Antimicrobial resistance in *Streptococcus pneumoniae* isolates from community acquired pneumonia in Belgium, with special reference to efflux mechanism.

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1. INTRODUCTION

1.1. *Streptococcus pneumoniae*

Streptococcus pneumoniae or pneumococcus was and remains one of the major human pathogens worldwide. *S. pneumoniae* is responsible of invasive pneumococcal diseases (IPD) mainly in elderly and in young children. It is the leading cause of community-acquired pneumonia (CAP) in many countries, including Belgium.

S. pneumoniae (Figure 1) is a Gram positive coccus that is catalase-negative, non-motile and non-sporing. *S. pneumoniae* tends to grow in pairs or small chains.

Streptococcus pneumoniae can be differentiated from other Streptococci through the following characteristics: alpha-haemolysis on blood agar witch allow differentiation from beta-haemolytic *Streptococcus pyogenes* but not from other commensal streptococci, for those a combination of tests is used, such as inulin fermentation, bile solubility, susceptibility to optochin or capsular polysaccharide serotyping (the capsular reaction to diagnostic pneumococcal sera).

(a) (b) (c)

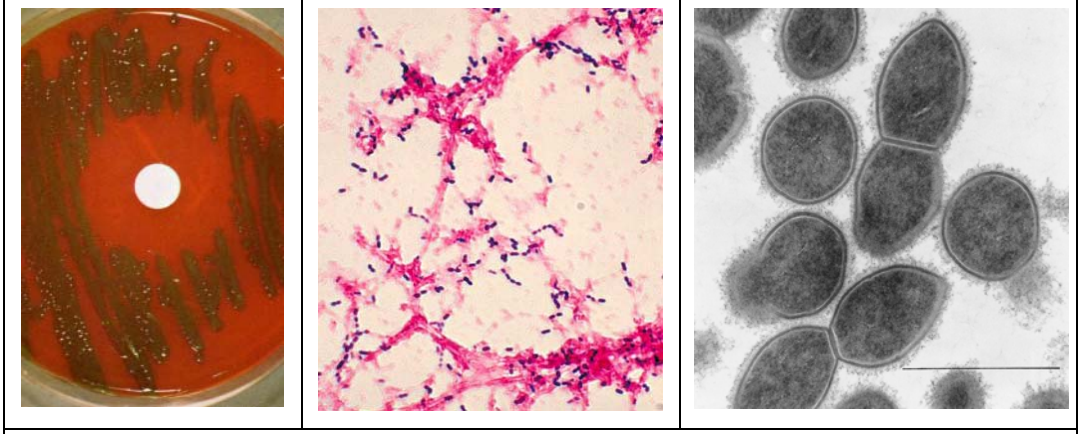


Figure 1: ***S. pneumoniae***

(a) demonstration of hemolysis and susceptibility to optochin on a blood agar plate

(b) optical microscopy after Gram staining showing grow in chains

(c) electron microscopy, showing grow as diplococcus, thick cell wall, and capsule

(a) http://textbookofbacteriology.net/Spalpha.jpeg
(b) http://textbookofbacteriology.net/PHILspbloodGram.jpg
(c) http://www.ppdictionary.com/bacteria/gpbac/pneumoniae.htm

1.1.1. Genetic Characteristics of *S. pneumoniae*: Competence and Transformation

The pneumococcal genome contains about 2,100,000 bp and more than 2,000 genes. The mosaic genome structure of *Streptococcus pneumoniae* explains the remarkably adaptive nature of this organism (Tettelin and Hollingshead, 2004). Its genome plasticity is a result of the abundance and genome-wide density of repeats which contribute to genomic rearrangements. Of interest, next to capsular region, there are multiple copies of repeating elements directly adjacent to each other. (Tettelin and Hollingshead, 2004)
Griffith, in 1928, reported that heat-killed encapsulated "smooth" *S. pneumoniae* could transfer the ability to infect mice when injected together with unencapsulated "rough" strain. He called this phenomenon transformation. Later, in 1944, Avery demonstrated that the agent transferred was DNA. (Lacks, 2004)

Artificial transformation is accomplished by shocking cells either electrically, as in electroporation, or by ionic and temperature shifts. In contrast, natural transformation by free DNA is considered as a sexual-like process that requires a set of specific genes. In *S. pneumoniae* the amounts of DNA introduced by natural transformation are a million-fold greater than by artificial transformation. *S. pneumoniae* can take up as much as 10% of its cellular DNA content. (Lacks, 2004)

The ability to take up free DNA, called competence and measured by transformation frequency, varies during the culture growth cycle. Competence is dependent of the accumulation of an extracellular polypeptide (see figure 2 for an illustration of the regulation of competence). The donor DNA must be in double-stranded form. Although single-stranded DNA can be taken up by *S. pneumoniae*, its ability to transform is <0.1% of that of native double-stranded DNA. The double-stranded DNA is converted to single-stranded DNA upon entry into the bacteria. (Lacks, 2004) If this strand segment has homologies with the chromosome, it is rapidly integrated. DNA lacking homology will mainly fail to be integrated and will eventually be degraded. Illegitimate recombination, at point lacking extensive homology, may occur but is rare (Lacks, 2004) making the genetic barrier between *S. pneumoniae* and closely related species somewhat porous (Spratt *et al.*, 2004). This can be a selective advantage when acquiring resistance against antimicrobial (such as genes coding for PBP 2b and resistance to β-lactams) (Johnsborg and Havarstein, 2009).

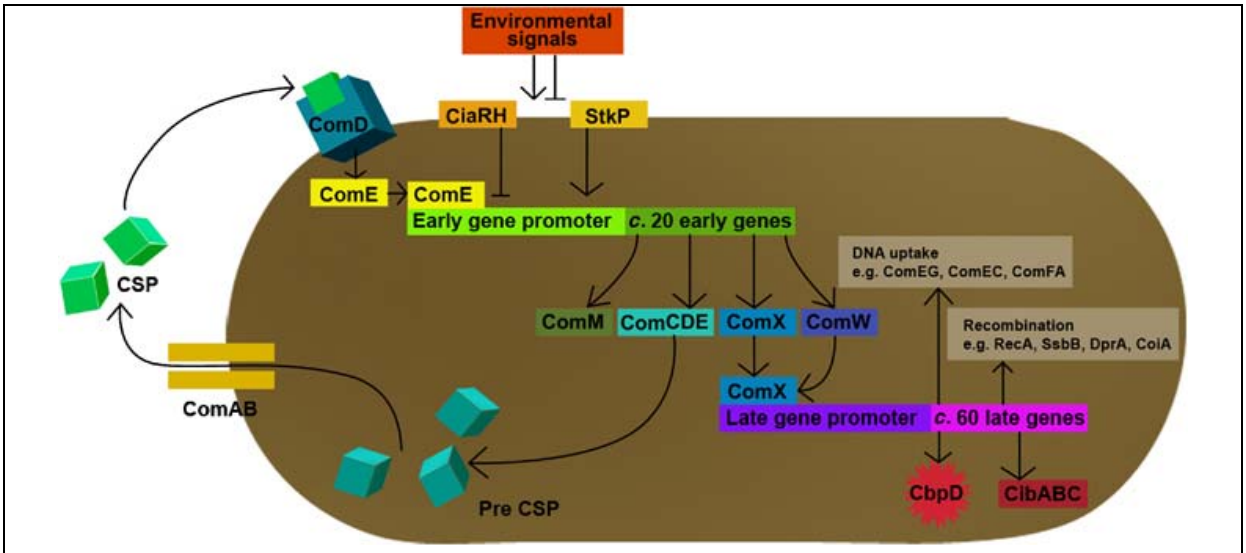


Figure 2: Schematic representation of competence regulation in *Streptococcus pneumoniae*. The CSP precursor, which is encoded by the *comC* gene, is processed and secreted by the dedicated ComAB transporter, resulting in extracellular accumulation of mature CSP. Basal transcription of the *comCDE* operon is subjected to regulation by global regulators such as the serine/threonine protein kinase StkP and the CiaRH two-component system (see text for details). Binding of CSP to its ComD receptor is believed to result in autophosphorylation of ComD and subsequent transfer of the phosphoryl group to the ComE response regulator. ComE then binds to and activates transcription from the various early gene promoters. ComE binding sets off increased transcription of the *comCDE* operon, leading to a boost in the production of CSP, ComD and phosphorylated ComE. This auto-induction loop ensures rapid accumulation of the alternative σ factor ComX, ComW and the ComM fratricide immunity protein. ComW protects ComX from proteolytic cleavage and stimulates the latter protein to activate transcription of the late genes encoding the fratricide trigger factors CbpD and CibAB as well as the protein apparatus for DNA uptake and recombination. While *cibAB* is cotranscribed with a cognate immunity gene (*cibC*), competent cells are protected from the CbpD murein hydrolase by the product of the early gene *comM*. (Johnsborg and Havarstein, 2009)

S. pneumoniae competence varies during growth cycle, as competence is under a two-level regulatory control (Lacks, 2004). A quorum-sensing mechanism constitutes the first level of regulation and involves the product of 5 genes (from 2 different operons: *comAB* and *comCDE*). The product of *comC* is cleaved to give a secreted 17-mer oligopeptide called Competence-Stimulating Peptide (CSP) which can induce competence in *S. pneumoniae* at a level of ~10nM (Havarstein *et al.*, 1995; Lacks, 2004).

A majority of the strains isolated from patients encode the CSP1 sequence, nearly all the rest encode a distinct but similar sequence, CSP2, that differs in 8 amino acid residues (Pozzi *et al.*, 1996). Corresponding to these two alternative forms of CSP, the sequence of the corresponding receptor, encoded by *comD*, differs leading to a specific recognition of the CSP (Lacks, 2004).

Both operons have a low basal level of transcription ensuring the production and release of CSP at a low rate, slowly accumulating in the external medium. When external concentration of CSP reaches a sufficient level it acts on the receptor which activates the response

regulator, encoded by *comE*, that will enhance greatly the transcription from both operons (Lacks, 2004).
The response regulator will also affect a third operon containing the single gene *comX* coding for an alternative sigma factor which replace SigA in RNA polymerase during competence. ComX does not recognize the usual promoter but a different sequence found upstream of operons containing nearly all other genes required for competence for DNA uptake and other functions associated with transformation (Lacks, 2004).

When CSP is added to a noncompetent culture of *S. pneumoniae*, early competence gene products depending on the response regulator ComE will reach a peak after ~5min, while the late competence gene transcripts depending on ComX will reach a peak ~10min after the addition of CSP. (Alloing *et al.*, 1998; Peterson *et al.*, 2004) In the laboratory strain Rx, competence lasts for about ~30 min while in strain R6, competence can last for several generations of bacterial growth. It is not know why competence ceases but it is probably due to one of the late competence genes that blocks competence development. It was observed that mutations in the CSP receptor or the response regulator can lead to constitutive competence (Lacks, 2004).

In addition to natural competence mechanism, the pneumococcus can also acquire new genes located on plasmid or transposons. Plasmid carrying drug resistance genes are rare in *S. pneumoniae*. Drug resistance genes are frequently located on conjugative transposons, which are large chromosomal elements ranging from 15 to 60 kb that contain mobilization factors for their self-transmission to other cells (Lacks, 2004).

1.1.2. Virulence factors, Capsules and Serotypes

Streptococcus pneumoniae has many virulence factors, the main one being the polysaccharide capsule. Traditionally *S. pneumoniae* strains were characterized by serology which divides the population into more than 90 immunologically distinct types (Spratt *et al.*, 2004). The structure of the capsule differs among strains with respect to the sugar composition and the linkage. Most structures are complex containing multiple sugars, linkages, and often, side chains. Few are simple like serotype 3 or 37 (Yother, 2004).
The main function of the capsule is to reduce the opsonophagocytosis by limiting access of phagocytic receptor to complement bound to pneumococcal cell wall, resulting in a resistance to phagocytosis. Spontaneous mutants that loose the capsule, also loose the resistance to phagocytosis and their virulence (Yother, 2004) .

The capsule locus is transcribed as a single operon and often contains insertion sequences elements and nonfunctional genes or genes fragments. The organisation of the capsule locus and the mechanism of capsule synthesis are similar in most strains (Yother, 2004).
S. pneumoniae has the ability to regulate capsule expression which is critical for its survival in the various host niches, and allows a switch from a colonizing to an invasive phenotype. The expression of capsule is reduced in the nasopharynx to allow exposition of the adhesins which are necessary for colonisation. On the opposite, its expression is increased in systemic infections to avoid complement-mediated opsonophagocytosis (Weiser *et al.*, 1994). The expression of the capsule also varies in vitro. Transparent-phase variants have a reduced expression of capsule as opposed to opaque-phase variants (Yother, 2004); they show increased adherence to epithelial cells but lower production of biofilm than there opaque counterparts (Romero-Steiner *et al.*, 2003; Trappetti *et al.*, 2011).

The capsule type will affect the ability to colonize as well as the virulence. There are more than 90 different serotypes but only a few of them cause invasive pneumococcal diseases (Yother, 2004). For example, serotype 1 is rarely found in carriage, but is frequent in IPD. Serotypes 3, 6A and B, 9N, and 19F are associated with a higher risk of mortality during bacteraemic pneumonia (Dockrell *et al.*, 2012). In young children, before introduction of the PCV7, serotype 3 was frequent in otitis media, but rare in invasive diseases (Feikin and Klugman, 2002; Brueggemann *et al.*, 2003; Yother, 2004; Flamaing *et al.*, 2008) and was therefore not included in the PCV7 (Hausdorff *et al.*, 2000a; Hausdorff *et al.*, 2000b). However, with the wide use of PCV7, AOM (Alonso *et al.*, 2013) and IPD (Ciruela *et al.*, 2013; Shen *et al.*, 2013) caused by serotype 3 increased dramatically, supporting its presence in the PCV13 formulation. Serotyping is important since existing vaccines are mimicking the capsular polysaccharides of those serotypes most commonly associated with invasive diseases (Hausdorff *et al.*, 2000a; Hausdorff *et al.*, 2000b; Spratt *et al.*, 2004).

