



## 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  $\leq 0.5$ . 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.

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### 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<sub>2</sub> 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  $FICI \leq 0.5$ .

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  $FICI \leq 0.5$ : 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

**Table 1**  
Results of in vitro synergy experiments of 65 antimicrobial combinations in four *Neisseria gonorrhoeae* isolates using the Etest method.

Antimicrobial A	Geometric mean FICI <sup>a</sup> (no. of strains with FICI < 1.0)										
	Antimicrobial B										
	Cefixime	Ceftriaxone	Colistin	Ertapenem	Fosfomycin	Gentamicin	Minocycline	Moxifloxacin	Rifampicin	Spectinomycin	Tigecycline
Azithromycin	0.93 (2)	1.59 (1)	1.54 (1)	1.42 (0)	1.75 (0)	1.75 (0)	1.17 (1)	1.31 (1)	1.51 (0)	1.55 (1)	1.29 (0)
Cefixime		– <sup>b</sup>	1.04 (2)	0.82 (3)	1.02 (1)	0.87 (3)	1.10 (0)	0.93 (3)	1.44 (0)	1.04 (1)	1.10 (1)
Ceftriaxone			1.23 (1)	0.91 (3)	1.01 (2)	1.30 (1)	1.58 (0)	1.47 (0)	1.78 (0)	1.33 (0)	2.00 (0)
Colistin				0.98 (2)	1.10 (0)	1.27 (0)	1.08 (2)	1.07 (1)	1.45 (0)	1.65 (0)	1.09 (1)
Ertapenem					0.90 (3)	1.18 (1)	1.66 (0)	0.96 (2)	1.70 (0)	1.48 (0)	1.50 (0)
Fosfomycin						1.29 (1)	1.58 (0)	1.35 (0)	1.38 (1)	1.28 (1)	1.80 (0)
Gentamicin							1.06 (0)	1.20 (0)	1.49 (0)	1.60 (0)	1.37 (1)
Minocycline								1.20 (1)	1.19 (1)	1.37 (0)	1.08 (2)
Moxifloxacin									1.54 (0)	1.28 (0)	1.08 (0)
Rifampicin										1.62 (0)	1.53 (1)
Spectinomycin											1.21 (1)

FICI, fractional inhibitory concentration index.

<sup>a</sup> Geometric mean FICI of four varying *N. gonorrhoeae* isolates per combination, each combination containing ceftriaxone-resistant strain F89.<sup>b</sup> Combination was not tested.**Table 2**  
Geometric mean MIC and FICI of six antimicrobial combinations against 11 *Neisseria gonorrhoeae* isolates using the Etest method.

Antimicrobial combination	Mean MIC (range) (mg/L)				Mean FICI (range)
Azithromycin + cefixime	Azithromycin Alone	0.26 (0.094–8.0)	Combination	Cefixime Alone	0.83 (0.49–1.41)
	Azithromycin + cefixime	0.106 (0.023–3.0)		Cefixime + cefixime	
Azithromycin + ceftriaxone <sup>a</sup>	Azithromycin Alone	0.27 (0.047–8.0)	Combination	Ceftriaxone Alone	1.18 (0.58–2.0)
	Azithromycin + ceftriaxone	0.153 (0.016–4.0)		Ceftriaxone + ceftriaxone	
Cefixime + ertapenem	Cefixime Alone	0.11 (0.016–2.0)	Combination	Ertapenem Alone	0.77 (0.49–1.27)
	Cefixime + ertapenem	0.047 (0.012–0.75)		Ertapenem + ertapenem	
Cefixime + gentamicin	Cefixime Alone	0.11 (0.016–2.0)	Combination	Gentamicin Alone	0.97 (0.51–2.0)
	Cefixime + gentamicin	0.052 (0.016–1.5)		Gentamicin + gentamicin	
Cefixime + moxifloxacin	Cefixime Alone	0.11 (0.016–2.0)	Combination	Moxifloxacin Alone	1.13 (0.66–1.43)
	Cefixime + moxifloxacin	0.065 (0.012–1.5)		Moxifloxacin + moxifloxacin	
Ertapenem + fosfomycin	Ertapenem Alone	0.036 (0.004–0.125)	Combination	Fosfomycin Alone	0.86 (0.51–1.45)
	Ertapenem + fosfomycin	0.014 (0.001–0.064)		Fosfomycin + fosfomycin	

MIC, minimum inhibitory concentration; FICI, fractional inhibitory concentration index.

