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ORIGINAL ARTICLE

Comparison of Disk Diffusion and Agar Dilution for *Neisseria gonorrhoeae* Susceptibility Testing and Update

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ABSTRACT

Background: **The aim of this study was to evaluate the validity of the disk diffusion for antimicrobial susceptibility testing of clinical *Neisseria gonorrhoeae* isolates, using the agar dilution as the reference standard. Four antimicrobial agents were assessed: penicillin, ciprofloxacin, ceftriaxone, and spectinomycin, with updated susceptibility data provided.**
Methods: **A total of 185 *Neisseria gonorrhoeae* isolates, identified by 16S rRNA sequence analysis, were collected from clinical specimens obtained from patients in four general hospitals in Changsha, China, between 2014 and 2018. Antimicrobial susceptibility to the four agents was determined by both disk diffusion and agar dilution. Correlation and agreement between the two methods were analyzed by plotting inhibition zone diameters against their corresponding minimal inhibitory concentrations (MICs) and performing linear regression analysis.**
Results: **By disk diffusion, the susceptibility rates were 1.1% (penicillin), 0.6% (ciprofloxacin), 98.4% (ceftriaxone), and 100% (spectinomycin), while by agar dilution the rates were 1.6% (penicillin), 0% (ciprofloxacin), 95.7% (ceftriaxone), and 100% (spectinomycin). The disk diffusion showed high categorical agreement with the agar dilution, with concordance rates of 98.9%, 99.4%, 96.2%, and 100% for penicillin, ciprofloxacin, ceftriaxone, and spectinomycin, respectively. Pearson correlation coefficients were -0.78, -0.65, -0.47, and -0.61 (all $p < 0.001$). Although discrepancies between the two methods were acceptable, further analysis is required to clarify discordant results observed for ceftriaxone in some isolates.**
Conclusions: **The disk diffusion remains a reliable qualitative approach for antimicrobial susceptibility testing of *Neisseria gonorrhoeae*, with results largely comparable to the agar dilution reference method.**
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INTRODUCTION

Neisseria gonorrhoeae (*N. gonorrhoeae*) is the etiological agent of gonorrhea, a sexually transmitted infection that poses a significant threat to global public health. The World Health Organization (WHO) estimates that approximately 78 million new cases of *N.* gonorrhea occur annually [1]. *N. gonorrhoeae* infection induces local inflammation, leading to genital pain and discomfort,

and is associated with enhanced susceptibility to HIV acquisition and transmission. If left untreated, *N.* gonorrhoeae may cause severe complications, including pelvic inflammatory disease, ectopic pregnancy, and infertility [2-4]. Therefore, timely diagnosis and effective treatment of *N. gonorrhoeae* infections are of particular importance.

At present, antibiotics remain the cornerstone of *N.* gonorrhea treatment. However, the effectiveness of nearly all antimicrobial classes has been compromised by the rapid emergence of resistance. Antimicrobial resistance in *N. gonorrhoeae* is now recognized as a major global public health concern. Since 2010, dual therapy with a cephalosporin plus either azithromycin or doxycycline has been recommended to improve treatment efficacy and mitigate resistance development [5]. Nevertheless, treatment failures have been reported in Japan [6], Australia [7], and South Africa [8]. Furthermore, extensively drug-resistant strains (H041 and F89), termed "superbugs" were first identified in Japan and France, both exhibiting high-level resistance to cephalosporins [9, 10]. Declining susceptibility to cefixime in the United States and increasing reports of treatment failures prompted the Centers for Disease Control and Prevention (CDC) to withdraw cefixime as a first-line regimen [11]. Since 2015, the CDC has recommended only the combination of ceftriaxone and azithromycin as empirical first-line therapy for uncomplicated *N. gonorrhoeae* [12]. Alarmingly, in 2018, the first *N.* gonorrhea strain resistant to ceftriaxone and displaying high-level azithromycin resistance was identified in England [13]. Spectinomycin, an aminocyclitol antibiotic, has historically been effective against *N. gonorrhoeae* and remains an alternative treatment option for patients with resistance or allergy to first-line agents. The developments highlight the urgent need for continuous surveillance of *N. gonorrhoeae* antimicrobial susceptibility.

Currently, the agar dilution is recommended by the Clinical and Laboratory Standards Institute (CLSI) as the reference standard for antimicrobial susceptibility testing (AST) of *N. gonorrhoeae*. However, the disk diffusion, originally described by Bauer et al. in 1966 [14], remains one of the most widely applied methods in clinical laboratories due to its simplicity and cost-effectiveness. The accuracy and reliability of the disk diffusion are therefore of critical importance. In the present study, we collected 185 clinical isolates of *N.* gonorrhoeae and compared the results of disk diffusion with those obtained by agar dilution. The objectives were to assess the accuracy of the disk diffusion, evaluate its applicability in clinical practice, and provide updated data on *N. gonorrhoeae* antimicrobial susceptibility.