Yet, virulence is a multifactorial process. Beside capsule, other virulence factors include the pore-forming toxin pneumolysin (PLY) (Mitchell, 2004), the choline binding proteins such as the pneumococcal surface protein A (PspA) (Swiatlo *et al.*, 2004), the autolysin (LytA) (Lopez *et al.*, 2004), the pilus (Barocchi *et al.*, 2006) and many other potential ones, but their roles and contributions to virulence differ among strains (Mitchell, 2004). A recent study demonstrates that the genes coding for autolysins (*lytC, lytA),* adhesion (*pavA*) and competence (*comD*) are the most highly expressed in the nasopharynx of healthy children, making these proteins attractive targets for vaccine development (Sakai *et al.*, 2013).

1.1.3. Diseases, Carriage, Burden

Transmission of *S. pneumoniae* occurs via respiratory droplets from healthy persons carrying the organism in the nasopharynx or from person with pneumococcal disease. Following exposure, the organism may establish itself in the nasopharynx of its new host, usually resulting in asymptomatic colonisation. The organism can be carried for a period of weeks to months. However sometimes, the newly acquired pneumococcus evades host defensive mechanisms and causes illness (Butler, 2004).

First colonization generally happens around 6 months of age, but some may acquire their first pneumococcus within the first weeks of life (Musher, 2004). The colonization rate rises from birth until it peaks around the age of 1-2 years (40-60%), then an age related decline is observed (20-40% colonization in older children, 5-10% in adults) (Musher, 2004 Children will acquire several different strains and serotypes over time (Donkor, 2013). Duration of carriage decreases with successive strain acquisition and inversely correlates with age (Obaro and Adegbola, 2002). In adults, pneumococcal strains usually persist for 2 to 4 weeks, which is shorter than in children (7-8 weeks) (Melegaro *et al.*, 2004). Some reports indicate carriages longer than 30 weeks (Donkor, 2013). Carriage duration is also very depending on the serotype (Melegaro *et al.*, 2004; Abdullahi *et al.*, 2012) as the less immunogenic serotypes tend to be carried longer in the nasopharynx (Obaro and Adegbola, 2002).

For healthy carriers, colonisation can be considered as an immunizing event resulting in production of antibodies directed against the capsular polysaccharides of the carried strain. Even if colonisation appears as a peaceful and transient coexistence between host and potential pathogen, the situation is not entirely benign as the carried *S. pneumoniae* can be transmitted to other hosts and can cause disease in susceptible persons. Furthermore, if the colonized person receives antimicrobial agents, the carried strain may develop drug resistance (Butler, 2004).

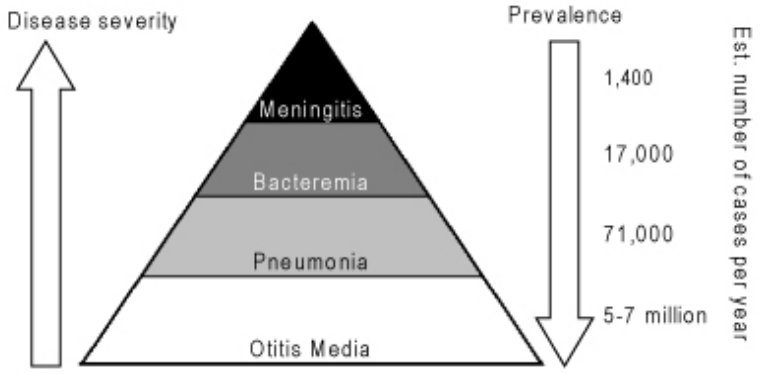
The large burden of disease caused by *S. pneumoniae* is mainly due to its ubiquity in human populations (Butler, 2004). Many host factors are known to be associated with pneumococcal disease such as age (mainly young children and elderly are infected), underlying medical conditions or immune system not functioning properly (diabetes mellitus, cancer, HIV infection), inflammatory conditions (smoking, asthma, chronic obstructive pulmonary disease), preceding or coincident respiratory viral infection, alcohol and tobacco use, living conditions (crowding, daycare centers), and socioeconomic status (Nuorti *et al.*, 2000; Chidiac, 2012; Ruoff and Bisno, 2013).

Two major categories of clinical manifestations do exist: mucosal infections, most often involving the upper respiratory tract, and invasive infections, where *S. pneumoniae* is isolated from normally sterile body site (Butler, 2004).

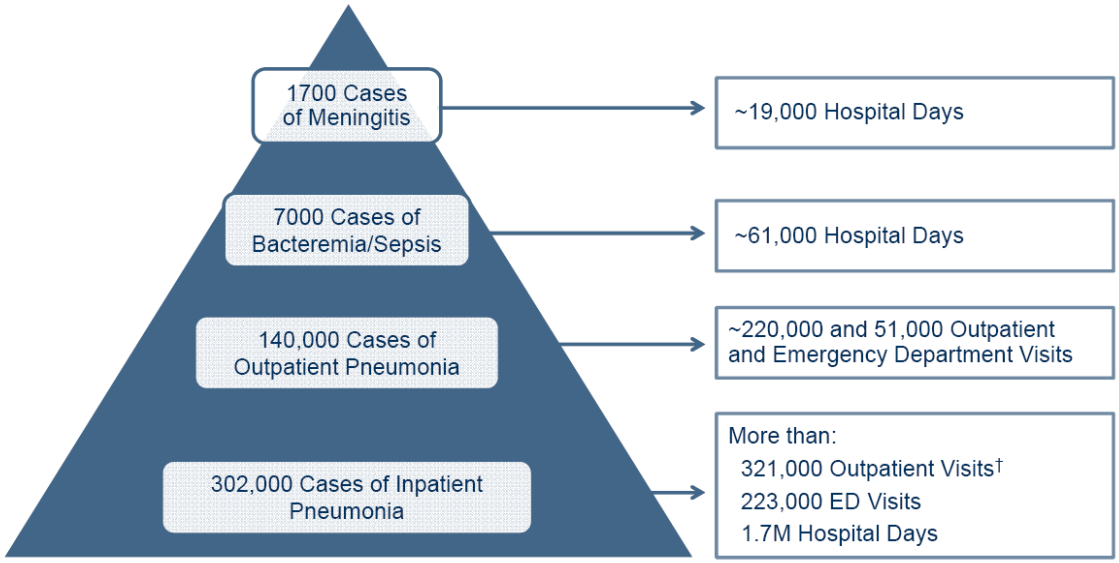
In all cases, colonization is a first and necessary step to pneumococcal infection (Blasi *et al.*, 2012) From the nasopharynx, pneumococci can spread to the respiratory tract, causing sinusitis, otitis media, or pneumonia. Moreover, the bacteria can also cross epithelial barriers and gain access to the blood, the pleura, or the meninges to cause invasive diseases (Dockrell *et al.*, 2012).

Figure 3 illustrates the prevalence and the impact of severe pathologies caused by *S. pneumoniae* in the most targeted populations (children and elderly).

Figure 3: main severe pathologies caused by *S. pneumoniae* and their impact in the US



(a) in children (Edwards, 2004)



(b) in adults older than 50 years
http://www.ct.gov/dph/lib/dph/infectious_diseases/immunization/naicp/3-april-2012-immunization_coordinators_teleconference_%5Bread-only%5D_%5Bcompatibility_mode%5D.pdf

With respect to respiratory tract infections, pneumococcus is a leading cause of community-acquired pneumonia (CAP). When gaining access to the alveola, bacteria will proliferate, activate complement and cytokine production, inducing an inflammatory reaction, and the filling of alveola by bacteria, white blood cells, and inflammatory exudate. Major symptoms include high fever, cough, fatigue, chills, and shortness of breath (Ruoff and Bisno, 2013).
The incidence of pneumococcal CAP is varying among countries and over time. In 1998-1999, Lim *et al*. found that *Streptococcus pneumoniae* was responsible of 48% of CAP in adult inpatients in Nottingham City Hospital, followed by influenza A virus (19%), *Chlamydia pneumoniae* (13%), *Haemophilus influenzae* (7%), *Mycoplasma pneumoniae* (3%), *Legionella pneumophilia* (3%), other *Chlamydia* spp (2%), *Moraxella catarrhalis* (2%), *Coxiella burnetii* (0.7%), others (3%) (Lim *et al.*, 2001). A recent European survey indicates a mean isolation rate of pneumococcus of 38% of outpatient cases and 27% of inpatient cases (Welte *et al.*, 2010).

In children, another frequent clinical manifestation of pneumococcal infection is acute otitis media. *S. pneumoniae* has classically been indeed isolated in about half of the microbiologically-documented cases (Ruoff and Bisno, 2013). However, the picture may change in the future due the spreading of anti-pneumococcal vaccination (Pichichero, 2013). Serotypes with higher ability to adhere to epithelial cells (6, 14, 19F, 23F) are more prevalent (Ruoff and Bisno, 2013).
Eustachian tube dysfunction or prior viral infection (e.g.: influenza, RSV) are predisposing factors, by favouring accumulation of secretions and congestion of mucosa. The pathology causes fever, pain and impairs hearing (Klein, 2005).

Acute sinusitis is the third type of infection where *S. pneumoniae* is highly prevalent, being responsible for about 30 % of cases in both children and adult populations (Gwaltney, 2005). It is often superimposed to bacterial infection, with has main symptoms fever, facial pain, sneezing and purulent nasal discharge (Gwaltney, 2005).

Finally, *S. pneumoniae* is also one of the most prevalent causes of infectious acute exacerbation in patients suffering from chronic obstructive bronchitis. The chronical inflammation of the bronchi is accompanied by an hypersecretion of mucus and the proliferation of bacteria. *S. pneumoniae* is found again in about 30 % of cases. This pathology being chronic and irreversible, these patients will require repetitive courses of antibiotics, with a higher risk of selection of resistant strains (Nseir and Ader, 2008).

With respect to invasive diseases, pneumococcus can invade the bloodstream (causing bacteremia) and pass through endothelial cells causing hematogenous infections (such as meningitis, peritonitis, pericarditis) (Musher, 2004). Of note, bacteraemia is present in about 10-30 percent of patients suffering from pneumococcal community-acquired pneumonia (Blasi *et al.*, 2012).

1.1.4. Vaccines and antibiotics

Preventive treatment includes two different types of vaccines: a capsular polysaccharide and a conjugate vaccine (Pletz *et al.*, 2008). Both are a mixture of various numbers of serotypes, selected based on their prevalence (Yother, 2004) and virulence in pneumococcal diseases in the target population (Käyhty and Mäkelä, 2004). The distribution of serotypes can be influenced by the age as well as by the immune status of the host, the type of disease, and geographic region (Butler, 2004).

Serotypes	PPV23	PCV7	PCV13
1	x		x
2	x		
3	x		x
4	x	x	x
5	x		x
6A			x
6B	x	x	x
7F	x		x
8	x		
9N	x		
9V	x	x	x
10A	x		
11A	x		
12F	x		
14	x	x	x
15B	x		
17F	x		
18C	x	x	x
19A	x		x
19F	x	x	x
20	x		
22F	x		
23F	x	x	x
33F	x		

Table 1:
Serotypes included in the PPV23, PCV7 and PCV13 vaccines.

The 23-valent pneumococcal polysaccharide vaccine (PPV23) contains the 23 most common capsular polysaccharide antigens (Table 1): 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. In early studies, the 23-

valent vaccine showed good efficacy in immunocompetent adults against invasive infections, especially bacteraemia and meningitis (overall 60 to 75%) (Bolan *et al.*, 1986). However, in patients from high-risk groups (HIV, elderly,...) the antibodies concentrations were lower and the response duration was shorter (Käyhty and Mäkelä, 2004). When developing this vaccine, pneumococcal pneumonia was the major target, however the PPV23 did not show efficacy towards overall pneumonia, and due to uncertainties in the aetiological diagnosis, it was not possible to determine whether or not this vaccine protected from pneumococcal pneumonia (Käyhty and Mäkelä, 2004). The PPV23 induces a short-term memory immune response based on B-cells that is efficient against IPD in adults (depending on the studies between 40 and 80%) (Pletz *et al.*, 2008; Van Steenkiste, 2013) while not conferring protection against mucosal infections. However PPV23 still decreases the severity of pneumonia and so decreases the risk of mortality due to CAP. Population that should be vaccinated are elderly (≥65 years) and persons at risk for invasive pneumococcal infections (Pletz *et al.*, 2008) such as adults with chronic diseases (heart, lung, liver, renal) or immunocompromised, or HIV infected patients. The PPV23 is used in adults only.
Due to their immature immune system, the PPV23 does not induce immunity in children under 2 years old. To solve this problem, the pneumococcal polysaccharides are conjugated to a carrier antigenic protein resulting in a T-cell-dependant immune response. In addition, conjugate vaccines induce high-avidity antibodies. It triggers B and T dependant immune response as well as mucosal immunity (Käyhty and Mäkelä, 2004).

