Clarithromycin alone and in combination with ceftriaxone inhibits the production of pneumolysin by both macrolide-susceptible and macrolide-resistant strains of Streptococcus pneumoniae

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Objectives: To investigate the effects of clarithromycin (0.01–0.5 mg/L) alone or in combination with ceftriaxone (0.1 and 0.25 mg/L) on pneumolysin production by both macrolide-susceptible and -resistant [2 erm(B) positive and 2 mef(A) positive] strains of Streptococcus pneumoniae.

Methods: The bacteria were cultured for 6 h at 37°C/5% CO_2 in tryptone soy broth, washed, enumerated and resuspended to 0.5–3 x 10^8 cfu/mL in tissue culture medium, RPMI 1640. After 16 h of incubation at 37°C / 5% CO_2, pneumolysin was assayed in the bacteria-free supernatants, as well as in lysates, using a functional assay based on the influx of calcium into human neutrophils.

Results: Exposure of not only macrolide-susceptible strains, but also the macrolide-resistant strains, of S. pneumoniae to sub-MICs of clarithromycin resulted in dose-related inhibition of the pneumolysin production, whereas production of the toxin was unaffected by ceftriaxone.

Conclusions: These observations demonstrate that even in the setting of macrolide resistance the production of pneumolysin, a key virulence factor of the pneumococcus, is attenuated by exposure of this microbial pathogen to clarithromycin.

Introduction

Many international societies currently recommend combination therapy in their guidelines on the management of community-acquired pneumonia (CAP), most commonly the addition of a macrolide to standard β-lactam therapy, especially for the treatment of the more severely ill hospitalized patient.[1–3] Nevertheless, the mechanism for the benefit of combining a macrolide with a β-lactam is uncertain, and may be multifactorial, such as providing cover for atypical pathogens, unrecognized polymicrobial infection, and/or additional cover for drug-resistant infections, or synergy between these two classes of agents.[4] In addition to these direct antimicrobial actions, macrolides also possess secondary anti-inflammatory / immunomodulatory properties directed at both the host and the microbial pathogen, which may be of potential benefit to patients with CAP.[4–8] The latter includes the ability of these agents to interfere with quorum sensing mechanisms in microorganisms, which has been most comprehensively studied in Pseudomonas aeruginosa.

Pneumolysin, a toxin produced by all clinically-relevant strains of S. pneumoniae, possesses both cytotoxic and pro-inflammatory properties and is a major virulence determinant of the pneumococcus.[9–11] Excessive levels of pneumolysin in the airways, as is likely to occur in severe pneumococcal pneumonia, results in

damage to vascular endothelium as a consequence of the cytolytic actions of the toxin.[12–14] This favours not only extrapulmonary dissemination of the pneumococcus, but also poorly controlled influx of neutrophils into the lungs and development of a potentially harmful, hyperacute inflammatory response. Because of its involvement in the pathogenesis of severe, invasive pneumococcal disease, pneumolysin has been identified as a potential target for adjunctive pharmacotherapy to β-lactam antimicrobial agents.[10,11] One such strategy is to combine a selective inhibitor of bacterial protein synthesis, such as clarithromycin, with a β-lactam, since the latter is less efficient than agents such as clindamycin and rifampicin in suppressing the production of pneumolysin both in vitro and in vivo.[15]

In the current study, we have investigated the in vitro effects of clarithromycin alone and in combination with ceftriaxone, on the production of pneumolysin by both macrolide-susceptible and -resistant strains of the pneumococcus.

Materials and methods

Antimicrobial agents

Pure substances of clarithromycin and ceftriaxone were provided by Abbott Laboratories, North Chicago, IL, USA, and Roche Products (Pty) Ltd, Johannesburg, South Africa, respectively, and made into stock solutions of 1 mg/L in sterile distilled water.