MATERIALS AND METHODS

Isolation and identification of *N. gonorrhoeae*

A total of 185 *N. gonorrhoeae* isolates were collected from clinically relevant specimens of patients attending four general hospitals in Changsha, China. Isolates were obtained from cervical secretions, urethral secretions, and prostatic fluids of patients with suspected gonococcal infection. Initial identification was performed using Gram staining, the superoxol test (30% hydrogen peroxide), and carbohydrate utilization reactions. For culture, isolates were subcultured on chocolate agar containing polymyxin B (25 μg/mL) and vancomycin (3.3 μg/mL) and incubated for 18 - 24 hours at 35°C in a humidified atmosphere enriched with 5% CO_2. Phenotypic identification of colonies was confirmed by morphology, Gram staining, oxidase, and catalase activity tests. Molecular identification was performed by polymerase chain reaction (PCR) targeting the 16S rRNA gene. PCR products were sequenced bi-directionally using an MGISEQ-200 platform (BGI, China). Sequence homology was analyzed with BLAST v2.0 (http://www.ncbi.nlm.nih.gov/BLAST/), and isolates showing ≥ 99.0% identity with the reference sequence (*N. gonorrhoeae* ATCC 49226) were confirmed as *N. gonorrhoeae* [15].

Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined by agar dilution according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, which served as the reference method [16]. Four antimicrobial agents - penicillin (0.015 - 8 U/mL), ciprofloxacin (0.125 - 32 μg/mL), ceftriaxone (0.008 - 0.5 μg/mL), and spectinomycin (8 - 64 μg/mL) were obtained from Sigma (St. Louis, MO, USA). Agar plates with serial twofold dilutions of each antibiotic and control plates without antibiotics were prepared in GC medium base (Difco, USA) supplemented with IsoVitaleX enrichment (BD, USA), which was added to provide essential nutrients required for the optimal growth of *N. gonorrhoeae*. Bacterial suspensions equivalent to 0.5 McFarland were prepared in saline, and approximately 5 x 10^5 CFU per spot were inoculated onto agar plates using a multipoint inoculator. Plates were incubated for 20 - 24 hours at 35°C in a humidified 5% CO_2 atmosphere. Minimal inhibitory concentrations (MICs) were interpreted according to CLSI criteria.

In parallel, susceptibility was assessed by disk diffusion following CLSI guidelines [16]. The same 0.5 McFarland suspension was applied to GC agar plates. Disks containing penicillin (10 U), ciprofloxacin (5 μg), ceftriaxone (30 μg), and spectinomycin (100 μg) (Oxoid Ltd., Basingstoke, UK) were placed on the inoculated plates. After incubation under the same conditions as the agar dilution, inhibition zone diameters were measured to the nearest millimeter with calipers. Interpretive zone breakpoints were determined according to CLSI standards. *N. gonorrhoeae* ATCC 49226 was used as a quality control strain in all experiments [17]. Each antimicrobial agent was tested in triplicate under identical conditions. According to CLSI, isolates with MIC ≤ 0.25 μg/mL are considered susceptible to ceftriaxone.

Table 1. Basic source information of 185 isolates.

Source of isolates	Specimen type of male		Specimen type of female	
	Prostatic fluid	Urethral secretions	Cervical secretions	Urethral secretions
Numbers	105	10	67	3

Table 2. *In vitro* activities of 4 antimicrobial agents against *N. gonorrhoeae* clinical isolates by disk diffusion and agar dilution (n = 185).

Antimicrobial agents	Disk diffusion (number (%) of isolates)			Agar dilution (number (%) of isolates)		
	Resistant	Intermediate	Susceptible	Resistant	Intermediate	Susceptible
Penicillin	121 (65.4%)	62 (33.5%)	2 (1.1%)	140 (75.7%)	42 (22.7%)	3 (1.6%)
Ciprofloxacin	181 (97.8%)	3 (1.6%)	1 (0.6%)	183 (98.9%)	2 (1.1%)	0 (0%)
Ceftriaxone	3 (1.6%)	0 (0%)	182 (98.4%)	8 (4.3%)	0 (0%)	177 (95.7%)
Spectinomycin	0 (0%)	0 (0%)	185 (100%)	0 (0%)	0 (0%)	185 (100%)

Table 3. MIC_{50} and MIC_{90} of 185 isolates to 4 antimicrobial agents.