From January 2007 to August 2011, children in Belgium received the heptavalent conjugate vaccine Prevenar (PCV7, Wyeth Lederle Vaccines S.A., Belgium). From September 2011 onwards, the Prevenar13 (PCV13) has been used in Belgium. During the period of this thesis, only PCV7 was used, (the PCV7 vaccine includes seven serotypes (4, 6B, 9V, 14, 18C, 19F and 23F) linked to the carrier protein CRM197, a nontoxic variant of diphteria toxin). This vaccine appeared safe and very efficient against IPD (97,4%), but less against pneumonia (21%) and even less against acute otitis media (7,8%) (Black *et al.*, 2000; Whitney *et al.*, 2003). The PCV7 was licensed in 2000 in the United States and it was launched on the market in 2001 in the European Union (Käyhty and Mäkelä, 2004).
Since its introduction, the incidence of IPD has decreased in children, and there was also a marked decrease of AOM (Eskola *et al.*, 2001) and of visits to the general practitioner. On the opposite of the PPV23, the PCV7 had an effect on the pneumococcal carriage. The reduction of carriage is specific to the serotypes included in the conjugate vaccine, and therefore their transmission to other non-vaccinated person is also reduced, leading to an overall reduction of infections caused by those serotypes in the population, which is called herd immunity. On the other hand, as this effect was specific to the serotypes included in the

vaccine, it was also accompanied by an increase of both the incidence and the prevalence of the non-vaccine types (Käyhty and Mäkelä, 2004). Last decade, we have assisted to a shift of the serotypes in the population by the ones non included in the PCV7. This serotypes replacement is not entirely due to the use of PCV7 (Van Steenkiste, 2013) as it already started before the latter was on the market and it is part of the population evolution of *S. pneumoniae* (Harboe *et al.*, 2010).

For treatment, various antibiotics from different classes can be used. These are chosen according to the host (age, allergy, localisation of the infection) and the epidemiology of antibiotic resistance of pneumococcus strains (MIC of the isolate or trends in antimicrobial resistance in the region or country).

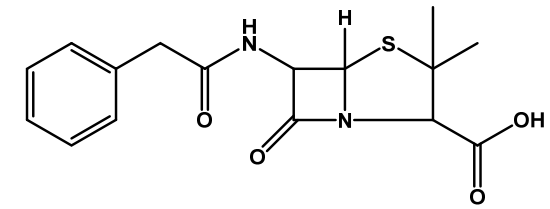
1.2. Antimicrobials used for treating pneumococcal infections

1.2.1. Penicillins and Cephalosporins

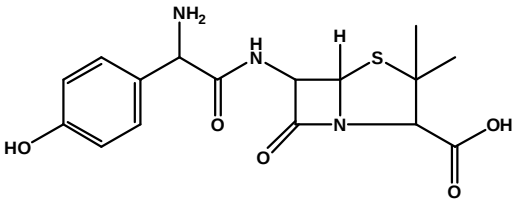
Aminopenicillins and penicillins are widely used for the treatment of pneumococcal infection. These antimicrobial drugs are the first line antimicrobials in many countries to treat acute otitis media and community-acquired pneumonia (File, Jr. *et al.*, 2004).
Beta-lactams inhibit the peptidoglycan synthesis of the bacterial cell wall by forming a covalent bond with the active site of penicillin-binding proteins (PBP) leading to hydrolysis of the bacteria. Penicillin-binding proteins are membrane proteins catalyzing late steps of murein biosynthesis outside the cytoplasmic membrane (Bergmann *et al.*, 2004). Mutations crucial for the development of resistance against beta-lactams are located in the penicillin-binding domain common to all PBP to perform the penicillin-sensitive reaction (a transpeptidation between two muropeptides) resulting in the cross-linked structure of the murein network. Mutations result in a decrease affinity to beta-lactams. However, the inhibition of those low-affinity PBP variant can be reached with higher antibiotic concentrations. There are six PBPs in *S. pneumoniae* : PBP1a, -1b, -2a, -2b, -2x and -3 (Bergmann *et al.*, 2004). In all of them, low affinity variants associated to beta-lactam resistance have been described. PBP2x and PBP2b are primary resistance determinants: the low affinity variant of each of them alone is enough to confer resistance (Grebe and Hakenbeck, 1996). Both are essential proteins which is not the case of the other pneumococcal PBP (Bergmann *et al.*, 2004).

In resistant clinical isolates, genes coding for low-affinity forms of PBP2x, PBP2b and PBP1a contain sequence blocks with up to over 20% divergence compared to those in sensitive genes, resulting in approximately 10% amino acid difference. Those sequence blocks have not evolved by mutations but are the result of gene transfer events most probably from commensal streptococci followed by recombination into the pneumococcal chromosome resulting in mosaic genes (Bergmann *et al.*, 2004). A new cephalosporin recently registered in the US and in Europe, ceftaroline, shows high activity against *S. pneumoniae* and keeps reasonably low MIcs against strains resistant to currently-used beta-lactams (Lemaire *et al.*, 2013).

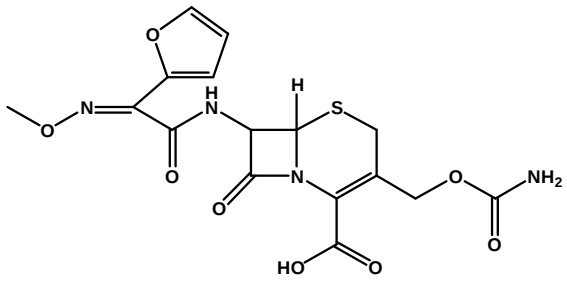
For this thesis, susceptibilities to the following molecules were tested: penicillin, amoxicillin, cefuroxime, ceftriaxone (see figure 4). Penicillin was mainly used as a marker of resistance, and it is also used in the clinics by i.v. route. Amoxicillin is the drug of choice to treat community-acquired pneumonia, and is available both for oral and i.v. treatment. It is often combined with clavulanic acid to extend the spectrum to β-lactamase producers. The combination of ampicillin (another aminopenicillin) with sulbactam is also available in some countries. Yet, ampicillin shows a lower oral bioavailability than amoxicillin (Gordon *et al.*, 1972), while sulbactam is less active than clavulanic acid on some class A β-lactamases (Akova, 2008), which are the most frequent in community acquired pathogens (Schito *et al.*, 1994). Cefuroxime is a very frequent alternative to amoxicillin. The molecule is also available in i.v. and orally (as a prodrug called cefuroxime-axetil). Ceftriaxone is another alternative to amoxicillin and is only available in i.v (also in IM form or even for SC administration in elderly patients with systemic infection); its interest lies in its long half life allowing a once-a-day administration.



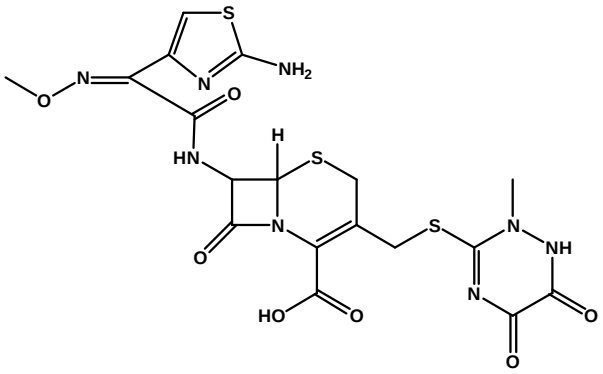
penicillin G



amoxicillin



cefuroxime



ceftriaxone

Figure 4: chemical structure of beta-lactam antibiotics used in the present study.

1.2.2. Macrolides and related antimicrobials

Macrolides are bacteriostatic antibiotics composed of amino and/or neutral sugar attached to 14-membered lactone rings in the case of erythromycin and clarithromycin, or 15- or 16-membered rings (Ambrose and Stephens, 2004). Their binding to the 50S ribosomal subunit stimulates the dissociation of the peptidyl-tRNA molecule from the ribosome and results in premature release of the peptide chain blocking the elongation step of protein synthesis (Weisblum, 1995a). Although structurally very different, lincosamides (such as clindamycin) and streptogramins share a similar mechanism of action with macrolides (Edelstein, 2004).

In *S. pneumoniae*, resistance to macrolides can occur by modification of the target either by methylation or by mutations, or by active efflux of the antibiotic.

Methylation of the 23S rRNA is a posttranscriptional modification done by adenine-N^6 methyltransferase. This methylase adds one or two methyl groups to an adenine residue within domain V of the 23S rRNA, which is the peptidyl transferase center, while conserving the secondary structure of this region. Methylation confers cross-resistance to macrolides, lincosamides and streptogramin B antibiotics (MLS_B resistance phenotype) as their binding sites overlap. Methylation usually results in high MIC (≥64 µg/mL). Many Gram-positive

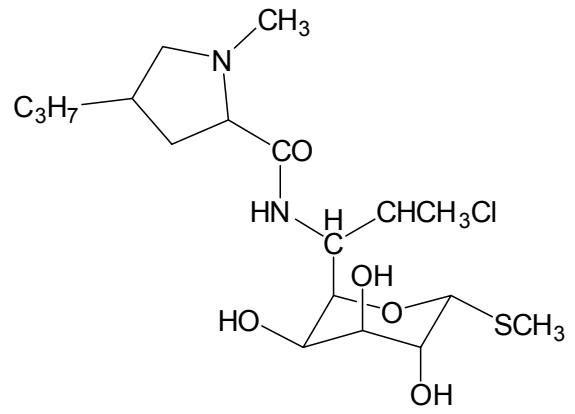
bacteria possess the genes encoding these methylases which have been designated *erm* for erythromycin ribosome methylase (Ambrose and Stephens, 2004). Those methylase genes are acquired via conjugative transposons. In pneumococci, *erm*(B) is the most frequently found, followed by *erm*(A) (Roberts *et al.*, 1999). The expression of these methylases is either constitutive or inducible by the antibiotic itself at the mRNA level (Weisblum, 1995b; Ambrose and Stephens, 2004).

Mutations altering the 23S rRNA subunit or the ribosomal protein L4 or L22 genes can lead to macrolide resistance in pneumococci. The pneumococcal genome has four copies of the 23S rRNA subunit, macrolides resistance occurs when at least two copies carry mutations. Point mutations can result in resistance (Canu *et al.*, 2002; Leclercq and Courvalin, 2002; Reinert *et al.*, 2003). Some particular unusual resistance phenotypes such as ML (macrolides and lincosamides), $M_{16}S$ (16-membered rings macrolides and streptogramins), or MSK (macrolides, streptogramins and ketolides) were also described (Tait-Kamradt *et al.*, 2000; Depardieu and Courvalin, 2001).

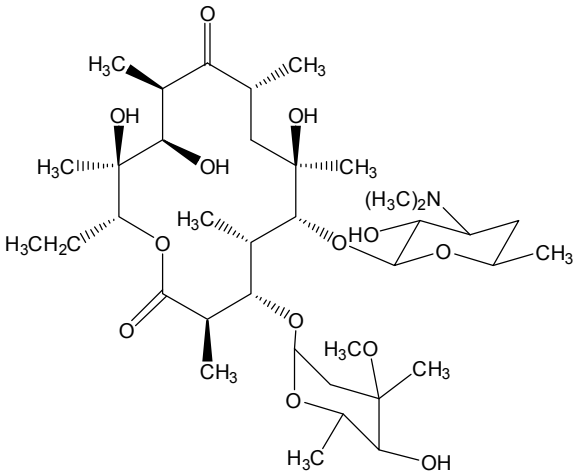
Active efflux of macrolides is due to the expression of the Mef efflux pump. The *mef* gene typically provide low level resistance, with erythromycin MIC of 2 to 16 µg/mL (Luna *et al.*, 1999). This pump belongs to the Major Facilitator Superfamily (MFS) class of transporter which use the proton motive force to drive efflux (Tait-Kamradt *et al.*, 1997). It is specific for 14- and 15-membered lactone rings macrolides, resulting in the so-called M resistance phenotype (Tait-Kamradt *et al.*, 1997). Three variants have been described in *S. pneumoniae*: *mefE*, the most frequent, *mefA* and the very rare *mefI* (Cochetti *et al.*, 2005). The *mefE* gene is present on the 5.4- or 5.5-kb macrolide efflux genetic assembly (mega) element which was found to be inserted in four different sites within pneumococcal chromosome (Gay and Stephens, 2001; Del Grosso *et al.*, 2002). Additionally, it was found that the presence of erythromycin induces MefE efflux resistance (Leclercq and Courvalin, 2002). The *mefA* gene is located on a 7.2-kb defective transposon (*Tn1207.1*) (Santagati *et al.*, 2000) found to be integrated at a single specific chromosomal site (*celB*) in all strains examined (Gay and Stephens, 2001; Del Grosso *et al.*, 2002). Interestingly, insertion of *Tn1207.1* in this gene impaired competence in those pneumococcal isolates (Gay and Stephens, 2001). Of note, however, M phenotype could actually cover strains expressing Mef efflux systems, but also strains with an inducible MLS_B phenotype. This is illustrated in a study performed on *S. pyogenes,* in which inducible MLS_B strains showed low or high level resistance level to macrolides but remained susceptible to clindamycin (Bemer-Melchior *et al.*, 2000).

Isolates carrying both an *erm* and a *mef* gene are of MLS_B phenotype (Luna *et al.*, 1999).
Ketolides, such as telithromycin, are semisynthetic derivatives of the 14-membered macrolides. Ketolides inhibit protein synthesis by interacting with domain V, like macrolides, but also with domain II of 23S rRNA (Douthwaite, 2001; Ackermann and Rodloff, 2003). Usually acquisition of *erm* alone is not sufficient for resistance and additional mechanisms, such as mutation in ribosomal protein L4, are needed to confer high-level resistance to ketolides (Ackermann and Rodloff, 2003).

