the hypothesis that a diagnostic that accurately reports susceptibility to ciprofloxacin may allow its reintroduction into clinical use. One diagnostic approach is to identify codon 91 in *gyrA*, where coding for the wildtype serine is associated with ciprofloxacin susceptibility and phenylalanine with resistance. Here, we found that escape from this diagnostic method can occur through *gyrA* F91S reversion and expansion of pre-existing ciprofloxacin non-susceptible *N. gonorrhoeae* lineages with *gyrA* 91S. Furthermore, we found that mutations conferring ciprofloxacin resistance in *gyrA* 91S isolates led to cross-resistance with zoliflodacin, an antibiotic in phase 3 trial for treatment of gonorrhea. These findings define *N. gonorrhoeae* mutations that should be monitored via surveillance systems, particularly in populations and regions deploying *gyrA* 91-based diagnostics, and demonstrate how diagnostics that guide antibiotic therapy can have unintended consequences.

38 **Introduction**

39 *Neisseria gonorrhoeae*, the causative agent of the sexually transmitted disease gonorrhea, has
40 developed resistance to each of the first-line antibiotics used to treat it[1]. This rise in antibiotic
41 resistance has led to fears of untreatable highly drug resistant gonorrhea[2], a concern further
42 heightened by the international circulation of strains resistant to the current first-line antibiotic,
43 ceftriaxone[3-5].

44

45 Due to high levels of drug resistance in *N. gonorrhoeae*, there has been substantial interest in the
46 development of new approaches for management of gonorrhea[6]. One promising avenue has been
47 the use of point-of-care diagnostics that test for susceptibility. By understanding alleles associated
48 with antibiotic susceptibility[7], sequence-based diagnostics may enable diagnosis of gonorrhea
49 and inform treatment decisions by using genotype to predict antibiotic susceptibility phenotype.

50

51 An early target for this approach has been the fluoroquinolone class antibiotic ciprofloxacin[8,9].
52 Once first-line treatment of *N. gonorrhoeae* infections, rising levels of resistance and concern for
53 failure of empiric therapy resulted in recommendations against its empiric use[10]. However, sur-
54 veillance data from the US, the UK, and Europe indicate that ciprofloxacin resistance is present
55 in a minority of cases[11-13], suggesting that a diagnostic that can inform on ciprofloxacin suscepti-
56 bility would allow for the drug's reintroduction to gonorrhea treatment and thereby aid in reducing
57 the selective pressure on ceftriaxone[14].

58

59 Ciprofloxacin resistance in *N. gonorrhoeae* is mainly mediated by mutations in the A subunit of
60 DNA gyrase (encoded by *gyrA*) and the A subunit of topoisomerase IV (encoded by *parC*). Though
61 multiple mutations contribute to ciprofloxacin resistance, including position 95 in GyrA and multi-
62 ple sites at positions 86-91 in ParC[15], position 91 in GyrA is highly predictive of clinical suscepti-
63 bility (if a serine) and resistance (if a phenylalanine) to ciprofloxacin[16,17].

The SpeeDx ResistancePlus GC diagnostic test reports the presence of *N. gonorrhoeae* and ciprofloxacin susceptibility. The ResistancePlus GC test consists of a multiplex qPCR, incorporating channels for the wild-type (WT) *gyrA* 91S allele, the mutant *gyrA* 91F allele, the *N. gonorrhoeae opa* gene, the *N. gonorrhoeae porA* gene, and a PCR positive control. Across multiple studies, the ResistancePlus assay detects gonorrhea with a sensitivity of 94-100%[8,18,19] and specificity of 99-100%[8,19]. The test is also highly sensitive (>99%) and specific (>97%) for ciprofloxacin resistance[18].The ResistancePlus GC assay has been approved in Europe and received a breakthrough designation from the FDA[20].

