Susceptibility trends of zoliflodacin against multidrug-resistant Neisseria gonorrhoeae clinical isolates in Nanjing, China (2014-2018)

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Susceptibility trends of zoliflodacin against multidrug-resistant *Neisseria gonorrhoeae* clinical isolates in Nanjing, China (2014-2018)

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ABSTRACT

Previously, we reported potent activity of a novel spiropyrimidinetrione, zoliflodacin, against *N. gonorrhoeae* isolates from symptomatic men in Nanjing, China, collected in 2013. Here, we investigated trends of susceptibilities of zoliflodacin in 986 isolates collected from men between 2014 and 2018. *N. gonorrhoeae* isolates were tested for susceptibility to zoliflodacin and seven other antibiotics. Mutations in *gyrA*, *gyrB*, *parC*, *parE* and *mtrR* genes were determined by PCR and sequencing. The MICs of zoliflodacin ranged from \( \leq 0.002 \) to 0.25 mg/L; the overall MIC\(_{50}\)s and MIC\(_{90}\)s were 0.06 mg/L and 0.125 mg/L in 2018, increasing two-fold from 2014. However, the percent of isolates with lower zoliflodacin MICs declined in each year sequentially while the percent with higher MICs increased yearly \((P \leq 0.00001)\). All isolates were susceptible to spectinomycin but resistant to ciprofloxacin \((\geq 1 \text{ mg/L})\); 21.2% \((209/986)\) were resistant to azithromycin \((\geq 1 \text{ mg/L})\), 43.4% \((428/986)\) were penicillinase-producing (PPNG), 26.9% \((265/986)\) tetracycline-resistant (TRNG) and
19.4% (191/986) were multi-drug resistant (MDR) isolates. Among 202 isolates tested, all were quinolone resistant with double or triple mutations in \textit{gyrA}; One hundred ninety three (193/202; 95.5\%) also had mutations in \textit{parC}. There were no D429N/A and/or K450T mutations in GyrB identified in the 143 isolates with higher zoliflodacin MICs; a S467N mutation in GyrB was identified in one isolate. We report that zoliflodacin continues to have excellent \textit{in vitro} activity against clinical gonococcal isolates, including those with high-level resistance to ciprofloxacin, azithromycin and extended spectrum cephalosporins.

INTRODUCTION

\textit{Neisseria gonorrhoeae}, the causative agent of the sexually transmitted infection gonorrhea, has developed resistance to all previously recommended antimicrobial agents for treatment, including sulfonamides, penicillins, tetracyclines and fluoroquinolones\cite{1}. Currently, dual antimicrobial therapy with ceftriaxone 250 mg or cefixime 400 mg plus azithromycin 1g is recommended as first-line treatment of uncomplicated gonorrhea by the World Health Organization (WHO)\cite{2} and ceftriaxone plus azithromycin by the U. S. Centers for Disease Control and Prevention (CDC)\cite{3}. Resistance to extended-spectrum cephalosporin (ESCs) and azithromycin is increasing worldwide. Gonococcal isolates with decreased susceptibility to cefixime and/or ceftriaxone have been reported in China\cite{4}, Japan\cite{5}, Australia\cite{6}, European countries\cite{7} and the United States\cite{8} and isolates with high-level resistance to ceftriaxone have
been identified in Japan, Australia, France, Spain, Denmark, Canada Ireland and China\textsuperscript{[9,10,11]}.

The reported prevalence of azithromycin-resistant \textit{N. gonorrhoeae} isolates is 18.6\% in China\textsuperscript{[4]}, 14.5\% in Japan\textsuperscript{[5]}, 6.2\% in Australia \textsuperscript{[6]}, 7.5\% in 25 European countries \textsuperscript{[7]}, 4.6\% in the United States \textsuperscript{[8]}, and 6.1\% in Western Africa\textsuperscript{[12]}. The first documented case that failed treatment with the recommended dual therapy was reported from the UK in 2016 \textsuperscript{[13]} and the first gonococcal isolates (the A2543 clone) with combined ceftriaxone plus high-level azithromycin resistance were identified in the UK\textsuperscript{[14]} and Australia\textsuperscript{[15]} in 2018.

