

Comparison of Microdilution Method with Agar Dilution Method for Antibiotic Susceptibility Test of *Neisseria gonorrhoeae*

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Introduction: Antimicrobial resistance (AMR) of *Neisseria gonorrhoeae* (*N. gonorrhoeae*) becomes a grave public health problem in the world. A strengthened Antimicrobial Resistance Surveillance Program is needed to track the trend of AMR development. However, the lack of a proper antimicrobial susceptibility test (AST) method is a barrier to expand the AMR surveillance in China. Traditional agar dilution (AD) method is laborious and E-test strips have no approval license for clinical use. Herein, a Chinese group modified the microdilution (MD) method for clinical ASTs. The objective of this study is to compare the MD method with the AD method for *N. gonorrhoeae* AST.

Materials and Methods: A total of 166 clinical isolates were tested for antimicrobial susceptibility of ceftriaxone, spectinomycin, azithromycin, ciprofloxacin, tetracycline, and penicillin using MD and AD method simultaneously. Results of MD method were read manually or automatically. Rates of essential agreement (EA), category agreement (CA), minor error, and very major error were compared.

Results: The total EAs (compared with results read manually) of penicillin, tetracycline, ciprofloxacin, spectinomycin, ceftriaxone, and azithromycin were 90.4%, 97.0%, 85.5%, 100.0%, 94%, and 72.3%; and CAs were 82.5%, 94.0%, 100%, 100%, 95.2%, and 94%, respectively.

Conclusion: We conclude that the MD method might be an alternative for clinical AST of *N. gonorrhoeae* in China. In particular, MD method has the potency of accurate differentiation of isolates resistant to ceftriaxone or azithromycin, which were empirically recommended for gonococcal treatment, but its quality remained suboptimal, and further improvement is needed for clinical use.

Keywords: *Neisseria gonorrhoeae*, antimicrobial susceptibility test

Introduction

Gonorrhea is one of the most prevalent sexually transmitted infections in the world. It has become a public health concern globally due to the antimicrobial resistance (AMR) to nearly all antimicrobials used for gonorrhea treatment.¹ AMR mitigates the management and control of gonorrhea. Nowadays, gonorrhea resistant to ceftriaxone, the first-line empiric treatment option, has been emerged and spread in many countries, including China.² Ideally, a strengthened gonococcal antimicrobial surveillance program is crucial to monitor the AMR trends and inform the adjustment of treatment guidelines.³ However, *Neisseria gonorrhoeae* (*N. gonorrhoeae*) is a fastidious bacterium that needs appropriate temperature and carbon dioxide concentration, and it is

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difficult to culture in liquid medium. These characteristics erect a barrier to its antimicrobial susceptibility tests (ASTs). ASTs in *N. gonorrhoeae* include qualitative tests (including disc diffusion assay and molecular tests) and quantitative tests (including agar dilution (AD) method and E-test method). The AD method is a global gold standard method for AST in *N. gonorrhoeae* and is now used in China Gonococcal Resistance Surveillance Program (China-GRSP). However, this traditional method is laborious and can only be used in regional or reference laboratories for a large number of isolates tests. E-test method is also a standardized AST method that can take stead of AD method and has benefits in clinical tests.⁴ However, the E-test strips are not commercially available for clinical use because it is not approved by the China Food and Drug Administration (CFDA) in China.⁵ Moreover, with the decreasing susceptibility to the currently recommended first-line treatment option ceftriaxone, it urgent to determine susceptibility profiles qualitatively or quantitatively within minimal time. Therefore, a quality-assured, quantitative, timely, and scalable method is needed in clinics to determine the antimicrobial susceptibility of *N. gonorrhoeae* isolates, especially to find out clinical ceftriaxone resistant isolates promptly.

The broth microdilution (MD) method has been applied for ASTs in many bacteria. It may become a promising quantitative method in *N. gonorrhoeae*.^{6,7} Several studies have attempted to develop MD methods for *N. gonorrhoeae*, but most tests had less accuracy.^{6,8} Recently, an MD method has been developed by a Chinese group and has been used for AMR surveillance in Guangdong province.⁹ However, the method was developed and evaluated in one lab and bias may be existed. Before scaling up the use of this method in China-GRSP, an objective evaluation is needed. Herein, we evaluated the broth MD method using reference strains and randomly selected clinical isolates. Furthermore, we evaluate the results read by an automatic reader by comparison with the results read manually for further investment of formulating a fully automatic detection kit.

Materials and Methods

Ethics Approval

The ethics approval for China-GRSP was obtained from 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 at Nanjing

(2014-LS-026) for the use of anonymized specimens collected annually from patients attending local dermatology or STD clinics.¹⁰ The isolates detected in our study were randomly selected from the China-GRSP sample bank collected in 2017.

