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Comparison of *Neisseria gonorrhoeae* minimum inhibitory concentrations obtained using agar dilution versus microbroth dilution methods

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ABSTRACT

With increasing antibiotic resistance observed amongst clinical isolates of *Neisseria gonorrhoeae*, the second most prevalent sexually transmitted bacterial disease in the United States, there is still a need for antimicrobial susceptibility testing (AST). The current method recommended by the Clinical and Laboratory Standards Institute is agar dilution.

In this study, we show that a commercially available version of Fastidious Broth is capable of supporting *N. gonorrhoeae* in the evaluation of minimum inhibitory concentrations of 4 antibiotics (ceftriaxone, azithromycin, ciprofloxacin, and tetracycline), when comparing the agar dilution (AD) versus microbroth dilution (MBD) method and the susceptibilities obtained for 32 *N. gonorrhoeae* isolates. Herein, 3 out of the 4 antibiotics tested showed 94% or greater essential agreement (EA) and 91% or greater categorical agreement (CA) respectively, when comparing the MBD versus AD methods.

1. Introduction

Neisseria gonorrhoeae is a fastidious Gram-negative bacterium surviving solely as a human pathogen, infecting the pharynx, rectum, and genitourinary tissues. Left untreated, gonorrhoea infections are associated with infertility, a high rate of morbidity and more rarely, mortality (CDC 2013a, b; Liu et al. 2014; Kirkcaldy et al. 2016). The current primary antibacterial regimen to treat uncomplicated gonorrhoea infections is a combination therapy of both intramuscular ceftriaxone (250 mg) and oral azithromycin (1 g) (Workowski and Bolan, 2015). This dual therapy approach was implemented proactively as an attempt to impeded the emergence of resistance to the extended-spectrum cephalosporins, cefixime and ceftriaxone (CDC 2013a, b). Recently, there have been several cases outside the United States that report an increase in the minimum inhibitory concentrations (MICs) observed to the two first-line antibiotics underscoring this global health concern (Day et al., 2018; Eyre et al. 2018; Lefebvre et al., 2018; Unemo and Nicholas, 2013).

In the United States, the prevalence of antimicrobial resistance (AMR) amongst clinical isolates of *N. gonorrhoeae* is tracked by the Gonococcal Isolate Surveillance Project (GISP), which is funded by the Centers for Disease Control and Prevention (CDC) and includes participating clinics in 27 sites (Centers for Disease Control and Prevention (CDC), 2013a, b, 2015a, 2015b). More recently, two other CDC-funded programs have joined the cause to surveil and track AMR in *N. gonorrhoeae*, the Enhanced Gonococcal Isolate Surveillance Project (eGISP) and Strengthening the United States Response to Resistant Gonorrhoea (SURRG). Both programs include testing of strains isolated from female patients' as well as strains obtained from extra-genital sites. SURRG conducts AST using Etests for an accelerated detection of elevated MICs to cefixime, ceftriaxone, and azithromycin (CDC 2017). It is imperative to track levels of antimicrobial resistance in clinical isolates to ensure current treatment methods are adequate, to monitor gradual changes in susceptibilities, and to drive the discovery of novel treatment options.

At present, the AD method is the global gold-standard for AST of *N. gonorrhoeae*. However, since these fastidious organisms require several nutritional supplements in the growth medium and relatively high volumes of antibiotic to be tested it can be considered costly compared to other AST methods. The preparation of AD plates is also labor-intensive as the manual preparation of GC agar plus additional supplements needed for every test plate requires several steps including; sterilization, cooling, and the individual addition of antimicrobial test agent at various concentrations to determine an MIC. There is an urgency to develop a more efficient method of AST for testing *N. gonorrhoeae* in order to detect both increases in antimicrobial resistance to currently provided drugs and the efficacy of potential candidates in drug discovery. When considering strains to include in evaluating two different MIC methodologies, we wanted a collection of strains representative of

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isolates currently found circulating within the local community. The CDC/FDA AR bank was selected as they specifically collate strains from reference laboratories in North America to create their AR-panels designed to aid in strengthening the development and validation of novel tests and assays (CDC and FDA, 2018).

The 31 isolates selected from the CDC/FDA *N. gonorrhoeae* AR-panel were chosen based on the MIC information supplied by the CDC to cover a board concentration range for the 4 antibiotic to be tested in a side-by-side comparison of AD versus MBD dilution method. Herein, we show that a more efficient method can be implemented without sacrificing accuracy and reproducibility of data.

2. Material and methods

2.1. Bacterial strains, media and antibiotics

Clinical *N. gonorrhoeae* isolates used in this study were obtained from the CDC (CDC and FDA antibiotic resistance isolate bank) and are listed in Table 2. The panel of strains tested included a range of susceptibility profiles (azithromycin: 0.25–256 μg/mL, ceftriaxone: 0.008–0.5 μg/mL, ciprofloxacin: 0.015–16 μg/mL, and tetracycline: 1–8 μg/mL). All MIC information provided by the CDC was obtained from testing each isolate several times and reporting the modal MIC value (the MIC value that occurs most often; M. Machado, personal communication, November 15th 2018). All strains were plated onto GC agar (Remel, R453502) supplemented with 1% hemoglobin (Remel, R451402), and 1% IsoVitaleX Enrichment (BD BBL, 211876). The plates were incubated at 37 °C with 5% CO_2 for 20–24 h and representative isolates from each strain cultured were frozen at −80 °C in GC broth containing 1% hemoglobin, 1% IsoVitaleX Enrichment, and 20% glycerol. The quality control strain recommended by the CLSI was procured from the American Type Culture Collection (ATCC 49226™) and propagated according to ATCC guidelines.

