International Journal of Antimicrobial Agents – In press (IJAA-D-14-00232)
(with Supplementary Material)

Characterization of a collection of *Streptococcus pneumoniae* isolates from patients suffering from acute exacerbations of chronic bronchitis: *in vitro* susceptibility to antibiotics and biofilm formation in relation with antibiotic efflux and serotypes/serogroups.

Nathalie M. Vandevelde [a], Paul M. Tulkens [a,*], Yvan Diaz Iglesias [a], Jan Verhaegen [b], Hector Rodriguez-Villalobos [c], Ivan Philippart [d], Julie Cadrobbi [e], Nathalie Coppens [f], An Boel [g], Kristien Van Vaerenbergh [g], Hugo Francart [h], Raymond Vanhoof [i], Giuseppe Liistro [j], Paul Jordens [k], Jean-Paul d'Odemont [l], Yvan Valcke [m], Franck Verschuren [n] and Françoise Van Bambeke [a]

[a] *Pharmacologie cellulaire et moléculaire & Centre de Pharmacie Clinique, Louvain Drug Research Institute, Université catholique de Louvain, Brussels;* [b] *Laboratorium microbiologie, Universitair Ziekenhuis Gasthuisberg, Leuven;* [c] *Laboratoire de microbiologie, Cliniques universitaires St Luc, Brussels;* [d] *Laboratoire de microbiologie, Centre hospitalier régional Mons-Warquignies, Warquignies;* [e] *Laboratoire de microbiologie, Clinique et Maternité Ste Elisabeth, Namur;* [f] *Service de pneumologie, Clinique et Maternité Ste Elisabeth, Namur;* [g] *Laboratorium microbiologie, Onze Lieve Vrouw Ziekenhuis, Aalst;* [h] *Laboratorium microbiologie, Algemeen Ziekenhuis Nikolaas, St Niklaas;* [i] *Institut Scientifique de Santé Publique, Brussels;* [j] *Service de pneumologie, Cliniques universitaires St Luc, Brussels;* [k] *Pneumologie, Onze Lieve Vrouw Ziekenhuis, Aalst;* [l] *Service de pneumologie, Centre hospitalier régional Mons-Warquignies, Warquignies;* [m] *Longziekten en allergieën, Algemeen Ziekenhuis Nikolaas, St Niklaas;* [n] *Service des Urgences, Cliniques universitaires Saint-Luc, Brussels*

Running title: *S. pneumoniae*, COPD, serotype, biofilm and resistance

* Corresponding author: Tel: + 32 2 764 7371

E-mail address: tulkens@facm.ucl.acbe (P.M. Tulkens)

Keywords: Chronic obstructive lung disease (COPD); acute exacerbations of chronic bronchitis (AECB); *S. pneumoniae*; susceptibility; serotype; biofilm; efflux

Paper metrics:

Text Word count (from Abstract [included] to References [excluded]): 3638 (max. 4000)

References: 30 (max. 30)

Abstract: 249 words (max. 250)

Figures: 5

Tables: 3

Supplementary material: 4 Figures and 1 Table

A B S T R A C T

The correlation between *S. pneumoniae* serotypes, biofilm production, susceptibility to antibiotics and drug efflux in isolates from patients suffering from acute exacerbations of chronic bronchitis (AECB) remains largely unexplored. Using 101 isolates collected from AECB patients for whom partial (n=51) or full (n=50) medical details were available, we determined the serotypes (ST)/serogroups (SG) (Quellung test), the antibiotic susceptibility pattern (MIC [microdilution] with EUCAST and CLSI interpretive criteria), and the ability to produce biofilm *in vitro* (10 days model; crystal violet staining). The majority of patients were 55-75 years old and <5% of them were vaccinated against *S. pneumoniae*. Fifty-four percent showed high severity scores (GOLD 3-4), and comorbidities were frequent including hypertension (60%), cancer (24%) and diabetes (20%). Alcohol and/or tobacco dependence was >30%. Isolates of SG6-11-15-23, known for large biofilm production and for causing chronic infections, were the most prevalent (>15% each), but other isolates also produced biofilm (SG9-18-22-27 and ST8-20 being most productive), except SG7, SG29 and ST5 (<2% of isolates each). Resistance (EUCAST breakpoints) was 8-13 % for amoxicillin and cefuroxime, 35-39% for macrolides, 2-8 % for fluoroquinolones, and 2% for telithromycin. ST19A isolates showed resistance to all antibiotics, ST14 to all except moxifloxacin, SG9 and SG19 to all except telithromycin, moxifloxacin and ceftriaxone (SG19 only). Solithromycin and telithromycin MICs were similar. No correlation was observed between biofilm production and MIC or efflux (macrolides, fluoroquinolones). *S. pneumoniae* serotyping may improve AECB treatment by avoiding antibiotics with predictable low activity, but is not predictive of biofilm production.

1. Introduction

Chronic obstructive pulmonary disease (COPD) remains one of the major causes of morbidity and mortality worldwide, occupying the 4th place for death since 2000 and predicted to reach the 3^{d} one in 2020 [1;2]. At increasingly closer intervals, COPD patients suffer from acute exacerbations of chronic bronchitis (AECB), which contribute to alteration of their respiratory function. These episodes are characterized by increased dyspnoea, coughing, and sputum production witnessing infections of the airways [3]. Bacterial pathogens are found in 50 to 80% of cases of AECB [4], with *Streptococcus pneumoniae* being one of the dominant species [1;4]. Recurrence of infections associated with bacterial persistence results in frequent antibiotic courses. This favours the emergence of multi-resistance of *S. pneumoniae* [5] through a variety of non-mutually exclusive mechanisms such as alterations in the antibiotic targets for β-lactams and macrolides and over-expression of efflux pumps for macrolides and fluoroquinolones [6]. Biofilm formation also favours the persistence of *S. pneumoniae* in the airways [7]. Up to 80% of chronic infections involve pneumococcal growth and survival within biofilms [8;9], in direct relation to the ability of this organism to colonize the nasopharynx [10], which may be dependent on its serotype (ST) /serogroup (SG) [11]. While more than 90 distinct ST have been described for *S. pneumoniae* [12], few studies have attempted to examine which correlations exist between ST and/or SG and the ability to form thick biofilms in clinical isolates from patients with COPD [1]. Moreover, none of these studies have extended the correlations to key properties of the isolates such as their susceptibility to commonly recommended antibiotics and the expression of efflux transporters. Since efflux is critical in other bacteria for liberating quorum sensing signalling molecules involved in biofilm formation [13], we also investigated the relationship between the ability of *S. pneumoniae* to build up biofilm and the presence of phenotypic efflux for macrolides and ciprofloxacin.

In the present report, we show (i) that the susceptibility of *S. pneumoniae* isolates from COPD patients to β-lactams and fluoroquinolones is lower than what was seen for patients with a confirmed diagnostic of bacterial community-acquired pneumonia [14], (ii) that most of these isolates produce large amounts of biofilm irrespective of their ST/SG, and (iii) that there is no correlation between ability of biofilm formation *in vitro* and susceptibility to antibiotics or efflux towards macrolides or fluroquinolones amongst the isolates investigated.

2. Materials and Methods

2.1. General outline of the clinical study, patient's selection and medical data acquisition

A first series of isolates consisted of 48 non-duplicate *S. pneumoniae* strains obtained between March 2006 and December 2008 from patients with a declared diagnosis of AECB and was assembled at the Belgian Scientific Institute of Public Health, Brussels, Belgium. Samples from this collection were equally distributed between the Belgian provinces in relation to their population. The second series of isolates consisted of 53 non-duplicate strains obtained in a prospective fashion between November 2010 and May 2013. For this purpose, 5 hospitals (one teaching and 4 non-teaching) were contacted and asked to enrol patients with a suspicion of AECB whether self-referred or referred by a general practitioner (GP). Patients were enrolled upon obtaining a sample of sputum from the lower respiratory tract fulfilling the microbiological interpretive criteria of an acceptable specimen for culture (abundance of white blood cells [WBCs], few epithelial cells at low-power magnification and ≥10–25 WBCs with no epithelial cells under 1000× magnification). Only patients with samples yielding a positive culture for *S. pneumoniae* and with a confirmed diagnosis of AECB based on Anthonisen's criteria [3] were retained. For 50 of these patients, the whole medical data could be collected and was anonymised. Patients were stratified based on the severity scores A2 (1 to 4 classification) of the 2013 edition of "Global Initiative for Chronic Obstructive Lung Disease" (GOLD) report [15], gender, age, length of hospitalization, geographical location, co-morbidities, smoking habit and therapeutic treatment at admission. Smoking habits were obtained from patient's declaration. Tobacco usage was converted into "pack x year" units by multiplying the number of packs smoked per day by the number of years as a smoker (using a threshold of > 20 for increased risk of tobacco-related cancer [16]).

2.2. Bacterial strains and growth conditions

After identification in each clinical laboratory, strains were stored at −80°C for transfer to a central laboratory until used for our experiments. Confirmation of identification was made by growth inhibition by optochin (Oxoid Ltd, Basingstoke, UK) and serotyping was performed as previously described [17]. ATCC 49619 strain (capsulated ST19F [18]) was used for quality control in each set of experiments. All strains were grown on Mueller Hinton blood agar plates supplemented with 5% defibrinated horse blood incubated at 37°C in a 5% CO_2 atmosphere.

2.3. Susceptibility testing

Minimal inhibitory concentrations (MICs) were determined by microdilution following the guidelines of the Clinical and Laboratory Standards Institute (2013 edition) [19]. MICs were read after 18-24h of incubation at 37°C. To improve accuracy, concentrations at half a value of each standard geometric progression were used as previously described [14] over the whole concentration range investigated. The MIC values were categorized as susceptible, intermediate or resistant according to the CLSI and the EUCAST interpretative breakpoints [19;20].