Antimicrobial Agents	MIC (μg/mL)		
	MIC_{50}	MIC_{90}	Range
Penicillin	4	8	0.015 to 8
Ciprofloxacin	16	32	0.125 to 32
Ceftriaxone	0.03	0.125	0.008 to 0.5
Spectinomycin	16	32	8 to 32

MIC: minimal inhibitory concentration, $MIC_{50/90}$: MIC of antibiotic capable of inhibiting 50% and 90% of tested isolates, respectively.

Table 4. Disk diffusion results compared with reference agar dilution using the breakpoints established by the linear least-square regression method.

Antimicrobial agents	Number (%) of errors			r	r^2
	Minor error	ME	VME		
Penicillin	54 (29.1%)	1 (0.6%)	1 (0.6%)	-0.784	0.614
Ciprofloxacin	3 (1.6%)	2 (1.1%)	1 (0.6%)	-0.649	0.421
Ceftriaxone	0 (0%)	2 (1.1%)	7 (3.8%)	-0.470	0.220
Spectinomycin	0 (0%)	0 (0%)	0 (0%)	-0.606	0.368

ME: major error, VME: very major error.

Statistical analysis

MIC_{50} and MIC_{90} values were calculated for each antimicrobial agent. Data analysis was performed using Microsoft Excel 2010 and GraphPad Prism 5.0 software. Correlation between agar dilution (reference method) and disk diffusion was assessed by plotting inhibition zone diameters against corresponding MICs, followed by linear regression analysis. Pearson correla-

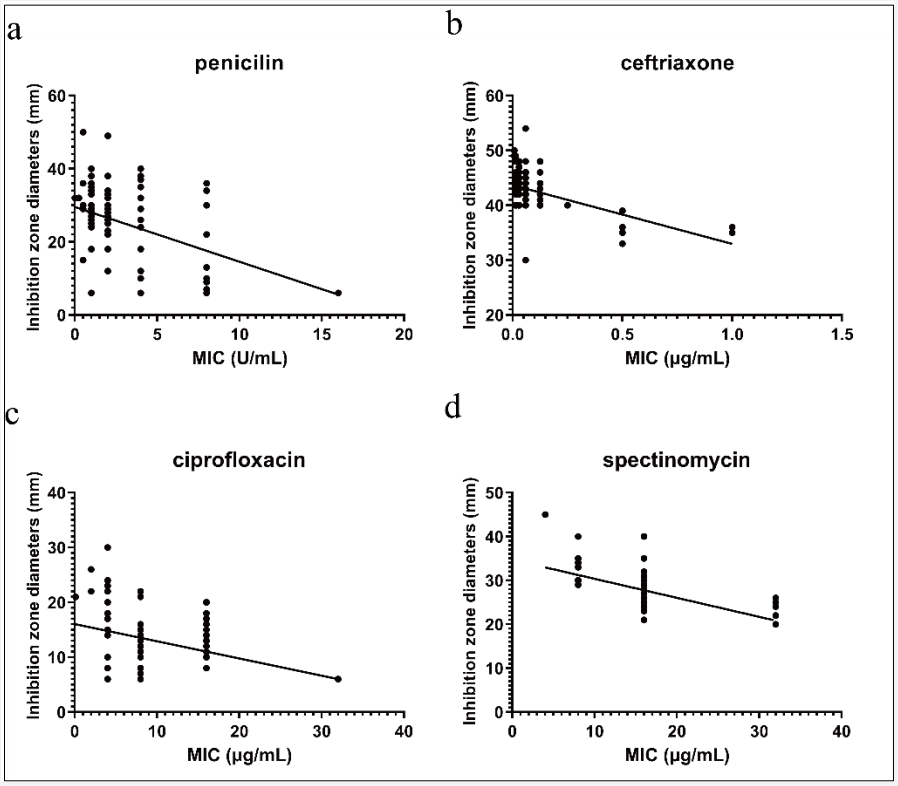

Figure 1. Plot of disk-diffusion zone diameter (mm) against agar-dilution minimum inhibition concentration (MIC, μg/mL), with CLSI threshold cutoffs for susceptibility.

A total of 185 isolates were tested. a: Penicillin, b: Ceftriaxone, c: Ciprofloxacin, d: Spectinomycin. X-axis: Agar-dilution (MIC, U/mL or μg/mL). Y-axis: Disk-diffusion zone diameter (mm).

tion coefficients (R) were calculated, with $p < 0.05$ considered statistically significant.

To evaluate categorical agreement, interpretations of results as susceptible (S), intermediate (I), or resistant (R) were compared between the two methods. Discrepancies were classified as minor errors (S vs. I or I vs. R), major errors (R by disk diffusion but S by agar dilution), and very major errors (S by disk diffusion but R by agar dilution).

RESULTS

Bacterial identification and source of isolates

All 185 clinical isolates were confirmed as *N.* gonorrhoeae by PCR amplification and sequencing of the 16S rRNA gene, showing ≥ 99% sequence homology with reference strains. The isolates were obtained from cervical secretions (36.2%), urethral secretions (7.0%), and prostatic fluids (56.8%) (Table 1).

