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Antimicrobial susceptibility of *Streptococcus pneumoniae* isolates from vaccinated and non-vaccinated patients with a clinically confirmed diagnosis of community-acquired pneumonia in Belgium

Ann Lismond[a], Sylviane Carbonnelle[a,1], Jan Verhaegen[b], Patricia Schatt[c], Annelies De Bel[d], Paul Jordens[e], Frédérique Jacobs[f], Anne Dediste[g], Frank Verschuren[h], Te-Din Huang[i,2], Paul M. Tulkens[a,*], Youri Glupczynski[j], Françoise Van Bambeke[a]

[a] *Pharmacologie cellulaire et moléculaire, Louvain Drug Research Institute, Université catholique de Louvain, Brussels, Belgium*
[b] *Laboratorium microbiologie, Universitair Ziekenhuis Gasthuisberg, Leuven, Belgium*
[c] *Laboratoire de microbiologie, Cliniques Notre-Dame de Grâce, Gosselies, Belgium*
[d] *Microbiologie en ziekenhuishygiëne, Universitair Ziekenhuis Brussel, Brussels, Belgium*
[e] *Afdeling pneumologie, O.L.V. Ziekenhuis, Aalst, Belgium*
[f] *Clinique des maladies infectieuses, Hôpital Erasme, Brussels, Belgium*
[g] *Laboratoire de microbiologie, CHU Saint-Pierre, Brussels, Belgium*
[h] *Service des urgences, Cliniques universitaires Saint-Luc, Brussels, Belgium*
[i] *Laboratoire de microbiologie, Cliniques universitaires Saint-Luc, Brussels, Belgium*
[j] *Laboratoire de microbiologie, CHU Mont-Godinne, Yvoir, Belgium*

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ABSTRACT

We assessed the in vitro susceptibility of *Streptococcus pneumoniae* isolates from patients with confirmed community-acquired pneumonia (CAP) to β-lactams, macrolides and fluoroquinolones and the association of non-susceptibility and resistance with serotypes/serogroups (STs/SGs), patient's risk factors and vaccination status. Samples (blood or lower respiratory tract) were obtained in 2007–2009 from 249 patients (from seven hospitals in Belgium) with a clinical and radiological diagnosis of CAP [median age 61 years (11.6% aged <5 years); 85% without previous antibiotic therapy; 86% adults with level II Niederman's severity score]. MIC determination (EUCAST breakpoints) showed for: (i) amoxicillin, 6% non-susceptible; cefuroxime (oral), 6.8% resistant; (ii) macrolides: 24.9% erythromycin-resistant [93.5% *erm*(B)-positive] but 98.4% telithromycin-susceptible; and (iii) levofloxacin and moxifloxacin, all susceptible. Amongst SGs: ST14, all resistant to macrolides and most intermediate to β-lactams; SG19 (>94% ST19A), 73.5% resistant to macrolides and 18–21% intermediate to β-lactams; and SG6, 33% resistant to clarithromycin. Apparent vaccine failures: 3/17 for 7-valent vaccine (children; ST6B, 23F); 16/29 for 23-valent vaccine (adults ST3, 7F, 12F, 14, 19A, 22F, 23F, 33F). Isolates from nursing home residents, hospitalised patients and patients with non-respiratory co-morbidities showed increased MICs for amoxicillin, all β-lactams, and β-lactams and macrolides, respectively. Regarding antibiotic susceptibilities: (i) amoxicillin is still useful for empirical therapy but with a high daily dose; (ii) cefuroxime axetil and macrolides (but not telithromycin) are inappropriate for empirical therapy; and (iii) moxifloxacin and levofloxacin are the next 'best empirical choice' (no resistant isolates) but levofloxacin will require 500 mg twice-daily dosing for effective coverage.

1. Introduction

Streptococcus pneumoniae remains a major cause of community-acquired pneumonia (CAP) [1], with antimicrobial resistance now becoming a major concern [2–4]. Whilst geographical variability in the susceptibility of *S. pneumoniae* to β-lactams, macrolides and tetracyclines is large [5], this is not the case for fluoroquinolones [6]. However, few studies have attempted to establish a direct link between microbiological characteristics of isolates and patients' actual clinical data. Moreover, recent introduction of the 7-valent

* Corresponding author at: Unité de pharmacologie cellulaire et moléculaire, Université catholique de Louvain, Avenue E. Mounier 73 Bte B1.73.05, B-1200 Brussels, Belgium. Tel.: +32 2 762 2136/764 7371; fax: +32 2 764 7373.
E-mail address: tulkens@facm.ucl.ac.be (P.M. Tulkens).
[1] Present address: Federal Agency for Nuclear Control, Brussels, Belgium.
[2] Present address: Laboratoire de microbiologie, CHU Mont-Godinne, Yvoir, Belgium.

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vaccine in children has led to an important shift in the prevalence of serotypes (STs) with specific resistance patterns [7,8]. Therefore, we undertook a survey in a cohort of patients admitted to hospital with a clinically confirmed diagnosis of CAP, aiming to correlate their clinical presentation with microbiological data regarding serogroups (SGs)/STs and susceptibility to β-lactams, macrolides and fluoroquinolones. We also compared the clinical breakpoints and interpretative criteria of the European Committee on Antibiotic Susceptibility Testing (EUCAST) (http://www.eucast.org), which are now gaining acceptance and popularity in Europe, with those of the US-based Clinical and Laboratory Standards Institute (CLSI) (http://www.clsi.org).

2. Materials and methods

2.1. General outline of the study, selection of patients and clinical data acquisition

The study involved seven hospitals (five teaching, two non-teaching; four in a large city, two in small cities and one rural, all within an area of ca. 200 km^2 around Brussels, Belgium), was observational, with isolate collection between April 2007 and March 2009. Patients arriving self-referred or referred by a general practitioner (GP) and with a suspicion of pneumonia were enrolled following isolation of *S. pneumoniae* from blood culture or from a lower respiratory tract specimen fulfilling the microbiological interpretative 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]. The diagnosis of CAP was confirmed retrospectively based on a clinical picture of lower respiratory tract infection associated with evidence of chest radiographic infiltrate(s), and no hospitalisation within the previous 48 h. Clinical data and information regarding antibiotic use within 1 month prior to hospitalisation were obtained by review of the medical charts and, if needed, by direct telephone contact with the referring GP (if any). Patients were stratified based on a severity score adapted from Niederman et al. [9] [level I, discharge from hospital with treatment after blood or respiratory sampling and clinical and radiological examination; level II, inpatients not admitted to the Intensive Care Unit (ICU); and level III, inpatients admitted to the ICU]. All data were anonymised after pertinent information had been collected.

