In vitro efficacy of gentamicin against multidrug-resistant *Neisseria gonorrhoeae*: synergy of three gentamicin antimicrobial combinations

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Running Head: gentamicin against multidrug-resistant gonococci

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ABSTRACT

Objectives: To determine *in vitro* activities of gentamicin alone and in combination with ceftriaxone, ertapenem and azithromycin against multidrug-resistant (MDR) *N. gonorrhoeae* isolates.

Methods: 407 isolates from Nanjing, China, obtained in 2016 and 2017, had minimum inhibitory concentrations (MICs) determined for gentamicin using the agar dilution method. Antimicrobial combinations were also tested in 97 MDR strains using the antimicrobial gradient epsilometer test (Etest); results ranging from synergy to antagonism were interpreted using the fractional inhibitory concentration (FICI).

Results: All 407 gonococcal isolates were susceptible to gentamicin. MICs ranged from 2 mg/L to 16 mg/L. Synergy was demonstrated in 16.5%(16/97), 27.8%(27/97) and 8.2%(8/97) MDR strains when gentamicin was combined with ceftriaxone [geometric mean (GM) FICI; 0.747], ertapenem (GM FICI; 0.662) and azithromycin (GM FICI; 1.021), respectively. No antimicrobial antagonism was observed with any combination. The three antimicrobial combinations were indifferent overall. The overall GM MICs of gentamicin were reduced by 2.63-, 3.80- and 1.98-fold when tested in combination with ceftriaxone, ertapenem and azithromycin, respectively. The GM MICs of the three antimicrobials by themselves were reduced by 3-, 2.57-

and 1.98-fold respectively, when each was tested in combination with gentamicin. No antimicrobial antagonism was observed with any combination.

Conclusions: Gentamicin alone was effective *in vitro* against MDR *N. gonorrhoeae* and in combination with ceftriaxone, ertapenem or azithromycin. Combination testing of resistant strains, overall, showed lower effective MICs against gentamicin itself and each of the three antimicrobials when used in combination with gentamicin.

Keywords: *Neisseria gonorrhoeae*, multidrug resistance, combination, gentamicin

INTRODUCTION

Gonorrhea, caused by *Neisseria gonorrhoeae*, is a common bacterial sexually transmitted infection (STI), which is responsible for an increasing burden of disease globally. In 2016, the World Health Organization (WHO) estimated 87 million new cases of gonorrhea worldwide in adults, aged 15-49(1). Common features of gonococcal infection include urethritis and cervicitis; more serious manifestations include pelvic inflammatory disease, chronic pelvic pain, infertility, chorioamnionitis in women and epididymitis in men. Disseminated gonococcal infections (DGI) occurs occasionally in adults and neonates born to infected mothers; in the pre-antibiotic era endocarditis and meningitis sometimes resulted from gonococcal bacteremia but is rarely seen today. Concomitant gonococcal and HIV infections also increase the risk of HIV transmission (2).

The emergence of antibiotic resistance among *N. gonorrhoeae* is a global public health threat. *N. gonorrhoeae* has developed resistance to all antimicrobials that have been used for treatment, including penicillin, tetracycline, ciprofloxacin and azithromycin, leading to the emergence of multidrug-resistant (MDR) isolates, which are difficult to treat (3). Currently, dual antimicrobial therapy with an extended spectrum cephalosporin (ESC), either ceftriaxone 250 mg intramuscularly or cefixime 400 mg orally, plus azithromycin 1g orally, is recommended as first-line treatment of uncomplicated gonorrhea by the World Health Organization (WHO) (4). Increased prevalence of azithromycin resistance globally prompted a revision of prior recommendations in the United Kingdom (U.K.) (5) in 2018 and the United States (U.S.) (6) in 2020 from dual therapy with ceftriaxone and azithromycin to recommendations of higher doses of ceftriaxone: 1 gram (U.K.) and 500 mg (U.S). Unfortunately, resistance to ESCs (7-9), the last remaining option for empirical first-line monotherapy, threatens future use of this class of antimicrobials. Treatment failures with both mono- and dual-therapy (including azithromycin) have been reported in recent years (10, 11).

