# Comparative evaluation of synergy of combinations of β-lactams with fluoroquinolones or a macrolide in *Streptococcus pneumoniae*

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**Objectives:** *Streptococcus pneumoniae* has shown a great ability to develop efficacious mechanisms of resistance to the main drugs for the treatment of pneumonia, such as β-lactams, macrolides and fluoroquinolones. The present study aimed to compare the antipneumococcal activity of combinations of respiratory fluoroquinolones with cephalosporins (either parenteral or oral) or protected penicillin versus the standard combinations (i.e. a macrolide with a protected penicillin or cephalosporin) against 100 isolates with different susceptibilities to macrolides and/or penicillin.

**Methods:** Chequerboard assays for all isolates and time–kill curves for nine isolates with different patterns of susceptibility were performed. Synergy between antibiotics at serum peak concentrations was also determined.

**Results:** The combination of levofloxacin with ceftriaxone produced the highest rate of synergy (54/100), mainly against macrolide-resistant strains (22/30). Antagonism was not observed for any tested combination apart from clarithromycin with amoxicillin/clavulanic acid (22/100 isolates). Although the killing activities of all antibiotics improved when they were tested in combination, synergy was observed only for some combinations after 12 and/or 24 h. Serum concentrations were effective in inhibiting the growth of the tested strains.

**Conclusions:** Combinations of levofloxacin with parenteral cephalosporins were the most active among all the tested combinations, while antagonism occurred when clarithromycin and amoxicillin/clavulanic acid were tested.

**Keywords:** pneumonia, respiratory tract infections, levofloxacin, moxifloxacin

## Introduction

Lower respiratory tract infections (LRTIs), particularly community-acquired pneumonia (CAP), represent the major cause of death among infectious diseases.[1,2] Thus, there is a pressing need to monitor the antimicrobial susceptibilities of the causative pathogens in order to provide proper treatment to patients.[3]

In adults, the most frequent pathogen isolated is *Streptococcus pneumoniae* (25%–60%), thus it is mandatory to treat empirically with an antibiotic with high activity against this pathogen.[2,3] However, over the years, *S. pneumoniae* has shown a great ability to develop efficacious mechanisms of resistance to the main drugs for the treatment of LRTIs, such as β-lactams, macrolides and fluoroquinolones.[4]

Resistance to penicillin is considered extremely variable in different geographical areas. In Italy, strains classified as resistant, according to CLSI (formerly NCCLS) criteria (MIC > 4 mg/L), are rather infrequent.[5,6] In contrast, the wide dissemination of resistance to macrolides in many countries, particularly in the Mediterranean and Asiatic regions, is quite alarming, reaching 30%–40% in our geographical area. With regard to fluoroquinolones, the development of resistance in *S. pneumoniae* represents a low-impact phenomenon worldwide.[7]

Data from retrospective analyses of patients with bacteraemic pneumococcal pneumonia suggest that combination antibiotic therapy is associated with reduced mortality as compared with that seen among those who receive monotherapy only.[8,9] Combinations of antibiotics are usually adopted with the double aim of widening coverage and increasing antibacterial activity, limiting the occurrence and spread of resistant strains.[10] A highly bactericidal antibiotic combination with excellent tissue penetration, which does not lead to the emergence of resistance, would be a major advantage in the treatment of pneumococcal diseases, particularly for the most severe forms.

Fluoroquinolones plus β-lactams are now recommended as an alternative option to macrolides plus β-lactams in the treatment of severe pneumonia by the latest international guidelines [Infectious Diseases Society of America (IDSA)/American Thoracic Society (ATS), 2007].[11] With respect to the standard combinations (i.e. macrolides plus β-lactams) the use of fluoroquinolones plus β-lactams may be synergistic against pneumococci.

However, these recommendations are still not strongly supported either by *in vitro* studies or by clinical prospective comparisons between different combinations in the therapy of severe CAP.

Among respiratory fluoroquinolones, levofloxacin has a double route of administration (oral and intravenous) and a good safety profile.[12] Thus, in cases of severe CAP (i.e. patients admitted to intensive care units or with very complicated infections), only levofloxacin is recommended in combination with a β-lactam (a third-generation cephalosporin or a carbapenem) as an alternative to standard combinations according to the Italian guidelines.[12] The use of moxifloxacin in CAP is recommended only when the other antibacterials commonly used for initial treatment of CAP are considered inappropriate.[12] Thus, in order to widen the knowledge on the potential use of levofloxacin in combination therapy with β-lactams, it would be interesting to evaluate its antibacterial activity in combination with parenteral and/or oral β-lactams.

