In vitro assessment of gentamicin and azithromycin-based combination therapy against Neisseria gonorrhoeae isolates in India

S. Sood1*, S. K. Agarwal1, R. Singh1, S. Gupta2 and V. K. Sharma2

Abstract

Purpose. The public health burden of infections caused by Neisseria gonorrhoeae is magnified due to high rates of resistance to traditional antimicrobials. The aim of this study was to evaluate the in vitro efficacy of an alternative dual therapy comprising gentamicin and azithromycin.

Methodology. The E-test method was used to determine the minimum inhibitory concentrations (MICs) of gentamicin and azithromycin individually prior to testing in combination using the cross or 90° angle formation method. A total of 70 clinical isolates of N. gonorrhoeae displaying varying ceftriaxone MICs along with 2 reference strains (WHO K and P) and 1 ceftriaxone-resistant QA isolate were examined. The fractional inhibitory concentration index (FICI) was calculated and the results were interpreted using the following criteria: synergy, FICI ≤0.5; indifference or additive, FICI >0.5 to ≤4.0; and antagonism, FICI >4.0.

Results. A total of 54 (77.1%) isolates displayed indifference, while 16 (22.9%) demonstrated synergy. When azithromycin was tested alone, the MICs ranged from 0.016 to 2 µg ml⁻¹. However, in combination with gentamicin, the mean MIC value of all isolates decreased from 0.275 µg ml⁻¹ to 0.090 µg ml⁻¹ (P=0.05). When gentamicin was tested alone, the MICs ranged from 0.25 to 8 µg ml⁻¹, with a mean MIC of 4.342 µg ml⁻¹, whereas in combination with azithromycin, it decreased significantly to 2.042 µg ml⁻¹ (P=0.04).

Conclusion. No antagonism was observed in this combination, suggesting that it could be a future treatment option as we prepare for a post-cephalosporin era. However, comprehensive in vivo evaluations are warranted and recommendations should be made based on clinical trials.

INTRODUCTION

The public health burden of infections caused by Neisseria gonorrhoeae is magnified due to high rates of resistance to traditional antimicrobials. As per the recent Centers for Diseases Control and Prevention (CDC) report, due to the serious consequences and significant risk associated with it, drug-resistant N. gonorrhoeae has been classified under the hazard level ‘urgent’ [1]. Due to alarming rates of resistance to classical antimicrobials agents such as penicillin, tetracycline and ciprofloxacin, extended-spectrum cephalosporins (ESCs) were considered to be the first-line monotherapy in the recent past [2]. Of the ESCs, injectable ceftriaxone and oral cefixime are the most prominent. However, the last decade has witnessed a trend of increasing minimum inhibitory concentrations (MICs) for N. gonorrhoeae for both agents globally and reports of the emergence of multi-drug resistant and extensively-drug resistant strains from some parts of the world [3–7]. This has threatened monotherapy for gonorrhoea and it is no longer recommended. Further, strains with decreased susceptibility (DS) to ceftriaxone also have high MICs for penicillin, tetracycline and quinolones, which rules out reintroducing these in the current era. Therefore, the CDC treatment guidelines now recommend dual antimicrobial therapy (ceftriaxone plus azithromycin) as a primary treatment option for uncomplicated gonococcal infections [8].
There are no new anti-gonococcal drugs on the horizon and the pharmaceutical industry is not investing in new molecules. Therefore, our search is restricted to the available stockpile with the hope of using the ‘old drugs’ with ‘new tricks’. There is renewed interest in aminoglycosides, particularly gentamicin, which has been used extensively in Malawi for over two decades in syndromic management of urethritis and has shown high clinical efficacy [9, 10]. Gentamicin exhibits concentration-dependent bactericidal activity and is economical, while the safety data for it, although several decades old, are encouraging. It also exhibits a post-antibiotic effect in which there is no or very little drug detectable in the blood, but there still seems to be an inhibition of bacterial regrowth. A recent study from India observed high in vitro efficacy of gentamicin against multidrug-resistant (MDR) strains and strains with DS to ESCs [11]. Gonococci that are resistant to cefalosporins are less likely to exhibit cross-resistance to gentamicin, as cefalosporins inhibit cell wall synthesis, whereas gentamicin disrupts the protein synthesis. Gentamicin is being considered as an alternative treatment option in dual therapy for gonorrhoea, along with other antimicrobials, particularly azithromycin. It was predicted that antibiotic synergistic activity may occur with this combination [12]. According to recent CDC guidelines, combination therapy comprising gentamicin and azithromycin is recommended in patients with cefalosporin allergy [8].

The potential advantage of combination antimicrobial therapy for gonococcal infections is the improvement of treatment effectiveness, possibly due to a synergistic effect afforded by the use of two agents. Further, it retards the emergence of resistance and provides co-treatment of other pathogens, e.g. Chlamydia trachomatis and Mycoplasma genitalium. Limited number of studies have analysed the in vitro efficacy of the combination of gentamicin and azithromycin in resource-limited settings such as ours [13]. Therefore, this study aimed to evaluate the in vitro efficacy of gentamicin and azithromycin in clinical isolates of N. gonorrhoeae in order to shed some light on the usefulness of this dual therapy regimen in the management of gonorrhoea in the post-cefalosporin era.

