Comparison of disk diffusion and agar dilution methods for gentamicin susceptibility testing of Neisseria gonorrhoeae

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ABSTRACT

Gentamicin is a promising antibiotic for the treatment of multidrug-resistant gonorrhea. The aim of this study was to analyze the suitability and reliability of disk diffusion to monitor the susceptibility to gentamicin. We studied 237 Neisseria gonorrhoeae isolates obtained in 2013 and 2015. Reference MICs were correlated with inhibition zone diameters (in millimeters) of gentamicin 10 µg disks manufactured by BBL and Oxoid. The Pearson correlation between disk diffusion and agar dilution was $r = -0.68$ ($P < 0.001$) for BBL disk and $r = -0.71$ ($P < 0.001$) for Oxoid disk. No very major or major discrepancies were detected. However, a high percentage of minor discrepancies was observed (44.7%, BBL disk) and (21.9%, Oxoid disk). By adjusting the susceptible breakpoint to $S ≥ 17$ mm, the minor discrepancies rate was reduced to 19.4% (BBL disk) and 10.1% (Oxoid disk). The disk diffusion may be a screening method in clinical laboratories to detect the gentamicin susceptibility of N. gonorrhoeae.

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1. Introduction

Antimicrobial resistance in Neisseria gonorrhoeae is a major public health concern globally. Almost all antibiotic classes used for treatment of gonorrhoea have lost much of their efficacy owing to emergence of resistance. The extended-spectrum cephalosporin (ESC) ceftriaxone and azithromycin are the last remaining options for first-line gonorrhea treatment. However, these antibiotics may become inadequate against gonorrhoea as reports of resistance to ESC or azithromycin have increased worldwide in recent years (Unemo et al., 2017; Wi et al., 2017). Dual antimicrobial therapy with an ESC plus azithromycin has been introduced for empirical first-line gonorrhoea treatment in many countries, but the first case of treatment failure with a dual therapy has recently been reported (Bignell et al., 2012; Fifer et al., 2016; Workowski et al., 2015). Moreover, the emergence of N. gonorrhoeae isolates with a multidrug resistant (MDR) and extremely drug resistant (XDR) profile highlights the need to consider alternatives for future therapeutic use (Unemo, 2015). MDR is defined as resistance to ESCs or resistance to 1 type of ESC and spectinomycin, in addition to 3 or more of penicillins, fluoroquinolones, macrolides, aminoglycosides, and carbapenems (Tapsall et al., 2009). In Argentina, N. gonorrhoeae isolates with decreased susceptibility and resistance to ESC have been detected (Gianecini et al., 2016, 2017). Therefore, the knowledge of the susceptibility to new antimicrobial options for gonorrhea treatment is urgently needed.

The aminoglycoside gentamicin has been considered a promising treatment option for gonorrhea, particularly in dual therapy (Dowell and Kirkcaldy, 2012). Gentamicin has been used successfully for several years in the treatment of gonococcal urethritis in Malawi, and a study has shown that the susceptibility of N. gonorrhoeae isolates to gentamicin did not change considerably after >10 years of use (Brown et al., 2010). If gentamicin is proposed as an alternative treatment for gonorrhea, the knowledge of the susceptibility of the isolates of N. gonorrhoeae will be necessary. So far, the Clinical and Laboratory Standard Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST) do not list breakpoints for gentamicin (CLSI, 2017). However, in the scientific literature, interpretative criteria have been proposed for MIC (susceptible, ≤4 µg/mL; intermediate susceptible, 8–16 µg/mL; and resistant, ≥32 µg/mL) (Brown et al., 2010). Furthermore, a recent report comparing the disk diffusion method with antibiotic concentration gradient strips (Etest;
bioMérieux, Marcy-l’Étoile, France), which is not the reference method, has established tentative gentamicin zone breakpoints for the CLSI method (Bala et al., 2016). The MIC determination by agar dilution method is the reference method for antimicrobial susceptibility in *N. gonorrhoeae* but is mostly used by reference laboratories. The disk diffusion test may be used as an alternative for *N. gonorrhoeae* antimicrobial susceptibility since it is easy to perform in clinical laboratories in microbiology. Having said that, studies which analyze the suitability and reliability of disk diffusion to monitor susceptibility of *N. gonorrhoeae* isolates to antimicrobial agents are essential.