Treatment options for gonorrhea including infections caused by MDR organisms are diminishing; there is an urgent need to explore new or repurposed antimicrobial agents and/or therapeutic strategies. Gentamicin, an aminoglycoside antibiotic that inhibits protein synthesis by binding to the 30S ribosomal subunit, has been used as first-line therapy for treatment of gonorrhea in several countries, including Malawi,

where it has been used officially for nearly 30 years (12). Numerous *in vitro* susceptibility studies have shown that gentamicin is active against *N. gonorrhoeae* (13-16), suggesting that gentamicin is appropriate for treatment of uncomplicated gonococcal infection. A single 240 mg dose of intramuscular gentamicin was 95% efficacious in a randomized controlled clinical trial to treat urethritis (17). In multicenter clinical trials, dual therapy with gentamicin plus azithromycin was 94%-100% effective compared to 98% for ceftriaxone plus azithromycin (18, 19). Little is known about *in vitro* susceptibility of gentamicin in Chinese isolates where this antimicrobial has not been used to treat gonorrhea. Use of antimicrobial combinations is a therapeutic strategy intended to increase efficacy and slow the development of resistance (20). Antimicrobial combinations may prove useful to successfully manage MDR *N. gonorrhoeae* infections and they are included in current WHO and CDC guidelines (4, 6).

Initially, we evaluated gentamicin susceptibility of gonococcal strains isolated between 2016 and 2017 in Nanjing, China. Second, we carried out studies with antimicrobial combinations that included gentamicin to evaluate possible *in vitro* enhancement of gentamicin activity against MDR strains when tested in combination with either ceftriaxone (an ESC), ertapenem (a carbapenem) or azithromycin (a macrolide). Third, we determined the MICs of each of the three antimicrobials by themselves when tested in combination with gentamicin.

RESULTS

All 407 *N. gonorrhoeae* strains were sensitive to gentamicin by agar dilution; minimum inhibitory concentrations (MICs) ranged from 2mg/L to 16mg/L; the MIC_{50} was 8 mg/L and the MIC_{90} was 16mg/L. Among the 407 isolates, 34 (8.4%) were susceptible (MIC ≤ 4mg/L), 373 (91.6%) were intermediately susceptible (MIC range, 8-16 mg/L) and none was resistant (MIC ≥ 32 mg/L) (Fig.1). Among the 34 fully susceptible isolates, 6 (17.6%) had decreased susceptibility to ESCs i.e. ceftriaxone or cefixime or both and 1 (2.9%) was fully resistant to ceftriaxone (MIC; 1mg/L) and cefixime (MIC; 2mg/L). There was no change in intermediate susceptibility to gentamicin of isolates between 2016 and 2017 (89.3% vs. 93.4%; χ^2=2.225, P=0.136) (Fig. 2).

The distribution of antimicrobial susceptibility patterns for the 97 MDR strains selected for antimicrobial combination testing showed 6 unique patterns of susceptibility (Table S1). 93 (95.9%) strains had decreased susceptibility to ceftriaxone or cefixime or both plus resistance to ciprofloxacin and penicillin, and 4 (4.1%) strains had decreased susceptibility to ceftriaxone or cefixime or both plus resistance to ciprofloxacin, penicillin and azithromycin. Resistance to gentamicin in MDR isolates was not detected by either the agar dilution or the Etest method. Agreement of MICs between agar dilution and Etest among MDR isolates is summarized in Table S2. Overall agreement of MICs (≤ 2-fold different) between the two methods was 93.8%. Etest always resulted in one to two dilutions lower MIC values than agar dilution.

Gentamicin agar dilution versus Etest MIC results (mg/L) were as follows: MIC_{50} = 8

versus 6; MIC_{90} =16 versus 8; geometric mean [GM] MIC =11.3 versus 5.84. Additionally, there was a discrepancy in the full susceptibility category (29.9% fully susceptible by Etest versus 9.3% by agar dilution; χ^2=13.090, P<0.001).