Increased antimicrobial resistance (AMR) in \textit{N. gonorrhoeae} poses an emerging global public health threat of untreatable gonococcal infections. New oral antimicrobial agents with activity against \textit{N. gonorrhoeae} are needed urgently.

WHO includes \textit{N. gonorrhoeae} on its list of “priority pathogens” that require new antibiotics for treatment\textsuperscript{[16]} and the U.S. CDC has designated drug-resistant \textit{N. gonorrhoeae} as an urgent threat \textsuperscript{[17]}.

Zoliflodacin (also known as AZD0914 and ETX0914) is a novel spiropyrimidinetrione bacterial DNA gyrase/topoisomerase inhibitor with broad-spectrum \textit{in vitro} activity against gram-positive and fastidious gram-negative organisms, including \textit{N. gonorrhoeae}\textsuperscript{[18,19]}. A recent multicenter, randomized, phase 2 clinical trial demonstrated that zoliflodacin was effective in treating gonococcal urogenital and rectal infections and supports a larger, more definitive study of zoliflodacin for the treatment of uncomplicated gonorrhea\textsuperscript{[20]}. We showed previously that zoliflodacin was highly effective against clinical isolates of \textit{N. gonorrhoeae in vitro}, including high-level ciprofloxacin-resistant and multidrug-resistant.
resistant isolates, collected in 2013 in Nanjing, China[21]. Here, in vitro activities and
trends of zoliflodacin susceptibilities were determined for clinical gonococcal isolates
(including multidrug resistant isolates), collected between 2014 and 2018 in Nanjing.
Mutations in the quinolone-resistance-determinant regions (QRDRs) of gyrA, parC, gyrB, parE and mtrR genes in were also determined for isolates across the
zoliflodacin MIC distribution range.

RESULTS

Susceptibilities to zoliflodacin and other antimicrobials

Susceptibilities (MICs) of N. gonorrhoeae to zoliflodacin and seven antimicrobials
previously or currently used for the treatment of gonorrhea are summarized for the
986 clinical isolates in Table 1. All isolates except one were inhibited by ≤0.125 mg/L
of zoliflodacin (the remaining isolate had an MIC of 0.25mg/L). MICs to zoliflodacin
ranged from ≤0.002 to 0.25mg/L overall, with an MIC50 and MIC90 of 0.06 mg/L and
0.125 mg/L, respectively. One hundred forty three (14.5%) isolates had zoliflodacin
MICs at the upper end of the distribution range (0.125-0.25 mg/L) and 59 (6%)
isolates had MICs in the lower end of the MIC distribution range (≤0.002
-0.015mg/L). The percent of isolates with an MIC of 0.03 mg/L to zoliflodacin
declined in each year sequentially (χ² = 82.237, P=0.000) while the percent with MICs
of 0.06 and 0.125 mg/L increased correspondingly (χ² = 20.739 and 41.717,
respectively; P≤0.00001; Chi square test for linear trend), shown in Figure 1. Overall,
the proportion of isolates with zoliflodacin MICs 0.125-0.25 mg/L increased from 3.1% (6/197) in 2014 to 23.0% (47/204) in 2018 ($\chi^2 = 43.112$, $P<0.0001$).

All 986 isolates were resistant to ciprofloxacin; 777 (78.8%) showed high level resistance ($\geq$16 mg/L)\(^{[22]}\). During the five year study period, the annual percentage of ciprofloxacin resistant isolates at each MIC point (from 1 mg/L to $\geq$16 mg/L) did not shift in either direction in the 5-year period. MICs of gonococcal isolates for zoliflodacin were lower than ciprofloxacin ($P<0.0001$), with a median difference of at least 267-fold. Four hundred and twenty eight isolates (43.4%) were PPNG and 265 (26.9%) were TRNG. The percent of penicillin-resistant isolates increased from 70% to 86.3% over the five years ($\chi^2 = 17.641$, $P<0.0001$). Although all isolates were susceptible to spectinomycin, the percent of isolates with lower spectinomycin MICs (8 mg/L and 16 mg/L \(\geq\)) declined ($\chi^2 = 16.35$ and 93.71, $P=0.0001$ and $P<0.0001$), respectively while the percent with higher MICs (32 mg/L) increased over the five years ($\chi^2 = 112.514$, $P<0.0001$).