The present study aims to compare the antipneumococcal activity of combinations of respiratory fluoroquinolones with cephalosporins (either parenteral or oral) or protected penicillin versus the standard combinations (i.e. a macrolide with a protected penicillin or cephalosporin).

## Materials and methods

### Microorganisms

*S. pneumoniae* strains isolated from respiratory infections in patients attending the IRCCS Galeazzi Hospital (Milan, Italy) and nursing home residences in the north area of Milan were included in the study. According to their patterns of resistance, they were divided into fully susceptible ($n=50$), macrolide resistant ($n=30$) and penicillin non-susceptible with MIC $\geq 2$ mg/L ($n=20$). Only one isolate per patient was considered, in order to avoid duplicates.

All isolates were stored in brain heart infusion broth containing 10% (w/v) glycerol at $-80°C$ until use.

### Antibiotics

Levofloxacin, moxifloxacin, cefotaxime, clarithromycin, cefpodoxime, ceftriaxone and amoxicillin/clavulanic acid as powders of stated potency were used to prepare stock solutions.

The following combinations were evaluated against 100 strains of *S. pneumoniae* with different patterns of susceptibility by the chequerboard assay and time–kill curves. Fluoroquinolone plus β-lactam combinations: levofloxacin and ceftriaxone; levofloxacin and cefotaxime; levofloxacin and amoxicillin/clavulanic acid; levofloxacin and cefpodoxime; moxifloxacin and ceftriaxone; moxifloxacin and cefotaxime. Standard combinations: clarithromycin and amoxicillin/clavulanic acid; clarithromycin and ceftriaxone.

Besides determining multiples and sub-multiples of MICs to evaluate the activity of the studied combinations, peak serum concentrations of each antibiotic, chosen from the available literature were assessed: 5.29 mg/L levofloxacin;[13] 3.22 mg/L moxifloxacin;[14] 209 mg/L cefotaxime;[15] 11.23 mg/L amoxicillin/clavulanic acid;[16] 2 mg/L clarithromycin;[17] 2.18 mg/L cefpodoxime;[18] and 260 mg/L ceftriaxone.[19]

### Determination of MICs

MICs were determined by means of the microdilution broth method (microwell method) in accordance with the CLSI criteria.[5,20] Briefly, serial 2-fold dilutions of a starting antibiotic concentration were inoculated into wells of a microtitre plate containing cation-adjusted Mueller–Hinton broth supplemented with 5% lysed horse blood, so that each well contained $\sim 5 \times 10^5$ cfu/mL. The MIC was defined as the lowest antibiotic concentration able to inhibit visible bacterial growth after 18–20 h of incubation in ambient air at 37°C.

### Evaluation of synergy

#### Chequerboard assay

For each combination, a synergy test was performed in a 96-well microtitre plate containing two antimicrobial agents in 2-fold dilutions ($2 \times$ MIC, $1 \times$ MIC, $1/2 \times$ MIC, $1/4 \times$ MIC and $1/8 \times$ MIC) dispensed in a chequerboard fashion on the day of the assay. Suspensions with turbidities equivalent to that of a 0.5 McFarland standard were prepared to yield final inocula of $\sim 3 – 5 \times 10^5$ cfu/mL. MICs were read after overnight incubation.

Each test was performed in duplicate at the stated antimicrobial concentration. Growth and sterility controls were included in each plate.

For the first clear well in each row of the microtitre plate containing both antimicrobial agents, the fractional inhibitory concentration (FIC) was calculated and the sum of both FICs in each well was used to classify the effects of combinations of antimicrobial agents as: synergistic, for FIC indexes $\leq 0.5$; no interaction, for FIC indexes $>0.5–4$; and antagonistic, for FIC indexes $>4$.[21]

#### Time–kill curves

An inoculum of each strain was prepared from a 24 h culture on blood agar plates, and then adjusted to a turbidity equivalent to that of a 0.5 McFarland standard ($\sim 1 \times 10^8$ cfu/mL) in sterile saline. It was subsequently diluted to a final cell concentration of $\sim 1 – 3 \times 10^6$ cfu/mL, which was confirmed by colony counts in agar plates, by addition of Mueller–Hinton broth supplemented with lysed horse blood containing no antibiotic (growth control). Each antibiotic was tested alone and the same amounts of antibiotics in combination at the stated concentrations.

Bacterial counts were performed at 0, 3, 6, 12 and 24 h of incubation at 37°C by plating aliquots of 10 and 100 μL after dilution in sterile saline onto Columbia blood agar plates.