Bacterial Strains and Broth Microdilution Assay

Clinical samples were collected from China-GRSP sentinel sites in 2017. Urethral swabs from males and endo-cervical swabs from females were collected and subsequently cultured on selective gonococcal culture media. Microscopy, rapid oxidase reaction and carbohydrate utilization test were used for species verification of *N. gonorrhoeae*. All strains were preserved in skimmed milk and stored at -70°C before tests. The samples used for this evaluation were randomly selected from the China-GRSP sample bank. Broth MD assay was performed according to the manufacturer's instruction and the reference.⁹ The 2008 World Health Organization (WHO) *N. gonorrhoeae* reference strains panel (D, G, J, K, L, and P) and ATCC49226 were included as outer quality control in every batch of experiments.¹¹ The results of each microplate were read manually and automatically by a plate reader after 24-hour incubation.

Agar Dilution Test

The minimal inhibitory concentrations (MICs) were determined according to the AD method recommended by WHO as previously described.¹⁰ The identical reference strains used in MD method were included in every batch of testing.¹¹

Essential Agreement and Categorical Agreement with Agar Dilution Test

Reference strains and contaminated strains were excluded from the data analysis procedure to avoid bias. The method reproducibility was assessed by calculating the essential agreement (EA), which was defined as the percentage of strains with predicted MICs that did not deviate by more than ± 1 doubling dilution from AD MICs. Ideally, an EA between different tests should be $>90\%$.¹²

The performance of the assays was evaluated by determining the categorical agreement (CA). Breakpoints from the European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.eucast.org/clinical_breakpoints/) were used to classify their interpretive categories as susceptible (S), intermediate (I) or resistant (R). Minor errors

were defined as misclassifications of intermediate strains as susceptible or resistant as previously described.¹² Very major errors were resistant strains that were misclassified as susceptible.

Results

During every batch of tests, MIC values obtained from the WHO *N. gonorrhoeae* reference strains were identical or within 1 MIC dilution of those previously reported.¹¹ A total of 166 clinical isolates were tested their antimicrobial susceptibility by AD and MD method, whereas one plate was omitted during the automatic read process. Hence, only 165 isolates were compared to their results read by naked eyes or the microplate reader machine.

The MIC results (AD method, MD method read manually, and MD method read automatically) along with distribution histograms were categorized by different antimicrobials and shown in the [supplemental file](#).

Comparing the MD method results read manually with the results of standard AD method, the total EAs of penicillin, tetracycline, ciprofloxacin, spectinomycin, ceftriaxone and azithromycin broth MD assay were 90.4%, 97.0%, 85.5%, 100.0%, 94% and 72.3% (Table 1). The CAs of the six antimicrobials were 82.5%, 94.0%, 100%, 100%, 95.2% and 94% separately, showing the MD method can accurately distinguish resistant strains for ceftriaxone and azithromycin, two kinds of antibiotics routinely prescribed for gonococcal treatment. Minor errors resulting from misclassifications of intermediated susceptible strains were found for 17.5% of the samples for penicillin MIC tests. Very major errors occurred for tetracycline (6.0%), ceftriaxone (4.8%), and azithromycin (6.0%) (Table 2).

For further investment to formulate automatic detection kits, results read by microplate reader automatically were

compared with the MICs read manually. Total EAs of penicillin, tetracycline, ciprofloxacin, spectinomycin, ceftriaxone and azithromycin were 81.8%, 95.2%, 92.7%, 95.2%, 88.5% and 92.1% (Table 3). CAs were 77.0%, 94.5%, 95.8%, 100%, 98.8% and 95.8%, respectively. Minor errors were found from 23% of the samples for penicillin MIC tests and 3.6% for ciprofloxacin MIC tests. Very major errors existed for tetracycline (5.5%), ciprofloxacin (0.6%), ceftriaxone (1.2%), and azithromycin (4.2%) (Table 4).

Discussion

Monitoring the trend of *N. gonorrhoeae* AMR is essential for public health agencies to control the gonorrhea epidemic. With the decreasing susceptibility to the currently recommended first-line treatment option cephalosporins, there is an urgent need to develop a simplified and sensitive method for clinical use to determine the antimicrobial susceptibility of *N. gonorrhoeae* qualitatively or quantitatively. At present, AD method is the gold standard for quantitatively determining MICs of a batch of isolates. However, the test is laborious and time-consuming. Besides, agar plates containing antimicrobials have a limited shelf-life (no more than 5 days).¹³ It is not feasible for laboratories that receive limited numbers of specimens or clinical laboratories that need to determine in time to perform AD tests routinely. An alternative method is E-test, for which antibiotic gradients have been applied to a plastic strip. E-test method has a potent prospect for future clinical surveillance to specific antimicrobial. However, this test is costly, requiring the use of strips that are expensive because of the manufacturer's patent protection, and these materials were not available in China because of no approved license by CFDA.^{4,5} Disk