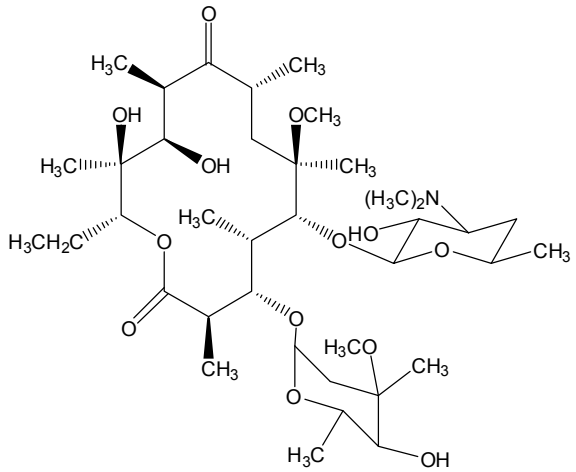
For this thesis, susceptibilities to the following molecules were tested: erythromycin , clarithromycin, clindamycin, telithromycin, and solithromycin (see Figure 5).
Erythromycin and clindamycin were used as antibiotic-resistance indicators and efflux indicators. Clarithromycin is currently the main macrolide used to treat pneumonia. Telithromycin is the only ketolide on the market, and solithromycin (formerly called CEM-101) is a promising molecule having completed phase II of clinical development (Oldach *et al.*, 2013) and currently in phase III for the treatment of moderate to moderately-severe community-acquired bacterial pneumonia (see http://www.clinicaltrials.gov – study NCT01756339).



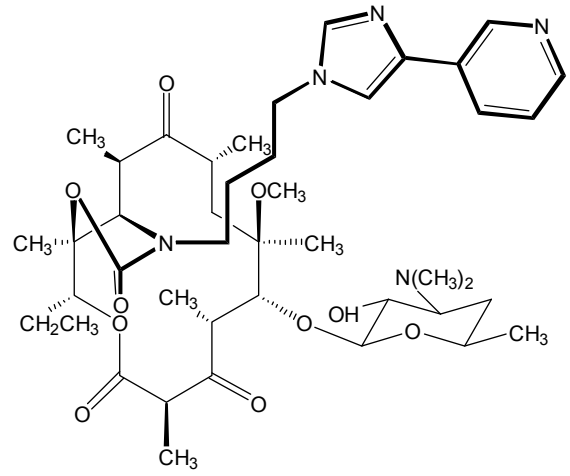
Clindamycin



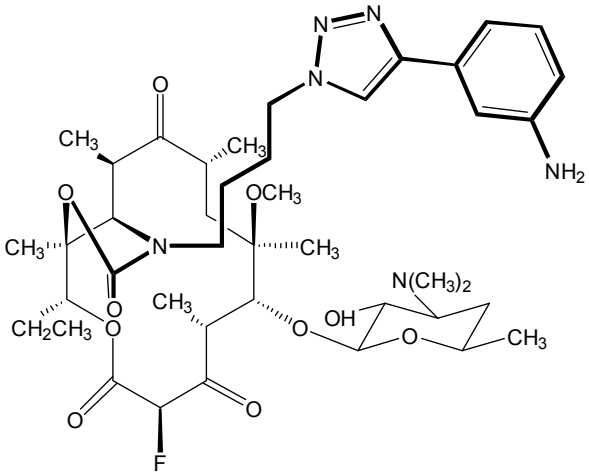
Erythromycin



clarithromycin



Telithromycin



solithromycin

Figure 5: chemical structure of lincosamides, macrolides and ketolides used in the present study.

1.2.3. Fluoroquinolones

Quinolones are broad-spectrum bactericidal antibiotics targeting bacterial type II topoisomerases: DNA gyrase and topoisomerase IV. The main function of these enzymes is to maintain the correct level of DNA supercoiling. The DNA gyrase, composed of two GyrA and two GyrB subunits, facilitates DNA unwinding during replication and transcription. The topoisomerase IV, composed of two ParC and two ParE subunits, is responsible for the unlinking of daughter chromosomes following DNA synthesis. Quinolones form a tripartite complex with the enzyme and the DNA to stimulate DNA cleavage and to inhibit religation of the resulting cut DNA, leading to dissociation of the enzyme subunits (Ambrose and Stephens, 2004; Van Bambeke *et al.*, 2010) In this class, and depending on their chemical structure, some molecules have a spectrum rather oriented towards gram-negative bacteria, like norfloxacin or ciprofloxacin, while other are much more active on Gram-positive bacteria, like moxifloxacin (Van Bambeke *et al.*, 2005). The latter type of molecule is thus recommended for pneumococcal pneumonia, mainly in case of resistance to other antibiotics.

In *S. pneumoniae*, resistance to fluoroquinolones mostly occurs by mutations in topoisomerases or by efflux. Mutations occur in the quinolone resistance-determining regions (QRDR) of mainly *gyrA* or *parC* depending on the antibiotic used to select resistance. (Ambrose and Stephens, 2004; Avrain *et al.*, 2007) It is a stepwise mechanism eventually leading to a high-level resistance (Nagai *et al.*, 2001).

Two major efflux systems have been described so far in *S. pneumoniae* for fluoroquinololones (Figure 6). PmrA, a member of the major facilitator superfamily proton-dependent pumps, was the first described fluoroquinolone transporter (Gill *et al.*, 1999). It provides low-level resistance to norfloxacin, mainly in engineered strains overexpressing this transporter (Gill *et al.*, 1999). Soon after, other studies have suggested the presence of another pump conferring resistance to a broader range of substrates (Piddock *et al.*, 2002; Pestova *et al.*, 2002; Martinez-Garriga *et al.*, 2007), which was eventually identified has the PatA-PatB efflux system (Marrer *et al.*, 2006a). PatA and PatB belong to the ATP binding cassette (ABC) superfamily (Marrer *et al.*, 2006b). The system confers resistance to both norfloxacin and ciprofloxacin (Marrer *et al.*, 2006b) and has been found associated with resistance in clinical isolates (Garvey *et al.*, 2010).

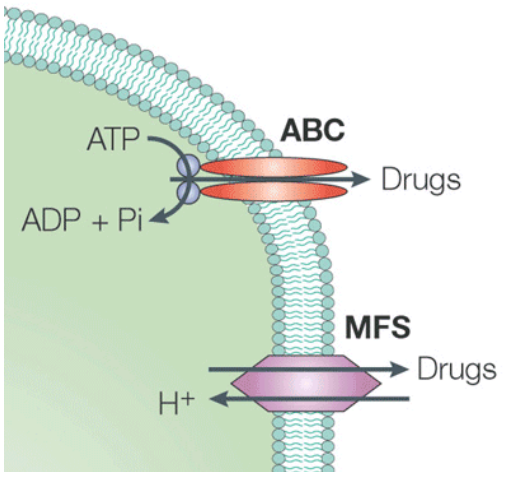


Figure 6: Major efflux systems to fluoroquinolones expressed by *S. pneumoniae.*

PatA-PatB belongs to the ABC superfamily (using ATP-hydrolysis as source of energy)

PmrA belongs to the MFS superfamiliy (proton antiporters).

In other bacterial species, the homologues of PatA and PatB are interdependent and function as heterodimers (Lubelski *et al.*, 2004). The first studies in pneumoccocus suggested that PatA and PatB function together to confer intrinsic resistance to fluoroquinolones (Robertson *et al.*, 2005) while representing two independent systems (Marrer *et al.*, 2006b). It has been recently demonstrated that, like other bacterial ABC transporters, they work as heterodimers (Boncoeur *et al.*, 2012).

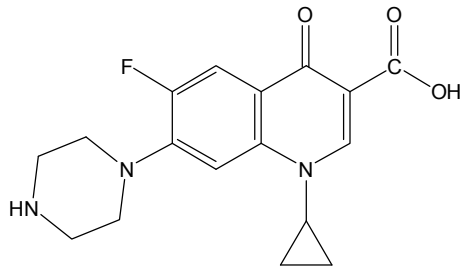
The genes encoding PmrA, PatA and PatB are all naturally present in the pneumococcal chromosome. However, it is the overexpression of those genes that confers the resistance. (Marrer *et al.*, 2006b; Avrain *et al.*, 2007; Garvey and Piddock, 2008) All fluoroquinolones act as inducers on the expression of *patA* and *patB*. (Marrer *et al.*, 2006a; Avrain *et al.*, 2007; see also the results of our own studies [El Garch *et al.*, 2010]).

Recently, the DinF transport system (SP1939) from the MATE family has been shown to confer increased susceptibility to moxifloxacin, ciprofloxacin, and levofloxacin (Tocci *et al.*, 2013). Its expression or impact in clinical strains has not yet been documented.

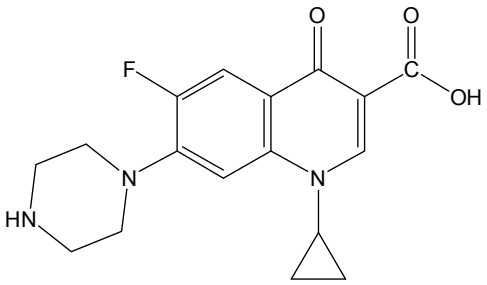
For this thesis, the following molecules were used: ciprofloxacin, norfloxacin, levofloxacin, moxifloxacin, garenoxacin and gemifloxacin (see figure 7).

Ciprofloxacin and norfloxacin are second generation fluoroquinolones, both were used as resistance and efflux indicators (ciprofloxacin and norfloxacin being used to treat infections due to Gram-negative bacteria). Levofloxacin and moxifloxacin are called "respiratory" fluoroquinolones due to their spectrum rather oriented towards Gram-positive infections, including pneumococci (also sometimes classified as third generation fluoroquinolones). Garenoxacin (not used in the clinics) was taken as an example of des-fluorquinolonone (meaning a molecule were the F substituant present in fluoroquinolones as been removed without loss of potency) (Van Bambeke *et al.*, 2005; Keam *et al.*, 2005). Gemifloxacin is a

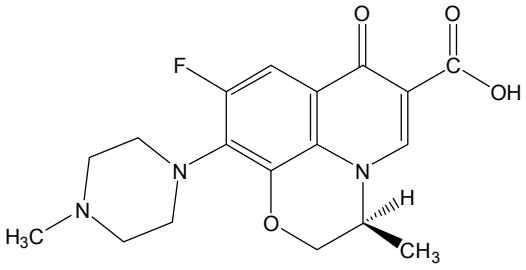
fourth generation respiratory fluoroquinolone (Van Bambeke *et al.*, 2005; Blondeau and Tillotson, 2008), it is not used in Belgium.



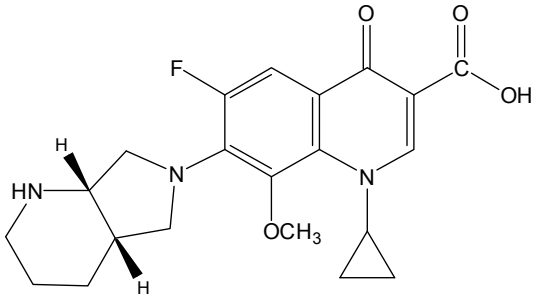
norfloxacin



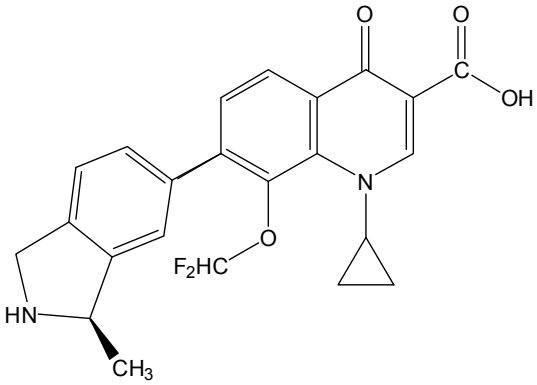
ciprofloxacin



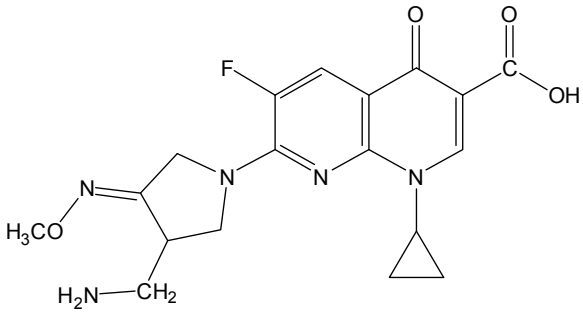
levofloxacin



moxifloxacin



garenoxacin



gemifloxacin

Figure 7: chemical structure of fluoroquinolones used in the present study.

1.2.4. Other antimicrobials

Tetracyclines are broad-spectrum antimicrobials agents that inhibit protein synthesis by binding to the 16S rRNA part of the 30S subunit (Van Bambeke *et al.*, 2010) of the bacterial ribosome. Tetracycline resistance results by acquisition of *tet(M)* and occasionally *tet(O)* genes conferring a ribosomal protection (Ambrose and Stephens, 2004). This is the only

resistance mechanism known in the pneumococcus (Ambrose and Stephens, 2004). Due to a high prevalence of resistance to these antibiotics, there are not used anymore for pneumococcal infections.

Trimethoprim-sulfamethoxazole or co-trimoxazole is a broad spectrum antimicrobial agent that acts by inhibiting the folate pathway by competition with the bacterial molecules. Resistance occurs via enzymes mutations preventing the binding to the dihydrofolate reductase (DHFR) for trimethoprim or to the dihydropteroate synthase (DHPS) for sulfamethoxazole (Ambrose and Stephens, 2004). It is still proposed as an alternative for pneumococcal infections for children, where fluoroquinolones cannot be used. However, in US and Europe, there is a high prevalence of non-susceptible strains: 36.3 and 26.7 % respectively (Riedel *et al.*, 2007; Farrell *et al.*, 2011).