The use of sequence-based diagnostics raises the possibility of diagnostic escape, with a precedent in *N. gonorrhoeae* of mutations that resulted in failure of clinical identification by PCR [21,22]. In this case, the diagnostic applies selective pressure for *N. gonorrhoeae* to develop resistance through a pathway other than the *gyrA* 91 mutation it targets. False negative tests for antibiotic susceptibility testing may result in clinical treatment failure for the individual and increased burden of disease and accelerated spread of resistance in the population[23]. The risk of ciprofloxacin resistance arising through novel pathways is especially pressing due to concern for cross-resistance to the new *N. gonorrhoeae* antibiotic zoliflodacin, a first-in-class spiropyrimidinetrione antibiotic that primarily targets the B subunit of DNA gyrase (GyrB)[24] and is in Phase 3 trials for treatment of gonorrhea[25].

In this study, we investigated the pathways of diagnostic escape from tests that rely on GyrA 91. We found that some clinical isolates with a 91S allele of GyrA maintain ciprofloxacin resistance at a level that has been associated with treatment failure, with one such isolate (GCGS0481) acquiring high-level ciprofloxacin resistance while maintaining the 91S allele. Finally, though these strains are zoliflodacin naïve, we found that, in the presence of ciprofloxacin, clinical isolates

90 readily evolve a D429N mutation in GyrB that confers ciprofloxacin resistance and that has been
91 reported to confer zoliflodacin resistance[26]. Taken together, these results indicate that there are
92 evolutionary pathways to diagnostic escape from *gyrA* 91-based diagnostics and that one of these
93 pathways leads to cross-resistance between ciprofloxacin and zoliflodacin. Finally, these results
94 provide experimental validation for the hypothesis that single antibiotic sequence-based diagnos-
95 tics may lead to unanticipated consequences, including novel resistance determinants and cross-
96 resistance.
97
98 **Methods**
99
100 **Bacterial strains, MIC measurements and Cloning**
101 The strains used in this study are listed in Supplementary Table 1. *N. gonorrhoeae* was grown on
102 GCB Agar (Difco) supplemented with Kellogg's supplements[27] at 37°C in 5% CO_2. Antibiotic sus-
103 ceptibility testing was performed on GCB-K agar via agar dilution for zoliflodacin (using the range
104 0.008 to 4 µg/mL) and Etest (BioMerieux) for ciprofloxacin, ceftriaxone, erythromycin, benzylpen-
105 icillin, and tetracycline.
106 Cloning was performed using primers and plasmids listed in Supp. Table 1 and Gibson assembly
107 [28] into a pUC19[29] backbone using a kanamycin cassette from pDR1[30]. Fragments were amplified
108 using Phusion (NEB), checked for appropriate size by gel electrophoresis, column purified (Qi-
109 agen PCR Purification kit), assembled with Gibson Master Mix (NEB), and finally transformed into
110 chemically competent DH5α *E. coli*. Finally, individual colonies were selected on LB agar with 50
111 µg/mL kanamycin, picked, and grown overnight. Plasmids were isolated using spin columns (Qi-
112 agen Spin Miniprep Kit). The resulting plasmids were checked by Sanger sequencing. For inser-
113 tion of GyrA alleles into *N. gonorrhoeae*, strains of *N. gonorrhoeae* in Supp. Table 2 were grown
114 overnight on GCB-K. After 16-20 hours, strains were scraped into 0.3M sucrose, electroporated

115 (V=1.8 kV, exponentially decaying wave) with 200 ng of plasmids DRE77-82, and rescued with
116 GCP medium (15 g/L proteose peptone 3 (ThermoFisher), 1 g/L soluble starch (ThermoFisher),
117 4 g/L K_2HPO_4, 1 g/L KH_2PO_4, 5 g/L NaCl)[31] with Kellogg's supplement. After 10 minutes of rescue,
118 transformants were then plated on nonselective GCB-K agar for 4 hours followed by selection on
119 GCB-K supplemented with 70 µg/mL kanamycin. Finally, single colonies were re-streaked on
120 GCB-K agar and checked for GyrA allele by Sanger sequencing. For GyrB cloning, fragments of
121 GyrB were amplified using primers DR542 and DR543. Electroporation was performed as above,
122 and single colonies were selected on GCB-K agar supplemented with 0.5 µg/mL ciprofloxacin.
123