Table 1 Essential Agreement (EA) of Microdilution Method (Read Manually) and Agar Dilution Method (Manually vs Agar Dilution)

Difference in MIC (n=166)	Penicillin		Tetracycline		Ciprofloxacin		Spectinomycin		Ceftriaxone		Azithromycin	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
<-2	2	1.2%	3	1.8%	2	1.2%	0	0.0%	0	0.0%	4	2.4%
-2	1	0.6%	2	1.2%	22	13.3%	0	0.0%	3	1.8%	42	25.3%
-1	9	5.4%	43	25.9%	100	60.2%	1	0.6%	17	10.2%	75	45.2%
0	80	48.2%	115	69.3%	41	24.7%	101	60.8%	74	44.6%	39	23.5%
1	61	36.7%	3	1.8%	1	0.6%	64	38.6%	65	39.2%	6	3.6%
2	13	7.8%	0	0.0%	0	0.0%	0	0.0%	6	3.6%	0	0.0%
>2	0	0.0%	0	0.0%	0	0.0%	0	0.0%	1	0.6%	0	0.0%
EA	150	90.4%	161	97.0%	142	85.5%	166	100.0%	156	94.0%	120	72.3%

Abbreviations: MIC, minimal inhibitory concentration; No., number; EA, essential agreement, deviation of MICs within 1 doubling dilution.

Table 2 Comparison Between Microdilution Method (Read Manually) and Agar Dilution Method (Manually vs Agar Dilution)

n=166	Penicillin		Tetracycline		Ciprofloxacin		Spectinomycin		Ceftriaxone		Azithromycin	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
EA	150	90.4%	161	97.0%	142	85.5%	166	100.0%	156	94.0%	120	72.3%
CA	137	82.5%	156	94.0%	166	100.0%	166	100.0%	158	95.2%	156	94.0%
VME	0	0.0%	10	6.0%	0	0.0%	0	0.0%	8	4.8%	10	6.0%
ME	29	17.5%	NA	NA	0	0.0%	NA	NA	NA	NA	NA	NA

Abbreviations: No., number; EA, essential agreement, deviation of MICs within 1 doubling dilution; CA, category agreement; VME, very major error, resistant strain being misclassified as susceptible; ME, minor error, intermediate strains being misclassified as susceptible or resistant; NA, not available.

Table 3 Essential Agreement (EA) of Microdilution Method Read Manually and Automatically (Manually vs Automatically)

Difference in MIC (n=165)	Penicillin		Tetracycline		Ciprofloxacin		Spectinomycin		Ceftriaxone		Azithromycin	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
<-2	8	4.8%	3	1.8%	1	0.6%	5	3.0%	7	4.2%	5	3.0%
-2	22	13.3%	5	3.0%	1	0.6%	3	1.8%	11	6.7%	8	4.8%
-1	74	44.8%	40	24.2%	31	18.8%	42	25.5%	79	47.9%	52	31.5%
0	61	37.0%	116	70.3%	115	69.7%	115	69.7%	67	40.6%	99	60.0%
1	0	0.0%	1	0.6%	7	4.2%	0	0.0%	0	0.0%	1	0.6%
2	0	0.0%	0	0.0%	4	2.4%	0	0.0%	0	0.0%	0	0.0%
>2	0	0.0%	0	0.0%	6	3.6%	0	0.0%	1	0.6%	0	0.0%
EA	135	81.8%	157	95.2%	153	92.7%	157	95.2%	146	88.5%	152	92.1%

Abbreviations: MIC, minimal inhibitory concentration; No., number; EA, essential agreement, deviation of MICs within 1 doubling dilution.

diffusion method is relatively easy to perform; however, the results were qualitative and it is unable to measure differences in antimicrobial susceptibilities.

In China, gonorrhea was the fourth most commonly reported notifiable communicable disease, with 133,156 reported cases in 2018.¹⁴ However, the isolates collected for ASTs by China-GRSP accounted for less than 1.8% (2344/133156). The scale of surveillance should be enlarged to screen resistant isolates. Notably, the rates of isolates with decreased susceptibility to ceftriaxone or resistance to azithromycin were high in China.¹¹ Moreover, non-

adherence to national guidelines and empirical treatments with high doses of ceftriaxone may lead to antimicrobial resistance.¹⁵ Hence, the AST for clinical use is critical for appropriate prescription and individualized treatment. Furthermore, clinics in small and middle-sized cities have limited labor force and experimental consumables availability for AD and E-test methods. And it is time-consuming to preserve and transport strains to central laboratories for further AST. Therefore, a simplified method that is affordable, specific, sensitive, rapid, robust, and even automatic is urgently needed.