2.4. Assessment of efflux phenotypes

The efflux resistance phenotype to macrolides was determined by examining the dissociation of susceptibilities between clarithromycin and clindamycin (substrate and non-

substrate of the macrolide efflux transporters, respectively [14]). Efflux of fluoroquinolones was detected by the decrease in MIC upon addition of reserpine (10 mg/L), an inhibitor of both PatA/B and PmrA fluoroquinolone efflux transporters in *S. pneumoniae* [14].

2.5 In vitro development of biofilms and determination of biofilm mass

Ninety-six well plates (VWR, Radnor, PA; European cat. number 734-2327) were used as support for the biofilm growth. In each well, 25 µL of bacterial culture (OD_{620nm} = 0.1) were added to 175 µL of CA-MHB supplemented with 5% lysed horse blood (Oxoid) and 2% glucose as previously described [18]. Biofilm development was obtained by incubation for 2 to 10 days at 37°C in a 5% CO_2 atmosphere with medium replacement every 48 h. Biofilms examined after 2 or 10 days are referred as young and mature biofilms, respectively. Biofilm mass was evaluated by staining with crystal violet followed by measuring its absorbance exactly as previously described [18]. Each isolate was tested twice at different dates, with each assay using 3 to 6 measures. The mean coefficient of variation of our assay was 12.3 % (extremes: 0.01 to 33.1). Data of all determinations for each isolate were pooled, and ST belonging to a given SG were regrouped after observing no significant differences in their capacity to form biofilm.

2.6. Antibiotics and other products

Amoxicillin, cefuroxime, and ceftriaxone were obtained as the corresponding branded product for human parenteral use complying with the prescriptions of the European Pharmacopoeia (> 90% purity) and distributed for clinical use in Belgium as CLAMOXYL® and ZINACEF® by GlaxoSmithKline s.a/n.v. (Genval, Belgium), and as ROCEPHINE® by Roche s.a/n.v., Brussels, Belgium. Clindamycin hydrochloride (potency 92.1%) was

obtained from Sigma-Aldrich, Saint-Louis, MO. Clarithromycin and azithromycin (potencies 100%) were from Teva Pharmaceutical Industries (Petah Tikva, Israel); telithromycin (potency 100%) and levofloxacin hemihydrate (potency 97.5%) from Sanofi-Aventis Deutschland GmbH (Frankfurt, Germany), solithromycin (potency 100%) from Cempra Pharmaceuticals (Chapel Hill, NC); and moxifloxacin chlorhydrate (potency 90.9%) from Bayer AG (Leverkusen, Germany). Reserpine was obtained from Fluka (Buchs, Switzerland). All other products were obtained from Sigma-Aldrich or E. Merck AG (Darmstadt, Germany).

Statistical analyses

Unpaired t-test, one-way ANOVA and contingency tables were made with GraphPad Prism® 4.02 and GraphaPad Instat® 3.10 (GraphPad software, San Diego, CA) and recursive partitioning analyses with JMP® 10.0.2 (SAS Institute Inc., Cary, NC).

3. Results

3.1. Patient's main characteristics

Table 1 shows the demographic characteristics of the whole patient population. Most patients were between 55 and 75 years old. For samples prospectively collected, two hospitals provided a number of samples markedly above the average (in proportion to their bed capacity) due to their location in or close to industrial areas. Most of these patients were living at home prior hospitalization and were smokers (2/3 active). Most patients remained hospitalized after reporting. The severity of their disease was equally distributed between low (1-2) and high (3-4) GOLD scores. Comorbidity percentages ranged from 20 to 30% for diabetes, cancer and alcoholism, and to 60% for arterial hypertension.

3.2. Correlations between demographic, clinical and pharmaceutical parameters

Associations between demographic factors, severity of disease, comorbidities, and drug usage were examined in 50 patients for whom full medical records were available. Table 2 shows that the length of hospitalization was clearly correlated with the severity of the disease and with tobacco dependence. Aged patients had a more severe obstructive syndrome and were more often hypertensive, were poorly vaccinated, and showed less incidence of alcoholism.

3.3. Serotype (ST)/ serogroup (SG) analyses

Serotyping was performed on all isolates (n=101). Figure 1 (upper panel) shows the distribution of the ST/SG amongst the two successive series of isolates (2006-2008 and

2010-2013) stratified by level of coverage (partial of total) with the PCV7 and PPV23 vaccines (the two vaccines in usage at the time during which most isolates were obtained), and for each of these groups by frequency. While there were some changes in ST/SG frequencies between the two series of isolates, SG6 and SG23 were the most prevalent throughout. The second series of isolates contained also a large proportion of SG11 and SG15 strains. There was no marked heterogeneity in ST/SG between the contributing regions (based on patients' living place records; see Figure SP1 in the Supplementary Material). Globally speaking, only 5% of patients hospitalized during the 2010-2013 period had been vaccinated but most isolates were actually from a ST/SG not fully covered by the two vaccines examined (adding the ST covered by the PCV13 did not much change this pattern). Figure 1B (lower panel) shows the ST/SG of all isolates when stratified as a function of their ability reported in the literature of being (i) high biofilm producers and to cause chronic infections [1;9] (ii) low biofilm producers and to cause acute infections [9;14;21] or (iii) with undescribed ability for these characteristics. About half of the isolates were found in the first group.

3.4. Characterization of the in vitro susceptibility to antibiotics and correlation with the severity of the disease and serotypes/serogroups

All strains were characterized for their susceptibility to antibiotics focusing on (i) drugs commonly recommended for the treatment of bacterial exacerbations of chronic bronchitis in Belgium ([22]; namely, for β-lactams, amoxicillin, cefuroxime and ceftriaxone; for macrolides, clarithromycin and azithromycin; and for fluoroquinolones, moxifloxacin and levofloxacin). We also added two ketolides because of their reported good activity against *S. pneumoniae* resistant to macrolides [23] and tested clindamycin and ciprofloxacin for efflux diagnostic purposes. Cumulative MIC distributions are shown in Figure 2 (see also Figure SP2 in the

supplementary material). MIC_{50} and MIC_{90} and analysis of the MIC profiles according to both EUCAST and CLSI interpretive criteria are presented in Table 3 A1-1 for all strains and for each collection individually. There was no significant differences in susceptibilities between the two strains collections. Moreover, largely similar distributions were observed for all three β-lactams except in the zones corresponding to their clinical breakpoints, with 6 to 8 % of all isolates falling into the intermediate category for the 3 drugs, and 8-13 % in the full resistant category for amoxicillin and cefuroxime, but only 1 % for ceftriaxone, based on EUCAST interpretive criteria (using CLSI interpretive criteria essentially resulted in having no or only 1 isolate in the intermediate category). For macrolides, isolates within the first half of the cumulative distribution were globally more susceptible to clarithromycin than azithromycin. The difference, however, largely disappeared for isolates with higher MICs. Resistance rates to both macrolides reached 35-39% or 27-29% using EUCAST or CLSI interpretive criteria, respectively. Isolates categorized as having intermediate susceptibility were rare (6 to 8 %) and observed only if using CLSI interpretive criteria. Cumulative clindamycin MIC distribution was essentially similar to that of azithromycin, indicating that most of the strains categorized as resistant to this macrolide were of the MLS_B type. For fluoroquinolones, moxifloxacin was systematically more active than levofloxacin, but due to the lower breakpoint set by EUCAST for moxifloxacin, more strains (8%) were categorized as being resistant compared to levofloxacin (3%). No meaningful difference with respect to susceptibility was seen if using CLSI breakpoints. For ciprofloxacin, the majority of isolates had MIC in the intermediate category of EUCAST (no breakpoint set for CLSI). We examined the occurrence of efflux for ciprofloxacin by addition of reserpine. As illustrated in Figure 3, there was a shift of the whole population towards lower MICs, with 38% and 35% of the isolates showing a 1 or ≥ 2 $\log_2$ dilution changes, respectively. Lastly, the cumulative MIC distributions of telithromycin and solithromycin were very similar, with few (EUCAST) or no isolate (CLSI) categorized as resistant (breakpoints for solithromycin have not yet been set).

We then examined the correlation between the severity of the disease and resistance of the isolates to amoxicillin and cefuroxime by stratifying patients by low (1 and 2) and high (3 and 4) GOLD scores, respectively, and performing a recursive partitioning analysis *vs*. the MICs of their isolates. While this allowed determination of a best MIC split value at 4 mg/L for amoxicillin and at 1 mg/L for cefuroxime, this was not statistically significant (LogWorth values = 0.18 and 0.17, respectively, corresponding to p-values of 0.66 and 0.68). This was further confirmed by 2 x 2 contingency table analysis (p-values of 0.18 and 0.31).

Figure 4 shows the susceptibilities of the strains grouped by ST/SG for each of the antibiotics tested in Figure 2 and ranked by their mean MIC value (from highest to lowest) with the corresponding EUCAST and CLSI intermediate susceptibility zones. Although the ranking of ST/SG varied between antibiotics, global trends emerged with ST14, ST19A, SG9, SG29, and SG15 having the highest mean MIC values for β-lactams, ST19A, SG9, ST14, SG 19, and SG 33 for macrolides, ST14, ST19A, SG19, SG9, and SG17 for both ketolides, and ST19A, SG33, ST4, ST5, and SG15 for moxifloxacin and levofloxacin, respectively. All ST19A isolates had MIC above the EUCAST R breakpoint for amoxicillin, cefuroxime, clarithromycin, azithromycin, moxifloxacin and levofloxacin. Conversely, SG7 and ST8 isolates were fully susceptible to all antibiotics (see Table SP1 for a ranking of all isolates stratified by ST/SG, MICs and resistance pattern).