124 **Genomic analysis**

125 The genomic pipeline was conducted with methodology as published.[32] Reads and metadata for
126 the sequenced *N. gonorrhoeae* isolates were accessed from multiple sources[33-62]. Reads were
127 then inspected using FastQC (v0.11.7) (https://www.bioinformatics.babraham.ac.uk/pro-
128 jects/fastqc/) and removed based on divergent GC content (from ~52-54%) or poor base quality.
129 The remaining reads were mapped to the genome of *N. gonorrhoeae* strain NCCP11945 (RefSeq
130 accession NC_011035.1) with BWA-MEM (v0.7.17-r1188)[63]. Mapped reads were then dedupli-
131 cated with Picard (v2.20.1) (https://github.com/ broadinstitute/picard). Samples with <40% cover-
132 age or <70% reads aligned were discovered with BamQC in Qualimap (v2.2.1)[64] and discarded.
133 Variants, including those in *gyrA* codons 91 and 95, were called with Pilon (v1.23)[65] with a mini-
134 mum mapping quality of 20 and a minimum depth of 10. Pseudogenomes were generated from
135 the resulting Pilon VCFs with inclusion of all PASS sites and alternate alleles with allele frequency
136 greater than 0.9. All other sites were set to "N", and finally samples with >15% of sites called as
137 missing were excluded. SPAdes (v3.12.0, with 8 threads, the –careful flag, and paired end reads
138 where available)[66] was used to create *de novo* assemblies, followed by contig filtering for quality
139 (coverage >10X, length >500 bp, total genome size ~2.0-2.4 Mbp). These assemblies were an-
140 notated using Prokka (v1.14.6)[67]. Finally, the phylogenetic tree in this study was assembled in a

141 recombination-corrected manner using Gubbins (v2.3.4)[68] from all sequenced isolates from Reim-
142 che *et al.*[55], representing lineages recently transmitting in the US, as well as the five genetically
143 manipulated strains in Table 1 and all strains that had GyrA^{91S} and GyrA$^{95G/A/N}$. The phylogeny
144 was visualized in iTOL (v6)[69].
145 Transformants in this study were sequenced at SeqCenter (seqcenter.com). Sample libraries
146 were assembled using the Illumina DNA Prep Kit and IDT 10bp UDI indices. The resulting libraries
147 were sequenced with an Illumina NextSeq 2000. The paired-end 2x151 bp reads were demulti-
148 plexed, followed by quality control and adapter trimming, using bcl-convert (v3.9.3) (https://sup-
149 port.illumina.com/sequencing/sequencing_software/bcl-convert.html). Resulting reads were
150 merged using BBMerge (v38.97)[70]. Reads were mapped to the parental genomic DNA assembly
151 and variants predicted in Geneious 2021.0 (www.geneious.com) using default settings.
152
153 **Experimental evolution**
154 Strain GCGS0481_SG (Supp. Table 1) was grown overnight on GCB-K agar. The overnight
155 streak was scraped, diluted to ~OD 0.1, then plated as 14 replicates overnight onto GCB-K agar
156 supplemented with 0.125 µg/mL ciprofloxacin. Each replicate was sequentially passaged, follow-
157 ing the same dilution pattern, onto double the concentration of ciprofloxacin overnight until there
158 was no visible growth or until 2 µg/mL was reached. Single colonies from the last resulting viable
159 passage were selected for further analysis.
160
161 **Results**
162
163 We selected five clinical *N. gonorrhoeae* isolates (Supp. Table 2) based on three major factors:
164 high-level ciprofloxacin resistance, genetic and geographic diversity, and varying sets of re-
165 sistance alleles (Figure 1A). All five of the strains encode GyrA^{S91F} as well as an additional muta-
166 tion at GyrA position 95. All five strains carried mutations in ParC that are known to cause reduced

