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In vitro interference of β -lactams with biofilm development by prevalent community respiratory tract isolates [☆]

J.R. Maestre ^a, M. Mateo ^a, M.L. Méndez ^a, L. Aguilar ^{b,*}, M.J. Gimenez ^b, L. Alou ^b, P. Coronel ^c, J.J. Granizo ^d, J. Prieto ^b

^a *Microbiology Department, Hospital Central de la Defensa Gómez-Ulla, Gta. del Ejército s/n, 28007 Madrid, Spain*

^b *Microbiology Department, School of Medicine, Universidad Complutense, Avda. Complutense s/n, 28040 Madrid, Spain*

^c *Scientific Department, Tedec-Meiji Farma S.A., Ctra. M-300, Km 30,500, 28802 Alcalá de Henares, Madrid, Spain*

^d *Grana Datos SL, Demetrio de la Guerra 4, 28223 Pozuelo de Alarcón, Madrid, Spain*

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* Corresponding author. Tel.: +34 913 941 505; fax: +34 913 941 511.

E-mail address: laguilar@med.ucm.es (L. Aguilar).

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ABSTRACT

Interference of cefditoren (CDN) and amoxicillin/clavulanic acid (AMC) with biofilm production was studied using 11 *Streptococcus pneumoniae* isolates with minimum inhibitory concentrations (MICs) ranging from 0.015 µg/mL to 0.5 µg/mL for CDN and from 0.06 µg/mL to 2 µg/mL for AMC (except for one isolate with an AMC MIC of 8 µg/mL) and 5 *Haemophilus influenzae* isolates with MICs of 0.03–0.06 µg/mL for CDN and 0.5–16 µg/mL for AMC. Slime production was assessed in antibiotic-free medium and with 0.03 µg/mL CDN or 1/0.5 µg/mL AMC by measuring the optical density at 450 nm (OD₄₅₀). Significantly lower mean OD₄₅₀ values were obtained for *S. pneumoniae* with antibiotics compared with controls (CDN, 0.088 vs. 0.118, $P = 0.003$; and AMC, 0.095 vs. 0.112, $P = 0.003$), with significant correlation between both antibiotics ($r = 0.752$; $P = 0.008$). Percent reduction in OD₄₅₀ values was higher for CDN compared with AMC (24.02% vs. 15.92%; $P = 0.008$). For *H. influenzae*, significantly lower mean OD₄₅₀ values were obtained with CDN compared with controls (0.083 vs. 0.096; $P = 0.043$) but not with AMC (0.086 vs. 0.095; $P = 0.08$). Comparing percent reductions in *S. pneumoniae* versus *H. influenzae* for each antibiotic, no differences were found for AMC (15.92% vs. 9.40%; $P = 0.36$), with a tendency for CDN (24.02% vs. 13.79%; $P = 0.069$). Different β-lactams may have different capabilities of interfering with *S. pneumoniae* biofilm development when tested under the same experimental conditions.

1. Introduction

Chronic ailments caused by normal commensal flora are now prevalent infectious diseases [1]. These types of microorganisms, such as *Streptococcus pneumoniae* and *Haemophilus influenzae*, which form part of the nasopharyngeal microbiota, make full use of the biofilm strategy when causing non-invasive disease. Biofilms are 'a structured community of bacterial cells enclosed in a self-produced polymeric matrix adherent to an inert or living surface' [2]. Alterations in the metabolism of biofilm bacteria make them difficult to recover with standard culture techniques [1] as well as more resistant to antibiotic therapy than planktonic bacteria [3]. Thus, bacterial biofilms play a relevant role in chronic infections such as sinusitis, chronic otitis, otitis media with effusion and chronic bronchitis, where antibiotic therapy may overcome the symptoms caused by waves of planktonic cells released from the biofilm during exacerbations of the disease but fails to eradicate infection as sessile cells are inherently less affected [4]. Owing to these facts, not only is time to eradication of symptoms an endpoint in clinical trials with antibiotics against exacerbations, but time to relapse is also increasingly used.