Bacteria

Six clinical macrolide-susceptible and -resistant pneumococcal strains isolated in South Africa and known to be serotype 23F were selected (isolates 2506 and 2507 were part of a collection previously described[16]). Identification of the isolates was confirmed using standardized techniques;[17] capsular serotyping was performed using specific antisera (Statens Serum Institut, Copenhagen, Denmark) to demonstrate the Quellung reaction. MICs were determined using agar dilution and Etest® (AB-Biodisk, Solna, Sweden) methodologies, and interpreted according to CLSI (formerly NCCLS) guidelines.[18]

Chromosomal DNAs were extracted from bacterial cells using a previously described method.[19] This purified DNA then served as template DNA in all further PCR reactions. Bacterial strains were genotyped using BOX-PCR fingerprinting,[20] and screened for the presence of erm(B) and mef(A) genes using PCR.[21]

To investigate the effects of the test antimicrobial agents on pneumolysin production—uncomplicated by their inhibitory effects on microbial proliferation—the various macrolide-susceptible and -resistant strains of S. pneumoniae were cultured in tryptone soy broth [TSB, Biolab Diagnostics (Pty) Ltd, Johannesburg, South Africa], enumerated and transferred to tissue culture medium in which they were exposed to the antibiotics. Briefly, the bacteria were grown for 6 h at 37°C in an atmosphere of 5% CO_2 after which they were harvested by cen-trifugation (800 g for 15 min) then transferred to, and washed in indicator-free tissue culture medium, RPMI 1640 (Highveld Biological Pty Ltd, Johannesburg, South Africa). The bacterial suspensions were then adjusted spectrophotometrically at a wavelength of 540 nm to an optical density of 0.1, which equated to approximately 0.5-3 x 10^8 cfu/mL, depending on the strain. The bacteria were then incubated for 1 h at 37°C in an atmosphere of 5% CO2 with or without the antimicrobial agents. Clarithromycin was used at concentrations of 0.01-0.5 mg/L, whereas ceftriaxone was used at fixed, final concentrations of 0.1 mg/L or 0.25 mg/L. Following this 1 h incubation, bovine serum albumin (5 g/L, final;

Sigma Chemical Co., St Louis, MO, USA) was added to each tube, after which the tubes were incubated for a further 16 h. On completion of incubation, the bacteria were pelleted by centrifugation (800 g for 15 min) and the bacteria-free supernatants and lysates (prepared by sonication) assayed for pneumolysin as described below. Pneumolysin was also measured in both the bacteria-free supernatants, as well as in the lysates (sonicates) of each strain at the outset, immediately prior to exposure to the antimicrobial agents.

Pneumolysin assay

The concentrations of pneumolysin in the bacteria-free supernatants were measured using an assay procedure based on the pore-forming interactions of the toxin with isolated human blood neutrophils, which results in influx of extracellular Ca^{2+}. Neutrophils were isolated from the blood of healthy, adult human volunteers and resuspended to 1×10^7 cells/mL in PBS (0.15 M, pH 7.4), as described previously.[22] The cells were then loaded with the Ca^{2+}-sensitive, fluorescent dye, fura-2/AM (Sigma Chemical Co.; 2 (μM final) for 30 min at 37°C after which the loaded cells were resuspended in indicator-free Hanks' balanced salt solution (HBSS, pH7.4; 1.25 mM $CaCl_2$; Highveld Biological Pty, Ltd), preincubated for 10 min at 37°C then transferred to reaction cuvettes which were maintained at 37°C in a Hitachi 650 10S fluorescence spectrophotometer with excitation and emission wavelengths set at 340 and 500 nm respectively. After a stable baseline was obtained, bacteria-free supernatants (maximum volume of 200 (μL) were added to the cuvettes and alterations in neutrophil cytosolic Ca^{2+} concentrations monitored over a 5 min period. The final volume in each cuvette was 3 mL containing a total of 3×10^6 neutrophils.

Recombinant pneumolysin (1-16.78 (μg/L) was used to construct standard curves from which concentrations of the toxin in the supernatants were calculated. Specificity of the assay was based on the following criteria: (i) complete absence of Ca^{2+} influx following the addition to neutrophils of culture supernatants from a mutant of the D39 strain of *S. pneumoniae* in which the pneumolysin-encoding gene had been selectively disabled; (ii) complete neutralization of induction of Ca^{2+} influx by supernatants which had been mixed with a monoclonal antibody to pneumolysin (mAb PLY 7) prior to addition to neutrophils,[23] as well as by removal of extracellular Ca^{2+} from the cell suspending medium by addition of a calcium chelating agent (10 mM EGTA) to the cells immediately prior to addition of the test supernatant fluids; and (iii) lack of effect of pre-treatment of neutrophils with the selective phospholipase C inhibitor, U-73122,[24] on the increased neutrophil cytosolic Ca^{2+} concentrations caused by addition of the test supernatants to the cells, excluding possible receptor-mediated mobilization of both stored and extracellular Ca^{2+} by bacterially derived N-formylated poly-peptides. Moreover, neither clarithromycin nor ceftriaxone at fixed, final concentrations of 5 (μg/mL antagonized the pore-forming interactions of recombinant pneumolysin (8.37 ng/mL) with neutrophils (not shown).