Antimicrobial powders of tetracycline (Sigma, T3383), ceftriaxone (Sigma, C5793), ciprofloxacin (Acros Organics, 456880250), and azithromycin (USP, 1046056) were stored in a desiccator at the appropriate temperature recommended by the manufacturer. Each drug was dissolved in DMSO (Fisher, BP231) to prepare 12.8 mg/mL working stocks that were frozen at −20 °C until use. A new aliquot was used for each assay to avoid repeated freeze-thaw cycles.

2.2. Growth analysis using FB or GC broth

After 24 h incubation at 37 °C with 5% CO_2, colonies were immersed to the equivalent of a 0.5 McFarland in 0.9% (w/v) saline. Bacterial suspensions were further diluted 1:100 in pre-warmed Fastidious Broth (FB, Remel, R07664) or GC broth supplemented with 1% hemoglobin (Remel, R451402), and 1% IsoVitaleX Enrichment (BD BBL, 211876) and used to inoculate 100 μL/well in a 96-well assay plate (Corning 3370) already containing the same test media with or without 0.5% (*v*/v) DMSO. Growth plates were incubated at 37 °C with 5% CO_2 for 24 h and aliquots were removed at several time points, serially diluted, and plated onto GC agar supplemented with 1% hemoglobin (Remel, R451402), and 1% IsoVitaleX Enrichment (BD BBL, 211876). CFUs were enumerated after 24 h incubation 37 °C with 5% CO_2.

2.3. Agar dilution method

The agar dilution method was performed according to previously established methods described by the CLSI in M07A10E (CLSI, 2015). Briefly, GC agar (without blood product) was prepared as follows; 15 g/L proteose peptone no. 3 (BD, 211693), 1 g/L corn starch (Fisher, S25580), 4 g/L dibasic potassium phosphate (Fisher, BP363), 1 g/L monobasic potassium phosphate (Acros Organics, 271,750,010), 5 g/L sodium chloride (Fisher, S27–1), 4.25 g/L agar (BD, 214510), and 1% IsoVitaleX enrichment. Plates containing a doubling dilution of the 4 tested antimicrobials were prepared from a 12.8 mg/mL stock solution with test concentrations of 64–0.031 μg/mL for tetracycline and azithromycin respectively, and 0.002–0.5 μg/mL and 0.002–16 μg/mL for ceftriaxone and ciprofloxacin, respectively. Bacterial strains were plated from glycerol stocks onto GC agar with 1% (w/v) hemoglobin, plus 1% (w/v) IsoVitaleX enrichment and incubated at 37 °C with 5% CO_2 for 20–24 h. A bacterial suspension equivalent to a 0.5 McFarland in 0.9% (w/v) saline was prepared from the overnight growth and was further diluted 1:10 before 2 μL was spotted onto each agar plate with a multichannel pipette, in duplicate, resulting in an approximate final inoculum of 1×10^7 CFU/spot. The MIC was recorded as the lowest concentration of drug that inhibited bacterial growth by visual inspection.

2.4. Microbroth dilution method

Bacterial strains were cultured as described above and an inoculum suspension from overnight growth was prepared equivalent to a 0.5 McFarland in 0.9% (w/v) saline, diluted 1:100 in pre-warmed Fastidious Broth (FB, Remel, R07664) and used to inoculate 100 μL/well in a 96-well assay plate (Corning 3370) already containing FB medium and drug at a 0.5% (v/v) final DMSO concentration. Assay plates were incubated at 37 °C with 5% CO_2 for 24 h after which, plates were cooled to room temperature and gently mixed with a multichannel pipette. Each assay plate was sealed (Thermo, 8,408,240) and read for an endpoint absorbance at 600 nm using the monochromator optical setting on a Spectramax i3 plate reader (Molecular Devices). All strains were tested in duplicate and prepared bacterial suspensions were enumerated in order to validate a final inoculum of 1×10^5–5×10^5 CFU/mL. MICs for every strain tested were recorded relative to the positive growth (inoculated FB media with 0.5% (*v*/v) DMSO) and no growth control (FB media and 0.5% (*v*/v) DMSO) wells thus allowing for a percent growth and an MIC_{90} to be determined. Percent growth was generated by calculating percent inhibition using the following equations:

$$\%\text{Inhibition} = 100 * (X - Y)/(Z - Y)$$

Where X = $OD_{600\ nm}$ of sample well.

Y = average $OD_{600\ nm}$ of negative controls (FB media and 0.5% (*v*/v) DMSO)

Z = average $OD_{600\ nm}$ of positive controls (inoculated FB media and 0.5% (v/v) DMSO)

$$\%\text{Growth} = 100 - \%\text{inhibition}$$

2.5. Etests MIC determination

The Etest strips for azithromycin; 0.016–256 μg/mL (412256), ceftriaxone; 0.002–32 μg/mL and 0.016–256 μg/mL (412,302 and 412,300), ciprofloxacin; 0.002–32 μg/mL (412310), and tetracycline; 0.016–256 μg/mL (412470) were procured through bioMérieux. Strains were cultured and incubated as described above and the Etest MICs were determined in accordance with the manufacturer's instructions on commercially available GC plates (Remel, R01460).