The three antimicrobial combinations used to test each of the 97 strains were examined for effects that were classified as: synergistic, indifferent or antagonistic-- summarized in Table 1. For example, the gentamicin GM MIC, when tested alone, was 5.840 mg/L; when combined with ceftriaxone, the GM MIC was reduced to 2.217 mg/L (2.63-fold reduction, P <0.001) (Table 2). Together with ceftriaxone, gentamicin exhibited synergy against 16.5% (16/97) MDR strains; overall, the combination was indifferent; FICI, 0.747. When tested alone, MICs of ceftriaxone ranged from 0.016-0.75 mg/L; in combination with gentamicin, the GM MIC against ceftriaxone decreased from 0.078 to 0.026 mg/L (3-fold reduction, P <0.001).

The gentamicin GM MIC when combined with ertapenem decreased to 1.536 mg/L (3.80-fold reduction, P <0.001) (Table 2). Gentamicin together with ertapenem was the most synergistic combination, displaying synergy against 27.8% (27/97) of MDR gonococcal isolates; overall, this combination was indifferent; FICI, 0.662. Ertapenem MICs of 97 MDR isolates, when tested alone, ranged from 0.006 to 0.064 mg/L (GM MIC, 0.018 mg/L); when combined with gentamicin, the GM MIC decreased to 0.007 mg/L (2.57-fold reduction, P <0.001).

The gentamicin GM MIC when combined with azithromycin decreased to 2.949 mg/L (1.98-fold reduction, P <0.001) (Table 2). Together with azithromycin, gentamicin

exhibited synergy against 8.2% (8/97) MDR strains; overall, the combination was indifferent; FICI, 1.021. When tested alone, MICs of azithromycin ranged from 0.047-8 mg/L; in combination with gentamicin, the GM MIC against azithromycin decreased from 0.347 to 0.175 mg/L (1.98-fold reduction, P <0.001).

DISCUSSION

Our study provides data on gentamicin susceptibility against *N. gonorrhoeae* isolated in Nanjing (Jiangsu Province), China. On agar dilution testing, most strains displayed intermediate susceptibility to gentamicin (MIC 8-16 mg/L), similar to a study that examined gonococcal isolates from seven hospitals in a neighboring eastern Chinese province; in that study 97.8% (493/504) of strains possessed gentamicin MICs of 8-16 mg/L (21). European and U.S. studies have reported 82.7% (15) and 73% (18), intermediate susceptibility, respectively, of *N. gonorrhoeae* isolates to gentamicin. A recent report of gentamicin susceptibility of *N. gonorrhoeae* in which 86.0% of 470 isolates were fully susceptible (MICs $\leq$ 4 mg/L), examined isolates from seven geographically distributed Chinese provinces as part of the China Gonococcal Resistance Surveillance Programme (China-GRSP) (22) similar to an Indian study where 90.7% of isolates were reported as fully susceptible (15).

Our study compared gentamicin MICs using agar dilution and Etest methods for 97 multidrug-resistant (MDR) *N. gonorrhoeae* isolates. Similar to previous studies (13, 23), we found that over 90% of gentamicin MICs determined by agar dilution and

166 Etest were ≤ 2-fold different; typically, Etest resulted in lower MICs and identified a
167 larger proportion of fully susceptible isolates. In particular, all MDR isolates were
168 fully or intermediately susceptible to gentamicin.
169 Synergistic or additive effects of combining antimicrobials for treatment may slow the
170 development of antimicrobial resistance of *N. gonorrhoeae* (24). We assessed the *in*
171 *vitro* activity of gentamicin in combination with 3 antibiotics. Determining synergy
172 has several challenges. Several test methods are available to evaluate synergistic
173 effects of antimicrobial combinations, however, they are not well standardized. We
174 chose Etest because it is practical and also correlates well with agar dilution, time–kill
175 curves and checkerboard testing in demonstrating synergy for two-drug combination
176 (25-27). Nonetheless, FICI interpretation depends on the criteria used. FICI values
177 between 0.5 and 1 have been interpreted as additive in Indian (28) and Japanese (29)
178 studies, differing from our criteria, which classifies FICI values in this range as
179 indifferent.
180 Ceftriaxone, in higher doses, is now recommended as single therapy by the U.S. and
181 the U.K. for treatment of uncomplicated gonorrhea (5, 6). Our study showed that the
182 combination of ceftriaxone and gentamicin exhibited an indifferent effect overall in
183 >80% of MDR strains (< 20% synergy). In the Indian study by Singh *et al* (28), 14.7%
184 synergy and 6.3% antagonism were reported for this combination against 95 *N.*
185 *gonorrhoeae* strains including 79 MDR and one extensively drug-resistant (XDR)
186 strain. In a Canadian study, a mean $FICI_{50}$ value of 1.2 (0.8-2.0) was shown for nine