3.5. Biofilm production in relation to pneumococcal susceptibility to antibiotics (MICs and occurrence of efflux)

No significant correlation (one-way ANOVA with Tukey post-test) was seen between biofilm production (crystal violet staining), and antimicrobial susceptibility (MIC) for each of the antibiotics tested (see data in Figure SP3 in the Supplementary Material). Likewise, there was no statistically significant correlation (unpaired t-test Welch corrected) between

biofilm production and expression of efflux for macrolides or for ciprofloxacin (see data in Figure SP4 in the Supplementary Material).

3.6. Characterization of biofilm formation, in relation to pneumococcal serotype/serogroup

Figure 5 shows the amount of biomass observed at day 10 for the reference strain ATCC 49619 (ST19F) and for the clinical isolates ranked by inverse amount of production and regrouped by ST/SG. Globally, all isolates, except SG7, SG29, and ST5 produced biofilm in a similar fashion to the reference strains ATCC 49619 with SG22, ST20, SG9, SG27, SG18, and ST8 being the most productive (SG9 and SG22 have been previously reported to be associated with chronic infections [1;9]). Conversely, SG7, SG29, and ST5 were the lowest producers in our collection, and these have been reported as poor producers with ST5 and SG7 claimed to be associated with acute infections [9].

4. Discussion

To the best of our knowledge, the present study is one of the first examining in a systematic fashion and correlating the serotypes/serogoups, the resistance pattern and the *in vitro* biofilm formation ability of *S. pneumoniae* isolates collected from COPD patients with a confirmed diagnosis of AECB. The number of patients and corresponding microbiological samples were limited due to the design of the study, which implied access to the medical history of the patients on the one hand, and the low rate of successful isolation of *S. pneumoniae* in this patient population.

Within these limits, we first confirm and strengthen the close link existing between the severity of COPD and cardiovascular and diabetes pathologies already described in the literature [2;24]. A decreased ability to breathe reduces, indeed, mobility, favoring thereby a sedentary lifestyle and weight gain. We next confirm that β-lactams, levofloxacin and moxifloxacin maintain useful activity against *S. pneumoniae* isolates from this population, although to a lesser extent than what we saw for isolates obtained in Belgium from patients suffering from community acquired pneumonia (CAP) during the 2006-2009 period [14]. While the two patient's populations cannot be directly compared, they nevertheless originate from the same small geographical area, suggesting that we deal, at least partially, with distinct bacterial populations. The lower susceptibility of isolates in the present patients' population probably reflects the large and prolonged use of antibiotics before they eventually report to the hospital (most patients suffering from CAP had not received any antibiotic when enrolled [14]). Our findings, therefore, call for caution against the non-documented use of β-lactams (especially cefuroxime) in COPD patients. For macrolides, the situation is even more critical as resistance patterns are appalling. Telithromycin, recommended for treatment of infections caused by macrolide-resistant strains, maintains a very high level of activity, probably because of its very low use in Belgium due to its non-inclusion as a recommended

antibiotic for the treatment of AECB in local guidelines for the treatment of AECB [22] (solithromycin is still an experimental compound). Of note is the larger prevalence of efflux for ciprofloxacin, especially if considering the proportion of isolates where an MIC change of ≥ 2 $\log_2$ dilutions could be observed upon addition of reserpine. While moxifloxacin and levofloxacin were not significantly affected, we know from previous studies that efflux for ciprofloxacin can herald similar changes in MICs for other fluroquinolones proposed for the treatment of respiratory tract infections such as gemifloxacin and garenoxacin [25].

The analysis of the susceptibility pattern in relation to their ST/SG shows that some of them (ST19A, ST14, SG9, and SG9) have a high level of resistance to β-lactams and macrolides (and decreased susceptibility to ketolides [ST19A and ST14 only]) but not to moxifloxacin (excepted for ST19A). This is largely akin to our previous findings for isolates from patients suffering from CAP [14], as well as with data from other countries in Europe [26] or the Far East [27;28]. Thus, determination of the prevalent ST/SG in patients may help in fine-tuning therapy by avoiding the use of antibiotics that are known to be poorly effective. The determination of the nucleotide sequence of the α-helical region of the pneumococcal surface protein (PspA), proposed also as a predictor of multiresistance [28], could not be examined in the context of the present study.

Turning now our attention to biofilm production, we see that most isolates obtained in this study were high producers (like the reference strain ATCC 49619 [ST19F] also known as a high producer [1;18]). In the present study, careful attention was paid to obtain data as reproducible as possible on biofilm production. Thus, A3 by and large, biofilm production seems to be a property shared by most isolates from COPD patients, suggesting that previous reports linking the poor production of biofilm by some of these strains to more acute infections may need some revisiting [9]. Conversely, we confirm that strains previously reported to be largely associated with acute infections, such as ST8 and ST3 [1], are indeed poor biofilm producers.

Lastly, we show no correlation between biofilm formation and intrinsic susceptibility or expression of macrolide or ciprofloxacin efflux in the isolates studied. This could explain why the determination of susceptibility by the reference methods (which use planktonic cells) may fail to truly predict the clinical outcome. Eradication of bacteria from biofilms, indeed, requires much larger antibiotic concentrations than those of breakpoints, especially if considering the activity of β-lactams and macrolides against mature biofilms [18]. The lack of correlation between biofilm production and ciprofloxacin efflux, which is in contrast with what is observed in Gram-negative bacteria, probably relates to differences in quorum sensing signalling pathways and the secretion of the corresponding mediators [13;29;30].

Acknowledgments

NMV is *boursière* of the Belgian *Fonds pour la Recherche dans l'Industrie et l'Agriculture* (FRIA) and FVB is *Maître de Recherches* of the Belgian *Fonds de la Recherche Scientifique* (F.R.S.-FNRS). This work was supported by the *Fonds de la Recherche Scientifique* (grants 3.4530.12 and T.0134.13) and the Interuniversity Attraction Poles Programme initiated by the Belgian Science Policy Office (program IAP P7/28) and by grants-in-aid from Cempra Pharmaceuticals and Bayer AG. F.V.B. and P.M.T. have received consultancy fees from Bayer AG.

Ethical approval

The entire study protocol was submitted and approved by the *Commission d'éthique facultaire* of the *Université catholique de Louvain* (unique Belgian no. 40320109783). The ethical committee of the participating hospital also gave their approval for the specific studies and access to medical files in the corresponding Institutions.

Supplementary data

Figures SP1 through SP4 and Tables SP1 are available as Supplementary

References

(1) Domenech A, Ardanuy C, Pallares R, Grau I, Santos S, De la Campa AG et al. Some pneumococcal serotypes are more frequently associated with relapses of acute exacerbations in COPD patients. PLoS One 2013; 8:e59027. Pubmed: PM:23536850.

(2) Sin DD, Anthonisen NR, Soriano JB, Agusti AG. Mortality in COPD: Role of comorbidities. Eur Respir J 2006; 28:1245-1257. Pubmed: PM:17138679.

(3) Anthonisen NR, Manfreda J, Warren CP, Hershfield ES, Harding GK, Nelson NA. Antibiotic therapy in exacerbations of chronic obstructive pulmonary disease. Ann Intern Med 1987; 106:196-204. Pubmed: PM:3492164.

(4) Eller J, Ede A, Schaberg T, Niederman MS, Mauch H, Lode H. Infective exacerbations of chronic bronchitis: relation between bacteriologic etiology and lung function. Chest 1998; 113:1542-1548. Pubmed: PM:9631791.

(5) Bronzwaer SL, Cars O, Buchholz U, Molstad S, Goettsch W, Veldhuijzen IK et al. A European study on the relationship between antimicrobial use and antimicrobial resistance. Emerg Infect Dis 2002; 8:278-282. Pubmed: PM:11927025.

(6) Van Bambeke F, Reinert RR, Appelbaum PC, Tulkens PM, Peetermans WE. Multidrug-resistant Streptococcus pneumoniae infections: current and future therapeutic options. Drugs 2007; 67:2355-2382. Pubmed: PM:17983256.

(7) Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science 1999; 284:1318-1322. Pubmed: PM:10334980.

(8) Moscoso M, Garcia E, Lopez R. Pneumococcal biofilms. Int Microbiol 2009; 12:77-85. Pubmed: PM:19784927.

(9) Dagan R, Leibovitz E, Greenberg D, Bakaletz L, Givon-Lavi N. Mixed Pneumococcal-Nontypeable Haemophilus influenzae Otitis Media Is a Distinct Clinical Entity With Unique Epidemiologic Characteristics and Pneumococcal Serotype Distribution. J Infect Dis 2013; 208:1152-1160. Pubmed: PM:23842949.

(10) Marks LR, Parameswaran GI, Hakansson AP. Pneumococcal interactions with epithelial cells are crucial for optimal biofilm formation and colonization in vitro and in vivo. Infect Immun 2012; 80:2744-2760. Pubmed: PM:22645283.

(11) Brueggemann AB, Griffiths DT, Meats E, Peto T, Crook DW, Spratt BG. Clonal relationships between invasive and carriage Streptococcus pneumoniae and serotype- and clone-specific differences in invasive disease potential. J Infect Dis 2003; 187:1424-1432. Pubmed: PM:12717624.

(12) Li Y, Weinberger DM, Thompson CM, Trzcinski K, Lipsitch M. Surface Charge of Streptococcus pneumoniae Predicts Serotype Distribution. Infect Immun 2013. Pubmed: PM:24082068.

(13) Sivaneson M, Mikkelsen H, Ventre I, Bordi C, Filloux A. Two-component regulatory systems in Pseudomonas aeruginosa: an intricate network mediating fimbrial and efflux pump gene expression. Mol Microbiol 2011; 79:1353-1366. Pubmed: PM:21205015.

