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Determination of in vitro synergy for dual antimicrobial therapy against resistant *Neisseria gonorrhoeae* using Etest and agar dilution

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ABSTRACT

In response to antimicrobial resistance of *Neisseria gonorrhoeae* to last-resort extended-spectrum cephalosporins, combination therapy of azithromycin + ceftriaxone is now recommended. Dual therapy can be effective to treat monoresistant strains as well as multidrug-resistant strains, preferably employing the effect of in vitro synergy. As reports on in vitro synergy of azithromycin + ceftriaxone in *N. gonorrhoeae* are conflicting, in this study an evaluation of this combination was performed using a cross-wise Etest method and agar dilution. Synergy was defined as a fractional inhibitory concentration index (FICI) of ≤0.5. To identify other dual treatment options for gonorrhoea, in vitro synergy was evaluated for 65 dual antimicrobial combinations using Etest. Azithromycin, cefixime, ceftriaxone, colistin, ertapenem, fosfomycin, gentamicin, minocycline, moxifloxacin, rifampicin, spectinomycin and tigecycline were screened for synergy in all possible combinations. No synergy or antagonism was found for any of the 65 combinations. The geometric mean FICI ranged from 0.82 to 2.00. The mean FICI of azithromycin + ceftriaxone was 1.18 (Etest) and 0.55 (agar dilution). The difference between both methods did not result in a difference in interpretation of synergy. Ceftriaxone-resistant strain F89 was tested in all combinations and no synergy was found for any of them. Most importantly, the ceftriaxone minimum inhibitory concentration of F89 was not decreased below the breakpoint with any concentration of azithromycin.

1. Introduction

Gonorrhoea is the most prevalent bacterial sexually transmitted infection worldwide [1]. If left untreated it can cause severe illness such as pelvic inflammatory disease or infertility and it increases the transmission of human immunodeficiency virus (HIV). However, the causative bacterium *Neisseria gonorrhoeae* has now become resistant to the last-resort monotherapy of extended-spectrum cephalosporins [2]. With few new antimicrobial drugs in the pipeline, this renders gonorrhoea potentially untreatable in the future.

Therefore, the US Centers for Disease Control and Prevention (CDC) as well as UK and European treatment guidelines now recommend dual therapy of azithromycin and ceftriaxone [3–5]. Dual therapy can be effective even if the organism is resistant to one of the drugs, and in addition it can relieve the selection pressure on an organism to become resistant. Combination therapy with azithromycin has the advantage to treat possible co-infection with *Chlamydia trachomatis*. Another reason for dual therapy is synergy, where the combined effect of two drugs is greater than the mere sum of the effects of both drugs alone [6].

In vitro synergy has been demonstrated with different antimicrobials in various Gram-negative bacteria [7,8]. In *N. gonorrhoeae*, this has been described for azithromycin + cefixime [9]. However, more recently Pereira et al. and Barbee et al. did not find synergy for azithromycin + cefixime or ceftriaxone [10,11]. If synergy in *N. gonorrhoeae* can be demonstrated, multidrug-resistant strains could be treated with earlier empirically proven effective treatment options. Therefore, the aim of this study was to determine in vitro synergy in *N. gonorrhoeae* for azithromycin + ceftriaxone as well as to evaluate synergy in other possible dual antimicrobial combinations.

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2. Materials and methods

2.1. Synergy testing for azithromycin + ceftriaxone

2.1.1. Bacterial isolates

This study included 12 clinical *N. gonorrhoeae* isolates, reference strains WHO K and L and ceftriaxone-resistant strain F89 (isolated in France in 2010) [2,12]. These 15 isolates were selected for their highest minimum inhibitory concentration (MIC) of both azithromycin (0.047–8.0 mg/L) and ceftriaxone (0.008–1.0 mg/L) determined by Etest as described by the manufacturer (bioMérieux SA, Marcy-l'Étoile, France).

2.1.2. Synergy testing, definition and interpretation

To determine synergy for azithromycin + ceftriaxone, two previously described methods were used, one using double Etests (positioned cross-wise at a 90° angle) and one using agar dilution [13–15]. In the latter method, azithromycin (0.032–32 mg/L in 11 two-fold dilutions) and ceftriaxone (0.008–4.0 mg/L in 10 two-fold dilutions) were added to GC agar (prepared in-house at Onze Lieve Vrouwe Gasthuis General Hospital, Amsterdam, The Netherlands). Then, 10 μL of 0.5 McFarland standard prepared in phosphate-buffered saline each of 15 *N. gonorrhoeae* isolates was inoculated onto the GC agar plates (120 × 120 mm).

With either method, MICs were determined for both antimicrobials alone (MIC_A^{alone} and MIC_B^{alone}) and in combination with the other (MIC_A^{combi} and MIC_B^{combi}). MICs were read following incubation at 37 °C in 5% CO_2 for 16–18 h (Etest) or 24 h (agar dilution). All experiments were performed in duplicate.

The fractional inhibitory concentration index (FICI) was calculated using the following formula: $FICI = (MIC_A^{combi}/MIC_A^{alone}) + (MIC_B^{combi}/MIC_B^{alone})$. A FICI of ≤0.5 was defined as synergy, a FICI of >0.5 but ≤4.0 was defined as no interaction, and a FICI of >4.0 was defined as antagonism [6].