Rifampin, an ansamycin, is used with vancomycin or a broad-spectrum cephalosporin for treatment of meningitis (Ambrose and Stephens, 2004). Vancomycin, a glycopeptide, acts by inhibiting the peptidoglycan cell wall synthesis (Van Bambeke *et al.*, 2010). Linezolid, an oxazolidinone, acts on protein synthesis by preventing 30S and 50S subunits of the ribosome from binding to each other. Vancomycin and linezolid are considered as reserve drugs for life threatening infections by mutiresistant organisms (Van Bambeke *et al.*, 2007).

1.2.5. Efflux as a resistance mechanism to antibiotics

Efflux pumps are transmembrane proteins involved in the transport of toxic substrates from within cells into the external environment. There are ubiquitous: they can be found in prokaryotes and eukaryotes. All bacterial genomes studied contains various efflux pumps (Webber and Piddock, 2003). These transporters most probably play a major role in the protection of bacterial cells from toxic polar or charged substances (including those produced by their own metabolism) that cannot easily diffuse out of the bacteria (Van Bambeke *et al.*, 2003; Webber and Piddock, 2003). So they have both physiological and self-protecting roles (Van Bambeke *et al.*, 2003). In this context, antibiotics appear as occasional substrates of transporters as, by design, they share the necessary basic structural features for effective recognition: an amphipathic character and the presence of an ionizable function (Van Bambeke *et al.*, 2003).

In the prokaryotic kingdom there are five major families of efflux transporter: the ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance (SMR) family and the

resistance-nodulation-division (RND) superfamily (Li and Nikaido, 2009). The ABC superfamily utilizes ATP hydrolysis to drive the export of substrates. The four others families utilize the proton motive force as energy source (Webber and Piddock, 2003).

Generally speaking, efflux alone often does not confer high-level, clinically significant resistance to antibiotics. However bacteria overexpressing efflux pump are better equipped to survive antibiotic pressure and develop further mutations in genes encoding the target sites of antibiotics.

Efflux mechanism can cooperate with other resistance mechanisms in the bacteria to reach a clinically significant resistance. As an example, a single mutation in DNA gyrase or topoisomerase IV confers only a low level of resistance, but the reduction in the intrabacterial concentration of fluoroquinolones through expression of one or several efflux pumps may result in MICs exceeding breakpoints (Van Bambeke *et al.*, 2003). Efflux also can favor the selection of mutants more resistant by exposing the targets to insufficient drug concentrations (Avrain *et al.*, 2007).

Pumps are further classified by their substrate specificity. Efflux pumps usually are specific for one class of antibiotics, but some may transport a range of structurally dissimilar compounds (antibiotics of more than one class as well as some dyes, detergents and disinfectants, including some commonly used biocides), such pumps can be associated with multiple drug resistance (MDR). This is especially the case for transporters of the RND superfamily in Gram-negative bacteria, such as the Mex-Opr systems of *Pseudomonas aeruginosa* (Van Bambeke *et al.*, 2003). Exposure to one substrate of the pump would favor its overexpression and result in cross-resistance to structurally unrelated drugs. As an example, MexAB-OprM overexpression confers resistance to a range of antibiotics (including β-lactams, tetracyclines, macrolides, lincosamides, chloramphenicol, fluoroquinolones) but also to triclosan, a common biocide (Webber and Piddock, 2003).

Many different transporters can be expressed in one bacterium. They can have different substrates, or they can share some of them, leading to a high-level resistance phenotype by concomitant expression of several pumps (typically the Mex-Opr systems of *P. aeruginosa*). In order to suppress the resistance to the shared substrates, all these pumps need to be inactivated simultaneously otherwise overexpression of one will compensate for the other (Van Bambeke *et al.*, 2003).

Some classes of antibiotics are particularly often recognized by efflux pumps, this is the case of fluoroquinolones, tetracyclines, macrolides and chloramphenicol. However, individual molecules can show different behavior than the rest of their class, such as moxifloxacin which is a poor substrate compared to other fluoroquinolones (Avrain *et al.*, 2007).

Expression of the transporter can be constitutive or inducible. Antibiotics can induce and regulate the expression of some efflux pumps, and, via the same regulon, also regulate the

expression of several independent genes as, in many cases, efflux pump genes are part of an operon (Webber and Piddock, 2003). Genes encoding efflux pumps can be on the chromosome providing an intrinsic mechanism that allows survival of the bacteria in a hostile environment (no need for new genetic material, the overexpression of the pump may be enough). Or they can also be found on plasmids or on transposons and be easily disseminated between species, even phylogenetically very distant. A good example is given by the macrolide efflux pumps (Mef) that has been transferred between streptococci (*S. pyogenes* and *S. pneumoniae*) (Santagati *et al.*, 2000) but also to other Gram-positive (Luna *et al.*, 1999) and even to Gram-negative bacteria (Luna *et al.*, 2000). If these genes are present on large mobile genetic elements, they can be transferred along with other resistance or virulence determinants.

1.2.6. Clinical relevance of resistance

In case of pneumococcal pneumonia, clinical failure of therapy due to resistance to antimicrobial agents has mainly been reported for macrolides (Pallares *et al.*, 2003; Perez-Trallero *et al.*, 2003; Iannini *et al.*, 2007), fluoroquinolones (Davidson *et al.*, 2002; Pallares *et al.*, 2003; Fuller and Low, 2005), trimethoprim-sulfamethoxazole (Klugman, 2004), tetracyclines (Klugman, 2004), or streptogramins (Klugman, 2004).
For some of those antibiotics, the pneumococci actually develop resistance during the therapy (or following a prophylaxis therapy), as demonstrated for macrolides (Perez-Trallero *et al.*, 2003), fluoroquinolones (Davidson *et al.*, 2002; Perez-Trallero *et al.*, 2003), or trimethoprim-sulfamethoxazole (Klugman, 2004).

At this stage, however, (Klugman, 2004; Jacobs, 2007; Klugman, 2007; Ho *et al.*, 2009), failure of therapy could not be linked to resistance with beta-lactams (penicillin, ampicillin, amoxicillin, cefotaxime or ceftriaxone), provided high doses were used; it is rather related to the severity of the disease in patients. Some patients are at high risk of mortality and may die regardless of the susceptibility of the organism (Klugman, 2004). However therapy with beta-lactam agents less active against pneumococcus, such as cefazolin, cefuroxime and ticarcillin, have already been associated to clinical failures (Klugman, 2004).

2. OBJECTIVES

This study was set up to answer the two main questions:

- Were the guidelines to treat pneumococcal pneumonia still accurate and proposing the most appropriate antimicrobials?
- What was the situation of antibiotic resistance in *Streptococus pneumoniae* coming from community-acquired pneumonia?

We also had secondary questions:

- Does a first treatment leading to failure increase the risk of selecting antibiotic resistance?
- Were the vaccines still targeting the most important serotypes?
- What was the proportion of macrolide resistance due to active efflux?
- What was the prevalence and the clinical relevance of active efflux to fluoroquinolones in clinical isolates?
- Does this efflux also affect other quinolones than ciprofloxacin and norfloxacin which are good markers for this mechanism of resistance but not used clinically for pneumococcal infections?
- Are there new molecules with potential to treat pneumonia?

To answer these questions, we collected pneumococci isolated in Belgian hospitals from patients with confirmed diagnosis of community-acquired pneumonia over the 2007-2009 period and used them to investigate:

1) the epidemiology of antimicrobial resistance of *Streptococcus pneumoniae* in this collection. We wanted to see if amoxicillin is still the drug of choice to treat CAP in Belgium, to study the effect of a previous antibiotic treatment on antimicrobial resistance in this population, in particular the prevalence of efflux, and to analyze if the resistance could be linked to some epidemiological factors.

2) the prevalence and clinical relevance of fluoroquinolone efflux as a resistance mechanism in *Streptococcus pneumoniae.* To this effect, molecular studies aimed at investigating the specificity of the transport for different fluoroquinolones, the expression levels of the efflux systems, and the inducibility of this expression were run in parallel, using both clinical isolates and reference strains.

3. RESULTS

3.1. Antimicrobial susceptibility of *S. pneumoniae* in CAP isolates in Belgium

3.1.a. susceptibility to currently used antibiotics, in relationship with serotypes

In this first part of the study, we collected clinical isolates of *S. pneumoniae* from patients diagnosed with community-acquired pneumonia in various hospitals from Belgium. The study physician associated to this work, Sylviane Carbonnelle, confirmed the CAP diagnosis and collected some relevant data from the patients' files (previous antibiotic treatment, anti-pneumococcal vaccination, comorbidities, clinical outcome of the infectious episode…). The pneumococcal strains were shipped to our laboratory where the susceptibility to three main classes of antibiotics (beta-lactams, macrolides, fluoroquinolones) were assessed. Capsular polysaccharide serotyping was done by J. Verhaegen, National Reference Center for Pneumococci) at the Laboratorium microbiologie, Universitair Ziekenhuis Gasthuisberg, Leuven, in order to correlate resistance patterns with serotypes.

> Article: Antimicrobial susceptibility of *Streptococcus pneumoniae* isolates from vaccinated and non-vaccinated patients with a clinically confirmed diagnosis of community-acquired pneumonia in Belgium.
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Originally, it was thought that patients coming to the hospital for pneumonia were sent by their general practitioner for severe cases or after a first treatment failure. The study was designed with the assumption that a majority of the patients would have received antibiotics at home and would therefore come to the hospital in situation of therapeutic failure, possibly related to resistance. In practice, it was not the case, as almost all patients were directly coming to the emergencies without having taken before any antibiotic.

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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

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Breakpoints

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

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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 ($P = 0.021$).

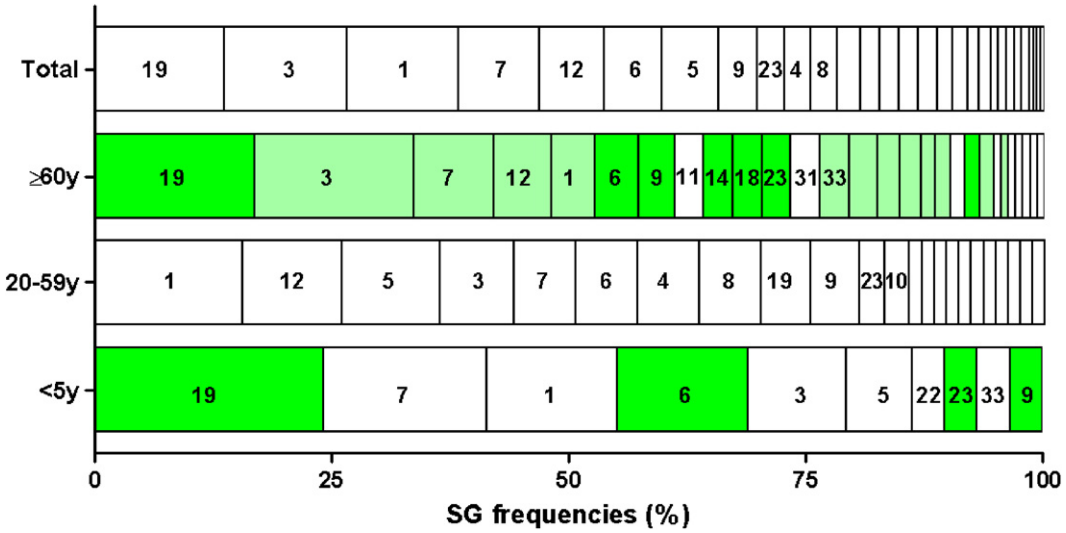

Fig. 1. Distribution of the serogroups (SGs)/serotypes (STs) amongst *Streptococcus pneumoniae* isolates ($n = 249$) used in this study, with subdivision by age group. Green boxes correspond to the SGs included in the 23-valent vaccine (used in adults), with those included in the 7-valent vaccine (used 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

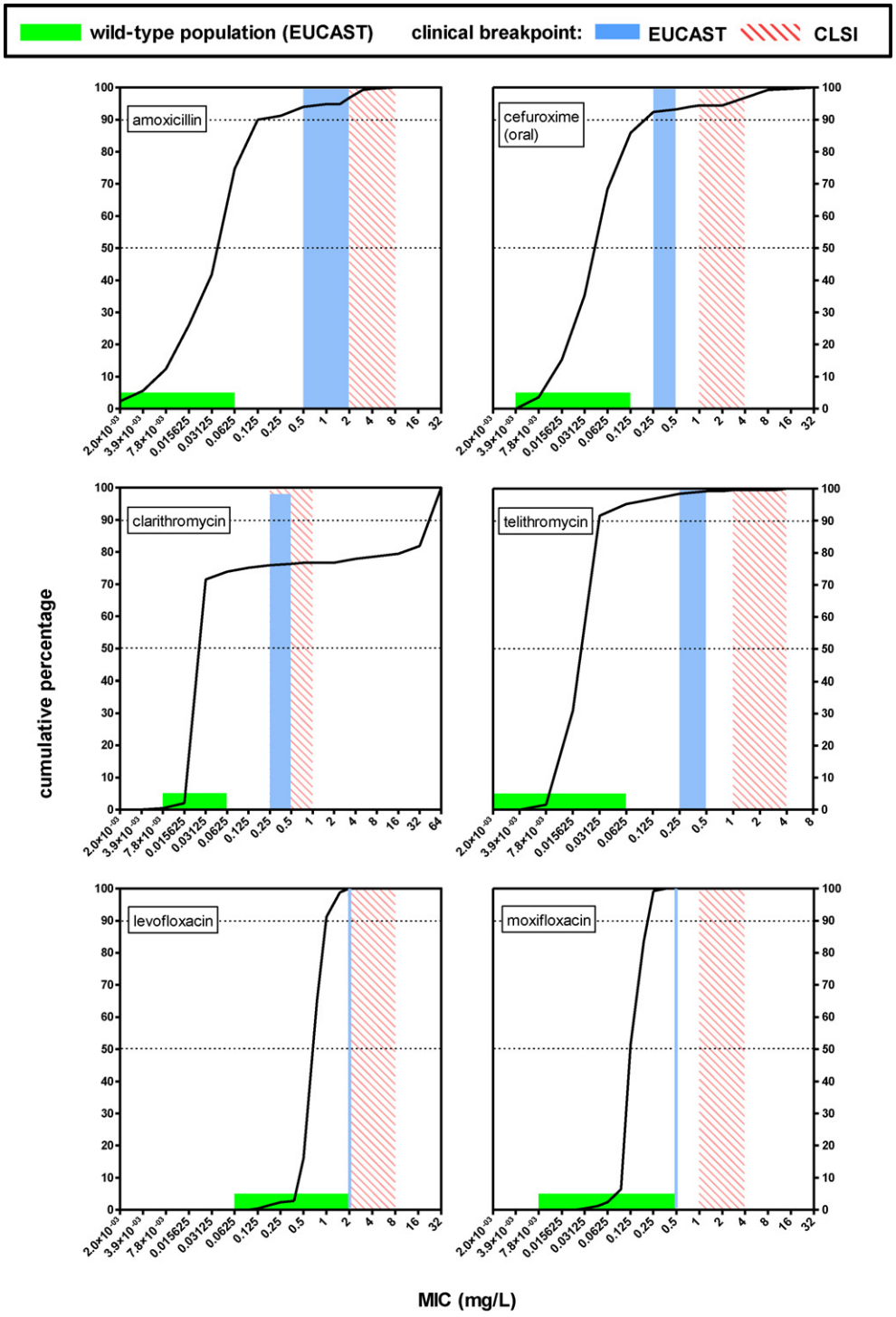

Fig. 2. Minimum inhibitory concentration (MIC) distributions (cumulative percentages) of non-duplicate *Streptococcus pneumoniae* isolates ($n = 249$) from all patients enrolled in the study. The horizontal green zone in the MIC scale shows the range (mg/L) covered by the wild-type population as defined 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.)