Synergy was defined as a 2 $\log_{10}$ decrease in colony count when the combinations were compared with the bacterial count obtained at the same timepoint with the most active single agent of the combination. Indifference was defined as a $<10$-fold change in colony count at each timepoint by the combination compared with that by the most active agent. Antagonism was defined as a $>100$-fold increase in colony count at each timepoint by the combination compared with that by the most active drug alone.[22]

## Results

### Antimicrobial activities of single drugs

MIC values were determined in order to classify each strain according to its susceptibility pattern: susceptible; penicillin non-susceptible; and macrolide resistant. Results are reported

in Table 1. Penicillin-non-susceptible and macrolide-resistant strains showed higher MIC values of cephalosporins than penicillin- and macrolide-susceptible strains. Penicillin resistance was associated with macrolide resistance in 15 strains.

### Evaluation of synergy

#### Chequerboard assay

Results are shown in Figure 1. The combination of levofloxacin with ceftriaxone produced the highest rate of synergy (54/100), mainly against macrolide-resistant strains (22/30). Levofloxacin and cefotaxime yielded synergy in 50% of the strains: 24/50 fully susceptible; 16/30 macrolide resistant; and 10/20 penicillin non-susceptible. Levofloxacin and cefpodoxime showed synergy against 14/100 strains: 5/50 susceptible; 7/30 macrolide resistant; and 2/20 penicillin non-susceptible. Antagonism was not observed for any tested combination. Moxifloxacin and ceftriaxone showed synergy against 41/100 strains: 20/50 susceptible; 13/30 macrolide resistant; and 8/20 penicillin non-susceptible. Synergy between moxifloxacin and cefotaxime was observed against 16/50 susceptible strains, 12/30 macrolide-resistant strains and 11/20 penicillin-non-susceptible strains.

With regard to standard combinations, clarithromycin and ceftriaxone yielded synergy against 19/100 strains: 10/50 fully susceptible; 7/30 macrolide resistant; and 2/20 penicillin non-susceptible. Synergy between clarithromycin and amoxicillin/clavulanic acid was observed against only 10/100 strains (4/50 susceptible, 3/30 macrolide resistant and 3/20 penicillin non-susceptible). Moreover, this combination was antagonistic against 22/100 strains. Antagonism was not observed for any other combination.

Thus, when pneumococcal strains were stratified according to their susceptibility profiles, combinations of levofloxacin with cefotaxime were the most active against fully susceptible strains, while levofloxacin with ceftriaxone gave the highest degree of synergy against macrolide-resistant *S. pneumoniae*. Against penicillin-non-susceptible strains the activity of the combination of levofloxacin and ceftriaxone was comparable to that of moxifloxacin plus cefotaxime and higher than all other combinations.

All the combinations were effective in inhibiting growth of the tested *S. pneumoniae* strains at concentrations equal to half the corresponding serum $C_{max}$.

#### Time–kill curves

Three strains for each group were chosen for time–kill assays. Results are summarized in Table 2, where interactions of each combination are reported. Although the killing activities of all antibiotics improved when they were tested in combination, synergy was observed only after 12 and/or 24 h. However, differently from the chequerboard assay, synergy was mainly observed against resistant strains rather than against susceptible strains.

Clarithromycin plus amoxicillin/clavulanic acid failed to result in synergy against all the tested groups and, in some cases, interaction between the compounds yielded antagonistic activity. In contrast, no antagonism was observed for combinations of fluoroquinolones with cephalosporins. Combinations of levofloxacin and moxifloxacin with ceftriaxone or cefotaxime and of levofloxacin plus cefpodoxime showed synergy when subinhibitory concentrations were tested, although they did not produce bactericidal effects in all cases. Moxifloxacin plus cefotaxime and levofloxacin plus amoxicillin/clavulanic acid showed indifference against fully susceptible strains. Combination of clarithromycin and ceftriaxone yielded synergy only against penicillin-non-susceptible strains. Levofloxacin plus cefotaxime showed synergy against all types of *S. pneumoniae*.

The antimicrobial activity of combinations with fluoroquinolones at $C_{max}$ was notably enhanced when compared with that of single agents, although failing to result in synergy. In particular, more rapid killing was observed after 6 h by the combinations of levofloxacin and moxifloxacin with cefotaxime, ceftriaxone and cefpodoxime against penicillin-non-susceptible strains and susceptible pneumococci. A similar trend was observed for ceftriaxone in association with moxifloxacin and, to a lesser extent, with clarithromycin. In contrast, the combination of clarithromycin with amoxicillin/clavulanic acid did not improve killing, and, in some cases, reduced it, resulting in antagonistic activity (data not shown).