Antimicrobial susceptibility by disk diffusion and agar dilution

All isolates were tested for susceptibility to penicillin, ciprofloxacin, ceftriaxone, and spectinomycin using both disk diffusion and agar dilution methods (Table 2). Because CLSI does not provide resistant breakpoints for ceftriaxone, EUCAST criteria were applied [18].

High resistance rates were observed for penicillin (75.7%) and ciprofloxacin (98.9%), while ceftriaxone (95.7%) and spectinomycin (100%) retained high activity. Notably, 4.3% of isolates exhibited reduced susceptibility to ceftriaxone, raising concerns about potential treatment failure. The MIC_{50}, MIC_{90}, and MIC ranges determined by agar dilution are shown in Table 3. Ceftriaxone continued to display potent antibacterial activity overall.

Correlation and discrepancies between disk diffusion and agar dilution

The inhibition zone diameters measured by disk diffusion correlated strongly with MIC values obtained by

agar dilution. Pearson correlation coefficients were -0.78, -0.65, -0.47, and -0.61 ($p < 0.001$) for penicillin, ciprofloxacin, ceftriaxone, and spectinomycin, respectively (Figure 1a - d). Categorical agreement between the two methods was 70% for penicillin, 96.8% for ciprofloxacin, 95.1% for ceftriaxone, and 100% for spectinomycin (Table 4). Discrepancies occurred mainly for penicillin, ciprofloxacin, and ceftriaxone, while no discrepancies were observed for spectinomycin. Although the discrepancies were generally acceptable, the misclassification of several ceftriaxone non-susceptible strains as susceptible warrants further investigation.

DISCUSSION

Antimicrobial resistance in *N. gonorrhoeae* has become a critical global health issue. Although dual therapy with ceftriaxone and azithromycin remains the recommended first-line regimen, isolates resistant to both agents have recently been reported [19-22]. In this study, several isolates displayed reduced susceptibility to ceftriaxone, consistent with international findings [23]. Ceftriaxone resistance is primarily associated with chromosomal mutations, particularly mosaic penA alleles, as well as alterations in penB and mtrR genes. However, the underlying mechanisms are not yet fully understood [24]. These developments highlight the urgent need to strengthen resistance surveillance, promote rational antibiotic use, and accelerate the development of new therapeutic options.

In China, antimicrobial susceptibility testing of *N.* gonorrhoeae commonly uses disk diffusion, agar dilution, and, less frequently, the E-test. While agar dilution remains the CLSI´s reference method, it is time-consuming, labor-intensive, and requires specialized facilities, limiting its use in routine clinical laboratories. The E-test offers a simpler alternative but is restricted by high costs and limited commercial availability of reagents in China. Consequently, disk diffusion is the most widely adopted method in primary and general hospitals.

Our findings confirm that disk diffusion demonstrates good overall concordance with agar dilution for penicillin, ciprofloxacin, ceftriaxone, and spectinomycin. Nevertheless, discrepancies were noted, particularly for ceftriaxone, where seven non-susceptible isolates were misclassified as susceptible. Minor errors were frequent for penicillin, while major and very major errors occured occasionally for penicillin and ciprofloxacin. Possible explanations include the following: 1) The qualitative nature of disk diffusion compared to the quantitative agar dilution method; 2) difficulties in interpreting inhibition zone boundaries; 3) lower drug purity in commercial disks compared with agar-prepared antibiotics; or 4) inter-operator variability in interpreting zone diameters. Given these limitations, we recommend that isolates with inhibition zones near clinical breakpoints, especially for ceftriaxone, be re-tested by agar dilution to minimize the risk of false-susceptible results. Clinicians should also be cautious when ceftriaxone treatment fails, as disk diffusion may overestimate susceptibility. Furthermore, the lack of azithromycin susceptibility testing is a limitation of this study, as azithromycin is recommended in dual therapy regimens. Future work should include azithromycin testing. In conclusion, the disk diffusion remains a reliable and practical tool for routine *N. gonorrhoeae* antimicrobial susceptibility testing, particularly in resource-limited laboratories. However, agar dilution should be prioritized in reference laboratories where higher accuracy is required. Enhanced personnel training and continuous surveillance are essential to improve diagnostic reliability and guide effective treatment strategies.

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Ethical Considerations:
The laboratory ID was anonymized before handling. Information about the date of sampling and site of sampling was available. Only subcultured bacterial isolates were stored; no tissue material or other biological material was kept. There was no personal data available, meaning no possibility of tracking patients or connecting them to the samples.

Declaration of Interest:
The authors declare no conflicts of interest.

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