(14) Lismond A, Carbonnelle S, Verhaegen J, Schatt P, De Bel A, Jordens P et al. Antimicrobial susceptibility of Streptococcus pneumoniae isolates from vaccinated and non-vaccinated patients with a clinically confirmed diagnosis of community-acquired pneumonia in Belgium. Int J Antimicrob Agents 2012; 39:208-216. Pubmed: PM:22245497.

(15) Global Initiative for Chronic Obstructive Lung Disease. http://www.goldcopd.org/uploads/users/files/GOLD_Pocket_2013_Mar27.pdf. Last accessed: 2013.

(16) Lubin JH, Caporaso N, Wichmann HE, Schaffrath-Rosario A, Alavanja MC. Cigarette smoking and lung cancer: modeling effect modification of total exposure and intensity. Epidemiology 2007; 18:639-648. Pubmed: PM:17700253.

(17) Flamaing J, Verhaegen J, Vandeven J, Verbiest N, Peetermans WE. Pneumococcal bacteraemia in Belgium (1994-2004): the pre-conjugate vaccine era. J Antimicrob Chemother 2008; 61:143-149. Pubmed: PM:17999974.

(18) Vandevelde NM, Tulkens PM, Van Bambeke F. Antibiotic activity against naive and induced Streptococcus pneumoniae biofilms in an in vitro pharmacodynamic model. Antimicrob Agents Chemother 2013. Pubmed: PM:24342635.

(19) Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. 23th informational supplement (MS100-S22): Clinical and Laboratory Standard Institute, Wayne, PA (2013).

(20) Anonymous. Breakpoint tables for interpretation of MICs and zone diameters (version 4.0). http://www.eucast.org. Last accessed: 2014.

(21) Almeida ST, de Lencastre H, Sa-Leao R. Epidemiology and Population Structure of Serotypes 1, 5 and 7F Carried by Children in Portugal from 1996-2010 before Introduction of the 10-Valent and 13-Valent Pneumococcal Conjugate Vaccines. PLoS One 2013; 8:e75442. Pubmed: PM:24058686.

(22) Anonymous. Guide belge des traitements anti-infectieux en pratique ambulatoire. http://www.health.belgium.be/eportal/Myhealth/Properuse/Antibiotics/15616531_FR?ie2Term=BAPCOC&ie2section=83. Last accessed: 2008.

(23) Farrell DJ, Sader HS, Castanheira M, Biedenbach DJ, Rhomberg PR, Jones RN. Antimicrobial characterisation of CEM-101 activity against respiratory tract pathogens, including multidrug-resistant pneumococcal serogroup 19A isolates. Int J Antimicrob Agents 2010; 35:537-543. Pubmed: PM:20211548.

(24) Lindberg A, Larsson LG, Ronmark E, Lundback B. Co-morbidity in mild-to-moderate COPD: comparison to normal and restrictive lung function. COPD 2011; 8:421-428. Pubmed: PM:22149402.

(25) Lismond A, Carbonnelle S, Tulkens PM, Van Bambeke F. Efflux of novel quinolones in contemporary Streptococcus pneumoniae isolates from community-acquired pneumonia. J Antimicrob Chemother 2011; 66:948-951. Pubmed: PM:21393137.

(26) Gajic I, Mijac V, Ranin L, Andjelkovic D, Radicevic M, Opavski N. [Invasive isolates of Streptococcus pneumoniae in Serbia: antimicrobial susceptibility and serotypes]. Srp Arh Celok Lek 2013; 141:48-53. Pubmed: PM:23539910.

(27) Liu C, Xiong X, Xu W, Sun J, Wang L, Li J. Serotypes and patterns of antibiotic resistance in strains causing invasive pneumococcal disease in children less than 5 years of age. PLoS One 2013; 8:e54254. Pubmed: PM:23342111.

(28) Hotomi M, Togawa A, Kono M, Ikeda Y, Takei S, Hollingshead SK et al. PspA family distribution, antimicrobial resistance and serotype of Streptococcus pneumoniae isolated from upper respiratory tract infections in Japan. PLoS One 2013; 8:e58124. Pubmed: PM:23483982.

(29) Vallet I, Olson JW, Lory S, Lazdunski A, Filloux A. The chaperone/usher pathways of Pseudomonas aeruginosa: identification of fimbrial gene clusters (cup) and their involvement in biofilm formation. Proc Natl Acad Sci U S A 2001; 98:6911-6916. Pubmed: PM:11381121.

(30) Li YH, Tian X. Quorum sensing and bacterial social interactions in biofilms. Sensors (Basel) 2012; 12:2519-2538. Pubmed: PM:22736963.

Table 1. Patients' demographic characteristics and environmental and medical conditions

1. Whole population (2006-2008 and 2010-2013) (n=101)

age and no. enrolled	mean	<55 y	≥55 to <65	≥65 to <75	≥75 to <85	≥85 y
	67.2 ± 12.7	11 (10.9%)	33 (32.7%)	30 (29.7%)	23 (22.8%)	4 (3.96%)

2. Prospectively assembled population (2010-2013) (n=53)

hospital	A	B	C	D	E	Total
no. enrolled	8	8	3	20	14	53
% of capacity [a]	0.8	0.95	0.36	3.6	4.7	2.1 ± 1.94

3. Patients from prospectively assembled population and with available medical data (n=50)

general and environmental conditions	gender % (M - F)	living place % (home - nursing home - psychiatric institutions)	smoking habits % [b] (active - former - non smoker - unknown)
	74 - 26	88 - 4 – 8	56 – 28 – 6 -10

disease severity	hospitalization % (Yes – No)	GOLD score % (1/2 – 3/4)
	80 - 20	46 - 54

comorbidities	hypertension [c] %	diabetes [d] %	cancer [e] %	alcoholism [f] %
	60	20	24	30

[a] no. of enrolled patients / no. of beds of the hospital x 100

[b] according to patient's declaration

[c] systolic blood pressure > 120mm Hg

[d] fasting glycaemia > 1.26g/L

[e] tissue biopsies and/or chest x-rays.

[f] according to patient's declaration, evidence at admission (inebriated condition), or presence of alcoholic cirrhosis.

Table 2. Associations between length of hospitalization, age, comorbidities and vaccine serotype coverage (variables #1) with disease severity (GOLD score 3 or 4), prolonged hospitalization, age and tobacco addition (variables #2) in the population of patients with fully accessible medical records (n=50). Associations were tested by means of 2×2 contingency tables (Fisher's exact two-tailed test). The table shows the odd ratios (with their 95% confidence interval) and appear in bold if the p-value is ≤ 0.05 (some associations with a p-value between 0.05 and 0.1 are shown in italic if considered potentially medically important).

	variable # 2			
variable #1	**disease severity** [a]	**hospitalization > 10 days**	**Age > 65 years**	**Tobacco addiction** [b]
	odds ratios (95% confidence interval)			
Hospitalization >10 days	**2.987 (1.242-7.182**) p<0.05		1.289 (0.571-2.91) ns	**3.201 (0.9928-10.322)** p<0.05
Age >65 years	**2.963 (1.296-6.772)** p<0.05	1.289 (0.571-2.91) ns		*0.408 (0.16-1.039)* *p=0.0761*
High blood pressure [c]	0.641 (0.284-1.451) ns	0.835 (0.369 -1.889) ns	**3.947 (1.688-9.233)** p<0.01	*2.545 (0.9643-6.719)* *p=0.07*
Alcoholism [d]	*0.424 (0.180-1.000)* *p=0.0541*	1.128 (0.469 -2.712) ns	**0.087 (0.031-0.246)** p<0.0001	1.071 (0.389-2.952) ns
Cancer [e]	*2.7 (0.966 -7.548)* *p=0.06*	0.767 (0.292 -2.013) ns	*2.700 (0.966 -7.548)* *p=0.06*	*0.353 (0.117 -1.058)* *p=0.07*
Vaccine coverage [f]	1.250 (0.416-3.758) ns	0.490 (0.146-1.649) ns	**0.185 (0.0549-0.625)** p<0.01	1.071 (0.316 -3.634) ns

[a] GOLD score 3 or 4 [15].

[b] > 20 pack x years

[c] systolic blood pressure > 120mm Hg

[d] according to patient's declaration, evidence at admission (inebriated condition), or presence of alcoholic cirrhosis.

[e] tissue biopsies and/or chest x-rays.

[f] Vaccine PCV13 (13-valent pneumococcal conjugate vaccine (Prevenar 13® [Wyeth]) covers serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F and vaccine PPV-23 (23-valent pneumococcal polysaccharide vaccine (Pneumo23® [Sanofi-Pasteur MSD]) covers serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, 33F).