Two hundred and nine (21.2%) isolates were resistant to azithromycin (MIC $\geq$1 mg/L), and 62 (6.3%) displayed high-level resistance (MIC $\geq$256 mg/L). The percent of isolates with lower azithromycin MICs (0.06 mg/L and 0.125 mg/L) increased over the five years ($\chi^2 = 16.916$ and 22.099, respectively; $P<0.0001$) while the percent with higher MICs (0.5 mg/L and $\geq$1024 mg/L) declined yearly ($\chi^2 = 15.403$ and 12.268, respectively; $P<0.001$). Overall, the percent of azithromycin-resistant isolates (MIC $\geq$1 mg/L) decreased from 27.9% to 15.2% over the five years and the percent of azithromycin-susceptible isolates increased from 72.1% to 84.8% ($\chi^2 = 14.618$, $P<0.0001$).
One hundred and fifty eight isolates (15.2%) exhibited decreased susceptibility (MIC 0.125-0.25 mg/L, n=156) or resistance (MIC = 1 mg/L, n=2) to ceftriaxone, and 102 isolates (10.1%) displayed decreased susceptibility (MIC 0.25 mg/L, n=64) or resistance (MIC 0.5 mg/L, n=36; MIC>2 mg/L, n=2) to cefixime. The percent of isolates with lower ceftriaxone MICs (≤ 0.03 mg/L) declined in each year sequentially (χ²= 10.512, P<0.01) while the percent with higher MICs (0.06 mg/L and 0.125 mg/L) increased yearly (χ² = 10.18 and 4.231, P<0.01 and P<0.05, respectively). The percent of isolates with lower cefixime MICs (0.015 mg/L and 0.03 mg/L) declined (χ² = 23.324 and 10.734, P<0.001 and P<0.01, respectively) while the percent with higher MICs (0.06-0.5 mg/L) increased over the five years (χ² = 10.734, 8.68, 14.683 and 20.056, P<0.05, ~P<0.0001, respectively). One hundred ninety one (19.4%) isolates showed multidrug resistance (MDR). The proportion of MDR isolates increased from 7.1% in 2014 to 27% in 2016, then decreased to 21.1% in 2018 (χ² = 12.82, P=0.00034). The two MDR isolates with high level resistance to ceftriaxone (MIC 1.0 mg/L), cefixime (MIC ≥ 2.0 mg/L), ciprofloxacin (MIC ≥ 16 mg/L), penicillin (MIC 4 mg/L) and tetracycline (MIC 4 mg/L) had low zoliflodacin MIC values (0.03 and 0.06 mg/L, respectively).

Characterization of amino acid substitutions in GyrA, GyrB, ParC and ParE

All 202 isolates tested were ciprofloxacin-resistant (MICs 2 to ≥ 16 mg/L). All isolates had double or triple mutations in the gyrA gene. Both S91F and D95A/G/N/Y
amino acid substitutions in GyrA were identified in the 202 isolates. 16 (11.2%) of isolates in the higher zoliflodacin MIC distribution group and 2 (3.4%) in the lower MIC group also had an additional A92P amino acid substitution in GyrA. ParC substitutions were observed in 97.2% of the isolates in the higher zoliflodacin MIC distribution group and 91.5% in the lower MIC group. Single, double and triple ParC substitutions were identified in 114 (79.7%), 22 (15.4%) and 3 (2.1%) of the isolates in higher MIC distribution group and 66.1%, 25.4% and 0 in the lower MIC group, respectively. The amino acid substitution at position S87 in the ParC, including S87C, S87I, S87N and S87R was present in 79.7% isolates in the higher MIC distribution group and 81.4% in the lower MIC group, respectively. The most common double substitutions in ParC were S87R plus S88P (10.7%) in the higher MIC group, and S87R plus G85D (15.3%) in the lower MIC group. The three isolates in higher MIC group had the same triple substitutions (S87R, A123V and A129V). A89T, G120R, A123V and A129V mutations in ParC are newly described here. GyrB substitutions/insertions were identified in four isolates (two with V470I substitutions, one with a S467N substitution and one with an arginine (A) insertion at 480 [480A]) in the upper end of the MIC distribution group but none in low MIC group. All four isolates with a GyrB mutation had MIC values of 0.125 mg/L for zoliflodacin and 4 mg/L or greater for ciprofloxacin. Amino acid substitutions in ParE were identified in 57 isolates (39.9%) in the high zoliflodacin MIC distribution group. The most common single substitution in ParE was D437N, which was greater in isolates with MICs in the upper end of the zoliflodacin MIC distribution range (23.1%) than in the lower end of the range (6.78%)
The overall frequency of amino acid substitutions in GyrA, GyrB, ParC and ParE was no different across the MIC distribution range (Table 2).