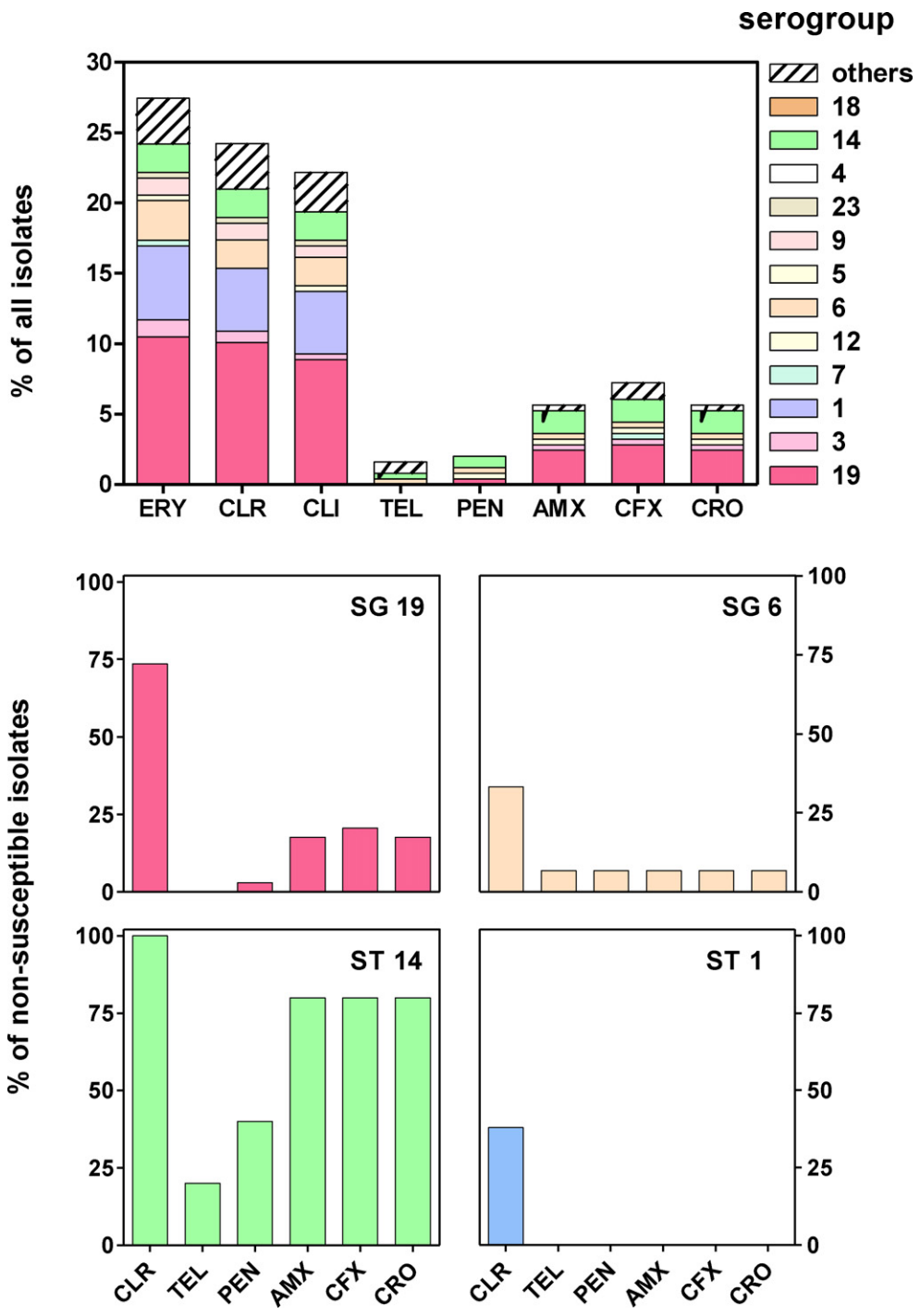

Fig. 3. Non-susceptibility [defined as a minimum inhibitory concentration (MIC) > the susceptible (S) clinical breakpoint of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (see values in the caption of Fig. 2)] of isolates according to the main serogroups/serotypes. Upper panel: 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.

References

[1] Welte T, Torres A, Nathwani D. Clinical and economic burden of community-acquired pneumonia among adults in Europe. Thorax 2012;67(1):71–9.
[2] Lode HM. Managing community-acquired pneumonia: a European perspective. Respir Med 2007;101:1864–73.
[3] Van Bambeke F, Reinert RR, Appelbaum PC, Tulkens PM, Peetermans WE. Multidrug-resistant *Streptococcus pneumoniae* infections: current and future therapeutic options. Drugs 2007;67:2355–82.
[4] Jones RN, Jacobs MR, Sader HS. Evolving trends in *Streptococcus pneumoniae* resistance: implications for therapy of community-acquired bacterial pneumonia. Int J Antimicrob Agents 2010;36:197–204.
[5] Riedel S, Beekmann SE, Heilmann KP, Richter SS, Garcia-de-Lomas J, Ferech M, et al. Antimicrobial use in Europe and antimicrobial resistance in *Streptococcus pneumoniae*. Eur J Clin Microbiol Infect Dis 2007;26:485–90.
[6] Pletz MW, van der Linden M, von Baum H, Duesberg CB, Klugman KP, Welte T. Low prevalence of fluoroquinolone resistant strains and resistance precursor strains in *Streptococcus pneumoniae* from patients with community-acquired pneumonia despite high fluoroquinolone usage. Int J Med Microbiol 2011;301:53–7.
[7] Kyaw MH, Lynfield R, Schaffner W, Craig AS, Hadler J, Reingold A, et al. Effect of introduction of the pneumococcal conjugate vaccine on drug-resistant *Streptococcus pneumoniae*. N Engl J Med 2006;354:1455–63.
[8] McGee L. The coming of age of niche vaccines? Effect of vaccines on resistance profiles in Streptococcus pneumoniae. Curr Opin Microbiol 2007;10:473–8.
[9] Niederman MS, Mandell LA, Anzueto A, Bass JB, Broughton WA, Campbell GD, et al. Guidelines for the management of adults with community-acquired pneumonia. Diagnosis, assessment of severity, antimicrobial therapy, and prevention. Am J Respir Crit Care Med 2001;163:1730–54.
[10] Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; 20th informational supplement. Document MS100-S20. Wayne, PA: CLSI; 2010.
[11] European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 1.3, January 5, 2011. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/EUCAST_breakpoints_v1.3_pdf.pdf [accessed 23 November 2011].
[12] Klaassen CH, Mouton JW. Molecular detection of the macrolide efflux gene: to discriminate or not to discriminate between *mef*(A) and *mef*(E). Antimicrob Agents Chemother 2005;49:1271–8.
[13] Baranova NN, Neyfakh AA. Apparent involvement of a multidrug transporter in the fluoroquinolone resistance of *Streptococcus pneumoniae*. Antimicrob Agents Chemother 1997;41:1396–8.
[14] 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;61:143–9.
[15] Woodhead M, Blasi F, Ewig S, Huchon G, Ieven M, Ortqvist A, et al. Guidelines for the management of adult lower respiratory tract infections. Eur Respir J 2005;26:1138–80.
[16] Mandell LA, Wunderink RG, Anzueto A, Bartlett JG, Campbell GD, Dean NC, et al. Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults. Clin Infect Dis 2007;44(Suppl. 2):S27–72.
[17] British Thoracic Society Standards of Care Committee. British Thoracic Society guidelines for the management of community acquired pneumonia in childhood. Thorax 2002;57(Suppl. 1):i1–24.
[18] Bonofiglio L, Regueira M, Pace J, Corso A, Garcia E, Mollerach M. Dissemination of an erythromycin-resistant penicillin-nonsusceptible *Streptococcus pneumoniae* Poland^{6B}-20 clone in Argentina. Microb Drug Resist 2011;17:75–81.
[19] Cooke B, Smith A, Diggle M, Lamb K, Robertson C, Inverarity D, et al. Antibiotic resistance in invasive *Streptococcus pneumoniae* isolates identified in Scotland between 1999 and 2007. J Med Microbiol 2010;59:1212–8.
[20] Felmingham D, Canton R, Jenkins SG. Regional trends in β-lactam, macrolide, fluoroquinolone and telithromycin resistance among *Streptococcus pneumoniae* isolates 2001–2004. J Infect 2007;55:111–8.
[21] Fasola EL, Bajaksouzian S, Appelbaum PC, Jacobs MR. Variation in erythromycin and clindamycin susceptibilities of *Streptococcus pneumoniae* by four test methods. Antimicrob Agents Chemother 1997;41:129–34.
[22] Vanhoof R, Camps K, Carpentier M, De Craeye S, Frans J, Glupczynski Y, et al. 10th survey of antimicrobial resistance in noninvasive clinical isolates of *Streptococcus pneumoniae* collected in Belgium during winter 2007–2008. Pathol Biol (Paris) 2010;58:147–51.
[23] Van Bambeke F, Tulkens PM. Safety profile of the respiratory fluoroquinolone moxifloxacin: comparison with other fluoroquinolones and other antibacterial classes. Drug Saf 2009;32:359–78.
[24] Reinert R, Jacobs MR, Kaplan SL. Pneumococcal disease caused by serotype 19A: review of the literature and implications for future vaccine development. Vaccine 2010;28:4249–59.
[25] Imohl M, Reinert RR, Mutscher C, van der Linden M. Macrolide susceptibility and serotype specific macrolide resistance of invasive isolates of *Streptococcus pneumoniae* in Germany from 1992 to 2008. BMC Microbiol 2010;10:299.
[26] Imohl M, Reinert RR, van der Linden M. Serotype-specific penicillin resistance of *Streptococcus pneumoniae* in Germany from 1992 to 2008. Int J Med Microbiol 2010;300:324–30.
[27] Dortet L, Ploy MC, Poyart C, Raymond J. Emergence of *Streptococcus pneumoniae* of serotype 19A in France: molecular capsular serotyping, antimicrobial susceptibilities, and epidemiology. Diagn Microbiol Infect Dis 2009;65:49–57.
[28] Ducoffre, G. Surveillance des Maladies Infectieuses par un Réseau de Laboratoires de Microbiologie 2009–Tendances Epidémiologiques 1983–2008–*S. pneumoniae*. https://www.iph.fgov.be/epidemio/epifr/plabfr/plabanfr/09_030f_v.pdf [accessed 24 November 2011].
[29] Klugman KP. The successful clone: the vector of dissemination of resistance in *Streptococcus pneumoniae*. J Antimicrob Chemother 2002;50(Suppl. S2):1–5.
[30] Pletz MW, Maus U, Krug N, Welte T, Lode H. Pneumococcal vaccines: mechanism of action, impact on epidemiology and adaption of the species. Int J Antimicrob Agents 2008;32:199–206.