Table 4 Comparison Between Results Read Manually and Automatically (Manually vs Automatically)

n=165	Penicillin		Tetracycline		Ciprofloxacin		Spectinomycin		Ceftriaxone		Azithromycin	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
EA	135	81.8%	157	95.2%	153	92.7%	157	95.2%	146	88.5%	152	92.1%
CA	127	77.0%	156	94.5%	158	95.8%	165	100.0%	163	98.8%	158	95.8%
VME	0	0.0%	9	5.5%	1	0.6%	0	0.0%	2	1.2%	7	4.2%
ME	38	23.0%	NA	NA	6	3.6%	NA	NA	NA	NA	NA	NA

Abbreviations: No, number; EA, essential agreement, deviation of MICs within 1 doubling dilution; CA, category agreement; VME, very major error, resistant strain being misclassified as susceptible; ME, minor error, intermediate strains being misclassified as susceptible or resistant; NA, not available.

The innovation of the MD method is of clinical importance. The MD method made it possible to determine the MICs of several antimicrobials in clinical laboratories simultaneously. The comparison between MD with the AD method showed that these two methods were generally comparable in determining antimicrobial susceptibility and interpreting categories. However, it is vital to notice that the EAs of the MD method to ciprofloxacin (85%) and azithromycin (72%) were low. MICs tested by MD were higher than AD method. We hypothesized discrepancies in EAs for these two insoluble antimicrobials may be caused in the stock solutions preparation procedure, when the agents cannot be fully dissolved. Then, serial solutions with drugs in lower concentrations were lyophilized to manufacture microplates. Notwithstanding with lower CAs and higher minor errors for penicillin, we noted that distinctions were on account of the MICs distribution of some isolates. MICs of these isolates were one dilution higher or lower than the standard breakpoint recommended by EUCAST and the deviation of one dilution may cause different susceptibility categories. Comparison between results read manually and automatically shown universally higher MICs in results read manually. We speculated that the discrepancy could be due to the turbidity value of referenced growth controls, which was the mean of two wells with no antibiotics.⁹ Therefore, we neglected that the growth was partially refrained in some wells, causing an undistinguished decrease in turbidity by naked eyes.¹³ Compared with manual judgement, the automatic process is labor-saving and objective. Additionally, the microplate reader machine can be replaced by an enzyme-labeled instrument or a nephelometer, which is used for other experiments. With further amendment and improvement, manual labor time can be significantly reduced when performing a large-scale AMR screening by the full automation of several steps in the MD method such as preparing the microplates with a liquid handling system, as well as, adding bacterial suspension in broth using a liquid dispensing system.

The greatest advantage of the MD method is performing ASTs for several antimicrobials at the same time for relatively few isolates. It has proved to be a useful tool in ASTs, mainly to empirical therapy (ceftriaxone or azithromycin), and even contributing to the individualized treatment of gonococcal infections. Meanwhile, results of this method can be discerned by objective readouts automatically, with immense prospects of a high-throughput screening.

On the other hand, there are also a few limitations. Primarily, the results of antimicrobial susceptibilities to ciprofloxacin and azithromycin were comparably lower. More accuracy needs to be improved when producing serially diluted wells for these antibiotics. Furthermore, results read manually shown higher MICs, and sometimes they were difficult to distinguish. An indicator can be added in wells to form an apparent visual distinction, such as resazurin, which can convert into pink-fluorescent in the presence of the metabolically active cell.^{16,17} Lastly, the method bases on the cultivation of *N. gonorrhoeae* isolates and takes over 12 hours for the incubation of *N. gonorrhoeae* in the microplates, without superiority than other methods. More researches and innovations are needed to shorten the time, allowing susceptibility results to be obtained on the same day like other bacteria.¹⁸

Conclusion

In summary, the broth MD assay is an objective, high-throughput, cost-effective, and quantitative method for AST of clinical *N. gonorrhoeae* isolates, having generally comparable accuracy. Continuous surveillance is necessary for monitoring the trend of resistant isolates. For clinical laboratories that require testing MICs or interpreting categories of small numbers of isolates, especially to therapeutic antimicrobials, this innovative method can be a reliable tool after further improvement. In short, the MD method proves to be a capable assay for high-throughput screening and surveillance of ceftriaxone- or azithromycin-resistant *N. gonorrhoeae* isolates.

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Disclosure

The authors declare no conflicts of interest.

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