<sup>a</sup> For this combination, 15 *N. gonorrhoeae* isolates were used.

1.0 mg/L. The azithromycin MIC only decreased after adding ceftriaxone in concentrations of  $\geq 1.0$  mg/L, which was only 1 dilution below the MIC for ceftriaxone alone.

#### 4. Discussion

In this study, a lack of in vitro synergy was demonstrated for any of 65 dual antimicrobial combinations against *N. gonorrhoeae*. Moreover, no synergy was observed in ceftriaxone-resistant strain F89 with any of the tested antimicrobial combinations. The results of this study do not support the results of Furuya et al., who reported synergy for azithromycin + cefixime [9]. The lack of synergy found in novel combinations of a third-generation cephalosporin with gentamicin, rifampicin or fosfomycin supports recent findings by Barbee et al. [11]. The present results suggest that of the antimicrobial combinations using azithromycin, cefixime, ceftriaxone, colistin, ertapenem, fosfomycin, gentamicin, minocycline, moxifloxacin, rifampicin, spectinomycin and tigecycline, none are promising candidates for gonorrhoea dual therapy, if such therapy was based only on in vitro synergy. However, no antagonism was found for any of these combinations, indicating no objections to clinical studies of these combinations as dual therapy for gonorrhoea.

In addition to the lack of synergy with novel combinations, no synergy was also found for the recommended dual therapy

of azithromycin + ceftriaxone using either Etest or agar dilution. Only when using agar dilution were FICIs of  $\leq 0.5$  found in four individual isolates; one was 0.16 and the other three were  $\geq 0.44$ . However, this could be due to chance, and given the lack of synergy in the majority of isolates as well as the results of experiments using Etest, we consider the results of these individual isolates not convincing evidence for synergy. Overall, the current results support recent findings by Pereira et al. and Barbee et al. that no synergy for this combination can be demonstrated [10,11].

Measuring synergy has several challenges. First, evaluation of in vitro synergy depends on the method used. Different methods exist for using double Etests, yet it remains unclear which yields the most reliable results [7,8,11,13]. When using agar dilution, reproducibility can be a problem, something we however did not see in the current experiments [6].

Second, variation exists in how to calculate and interpret synergy. We used the most widely described FICI. This method was also used by other studies on synergy in *N. gonorrhoeae*, allowing comparison of results [9–11]. To avoid suggestive effects for FICIs of  $>0.5$  and  $\leq 1.0$ , we chose to define synergy more strictly, so all combinations within this range were defined as ‘no interaction’ [6].

There are some limitations to the current study. When testing the 65 dual combinations, isolates were selected based on highest

MICs for both antimicrobials, which resulted in a small panel of isolates. Whilst this experiment was conducted as a mere screening for synergy, this could have led to insufficient power to detect synergy. However, no synergy was found when re-testing combinations with FICIs <1.0 using more isolates. Ideal would be a larger panel with isolates showing high-level resistance to both drugs in a combination. However, only a few strains with overt resistance to ceftriaxone have been described so far, and no strains highly resistant to both azithromycin and ceftriaxone have been isolated.

Both Etest and agar dilution were used to test azithromycin+ceftriaxone. As in previous studies, a significant difference was found when comparing FICIs of both methods [7,8,11]. The most likely explanation for the higher FICIs when using Etest is the fact that lateral diffusion gradients of this method are not taken into account. Due to this gradient effect, the concentration of antimicrobial A can be lower than the MIC level at the location where MIC<sub>B</sub><sup>combi</sup> is read, leading to an overestimate of MIC<sub>B</sub><sup>combi</sup> and possibly an overestimate of the FICI. In addition, by using Etest intermediate MICs can also be determined, whilst in agar dilution only full two-fold dilutions are used. However, the difference between methods did not lead to a difference in interpretation of synergy.

In conclusion, no synergy was found for 65 dual antimicrobial combinations as well as no synergy for azithromycin + ceftriaxone, suggesting that for these combinations synergy might not play a significant role in the treatment of gonorrhoea. Therefore, research aimed at new treatment options against emerging resistant strains should move in other directions. One alternative could be to focus more on the clinical outcome of dual therapy, given that in vitro synergy is subject to uncertain testing methods and does not always correspond to a clinical effect, whilst dual therapy could be helpful to treat isolates resistant to one of the two antimicrobials and possibly decrease selection pressure. In addition, new treatment options are urgently needed and should be tested for the elimination of *N. gonorrhoeae* both in vitro and in clinical trials to sustain the treatment of gonorrhoea in the future.

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