2.2. Microbiological characteristics of the isolates

All *S. pneumoniae* isolates, first identified by the local clinical microbiology laboratory and stored at −20 °C/−80 °C, were sent to a central laboratory for identification confirmation [haemolysis on Mueller–Hinton II agar with 5% sheep blood (BD Diagnostics, Franklin Lakes, NJ) at 37 °C with 5% CO_2, and growth inhibition by optochin (Oxoid Ltd., Basingstoke, UK)]. Minimal inhibitory concentrations (MICs) were determined by broth microdilution [10], using interpretative criteria both of EUCAST [11] and of the CLSI [10]. To improve accuracy, concentrations at half a value of each standard geometric progression were used in the concentration range covering the susceptible to resistant EUCAST clinical breakpoints and/or the zone at which a change in MIC was expected to result from impairment of the activity of efflux transporters. Thus, taking amoxicillin as an example [for which the EUCAST breakpoints are susceptible (S) ≤ 0.5 mg/L and resistant (R) > 2 mg/L], susceptibility in the range 0.5–4 mg/L was tested using drug concentrations of 0.5, 0.75, 1, 1.5, 2, 3 and 4 mg/L. Likewise, when assessing the susceptibility of the isolates to ciprofloxacin [for which EUCAST breakpoints are S ≤ 0.125 mg/L and R > 2 mg/L and for which a change of MIC upon addition of reserpine was expected to be ca. 1 log_2 dilution within that range], we used a concentration progression of 0.125, 0.1875, 0.25, 0.375, 0.5, 0.75, 1, 1.5, 2, 3 and 4 mg/L to cover the 0.125–4 mg/L interval. *Streptococcus pneumoniae* ATCC 49619 was used for quality control in each set of determinations. The putative mechanisms of resistance to macrolides [ribosomal methylation (MLS_B phenotype) versus efflux-mediated (M phenotype) resistance] were inferred from dissociation of susceptibilities between clindamycin (not subject to efflux) and erythromycin [12] using the EUCAST non-susceptible (S) breakpoint [11] and were confirmed genotypically by polymerase chain reaction (PCR) assays targeting the corresponding *erm*(B) and *mef*(E) genes (see supplementary material). Efflux of fluoroquinolones was detected by measuring the MIC decrease in the presence of reserpine [13] (10 mg/L) [change of ≥1 log_2 dilution (made possible because determinations used a 0.5 log_2 concentration progression and differences proved highly reproducible)]. Serogrouping/serotyping was performed as described previously [14] [ST is used as an acronym for all serogroups containing only one serotype (e.g. 1, 3, 4, 5, 8 and 14) and SG is used for all others unless the specific serotype within that serogroup is known (e.g. ST19A)].

2.3. Assessment of apparent vaccination failures

Vaccination failure was defined as the occurrence of a CAP episode in a vaccinated patient with a causative *S. pneumoniae* isolate belonging to a ST included in the administered vaccine [adults, 23-valent pneumococcal polysaccharide vaccine (PPV-23) (Pneumo23®; Sanofi-Pasteur MSD, Lyon, France); children (aged <5 years), 7-valent pneumococcal conjugate vaccine (PCV-7) [Prevenar®; Wyeth (now Pfizer), New York, NY]; see note in Table 1 showing the STs/SGs covered by each vaccine]. These failures were qualified as apparent because the vaccination status as well as the compliance to the recommended scheme could only be inferred from declarations from the patients or their GP.

2.4. Antibiotics

Antibiotics were obtained (i) as the preparation for intravenous use (>90% purity; no excipient) for cefuroxime (CEFURIM®; Teva Pharma Belgium, Wilrijk, Belgium) and ceftriaxone (ROCEPHINE®; Roche s.a., Brussels, Belgium); (ii) as microbiological standards for telithromycin and levofloxacin (Sanofi-Aventis, Paris, France), ciprofloxacin and moxifloxacin (Bayer Healthcare, Leverkusen, Germany) and clarithromycin (Teva Pharmaceuticals, Petah Tikva, Israel); and (iii) as chemicals for in vitro investigations from Sigma-Aldrich (St Louis, MO) for penicillin G, amoxicillin, clindamycin and erythromycin. Reserpine was obtained from Fluka (Buchs, Switzerland).

2.5. Statistical analyses

Contingency tables, non-parametric analysis of variance (ANOVA) and other statistical analyses were made with JMP® v.8.0.2 (SAS Institute, Cary, NC).

3. Results

3.1. Patient characteristics

In total, 249 patients with a positive culture of *S. pneumoniae* were enrolled (Table 1). Mean and median ages were 55 years and 61 years, respectively, with 11.6% aged <5 years. Approximately one-half of the patients had not been referred by their GP (with wide variations between centres) and only ca. 15% had received an antibiotic prior to hospitalisation. Most patients remained hospitalised after diagnosis, but only ca. 10% of adults required admission

Table 1
General characteristics of patients.

Origin								
Hospital	A	B	C	D	E	F	G	Total
Bed size	677	196	858	420	1000	529	700	4380
No. enrolled	42	15	59	18	36	30	49	249
Ratio (% of capacity)	6.2	7.7	6.9	4.3	3.6	5.7	7.0	5.7 ± 1.5
Population characteristics (whole)								
	Years				Distribution (*n*)			
Age	Mean	Median			<5 years	≥5 and <60 years		≥60 years
	55.6	61.6			29	88		132
Pre-diagnosis history	Antibiotic treatment (*n*)[a]				Referral by GP (*n*)			
	Yes	No	Unknown		Yes	No		Unknown
	36	213	0		117	131		1
Post-diagnosis management	Hospitalisation (*n*)							
	Yes	No	Unknown					
	236	13	0					
Origin of the sample (*n*)	Blood	Lower respiratory tract						
	156	93						
Adult population (≥20 years; *n* = 209)								
Whole	Hospitalisation (*n*)[b]				Smokers (*n*)[c]			
	No	Ward	ICU		Yes	No		Unknown
	9	180	20		51	150		8
	Co-morbidities							
	Respiratory (*n*)[d]				Non-respiratory (*n*)[e]			
	Yes	No	Unknown		Yes	No		Unknown
	72	135	2		97	110		2
≥60 years (*n* = 132)	Vaccination (*n*)[f]				Nursing home (*n*)			
	Yes	No	Unknown		Yes	No		Unknown
	26	80	26		20	111		1
Children (<5 years: *n* = 29)[g]								
	Vaccination (*n*)[h]				Day-care centre (*n*)			
	Yes	No	Unknown		Yes	No		Unknown
	17	11	1		10	13		6

GP, general practitioner; CAP, community-acquired pneumonia; ICU, Intensive Care Unit; COPD, chronic obstructive pulmonary disease.

[a] Main antibiotics: β-lactams, 25; macrolides, 5; fluoroquinolones, 2; others, 2 (some patients received more than one antibiotic) as noted from the declaration of the patient and/or the GP and over a period of 1 month prior to diagnosis of CAP.

[b] no = outpatients; ward = inpatients not admitted to the ICU; ICU = inpatients admitted to the ICU.

[c] Smoking status based on patient's declaration and habit at the time of the onset of pneumonia.

[d] COPD if mentioned by the GP and/or in the patient's chart and based on the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria (dyspnoea, chronic cough or sputum production and/or a history of exposure to risk factors for the disease and, if available, spirometry data [FEV_1/FVC (ratio between the volume exhaled at the end of the first second of forced expiration and the forced vital capacity, also called Tiffeneau index) < 0.70]).

[e] Cancer, cardiovascular disease, diabetes mellitus, acquired immune deficiency syndrome (AIDS), epilepsy, liver failure and renal failure.

[f] 23-Valent pneumococcal polysaccharide vaccine (Pneumo23®; Sanofi-Pasteur MSD, Lyon, France) (covers serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F); vaccination was more frequent in adults with COPD ($P = 0.04$) and in adults aged ≥60 years and suffering from a combination of COPD and non-respiratory co-morbidity ($P = 0.007$).

[g] Children aged between 5 years and 19 years ($n = 11$) have not been included in this cohort because they belonged to pre-vaccine generations and because of their lower risk factors.