Statistical analysis

The results of each series of experiments are presented as the mean values ± standard errors of the means (SEMs). Levels of statistical significance were calculated using the Student's *t*-test (unpaired *t* statistic).

Results

All six pneumococcal isolates were from blood or sputum specimens and confirmed as serotype 23F. MICs for each strain of erythromycin, clarithromycin and ceftriaxone are shown in Table 1. The presence of macrolide resistance determinants [*erm*(B) and *mef*(A)] was confirmed using PCR (Table 1). BOX-PCR

fingerprinting, shown in Figure 1, was used to determine the relatedness of the pneumococcal strains to the Spain^{23F}-1 pneumococcal clone. Strains 171, 172, 2506 and 2507 showed an identical pattern to a representative of the Spain^{23F}-1 clone and were therefore related to this clone (Figure 1). Strains 521 and 14787 were unrelated to the Spain^{23F}-1 clone (data not shown).

Table 1. Phenotypic and genotypic characteristics of clinical pneumococcal strains confirmed as serotype 23F

		MIC (mg/L)			PCR confirmed macrolide resistance determinants		
Strain	Source	erythromycin	clarithromycin	ceftriaxone	*erm*(B)	*mef*(A)	Related on BOX-PCR to Spain^{23F}-1
171[a]	sputum	0.125 (susceptible)	0.064	0.38	negative	negative	yes
172[a]	sputum	0.094 (susceptible)	0.064	1	negative	negative	yes
2506[b]	sputum	>256 (resistant)	>256	0.38	positive	negative	yes
2507[b]	sputum	>256 (resistant)	>256	0.5	positive	negative	yes
521	blood culture	4 (resistant)	2	0.06	negative	positive	no
14786	blood culture	24 (resistant)	1	0.06	negative	positive	no

[a]Age was unknown for patient with strain 171 and 172, all other patients were adults.

[b]Part of collection of strains previously described.

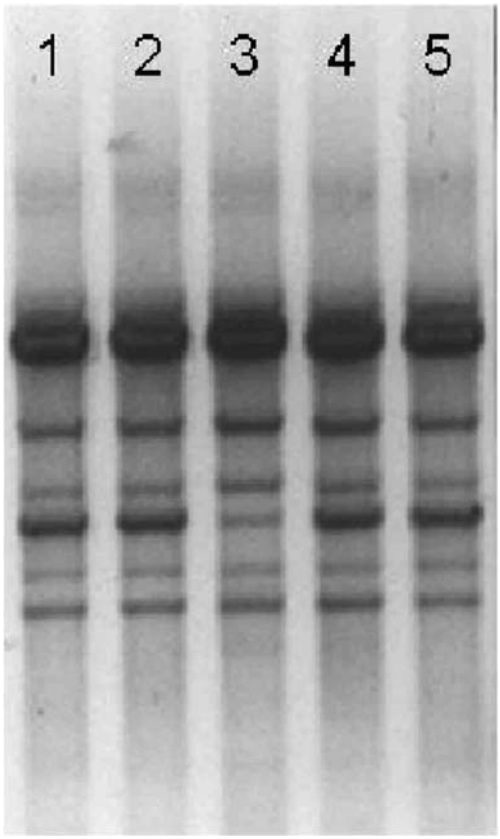

Figure 1. BOX-PCR fingerprint patterns of pneumococcal strains compared with a representative of the Spain^{23F}-1 clone. Lane 1, strain 2507; lane 2, strain 2506; lane 3, Spain^{23F} strain; lane 4, strain 172, lane 5, strain 171.

Time-course of production of pneumolysin in RPMI 1640

The time-course of production of pneumolysin by S. pneumoniae strain 172 in relation to bacterial viability (cfu/turbidity) is shown in Figure 2. At time 0, immediately following turbidometric adjustment (after 6 h of growth in tryptone soy broth), and transfer to RPMI 1640, pneumolysin could not be detected either in bacteria-free supernatants or sonicates in this experiment or all other experiments (data not shown). Significant release of pneu-molysin into the culture medium was evident at 12 h and maximal at 16 h, and was accompanied by a considerable decline in viable counts (Figure 2). Subsequent experiments using the various test strains of S. pneumoniae were performed using a fixed incubation time of 16 h.