2.6. Statistical analysis

All AST methods compared in this study were performed in parallel and for the purposes of testing reproducibility several of the assays were carried out in duplicate on separate days. The CLSI recommended *N. gonorrhoeae* strain, ATCC 49226™, was used to validate all tests as a necessary quality control. Assay reproducibility was assessed by calculating the essential agreement (EA) between MIC values obtained from different assays. EA was recorded by calculating the percentage of isolates that yielded an identical MIC, or a single 2-fold dilution difference for each antimicrobial agent tested. Ideally, an EA between

compared assays should be > 90% (Biedenbach and Jones, 1996).

Additionally, all MIC values obtained were assessed for quality performance by determining the categorical agreement (CA) between the conducted assays. To establish CA all strains tested were described as susceptible (S), intermediate (I) or resistant (R) to each antimicrobial in accordance with CLSI breakpoint criteria or the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint criteria in the event that data was otherwise unavailable from the CLSI (CLSI, 2016; EUCAST, 2017). If there was agreement between the test and reference method then categorical agreement was achieved. Minor errors were recorded when one method categorized an isolate as intermediate when the other method determined it as susceptible or resistant. Major errors were recorded when there was discordance between a susceptible or resistant determination dependent on the test method.

3. Results

Fastidious broth has previously been shown to support the cultivation of *N. gonorrhoeae* but it has yet to be demonstrated that the inclusion of 0.5% (v/v) DMSO has no marked effect on growth of this organism. A comparison of growth rates in GC broth plus supplement or FB showed no significant changes in growth observed for the 2 strains tested, ATCC 49226 and AR-172 in either the presence or absence of 0.5% (v/v) DMSO, Fig. 1.

To establish the inter-laboratory reproducibility of the reference AD method between this study and the data provided by the CDC, the MIC values obtained for 4 antimicrobial agents were compared for 31 *N. gonorrhoeae* isolates indicated in Table 1. Comparisons were made by using the MICs at which 90% of the strains visible growth was inhibited (MIC_{90}). EA was calculated for each antimicrobial agent tested, for every strain, when the MIC value was within a single 2-fold dilution obtained from the two separate test methods. Four of the antimicrobials (azithromycin, ceftriaxone, ciprofloxacin and tetracycline) tested had an EA > 90%, when comparing MICs values for this study and those provided by the CDC. When comparing the CA between the MIC values obtained for the two different AD assays, the CA was calculated using the breakpoints published in the CLSI M100-S26:2016 document for ceftriaxone, ciprofloxacin and tetracycline. For azithromycin, where CLSI reported breakpoints are not available, EUCAST criteria were utilized. The calculated CA was as follows for the four antimicrobial agents tested; 78% for azithromycin, 88% for tetracycline (minor errors - 4 out of 31), 100% for both ciprofloxacin and ceftriaxone (1 out of the 31 strains was classified as non-susceptible by CLSI criteria and therefore excluded from this analysis as an outlier). The exception was azithromycin which had 7 out of 31 major errors. However, this outcome was skewed by the EUCAST criteria which only provide two breakpoint criteria (susceptible or resistant) versus the susceptible, intermediate