A1-2.**Table 3.** MIC_{50} and MIC_{90} and percentages of susceptible, intermediate and resistant isolates according to EUCAST and CLSI interpretive criteria

Antibiotic	Strains collections	MIC_{50} mg/L	MIC_{90} mg/L	% non-susceptible isolates according to EUCAST [a]		% non-susceptible isolates according to CLSI [b]	
				Breakpoint (S / R) mg/L	% I / R [c]	Breakpoint (S / R) mg/L	% I / R [c]
amoxicillin	**global**	**0.046875**	**2**	**≤0.5 / >2**	**8/8**	**≤2 / ≥8**	**1/4**
	2006 - 2008	0.02344	1.5		10/4		0/0
	2010 - 2013	0.06250	4		6/11		2/9.4
cefuroxime	**global**	**0.03125**	**4**	**≤0.25 / >0.5 [d]**	**6/13**	**≤1 / ≥4 [d]**	**1/7**
	2006 - 2008	0.03125	4		6.3/14.6		2/8.4
	2010 - 2013	0.01563	4		5.7/11.3		0/5.7
ceftriaxone	**global**	**0.03125**	**0.75**	**≤0.5 / >2**	**8/1**	**≤2 / ≥4**	**0/1**
	2006 - 2008	0.03125	2		10.4/2		0/2
	2010 - 2013	0.01563	0.5		5.7/0		0/0
clarithromycin	**global**	**0.03125**	**48**	**≤0.25 / >0.5**	**1/27.7**	**≤0.25 / ≥1**	**7.9/26.7**
	2006 - 2008	0.03125	48		0/31.2		0/30.2
	2010 - 2013	0.03125	64		2/24.5		15.1/22.7
azithromycin	**global**	**0.125**	**64**	**≤0.25 / >0.5**	**1/38.6**	**≤0.5 / ≥2**	**6/28.7**
	2006 - 2008	0.125	64		0/31.2		0/31.2
	2010 - 2013	0.250	128		0/39.6		11.3/26.4
clindamycin [e]	**global**	**0.0625**	**32**	**≤0.5 / >0.5**	**0/35.7**	**na**	**na**
	2006 - 2008	0.046875	16		0/27		na
	2010 - 2013	0.0625	48		0/43.4		na
telithromycin	**global**	**0.015625**	**0.125**	**≤0.25 / >0.5**	**1/2**	**≤1 / ≥4**	**0/0**
	2006 - 2008	0.015625	0.0625		0/2		0/0
	2010 - 2013	0.015625	0.125		2/2		0/0
solithromycin	**global**	**0.01172**	**0.0625**	**na**	**na**	**na**	**na**
	2006 - 2008	0.01172	0.046875		na		na
	2010 -2013	0.00781	0.0625		na		na
moxifloxacin	**global**	**0.125**	**0.375**	**≤0.5 / >0.5**	**0/8**	**≤1 / ≥4**	**3/0**
	2006 - 2008	0.125	0.5		0/6.3		2/0
	2010 - 2013	0.09375	0.375		0/9.4		4/0
levofloxacin	**global**	**1**	**2**	**≤2 / >2**	**0/3**	**≤2 / ≥8**	**2/1**
	2006 - 2008	1	2		0/2		0/0
	2010 - 2013	0.75	1.5		0/4		3.8/2
ciprofloxacin [e]	**global**	**1**	**4**	**≤0.125 / >2**	**82.2/13.8**	**na**	**na**
	2006 - 2008	1	4		87.5/10.4		na
	2010 - 2013	1	4		77.4/17		na

na: not applicable (no breakpoint defined)

[a] European Committee for Antibiotic Susceptibility Testing [20]

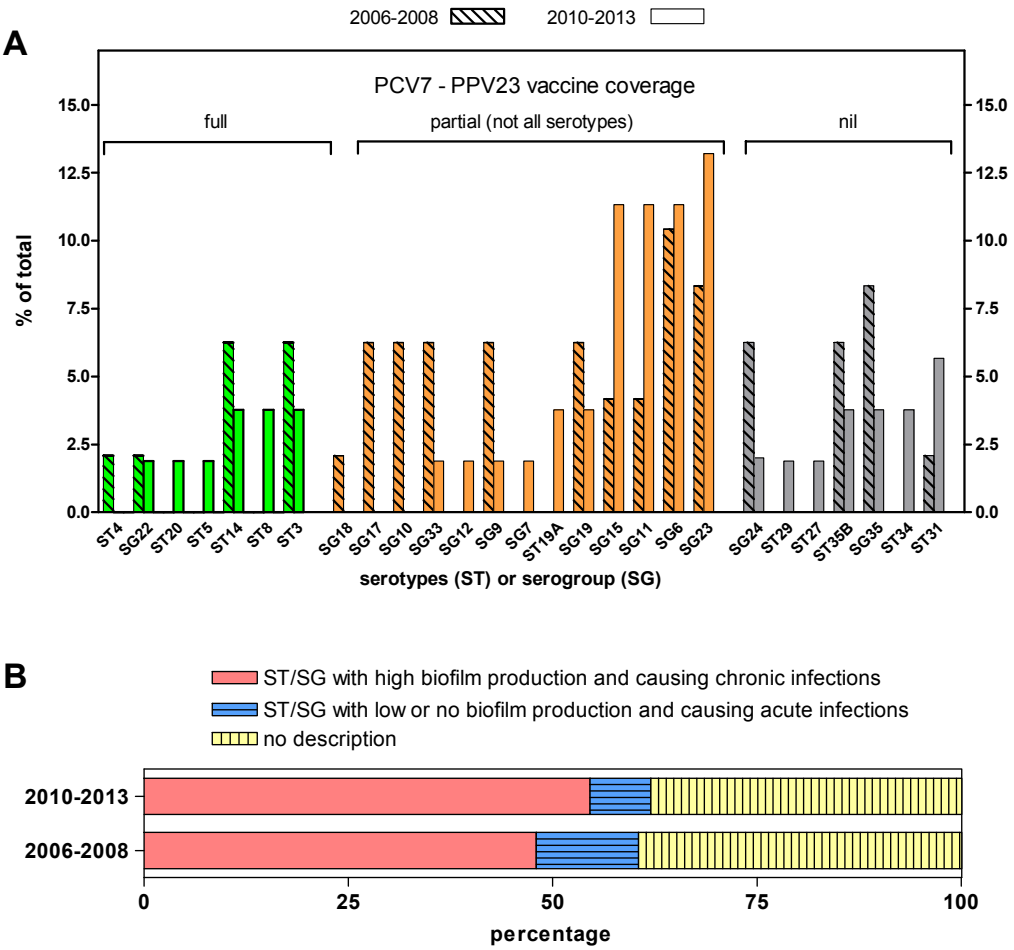
[b] Clinical Laboratory Standard Institute [19]

[c] I: intermediate; R: resistance

[d] oral form (cefuroxime axetil)

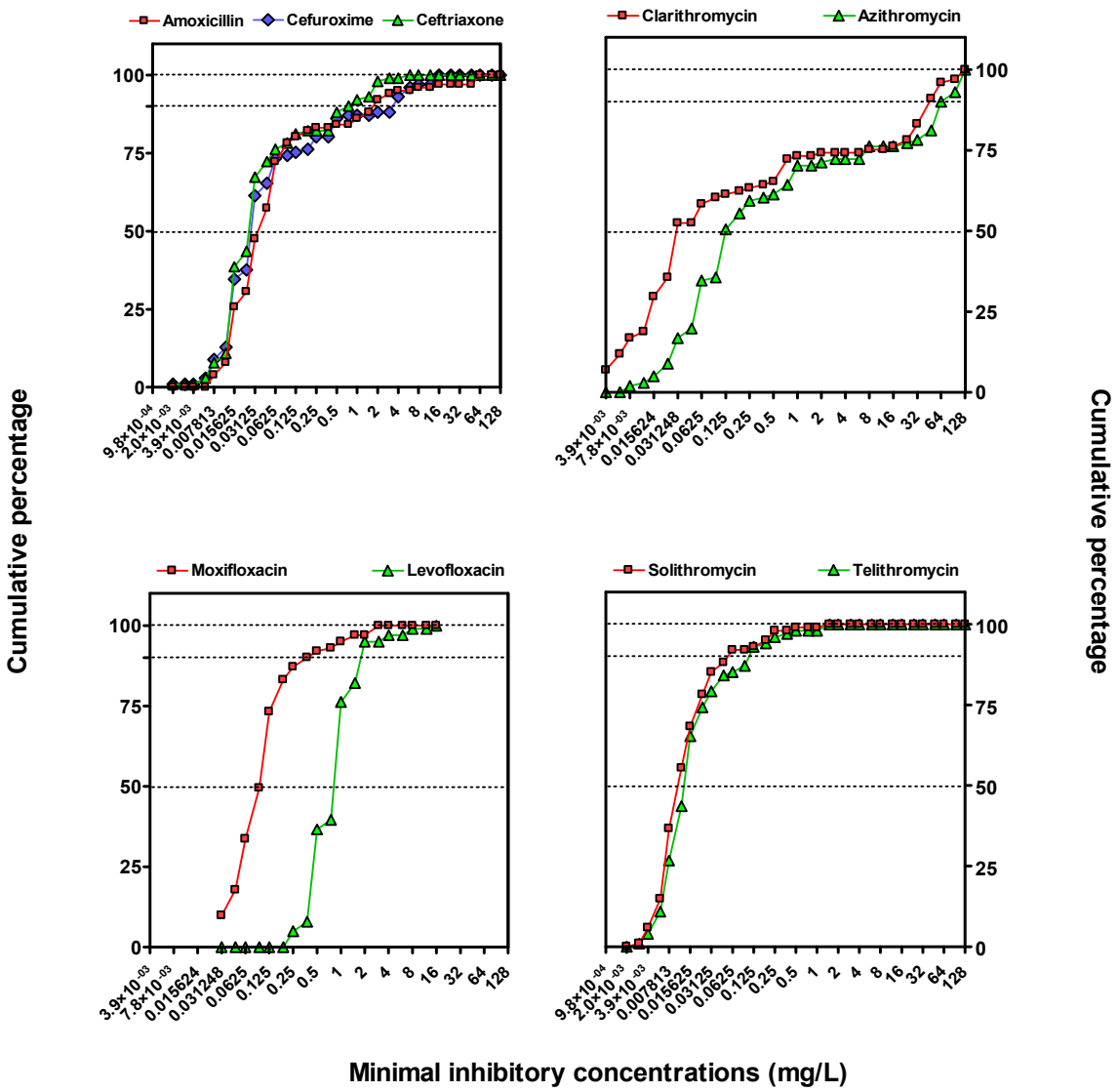
[e] not recommended for clinical use but tested here for epidemiological purposes