[h] 7-Valent pneumococcal conjugate vaccine [Prevenar®; Wyeth (now Pfizer), New York, NY] (covers serotypes 4, 6B, 9V, 14, 18C, 19F and 23F); children in day-care centres were more frequently vaccinated ($P = 0.021$).

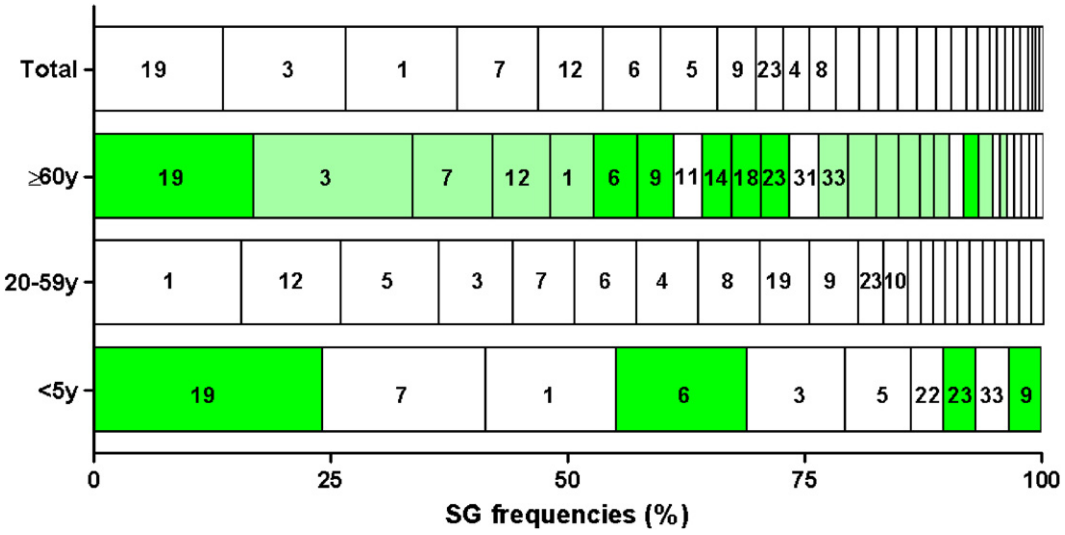

Fig. 1. Distribution of the serogroups (SGs)/serotypes (STs) amongst *Streptococcus pneumoniae* isolates ($n = 249$) used in this study, with subdivision by age group. Green boxes correspond to the SGs included in the 23-valent vaccine (used in adults), with those included in the 7-valent vaccine (used for children) in dark green. SGs 1 and 3 contain only one ST, and isolates categorised as SG19 were ST19A in >94% of 100 random isolates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

to the ICU, meaning that the population mainly showed a level II severity score [9]. Approximately two-thirds of the isolates were from blood. Respiratory (mainly chronic obstructive pulmonary disease; see criteria in Table 1) and non-respiratory co-morbidities (mainly hypertension, diabetes and heart failure) affected ca. one-third to one-half of the adult patients, respectively. Only 20% of adults aged ≥60 years, but ca. 60% of children (<5 years), had been vaccinated (paediatric vaccination was introduced in Belgium ca. 3 years before the beginning of the study).

3.2. Serogroups/serotypes

Fig. 1 shows the distribution of the main SGs amongst all isolates. Considering the whole population, SG19, ST3 and ST1 were the most frequent (13.7%, 12.9% and 11.7% of all isolates, respectively), with variations occurring between age groups. SG19 was the primary SG in adults aged ≥60 years and children (<5 years), followed by ST3 and SG7 in adults aged ≥60 years and SG7, ST1 and SG6 in children. ST1, SG12 and ST5 were dominant in adults in the age range 20–59 years.

3.3. Minimum inhibitory concentration distributions and in vitro susceptibility

MIC distributions were obtained for all antibiotics and the data for six antibiotics chosen as representative of their pharmacological class (β-lactams, amoxicillin and cefuroxime; macrolides/ketolides, clarithromycin and telithromycin; and fluoroquinolones, levofloxacin and moxifloxacin) are shown in Fig. 2 [see supplementary material for (i) MIC distributions of penicillin G, ceftriaxone, erythromycin, clindamycin and ciprofloxacin (Supplementary Fig. S1); (ii) MIC range, MIC_{50} and MIC_{90} values (MICs for 50% and 90% of the organisms, respectively), and percentage of non-susceptible isolates based on EUCAST and CLSI clinical susceptibility breakpoints (Supplementary Table S1) for all antibiotics]. For β-lactams, distributions were largely superimposable, but susceptibilities varied according to the breakpoint used [amoxicillin, 6% non-susceptible with EUCAST vs. 3.2% with CLSI; cefuroxime (oral), 6.8% and 5.6% resistant with EUCAST and CLSI, respectively]. For macrolides (erythromycin and clarithromycin) and clindamycin, resistance was observed in >20% of the isolates, but in only 0.8% and 0.4% of the isolates for telithromycin according to EUCAST and CLSI, respectively. For levofloxacin and moxifloxacin, all isolates were categorised as susceptible (corresponding entirely to the EUCAST wild-type population). Of note, the MIC_{50} and MIC_{90} values of levofloxacin were close to those of ciprofloxacin (0.5 and 1 $\log_2$ dilution difference only; see Supplementary Table S1).

3.4. Mechanisms of resistance to macrolides and efflux of fluoroquinolones

Dissociation of susceptibility between clindamycin and erythromycin was observed for ca. 20% of the erythromycin-non-susceptible isolates (5% of total). However, one-half of the isolates displaying an M-phenotype (susceptible to clindamycin but non-susceptible to erythromycin, and therefore assumed to harbour an efflux-mediated mechanism) were *mef*(E)-negative and *erm*(B)-positive (see Supplementary Fig. S2) and were therefore re-categorised as methylase-mediated-resistant. Clarithromycin MICs were always in close correlation with those of erythromycin. For fluoroquinolones, efflux (two-fold MIC reduction in the presence of reserpine) was present in most isolates when tested with ciprofloxacin but not with levofloxacin (no change in MIC_{90} or MIC_{50}) and in only a few isolates with moxifloxacin (MIC_{90} shift from 0.25 mg/L to 0.187 mg/L).

3.5. Serogroups/serotypes and antibiotic resistance

Fig. 3 (upper panel) shows the distribution of the main SGs amongst non-susceptible bacteria for each antibiotic tested. Fig. 3 (middle and lower panels) shows the resistance patterns for the four SG/STs with the highest level of resistance to macrolides. For SG19 (>94% ST19A based on a random selection of 100 isolates), non-susceptibility was highest for clarithromycin (73.5%) and was important (18–21%) for amoxicillin, cefuroxime and ceftriaxone, whilst being only 3% for penicillin G (based on EUCAST breakpoint values). For SG6 isolates as a whole, non-susceptibility was ca. 30% for clarithromycin but only 7% for β-lactams. All ST14 isolates were resistant to macrolides and 80% were intermediate to amoxicillin. For ST1 isolates, 38% were resistant to macrolides but all remained susceptible to β-lactams. For the other SGs, 30% of SG9, 15% of SG23