**METHODS**

**Bacterial isolates**

We at the All India Institute of Medical Sciences (AIIMS), New Delhi, have been conducting continuous surveillance of antimicrobial resistance (AMR) of [14] since 2007. We have also been participating in the EQAS programme of WHO, the Gonococcal Antimicrobial Surveillance Program (GASP), conducted by the Regional Reference Laboratories (RRL), New Delhi since 2005. All clinical isolates, WHO reference strains and QA strains have been stocked using glycerol broth at −70 °C in a deep freezer (Forma −86 °C ULT Freezer, model 991, Thermo Scientific) and also lyophilized using a suitable lyophilizer (Free Zone Triad Freeze Dry system, model 7400030, Labconco). We revived 70 isolates displaying varying cefalosporin MICs that had been obtained between 2013–2017 for the present investigation. However, all DS isolates, irrespective of the year of isolation, were included. These were revived on chocolate agar plates at 36 °C in an atmosphere containing 5 % CO₂. In addition, two N. gonorrhoeae reference strains, WHO K and P, and one ceftriazone-resistant QA isolate received as a part of an EQAS panel from WHO/GASP in 2015, were incorporated.

**Antimicrobial susceptibility testing**

Standard protocols were used for the identification and isolation of N. gonorrhoeae [15]. E-strips containing gentamicin (0.016–256 µg ml⁻¹) and azithromycin (0.016–256 µg ml⁻¹) were used to determine the MICs of each single antibiotic agent as per the manufacturer’s instructions (bioMérieux, France) prior to them being testing in combination. The MICs were read following incubation at 36 °C in 5 % CO₂ for 16–18 h. For azithromycin, isolates with an MIC <1 µg ml⁻¹ were categorized as susceptible and those with an MIC ≥1 µg ml⁻¹ as resistant. For gentamicin, the following breakpoints were used: susceptible (MIC ≤4 µg ml⁻¹), less susceptible (MIC 8–16 µg ml⁻¹) and resistant (MIC ≥32 µg ml⁻¹) [16]. All experiments were performed in duplicate.

**In vitro synergy testing**

The antimicrobial combination of gentamicin with azithromycin was investigated using the E-test MIC fixed ratio method. Synergy was tested by crossing pairs of E-test strips perpendicularly to each other with intersections at the points of their individual MICs using the cross or 90° angle formation method. The fractional inhibitory concentration (FIC) for each antimicrobial agent was calculated by using the results for the MICs of the antibiotics in combination divided by the MIC of the individual antibiotic. The fractional inhibitory concentration index (FICI) was determined by adding FIC<sub>gentamicin</sub> and FIC<sub>azithromycin</sub>. The FICI values were analysed with the following cut-off criteria: synergistic (≤0.5), additive or indifferent (>0.5 to≤4) and antagonistic (>4) effect [17].

**Statistical analysis**

All the isolates in antibiotic combination was calculated to determine mean value of MICs and geometric mean of FICIs. We stratified the results into strains with ceftriaxone MICs <16–18 h. For azithromycin, isolates with an MIC <1 µg ml⁻¹ were categorized as susceptible and those with an MIC ≥1 µg ml⁻¹ as resistant. For gentamicin, the following breakpoints were used: susceptible (MIC ≤4 µg ml⁻¹), less susceptible (MIC 8–16 µg ml⁻¹) and resistant (MIC ≥32 µg ml⁻¹) [16]. All experiments were performed in duplicate.

**RESULTS AND DISCUSSION**

The arrival of DS for ESCs worldwide has marked an end to monotherapy for gonorrhoea. In India, the rates of resistance to previously used antimicrobials are high and sustained [18]. We are currently relying on dual therapy as recommended by the National AIDS Control Organization (NACO) and its use is hypothesized to lessen the survivability of N. gonorrhoeae strains that have become resistant to one or more...
of the antibiotics. The combination therapy proposed for *N. gonorrhoeae* is based on a similar theory to the rationale for multidrug therapy for tuberculosis or malaria [19]. There are different methods for combination testing, such as time–kill curves, checkerboard techniques, agar dilution and E-test methods. The checkerboard method has been used in Japan to investigate the *in vitro* activities of β-lactams with azithromycin only [20]. Studies that have used agar dilution and E-test methods have demonstrated reasonable concordance between the two techniques [21, 22]. We do not have data on comparisons of these studies with time–kill curve studies or checkerboard methods of testing for synergy. However, as the E-test is more practical, we adopted it. Another advantage of the E-test is that it can be performed on isolates on an emerging basis and in our setting, where the number of isolates is small, the agar dilution assay is usually batched.