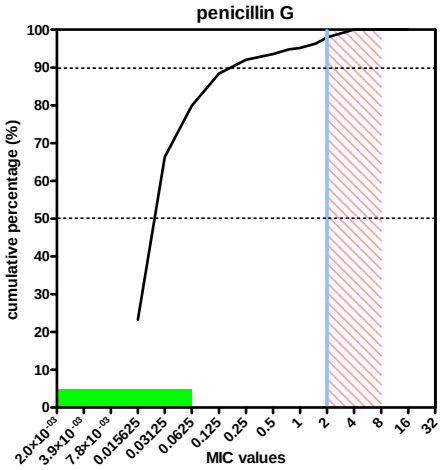
Supplementary material

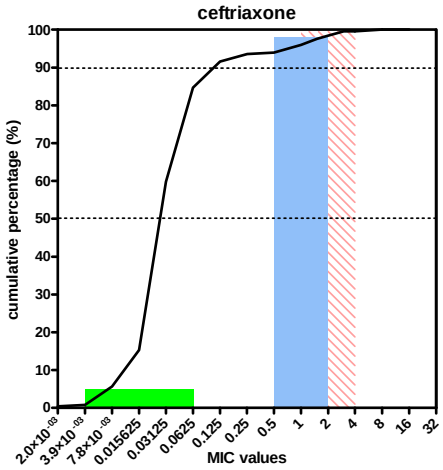
1. Determination of the mechanism of resistance to macrolides by polymerase chain reaction (PCR)

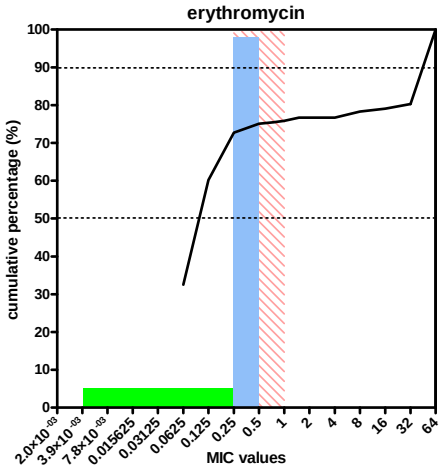
- Primers: 5'-CGTATTGGGTGCTGTGATTG-3' and 5'-TATGCACAGGCGTTCCATTA-3' amplifying equally 248 bp of *mef*(E) or *mef*(A) and 5'-TTGAGTGTGCAAGAGCAACC-3' and 5'-AAAGGGCATTTAACGACGAA-3' amplifying equally 327 bp of *erm*(B) or *erm*(A) (obtained from Eurogentec s.a., Seraing, Belgium).
- PCR mix composition (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.

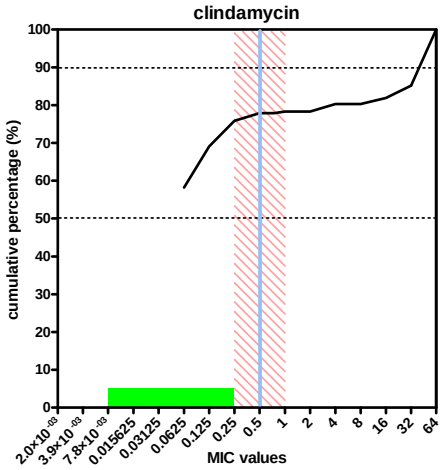
Supplementary Fig. S1. Minimum inhibitory concentration (MIC) distributions (cumulative percentages) of non-duplicate *Streptococcus pneumoniae* isolates (*n* = 249) from all patients enrolled in the study for penicillin G, ceftriaxone, erythromycin, clindamycin and ciprofloxacin (for penicillin G, erythromycin 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).

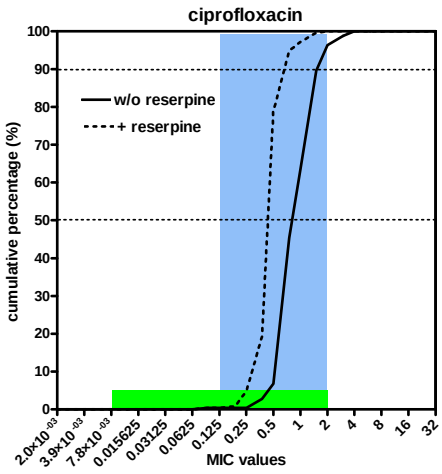
2


penicillin G
cumulative percentage (%)
MIC values

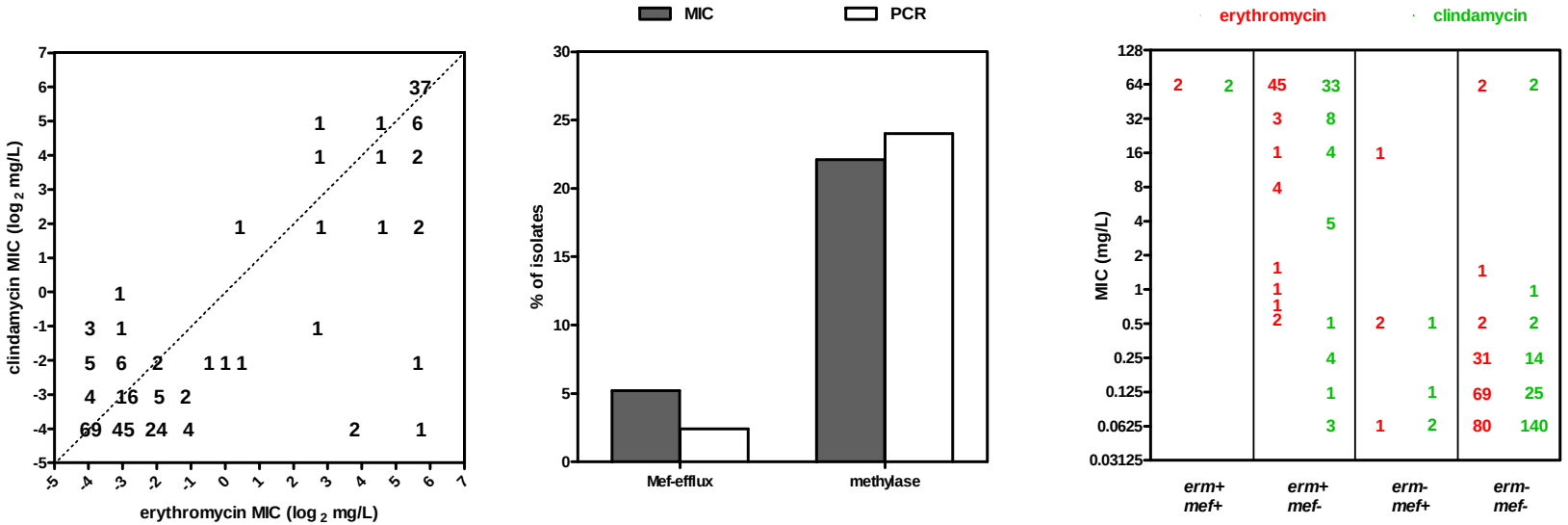

ceftriaxone
cumulative percentage (%)
MIC values


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]	–

5

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.

3.1.b. susceptibility to investigational antibiotics

Because of the high proportion of resistance to macrolides, this class cannot be recommended anymore for the treatment of pneumococcal infections. Ketolides may offer a useful alternative in this respect, but their use is limited by severe toxicity, as observed for telithromycin, the only molecule in this subclass currently on the market. This drug induces exacerbation of myasthenia gravis, visual disturbance, and liver failure, which have been recently suggested to be due to an inhibition of the nicotinic acetylcholine receptors (nAChR) in the organs targeted by toxicity (Bertrand *et al.*, 2010). Solithromycin, a fluoroketolide in phase III of clinical development, is less prone to interact with this receptor due to the absence of pyridine-imidazole group of the telithromycin side chain (Lewis, 1990). On a microbiological point of view, this drug shows potent activity against pathogens isolates in respiratory tract infections (Farrell *et al.*, 2010). We therefore undertook to compare its activity to that of telithromycin on isolates that were resistant to macrolides in our collection in order to delineate its potential interest in this specific situation.

Poster: Comparative activities of the novel ketolide CEM-101 and telithromycin (TEL) towards *Streptococcus pneumoniae* (SP) resistant to macrolides (ML) from patients with confirmed community-acquired pneumonia (CAP).
Ann Lismond, Françoise Van Bambeke, Paul M. Tulkens
19th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID); Helsinki, Finland, 16-19 May 2009

P1099

Comparative activities of the novel ketolide CEM-101 and telithromycin (TEL) towards *Streptococcus pneumoniae* (SP) resistant to macrolides (ML) from patients with confirmed community-acquired pneumonia (CAP).

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ABSTRACT (edited)

Background and aims: CEM-101 is a new fluoroketolide in development with activity against macrolide (ML)-resistant isolates. A dose of 400 mg qD yields an AUC_{24h} similar to that of telithromycin (TEL) 800 mg qD and shows similar protein binding properties in human serum (about 15 % free drug). Belgium is a country with high resistance of SP to ML (> 35 % for clarithromycin). Our aim was to compare the activity of CEM-101 to that of TEL against ML-resistant strains of SP obtained from patients with confirmed CAP. .

Methods: 29 first ML-R isolates (based on clarithromycin MICs determination; 19 MLS_B, 10 M-phenotype based on erythromycin and clindamycin resistance dissociation) were selected (for which 6 were TEL-I and 7 TEL-R based on EUCAST breakpoints [S ≤ 0.25 - R > 0.5]). MICs were determined by geometric microdilution in CAMH broth + 2.5% lysed horse blood according to CLSI, using SP ATCC-49619 as a control.

Results:
ATCC-49619 MICs were ≤0.008 mg/L for TEL and CEM-101. Data for ML-resistant isolates are shown in the Table.

Phenotype*	No.	TEL			CEM-101		
		range	geom. mean	MIC_{90}	range	geom. mean	MIC_{90}
TEL-S	16	0.008-0.25	0.021	0.25	0.008-0.063	0.022	0.063
TEL-I	6	0.5-0.5	0.5	0.5	0.063-0.5	0.223	0.5
TEL-R	7	1-3	1.426	3.0	0.5-1.0	0.906	1.0

* MLS_B for 7/16 of TEL-S, 5/6 of TEL-I, and 7/7 of TEL-R isolates
(S / I / R are defined based on EUCAST breakpoints (S ≤ 0.25 – R > 0.5)

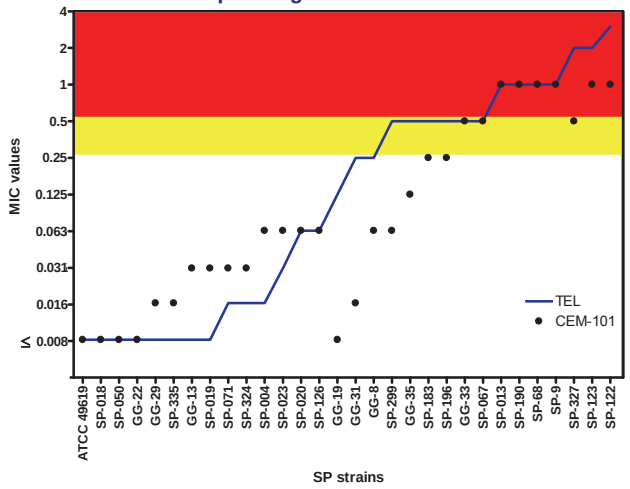
Conclusions: In this Belgian collection of *S. pneumoniae* from confirmed CAP resistant to macrolides, CEM-101 shows globally lower MICs compared to TEL, especially with respect to TEL-I and TEL-R isolates. CEM-101, therefore, has the potential to stand as an alternative to telithromcyin in areas with high ML resistance and emerging resistance to TEL.

Background and Aim

- CEM-101 is a new fluoroketolide in development with activity against macrolide (ML)-resistant isolates, yielding, at 400 mg qD, an AUC_{24h} similar to that of telithromycin at 800 mg qD. CEM-101 and TEL show similar protein binding in human serum (about 15 % free drug). Previous studies have shown that CEM-101, with MIC values ranging from 0.004 up to 1 µg/ml, can be up to four-fold more active than TEL against *S. pneumoniae* and that only ErmB strongly affects its activity (1).
- In Belgium, ~ 35% of *S. pneumoniae* isolates are resistant to macrolides and already 7.5% must be considered as having a "decreased susceptibility" to TEL telithromycin if using EUCAST breakpoints (2).
- Our aim was to compare the activity of CEM-101 to that of TEL against *S. pneumoniae* clinical strains selected for
  - decreased susceptibility to telithromycin (13 TEL-NS), and
  - distinct patterns of resistance to macrolides (7 MLS_B- and 9 M-phenotype) among TEL-S isolates.

Results

Strains ordered by increasing MIC for telithromycin with corresponding MICs for CEM-101



EUCAST breakpoint for TEL: S ≤ 0.25 (white) – I (yellow) – R>0.5 (red).

Range of CEM-101 MIC values and macrolides resistance phenotype according to telithromycin MICs.

	nbr of strains	MIC TEL (µg/ml)	range MIC CEM-101 (µg/ml)	Macrolides resistance phenotype	
				MLS_B	M
TEL-R	1	4	1	1	0
	3	2	0.5-1	3	0
	3	1	1	3	0
TEL-I	6	0.5	0.06-0.5	5	1
TEL-S	2	0.25	0.016-0.06	0	2
	1	0.125	≤0.008	0	1
	2	0.06	0.06	0	2
	1	0.03	0.06	1	0
	3	0.016	0.03-0.06	1	2
	7	≤0.008	≤0.008-0.03	5	2

MLS_B-phenotype (methylase Erm): resistance to macrolides, lincosamides and streptogramins B.

M-phenotype (efflux [Mef pump]): resistance to 14- and 15-membered-ring macrolides.

Methods

Bacteria: All of TEL-R (7) and TEL-I (5) isolates found in our collection of *S. pneumoniae* plus 16 TEl-S isolates with distinct macrolide resistance phenotypes (MLS_B or M) were also used for testing.

Susceptibility testing: CEM-101 was diluted in 0.1N HCl. MICs were determined by geometric microdilution in CAMH broth + 2.5% lysed horse blood following CLSI recommendations. *S. pneumoniae* ATCC 49619 was used as a quality control. Susceptibility was assessed according to EUCAST breakpoints. Clarithromycin and clindamycin were used to differentiate between MLS_B and M-phenotype. Active efflux of macrolides (M-phenotype) was evidenced by comparison with the MICs of CLR and CLI (only affected by ribosomal mutations or methylation).