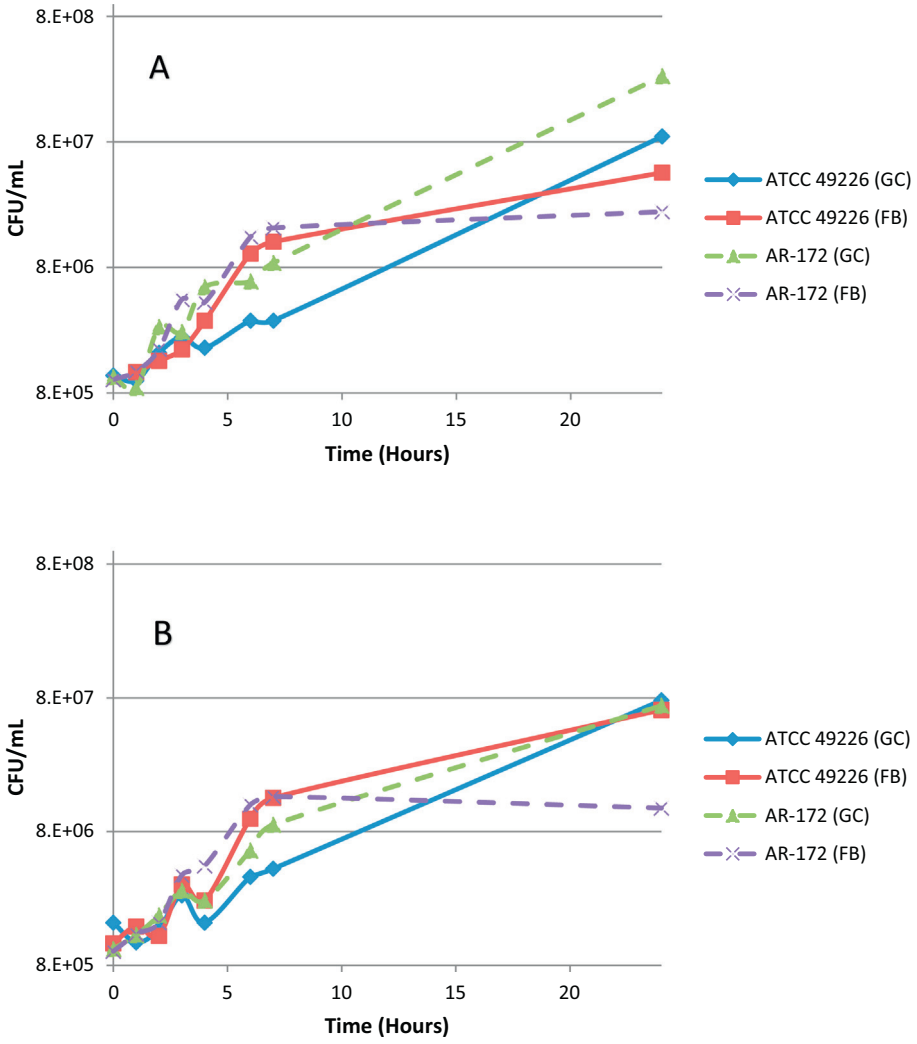

Fig. 1. (A) Growth of *N. gonorrhoeae* strains ATCC 49226 (solid line) and AR-172 (dashed line) in FB media or GC media with 0.5% (v/v) DMSO, or (B) without 0.5% (v/v) DMSO.

Table 1
Inter-laboratory MIC values obtained for the 31 *N. gonorrhoeae* strains when tested against four antimicrobial agents using the CLSI reference agar dilution method.

Strains	Azithromycin		Ceftriaxone		Ciprofloxacin		Tetracycline	
	This study	CDC AD[a]	This study	CDC AD	This study	CDC AD	This study	CDC AD
AR-0165	0.5 (R)[b]	1 (R)	0.125 (S)	0.063 (S)	> 16 (R)	8 (R)	4 (R)	8 (R)
AR-0166	0.5 (R)	1 (R)	0.125 (S)	0.125 (S)	16 (R)	8 (R)	2 (R)	4 (R)
AR-0168	0.25 (S)	0.5 (R)	0.063 (S)	0.063 (S)	8 (R)	16 (R)	2 (R)	4 (R)
AR-0169	0.5 (R)	1 (R)	0.125 (S)	0.063 (S)	16 (R)	16 (R)	2 (R)	4 (R)
AR-0170	0.5(R)	1 (R)	0.125 (S)	0.063 (S)	16 (R)	16 (R)	2 (R)	4 (R)
AR-0172	0.25 (S)	0.5 (R)	0.125 (S)	0.063 (S)	16 (R)	16 (R)	2 (R)	2 (R)
AR-0173	0.5 (R)	0.5 (R)	0.063 (S)	0.125 (S)	16 (R)	16 (R)	2 (R)	4 (R)
AR-0174	0.5 (R)	1 (R)	0.125 (S)	0.125 (S)	16 (R)	16 (R)	2 (R)	4 (R)
AR-0175	8 (R)	16 (R)	0.008 (S)	0.008 (S)	0.004 (S)	0.015 (S)	0.5 (I)	1 (I)
AR-0178	1 (R)	1 (R)	0.125 (S)	0.063 (S)	32 (R)	16 (R)	4 (R)	8 (R)
AR-0179	8 (R)	8 (R)	0.008 (S)	0.008 (S)	0.002 (S)	0.015 (S)	0.5 (I)	1 (I)
AR-0180	0.5 (R)	0.5 (R)	0.125 (S)	0.063 (S)	16 (R)	16 (R)	2 (R)	4 (R)
AR-0181	> 16 (R)	256 (R)	0.063 (S)	0.031 (S)	0.008 (S)	0.015 (S)	1 (I)	2 (R)
AR-0186	0.25 (S)	0.5 (R)	0.125 (S)	0.125 (S)	16 (R)	16 (R)	2 (R)	4 (R)
AR-0187	1 (R)	2 (R)	0.031 (S)	0.031 (S)	4 (R)	4 (R)	0.5 (I)	1 (I)
AR-0190	0.5 (R)	1 (R)	0.125 (S)	0.125 (S)	16 (R)	16 (R)	2 (R)	4 (R)
AR-0192	1 (R)	1 (R)	0.125 (S)	0.063 (S)	16 (R)	8 (R)	2 (R)	8 (R)
AR-0193	2 (R)	2 (R)	0.125 (S)	0.031 (S)	0.016 (S)	0.015 (S)	2 (R)	2 (R)
AR-0194	0.25 (S)	0.5 (R)	0.125 (S)	0.5 (NS)	0.002 (S)	0.015 (S)	0.5 (I)	1 (I)
AR-0198	1 (R)	1 (R)	0.125 (S)	0.063 (S)	16 (R)	16 (R)	2 (R)	4 (R)
AR-0200	0.5 (R)	0.5 (R)	0.125 (S)	0.125 (S)	16 (R)	16 (R)	2 (R)	2 (R)
AR-0201	0.5 (R)	0.5 (R)	0.125 (S)	0.125 (S)	16 (R)	8 (R)	2 (R)	2 (R)
AR-0203	0.5 (R)	0.5 (R)	0.125 (S)	0.125 (S)	16 (R)	16 (R)	2 (R)	4 (R)
AR-0205	0.5 (R)	0.5 (R)	0.125 (S)	0.063 (S)	16 (R)	16 (R)	2 (R)	2 (R)
AR-0207	0.5 (R)	0.5 (R)	0.125 (S)	0.063 (S)	16 (R)	16 (R)	2 (R)	4 (R)
AR-0209	1 (R)	1 (R)	0.125 (S)	0.063 (S)	16 (R)	16 (R)	2 (R)	2 (R)
AR-0210	0.25 (S)	0.25 (S)	0.125 (S)	0.063 (S)	16 (R)	16 (R)	2 (R)	1 (I)
AR-0211	0.5 (R)	1 (R)	0.125 (S)	0.063 (S)	16 (R)	16 (R)	2 (R)	2 (R)
AR-0212	0.25 (S)	0.5 (R)	0.125 (S)	0.063 (S)	16 (R)	16 (R)	2 (R)	4 (R)
AR-0213	0.25 (S)	0.5 (R)	0.25 (S)	0.063 (S)	16 (R)	16 (R)	1 (I)	4 (R)
AR-0214	0.25 (S)	0.5 (R)	0.125 (S)	0.125 (S)	16 (R)	16 (R)	1 (I)	4 (R)