**Mutations in mtrR**

A number of single or multiple mutations were identified in the 202 isolates, including an adenosine (A) deletion in the mtrR promoter region, and mutations in the mtrR coding region that resulted in amino acid changes in MtrR: A39T, A40D, G45D, F62L, D79N, and E117K mutations, singly or in combination (Supplemental Table 2). A total of 175 (86.6%) isolates carried the A deletion, 48 (81.4%) in the low zoliflodacin MIC group and 127 (88.8%) in the high group (P=0.2346). There were no significant differences in the rates of individual mutations (singly or combined) in MtrR accompanied (or not) by an A deletion in the promoter region, except for an H105Y mutation accompanied by an A deletion in the promoter, which accounted for 62.7% (37/59) of isolates with low zoliflodacin MICs and 41.3% (59/143) in the high zoliflodacin MIC group (P<0.01) (Supplemental Table 2).

**DISCUSSION**

We determined susceptibility trends in *in vitro* antibacterial activity of zoliflodacin and seven other antimicrobial agents against 986 clinical gonococcal isolates collected over a five-year period (2014-2018). The 986 gonococcal isolates were susceptible to zoliflodacin and all were resistant to ciprofloxacin. Nearly a quarter were resistant to azithromycin or were TRNG isolates. Greater than 40% were PPNG.
isolates and just under 20% were MDR isolates. All 986 isolates had zoliflodacin MICs below the breakpoint (MIC ≥ 0.5mg/L) that have been proposed, guided by clinical efficacy [20]. Similar to other reports [19,23], zoliflodacin exhibited an MIC range of 0.002 to 0.25 mg/L and there was no correlation between zoliflodacin MICs at the upper end of the MIC range and ciprofloxacin-resistance [19, 24, 25]. Furthermore, zoliflodacin exhibited low MICs (0.03 and 0.06mg/L) in two isolates that were fully resistant to ceftriaxone and cefixime. A modest temporal shift in the MICs to zoliflodacin was observed over the five year period.

Zoliflodacin is a novel spiropyrimidinetrione bacterial DNA gyrase/ topoisomerase inhibitor, which prevents bacterial DNA biosynthesis and results in accumulation of double-strand cleavages through a mechanism distinct from that in fluoroquinolones [18,24,26]. In our study, all the ciprofloxacin-resistant zoliflodacin-sensitive isolates tested, displayed double or triple mutations in GyrA; greater than 90% had additional amino acid substitutions in ParC.

In contrast to fluoroquinolones, zoliflodacin inhibits the GyrB subunit of type II topoisomerase; specific mutations in GyrB can result in increased resistance to zoliflodacin [24,25]. We did not find mutations such as D429N, D429A or K450T alterations in GyrB, which have been identified in vitro and select for resistant mutants that result in zoliflodacin MICs of 0.5–8 mg/L [24,25]. However, we found that 4/143 (2.8%) of gonococcal isolates at the upper end of the MIC distribution range MICs (0.125 and 0.25 mg/L) harbored a GyrB mutation, however the amino acid substitutions/insertions (S467N, V470I or 480A) were not associated with
resistance. An S467N amino acid substitution in GyrB, which did not result in reduced
susceptibility to zoliflodacin, has been reported in a clinical gonococcal isolate[19].

Mutations of V470I or 480A have not been reported previously in clinical isolates or
in *in vitro* selected resistant mutants.