187 reference strains of *N. gonorrhoeae* (WHO F, G, K, L, M, N, O, P and ATCC 49226)
188 with this combination (30). An American study reported a mean FICI of 1.25 (0.73-2)
189 using gonococcal isolates that displayed different cefixime MICs (26). No
190 synergistic/antagonistic effect (resulting in 100% indifference) was observed in either
191 study (26, 30).
192 We chose ertapenem as a candidate for *in vitro* synergy testing because its mechanism
193 of action differs from that of gentamicin and it has been used to treat infection with
194 combined high-level azithromycin and ceftriaxone resistant *N. gonorrhoeae* (11).
195 Ertapenem has demonstrated an advantage over ceftriaxone for MDR or ceftriaxone
196 resistant isolates and has also been suggested for possible use in a dual antimicrobial
197 regimen (31). We showed that gentamicin plus ertapenem in combination resulted in
198 synergistic and indifferent effects with no antagonism demonstrated in any MDR
199 strain. In the study by Singh *et al* (28), this combination displayed either synergy
200 (31.6%) or indifference (68.4%) in 100% of strains; no antagonism was seen.
201 Gentamicin in combination with azithromycin is currently recommended as an option
202 for retreatment by the WHO when dual therapy fails (4). Also, it is proposed as an
203 alternative CDC recommendation when higher dose ceftriaxone therapy cannot be used
204 (6). In our studies, this combination demonstrated synergy in fewer MDR isolates
205 (<10%) than combinations with either ceftriaxone or ertapenem and exhibited the
206 highest FICI value of the three combinations tested. Similar to our results, Sood *et al*
207 (32) demonstrated synergistic effects in 22.9% of isolates displaying different

208 ceftriaxone MICs and no antagonism for this combination. All isolates with differing
209 cefixime/ ceftriaxone MICs showed indifference with a mean FICI of 1.7/0.83 in
210 studies from the UK (33) and Japan (29). The separate study by Singh *et al* (28) differed
211 from these results, with 6.3% of strains exhibiting antagonism when this combination
212 was used.
213 A summary of results from these previous studies is shown in Table S3. No
214 synergistic/antagonistic effect (resulting in 100% indifference) was observed in studies
215 from Canada (30), Japan (29), the US (26) and the UK (33). None of these studies
216 incorporated isolates with multi-drug resistance. However, a certain proportion of
217 synergistic effects was observed in the two Indian studies (28, 32). Antagonism was
218 also observed in combinations of gentamicin with ceftriaxone and azithromycin in the
219 study by Singh *et al* (28). Sood *et al* (32) used the same Etest that we used. In contrast,
220 Singh *et al* (28) incubated the Etest strip of the first antimicrobial (antimicrobial A)
221 for 1 h, and then replaced it with the Etest strip of the second antimicrobial
222 (antimicrobial B) at the same location and looked for synergism. A mild degree of
223 antagonism may have been missed in our tests when the zone of inhibition ran under the
224 strips where they crossed and therefore was unreadable and interpreted as indifference
225 (25).
226 From a pharmacokinetic perspective, all antimicrobials in the 3 combinations in our
227 study result in peak levels of drug during the first 3 hours after administration (34-37).
228 However, azithromycin has a longer half-life than gentamicin (approximately 68 and

229 2 hours, respectively) (34, 37). Gentamicin in combination with azithromycin also
230 produced the lowest synergistic effects among the 3 combinations in our study so it
231 may not be optimal for clinical use where synergy would not be prolonged.
232 In conclusion, resistance to gentamicin was not observed in gonococcal isolates
233 examined in this study, including MDR isolates, indicating potential for its
234 therapeutic use. Antimicrobial combinations of gentamicin plus ertapenem,
235 ceftriaxone, and azithromycin showed no antagonistic effects; enhanced efficacy of
236 individual antimicrobials in the presence of other antimicrobials was also
237 demonstrated. Improved efficacy of antimicrobial combinations suggest that dual
238 therapy might be a promising approach to manage MDR *N. gonorrhoeae*. Further
239 studies to correlate *in vitro* results with clinical outcomes are warranted.
240