The aim of this study was to evaluate the susceptibility of *N. gonorrhoeae* isolates to gentamicin and to determine whether disk diffusion was a reliable method for assessing the susceptibility of *N. gonorrhoeae* to this antibiotic.

### 2. Materials and Methods

#### 2.1. N. Gonorrhoeae Isolates

A total of $N = 237 (n = 118$ from 2013 and $n = 119$ from 2015) consecutive, nonduplicate clinical isolates of *N. gonorrhoeae* were collected from Gonococcal Antimicrobial Susceptibility Surveillance Programme–Argentina (GASSP–AR) for use in the present study. Identification of the *N. gonorrhoeae* isolates was performed by Gram staining, oxidase test, superoxide test (30% hydrogen peroxide), carbohydrate utilization reactions, and the Phadebact GC Monoclonal Test (MKL Diagnostic AB, Solleårna, Sweden) (WHO, 2010). Isolates were stored at $-80^\circ$C in trypticase soy broth containing 20% glycerol. All isolates were subcultured on Difco GC medium base agar (BD, Franklin Lakes, NJ) supplemented with 1% Britalex enrichment supplement (Britания, Ltd, Argentina) for 18 to 24 h at 35°C in a humidified environment and enriched with 5% CO$_2$ prior to testing. All the isolates were evaluated for resistance to 6 antimicrobial agents, including penicillin, tetracycline, ciprofloxacin, ceftriaxone, cefixime, and azithromycin, by the agar dilution method according to the guidelines established by the CLSI (CLSI, 2012a). All antimicrobial agents for the agar dilution method were obtained from Richet Laboratories, Buenos Aires, Argentina, except cefixime (Bagó Laboratories, Buenos Aires, Argentina). The CLSI guideline (M100–2010) was used to interpret the results, except in the cases of azithromycin, for which EUCAST guidelines were followed (CLSI, 2017; EUCAST, 2017). The breakpoints for the definition of decreased susceptibility to ceftriaxone and cefixime were MICs of 0.06–0.25 μg/mL and 0.125–0.25 μg/mL, respectively (Gianecini et al., 2017). All the 2008 World Health Organization *N. gonorrhoeae* reference strains panel and *N. gonorrhoeae* ATCC 49226 were used as quality control strains in the testing (Unemo et al., 2009).

#### 2.2. Gentamicin Susceptibility by Agar Dilution and Disk Diffusion

Agar dilution and disk diffusion methods for gentamicin susceptibility were performed in the 237 *N. gonorrhoeae* isolates in accordance with CLSI guidelines (CLSI, 2012a, 2012b). For agar dilution testing, gentamicin research powder was obtained from Bagó Laboratories (Buenos Aires, Argentina). Plates containing 2-fold dilution of gentamicin (64–1 μg/mL) and control plates containing no antibiotic were prepared in Difco GC medium base agar supplemented with 1% Britalex. For each isolate, colonies were suspended in Mueller–Hinton broth (Biokar Diagnostics, Beauvais, France) to obtain the inoculum equivalent to 0.5 McFarland standard. The same suspension was used within 15 min to perform disk diffusion test as described below. A Steers replicator was used to deliver approximately $5 \times 10^5$ CFU/spot to the surface of the agar. The plates were incubated from 20 to 24 h at 35°C in a humidified environment and enriched with 5% CO$_2$. The interpretative criteria previously published were used: MIC ≤4 μg/mL, susceptible; MIC 8–16 μg/mL, intermediate susceptible; and MIC ≥32 μg/mL, resistant (Brown et al., 2010).

The disk diffusion test was realized for each isolate on Difco GM medium base agar supplemented with 1% Britalex enrichment supplement. Approximately 25 mL of GC medium base was poured into 90-mm diameter sterile Petri dishes to a depth of 4 mm. All isolates were tested with gentamicin 10 μg disk from 2 different companies (Oxoid, Basingstoke, UK, lot 1894871; BBL, Sparks, MD, lot 5180663). The plates were incubated in the same conditions as those indicated for MIC test. Using calipers, inhibition zone diameters were measured to the nearest millimeter at the inner zone edge. Tentative interpretative zone breakpoints for 10-μg gentamicin disk were used: ≤12 mm, resistant; 13–15 mm, intermediate susceptible; and ≥16 mm susceptible (Bala et al., 2016).