[a] Reference agar dilution method.
[b] Interpretive criteria according to the recommended CLSI or EUCAST breakpoint criteria (μg/mL) for azithromycin, ceftriaxone, ciprofloxacin, and tetracycline; S – susceptible, NS – non-susceptible, I – intermediate, and R –resistant.

and resistant classifications provided by the CLSI system. As a consequence the "minor" error category was not available for the calculation of CA values associated with azithromycin's activity.

To compare the reproducibility of the reference AD method to the MBD dilution method, the MIC values of 4 antimicrobials were compared for 32 *N. gonorrhoeae* isolates as indicated in Table 2. The EA was calculated for the two different assays conditions, with 97% achieved for azithromycin, 91% for tetracycline, and 97% for ciprofloxacin. The EA for ceftriaxone was only 28% although the data showed that the MBD dilution assay generally exhibited a greater sensitivity to ceftriaxone, consistently generating slightly lower MICs ($\pm$ 1 $\log_2$) than observed in our AD method. For 23 out of the 32 strains tested, only 2 had MIC values more than a $\pm$ 2 $\log_2$ (doubling dilution) between the two methods. When comparing the provided CDC AD MIC data to the MBD MIC data, only 75% EA is achieved (8 out of the 32 strains tested had a 2 $\log_2$ MIC value). A 100% CA for ceftriaxone was achieved as no major errors were observed when comparing the AD from this study and the MBD AST methods. The calculated CA was > 90% for ciprofloxacin and tetracycline, and 78% for azithromycin (major errors - 7 out of 32) (Table 3).

Interestingly, when comparing the EA and CA agreements between the reported CDC AD and the obtained MBD MIC values as seen in Table 3, there was a similar level of concordance observed with a calculated EA of 94% for azithromycin, 97% for tetracycline, 75% for ceftriaxone and 91% for ciprofloxacin. When considering CA, azithromycin again was lowest at 63% (major errors - 12 out of 32), 91% for tetracycline, and complete CA (100%) observed for ceftriaxone and ciprofloxacin.

N. gonorrhoeae strain ATCC 49226 was included in all testing for quality control purposes. The MIC values obtained for the 4 tested antimicrobials; azithromycin (0.25–1 μg/mL), ceftriaxone (0.001–0.008 μg/mL), ciprofloxacin (0.004–0.015 μg/mL), and tetracycline (0.25–1 μg/mL) were within range of the approved CLSI values (CLSI, 2017).

As an independent measure of quality assurance the susceptibilities of 25% of the strains included in this study were tested against the same 4 antimicrobials (azithromycin, ceftriaxone, ciprofloxacin, and tetracycline) by using Etest strips (bioMérieux, France). It has been previously demonstrated that Etests reliably correlate with more traditional methods such as AD and disk diffusion (Biedenbach and Jones, 1996; Liu et al., 2014; Singh et al., 2012; Unemo et al., 2016; Gose et al., 2013). If required, Etests MIC values were rounded up to match the nearest 2-fold dilution, a practice recommended by the manufacturer. When comparing calculated EA and CA for the MIC values obtained from the AD assay from this study versus the Etest, comparable percentages were obtained except in the case of ceftriaxone, which had a calculated EA of only 38%, as can be seen in Table 4. Comparison of the MIC values derived from Etest and MBD assays with the 4 antibiotics provided calculated EAs of 75%, 100%, 63% and 88% for azithromycin, ceftriaxone, ciprofloxacin, and tetracycline, respectively. The CAs are as follows; 50% for azithromycin (major errors – 4 out of 8), 50% for tetracycline (minor errors - 4 out of 8), and 100% for ceftriaxone and ciprofloxacin respectively.

4. Discussion

Presently treatment failures with empiric ceftriaxone and azithromycin for uncomplicated gonococcal infections have been recently

Table 2
MIC values obtained for the 32 *N. gonorrhoeae* strains when tested against four antimicrobial agents in either the CLSI reference AD or MBD method.