241 **MATERIALS AND METHODS**

242 **Bacterial strains** 407 gonococcal isolates were recovered from men with
243 symptomatic urethritis (urethral discharge and/or dysuria) attending the STD clinic at
244 the Institute of Dermatology, Chinese Academy of Medical Sciences in Nanjing,
245 between January 2016 and December 2017. Urethral specimens were collected with
246 cotton swabs and immediately streaked onto modified Thayer-Martin medium
247 (Zhuhai DL Biotech Co. Ltd.) and cultured in candle jars at 36°C for 24–48 h.
248 Gonococcal isolates were identified by colonial morphology, Gram's stain, and
249 oxidase testing and sub-cultured onto GC chocolate agar base (Difco, Detroit, MI)

supplemented with 1% IsovitaleX™ (Oxoid, USA); pure cultures were swabbed, suspended in tryptone-based soy broth and frozen (−80°C) until used for antimicrobial testing.

Antimicrobial susceptibility testing The MICs of the 407 isolates were determined using the agar dilution method for gentamicin, penicillin, tetracycline, ciprofloxacin, azithromycin, spectinomycin, cefixime and ceftriaxone, used singly, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (38). *N. gonorrhoeae* ATCC 49226, WHO reference strains F, G, L, O and P were used as quality control strains in susceptibility tests. Although formal susceptibility criteria for gentamicin have not been established by CLSI, criteria described by CDC (16) have used MICs of ≤4 mg/L as fully susceptible, 8 to 16 mg/L as intermediately susceptible and ≥32 mg/L as resistant. Resistance to azithromycin (MIC ≥1 mg/L) was determined using EUCAST criteria (39). Susceptibilities to other antibiotics were assessed based on CLSI standards (38). Decreased susceptibility to cephalosporins was determined according to WHO standards (40). Based on criteria proposed by Tapsall *et al* in 2009 (41), MDR isolates were defined as those resistant or with decreased susceptibility to one or more widely used antimicrobials (ceftriaxone and cefixime; category I) and resistant to two or more antimicrobials, which are used less frequently (penicillin; ciprofloxacin and azithromycin; category II) (Table S4).

Synergy testing and interpretation Of 407 clinical isolates of *N. gonorrhoeae*, 97 MDR isolates (determined below) were selected for antimicrobial combination testing

(synergy/ antagonism) according to MDR criteria. WHO-P was used as a quality control strain. Dual antimicrobial testing was performed to evaluate the efficacy of gentamicin in combination with either ceftriaxone, ertapenem or azithromycin, using the Etest method, described previously (25). Briefly, MICs for individual antimicrobials (MIC A_{alone} and MIC B_{alone}) against *Neisseria gonorrhoeae* isolates were determined using Etest strips (Liofilchem, Italy); *in vitro* activity of each combination was determined by placing E-test strips of the two antimicrobials on the agar plates at a 90° angle, with intersections at the points of their individual MICs (Fig. 3). Agar plates were inverted during incubation at 36°C in 5% CO_2 for 16–18 hours, and the MIC of each antimicrobial in the combination (MIC $A_{in\ combination}$ and MIC $B_{in\ combination}$) was read.

To determine whether each antimicrobial combination resulted in a synergistic, indifferent or antagonistic effect, the fractional inhibitory concentration index (FICI) was calculated using the following formula: FICI = (MIC $A_{in\ combination}$ / MIC A_{alone}) +(MIC $B_{in\ combination}$ / MIC B_{alone}) (25). FICI values were interpreted using the following criteria: synergy: ≤0.5; indifference: FIC >0.5 to ≤4.0 and antagonism: FICI of > 4.0(25).

Statistical Analysis The chi-square test was used to compare gentamicin susceptibility trend data and the categorical assignments of gentamicin susceptibility by the agar dilution or Etest method. Mean values of MICs and FICIs were calculated as geometric means (GM). The statistical significance of the difference between the MIC

of each of the three antimicrobials tested alone and in combination with gentamicin was determined using the nonparametric Mann-Whitney's U-test. P values less than 0.05 were considered statistically significant.