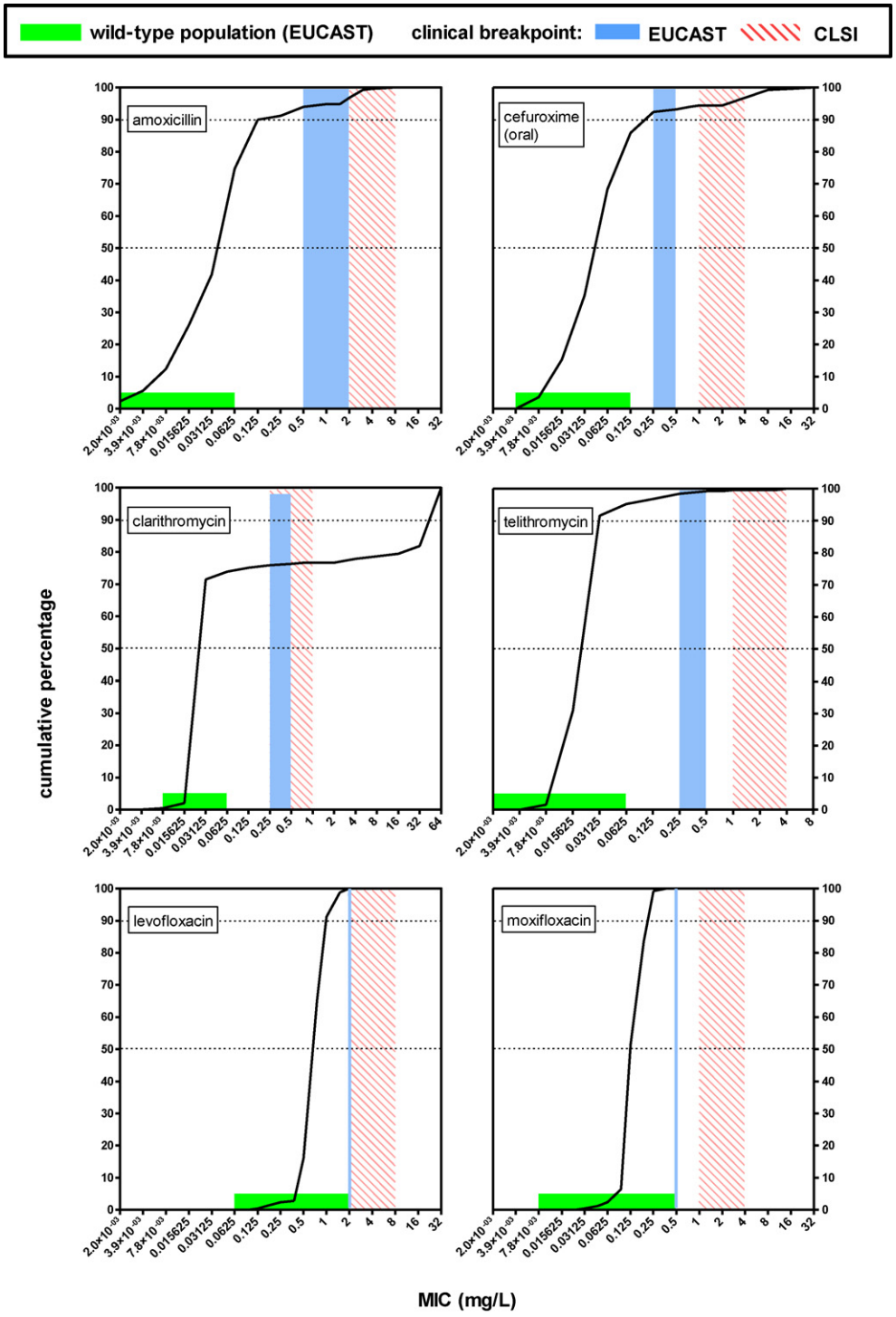

Fig. 2. Minimum inhibitory concentration (MIC) distributions (cumulative percentages) of non-duplicate *Streptococcus pneumoniae* isolates ($n = 249$) from all patients enrolled in the study. The horizontal green zone in the MIC scale shows the range (mg/L) covered by the wild-type population as defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (amoxicillin, ≤0.002–0.063; cefuroxime, 0.004–0.125; clarithromycin, 0.008–0.063; telithromycin, ≤0.002–0.063; levofloxacin, 0.063–2; and moxifloxacin, 0.008–0.5). The blue and hatched red vertical zones correspond to the MIC range (mg/L) of S (susceptible) to R (resistant) clinical breakpoints defined by EUCAST and the Clinical and Laboratory Standards Institute (CLSI), respectively [amoxicillin, 0.5–2 and 2–8; cefuroxime (oral), 0.25–0.5 and 1–4; clarithromycin, 0.25–0.5 and 0.25–1; telithromycin, 0.25–0.5 and 1–4; levofloxacin, 2–2 and 2–8; and moxifloxacin, 0.5–0.5 and 1–4; for EUCAST, S is ≤ and R is > the lowest and highest value, respectively; for CLSI, S is ≤ and R is ≥ the lowest and highest limit, respectively; the EUCAST breakpoint for levofloxacin is for the registered high-dose therapy (2×500 mg) in Europe]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