## Discussion

Fluoroquinolones plus β-lactams are now recommended as an alternative option to macrolides plus β-lactams in the treatment of severe pneumonia according to IDSA/ATS guidelines.[11] In some *in vitro* studies, combinations of fluoroquinolones with β-lactams showed synergy against pneumococci, and a wider spectrum of activity when compared with the standard combinations (i.e. macrolides plus β-lactams).[12] However, these recommendations are still not supported by *in vitro* studies or by clinical prospective comparisons between different combinations.

It is possible to hypothesize a grading of efficacy for the therapy of severe CAP as follows: a β-lactam plus a macrolide is superior to monotherapy with a β-lactam, but comparable to monotherapy with a quinolone. Combination of a quinolone plus a β-lactam could be superior to the other options, with consequent indication for the most severe cases.

**Table 1.** Susceptibility of *S. pneumoniae* to the tested antibiotics

| | MIC range (mg/L) | | | | | | |
|---|---|---|---|---|---|---|---|
| | LVX | MXF | CLR | CRO | CTX | AMC | CPD |
| Susceptible ($n=50$) | 0.06–1 | 0.03–0.25 | 0.008–0.25 | 0.008–0.125 | 0.008–0.125 | 0.008–0.06 | 0.016–1 |
| Macrolide resistant ($n=30$) | 0.5–2 | 0.06–0.5 | 2–512 | 0.016–4 | 0.016–4 | 0.016–8 | 0.125–8 |
| Penicillin non-susceptible ($n=20$) | 0.5–2 | 0.06–0.125 | 0.03–512 | 0.016–4 | 0.016–4 | 2–16 | 4–16 |

LVX, levofloxacin; MXF, moxifloxacin; CLR, clarithromycin; CRO, ceftriaxone; CTX, cefotaxime; AMC, amoxicillin/clavulanic acid; CPD, cefpodoxime.
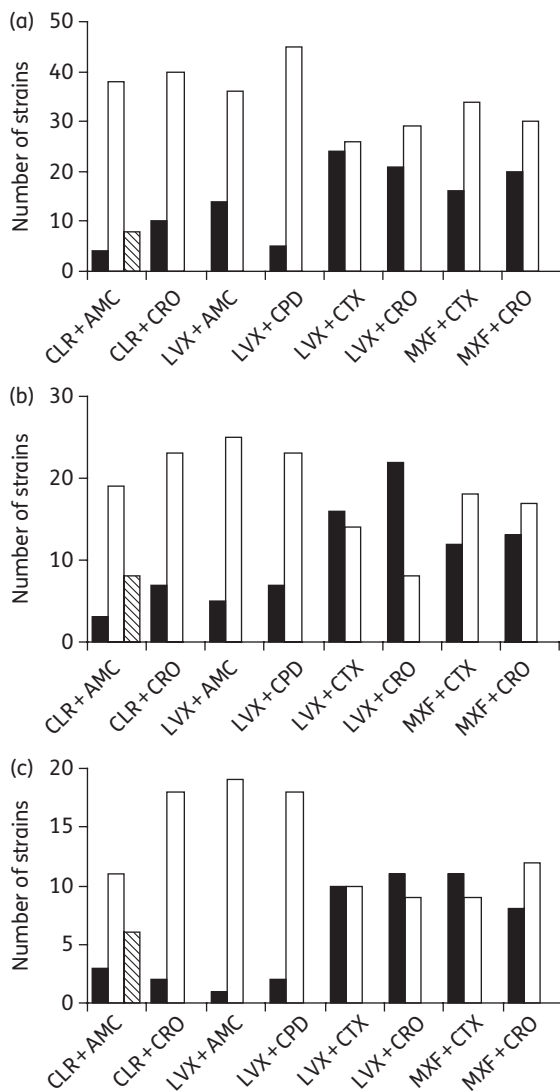
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**Figure 1.** Activity of antimicrobial combinations against susceptible (a), macrolide resistant (b) and penicillin non-susceptible (c) *S. pneumoniae* (n=100). Black bars, synergy; white bars, no interaction; diagonally striped bars, antagonism. LVX, levofloxacin; MXF, moxifloxacin; CLR, clarithromycin; CRO, ceftriaxone; CTX, cefotaxime; AMC, amoxicillin/clavulanic acid; CPD, cefpodoxime.