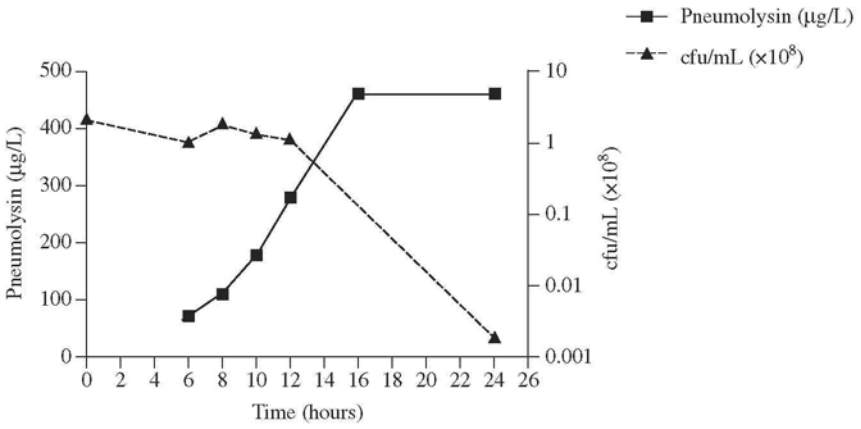

Figure 2. Time-course of production of pneumolysin by strain 172 of Streptococcus pneumoniae (macrolide-susceptible) in RPMI 1640 in relation to bacterial viability. The data are the mean values for each time-point from one experiment

Effects of clarithromycin and ceftriaxone alone on release of pneumolysin

The effects of clarithromycin (0.01, 0.025, 0.05 mg/L) and ceftriaxone (0.1 mg/L) alone on the release of pneumolysin from S. pneumoniae strain 172 are shown in Figure 3. Exposure of the bacteria to clarithromycin resulted in dose-related inhibition of pneumolysin production which was maximal at concentrations of 0.05 mg/L and higher concentrations (but undetectable at 0.1 mg/L), whereas production of the toxin was unaffected by ceftriaxone. Similar effects were observed with the other macrolide-susceptible strain of the pneumococcus, strain 171 (data not shown)

The effects of clarithromycin and ceftriaxone on pneumolysin production by the macrolide-resistant strains of the pneumococcus are shown in Figures 4 and 5. Somewhat surprisingly, clarithromycin, but again not ceftriaxone, caused dose-related inhibition of pneumolysin production by all four resistant strains, with toxin production by the *erm* strains 2506 and 2507 of S. pneumoniae being most susceptible to the macrolide, which in the case of extracellular toxin achieved statistical significance at concentrations of 0.01 and 0.025 mg/L respectively, whereas the corresponding concentrations for a significant reduction in total pneumolysin were 0.025 and 0.5 mg/L. Treatment with ceftriax-one significantly increased the concentration of total pneumolysin in strain 2507, but not in any other strain. The two *mef* strains were somewhat less sensitive to clarithromycin, with significant decreases in the concentrations of extracellular toxin observed at 0.05 and 0.1 mg/L for strains 14786 and 521, respectively; with both strains significant reductions in the total toxin concentration were observed at 0.1 mg/L.

The numbers of viable bacteria (cfu) ranged from 1.1 x 10^8 to 2.5 x 10 cfu/mL at the outset, depending on the strain, and decreased to between 5.9 x 10 and 7.9 x 10 cfu/mL after 16 h. Importantly, there were no significant differences between systems with or without clarithromycin.

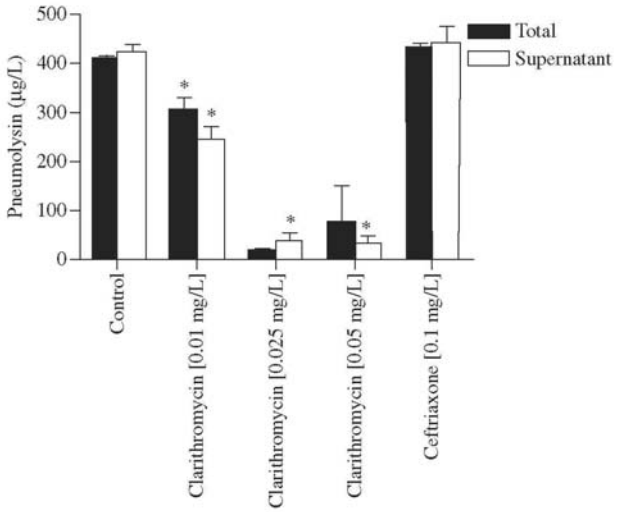

Figure 3. Effects of clarithromycin (0.01–0.05 mg/L) on the production of pneumolysin by strain 172 of Streptococcus pneumoniae. The results of two representative experiments (four in the series) with three replicates for each system in each experiment are expressed as the mean values ± SEM for both total and extracellular pneumolysin (left and right members of each pair of columns, respectively). *P < 0.05 for comparison with the corresponding antibiotic-free control systems