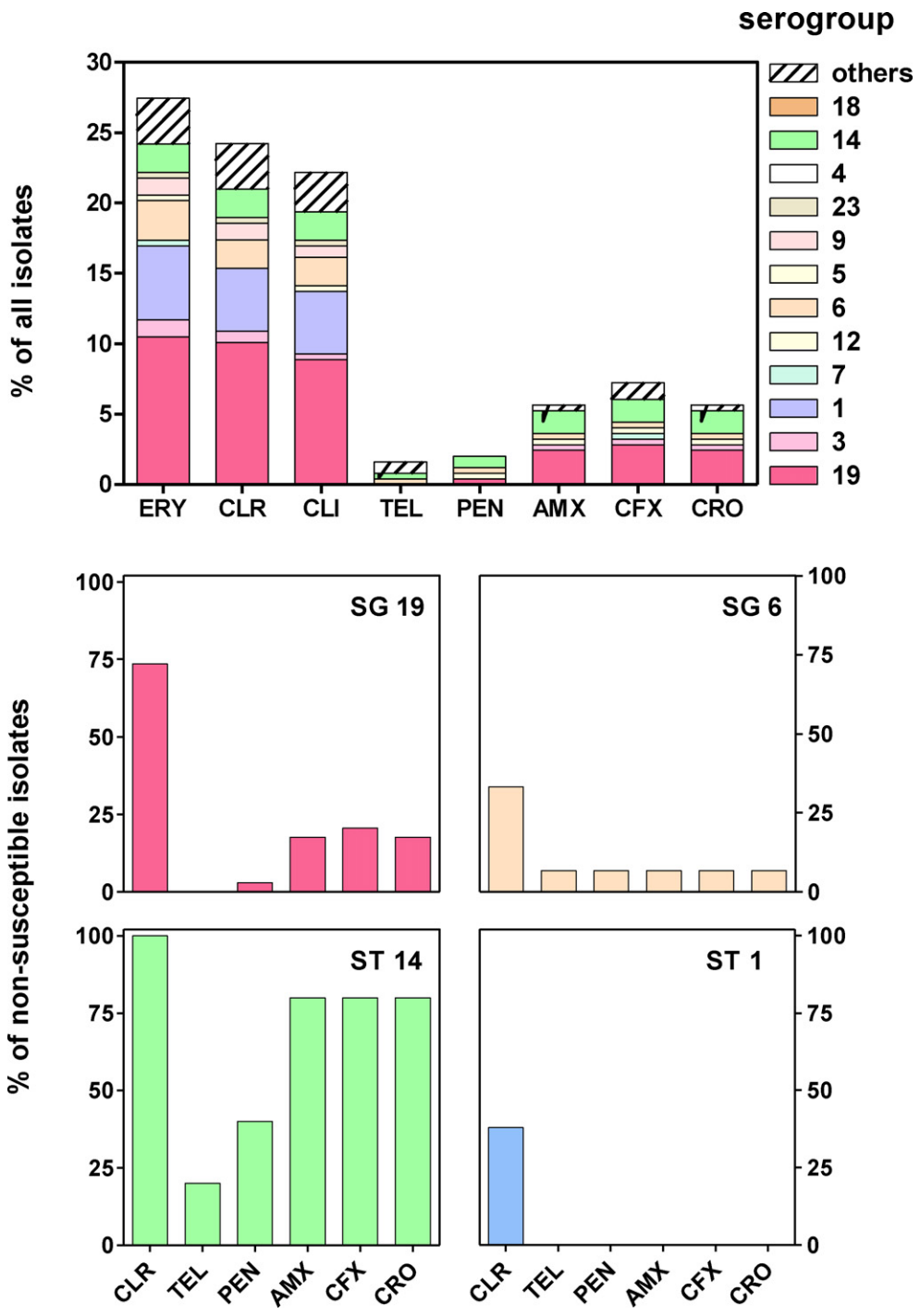

Fig. 3. Non-susceptibility [defined as a minimum inhibitory concentration (MIC) > the susceptible (S) clinical breakpoint of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (see values in the caption of Fig. 2)] of isolates according to the main serogroups/serotypes. Upper panel: non-susceptibility for all isolates as a function of each antibiotic [erythromycin (ERY), clarithromycin (CLR), clindamycin (CLI), telithromycin (TEL), penicillin G (PEN), amoxicillin (AMX), cefuroxime (oral form) (CFX) and ceftriaxone (CRO); there were no non-susceptible isolates for fluoroquinolones]. Lower panels: non-susceptibility for the four serogroups (SGs)/serotypes (STs) with the largest levels of non-susceptibility to macrolides towards the six clinically used antibiotics for which resistance could be detected (SG19 was ST19A in >94% of 100 random isolates).

and 6% of ST3 isolates were resistant to macrolides but most were susceptible to amoxicillin. Almost all ST5, SG7 and SG12 and all ST4 and SG18 isolates were susceptible to all antibiotics.

3.6. Apparent vaccine failures

For the 7-valent conjugate vaccine, apparent failures were limited to ST6B (2 cases) and 23F (1 case) out of 17 vaccinated children. For the 23-valent vaccine, 16 apparent failures (from a total of 29 vaccinated adults) were observed, corresponding to serotypes 7F (5 cases), 3 (4 cases), 19A (2 cases) and 12F, 14, 22F, 23F and 33F (1 case each).

3.7. Correlations between clinical, microbiological and serological observations

Table 2 shows the associations meeting criteria of statistical significance between patients' presentation, susceptibility testing

Table 2
Associations between variables related to patients' presentation, isolate susceptibility and vaccination failures (variables #1) and all pertinent variables recorded in the study (variables #2). Unless stated otherwise, variables considered were categorical. For those with only two possible values, associations were tested by means of 2 × 2 contingency tables to calculate odd ratios (ORs) [with the corresponding 95% confidence interval (CI) and *P*-value (Fisher's exact two-tailed test)]; for those with more than two possible values, a first analysis was performed using all values with significance assessed by χ^2 analysis; if significant, individual values were cross-tested in 2 × 2 contingency table to calculate the corresponding ORs, CIs and *P*-values. The table shows only associations for which the *P*-value was <0.05 (ordered from lowest to highest). Some associations with a *P*-value between 0.05 and 0.1 considered potentially medically important are also shown but appear in italic.

Variable #1	Variable #2	OR (95% CI)	*P*-value
1. Patient presentation			
1.1. Referral by a GP	Patient aged ≥60 years	3.53 (2.08–5.97)	<0.001
	Smoking patient	0.41 (0.22–0.81)	0.010
	Vaccinated child (PCV-7)	0.11 (0.02–0.63)	0.013
	Unknown vaccination status in elderly (PPV-23)[a]	*0.43 (0.20–0.95)*	*0.051*
1.2. Vaccination (adult)	SG23	5.21 (1.12–24.2)	0.041
1.3. Nursing home	SG19 (in patients aged ≥60 years)	3.41 (1.17–9.92)	0.045
1.4. Smoking (adult)	SG19	0.10 (0.01–0.79)	0.006
	ST5	3.84 (1.12–13.2)	0.033
1.5. Previous antibiotic treatment[b]	Isolate non-susceptible to erythromycin[c]	13.2 (2.32–75.0)	0.005
	Patient residing in a nursing home	*2.96 (0.98–9.00)*	*0.083*
1.6. Co-morbidity			
Any (adults)	ST1	0.24 (0.08–0.66)	0.006
Non-respiratory	COPD[d]	0.47 (0.26–0.84)	0.013
	Smoking patient	0.45 (0.23–0.87)	0.023
Respiratory	Smoking patient (adult aged <50 years)[e]	7.14 (1.07–47.42)	0.027
1.7. Isolate origin			
Respiratory tract	Respiratory co-morbidity	2.93 (1.62–5.29)	<0.001
	Vaccinated adult (PPV-23)	4.77 (1.79–12.71)	0.001
	ST3 isolate	3.28 (1.49–7.21)	0.004
Blood culture	ST1 isolate	5.91 (1.71–20.40)	0.001
	ST5 isolate	8.54 (1.09–66.57)	0.021
	Patient aged ≤20 years	2.31 (1.05–5.11)	0.049
1.8. Need for hospitalisation	ST3, ST5 or SG7	All hospitalised patients[f]	
	Patient aged ≥60 years[g]	15.0 (1.91–117)	<0.001
2. Susceptibility testing [ns = non susceptible[c]; only one antibiotic shown per class (see note[h] for other antibiotics)]			
2.1. Patient-related factors			
Non-respiratory co-morbidity	Non-susceptibility to amoxicillin	6.91 (1.49–32.0)	0.007
	Non-susceptibility clarithromycin	2.65 (1.29–5.41)	0.008
Any co-morbidity	Non-susceptibility to clarithromycin	2.47 (1.03–5.93)	0.039
	Higher MIC for levofloxacin [i]	*	0.026
>1 co-morbidity	Non-susceptibility to clarithromycin	3.98 (1.65–9.61)	0.003
Hospitalised patients	Non-susceptibility to β-lactams and telithromycin	All hospitalised patients[f]	
Patient from nursing home	Increased MIC for amoxicillin[j]	*	0.021
2.2. Serotype or serogroup of the isolate			
ST14	Non-susceptibility to amoxicillin	93.52 (9.52–912)	<0.001
	Non-susceptibility to clarithromycin[k]	All patients with ST14 isolates	
	Non-susceptibility to telithromycin	*20.0 (1.69–236)*	*0.079*
SG19	Non-susceptibility to clarithromycin	14.2 (6.11–33.0)	<0.001
	Non-susceptibility to amoxicillin	5.52 (1.78–17.1)	0.006
ST1	Non-susceptibility to erythromycin[k]	2.42 (1.10–5.35)	0.044
3. Apparent vaccination failures			
3.1. Failures for all patients	Respiratory culture	4.93 (1.21–15.4)	0.003
3.2. Failures of PVV-23 (adults) vs. PCV-7 (children)		6.15 (1.46–26)	0.014

GP, general practitioner; PCV-7, 7-valent pneumococcal conjugate vaccine; PPV-23, 23-valent pneumococcal polysaccharide vaccine; MIC, minimum inhibitory concentration; SG, serogroup; ST, serotype; EUCAST, European Committee on Antimicrobial Susceptibility Testing; COPD, chronic obstructive pulmonary disease.

