To the Editor: Neisseria gonorrhoeae (NG) with decreased susceptibility to ceftriaxone (CRO) and cefixime, the last option for first-line anti-microbial monotherapy, was spread globally. Until now, more than ten clinical treatment failures with CRO have been reported with minimum inhibitory concentration (MIC) range as 0.5 to 2.0 mg/L.\(^1\) CRO, thereafter, is no longer recommended as monotherapy for gonorrhea. Instead, dual therapy that combines CRO with azithromycin is currently exerted in many countries including China. However, the first treatment failure to dual therapy (500 mg CRO plus 1 g azithromycin) was identified in 2014 in London.\(^1\) The reasons for NG to gain the resistance to anti-microbial agents are either through spontaneous mutation and/or horizontal genetic transfer. Here, we applied whole-genome sequencing (WGS) to compare the mutation accumulation between NG isolates with susceptibility to CRO (CRO-S, MIC $< 0.125$ mg/L) and that with decreased susceptibility to CRO (CRO-DS, MIC $\geq 0.25$ mg/L).

NG isolates were collected from patients at the sexually transmitted infection clinics in Guangzhou, China, 2009 to 2013. Anti-microbial susceptibility was determined using the agar dilution method as previously described. Twenty-two CRO-DS NG isolates and 22 CRO-S NG isolates were included, which the selected isolates between the two groups were, respectively, matched as closely as possible by comparing their MICs for ciprofloxacin, spectinomycin, and azithromycin [Supplementary Table 1, http://links.lww.com/CMJ9/A238]. WGS was performed using Illumina HiSeq 4000 platform (Illumina, San Diego, CA, USA). The high-quality reads were aligned onto the publicly available reference genome (NG NCCP11945, NC_011035) with Burrows-Wheeler Aligner version 0.5.9-r16, and then single-nucleotide variants are identified using Sequence Alignment Map tools (version 0.1.19-44428cd). The gene mutations attributed to CRO-DS such as mtrR promoter 23 to 35 A deletion, mtrR Gly45, penA Ala501, penA Gly542 and penA Pro551, porB1b Gly120, and porB1b Ala121 were identified from the WGS data. A neighbor-joining phylogenetic tree of single-nucleotide variants was generated by MEGAN7.

After filtering low-quality bases and adapter sequences, the abundance of clean WGS reads shows no difference between CRO-S and CRO-DS NG ($P > 0.05$) [Figure 1A]. The point mutations in CRO-S NG strain as shown in Figure 1B are significantly more than that in CRO-DS NG isolates, illustrating by total (6206.46 ± 776.50 vs. 5420.73 ± 770.68, $P < 0.01$), homozygous (5694.32 ± 766.86 vs. 4968.59 ± 738.41, $P < 0.01$), and heterozygous mutation (512.14 ± 61.27 vs. 452.14 ± 69.85, $P < 0.01$), respectively. Moreover, a significant negative correlation was found between CRO MICs (range 0.004–0.500 mg/L) and the total number of point mutations ($r = 0.4631$, $P = 0.0013$, or heterozygous point mutations ($r = 0.3348$, $P = 0.0263$) [Figure 1C]. The point mutation types such as A $>$ G, G $>$ A, C $>$ T, and T $>$ C have high frequency in homozygous mutations as well as in heterozygous mutations; moreover, such types are significantly higher in CRO-S NG when compared with CRO-DS NG [Figure 1D]. The difference of point mutations between CRO-S and CRO-DS was also analyzed using circos plots [Figure 1E], which showed similar findings that CRO-S NG possessed more mutations than CRO-DS NG. In contrast, the point mutations within mtrR promoter 23 to 35 A deletion, at loci of penA (Ala501, Gly542, or Pro551) and porB1b...
Figure 1: Differences of point mutations between CRO-S NG (n = 22) and CRO-DS NG (n = 22). (A) The amount of clean whole genome sequencing data is not different in CRO-S and CRO-DS NG isolates group. (B) The number of point mutations (total, homozygous, and heterozygous) in CRO-S NG and CRO-DS NG. (C) The point mutations (total, homozygous, and heterozygous) are negatively correlated with CRO MICs, respectively. (D) The overall mutation types are shared or different between CRO-S and CRO-DS NG isolates. (E) The circos diagram summarizes the point mutations in NG genome. The tracks from outer to inner rings represent NG genome (in Mb), the point mutations in CRO-S NG, the point mutations in CRO-S NG minus that in CRO-DS NG (green represents more mutations in CRO-S NG, whereas red represents more mutations in CRO-DS NG), (F) Phylogeny of NG based on single nucleotide variants, with the tracks from outer to inner rings representing amino acid mutations at mtrR promoter 23–35 A deletion, mtrR Gly45, penA Ala501, penA Gly542, penA Pro551, porB1b Gly120, and porB1b Ala121. *P < 0.05 and **P < 0.01, as CRO-S NG compared to CRO-DS NG. CRO: Ceftriaxone; MIC: Minimum inhibitory concentration; NG: Neisseria gonorrhoeae.
(Gly120 or Ala121) with the exception of mtrR Gly45, were slightly lower in CRO-S NG than that in CRO-DS NG, although the difference was not statistically significant [Figure 1F and Supplementary Figure 1, http://links.lww.com/CM9/A239].

Interestingly, this study provides evidence that CRO-S NG has markedly more point mutations (homozygous and/or heterozygous) compared with CRO-DS NG. CRO-S NG evolving under certain selection may be required to acquire more mutations for conferring resistance, whereas CRO-DS NG may only need relatively lower mutations because it has acquired some degree of resistance evolution to cope with such selection; however, further studies are needed to clarify this hypothesis. Indeed in present study, several mutations such as mtrR promoter 23 to 35 A deletion, penA (Ala501, Gly542, or Pro551), and porB1b (Gly120 or Ala121), correlated well with CRO-DS, were more frequently observed in CRO-DS NG, although these mutations were also presented in CRO-S NG. A previous study has strongly suggested a positive relationship between hypermutable (mutator) bacteria and acquisition of antibiotic resistance.\(^2\) Wistrand-Yuen et al recently reported that the bacteria evolved high-level resistance in response to lethal selection are different from those in sub-MIC selection; only few strong-effect resistance mutations are observed during lethal selections and sub-MIC selection generates many small-effect resistance mutations, but combination of such small-effect mutations can cause high-level resistance as well. Therefore, due to its hypermutation and the potential occurrence of resistance mutations, molecular surveillance of CRO-S NG should be enhanced as well as CRO-DS NG in the future.

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**Conflicts of interest**

None.

**References**


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