All the 2008 WHO *N. gonorrhoeae* reference strains panel and ATCC 49226 were included on each occasion as quality control (Unemo et al., 2009).

#### 2.3. Data Analysis

MICs for 50% (MIC$_{50}$) and 90% (MIC$_{90}$) for the isolates tested were determined. All data analysis was performed using Microsoft Excel 2010 software (Microsoft Corporation, Redmond, VA) and GraphPad prism 5.0 (La Jolla, CA).

The correlation between the agar dilution reference method and disk diffusion test was determined by plotting the inhibition zones against their respective MICs, and a linear regression analysis was performed. The correlation coefficient was determined using Pearson’s R at a significance level of 0.05.

The resistance breakpoints used in this study were used to calculate very major, major, and minor errors between the agar dilution reference method and disk diffusion test. Very major errors occurred with *N. gonorrhoeae* isolates for which MICs indicated resistance by agar dilution method and susceptibility by disk diffusion method. Major errors occurred with *N. gonorrhoeae* isolates for which MICs indicated susceptibility by agar dilution method and resistance by disk diffusion method. Minor errors were defined as one value corresponding to intermediate and the other corresponding to resistant or susceptible. To evaluate whether the gentamicin disk diffusion reflected an acceptable or unacceptable rates of discrepancy, the CLSI M23–ED4 guideline was used (CLSI, 2016). The percentage of discrepancies considered acceptable within 1 log$_2$ dilution of the intermediate MIC value (gentamicin agar dilution MICs, 4–32 μg/mL) was <40% for minor error, <10% for major error, and <10% for very major error. At gentamicin MICs ≥64 μg/mL, discrepancies rates of <5% for minor error and <2% for very major error were considered acceptable, while at gentamicin MICs ≤2 μg/mL, discrepancies rates ≤5% for minor error and ≤2% for major error were considered acceptable.

The reproducibility of the disk diffusion method was evaluated by testing *N. gonorrhoeae* ATCC 49226 and all the 2008 WHO *N. gonorrhoeae* reference strains panel in triplicate for 3 different days. Categorical agreement was calculated by dividing the number of tests with no category discrepancy by the number of organism tested (Clark et al., 2009).

### 3. Results

The susceptibility profile of 237 *N. gonorrhoeae* isolates is presented in Table 1. A total of 75.5% of the isolates included in this study showed resistance to 1 or more of the following antibiotics: penicillin (40.5%), tetracycline (30.4%), and ciprofloxacin (59.9%). While all the strains were susceptible to ESCs, 13 (5.5%) isolates showed decreased susceptibility to ceftriaxone and cefixime. Moreover, 3 isolates with decreased susceptibility to ESCs demonstrated resistance to azithromycin (MIC: 1 μg/mL). Although *N. gonorrhoeae* isolates with MDR phenotype were not observed using the criteria mentioned above, 48 (20.2%) of the isolates showed resistance to 3 or more different antimicrobials.
3.1. Gentamicin Susceptibility

The gentamicin MICs for *N. gonorrhoeae* ATCC 49226 and the 2008 WHO *N. gonorrhoeae* reference strains were within 1 dilution of those previously reported (Bala et al., 2016; Unemo et al., 2016). MICs for the 237 isolates were observed in a narrow range of concentrations between 2 and 16 μg/mL. No differences were observed between the MIC50 and MIC90 value, which was for both at 8 μg/mL. A high percentage of *N. gonorrhoeae* isolates (69.2%, 164/237) was categorized as intermediate susceptibility (MIC = 8–16 μg/mL), and isolates with resistance to gentamicin were not detected.

3.2. Comparison of Gentamicin Susceptibility by Agar Dilution and Disk Diffusion

The correlation coefficient and the scatter plot of the MICs and the zone diameter of gentamicin evaluated in this study demonstrated a correlation between the reference agar dilution method and disk diffusion test. The Pearson correlation between disk diffusion and agar dilution methods was strong (r = −.68; P < 0.001) by using BBL disks and (r = −.71; P < 0.001) by using Oxoid disks (Fig. 1).