Strains	Azithromycin		Ceftriaxone		Ciprofloxacin		Tetracycline	
	AD[a]	MBD[b]	AD	MBD	AD	MBD	AD	MBD
AR-0165	0.5 (R)[c]	0.5 (R)	0.125 (S)	0.031 (S)	16 (R)	8 (R)	4 (R)	4 (R)[d]
AR-0166	0.5 (R)	0.5 (R)	0.125 (S)	0.031 (S)	16 (R)	8 (R)	2 (R)	2 (R)
AR-0168	0.25 (S)	0.25 (S)	0.063 (S)	0.016 (S)	8 (R)	8 (R)	2 (R)	2 (R)
AR-0169	0.5 (R)	0.5 (R)	0.125 (S)	0.031 (S)[d]	16 (R)	8 (R)	2 (R)	2 (R)
AR-0170	0.5(R)	0.5 (R)	0.125 (S)	0.031 (S)	16 (R)	8 (R)	2 (R)	2 (R)
AR-0172	0.25 (S)	0.5 (R)[d]	0.125 (S)	0.031 (S)	16 (R)	8 (R)	2 (R)	2 (R)[d]
AR-0173	0.5 (R)	0.5 (R)	0.063 (S)	0.031 (S)	16 (R)	8 (R)	2 (R)	2 (R)
AR-0174	0.5 (R)	1 (R)[d]	0.125 (S)	0.031 (S)	16 (R)	16 (R)[d]	2 (R)	2 (R)
AR-0175	8 (R)	8 (R)	0.008 (S)[d]	0.004 (S)[d]	0.004 (S)[d]	0.004 (S)[d]	0.5 (I)	1 (I)
AR-0178	1 (R)	0.5 (R)	0.125 (S)[d]	0.031 (S)[d]	32 (R)[d]	16 (R)[d]	4 (R)	4 (R)
AR-0179	8 (R)	8 (R)	0.008 (S)	0.004 (S)[d]	0.002 (S)	0.004 (S)	0.5 (I)	2 (R)
AR-0180	0.5 (R)	0.25 (S)	0.125 (S)	0.031 (S)[d]	16 (R)	8 (R)	2 (R)	2 (R)
AR-0181	≥16 (R)	> 64 (R)	0.063 (S)[d]	0.016 (S)	0.008 (S)	0.004 (S)	1 (I)	1 (I)
AR-0186	0.25 (S)	0.25 (S)	0.125 (S)[d]	0.031 (S)	16 (R)	16 (R)[d]	2 (R)	2 (R)
AR-0187	1 (R)	1 (R)	0.031 (S)	0.016 (S)	4 (R)	4 (R)[d]	0.5 (I)	1 (I)
AR-0190	0.5 (R)	0.5 (R)	0.125 (S)	0.063 (S)	16 (R)	8 (R)	2 (R)	4 (R)[d]
AR-0192	1 (R)	0.5 (R)	0.125 (S)	0.031 (S)[d]	16 (R)	8 (R)	2 (R)	2 (R)[d]
AR-0193	2 (R)	1 (R)	0.125 (S)[d]	0.008 (S)[d]	0.016 (S)[d]	0.016 (S)[d]	2 (R)	2 (R)
AR-0194	0.25 (S)	0.125 (S)	0.125 (S)[d]	0.125 (S)	0.002 (S)[d]	0.004 (S)[d]	0.5 (I)	1 (I)
AR-0198	1 (R)	0.5 (R)	0.125 (S)	0.031 (S)[d]	16 (R)	8 (R)	2 (R)	2 (R)
AR-0200	0.5 (R)	0.25 (S)	0.125 (S)	0.063 (S)	16 (R)	8 (R)	2 (R)	2 (R)
AR-0201	0.5 (R)	0.25 (S)	0.125 (S)	0.063 (S)	16 (R)	8 (R)	2 (R)	2 (R)
AR-0203	0.5 (R)	0.25 (S)	0.125 (S)	0.031 (S)[d]	16 (R)	16 (R)[d]	2 (R)	2 (R)
AR-0205	0.5 (R)	0.25 (S)	0.125 (S)[d]	0.031 (S)	16 (R)[d]	8 (R)[d]	2 (R)	2 (R)
AR-0207	0.5 (R)	0.25 (S)	0.125 (S)	0.031 (S)	16 (R)	8 (R)	2 (R)	2 (R)
AR-0209	1 (R)	0.25 (S)	0.125 (S)	0.031 (S)	16 (R)	8 (R)	2 (R)	2 (R)
AR-0210	0.25 (S)	0.25 (S)	0.125 (S)	0.031 (S)	16 (R)	8 (R)	2 (R)	2 (R)[d]
AR-0211	0.5 (R)	0.5 (R)	0.125 (S)	0.031 (S)	16 (R)	8 (R)	2 (R)	4 (R)[d]
AR-0212	0.25 (S)	1 (R)[d]	0.125 (S)	0.031 (S)	16 (R)	8 (R)	2 (R)	2 (R)
AR-0213	0.25 (S)	0.25 (S)	0.25 (S)	0.031 (S)	16 (R)	8 (R)	1 (I)	8 (R)[d]
AR-0214	0.25 (S)	0.25 (S)	0.125 (S)	0.063 (S)	16 (R)	8 (R)	1 (I)	8 (R)[d]
ATCC 49226	0.25 (S)	0.25 (S)[d]	0.031 (S)[d]	0.008(S)[d]	≤0.002 (S)[d]	0.004 (S)[d]	0.5 (I)	1 (I)[d]

[a] Reference agar dilution method from this study.
[b] Microbroth dilution method.
[c] Interpretive criteria according to the recommended CLSI or EUCAST breakpoint criteria (μg/mL) for azithromycin, ceftriaxone, ciprofloxacin, and tetracycline.
[d] $n = 2$ biological replicates performed on separate days.