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CONFLICTS OF INTEREST

No conflicts for all authors.

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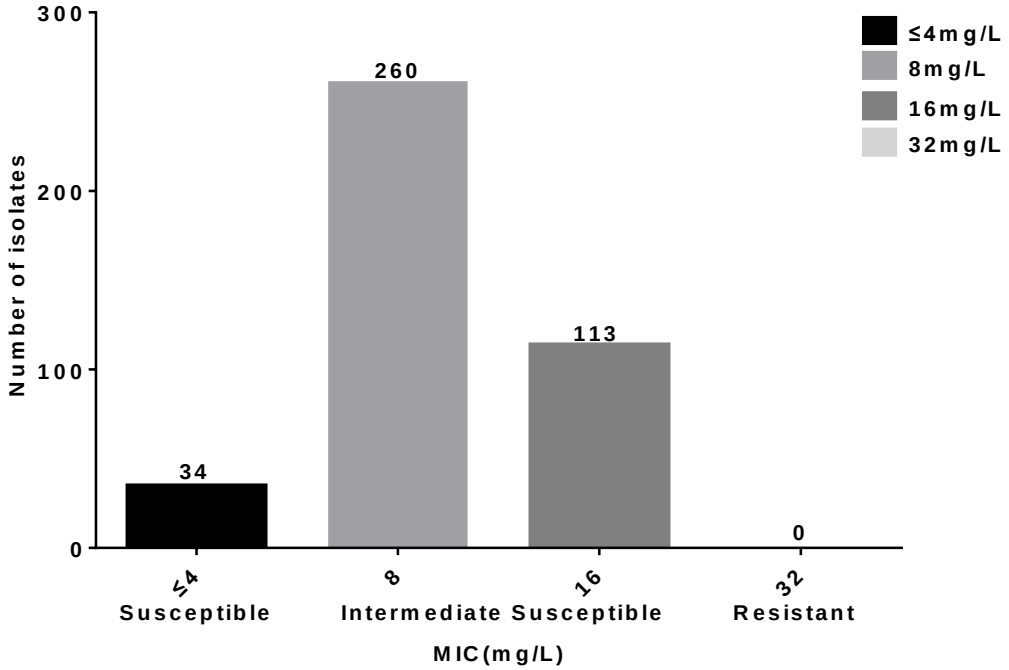

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454 Figure 1. Susceptibility of gentamicin against *N. gonorrhoeae* strains isolated in

455 Nanjing, China, 2016–2017 (n =407).

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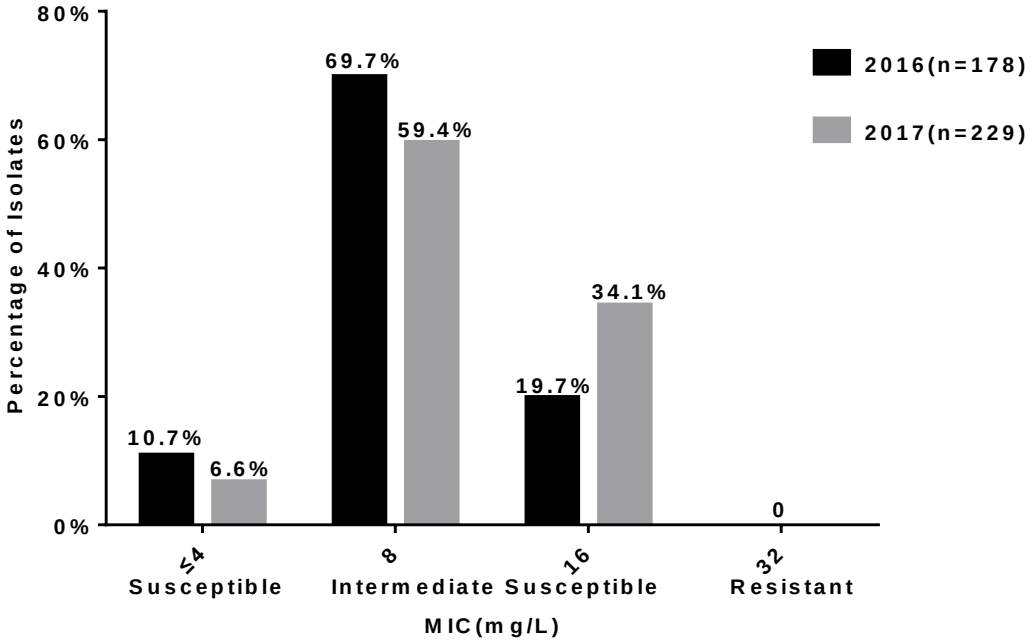

