

Evaluation of *Neisseria gonorrhoeae* Isolates Susceptibility to Tetracycline Antibiotics from 9 Provinces in China Since 2020

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Purpose: The increasing drug resistance of *Neisseria gonorrhoeae* has become a serious public health concern. This study investigated *N. gonorrhoeae* isolates susceptibility to tetracycline antibiotics and the correlation between minimum inhibitory concentrations (MICs) of different antibiotics. The presence of resistance determinants in *N. gonorrhoeae* strains displaying different levels of tigeicycline resistance was also compared.

Methods: The minimum inhibitory concentrations (MICs) of tetracycline, minocycline, tigeicycline, eravacycline, omadacycline on 412 *N. gonorrhoeae* isolates were measured by the agar dilution method. The MICs of ceftriaxone and azithromycin were also measured to determine the correlations between antibiotics by the value of the correlation coefficient *R*. The presence of resistance determinants was identified through polymerase chain reaction (PCR) and sequencing.

Results: The MIC₉₀ was 64mg/L for tetracycline, 64mg/L for minocycline, 0.5mg/L for tigeicycline, 0.5mg/L for eravacycline, 4mg/L for omadacycline, 0.25 mg/L for ceftriaxone, and 1mg/L for azithromycin. The MIC₉₀ and mode of tigeicycline and eravacycline were much lower than those of tetracycline and minocycline. A poor correlation between omadacycline, eravacycline and tetracycline susceptibility was observed. Minocycline has a strong correlation with tetracycline. *PorB1* typing, *TetM*-encoding plasmid, and *mtrR* promoter adenine deletion were significantly correlated with tigeicycline MIC > 0.25mg/L.

Conclusion: This study suggested that tigeicycline and eravacycline had better in vitro activity and might be alternative antibiotics against resistant *N. gonorrhoeae* infections. Nevertheless, further in vitro experiments and clinical studies are needed for verification.

Keywords: *Neisseria gonorrhoeae*, antibiotics susceptibility, tigeicycline, eravacycline, omadacycline

Introduction

Neisseria gonorrhoeae (*N. gonorrhoeae*) is the etiologic agent of gonorrhea which causes a sexually transmitted infection that presents a major global public health concern.¹ According to the WHO estimation, there were 86.9 million incident global cases of gonorrhea among 15 to 49-year-old individuals in 2016.² Currently, the treatment of *N. gonorrhoeae* infection mainly relies on a variety of antibiotics.³ Notably, as the sensitivity of *N. gonorrhoeae* to various therapeutic drugs decreases with time, multidrug-resistant (MDR) *N. gonorrhoeae* cases have been reported intermittently in many countries, including China.^{4–6} Hence, the detection of putative alternative antibiotics and search for new therapeutic options are the key to the accurate treatment of patients with gonorrhea and to hindering the progress of drug resistance.⁷

Tetracyclines are a type of antibiotic discovered in the 1940s. They were used in the early years to treat gonorrhea, especially in patients with penicillin allergies. With increasing drug resistance and the emergence of *tetM*-encoding

plasmids, the clinical status of these antibiotics has gradually declined.⁸ In the last two decades, tetracyclines have reappeared on the market as the third-generation tetracycline antibiotics (tigecycline, eravacycline, sarecycline and omadacycline) were discovered.^{9,10}

The existing studies on the susceptibility of these tetracycline antibiotics of *N. gonorrhoeae* remain limited. While research on tetracyclines has progressed, subsequent evaluation of these antibiotics against *N. gonorrhoeae* is imperative. In this study, we analyzed the susceptibility of 412 *N. gonorrhoeae* isolates to seven antibiotics including five tetracycline antibiotics, ceftriaxone, and azithromycin.

Materials and Methods

Gonococcal Isolates

Clinical strains of 412 *N. gonorrhoeae* isolates were collected from 9 Chinese provinces (Guangdong (n=68), Sichuan (n=43), Hainan (n=36), Yunnan (n=47), Shanxi (n=18), Chongqing (n=49), Jiangsu (n=46), Guangxi (n=64), Shaanxi (n=41)) between 1 January and 31 December 2020. All the isolated strains were from urogenital secretions of confirmed gonorrhea patients and identified as *N. gonorrhoeae* after isolation, identification and pure culture. This project was approved by the Medical Ethics Committee at the Institute of Dermatology, the Chinese Academy of Medical Sciences & Peking Union Medical College and the National Center for Sexually Transmitted Disease Control (2014-LS-026). This study complies with Declaration of Helsinki. Participants no less than 18 years of age who signed an informed consent form to provide urine, vaginal and rectal swabs were enrolled in the study. All the strains were stored in skim milk in a deep freezer at -80°C before antimicrobial agent susceptibility testing. The WHO reference *N. gonorrhoeae* strains G, J, K, O, P, V, X, Y, and Z were used for quality control.