In the present study, 51 (72.9 %) isolates were susceptible to gentamicin. Previous studies from India, Mongolia and Brazil have also reported high rates of gentamicin susceptibility, i.e. 90.7%, 100 and 100%, respectively [11, 23, 24]. Further, we observed that 27.1 % of the gonococcal isolates were less susceptible to gentamicin. This is in contrast to the studies from Europe and Canada, where most (83%) of the isolates were less susceptible. This discrepancy may have been due to differences in the methodology used in these studies and to geographical differences in isolates [25, 26]. No resistance to gentamicin was reported in the present study, which is in line with some other reports [11, 23, 25, 26]. In our study, two isolates (2.9%) that were resistant to azithromycin were susceptible to gentamicin. In these strains, the mean MICs of azithromycin and gentamicin alone were 1.5 µg ml−1 and 4.0 µg ml−1, respectively, but when they were used in combination they decreased to 0.095 µg ml−1 and 0.690 µg ml−1, respectively. The combination of azithromycin and gentamicin in these isolates showed synergy and the observed geometric mean FICI value was 0.218. On the other hand, all of the strains isolates showed synergy and the observed geometric mean FICI value was 0.794. Previous studies from India, Japan and the Netherlands reported mean FICI values of 1.499, 0.83 and 1.75, respectively [13, 22, 28]. We also observed that there were no significant differences in the geometric mean FICI values between isolates that were fully susceptible to ceftriaxone and those exhibiting DS to ceftriaxone (Table 1). Although gentamicin–azithromycin was additive/indifferent against most clinical isolates, the absence of antagonism and the low prevalence of strains that are less susceptible to gentamicin

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<th>Table 1. E-test MICs (mean) and range of gentamicin and azithromycin alone and in combination</th>
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<td><strong>Mean MIC (range) (µg ml −1)</strong></td>
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<tr>
<td>Gentamicin-susceptible (MIC ≤4 µg ml −1)</td>
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<td>Gentamicin less susceptible (MIC 8–16 µg ml −1)</td>
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<td>Ceftriaxone-susceptible (MIC &lt;0.06 µg ml −1)</td>
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<td>Ceftriaxone DS (MIC 0.06–0.25 µg ml −1)</td>
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and resistant to azithromycin may indicate clinical utility and justify routine use of this combination therapy in this region. The gonococcal population in our region is highly diverse and we have previously reported that the most common sequence type was ST6058 (n=15) [14].

A clinical trial in the United States has already validated the utility of the combination in men and women who presented with suspicion of urethritis or cervicitis [30]. However, this study did not determine the efficacy of the individual antibiotics or combination of these antibiotics for extragenital isolates. Overall, the combination of gentamicin and azithromycin had a geometric mean FICI value of 0.794 in our study and an enhancement of azithromycin activity in the presence of gentamicin was observed. When azithromycin was tested alone, the MICs ranged from 0.016 to 2 µg ml−1. However, in combination with gentamicin, the mean MIC value of all isolates decreased from 0.275 µg ml−1 to 0.090 µg ml−1 (P<0.05). Similarly, when gentamicin was tested alone, the MICs ranged from 0.25 to 8 µg ml−1, with a mean of 4.343 µg ml−1, whereas in combination with azithromycin it decreased significantly to 2.042 µg ml−1 (P=0.04). As gentamicin and azithromycin have a similar target, but different binding sites (azithromycin binds to the 50S ribosomal subunit and gentamicin to the 30S subunit), and consequently both inhibit protein synthesis, it was predicted that antimicrobial synergistic activity may occur. Further, the use of a single intramuscular dose of gentamicin paves the way for outpatient management and reduces the risk of vestibular and renal toxicity. However, the azithromycin dosage recommended in this combination, i.e. 2 gram, has raised concerns regarding adverse events related to the gastro-intestinal tract.

Our results help to provide a baseline for larger scale susceptibility and combinational studies to find agents that are active against antimicrobial-resistant *N. gonorrhoeae*. However, all these potential treatment regimens require comprehensive *in vivo* evaluations and recommendations that are made based on clinical trials, not only on *in vitro* results. The present study has a few limitations, i.e. small sample size and having been conducted at a single site, and more studies worldwide are required to advance our knowledge concerning gentamicin and azithromycin efficacy at the local, national and regional levels. Further, we have not undertaken the *in vitro* evaluation of the in-use dual therapy in the circulating gonococcal isolates.

To conclude, there is an urgent need to identify possible future treatment options in view of the small but increasing proportion of isolates with DS to ceftriaxone globally. Studies on a larger panel of isolates, including those with resistance to ceftriaxone, are warranted. No antagonism was observed in this combination, suggesting that it could be a future treatment option as we prepare for an era of increasing antimicrobial resistance in *N. gonorrhoeae*, a problem that is somewhat more evident in Men who have Sex with men (MSM). Unfortunately, studies comparing the antimicrobial susceptibilities of pharyngeal, rectal and urethral *N. gonorrhoeae* isolates are lacking. It will also be important to investigate the effectiveness of the gentamicin–azithromycin combination in the eradication of extragenital gonococcal infections.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**