* Tested by analysis of variance (Wilcoxon/Kruskal–Wallis rank-sum test) comparing the MIC of all isolates from the corresponding patient group versus those from all other patients.

[a] Status not known by the patient and his/her GP.

[b] Prescribed by an attending physician (or taken by the patient on her/his own initiative) before the patient was referred to or presented her/himself at the hospital.

[c] EUCAST interpretative criteria [MIC > the clinical susceptible (S) breakpoint; see Fig. 2 for values].

[d] See criteria for COPD in Table 1.

[e] Logistic fit of current smoking habit versus age showed a non-smoking probability ≥0.75 for patients aged ≥63.6 years (95% CI 52.7–77.10).

[f] No calculation possible since all patients positive for variable #1 were also positive for variable #2.

[g] All patients from nursing homes were hospitalised.

[h] ORs (with 95% CI) and *P*-value for association with non-susceptibility to other antibiotics:

ST14 isolates and β-lactams/macrolides: penicillin G, 53.3 (6.39–445), *P* = 0.003; cefuroxime, 65.4 (6.85–625), *P* < 0.001; ceftriaxone, 93.2 (9.52–912), *P* < 0.004; erythromycin, all isolates.

SG19 and β-lactams/macrolides: penicillin G, non-significant; cefuroxime, 4.78 (1.70–13.4), *P* = 0.005; ceftriaxone, 5.52 (1.78–17.1), *P* = 0.006; erythromycin, 13.3 (5.63–31.5), *P* < 0.001 (telithromycin, non-significant).

non-respiratory co-morbidity and β-lactams/macrolides: penicillin G, non-significant; cefuroxime, 5.52 (1.52–20.0), *P* = 0.007; ceftriaxone, 15.39 (1.96–121), *P* < 0.001; erythromycin, 2.75 (1.40–5.45), *P* = 0.004 (telithromycin, non-significant).

Any co-morbidity and macrolides: *erythromycin: 2.15 (0.97–4.77), P* = 0.07 (telithromycin, non-significant).

[i] All isolates remaining clinically susceptible according to the EUCAST interpretative criteria (MIC < S breakpoint).

[j] *P*-value for ceftriaxone, 0.016; for penicillin G, 0.023; trend only for cefuroxime.

[k] But not for other macrolides.

and data on apparent vaccination failures on the one hand, and all variables recorded in the study on the other hand. Concentrating on the most salient data regarding patient presentation, we see that: (i) GPs were more frequently involved in the referral of elderly patients but less in that of smoking adults and vaccinated children; (ii) that patients from nursing homes were more frequently infected by *S. pneumoniae* isolates of SG19 [contributing to the increased resistance observed in hospitalised patients (see susceptibility data)]; and (iii) that previous antibiotic treatment was associated with higher non-susceptibility to erythromycin (but not to other antibiotics). All patients with a ST3, ST5 or SG7 isolate were hospitalised. SG23 isolates were more frequently observed in vaccinated adults although the corresponding vaccine (PPV-23) covers one of its contributing STs (ST23F). With respect to susceptibility data, co-morbidities were associated with a global decrease in susceptibility to β-lactams and macrolides, which also affected more specifically ST14 and SG19 and, for erythromycin only, ST1 isolates. None of these factors affected the susceptibility of fluoroquinolones, except for a significant elevation in the MICs of levofloxacin in patients with co-morbidity. There was no significant correlation between absence of vaccination and altered susceptibility of the offending isolate. Lastly, patients with apparent vaccination failure more preferentially yielded positive respiratory samples, and the 23-valent non-conjugated vaccine for adults was significantly less effective than the 7-valent conjugated vaccine for children.

4. Discussion

CAP treatment has received considerable attention and has been the object of numerous guidelines aimed at optimising the management and use of antibiotics (see [15,16] for typical examples for adults and [17] for children). It nevertheless still remains a potentially life-threatening disease with ca. one-third of cases requiring hospitalisation, which leads to a marked increase in overall treatment costs [1]. The present study provides information on the potential usefulness of three main classes of antibiotics (included in most guidelines dealing with the treatment of CAP) for initiating treatment in patients reporting spontaneously to the hospital or referred by their primary care physician. We were also able to assess the associations between vaccination status and other clinical factors with the in vitro susceptibility of isolates. However, there are three main limitations to this study, namely: (i) we could only enrol patients admitted to hospital (making the study not pertinent to what may prevail with patients treated at home); (ii) it was restricted to a specific geographical area; and (iii) it was retrospective (making it uncertain that all necessary information had been collected, as it was entirely dependent upon the quality of the individual medical records and on the information obtained from GPs). The first limitation was by design as it is very difficult to obtain reliable microbiological samples from non-hospitalised patients. Most cases, however, were of moderate severity, therefore corresponding to situations where the same antibiotics as those used here will be used by the GPs for home therapy. The second limitation results from our desire to collect as meaningful and reliable clinical data as possible. This imposed close and repeated contacts between the investigators and the patients, the referring GPs and the local hospital team, including site visits for analysis of the patient's individual medical charts; this in-depth analysis inevitably limited the number of contributing centres that could be studied. Thus, whilst the conclusions of this study may be limited to Belgium, our assessment of the clinical status of the patients and the correlations made with the other parameters analysed go beyond what is usually obtained from larger studies. Lastly, there was no practical way to prospectively collect information as it would have, in many cases, interfered with the normal care of the patients and was therefore considered unethical in the context of an observational study.

Considering first the susceptibility analysis, the data indicate a risk of failure with macrolides (if given as monotherapy) in the population surveyed since resistance exceeds 20%, a value we consider a critical threshold in a context of empirical therapy. Resistance was higher for some SGs included in the 7-valent vaccine (especially SG19 and ST14) than in non-vaccine serogroups, although ST1 isolates were also often resistant. This differs from what has been observed in Argentina where ST6B, important in patients aged <5 years, shows 100% resistance to erythromycin [18] and, to some extent, in Scotland where 80% erythromycin resistance in ST14 isolates has been reported [19]. As most commonly found in Europe, macrolide resistance in *S. pneumoniae* was mainly mediated through ribosomal methylation [20]. However, for a small but significant number of isolates, the mechanism of resistance was incorrectly diagnosed as being due to efflux when using the clindamycin/erythromycin dissociation resistance test, an observation that has also been made by others [21]. Notably, telithromycin remained fully active against most *S. pneumoniae* isolates in the environment where the study has been conducted, using both the EUCAST [11] and CLSI [10] interpretative criteria.

The susceptibility of the collected isolates to β-lactams remains apparently favourable for penicillin G and amoxicillin if considering clinical resistance breakpoints only (but not for cefuroxime, because of differences in breakpoints related to pharmacokinetic considerations; see [11]). However, a significant proportion of these isolates must be categorised as intermediate for amoxicillin when using EUCAST breakpoints ($S \leq 0.5$ mg/L to $R > 2$ mg/L), implying the need for daily doses of 2–3 g [11]. This would not be the case if using CLSI breakpoints ($S \leq 2$ mg/L to $R \geq 8$ mg/L).