Antimicrobial Susceptibility Testing

Based on the WHO standard operation of the agar dilution method,¹¹ the antimicrobial susceptibility of all isolates was tested to seven antibiotics (ceftriaxone, azithromycin, tetracycline, minocycline, tigecycline, eravacycline and omadacycline). Eravacycline and omadacycline were obtained from Shanghai ZZBIO Co., Ltd (Shanghai, China). Tigecycline was obtained from Beijing BIOBW Co., Ltd (Beijing, China). Tetracycline, minocycline, ceftriaxone and azithromycin were obtained from the United States Pharmacopeia.

First, the *N. gonorrhoeae* isolates were revived on culture media overnight and then suspended in Mueller-Hinton (MH) broth. Subsequently, 2 μL of bacterial suspension at 10^7 CFU/mL was transferred onto antimicrobial medium which was a mixture of antibiotic and GC agar bases. Thereafter, the plates were incubated for 18–24 hours at 36°C in a 5% CO_2 -enriched atmosphere. Ultimately, the growth of *N. gonorrhoeae* in each concentration of antimicrobial-containing media was observed and recorded. The susceptibility test was conducted following the WHO guidelines¹¹ and the results were interpreted according to CLSI guidelines.¹²

Sequence Analysis of Resistance Determinants

There are no standardized criteria in the breakpoint of tigecycline against *N. gonorrhoeae*. According to the susceptibility test results, US Food and Drug Administration (FDA) interpretive criteria,¹³ and relevant reference,¹⁴ we chose 0.25mg/L as the breakpoint to distinguish the different levels of tigecycline resistance. Forty *N. gonorrhoeae* isolates with tigecycline MIC $>0.25\text{mg/L}$ and 40 *N. gonorrhoeae* isolates with tigecycline MIC $\leq 0.25\text{mg/L}$ were randomly selected to investigate the possible resistance determinants of tigecycline and the source of the correlation between the MICs of tigecycline and other antibiotics. PCR was utilized to amplify five loci (*rpsJ*, *mtrR*, *porB*, *tetM* and *23S rRNA*) using previously reported primers^{14,15} (Table 1). DNAs were sequenced using Sanger sequencing. DNA sequences were analyzed using Snapgene 5.3. Moreover, the AMR markers of *mtrR*, *porB*, and *23S rRNA* were analyzed via the NG-STAR website (<https://ngstar.canada.ca>).¹⁶

Table 1 Primers Implemented for PCR Assay and Target Mutations

Target	Primer Name	Primer Sequence
rpsJ	rpsJ_F	5'-GCCAAACCGGGTACTATCAACTCCT-3'
	rpsJ_R	5'-CCGATTTACCGACCGCACTGT-3'
mtrR	mtrR_F	5'-AACAGGCATTCTTATTTTCAG-3'
	mtrR_R	5'-TTAGAAGAATGCTTTGTGTC-3'
porB1b	porB1b_F	5'-CAAGAAGACCTCGGCAA-3'
	porB1b_R	5'-CCGACAACCACTTGGT-3'
tetM_A	tetM_A_F	5'-CTTCCCAACGGAAGCGGTGAT-3'
	tetM_A_R	5'-GGTCCGTCTGAACTTTGCGG-3'
tetM_D	tetM_A_F	5'-CTCCCTAATGGAAGCGGTGC-3'
	tetM_A_R	5'-GGTCAGTCTGAACTTTGCGG-3'
23S rRNA	23SrRNA_F	5'-ACGAATGGCGTAACGATGGCCACA-3'
	23SrRNA_R	5'-TTCGTCCACTCCGGTCTCTCGTA-3'

Statistical Analysis

Descriptive statistics (range, mode, MIC₅₀, MIC₉₀) were calculated to characterize the distribution of antibiotic MIC results. Furthermore, the correlations between antibiotics were determined by the value of the correlation coefficient *R* after logarithmic conversion of the MIC value. More specifically, poor, medium and strong correlations correspond to *R* values between 0.3–0.5, 0.5–0.8 and 0.8–1 respectively. The relation between gene mutation and drug resistance was interpreted through the chi-square test or Fisher's exact test, in which *P* values lower than 0.05 indicated statistical significance. SPSS software 22.0 (SPSS Inc., Chicago, USA) and Excel 2019 (Microsoft, Washington, USA) were applied for data analysis. Figures were made in RStudio.