167 susceptibility to ciprofloxacin, including ParC$^{S87R/N}$ and ParC$^{E91G/K}$ [71,72]. Finally, all five strains had
168 the GyrB^{429D} allele associated with zoliflodacin susceptibility[26].
169
170 We introduced six different alleles of GyrA into each strain using kanamycin co-selection, includ-
171 ing pairwise combinations of codon 91 (91S – susceptible, 91F – resistant) and codon 95 (95D –
172 susceptible, 95G, 95N – resistant). We were particularly interested in strains with the 91S codon,
173 as these isolates would be reported as ciprofloxacin susceptible by assays focused on this codon.
174 In all backgrounds, introduction of the GyrA$^{91S/95D}$ allele, containing the susceptible variant at both
175 codon 91 and 95, conferred susceptibility to ciprofloxacin. There was substantial MIC variation
176 even at this baseline, with MICs varying almost 10-fold (Table 1).
177
178 Introduction of a mutation at the 95 codon, either GyrA^{95G} or GyrA^{95N}, increased the MIC in all
179 backgrounds by approximately 10-fold. Some isolates, including NY0842, a 2013 urethral isolate
180 from New York City, and GCGS0481, a 2006 urethral isolate from U.S. Department of Health and
181 Human Services Region 10 (Alaska, Idaho, Oregon, and Washington), reached an intermediate
182 level of ciprofloxacin resistance by the Clinical and Laboratory Standards Institute (CLSI) break-
183 point (≥0.125 μg/mL)[73]. Notably, MICs for ciprofloxacin ≥0.125 μg/mL are associated with sub-
184 stantially increased risk of treatment failure[74]. Finally, introduction of GyrA$^{91F/95G/N}$ into all five back-
185 grounds did not substantially affect MICs compared to the original isolates (Table 1, Supp. Table
186 2), indicating that the co-selected kanamycin cassette minimally affected the ciprofloxacin re-
187 sistance profile.
188
189 These data suggest that some clinical isolates still retain ciprofloxacin non-susceptibility even
190 after reversion of the GyrA^{91F} allele to GyrA^{91S}. To evaluate whether examples with this genotype
191 and phenotype have been observed, we investigated *gyrA* genotypes in 11,355 clinical isolates
192 with published genomes and ciprofloxacin MICs. We identified 30 isolates bearing GyrA$^{91S/95G/N/A}$,

193 distributed across the *N. gonorrhoeae* phylogeny as singletons and closely related isolates (Fig-
194 ure 1A), suggesting that the GyrA$^{91S/95G/N/A}$ alleles can arise in multiple genetic backgrounds and
195 are sufficiently fit for transmission. The reported MICs for these isolates varied from 0.023 µg/mL
196 to 0.25 µg/mL, including four with MICs above the clinically defined threshold where treatment
197 failure is likely to occur (Figure 1B). Thus, diagnostic escape could occur either through reversion
198 or expansion of specific clades with GyrA^{91S} and reduced susceptibility to ciprofloxacin.
199
200 We investigated the possibility that GyrA^{91S} isolates could develop higher level ciprofloxacin re-
201 sistance through experimental evolution. We focused on the GyrA$^{91S/95G}$ mutant of GCGS0481,
202 differing from the parental isolate by only the kanamycin cassette and the 91S allele of *gyrA* as
203 validated by whole-genome sequencing (WGS). We sequentially passaged GCGS0481
204 GyrA$^{91S/95G}$ on increasing levels of ciprofloxacin, starting at ½ MIC (0.125 µg/mL) and increasing
205 two-fold up to 2 µg/mL. Of the 14 parallel cultures, 10 (78%) had no viable colonies at the MIC.
206 Single colonies from the four remaining cultures were then re-streaked and tested for ciprofloxacin
207 susceptibility. One colony had a high ciprofloxacin MIC (>32 µg/mL), attributable to an S91F mu-
208 tation; this colony was excluded from further analysis.
209
210 All three remaining mutants were isolated at 1 µg/mL ciprofloxacin, suggesting decreased sus-
211 ceptibility as compared to the GCGS0481 GyrA$^{91S/95G}$ strain (0.25 µg/mL; Table 2) and clinical
212 resistance to ciprofloxacin. WGS revealed that all three mutants had maintained GyrA^{91S} and
213 acquired mutations in GyrB. One isolate had a missense mutation encoding a GyrB^{E469D} mutation.
214 Introduction of this single-nucleotide polymorphism (SNP) in the parental isolate recapitulated the
215 decreased susceptibility to ciprofloxacin and also conferred a two-fold increase in MIC for zoli-
216 flodacin (Table 2).
217