2.2. Synergy testing for 65 antimicrobial dual combinations

2.2.1. Antimicrobial combinations

Based on in vitro synergy described in other Gram-negative bacteria, 12 antimicrobial agents were selected, namely azithromycin, cefixime, ceftriaxone, colistin, ertapenem, fosfomycin, gentamicin, minocycline, moxifloxacin, rifampicin, spectinomycin and tigecycline [7–9]. With the exception of cefixime + ceftriaxone, all possible dual combinations were tested ($n = 65$).

First, these 65 combinations were screened for synergy using the double Etest method [13]. This screening was performed on four isolates per combination and was used as a crude selection method. If the FICI was <1.0 in at least 3 of the 4 tested isolates for a specific combination, that combination was re-tested using 11 isolates.

Combinations with cefixime were selected over combinations with ceftriaxone, as oral administration of cefixime has practical advantages, especially in general practitioner settings. Azithromycin + cefixime was included in any case; azithromycin + ceftriaxone was already tested as described in Section 2.1. All experiments were performed in duplicate. If synergy was inconsistent between both experiments, it was performed a third time.

2.2.2. Bacterial isolates

For each antimicrobial combination, four *N. gonorrhoeae* isolates were selected from a panel consisting of WHO strains K, L, M, O, P and G, control strain ATCC 49226, strain F89 and 24 clinical isolates. For each combination, ceftriaxone-resistant strain F89 was selected and the other three isolates were selected based on the highest MICs for that specific combination.

The panel of 11 isolates used for re-testing was identical for all combinations and included WHO strains K and L, strain F89 and 8 of the clinical isolates described in Section 2.1.1.

2.3. Statistical analysis

MICs and FICIs were calculated as geometric means of all isolates and duplicate experiments in each antimicrobial combination. The difference in FICI between Etest and agar dilution was defined using a Wilcoxon signed-rank test. A P-value of <0.05 was considered statistically significant. Analyses were performed using SPSS Statistics for Windows v.21.0 (IBM Corp., Armonk, NY).

3. Results

3.1. Synergy of azithromycin + ceftriaxone

When testing azithromycin + ceftriaxone using Etest, the geometric mean MIC decreased for azithromycin from 0.27 mg/L to 0.15 mg/L and for ceftriaxone from 0.062 mg/L to 0.037 mg/L. The mean FICI of all isolates was 1.18 (range 0.58–2.00), indicating no interaction. No individual isolates showed a FICI ≤ 0.5.

When using agar dilution, the mean MIC (range) decreased for azithromycin from 0.56 mg/L (0.125–16.0 mg/L) to 0.092 mg/L (0.032–0.5 mg/L) and for ceftriaxone from 0.082 mg/L (0.016–2.0 mg/L) to 0.025 mg/L (0.008–1.0 mg/L). The mean FICI was 0.55 (range 0.16–0.76), indicating no interaction. Four of the 15 individual isolates showed a FICI ≤ 0.5: three isolates with a FICI between 0.44 and 0.50, and one isolate with a FICI of 0.16.

When comparing the mean FICI of the Etest and agar dilution methods, a significant difference ($P = 0.001$) was found, with agar dilution resulting in lower FICIs than the Etest method.

3.2. Synergy of 65 dual antimicrobial combinations

Results of the screening of 65 dual combinations showed no synergy for any combination; the mean FICI ranged from 0.82 to 2.00 (Table 1). Five combinations showed a FICI < 1.0 in three of the four tested isolates: cefixime + ertapenem; cefixime + gentamicin; cefixime + moxifloxacin; ceftriaxone + ertapenem; and ertapenem + fosfomycin.

When these combinations, plus azithromycin + cefixime and azithromycin + ceftriaxone, but without ceftriaxone + ertapenem, were tested on 11 isolates, mean FICIs were: azithromycin + cefixime, 0.83; cefixime + ertapenem, 0.77; cefixime + gentamicin, 0.97; cefixime + moxifloxacin, 1.13; and ertapenem + fosfomycin, 0.86; all indicating no interaction (Table 2).

3.3. Synergy in ceftriaxone-resistant strain F89

Ceftriaxone-resistant strain F89 was used in all experiments in this study. None of the 65 combinations tested showed synergy with this isolate. When using Etest for the combinations as described in Table 2, this resulted in the following mean MICs of antimicrobials alone: azithromycin, 0.22 mg/L; cefixime, 1.73 mg/L; ceftriaxone, 0.87 mg/L; ertapenem, 0.004 mg/L; fosfomycin, 16.0 mg/L; gentamicin, 2.0 mg/L; and moxifloxacin, 1.50 mg/L. The mean FICIs were: azithromycin + cefixime, 1.00; azithromycin + ceftriaxone, 1.20; cefixime + ertapenem, 0.69; cefixime + gentamicin, 1.46; cefixime + moxifloxacin, 1.37; and ertapenem + fosfomycin, 0.69; all indicating no interaction.

Testing of azithromycin + ceftriaxone using agar dilution resulted in a mean MIC alone of 0.5 mg/L and 2.0 mg/L, respectively, and a FICI of 0.56, indicating no synergy. Adding azithromycin in any dosage did not decrease the ceftriaxone MIC for strain F89 below