Results

Antimicrobial Susceptibility Results of 412 *N. gonorrhoeae* Isolates

The susceptibility of the 412 clinical isolates was summarized in Tables 2 and S1. In total, tetracycline MIC values of 84.7% of these isolates were greater than or equal to 2mg/L, which was considered a clinical breakpoint for tetracycline resistance.¹⁷ In comparison, the MIC range of minocycline was 0.25–64mg/L, while its MIC₉₀ was the same as that of tetracycline. Meanwhile, the MIC₉₀ and mode of tigecycline and eravacycline were 0.5mg/L and 0.25mg/L respectively, which were much lower than those of tetracycline and minocycline. 70.1% of the isolates showed a tigecycline MIC ≤ 0.25mg/L and 88.3% showed an eravacycline MIC ≤ 0.25 mg/L. However, only 3 of 412 clinical isolates had a tigecycline MIC of 1mg/L. All the strains displayed a MIC ≤ 0.5mg/L for eravacycline. Furthermore, the MIC₉₀ and mode of omadacycline were 4mg/L, which was higher than those of other third-generation tetracyclines.

Table 2 Antimicrobial Susceptibility Results of 412 *N. gonorrhoeae* Isolates (Number of Strains Were Indicated in Parentheses)

Antimicrobial	MIC Range (mg/L)	Mode (mg/L)	MIC ₅₀ (mg/L)	MIC ₉₀ (mg/L)
Ceftriaxone	≤0.008–≥1	0.06 (139)	0.06 (139)	0.25 (20)
Azithromycin	≤0.03–≥8	0.25 (180)	0.25 (180)	1 (65)
Tetracycline	≤0.5–128	2 (186)	4 (148)	64 (59)
Minocycline	≤0.25–64	1 (141)	1 (141)	64 (45)
Tigecycline	0.03–1	0.25 (206)	0.25 (206)	0.5 (120)
Eravacycline	0.06–0.5	0.25 (244)	0.25 (244)	0.5 (48)
Omadacycline	0.5–8	4 (267)	4 (267)	4 (267)

Cross-Resistance Analysis for Different Drugs

To detect potential cross-resistance of *N. gonorrhoeae* between different antibiotics, the correlation between the MICs of four tetracycline antibiotics (minocycline, tigecycline, eravacycline and omadacycline) with tetracycline, ceftriaxone and azithromycin was compared (Figure 1). Among them, only minocycline and tetracycline showed a strong correlation ($R=0.96$). Apart from that, only weak correlations were observed: tigecycline with azithromycin ($R=0.44$), omadacycline with tetracycline ($R=0.36$) and eravacycline with tetracycline ($R=0.38$).

Possible Resistance Determinants of Tigecycline

Considering the susceptibility test results, US Food and Drug Administration (FDA) MIC interpretative breakpoints,¹³ and relevant reference,¹⁴ 0.25mg/L was chosen as the breakpoint of different levels of tigecycline resistance to delve into possible resistance determinants (Table 3). All *porb1a* strains were detected to be tigecycline sensitive through drug resistance gene scanning. In particular, susceptibility to *porb1b* mutations significantly correlated with tigecycline resistance (MIC>0.25mg/L). Moreover, the *tetM* plasmid correlated significantly with tigecycline (MIC>0.25mg/L), while *tetM* typing demonstrated no correlation with tigecycline susceptibility. Further typing of *mtrR* and *23S rRNA* genes showed that *mtrR* promoter-35ΔA was significantly correlated with tigecycline MIC > 0.25mg/L.