218 Two of the evolved strains gained GyrB^{D429N} mutations, which have been shown to cause de-
219 creased susceptibility to zoliflodacin[24,75]; we validated by WGS that these GyrB^{D429N} strains had
220 not acquired other genomic mutations. Introduction of the GyrB^{D429N} mutation into the parental
221 isolate recapitulated the phenotype of decreased susceptibility to ciprofloxacin and led to an 8-
222 fold increase in the MIC for zoliflodacin as compared to the wildtype and parental GCGS0481
223 isolates (Table 2). These changes in antibiotic susceptibility are specific to ciprofloxacin and zoli-
224 flodacin (Supp. Table 3), suggesting that the differences in MIC are not due to general mecha-
225 nisms of reduced drug susceptibility.
226
227 **Discussion**
228
229 Our results indicate that GyrB^{D429N} is readily acquired by *N. gonorrhoeae* in the presence of ciprof-
230 loxacin and that GyrB^{D429N} increases ciprofloxacin resistance in at least some backgrounds. As
231 such, this study raises both conceptual and practical implications for the introduction of assays
232 using *gyrA* codon 91 to infer ciprofloxacin susceptibility. From a conceptual standpoint, this work
233 provides an example of how escape from a diagnostic used to guide antibiotic therapy can drive
234 the acquisition and possible spread of novel resistance determinants and how these pathways
235 may have unintended consequences for resistance profiles to other antibiotics[23]. The presence
236 of multiple clinical isolates with GyrA^{91S}, together with resistance-associated mutations at GyrA^{95},
237 strongly suggest that reversion of codon 91 is viable for pathogenesis. Our findings also indicate
238 that a test that detects both position 91 and position 95 would be less likely to select for reversion
239 to the susceptible allele, as all of the GyrA$^{91S/95D}$ strains in this study remained highly susceptible
240 to ciprofloxacin. Though the GyrB^{E469} mutation identified in this study has not been observed in
241 clinical isolates of *N. gonorrhoeae*, the corresponding GyrB^{E466D} mutation in clinical isolates of
242 *Salmonella enterica* serovar Typhimurium confers decreased susceptibility to ciprofloxacin[76]. This

243 finding provides further evidence consistent with the hypothesis that diagnostic escape from a
244 *gyrA* 91S assay may provide evolutionary pressure to drive novel resistance mechanisms.
245
246 The finding that $GyrB^{D429N}$ reduces susceptibility to both ciprofloxacin and zoliflodacin was unex-
247 pected. Earlier work on zoliflodacin showed that, in ciprofloxacin-resistant clinical isolates from
248 China, introduction of $GyrB^{D429N}$ led to a decrease in ciprofloxacin susceptibility or had no effect
249 on ciprofloxacin MIC[26]. Other studies have suggested that there is no cross-resistance between
250 ciprofloxacin and zoliflodacin in recently sampled clinical isolates[77,78], likely due to the finding that
251 $GyrB^{D429N}$ mutations have not been identified in sequences from ~13,000 clinical isolates[79]. In
252 contrast, enzymology suggests that ciprofloxacin has modestly decreased potency for the stability
253 of cleaved complexes with $GyrB^{D429N}$ as compared to $GyrB^{429D}$ (a CC_{50}, a measure of the potency
254 for stability of cleaved complexes, of 3 +/- 0.4 for the mutant vs. 1.9 +/- 0.7 for the WT)[75]. The
255 cleaved complex is the adduct of enzyme and DNA formed at DNA nicks that are unable to be
256 repaired in the presence of fluoroquinolones. As these cleaved complexes are thought to be cor-
257 related with, if not the mechanism of, fluoroquinolone-mediated cell death[80], these data are con-
258 sistent with zoliflodacin-ciprofloxacin cross-resistance. Further work is required to understand the
259 genetic background dependence of phenotypic outcomes following introduction of $GyrB^{D429N}$. One
260 intriguing possibility is that $GyrB^{D429N}$ is epistatic to $GyrA^{S91F}$ with respect to ciprofloxacin re-
261 sistance and is therefore less likely to arise in highly ciprofloxacin resistant backgrounds.
262
263 The findings in this work have implications for the introduction of zoliflodacin, pending a successful
264 phase III trial. From the perspective of zoliflodacin resistance, testing for the $GyrB^{D429N}$ allele
265 should be prioritized in locations where ciprofloxacin is used, as well as in settings where assays
266 targeting *gyrA* codon 91 inform treatment decisions. From the perspective of ciprofloxacin use,
267 the emergence of zoliflodacin resistant isolates may also lead to an increase in ciprofloxacin MIC.