Mutations in *mtrR*, which result in overexpression of the MtrCDE efflux pump, can
increase efflux of antimicrobials and reduce the susceptibility to numerous
antimicrobials [26,27]. The MtrCDE efflux pump can also influence susceptibility to
zoliflodacin[25]. Inactivation of the MtrCDE efflux pump has been shown to
decrease the MIC of zoliflodacin in *N. gonorrhoeae* strain H041 strain from 0.125 to
0.004 mg/L[25]. In our study, an adenine (A) deletion in the *mtrR* promoter and a
number of mutations in MtrR (or both), were identified in isolates that possessed
either lower or higher zoliflodacin MICs. A single H105Y amino acid substitution
was the most common substitution present in MtrR; this change was identified in 50%
of the isolates. The single H105Y amino acid substitution, which lies outside the
known DNA binding domain of MtrR, is generally thought not to be involved with
active repressor function of MtrR; it has also been shown to be associated with *N.
gonorrhoeae* isolates that are fully sensitive to ceftriaxone[28]. One possibility is that
the H105Y mutation may interfere with MtrR dimerization resulting in a reduction of
MtrR binding to target sequences[29]

Few studies have examined the impact of *parE* mutations on quinolone
resistance in *N. gonorrhoeae*[30,31]. Clinical gonococcal isolates with P439S amino acid
substitutions in ParE did not result in a significant increase in MIC to
ciprofloxacin\textsuperscript{[31,32]} . The clinical relevance of the ParE mutations identified in our study is unclear.

In conclusion, zoliflodacin demonstrated potent \textit{in vitro} antibacterial activity against a recent collection of clinical gonococcal isolates from China (2014 to 2018), including isolates with high-level resistance to ciprofloxacin, azithromycin and extended spectrum cephalosporins. Zoliflodacin MICs shifted upward temporally in the five-year period in the absence of clinical use. These results confirm the lack of pre-existing clinical resistance to zoliflodacin. Continued monitoring of antimicrobial susceptibility of zoliflodacin, a promising new oral antibacterial agent, for the treatment of uncomplicated gonorrhea is warranted.

**MATERIALS AND METHODS**

**Bacterial isolates** From January 2014 to December 2018, a total of 986 gonococcal isolates were collected from male patients with symptomatic urethritis (urethral discharge and/or dysuria) attending the STD clinic at the Institute of Dermatology, Chinese Academy of Medical Sciences, Nanjing, China. All men except one reported that they were heterosexual. Urethral exudates were collected with cotton swabs, then immediately inoculated onto Thayer-Martin medium (Zhuhai DL Biotech, China) and cultured in candle jars at 36°C for 24–48 h. Gonococcal isolates were identified by colonial morphology, Gram’s stain and oxidase testing and growth on GC chocolate agar base (Difco, Detroit, MI) supplemented with 1% IsovitaleX™ (Oxoid,
Gonococcal colonies were suspended in tryptone-based soy broth and frozen (−70°C) until used for antimicrobial testing.

**Antimicrobial susceptibility testing**  Zoliflodacin powder was provided by Entasis, Therapeutics, Waltham, MA. The minimum inhibitory concentrations (MICs; mg/L) of *N. gonorrhoeae* isolates to zoliflodacin, penicillin, tetracycline, ciprofloxacin, spectinomycin, azithromycin, cefixime and ceftriaxone were determined by the agar dilution method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines\[33\]. ATCC 49226, WHO reference strains F, G, L, O, and P were used as quality controls. The MIC ranges of zoliflodacin for quality control (QC) strain ATCC 49226 were 0.125-0.25mg/L in each antimicrobial susceptibility testing run in this study in accordance with the defined MIC QC ranges (0.06-0.5mg/L) for zoliflodacin\[34\]. Criteria for decreased susceptibility to ceftriaxone (MIC $\geq$0.125 mg/L) and cefixime (MIC $\geq$0.25 mg/L) were defined by WHO\[35\]. Using CLSI\[33\] and EUCAST \[36\] (for azithromycin only) criteria, the following MIC breakpoints were used to ascertain resistance: $\geq$128 mg/L, spectinomycin; $\geq$2 mg/L, penicillin and tetracycline and $\geq$1 mg/L, ciprofloxacin and azithromycin. The breakpoint for zoliflodacin of $\geq$0.5 mg/L was utilized as previously described \[20\]. Multi-drug resistant (MDR) *N. gonorrhoeae* was defined as decreased susceptibility or resistance to extended spectrum cephalosporins (ESCs), plus resistance to at least two of the following antimicrobials: penicillin; ciprofloxacin and azithromycin \[37,38\].