Discussion

According to some national and WHO current treatment guidelines,^{18–20} combination therapy with ceftriaxone and azithromycin is the recommended way to treat gonorrhea.²¹ However, the constant emergence of multidrug-resistant (MDR) *N. gonorrhoeae* isolates continues to hamper the curative effect of the aforementioned dual antibiotic therapy.²² Against the tendency of increased antibiotic resistance, suggestions from the WHO include “development of new

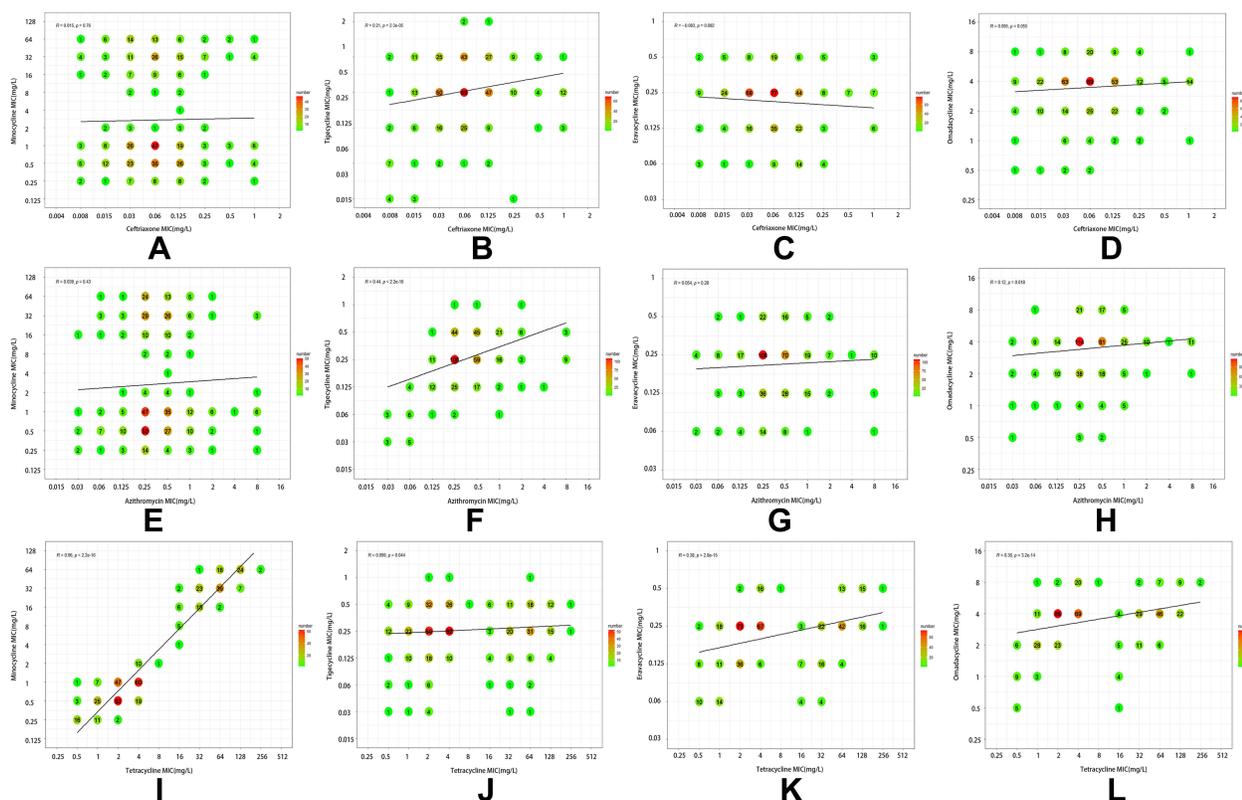


Figure 1 Correlation between MICs of four tetracycline antibiotics and tetracycline, ceftriaxone or azithromycin. Each symbol with specific numbers and colors represents one or multiple isolates. MIC values were log-transformed (\log_2) before calculating the regression line. (A–D) Correlation between MICs of minocycline, tigecycline, eravacycline, omadacycline and ceftriaxone. (E–H) Correlation between MICs of minocycline, tigecycline, eravacycline, omadacycline and azithromycin. (I–L) Correlation between MICs of minocycline, tigecycline, eravacycline, omadacycline and tetracycline.

Table 3 Presence of Resistance Determinants in *N. gonorrhoeae* Strains Displaying High Tigecycline MICs or Low Tigecycline MICs

Molecular Markers	MIC≤0.25mg/L	MIC>0.25mg/L	P
porBIA	11	0	0.013
porBIB	23	37	<0.001
GA120KD	20	36	0.153
A121D	16	33	0.086
rpsJ V57M	36	36	1
tetM	11	27	<0.001
tetM_A	1	3	1
tetM_D	10	24	1
mtrR	30	31	1
A39T	7	6	1
G45D	8	5	0.537
-35ΔA	16	26	0.02
23S rRNA	1	5	0.207
C261IT	1	4	0.364
A2059G	0	1	1

Abbreviations: tetM_A, tetM American type; tetM_D, tetM Dutch type.

antibiotics”, “recycling of old drugs”, etc.²³ With the listing of new tetracycline antibiotics, the effect of the second generation and third generation of tetracyclines against *N. gonorrhoeae* need to be further reevaluated. For this purpose, this study investigated for the first time all three generations of tetracyclines simultaneously, which can serve as an alternative therapy for *N. gonorrhoeae* infection.