Streptococcus pneumoniae and *H. influenzae* are the most prevalent bacterial isolates in the abovementioned infections. Specific resistance phenotypes have been described in these two species: (a) high resistance to macrolides in *S. pneumoniae* owing to the prevalent macrolide–lincosamide–streptogramin B (MLS_B) phenotype, implying high-level resistance (that cannot be overcome by increasing doses) [5]; (b) amoxicillin resistance related to specific clones among penicillin-resistant *S. pneumoniae* [6]; and (c) β -lactamase production and the recently increasing β -lactamase-negative ampicillin-resistant (BLNAR) and β -

lactamase-positive amoxicillin/clavulanic acid-resistant (BLPACR) phenotypes in *H. influenzae* [7]. Despite these facts, oral penicillins, cephalosporins and macrolides are commonly used antibiotics to treat these infections.

It has been hypothesised that compounds active against not only planktonic cells but also able to interfere or decrease biofilm development by these two capsulated bacteria may offer clinical advantages [8]. Cefditoren (CDN) is an oral third-generation cephalosporin with high intrinsic activity against *S. pneumoniae* [9] and *H. influenzae* [7] in planktonic cultures regardless of the resistance phenotype.

The aim of this study was to assess whether CDN [compared with amoxicillin/clavulanic acid (AMC)] is able to interfere with the ability of *S. pneumoniae* and *H. influenzae* to produce biofilms.

2. Material and methods

2.1. Strains

2.1.1. *Streptococcus pneumoniae*

Among 40 recent clinical isolates from non-invasive samples kindly supplied by the Spanish Reference Pneumococcal Laboratory (Instituto de Salud Carlos III, Majadahonda, Madrid, Spain), 12 isolates were chosen on the basis of their capability of biofilm production, spectrophotometrically measured, defined as an optical density at 450 nm (OD_{450}) ≥ 0.08 , the value corresponding to the OD_{450} produced by strain R6 (see below). Among the group of isolates producing biofilm, the 12 isolates were chosen based on their susceptibility to penicillin in order to

have 75% of them non-susceptible to penicillin according to Clinical and Laboratory Standards Institute (CLSI) breakpoints for oral penicillin [10].

2.1.2. *Haemophilus influenzae*

Among 40 recent clinical isolates from non-invasive samples kindly supplied by six Spanish hospitals in a previous study [7], 12 isolates were chosen on the basis of their capability of biofilm production, spectrophotometrically measured, defined as an $OD_{450} \geq 0.08$ (see below). Among the group of isolates producing biofilm, the 12 isolates were chosen based on their susceptibility to ampicillin in order to have 75% of them non-susceptible to ampicillin according to CLSI breakpoints [10].

2.2. Susceptibility testing

Minimum inhibitory concentrations (MICs) of CDN and AMC were determined three times by the microdilution method following CLSI guidelines [11]. Modal values were considered.

2.3. Biofilm production

Biofilm production was assessed by a spectrophotometric method as described previously [12] using 96-well polystyrene microtitre plates (Masterlab S.L., Madrid, Spain). Briefly, in antibiotic-free experiments (controls), wells were inoculated with 200 μ L of a bacterial suspension in Haemophilus Test Medium (HTM) broth (Dade Behring, West Sacramento, CA), corresponding to 0.5 McFarland standard, of *S. pneumoniae* strain R6 (strong slime producer), *S. pneumoniae* ATCC 6303 (negative slime producer) and each of the study strains. In experiments with