**Identification of gene mutations that resulted in amino acid substitutions in GyrA, GyrB, ParC and ParE**
One hundred forty three gonococcal isolates with zoliflodacin MICs (0.125mg/L and 0.25mg/L) at the upper end of the MIC distribution range and 59 isolates with lower zoliflodacin MICs (≤0.002-0.015mg/L) were selected for genetic resistance determinants study. Mutations in the quinolone-resistance-determining regions (QRDR) of \( \text{gyrA, gyrB, parC and parE} \) genes were determined by PCR and DNA sequencing using primers described previously \([39-41]\) (supplemental Table 1). Genomic DNA was extracted from gonococcal isolates using the Rapid Bacterial Genomic DNA Isolation Kit (DNA-EZ Reagents V All-DNA-Fast-Out, Sangon Biotech Co. Ltd, Shanghai). PCR amplification and sequencing of the genes were carried out by Nanjing Qingke Biotech Co. Ltd.

**Evaluation of mutations in the mtrR gene**

To identify mutations that potentially could cause enhanced expression of the MtrCDE-encoded efflux pump, mutations in the \( \text{mtrR} \) gene and promoter region were identified by PCR. Sequencing of \( \text{mtr} \) genes from 202 isolates was performed as described previously \([28]\).

**Data Analysis**

Chi-square (\( \chi^2 \)) testing was used to compare the rate of resistance in different years and Chi-square test for linear trends was used to assess the change in the MICs and the proportion of isolates resistant to antibiotics. SPSS version 19.0 was used for statistical analysis; P<0.05 was considered statistically significant.
ACKNOWLEDGEMENTS

We thank Dr. Unemo Magnus for providing WHO reference strains. This work was supported by the grants from the Chinese Academy of Medical Sciences Initiative for Innovative Medicine (2016-Ⅰ2M-3-021) and the U.S. National Institutes of Health (AI084048 and AI116969).

CONFLICTS OF INTEREST

One author is employed by the manufacturer of zoliflodacin but was not involved in the design or the execution of the study but rather in the writing/preparation of the manuscript. Other authors declare no conflicts.

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Table 1. Susceptibilities and MICs of zoliflodacin and seven antimicrobials previously or currently used for treatment of gonorrhea against 986 clinical *N. gonorrhoeae* isolates.

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>No. (%)</th>
<th>Intermediate</th>
<th>Resistant</th>
<th>Range</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>zoliflodacin</td>
<td>986 (100)</td>
<td></td>
<td></td>
<td>≤0.002 to 0.25</td>
<td>0.06</td>
<td>0.125</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>0</td>
<td>171 (17.3)</td>
<td>815 (82.7)</td>
<td>0.125 to ≥16</td>
<td>4</td>
<td>≥16</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>4 (0.4)</td>
<td>150 (15.2)</td>
<td>832 (84.4)</td>
<td>≤0.125 to ≥32</td>
<td>2</td>
<td>≥32</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0</td>
<td>0</td>
<td>986 (100)</td>
<td>1 to ≥16</td>
<td>≥16</td>
<td>≥16</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>551(55.9)</td>
<td>226 (22.9)</td>
<td>209 (21.2)</td>
<td>≤0.015 to ≥2048</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>986(100)</td>
<td></td>
<td></td>
<td>≤4 to 32</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>Cefixime</td>
<td>948(96.1)</td>
<td>-</td>
<td>38 (3.9)</td>
<td>≤0.002 to &gt;2</td>
<td>0.03</td>
<td>0.25</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>984(99.8)</td>
<td>-</td>
<td>2 (0.2)</td>
<td>≤0.002 to 1</td>
<td>0.03</td>
<td>0.125</td>
</tr>
</tbody>
</table>