268 GyrB^{429} should therefore be considered amongst the panel of mutations contributing to ciproflox-
269 acin resistance and informing antibiotic selection for clinical decision-making.
270

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280

281 **Conflicts of Interest**
282 Y. H. G. receives or has received support from Welcome Trust, Pfizer, and Merck, consulting
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285 mitted work.
286
287

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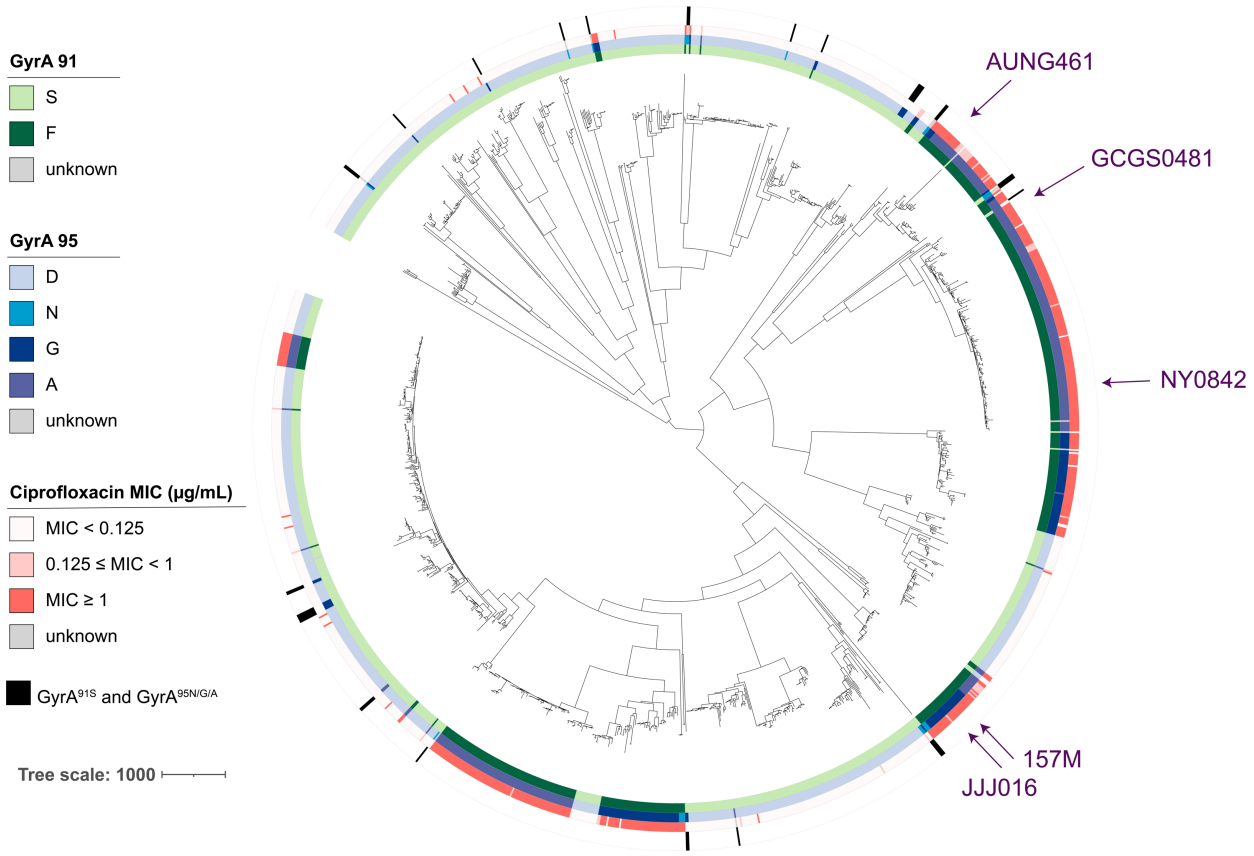
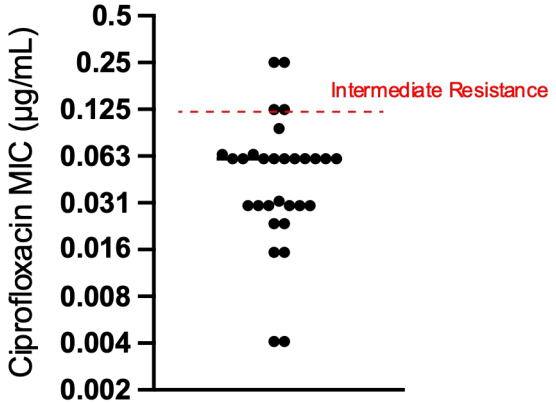
Figure 1