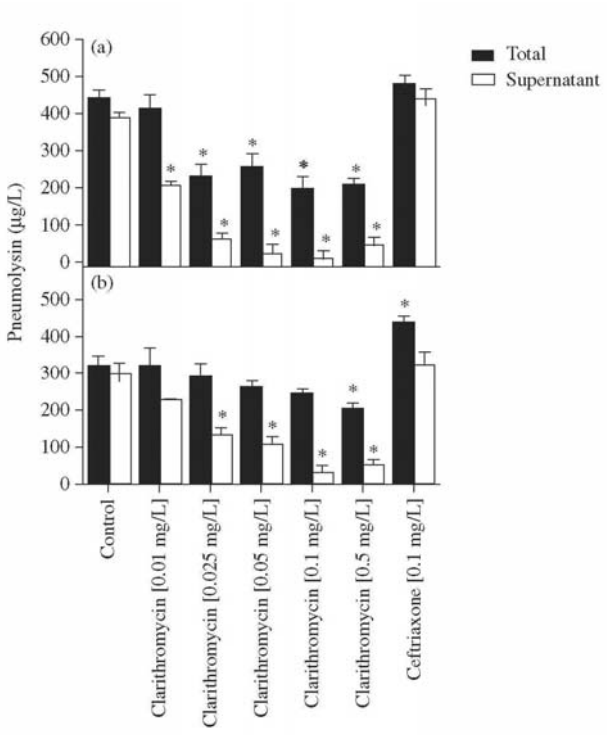

Figure 4. Effects of clarithromycin (0.01–0.5 mg/L) on the production of pneumolysin by strain 2506 (a) and strain 2507 (b) of Streptococcus pneumoniae (macrolide-resistant, erm). The results of three experiments with three replicates for each system in each experiment are expressed as the mean values ± SEM for both total and extracellular pneumolysin (left and right members of each pair of columns, respectively). *P < 0.05 for comparison with the corresponding antibiotic-free control systems

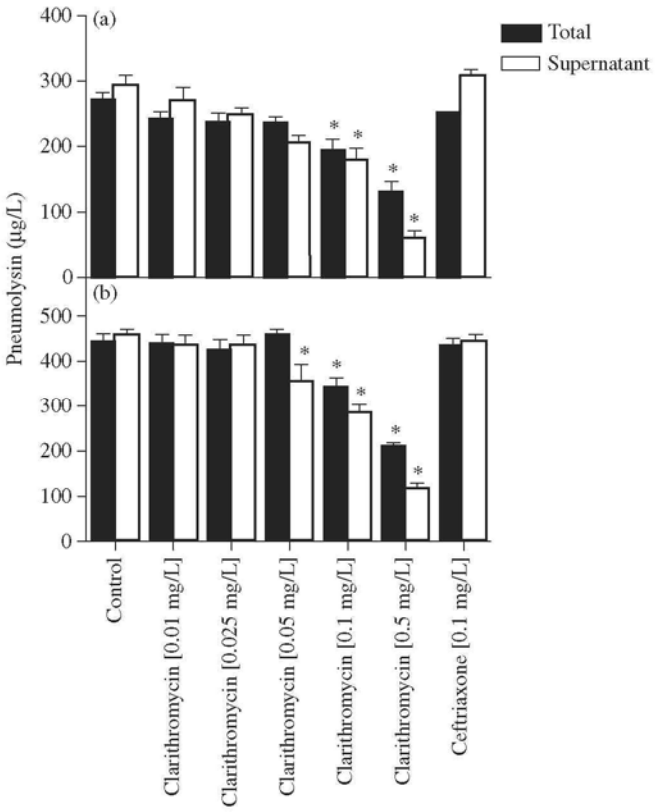

Figure 5. Effects of clarithromycin (0.01–0.5 mg/L) on the production of pneumolysin by strain 521 (a) and strain 14786 (b) of Streptococcus pneumoniae (macrolide-resistant, mef). The results of three experiments with three replicates for each system in each experiment are expressed as the mean values ± SEM for both total and extracellular pneumolysin (left and right members of each pair of columns, respectively). *P < 0.05 for comparison with the corresponding antibiotic-free control systems