Figure 1



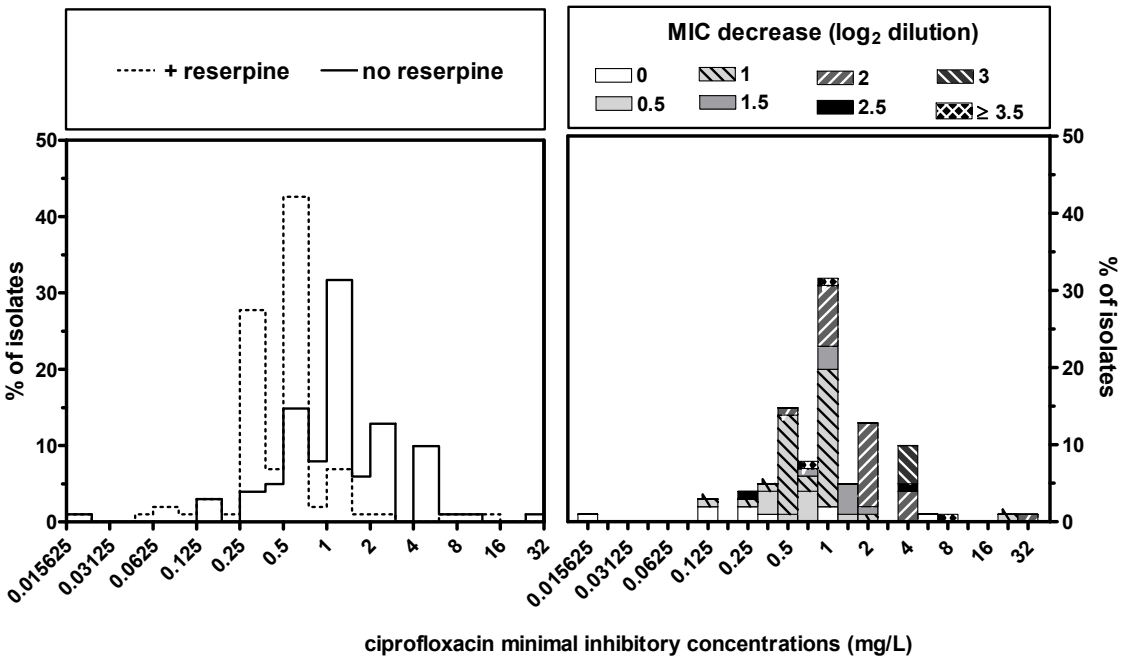
Caption to Figure 1: A (upper panel): distribution of isolates as a function of their period of collection (shaded blocs: 2006-2008; open blocks: 2010-2013) and serotype (ST) /serogroup (SG) and grouped following the vaccine coverage. Vaccine PCV7: 7-valent pneumococcal conjugate vaccine (Prevenar® [Wyeth]) covers ST4, 6B, 9V, 14, 18C, 19F, and 23F (discontinued in 2011); vaccine PCV13 (13-valent pneumococcal conjugate vaccine (Prevenar 13® [Wyeth]) covers ST1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F and vaccine PPV-23 (23-valent pneumococcal polysaccharide vaccine (Pneumo23® [Sanofi-Pasteur MSD]) covers ST1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, 33F). B (lower panel): percentages of ST/SG belonging to high biofilm producers and/or causing chronic infections (red bars; ST/SG 6, 9, 11, 15, 23, 33, 35B), low biofilm producers and/or causing acute infections (blue bars; ST/SG 3, 14, 19A) or with undescribed characteristics (all others; yellow bars; see text for references).

Figure 2



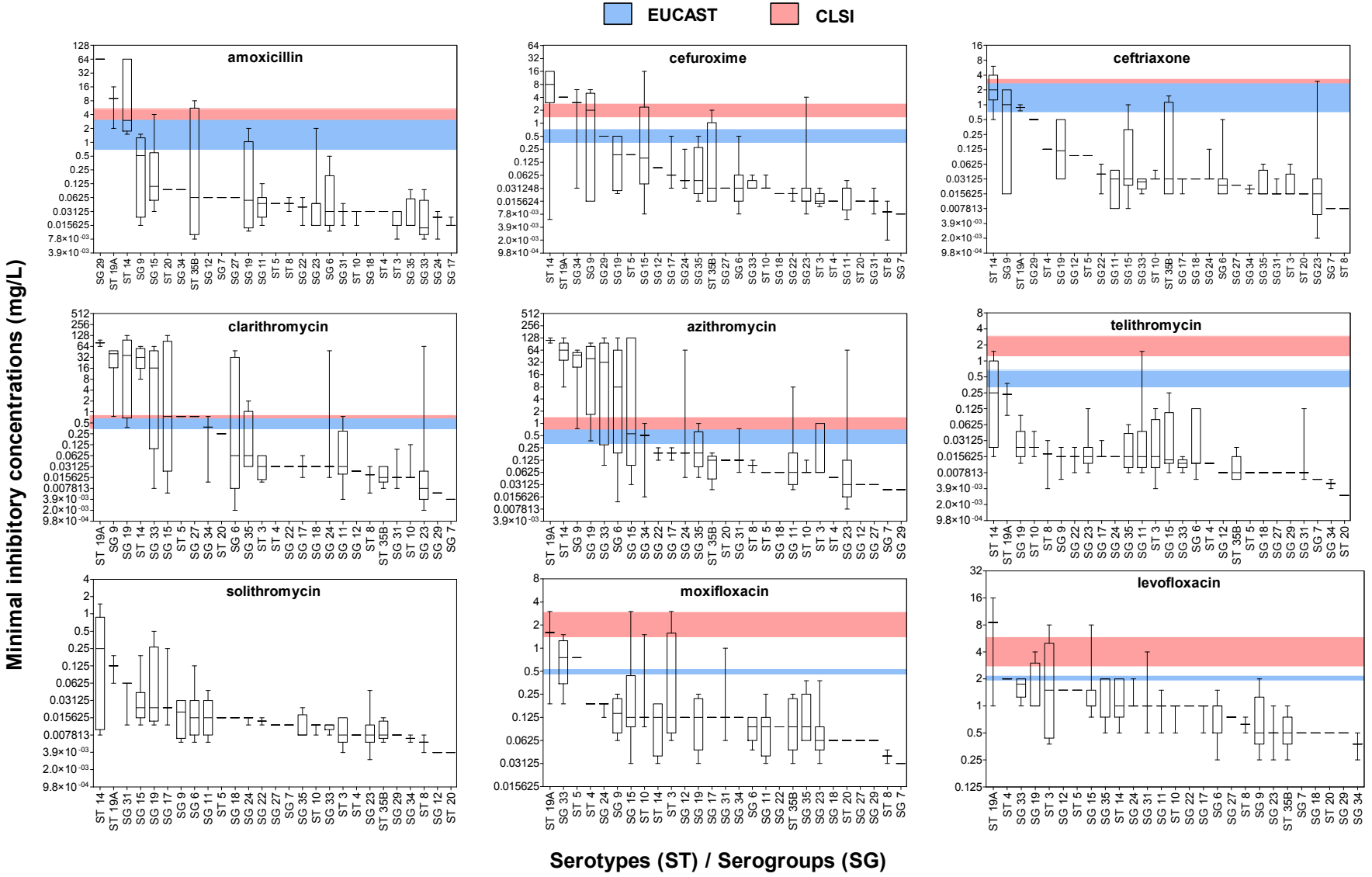
Caption to Figure 2: Minimum inhibitory concentration (MIC) distributions (cumulative percentages) of β-lactams (amoxicillin, cefuroxime and ceftriaxone; upper left panel), macrolides (clarithromycin and azithromycin; upper right panel), fluoroquinolones (moxifloxacin and levofloxacin; lower left panel) and ketolides (telithromycin and solithromycin; lower right panel) for 101 non-duplicate *Streptococcus pneumoniae* isolates from COPD patients. The horizontal dotted lines are drawn at values corresponding to the MIC_{50}, MIC_{90}, and MIC_{100}.