A


GyrA 91
S
F
unknown
GyrA 95
D
N
G
A
unknown
Ciprofloxacin MIC (µg/mL)
MIC < 0.125
0.125 ≤ MIC < 1
MIC ≥ 1
unknown
GyrA91S and GyrA95N/G/A
Tree scale: 1000
AUNG461
GCGS0481
NY0842
157M
JJJ016

B


Ciprofloxacin MIC (µg/mL)
0.5
0.25
0.125
0.063
0.031
0.016
0.008
0.004
0.002
Intermediate Resistance

1 **Figure 1. (A)** Recombination-adjusted phylogenetic tree of 1,510 *N. gonorrhoeae* clinical
2 isolates. On the innermost ring, the codon at position 91 of GyrA is coded by color, while on the
3 second ring, the codon at position 95 of GyrA is similarly marked. The ciprofloxacin MIC of each
4 isolate is shown on the third ring in red, while isolates with $GyrA^{91S}$ and $GyrA^{85N/G/A}$ are shown on
5 the outermost ring in black. Isolates from this study are marked by arrows in purple. Tree scale
6 represents recombination-adjusted number of SNPs. **(B)** Ciprofloxacin MICs of $GyrA^{91S}$ and
7 $GyrA^{95N/G/A}$ strains indicated in **(A)**, with the CLSI threshold for intermediate resistance marked
8 by a red dashed line.
9

Table 1

Strain	GyrA$^{91S/95D}$	GyrA$^{91S/95G}$	GyrA$^{91S/95N}$	GyrA$^{91F/95D}$	GyrA$^{91F/95G}$	GyrA$^{91F/95N}$
JJJ016	.006	.064	.19	.25	>32	>32
NY0842	.012	.19	.25	.25	>32	>32
AUNG461	.004	.064	.064	.19	12	12
GCGS0481	.023	.25	.25	.50	>32	>32
157M	.004	.047	.094	.25	>32	>32

10 **Table 1.** Ciprofloxacin MICs in µg/mL of five clinical isolates of *N. gonorrhoeae* following

11 introduction of GyrA alleles via kanamycin co-selection.

12

Table 2

Strain	Ciprofloxacin MIC (µg/mL)	Zoliflodacin MIC (µg/mL)	Ciprofloxacin concentration at isolation
GCGS0481 GyrA$^{91F/95G}$ (parent)	>32	0.25	N/A
GCGS0481 GyrA$^{91S/95G}$	0.25	0.25	N/A
GCGS0481 GyrA$^{91S/95G}$ Cip^{mut} 1	1.5	0.5	1 µg/mL
GCGS0481 GyrA$^{91S/95G}$ GyrB^{E469D}	1.5	0.5	N/A
GCGS0481 GyrA$^{91S/95G}$ Cip^{mut} 2	1.5	2	1 µg/mL
GCGS0481 GyrA$^{91S/95G}$ Cip^{mut} 3	1.5	2	1 µg/mL
GCGS0481 GyrA$^{91S/95G}$ GyrB^{D429N}	1.5	2	N/A

13 **Table 2.** Ciprofloxacin and zoliflodacin MICs of *N. gonorrhoeae* isolates with GyrA$^{91S/95G}$

14 following experimental evolution in the presence of ciprofloxacin.

15