Effects of clarithromycin and ceftriaxone in combination on the production of pneumolysin by strains 172, 2507 and 14786 of S. pneumoniae

In the case of strain 172, the mean total pneumolysin values ± SEM for the control systems and for systems containing clarithromycin (0.05 mg/L) and ceftriaxone (0.25 mg/L), individually or in combination, were 420 ± 15, 269 ± 15, 435 ± 0.0 and 313 ± 13 µg/L respectively (data from two experiments with three replicates for each system). Similar results were obtained with the two macrolide-resistant strains (not shown).

Discussion

The effects of clarithromycin and ceftriaxone on pneumolysin production by both the macrolide-susceptible and -resistant strains of the pneumococcus, uncomplicated by the inhibitory effects of this agent on growth, were investigated by exposing numerically standardized suspensions of the bacteria to the antibiotics in RPMI 1640, a tissue culture medium which does not support proliferation of the pneumococcus. Ceftriaxone, at the concentrations used, did not affect pneumolysin production by any strain of pneumococcus investigated in this system, whereas clarithromycin, as expected, caused dose-related inhibition of production of the toxin by all of the macrolide-susceptible strains that was maximal at concentrations of 0.05-0.1 mg/L. Importantly, however, clarithromycin also inhibited pneumolysin production by macrolide-resistant strains of the pneumococcus carrying either the *erm* gene, which is associated with the ribosomal

methylation mechanism of resistance, as well as those expressing the *mef* efflux pump genes. Isolates that express the *erm(B)* gene most commonly have high levels of macrolide resistance, usually with a macrolide MIC of ≥256 mg/L, as was the case for the isolates used in the current study, while those expressing the *mef(A)* genes are inhibited at lower MICs (intermediate macrolide resistance is defined as 0.5 mg/mL and these have MICs of 4-32 mg/mL).

In the case of the macrolide-resistant strains of the pneumococcus, extracellular pneumolysin was somewhat more sensitive to the inhibitory effects of clarithromycin than was the production of total toxin, compatible with a mechanism whereby the macrolide interferes not only with the synthesis of the toxin, but also with its release, possibly by inhibition of production of autolysin. Surprisingly, in view of the MIC data shown in Table 1, pneumolysin production by the *mef(A)* strains appeared to be somewhat less sensitive to the inhibitory effects of clarithromycin than that of the *erm(B)* strains. This may reflect the efficiency of the antibiotic efflux pump at the relatively low concentrations of the macrolide used in the current study.

There are similarities between the results of the current study and those reported by Lagrou and colleagues. These authors observed that exposure of a highly macrolide-resistant strain of *S. pneumoniae,* expressing the *erm(AM)* gene, to erythromycin in tissue culture medium was accompanied by reduced adherence to respiratory epithelial cells, as well as by a striking decrease in the production of pneumolysin.[25] More recently, Fukuda *et al.*[26] have reported that both azithromycin and clarithromycin inhibit the production of pneumolysin *in vitro* by macrolide-resistant strains of *S. pneumoniae,* while administration of these agents to mice prolongs survival following experimental infection with macrolide-resistant pneumococci in the setting of decreased activity of pneumolysin in the lungs. There are, however, several notable differences between our study and those of Lagrou *et al.* and Fukuda *et al.,* especially in respect of the number and diversity of isolates investigated, the relatively low concentrations of the macrolide used in the current study, as well as the measurement of both total and extracellular pneumolysin. Our findings also complement those of Spreer *et al.,*[15] who observed that clindamycin and rifampicin were more effective inhibitors of pneumolysin production than ceftriaxone *in vitro,* while rifampicin was also found to be more effective than the β-lactam agent in decreasing pneumolysin concentrations in cerebrospinal fluid in an animal model of experimental pneumococcal meningitis.[15]

In conclusion, our study, which documents the ability of clarithromycin, but not ceftriaxone, to inhibit pneumolysin production by both macrolide-susceptible and -resistant pneumococci, may provide an explanation for the benefit of combination therapy with these antibiotics on the outcome of bacteraemic pneumococcal pneumonia.

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