Figure 3



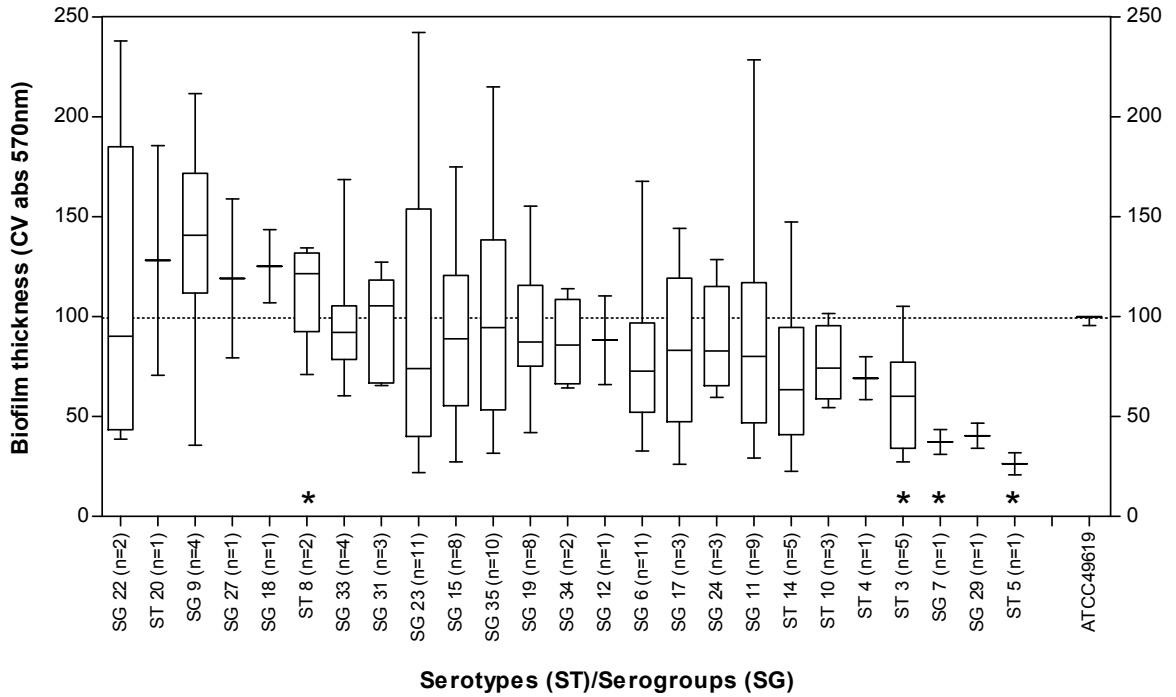
Caption to Figure 3: MIC distribution of ciprofloxacin for all isolates (n=101). Left: MIC distributions determined in the absence (control; plain line) or in the presence (dotted line) of 10 mg/L reserpine (statistical analysis: $p < 0.0001$ when comparing distributions in the absence and in the presence of reserpine by two-tailed paired tests [Wilcoxon signed rank test (non parametric) and t test (parametric)]). Right: reduction of MIC (in blocks of 0.5 $\log_2$ dilutions from 0 to ≥ 3.5 $\log_2$ dilutions) after addition of 10 mg/L reserpine and plotted as a function of the MIC distribution of the isolates in the absence of reserpine.

Figure 4


EUCAST
CLSI
amoxicillin
cefuroxime
ceftriaxone
clarithromycin
azithromycin
telithromycin
solithromycin
moxifloxacin
levofloxacin
Minimal inhibitory concentrations (mg/L)
Serotypes (ST) / Serogroups (SG)

Caption to Figure 4: Antibiotic susceptibility to β-lactams (amoxicillin, cefuroxime and ceftriaxone), macrolides (clarithromycin and azithromycin), ketolides (telithromycin and solithromycin) and fluoroquinolones (moxifloxacin and levofloxacin) for all isolates as a function of their serotype (ST) / serogroup (SG) ranked from less to more susceptible. Data are presented as "Box and whiskers plots" giving the 25, 50 and 75 quartiles (boxes and horizontal line) of the MIC distributions with the lower and upper bars extending from the lowest to the highest MIC value observed. The blue and pink horizontal ribbons show the intermediate zones of clinical susceptibility according the interpretive criteria of EUCAST (from > S to > R; [20]) and CLSI (from >S to < R [19]), respectively (see Table 3 for values; for clarithromycin, the intermediate zone is the same for EUCAST and CLSI; no clinical breakpoint has been set so far for solithromycin).

Figure 5



Caption to Figure 5: Biofilm production after 10 days of culture for all clinical isolates (as a function of their serotype (ST) / serogroup (SG) and ranked from the most to the least productive [using arithmetic means; the box and whiskers show the lowest, the 25 and 75 percentiles, the median and the highest value]; the number of isolates tested for each ST/SG is shown in the abscissa) and of the reference strain ATCC 49619 (ST19F). Each strain was tested twice with 3 to 6 measures each time. Strains with ST belonging to a single SG have been pooled (and marked as SG) after having observed no significant differences between these serotypes. The horizontal dotted line corresponds to the mean value for the reference strain. Strains marked ST correspond to isolates where there was only one serotype. ST/SG reported in the literature [1;9] as causing acute infections are marked with an asterisk.

Supplementary material

Table SP1. Isolates ranking for each serotype (ST)/serogroup (SG) as a function of their MICs. ST/SG are ranked from the least susceptible (small ranking value) to the most susceptible (high ranking value).

β-lactams [a]		Macrolides [b]		Ketolides [c]		Fluoroquinolones [d]	
ST/SG [e]	Ranking [f]	ST/SG [e]	Ranking [f]	ST/SG [e]	Ranking [f]	ST/SG [e]	Ranking [f]
ST 14	5	ST 19A	2	ST 14	2	ST 19A	2
ST 19A	7	SG 9	5	ST 19A	4	SG 33	5
SG 9	10	ST 14	6	SG 19	8	ST 4	6
SG 29	10	SG 19	7	SG 9	13	ST 5	10
SG 15	23	SG 33	10	SG 17	15	SG 15	15
SG 19	23	SG 15	12	SG 15	18	ST 3	15
SG 12	24	SG 34	16	SG 22	20	SG 19	16
ST 5	28	SG 6	17	SG 11	21	SG 24	16
SG 34	30	ST 20	24	ST 10	21	SG 12	17
ST 35B	34	ST 5	24	SG 24	22	ST 14	19
SG 27	43	SG 22	24	SG 6	24	ST 10	22
SG 22	43	SG 35	24	SG 31	27	SG 9	26
SG 11	45	SG 17	26	SG 35	27	SG 31	26
ST 10	49	SG 24	29	SG 23	29	SG 35	29
SG 6	50	SG 27	33	ST 5	30	SG 17	29
SG 17	51	ST 3	34	ST 8	30	SG 11	30
ST 4	52	ST 35B	35	SG 18	32	SG 22	33
SG 33	52	SG 18	35	ST 3	32	SG 6	33
ST 20	53	ST 4	36	SG 33	33	ST 35B	41
SG 24	53	ST 8	37	SG 27	36	SG 34	42
SG 18	54	SG 11	38	ST 4	37	SG 27	42
SG 35	56	SG 31	38	SG 7	40	SG 23	42
SG 23	61	SG 12	44	ST 35B	41	ST 8	45
SG 7	63	ST 10	44	SG 12	44	SG 18	46
SG 31	65	SG 23	48	SG 29	46	ST 20	48
ST 3	66	SG 29	53	SG 34	50	SG 7	50
ST 8	68	SG 7	53	ST 20	54	SG 29	51

[a] amoxicillin, cefuroxime and ceftriaxone

[b] clarithromycin and azithromycin

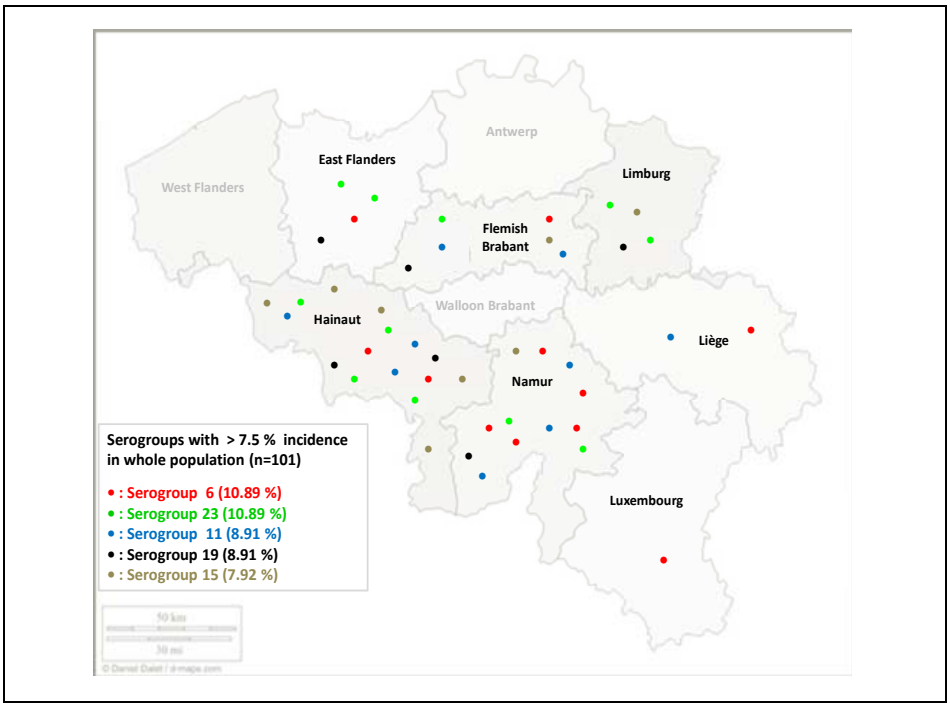
[c] telithromycin and solithromycin

[d] moxifloxacin and levofloxacin

[e] serotypes (ST) and serogroups (SG)

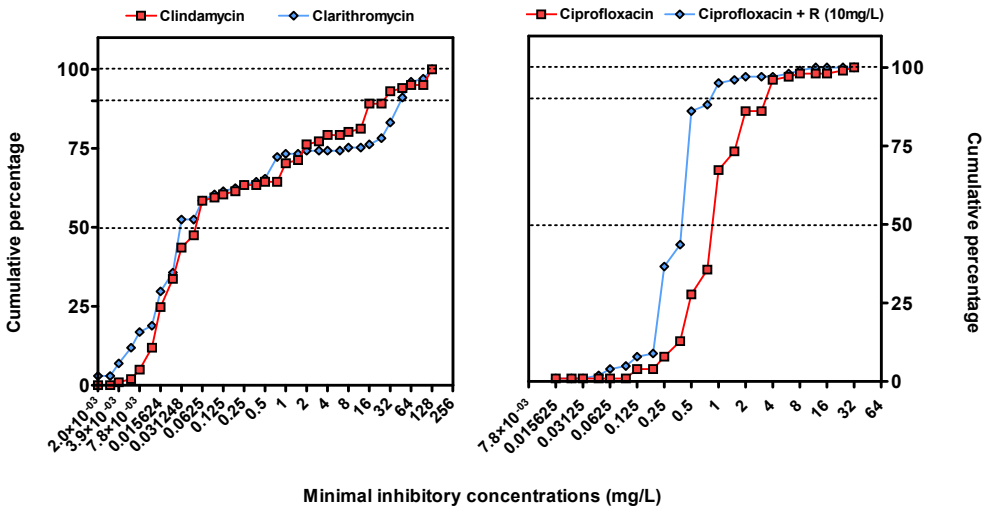
[f] ranking values were calculated by adding, for drugs belonging to the same antibiotic class, the numbers of ranking positions (from 1 to 27) of each serotype/serogroup, following their classification in Figure 3 (from the least to the most susceptible).The five most susceptible and resistant ST/SG are marked in colour. Similar colours indicate similarities between antibiotic classes.