antibiotics that were run in parallel, wells containing 50 μL of antibiotic concentrations in HTM were inoculated with 150 μL of bacterial suspension of study strains (0.5 McFarland standard) in HTM. Final antibiotic concentrations in the final 200 μL volume were 0.03 $\mu\text{g/mL}$ CDN and 1/0.5 $\mu\text{g/mL}$ AMC (free concentrations achievable in serum after standard oral doses) [13]. Plates were covered and incubated at 37 °C under a 5% CO_2 atmosphere for 24 h. Following incubation, the contents of each well were removed by decantation and the wells were washed with sterile distilled water. The empty wells were allowed to dry for 30 min and then 200 μL of crystal violet stain (25%) was added to each well. After 5 min, the excess stain was rinsed off by decantation and the plate was washed. After the well had been allowed to dry for 30 min, 200 μL of HCl 25% was added to each well and after 1 min the OD_{450} of stained adherent bacterial films was read using a Micro-ELISA auto-reader spectrophotometer (Labsystems Multiskan, Helsinki, Finland). As a negative control, non-inoculated wells were subjected to the same procedures. Experiments with each strain were performed 12 times and mean values were considered.

2.4. Statistical analysis

Per-strain percentages of reduction in OD_{450} in the presence of antibiotics compared with controls (antibiotic-free wells) were calculated. Mean $\pm$ standard deviation OD_{450} values for *S. pneumoniae* and *H. influenzae* strains were calculated for each antibiotic. Percentage reductions obtained with CDN and AMC as well as comparisons between OD_{450} values in antibiotic-free media and in the presence of antibiotics were compared using the Wilcoxon test.

For each antibiotic, percentage reductions obtained for *S. pneumoniae* were compared with those obtained for *H. influenzae* using the Mann–Whitney test. Correlations of CDN and AMC reductions for each species were analysed using the Spearman correlation coefficient.

3. Results

Negative controls (non-inoculated wells and those inoculated with *S. pneumoniae* ATCC 6303) exhibited a mean OD₄₅₀ of 0.053, whilst the positive control (*S. pneumoniae* R6) exhibited a mean OD₄₅₀ of 0.084.

3.1. *Streptococcus pneumoniae*

At screening, among the 40 pneumococci tested, 19 (47.5%) produced biofilms exhibiting OD₄₅₀ values similar to or higher than that of R6. Among them, 12 pneumococci were chosen for the study: 3 were penicillin intermediate and 9 were fully resistant to penicillin [10]. One strain could not be further recovered. In the study phase, the remaining 11 strains exhibited an OD₄₅₀ value ranging from 0.078 to 0.235 in antibiotic-free experiments (Table 1).

Table 1 shows the serotypes and susceptibility to CDN and AMC of pneumococcal study strains. All strains but one (strain 11) were susceptible to AMC, and the CDN MIC was ≤ 0.5 $\mu\text{g/mL}$ in all cases. Table 1 also shows OD₄₅₀ values for controls as well as percentage reduction by the presence of the antibiotics.

In the CDN experiments, global mean OD₄₅₀ values for CDN-treated strains were significantly lower than those for controls (0.088 ± 0.039 vs. 0.118 ± 0.052 ; $P = 0.003$). Percentage reduction in OD₄₅₀ by the presence of CDN ranged from 14.7% to 50.5% (Table 1).

In experiments carried out with AMC, global mean OD₄₅₀ values for AMC were significantly lower than those for controls (0.095 ± 0.053 vs. 0.112 ± 0.056 ; $P = 0.003$), although the reduction in the case of strain 4 was <5%. Excluding strain 4, the percentage reduction in OD₄₅₀ by the presence of AMC ranged from 8.5% to 36.7% (Table 1).

Comparing reductions in OD₄₅₀ obtained with both antibiotics, a significantly higher global mean percentage reduction was obtained with CDN than with AMC ($24.02 \pm 11.12\%$ vs. $15.92 \pm 8.64\%$; $P = 0.008$), with high and significant correlation between the reductions obtained with both antibiotics ($r = 0.752$; $P = 0.008$).