**Table 2.** Time–kill activities of antimicrobial combinations against 9 strains of *S. pneumoniae*

| | Number of strains | | | | | |
| | synergy | | indifference | | antagonism | |
| Drugs | 12 h | 24 h | 12 h | 24 h | 12 h | 24 h |
|---|---|---|---|---|---|---|
| CLR+AMC | 0 | 0 | 7 | 5 | 2 | 4 |
| CLR+CRO | 1 | 2 | 8 | 7 | 0 | 0 |
| LVX+AMC | 4 | 6 | 5 | 3 | 0 | 0 |
| LVX+CPD | 7 | 9 | 4 | 3 | 0 | 0 |
| LVX+CTX | 5 | 5 | 4 | 4 | 0 | 0 |
| LVX+CRO | 5 | 8 | 4 | 1 | 0 | 0 |
| MXF+CTX | 0 | 5 | 9 | 4 | 0 | 0 |
| MXF+CRO | 1 | 7 | 8 | 3 | 0 | 0 |

LVX, levofloxacin; MXF, moxifloxacin; CLR, clarithromycin; CRO, ceftriaxone; CTX, cefotaxime; AMC, amoxicillin/clavulanic acid; CPD, cefpodoxime.

Evidence from our study seems to suggest that combinations of antipneumococcal fluoroquinolones with cephalosporins are the most active against all phenotypes of *S. pneumoniae*, showing synergy or indifference against all the tested strains, while with combinations of β-lactams with a macrolide (i.e. clarithromycin) a risk of antagonism exists, particularly in combination with amoxicillin/clavulanic acid. Results obtained with the chequerboard assay for a total of 100 strains of *S. pneumoniae* with different susceptibility patterns were confirmed by time–kill assays for a restricted number of strains with all the tested combinations.

The highest rate of synergy was found with the combination of levofloxacin with ceftriaxone, which was particularly active against macrolide-resistant pneumococci; the combination of levofloxacin with cefotaxime was equally effective against the fully susceptible, macrolide-resistant or penicillin-non-susceptible strains tested. Interestingly, the two fluoroquinolones tested, chosen for their well-known activity against respiratory pathogens, showed a different rate of synergy with the parenteral cephalosporins. Since the mechanism underlying the activity of antibiotics in combination should be the same for levofloxacin and moxifloxacin, the difference observed could be attributable to differences in intrinsic activity of the two molecules. Synergy of levofloxacin with ceftriaxone and cefotaxime has been previously reported in animal models of meningitis caused by two penicillin-resistant pneumococci.[26,27] In contrast to these studies, we observed synergy only after 12 h of incubation. It is possible that this difference could be due to the tested strains or to the difference in the bacterial inocula used, which were ~1 log higher in the studies of Cottagnoud *et al.*[27] than in our study.

According to the Italian guidelines, levofloxacin is recommended for the therapy of severe CAP in combination with β-lactams. After reaching clinical stability, sequential therapy, whenever possible, or switch therapy, is suggested for both drugs.[10] With regard to moxifloxacin, recently its labelling recommends its use in CAP only when the other antibacterials commonly used for the therapy of CAP are considered inappropriate. Moreover, the intravenous form is still not available in Italy, further limiting its use in severe CAP.[12] Thus, only levofloxacin in combination with oral β-lactams was investigated in the present study.

In previous studies, combined therapy showed a better outcome compared with monotherapy, particularly in bacteraemic pneumococcal pneumonia.[10,22] However, there are some concerns about potential antagonism between β-lactams and macrolides, which are a matter of debate, since conflicting evidence has been obtained in *in vitro* studies.[23,24] In contrast, more consistent data have been obtained in studies assessing the activities of combinations involving fluoroquinolones and β-lactams, particularly cephalosporins, both *in vitro* and *in vivo*.[25–29] However, most of the cited studies limited their observations to a few strains of pneumococci and did not compare different combinations between them.

The present study was designed to compare the *in vitro* interactions of combinations between respiratory fluoroquinolones and β-lactams versus standard combinations.

Ceftriaxone showed superior activity compared with amoxicillin/clavulanic acid in combinations with both levofloxacin and clarithromycin, although associations of ceftriaxone with a fluoroquinolone were more active than combination with a macrolide. Moreover, amoxicillin/clavulanic acid in combination with a macrolide was the only antibiotic combination that yielded an antagonistic effect against some pneumococci, thus confirming the need to further investigate these results.

Synergy was also observed for the combination of levofloxacin with cefpodoxime, but only in a few cases, while the two antibiotics acted substantially as individuals in their activity against *S. pneumoniae*.

In conclusion, eight combinations of fluoroquinolones and a macrolide with different β-lactams have been assessed against *S. pneumoniae* strains with different patterns of susceptibility. Combinations of levofloxacin with parenteral cephalosporins were the most active among all the tested combinations, supporting their important role as an alternative to the standard combinations. Instead, antagonism occurred when clarithromycin and amoxicillin/clavulanic acid were tested. Data from prospective clinical trials are needed to evaluate the relevance of our findings in clinical practice.

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## Transparency declarations

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