Table 3
Comparison of the MIC, essential and categorical agreement values obtained from the AD and MBD dilution methods when testing four antimicrobial agents against 32 *N. gonorrhoeae*.

Antimicrobial agent	Test method	MIC (μg/ml)	% of agreement	
		90%	Essential	Categorical
Azithromycin	AD[a]/AD[b]	0.25– > 16	94%[a,c]/	63%/78%
	MBD[c]	0.125– > 64	97%[b,c]	
Ceftriaxone	AD/AD	0.008–0.25	75%/28%	100%/100%
	MBD	0.004–0.125		
Ciprofloxacin	AD/AD	0.002–32	91%/97%	100%/100%
	MBD	0.004–16		
Tetracycline	AD/AD	0.5–4	97%/94%	91%/91%
	MBD	1–8		

[a] CDC agar dilution.
[b] Reference agar dilution from this study.
[c] Microbroth dilution method.

Table 4
Comparison of the MIC, essential and categorical agreement values obtained when comparing reference AD to Etest or MBD to Etest when testing four antimicrobial agents against 32 *N. gonorrhoeae*.

Antimicrobial agent	Test method	% of agreement	
		Essential	Categorical
Azithromycin	AD[a]/Etest	88%	75%
	MBD[b]/Etest	75%	50%
Ceftriaxone	AD/Etest	38%	100%
	MBD/Etest	100%	100%
Ciprofloxacin	AD/Etest	100%	100%
	MBD/Etest	100%	100%
Tetracycline	AD/Etest	63%	63%
	MBD/Etest	88%	50%

[a] Reference agar dilution from this study.[b]Microbroth dilution method.

described outside of the United States (Fifer et al., 2016). This recent emergence of resistance highlights the necessity for a reliable and reproducible AST method, especially one that can be used in a pharmaceutical setting to test novel antimicrobial agents that may be initially of limited quantity. The current CLSI reference AD method is burdensome for testing large numbers of antimicrobial agents and the prepared agar plates containing test compound have a limited shelf-life (≤5 days), making it unsuitable for both a clinical and pharmaceutical setting (CLSI, 2015). The MBD method is simpler, less-labor intensive, has the ability to provide more data in the context of testing multiple antibiotics quickly, and has been found to be predictive of clinical resistance to specific antibiotics for other pathogenic bacteria. As a consequence it is used routinely as the reference method for AST determination in several bacterial species. However, it has not gained much traction for assessment of antibiotic resistance in *N. gonorrhoeae*, possibly due to liquid media constraints. It has previously been described that FB, developed by Cartwright et al., can support the growth of *N. gonorrhoeae* along with several other fastidious bacteria (Cartwright et al., 1994; Farrell et al., 2017; Takei et al., 2005). Additionally, several studies have shown that FB can be used for MBD AST of *N. gonorrhoeae* isolates with good correlation to the reference AD

method, for a variety of quinolones and an experimental antibiotic, Gepotidacin (Farrell et al., 2017; Takei et al., 2005). Based upon the outcomes of those studies and the current study, it appears that a FB based micro dilution AST will prove advantageous for screening large numbers of *N. gonorrhoeae* strains or samples (natural product or synthetic) in the search for new antibiotic treatments. This is especially compelling as it halves the turn-around time for data output and is considerably more cost effective when factoring in both labor and consumables costs. Manual labor time can be greatly reduced further if considering the evaluation of a large compound library screen by the automation of several steps in the MBD method including; pre-printing of the assay plates on a liquid handling system, as well as, the addition of bacterial suspension in FB media using a liquid dispensing system.

The review criteria used in this study for comparing the obtained or provided susceptibility data ensured quantitative statistical analysis. Four clinically relevant antimicrobial agents (azithromycin, ceftriaxone, ciprofloxacin, and tetracycline) were selected for testing against a total of 32 *N. gonorrhoeae* isolates; 31 clinical strains obtained from the CDC and the recommended ATCC QC strain. All 32 strains were tested in the two different test conditions; CLSI reference AD and a MBD method. Additionally, a subset of strains was chosen to be further interrogated in a third AST method, Etest which has previously been shown to be a simple and effective alternative to the reference AD method (Biedenbach and Jones, 1996; Gose et al, 2013). Inter-laboratory reproducibility of the reference AD method between this study and the data provided by the CDC was performed as the inherent assay variance in any MIC determination, regardless of the method, must be accounted for (Mouton et al. 2018). The performance of the two reference agar assays was excellent (> 90%) for azithromycin, ceftriaxone and tetracycline. Although the calculated EA for ciprofloxacin was 88%, the ISO 20776-2 acceptance criteria for MIC reproducibility is within a two 2-fold dilution range, thus when comparing the MIC values obtained for the two data sets, these fell well within that criteria. When considering the calculated CA, ciprofloxacin and ceftriaxone were exemplary (no errors), tetracycline was acceptable (minor errors), and azithromycin was passable (major errors). As noted previously the outcome for azithromycin was skewed by the EUCAST criteria which only provide two breakpoint criteria (susceptible and resistant) versus the susceptible, intermediate and resistant classifications provided by the CLSI system. As a consequence the “minor” error category was not available for the calculation of azithromycin's CA impacting 4 out of the 8 major error classifications. In the case of ceftriaxone, the CA was considered 100% even though one isolate, AR-194 had a reported MIC of 0.5 μg/mL and categorized as NS (non-susceptible) by CLSI criteria. NS is the assignment given by CLSI when test results exceed the susceptible breakpoint but there is a paucity of information from strains with resistance to this antimicrobial agent, therefore no established intermediate or resistant breakpoints exist. Because of this we categorized this strain as an outlier and excluded it from the CA calculation. The calculated agreements achieved when comparing the MIC values obtained in the reference AD method from this study and those from the MDB assay were > 90% for azithromycin, tetracycline, and ciprofloxacin.