Minocycline is a second-generation tetracycline antibiotic that was discovered in the 1970s. Its effectiveness against drug-resistant bacteria has made it favored in the clinic. In this study, the MIC range of minocycline was 0.25–64mg/L and the MIC₉₀ was the same as that of tetracycline. It could also be seen from the correlation analysis of the MIC values that minocycline has a strong correlation with tetracycline. Until then, no in vitro experiments on the resistance of *N. gonorrhoeae* to minocycline have been carried out. Only studies proved the synergistic effect of minocycline, azithromycin and ceftriaxone in vitro.²⁴ Therefore, further trials should be conducted to confirm the effect of minocycline.

Tigecycline is a third-generation tetracycline antibiotic that has been widely studied in recent years. The results showed that the MIC₉₀ of tigecycline was 0.5mg/l, and only 3 of 412 clinical isolates had a tigecycline MIC of 1mg/L. Through correlation analysis, tigecycline was only found to be weakly correlated with azithromycin and had no correlation with tetracycline and ceftriaxone. In the possible resistance determinants analysis, tigecycline (MIC>0.25mg/L) demonstrated no correlation with 23S rRNA mutation, which can lead to the medium and high resistance to azithromycin.^{25,26} This result indicates that tigecycline can be used in patients infected with ceftriaxone and tetracycline resistant *N. gonorrhoeae*. Our results were consistent with previous studies in Zhejiang/China and Canada,^{14,27} suggesting that tigecycline can be a candidate for gonococcal treatment in the future.

Eravacycline and omadacycline are both third-generation tetracycline antibiotics approved by the FDA in 2018.²⁸ Some countries have approved the former in the treatment of complicated intra-abdominal infections.²⁹ In our experiments, the MIC range of eravacycline against *N. gonorrhoeae* isolates was 0.06–0.5 mg/L. Approximately 88.3% of *N. gonorrhoeae* isolates had an eravacycline MIC below the susceptible breakpoint provided by the CLSI guidelines for *Staphylococcus aureus* (≤0.25 mg/L) and 100% had an eravacycline MIC below the susceptible breakpoint for *Escherichia coli* (≤0.5mg/L). Correlation analysis revealed a weak correlation with tetracycline and no correlation with ceftriaxone and azithromycin. These conclusions on antimicrobial susceptibility were similar to those proposed by other researchers.²⁷ Both in vitro results suggest the possibility of applying eravacycline in the treatment of gonococcal infection.

Omadacycline is a semisynthetic drug that is effective against Gram-positives and some Gram-negatives.³⁰ It is currently approved by the FDA for the treatment of acute bacterial skin, skin structure infection and community-acquired

bacterial pneumonia.³¹ As observed in our experiment, the MIC₉₀ of omadacycline was 4mg/L, which was higher than that of the other two third-generation tetracyclines. In consideration of the correlation with other antibiotics, only a weak correlation was found between it and tetracycline. In the pharmacokinetic study of Rodvold et al, omadacycline exhibited unsatisfactory in vivo availability, a single oral dose of 300 mg or intravenous injection dose of 100 mg of omadacycline resulted in a maximum plasma concentration of approximately 0.5–0.6 mg/L.³² Considering the difficult availability and high price of omadacycline, it is not suggested as a candidate drug to treat the gonococcal infection.

Conclusion

In this study, the activities of tetracycline antibiotics against *N. gonorrhoeae* were assayed in vitro. Compare to minocycline and omadacycline, tigecycline and eravacycline had better in vitro activity and might be alternative antibiotics against resistant *N. gonorrhoeae* infections, while the effect of minocycline and omadacycline needs further investigation. Hence, more in vitro experiments and clinical studies are needed to validate the findings in this paper and provide possible doses used for human clinical practice.

Data Sharing Statement

The data that supports the findings of this study are available in the [Supplementary Material](#) of this article.

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Disclosure

The authors declare that there is no conflict of interest regarding the publication of this article.

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