MIC: minimum inhibitory concentration
Table 2. Comparison of amino acid substitutions in GyrA, GyrB, ParC and ParE in isolates with lower zoliflodacin MICs versus isolates with higher MICs

<table>
<thead>
<tr>
<th>Amino acid substitutions</th>
<th>lower zoliflodacin MICs group (n=59)a</th>
<th>higher zoliflodacin MICs group (n=143)b</th>
<th>P-valuec</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GyrA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S91F</td>
<td>59(100.00%)</td>
<td>143(100.00%)</td>
<td>NA</td>
</tr>
<tr>
<td>D95A/G/N/Y</td>
<td>59(100%)</td>
<td>143(100%)</td>
<td></td>
</tr>
<tr>
<td>A92P</td>
<td>2(3.39%)</td>
<td>16(11.19%)</td>
<td>0.103</td>
</tr>
<tr>
<td>D80N</td>
<td>1(1.69%)</td>
<td>0</td>
<td>0.292</td>
</tr>
<tr>
<td>V81I</td>
<td>1(1.69%)</td>
<td>0</td>
<td>0.292</td>
</tr>
<tr>
<td><strong>ParC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G85C/D/A</td>
<td>14(23.73%)</td>
<td>7(4.90%)</td>
<td>0.001</td>
</tr>
<tr>
<td>D86N</td>
<td>3(5.08%)</td>
<td>20(13.99%)</td>
<td>0.088</td>
</tr>
<tr>
<td>S87C/I/N/R</td>
<td>48(81.36%)</td>
<td>114(79.72%)</td>
<td>0.943</td>
</tr>
<tr>
<td>S88P</td>
<td>1(1.69%)</td>
<td>10(6.99%)</td>
<td>0.181</td>
</tr>
<tr>
<td>A89T</td>
<td>1(1.69%)</td>
<td>1(0.70%)</td>
<td>0.499</td>
</tr>
<tr>
<td>E91G</td>
<td>2(3.39%)</td>
<td>7(4.90%)</td>
<td>1.000</td>
</tr>
<tr>
<td>G120R</td>
<td>0</td>
<td>2(1.40%)</td>
<td>1.000</td>
</tr>
<tr>
<td>A123V</td>
<td>0</td>
<td>3(2.10%)</td>
<td>0.557</td>
</tr>
<tr>
<td>A129V</td>
<td>0</td>
<td>3(2.10%)</td>
<td>0.557</td>
</tr>
<tr>
<td><strong>GyrB</strong></td>
<td>0</td>
<td>4(2.80%)</td>
<td>0.32</td>
</tr>
<tr>
<td>S467N</td>
<td>0</td>
<td>1(0.70%)</td>
<td>1.000</td>
</tr>
<tr>
<td>V470I</td>
<td>0</td>
<td>2(1.40%)</td>
<td>1.000</td>
</tr>
<tr>
<td>+480A</td>
<td>0</td>
<td>1(0.70%)</td>
<td>1.000</td>
</tr>
<tr>
<td><strong>ParE</strong></td>
<td>20(33.90%)</td>
<td>57(39.86%)</td>
<td>0.43</td>
</tr>
<tr>
<td>D437H/N</td>
<td>5(8.47%)</td>
<td>34(23.78%)</td>
<td>0.01</td>
</tr>
<tr>
<td>P456S</td>
<td>14(23.73%)</td>
<td>22(15.38%)</td>
<td>0.227</td>
</tr>
<tr>
<td>P469L</td>
<td>0</td>
<td>1(0.70%)</td>
<td>1.000</td>
</tr>
<tr>
<td>D425Y</td>
<td>1(1.69%)</td>
<td>0</td>
<td>0.292</td>
</tr>
<tr>
<td>L462I</td>
<td>1(1.69%)</td>
<td>0</td>
<td>0.292</td>
</tr>
</tbody>
</table>

a isolates with zoliflodacin MICs ≤0.002-0.015mg/L  

b isolates with zoliflodacin MICs 0.125-0.25mg/L  

c Determined by the χ² or fisher exact test
Figure 1. MIC distributions of zoliflodacin for 986 clinical *N. gonorrhoeae* isolates (2014-2018).