Figure SP1



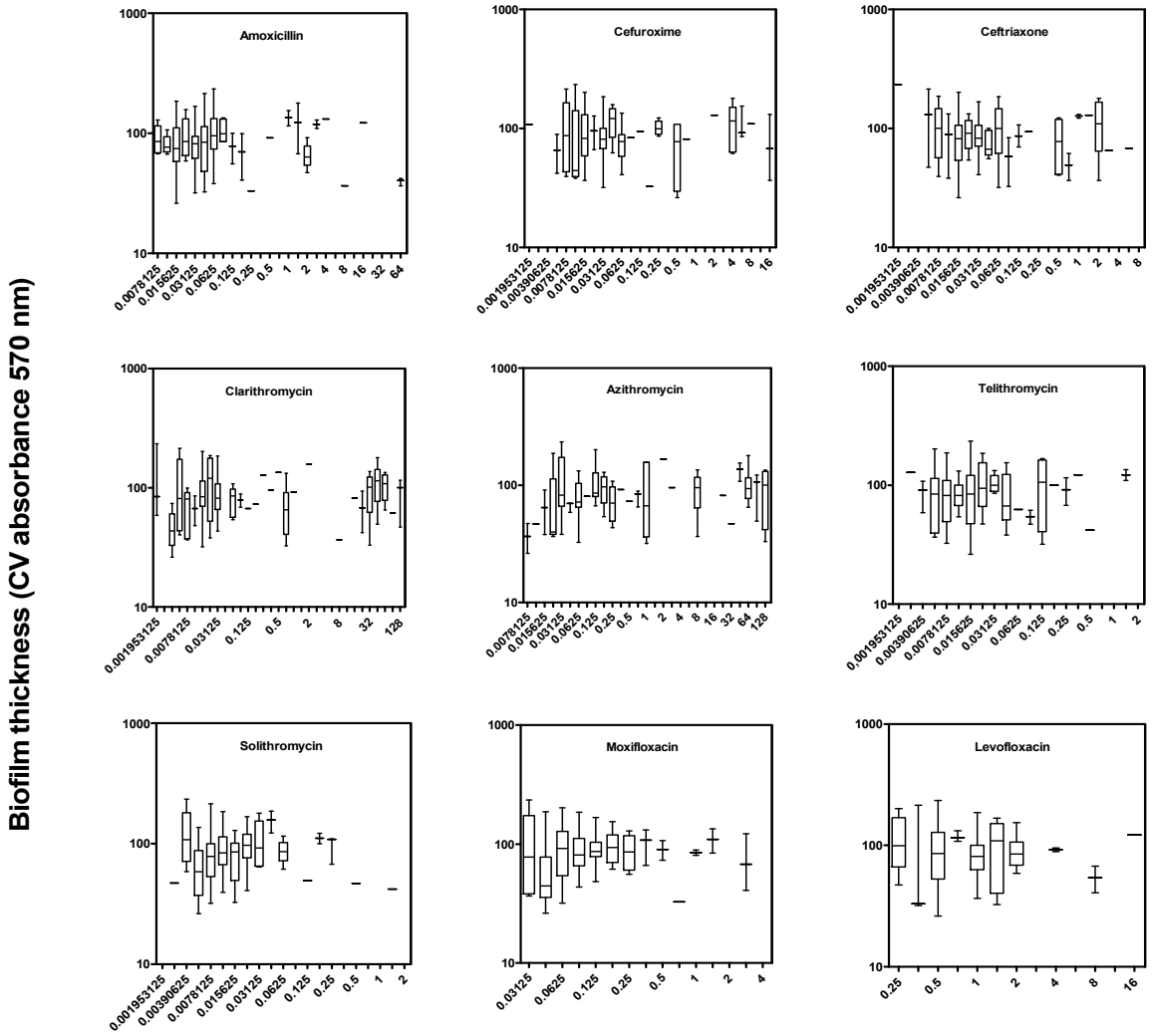
Caption to Figure SP1: Distribution of the serogroups with an incidence > 7.5% in the whole population (n=101) across the provinces where patients were living (provinces with no patients are labelled in grey).

Figure SP2



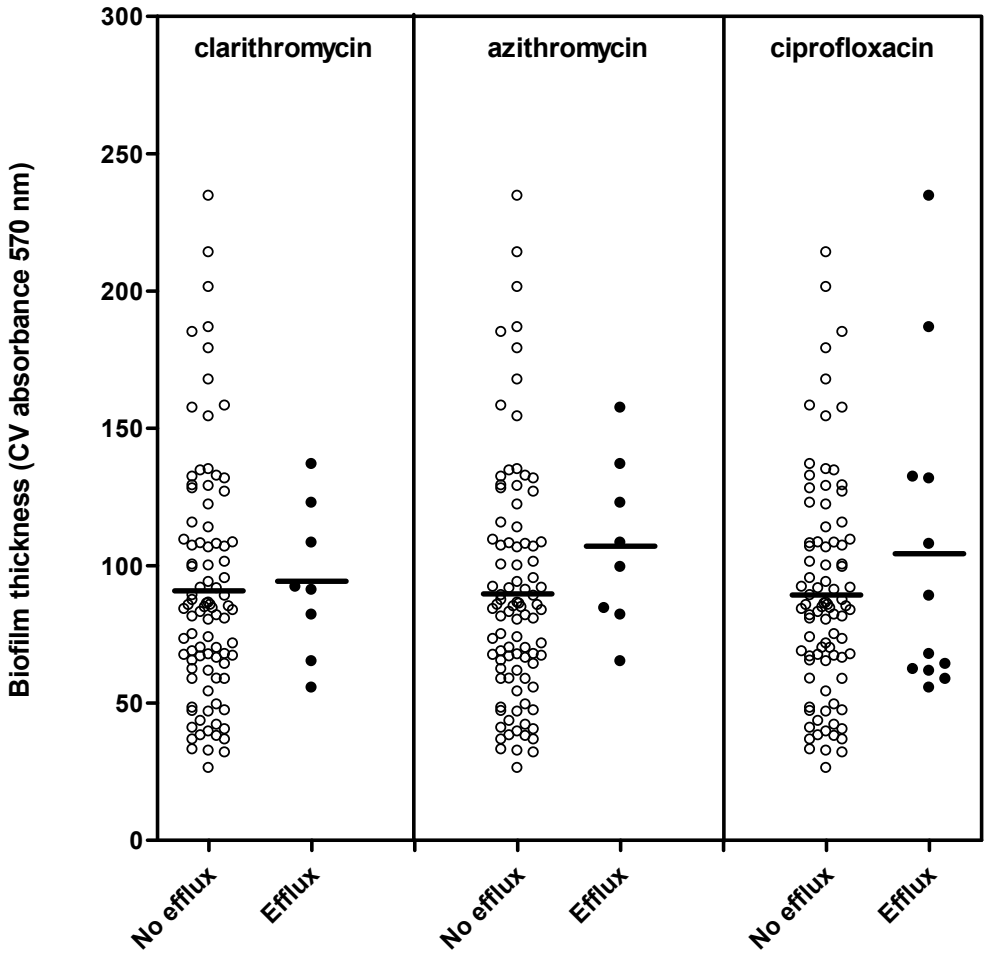
Caption to Figure SP2: Minimum inhibitory concentration (MIC) distributions (cumulative percentages) of the macrolide and fluoroquinolone markers of efflux, respectively, clindamycin vs. clarithromycin (left panel) and ciprofloxacin vs. ciprofloxacin + reserpine (R) (right panel) for 101 non-duplicate *Streptococcus pneumoniae* isolates from COPD patients. Three horizontal dotted lines are drawn at values corresponding to the MIC_{50}, MIC_{90}, and MIC_{100}.

Figure SP3



Caption to Figure SP3: Box and whisker plots represents the biofilm production after 10 days of culture for all isolates as a function of their MICs in broth for 3 β-lactams (amoxicillin, cefuroxime and ceftriaxone), 2 macrolides (clarithromycin and azithromycin), 2 ketolides (telithromycin and solithromycin) and 2 fluoroquinolones (moxifloxacin and levofloxacin). Data are presented as "Box and whiskers plots" giving the 25, 50 and 75 quartiles (boxes and horizontal line) and extending from 0 to 100 % of the isolates. No significant correlation was seen between MIC and biofilm thickness (one-way ANOVA with or without Tukey post-test).

Figure SP4. Distribution of isolate biofilm production as a function of phenotypic efflux



Caption to Figure SP4: Comparison of biofilm production after 10 days of culture for strains resistant to both clarithromycin and azithromycin and to ciprofloxacin using EUCAST interpretive criteria [20] and grouped according to the absence (open symbols) or the presence (closed symbols) of efflux as detected for clarithromycin and azithromycin by dissociation of susceptibilities with clindamycin, and for ciprofloxacin by a 2-fold decrease in MICs upon addition of reserpine (10 mg/L). No correlation between efflux and biofilm thickness was seen unpaired t-test (with or without Welch correction).