When comparing disk diffusion to agar dilution method and using the interpretation criteria previously reported for gentamicin, very major or major discrepancies were not found with the disk diffusion test (BBL and Oxoid disks). However, a high percentage of minor discrepancies (44.7%, BBL disk; 21.9%, Oxoid disk) was observed, with a high number of discrepancies consisting of susceptible isolates by disk diffusion and identified as intermediate by the agar dilution method (Table 2). According to CLSI recommendation, by using the BBL disk, the minor discrepancy rate for the IHigh + 1 to ILow − 1 population (45.1%) was not within approved limits (<40%). However, by adjusting the susceptible breakpoint for disk diffusion to S ≥ 17 mm, the minor discrepancies rate was reduced to 19.4% and 10.1% for BBL and Oxoid disk, respectively (Table 3). The following zone diameter breakpoints resulted in a considerable reduction in the number of errors: ≥17 mm (susceptible), 13–16 mm (intermediate susceptibility), and ≤12 mm.

### Table 1

In vitro activities of 6 antimicrobial agents against *N. gonorrhoeae* clinical isolates (N = 237).

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>No. (%) of isolates</th>
<th>MIC (μg/mL)</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt;</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>96 (40.5)</td>
<td>18 (7.6)</td>
<td>1</td>
<td>8</td>
<td>0.016–128</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>72 (30.4)</td>
<td>14 (5.9)</td>
<td>1</td>
<td>16</td>
<td>0.125–32</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>142 (59.9)</td>
<td>94 (39.7)</td>
<td>2</td>
<td>8</td>
<td>0.002–16</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>-</td>
<td>237 (100)</td>
<td>0.008</td>
<td>0.03</td>
<td>0.001–0.125</td>
</tr>
<tr>
<td>Cefixime</td>
<td>-</td>
<td>237 (100)</td>
<td>0.016</td>
<td>0.06</td>
<td>0.002–0.25</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>7 (2.9)</td>
<td>165 (68.7)</td>
<td>0.25</td>
<td>0.5</td>
<td>0.03–2</td>
</tr>
</tbody>
</table>

![Fig. 1. Gentamicin MICs versus zone inhibition diameters using gentamicin 10-μg BBL and Oxoid Disks for 237 isolates of *N. gonorrhoeae*. The horizontal and vertical lines represent MIC and zone diameter breakpoints previously reported, while the vertical dashed line represents the proposed susceptibility breakpoint (≥17 mm).](image-url)
(resistant). The MIC and zone diameters scattergrams using the zone diameter breakpoints proposed herein are presented in Fig. 1. All discrepancy rates were within CLSI-accepted limits; no very major or major discrepancies were detected.

To test for reproducibility, the gentamicin tentative zone breakpoints previously reported and the zone breakpoints proposed in this study were used. The categorical agreement by using these zone breakpoints ranged between 38.3% and 81.5%, with the lowest categorical agreement being found for BBL disk and the tentative zone breakpoints previously reported (Table 4). Very major or major errors were not detected.

4. Discussion

The goal of this study was to evaluate the susceptibility of N. gonorrhoeae isolates to gentamicin and to compare the disk diffusion test with the reference agar dilution method to determine the susceptibility of N. gonorrhoeae isolates to gentamicin. In this study, 97.1% of the isolates showed a MIC between 4 and 8 μg/mL and 30.8% of the isolates were categorized as susceptible. This is consistent with previous studies from Europe and Canada in which 90% of MICs to gentamicin were distributed within 4–8 μg/mL (Chisholm et al., 2011; Public Health Agency of Canada, 2014). Although previous studies using agar dilution showed a wide range in the MICs of gentamicin, MICs of 4–8 μg/mL were most frequently observed (Brown et al., 2010; Joesoef et al., 1994; Rice and Knapp, 1994). Although there are insufficient data to support the use of single-dose gentamicin as a first-line agent in the treatment of uncomplicated gonorrhea, a previous study showed a high efficacy of gentamicin plus azithromycin for treatment of uncomplicated urogenital gonorrhea (Kirkcaldy et al., 2014). Nowadays, CDC treatment guidelines recommend gentamicin as dual treatment with single dose of gentamicin plus azithromycin in case of suspected treatment failure with the recommended first-line regimen of cettri-axone plus azithromycin (Workowski et al., 2015). Moreover, a recent outbreak of azithromycin-resistant N. gonorrhoeae in South Australia resulted in a change in the treatment guidelines with gentamicin plus azithromycin as second-line regimen (Lahra et al., 2017). Therefore, gentamicin is a promising treatment option for gonorrhea. In this study, the absence of resistant N. gonorrhoeae isolates to gentamicin indicates that this antimicrobial may be a future therapeutic option in Argentina.