Lastly, the data show that the susceptibility of *S. pneumoniae* to fluoroquinolones, especially to moxifloxacin, remains excellent, as has also been found in other studies covering a similar period in Belgium [22] and Germany [6]. This brings into question the rationale of positioning/restricting moxifloxacin as a second-line antibiotic only, since its global safety profile (including the risk of emergence of resistance or of superinfections) seems as acceptable as that of most other antimicrobials once patients with known contraindications are excluded [23]. The situation may be less favourable for telithromycin since, whilst its susceptibility profile is similar to that of fluoroquinolones (based on the present data), its safety has been closely scrutinised by regulatory authorities which, however, still acknowledge its favourable benefit-to-risk ratio in treating CAP.

The association of resistance with given SGs/STs is clearly influenced by the introduction of vaccination. Thus, before vaccination was introduced, ST14 was most prevalent in young children and elderly patients and ST1 in non-elderly adults [14]. However, SG19 (mostly ST19A) has now emerged as the predominant strain in these populations, both in this study and elsewhere [24]. Isolates from this SG, together with those from ST14, were largely non-susceptible to β-lactams and resistant to macrolides, as found by others [25,26]. In contrast, ST1, reported as fully or largely susceptible to macrolides in France and Germany [25,27], showed >30% resistance, confirming another Belgian study [28]. This may perhaps result from local spread of restricted, successful clones [29,30] and indicates that region-specific surveillance is needed. Lastly, failures of the 23-valent polysaccharide vaccine were considerably more frequent than those of the 7-valent conjugated vaccine, as has been reported by others [30], demonstrating the need to improve the efficacy of adult vaccination.

In conclusion, the current in vitro susceptibilities of the main SGs of *S. pneumoniae* isolates associated with CAP in this study would suggest that: (i) amoxicillin can still be considered useful for empirical therapy but with higher daily doses than originally

proposed and, if using the target attainment rate values for efficacy proposed by EUCAST [11], of ≥0.5 g every 8 h; (ii) that cefuroxime axetil may have become inappropriate as its MIC distribution in the population analysed extends beyond the so-called clinically resistant breakpoint; (iii) that macrolides (but not telithromycin) are best avoided in the absence of demonstrated susceptibility of the causative isolate; and (iv) that moxifloxacin may constitute a next 'best empirical choice' since there is no evidence of significant emergence of a non-wild-type population in the considered environment. For levofloxacin, which has a less favourable MIC profile, the larger dose (500 mg twice daily, recommended by EUCAST to avoid dividing the MIC wild-type population distribution [11]) is advisable.

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Ethical approval: The protocol of this academic, non-commercial, observational study was approved by the Ethical Committee of the Faculty of Medicine of the co-ordinating institution (Université Catholique de Louvain, Brussels, Belgium) within the context of a grant application to the Belgian *Fonds de la Recherche Scientifique Médicale* (grant no. 3.597.06).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2011.11.011.

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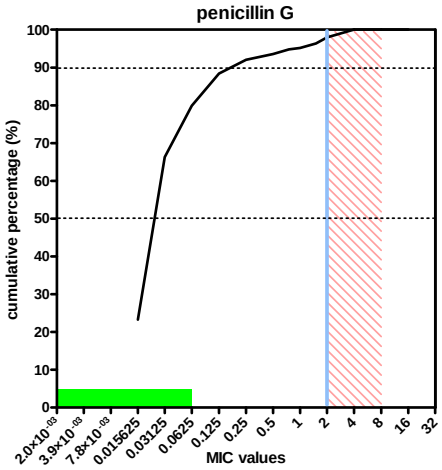
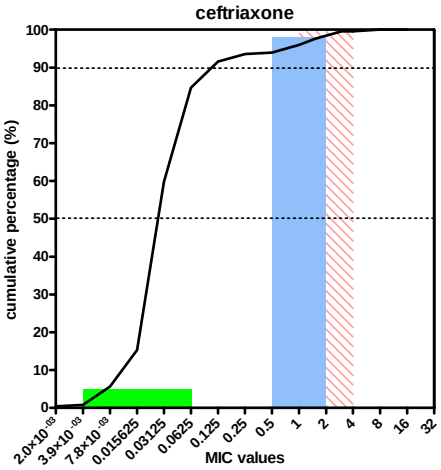
Supplementary material

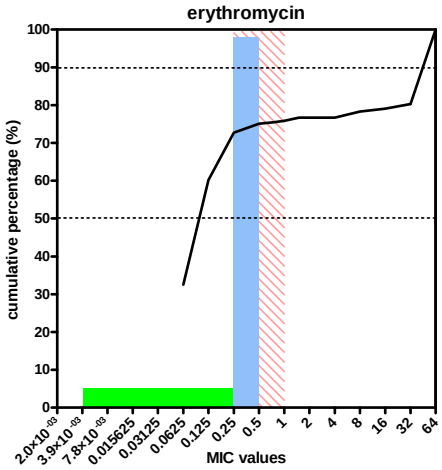
1. Determination of the mechanism of resistance to macrolides by polymerase chain reaction (PCR)

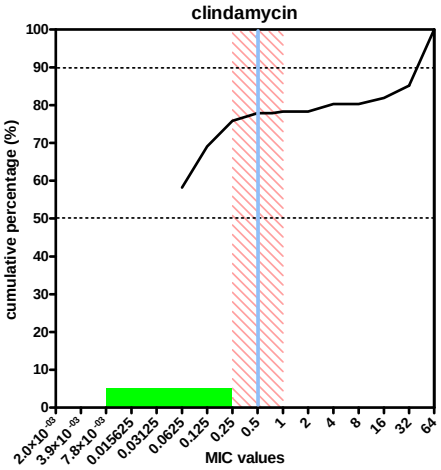
- Primers: 5'-CGTATTGGGTGCTGTGATTG-3' and 5'-TATGCACAGGCGTTCCATTA-3' amplifying equally 248 bp of *mef*(E) or *mef*(A) and 5'-TTGAGTGTGCAAGAGCAACC-3' and 5'-AAAGGGCATTTAACGACGAA-3' amplifying equally 327 bp of *erm*(B) or *erm*(A) (obtained from Eurogentec s.a., Seraing, Belgium).
- PCR mix composition (in 25 μL of sterile distilled water) was 0.5 μM primers (each), 2 mM $MgCl_2$, 0.2 mM dNTP (each), 0.05 U of BIOTAQ^{TM} Red DNA Polymerase (Bioline, London, UK), 1× buffer and the corresponding DNA template. Thermal cycles included an initial denaturation of 95 °C for 4 min, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 62 °C for 1 min and extension at 72 C for 30 s, and a final extension at 72 °C for 5 min.