3.2. *Haemophilus influenzae*

Among the 40 strains tested, only 12 (30%) produced biofilms exhibiting OD₄₅₀ values similar to or higher than that of *S. pneumoniae* R6 and were chosen for the study. Of them, nine strains exhibited ampicillin non-susceptibility ($\text{MIC} \geq 2 \mu\text{g/mL}$): three strains were β -lactamase positive, four were BLNAR and two were BLPACR exhibiting mutations in the *ftsI* gene [7]. Only 5 (41.7%) of the 12 strains exhibited a decrease (>5% reduction) in OD₄₅₀ in the presence of antibiotics, whether CDN or AMC. Phenotypically, three strains were susceptible to AMC (strains 1, 3 and 4)

and two were resistant (strains 2 and 5) following the current CLSI breakpoints [10]. Table 2 shows the resistance genotypes and susceptibility of the five *H. influenzae* strains.

In the CDN experiments, global mean OD₄₅₀ values for CDN were significantly lower than those for controls (0.083 ± 0.008 vs. 0.096 ± 0.008 ; $P = 0.043$). Per-strain percentage reductions in OD₄₅₀ compared with controls by the presence of CDN ranged from 6.1% to 23.1% (Table 2).

In experiments carried out with AMC, global mean OD₄₅₀ values were not significantly different from those for controls (0.086 ± 0.011 vs. 0.095 ± 0.006 ; $P = 0.08$).

Comparing reductions in OD₄₅₀ obtained with both antibiotics, no significant differences were found between the global mean percentage reduction obtained with CDN versus AMC (13.79 ± 6.65 vs. 9.40 ± 9.38 ; $P = 0.5$), with a weak and statistically non-significant correlation between the reductions obtained with both antibiotics ($r = 0.300$; $P = 0.624$).

3.3. *Streptococcus pneumoniae* versus *Haemophilus influenzae*

When comparing per antibiotic global mean percentage reductions in OD₄₅₀ between *S. pneumoniae* and *H. influenzae*, no differences were found for AMC ($15.92 \pm 8.60\%$ vs. $9.40 \pm 9.38\%$; $P = 0.36$), with a tendency to higher percentage

reductions in *S. pneumoniae* for CDN ($24.02 \pm 11.12\%$ vs. $13.79 \pm 6.65\%$; $P = 0.069$).

4. Discussion

One of the major goals in modern clinical microbiology is the development of strategies capable of reducing biofilm infections related to chronic conditions owing to the difficulty of antibiotics in eradicating bacteria within these structures. In this regard, macrolides/azalides and quinolones have been shown to interfere with biofilm formation [8,14], but the quinolones are not adequate in paediatric infections and resistance rates to azithromycin in *S. pneumoniae* and *H. influenzae* in Spain using pharmacokinetic/pharmacodynamic breakpoints [5] make them pharmacodynamically inadequate in the treatments of infections caused by these two microorganisms.

Whilst for highly charged antibiotics the glycocalyx affects their ability to reach bacterial cells that are deep within the biofilm, for β -lactams such limitation does not exist since they are uncharged agents [15]. Reduced susceptibility to β -lactams in biofilm bacteria is more related to the diminished growth rate in these structures, as in the case of *S. pneumoniae* (2–3 h for planktonic bacteria versus ca. 10 h in biofilms) [15]. Since β -lactams are widely used antibiotics, in the present study we explored the capability of suprainhibitory and subinhibitory physiologically achievable concentrations of a third-generation oral cephalosporin and AMC in interfering with biofilm formation in HTM media, since this media supported *S. pneumoniae* biofilm development better than trypticase soy broth or

brain–heart infusion (data not shown). Owing to the higher intrinsic activity of CDN, a concentration of 0.03 µg/mL of this drug was tested and compared with a concentration of 1/0.5 µg/mL AMC.

When antibiotics were present in the media, significantly lower OD₄₅₀ values were obtained for *S. pneumoniae*, with a significant correlation between both antibiotics in reducing biofilm formation. In any case, the reduction in OD₄₅₀ was significantly higher for CDN, although the reductions appeared not to be related to MIC values or the concentration:MIC ratio. The heterogeneity in results displayed by the individual isolates could probably be attributed to chemical and physical heterogeneity of specific biofilms (different serotypes), or the results may represent differential gene regulation patterns on a strain basis as has been suggested previously [8]. The significant correlation found in this study between both antibiotics in reducing biofilm development supports this strain basis theory.