Since clinical laboratories need easy, reliable, and non-time-consuming alternative methods for the determination of susceptibility to antibiotics, we investigated the disk diffusion as a method for testing the susceptibility of N. gonorrhoeae to gentamicin. Two sources of gentamicin 10-μg disks were evaluated, with the BBL disks providing zone diameters ≥1 mm than the Oxoid disks. Based on this, a high percentage of minor errors was observed using BBL disk, which was unacceptable according to CLSI recommendations. Adjusting the zone diameter limit of the susceptible range to the following tentative disk diffusion breakpoints generated the least number of errors: ≥17 mm (S), 13–16 mm (I), and ≤12 mm (R). Previous studies comparing gentamicin MICs by agar dilution method and Etest showed that the latter generated slightly lower values for gentamicin than the agar dilution method (Chisholm et al., 2011; Daly et al., 1997). Thereby, the differences observed in this study and Bala et al. may be due to the use of Etest of the latter, reporting a high percentage of isolates susceptible to gentamicin by MIC (75.9%) (Bala et al., 2016).

We found that the categorical agreement between disk diffusion and agar dilution method was <95% (Clark et al., 2009). However, the N. gonorrhoeae ATCC 49226 and all the 2008 WHO N. gonorrhoeae reference strains showed gentamicin MICs near to the breakpoint of 4 and 8 μg/mL, respectively. When data points for a large proportion of isolates are close to a breakpoint, a high percentage of minor discrepancies are expected to occur (CLSI, 2016). One limitation of this study was the absence of resistant and highly susceptible isolates to evaluate the performance of disk diffusion with these isolates.

<table>
<thead>
<tr>
<th>MIC range</th>
<th>Number of isolates</th>
<th>Interpretation differences, n (%)</th>
<th>MIC range</th>
<th>Number of isolates</th>
<th>Interpretation differences, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Very Major</td>
<td>Major</td>
<td>Minor</td>
<td></td>
</tr>
<tr>
<td>2I_High + 2a</td>
<td>0</td>
<td>0</td>
<td>N/A</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1I_High + 1 to Ilow - 1c</td>
<td>235</td>
<td>0</td>
<td>0</td>
<td>52 (22.1%)</td>
<td>235</td>
</tr>
<tr>
<td>1Ilow - 2d</td>
<td>2</td>
<td>N/A</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>237</td>
<td>0</td>
<td>0</td>
<td>52 (21.9%)</td>
<td>237</td>
</tr>
</tbody>
</table>

a: S: susceptible; I: intermediate; R: resistant.
b: Defined as MICs ≥2 two-fold concentrations above the higher intermediate MIC.
c: Defined as the higher and lower MICs in the intermediate MIC range.
d: Defined as MICs ≤2 two-fold concentrations below the lower intermediate MIC.

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In conclusion, Neisseria gonorrhoeae isolates with resistance to gentamicin were not observed, and gentamicin might be considered a future treatment option for gonorrhea in Argentina. Until gentamicin breakpoints become standardized, the disk diffusion method and interpretative criteria described in this study provided a feasible method that may be used for epidemiological surveillance and clinical situations in resource-limited settings in which MIC determinations methods are not readily available.

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GASSP-AR Working Group


Competing Interests

The authors declare that they have no competing interests.

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Ethical Approval

No institutional review board approval was necessary for this study because no personal information of the patients was collected during the investigation.

References