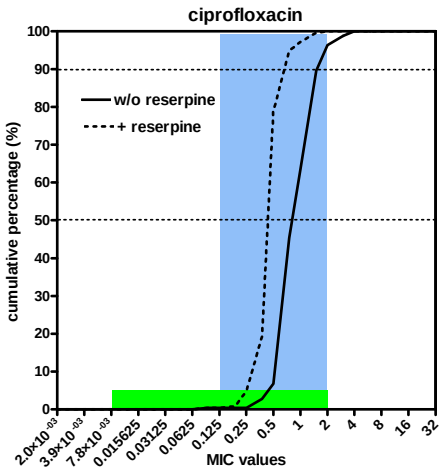
Supplementary Fig. S1. Minimum inhibitory concentration (MIC) distributions (cumulative percentages) of non-duplicate *Streptococcus pneumoniae* isolates (*n* = 249) from all patients enrolled in the study for penicillin G, ceftriaxone, erythromycin, clindamycin and ciprofloxacin (for penicillin G, erythromycin and clindamycin, investigations did not include concentrations lower than 0.0156, 0.0625 and 0.0625 mg/L, respectively). The horizontal green zone in the MIC scale shows the range (mg/L) covered by the wild-type population as defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (penicillin G, ≤0.002–0.063; ceftriaxone, 0.004–0.063; erythromycin, 0.004–0.25; clindamycin, 0.008–0.25; and ciprofloxacin, 0.008–2). The blue and hatched red vertical zones correspond to the MIC range (mg/L) of S (susceptible) to R (resistant) clinical breakpoints defined by EUCAST and the Clinical and Laboratory Standards Institute (CLSI), respectively (see Supplementary Table S1; there is no breakpoint defined for ciprofloxacin by the CLSI). For ciprofloxacin, testing was made in the absence and presence of reserpine (non-specific inhibitor of efflux).


penicillin G
cumulative percentage (%)
MIC values

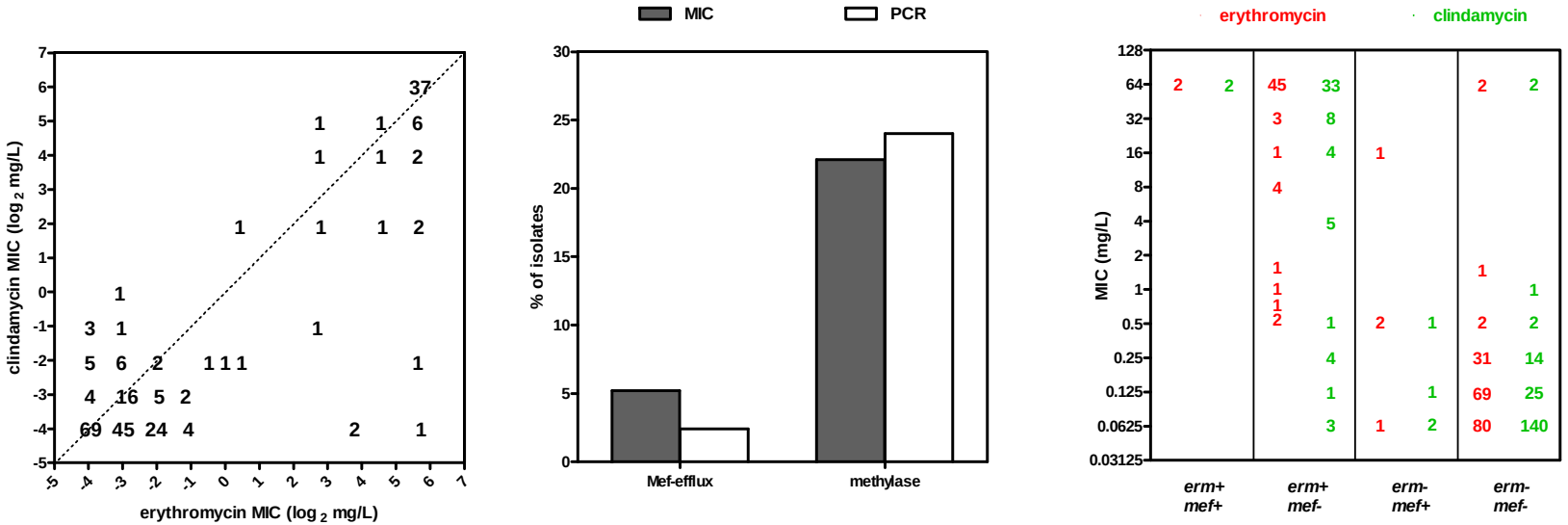

ceftriaxone
cumulative percentage (%)
MIC values


erythromycin
cumulative percentage (%)
MIC values


clindamycin
cumulative percentage (%)
MIC values


ciprofloxacin
w/o reserpine
+ reserpine
cumulative percentage (%)
MIC values

Supplementary Fig. S2. Analysis of the mechanism of resistance of non-duplicate *Streptococcus pneumonia*e isolates (*n* = 249) to erythromycin. Left: correlation between the minimum inhibitory concentrations (MICs) of erythromycin (abscissa) and clindamycin (ordinate); each figure is centred on its corresponding coordinate and shows the number of strains at these values. Middle: grey bars show the percentage of all isolates suggested to show efflux- or methylase-mediated resistance based on MIC dissociation between erythromycin and clindamycin; open bars show the percentage of isolates with positive genomic detection of the corresponding genes (*mef* or *erm*) by polymerase chain reaction (PCR). Right: MIC of isolates categorised as positive or negative for *mef* or *erm* by PCR (the figures indicate the number of strains: red, erythromycin; green, clindamycin).



Supplementary Table S1

Susceptibility pattern of *Streptococcus pneumoniae* isolates (*n* = 249) from patients enrolled in the study with a clinically and radiologically confirmed diagnosis of community-acquired pneumonia (CAP)

Antibiotic	MIC (mg/L)			% non-susceptible isolates [a] according to:			
	Range	MIC_{50}	MIC_{90}	EUCAST		CLSI	
				Breakpoint (≤ S/R >) (mg/L)	Isolates (I/R)	Breakpoint (≤ S/R ≥) (mg/L)	Isolates (I/R)
β-Lactams							
Penicillin G	0.016–4	0.03	0.25	2/2	2 [c]	2/8	2/0
Amoxicillin	0.001–8	0.06	0.125	0.5/2	2.8/3.2	2/8	2.8/0.4
Cefuroxime	0.008–32	0.06	0.25	0.25/0.5 [b]	0.8/6.8	1/4 [b]	0/5.6
Ceftriaxone	0.001–8	0.03	0.125	0.5/2	4.4/1.6	1/4	3.6/0.4
Macrolides/lincosamides							
Erythromycin	0.06–64	0.12	64	0.25/0.5	**2.4/24.9**	0.25/1	**2.8/24.5**
Clarithromycin	0.008–64	0.03	64	0.25/0.5	**0.4/23.7**	0.25/1	**0.8/23.3**
Clindamycin	0.06–64	0.06	64	0.5/0.5	**22.1** [c]	0.25/1	**2/22.1**
Telithromycin	0.008–4	0.03	0.03	0.25/0.5	0.8/0.8	1/4	0/0.4
Quinolones							
Ciprofloxacin	0.094–4	1	2	0.125/2	**96/3.6**	[d]	–

Levofloxacin	0.125–2	0.75	1	2/2	0 [c]	2/8	0/0
Moxifloxacin	0.03–0.38	0.125	0.25	0.5/0.5	0 [c]	1/4	0/0

I, intermediate; R, resistant; MIC, minimum inhibitory concentration; $MIC_{50/90}$, MICs for 50% and 90% of the organisms, respectively; EUCAST, European Committee on Antimicrobial Susceptibility Testing; CLSI, Clinical and Laboratory Standards Institute; S, susceptible.

[a] Figures in bold indicate situations in which non-susceptibility to a given antibiotic exceeds 20% of isolates based on the corresponding criteria of EUCAST (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/EUCAST_breakpoints_v1.3_pdf.pdf) or the CLSI (*Performance standards for antimicrobial susceptibility testing; 20th informational supplement*. Document MS100-S20. Wayne, PA: CLSI; 2010).

[b] Clinical breakpoints for the oral form (cefuroxime axetil).

[c] No intermediate category clinical breakpoints for this antibiotic.

[d] No clinical breakpoint defined.