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460 Figure 2. Distributions of MICs of gentamicin against *N. gonorrhoeae* isolates in 2016

461 (n=178) and 2017 (n=229).

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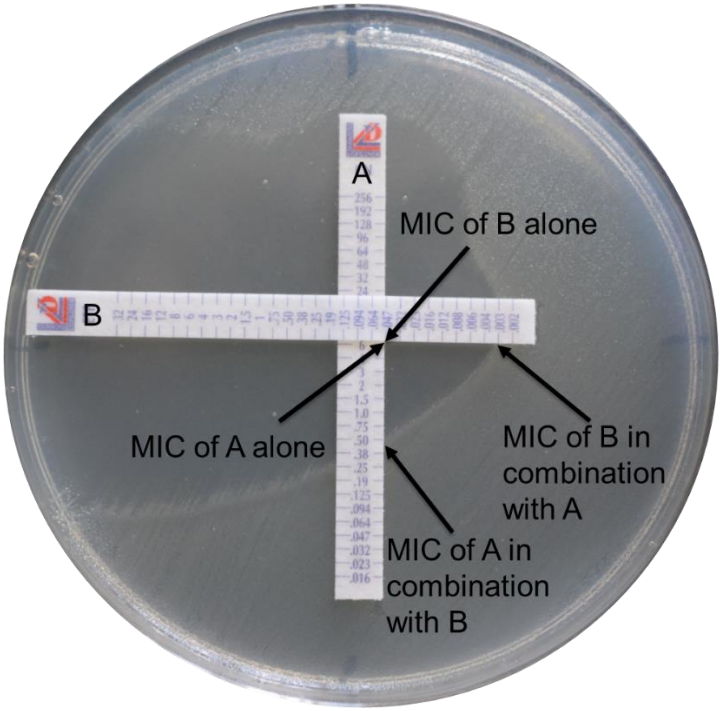

Figure 3. Photograph of strip placement for E-test synergy method.

Table 1. Synergy test results for combinations of gentamicin plus azithromycin, ceftriaxone, ertapenem against 97 MDR *N. gonorrhoeae* isolates

	Antimicrobial combinations		
Effect n (%)	GEN +CRO	GEN +ETP	GEN +AZM
Synergistic n (%)	16(16.5%)	27(27.8%)	8(8.2%)
Indifferent n (%)	81(83.5%)	70(72.2%)	89(91.8%)
Antagonistic n (%)	0	0	0
FICI (geometric mean)	0.747	0.662	1.021
Classification Overall	Indifferent	Indifferent	Indifferent

Abbreviations: n, number; GEN, gentamicin; CRO, ceftriaxone; ETP, ertapenem; AZM, azithromycin.

492 Table 2. *In vitro* antibacterial activity of tested antibiotics combinations against 97

493 MDR *N. gonorrhoeae* isolates

MIC	Antimicrobial combinations		
	GEN +CRO	GEN +ETP	GEN +AZM
MIC A $_{\text{alone}}$	5.840(2-12)	5.840(2-12)	5.840(2-12)
MIC A $_{\text{in combination}}$	2.217(0.38-6)	1.536(0.25-6)	2.949(1-8)
MIC B $_{\text{alone}}$	0.078(0.016-0.75)	0.018(0.006-0.064)	0.347(0.047-8)
MIC B $_{\text{in combination}}$	0.026(0.004-0.25)	0.007(0.002-0.047)	0.175(0.023-2)

494 Abbreviations: GEN, gentamicin; CRO, ceftriaxone; ETP, ertapenem; AZM,

495 azithromycin; MIC, minimum inhibitory concentration.

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