The situation was completely different for *H. influenzae* where antibiotics were able to reduce biofilm formation in only 5 of the 12 strains tested. Significantly lower mean OD₄₅₀ values were obtained compared with controls in the case of CDN but not in the case of AMC. Again, reductions appeared not to be related to the resistance genotype, MIC or the concentration:MIC ratio, although the number of strains tested is too low to draw conclusions.

As expected, lower mean OD₄₅₀ reductions were obtained in the current study with β-lactams compared with those obtained with a quinolone in a previous study [8], in part due to the intrinsic physicochemical characteristics of the drugs and

probably also due to the different methodology used (including the test media) since there is no standard reference methodology, making the results of both studies not comparable. However, the results of the present study may suggest that different β -lactams have different capabilities of interfering with *S.*

pneumoniae biofilm development when tested under the same experimental conditions. Based on this, in addition to the high in vitro bactericidal activity against *S. pneumoniae* and *H. influenzae* planktonic cells [7,9] exhibiting different resistance phenotypes, CDN may offer the advantage of a higher reduction in biofilm development in *S. pneumoniae*.

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Competing interests

PC is an employee of Tedec-Meiji Farma S.A., Madrid, Spain. All other authors declare no competing interests.

Ethical approval

Not required.

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Table 1

Streptococcus pneumoniae serotypes, minimum inhibitory concentrations (MICs) and percentage reduction in optical density at 450 nm (OD₄₅₀) compared with controls (in antibiotic-free media) by 0.03 µg/mL cefditoren (CDN) and 1/0.5 µg/mL amoxicillin/clavulanic acid (AMC)

Strain	Serotype	MIC (µg/mL)		CDN		AMC	
		CDN	AMC	OD ₄₅₀ (control)	% OD ₄₅₀ reduction	OD ₄₅₀ (control)	% OD ₄₅₀ reduction
1	21	0.015	0.06	0.232	34.5	0.214	20.5
2	23A	0.015	0.06	0.130	50.5	0.105	36.7
3	31	0.03	0.12	0.092	19.2	0.090	10.4
4	42	0.06	0.25	0.210	14.7	0.235	3.3
5	35B	0.25	1	0.080	20.1	0.086	20.9
6	6B	0.25	2	0.095	32.9	0.089	11.2
7	23F	0.5	2	0.101	16.3	0.087	15.6
8	19A	0.5	2	0.084	14.8	0.078	8.5
9	9V	0.5	2	0.091	23.5	0.088	17.0
10	9V	0.5	2	0.088	15.2	0.084	14.9
11	14	0.5	8	0.096	22.6	0.085	16.1

Table 2

Haemophilus influenzae resistance phenotypes, minimum inhibitory concentrations (MICs) and percentage reduction in optical density at 450 nm (OD₄₅₀) compared with controls (in antibiotic-free media) by 0.03 µg/mL cefditoren (CDN) and 1/0.5 µg/mL amoxicillin/clavulanic acid (AMC)

Strain	Genotype	MIC (µg/mL)		CDN		AMC	
		CDN	AMC	OD ₄₅₀ (control)	% OD ₄₅₀ reduction	OD ₄₅₀ (control)	% OD ₄₅₀ reduction
1	BL+	0.03	0.5	0.098	23.1	0.075	20.0
2	BL+	0.06	8	0.098	17.4	0.103	-2.2
3	BLNAR	0.06	2	0.091	12.9	0.082	18.0
4	BLPACR	0.06	4	0.107	9.5	0.091	4.5
5	BLNAR	0.06	16	0.085	6.1	0.080	6.7

BL+, β-lactamase-positive; BLNAR, β-lactamase-negative ampicillin-resistant; BLPACR, β-lactamase-positive amoxicillin/clavulanic acid-resistant.