The calculated percent EA for ceftriaxone when comparing either the reference AD method to MBD or Etest to MBD yielded 28% and 38% respectively. In this comparison slightly higher MIC values (two doubling dilutions) were consistently observed for ceftriaxone in the AD from this study relative to those obtained for Etest or MBD. This lower percentage of agreement for ceftriaxone when comparing these alternative AST methods to the reference method is likely due to the exquisite sensitivity of these strains to ceftriaxone (0.004–0.5 μg/mL) and the exposure of the bacterial strains to the antibiotic in different physical mediums (solid versus liquid). The CA was still 100% for all strains tested when comparing AD to MBD or MBD to Etest, and all were correctly categorized according to CLSI breakpoint criteria as susceptible. In 2016, only 0.3% of all the *N. gonorrhoeae* isolates screened in the GISP program had an elevated (defined as ≥0.125 μg/mL) ceftriaxone MICs (CDC, 2005). Therefore, in the United States there are very few strains available with elevated ceftriaxone MICs for use in evaluating AST methodologies. While there was an overall lower agreement observed for azithromycin and more so for ceftriaxone this is likely due to the diversity of strains available for testing. While it may be premature to robustly conclude that this method is fully comparative to the reference standard, it is encouraging to note that comparisons between the Etest and MBD AST consistently outperformed those obtained with the AD method. Suggesting the MBD method may be a more sensitive assay for the testing of ceftriaxone meriting consideration for implementation when needing to definitively identify creeping of MIC values to one of the frontline drugs used to treat *N. gonorrhoeae* infections worldwide.

In general MBD and Etest derived MIC results were in good agreement with one another. Especially considering the computed EA and CA for ciprofloxacin was 100% agreement between the two compared methods. The discrepancies in EA for azithromycin between the two methods was less than that achieved when comparing the reference AD to the MBD method (EA = 97%). This was a result of a two 2-fold dilution difference between 1 out of the 8 strains tested for the reference AD method compared to Etest, and 2 out of 8 strains for the MBD method compared to Etest. Perhaps a larger sample set with a more diverse MIC profile would have bolstered the statistical agreement. The EA for ceftriaxone when comparing MBD to Etest resulted in a 100% correlation, far greater than was achieved when comparing the reference AD MIC values from this study, which tended to be 2-fold greater than those obtained from Etest, highlighting the probability that proximity to the antimicrobial is vital. The lowest statistical agreement was observed when comparing MIC values obtained for tetracycline from either the reference AD in this study or the MBD assay to those from Etest. It has been reported that there is lower degree of reproducibility of results when testing tetracycline, especially when different media are used (Singh et al. 2012). While none of the obtained MIC were > 2-fold lower than one another, they were on the cusp of susceptible (≤0.25 μg/mL) versus intermediate (0.5–1 μg/mL) and hence the calculated CAs were extremely low.

Overall, the developed MBD assay using FB media was able to reliably discriminate between resistant, intermediate and susceptible strains as displayed by high degree of categorical agreement. This method was rapid to set-up and complete, comparative to the current gold-standard AD method for *N. gonorrhoeae* and had an overall excellent sensitivity for the 4 antimicrobials tested. Both the reference AD method and Etest are based on a subjective readout and are therefore limited to a lower throughput without tangible qualitative parameters. In the developed MBD method an absorbance at 600 nm is used to measure growth and consequently calculate a value for each test well as well as an average value for both the positive growth and no growth controls. Furthermore using this data we were able to calculate Z' (± 0.8), coefficient of variation (± 3.3), signal to background (± 3.4), and MIC values for all assay plates tested using automated data analysis software. Tracking this information allows for rapid analysis of assay performance and provides assurance that each assay is within a well-defined range by monitoring sensitivity and batch reproducibility. These properties coupled with the commercial availability of FB media are especially valuable when considering screening large collections of samples. For example libraries of clinical isolates or compounds, especially in a multicenter study were standardization is imperative. Even more compelling is the ability to use the MBD assay to determine the potency of antimicrobials relative to one another in combination therapy.

The data presented supports the use of liquid AST MBD using FB media for quantitative determination of antimicrobial susceptibility of *N. gonorrhoeae* providing a new approach for the evaluation of novel antimicrobials.

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