Results

- Population analysis (graph): At MICs values of up to 0.06 µg/ml, TEL was more effective than CEM-101, while the inverse situation was seen at higher TEL MICs, whith all isolates showing lower or similar MIC values for CEM-101.
- Analysis by isolates: Isolates with MICs of 1 mg/L were observed only for TEL-R isolates (all with MLS_B-phenotype). Only one TEL-I isolate displayed an M-phenotype and its MIC for CEM-101 was 0.06 mg/L.
- No correlation was found between the macrolide resistance phenotype in the TEL-S isolates and the MIC of CEM-101.

Conclusions

- In this Belgian collection of *S. pneumoniae* resistant to macrolides, CEM-101 showed globally lower MICs compared to telithromycin, especially with respect to TEL-I and TEL-R isolates.
- CEM-101 has the potential to stand as an alternative to telithromycin in areas with high macrolide resistance and emerging resistance to telithromycin.

References

1. McGhee *et al.* Comparative activity of CEM-101 against macrolide-susceptible and –resistant pneumococci. 48th ICAAC & 46th IDSA, 25-28 Oct. 2008; F1-3974
2. Lismond *et al.* Epidemiological survey of susceptibility to β-lactams (AMX, CFX, CRO), macrolides (CLR, TEL), and fluoroquinolones (LVX, MXF) in a Belgian collection of CAP isolates of *Streptococcus pneumoniae* (SP). 18th ECCMID 19-22 April 2008; P-1747.

A copy of this poster will be made available after the meeting at http://www.facm.ucl.ac.be/posters.htm

3.1.c. critical review of Community-Acquired Pneumonia guidelines

Antibiotic treatment guidelines are highly variable from one country to the other. We have therefore undertaken to review these guidelines, and to put them in perspective, considering the epidemiology of resistance in these countries, as well as the safety profile of the prescribed drugs.

Article to be submitted: Guidelines for antibiotic treatment of community-acquired pneumonia in general practice: a critical appraisal.
Sylviane Carbonnelle, Ann Lismond, Dominique Pestiaux, Françoise Van Bambeke, Paul M. Tulkens

To be submitted

Title:

Guidelines for antibiotic treatment of community-acquired pneumonia in general practice: a critical appraisal

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Running head: Guidelines for community-acquired pneumonia

Parts of the data shown here have been presented at:

- the 9th Annual meeting of the International Society of Pharmacovigilance (ISoP): "From Pharmacovigilance to Risk Management", Reims, France, October 6th-9th, 2009 (Poster no. 375: *Critical Analysis of the Risk/Benefit Ratio of First Line Antibiotics Included in Guidelines for the Treatment of Community-Acquired Pneumonia*)
- 21st European Congress of Clinical Microbiology and Infectious Diseases & 27th International Congress of Chemotherapy, Milan, Italy, 7-10 May 2011 (Invited lecture: *Improving usage by guidelines: a critical view* [Symposium: Bridging the gap of innovation – what we all could do ?])

Paper metrics

Title character count: 110 (incl. spaces; max. allowed: 150)

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Supplementary Material: 2 Tables, 1 Figure, 1 calculaitor (Excel file)

Abstract

Background: Guidelines for community-acquired pneumonia (CAP) should provide for effective, safe, and cost-contained therapy, while minimizing patient's risks. Yet, they are poorly followed by practicing physicians.

Methods and Findings: CAP guidelines from 23 countries and 5 regions were analyzed by confronting them with (i) levels of resistance of *S. pneumoniae* (the causative organism for which resistance has become worrisome), (ii) safety (drug labelling and literature survey), (iii) compliance with the AGREE instrument ("Appraisal of Guidelines for Research and Evaluation", assessing the quality of guidelines development and reporting; 2 observers with divergence assessment and reconciliation), (iv) drug acquisition costs (in Europe). Amoxicillin (+/- clavulanic acid or beta-lactams in general) is most often recommended as first line, followed by macrolides and tetracyclines (variable between countries) whereas fluoroquinolones are almost always second line or restricted. *S. pneumoniae* resistance to beta-lactams, macrolides and tetracyclines, although variable, may reach >10 % in several countries but remains <3% to fluoroquinolones. Main patient-related safety issues include allergy (beta-lactams), hepatotoxicity (clavulanic acid), cardiac arrhythmia and drug interactions (macrolides), phototoxicity (tetracyclines), and tendonitis (fluoroquinolones). The main weaknesses of guidelines based on AGREE criteria concern editorial independence, quality of data collection and programmed update, stakeholder involvement, and anticipation of potential risks, with huge variations between guidelines. Typical costs (in Europe) vary from 7 [min] to 75 [max] euros, with beta-lactam/macrolide combination (necessary for more effective coverage) as costly as fluoroquinolones.

Conclusions: Several CAP guidelines may be suboptimal for effective antimicrobial coverage in empiric therapy as well as in terms of patient's risk assessment. Weaknesses in design and construction and undue insistence on minimizing drug costs (low, if considering that treatment, when appropriate, is successful and without relapse in most cases), may also undermine confidence. Improving CAP guidelines construction may be critical for better compliance by practicing physicians.

Introduction

Community-acquired pneumonia (CAP) is a frequent disease [1–4]. Its severity at outset is of main prognostic value (and is the basis of Fine's classification and its long-standing success) [5–7]. Thus, while mortality of CAP is only < 2 % for low-risk patients, it can reach up to ~ 19 % for high-risk patients [8–10]. Globally, CAP is the main cause of death from infectious diseases in the Western hemisphere [11,12], the 6th most common cause in adults [13], and a leading cause in children worldwide [14]. *Streptococcus pneumoniae, Mycoplasma pneumoniae* and, depending on the studies, *Chlamydophyla* (most often referred to as *C. pneumoniae* in the surveys), are the most frequent bacterial species isolated in non-epidemic settings [15–17], with, however, huge variations among studies and a large number of cases (about 50 %) in which no pathogen could be isolated [17,18] (Table 1). Antibiotics remain at the forefront of CAP therapy and significantly contribute to reduce mortality, especially if started "as soon as possible after the diagnosis is considered likely" [19]. This should result in an improvement of the clinical situation within 3-4 days for the majority of immunocompetent patients [19,20]. Because most of these infections are initially taken care of out of the hospital [21], where a causal diagnostic is difficult to establish and where ineffective therapy may create an important risk, optimized treatments applicable by general practitioners are essential. Guidelines for antibiotic choice by primary care physicians have therefore been issued in most countries or regions. The aim of this paper is to examine how these guidelines cope with the current challenges of providing the patients with safe and efficacious first line therapies while avoiding unnecessary expenses. Bacterial resistance patterns are, indeed, rapidly evolving, new drugs are being introduced, and new information related to efficacy and safety of antibacterial agents becomes increasingly available and of concern to the general public. It must also be underlined that the rates of mortality due to pneumonia have not decreased significantly since penicillin became routinely available [6,22–24], with a 1-year mortality as high as 40% in elderly patients if admitted to the hospital [25]. Most guidelines include many other areas than treatment recommendations that are equally or even more important than antibiotic choices in terms of providing safe therapies (e.g., how to diagnose CAP, when to admit to hospital and so on). These aspects have not been examined here as their analysis would have largely exceeded what could be reasonably undertaken in a single study. Conversely,

although most guidelines do not include pharmaeconomic analysis, we examined drug acquisition costs because this is increasingly taken as an important basis for final choices in a context of public interventions for rational prescriptions and savings [26–29].

Methods

Search strategy and selection criteria

We followed a 3-step-approach consisting of a (i) search for guidelines, (ii) search for susceptibility patterns of *S. pneumoniae* to the antibiotics recommended in those guidelines, and (iii) retrieval of safety information for those antibiotics.

Retrieval of guidelines

Guidelines from European countries, from the United States and Canada, from selected countries from other parts of the world, and from the World Health Organization (for children only) were identified and retrieved (i) by consulting the US National Guideline Clearinghouse (NGC) website (www.guideline.gov), (ii) by searching for other guidelines as cited in those already identified, (iii) by use of the SCIRUS search engine (http://www.scirus.com), (iv) by examining the results of a systematic search through the Google web search engine (http://www.google.com) using "guidelines" and "community-acquired pneumonia" keywords in English and, if not already obtained, in the language of the target country, (v) by consultation of the websites of professional organisations dealing with infectious respiratory diseases; (v) by direct contact with colleagues from countries for which the previous steps had not allowed a clear identification of the most current guidelines. References to the public source(s) of each guideline used in our study are shown in Tables 2 and 3. Only guidelines issued by a *bona fide* scientific, medical or official organisation were accepted, and only information concerning empiric treatment of patients in the community setting was assessed (because the pathogen is unknown in most cases of CAP in ambulatory patients).

Susceptibility patterns of Streptococcus pneumoniae to the recommended antibiotics.

We concentrated *S. pneumoniae* because it is the organism most responsible for rapid deterioration of the health status of CAP patients [30–32] and for which resistance has become most worrisome [33,34]. We examined data obtained and published during the period 2005-2010 to take into account the rise in resistance observed since the early 2000's while only including studies that even the most recent guidelines could have

reasonably included in their analysis. First, original papers published in peer-reviewed journals were retrieved from PubMed (US National Library of Medicine - http://www.ncbi.nlm.nih.gov/sites/entrez) by using as keywords “resistance” and "*S. pneumoniae*" (as such or in full). Second, we examined the data from antimicrobial resistance surveillance programmes with wide geographic coverage but reporting data by countries and/or regions, namely (i) the European Union-supported programme EARSS [European Antimicrobial Surveillance system - http://www.rivm.nl/earss [a]]); (ii) several major Industry-supported programmes (PROTEKT [Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin] [35,36], SENTRY [Antimicrobial Surveillance Program] [37], MYSTIC [Meropenem Yearly Susceptibility Test Information Collection] [38], TEST [Tigecyline Evaluation Surveillance Trial] [39], the Doripenem surveillance program [40], ZAAPS [Zyvox Annual Appraisal of Potency and Spectrum] [41] and LEADER [Linezolid Surveillance Program] [42]). Third, we screened the abstracts and posters presented at the 18th-20st (2008-2010) European Congresses of Clinical Microbiology and Infectious Diseases (ECCMID) and at the 50th (2010) Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), using only those presenting relevant information and containing sufficient details (methods, origin of isolates [confirmed CAP, respiratory tract infection, or bacteraemia], and defined criteria to assess susceptibility [Clinical and Laboratory Scientific Institute (CLSI - http://www.clsi.org [formerly NCCLS]) or the European Committee for Antibiotic Susceptibility Testing (EUCAST - http://www.eucast.org]) [b].

Safety of recommended antibiotics

For each recommended antimicrobial, adverse effects were compiled from the corresponding labelling using official documents from the EMA (European Medicines Agency – http://www.ema.europa.eu), the FDA (US Food and Drug Administration –

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[a] This address is now re-routed to the new website of the EARS network operated by the European Center for Disease Control (http://www.ecdc.europa.eu/en/activities/surveillance/EARS-Net/Pages/index.aspx)

[b] for β-lactams, data were interpreted using the penicillin breakpoints as currently set by EUCAST (S ≤ 0.06 mg/L and R > 2 mg/L) [43] or by the CLSI prior to 2008 (S ≤ 0.06 mg/L and R ≥ 2 mg/L; reports using only the current higher CLSI breakpoints for penicillin [S ≤ 2 and R ≥ 8 mg/L] [44] with no indication of MIC values were not used).

http://www.fda.gov) complemented from the (i) the latest US Prescription Information document (labelling) available from the corresponding major US supplier, (ii) the official labelling applicable in the United Kingdom, Belgium, and Switzerland (retrieved from http://www.mhra.gov.uk, http://www.pharma.be and http://www.fagg-afmps.be, and http://www.kompendium.ch, respectively). Additional information was collected from original and review papers searched in PubMed, by entering the name of the corresponding antibiotics together with the keywords "safety", "side effect", "adverse effect", or "toxicity"", and “community-acquired pneumonia”. Abstracts from the same meetings as for the susceptibility patterns were also examined by reviewing the relevant specific sections. We did not attempt to collect accurate incidence rates since we did not have access to pertinent phamacovigilance data and also because the effects to consider were too numerous, too different, and often infrequent, making true estimations very difficult.

Analysis for compliance of guidelines with the AGREE Instrument

This analysis using the "Appraisal of Guidelines Research and Evaluation" instrument [45] was performed independently by two investigators (SC and FVB), using a score sheet assessing 6 main domains considered essential (scope and purpose; stakeholder involvement; rigour of development; clarity of presentation; applicability; editorial independence) through a series of evaluation criteria (available as supplemental material [Figure SP1] with comments about the revised version presented ina peer-reviewed journal at the end of 2010 [46]). Since our purpose was not to rank guidelines but to obtain a global assessment of their individual value against the instrument, and because of the number of guidelines analyzed, each item was judged against a simplified 3-levels score ("Disagree" [negative] , "Agree" [positive] and "neutral"). According to the recommendations of the AGREE Instrument, items for which no clear information could be found were given a negative score [45]. For guidelines written in a language not mastered by the investigators, score sheets were filled up by at least two colleagues proficient in that language who reported the results in English; these scores where thereafter reanalyzed by SC and FVB using an English translation of the same guidelines to ensure criteria homogeneity throughout the analysis process. Domain

scores were calculated by summing up all the scores of the individuals items in a domain and by standardising the total as the fraction of the maximum possible score for that domain. The agreement between the two investigators was assessed using the Cohen's kappa test (http://www.dmi.columbia.edu/homepages/chuangj/kappa).

Direct antibiotic treatments acquisition costs

Costs were calculated for the main variations of treatment recommended for adults in the analyzed guidelines and using, as a common base, the corresponding retail prices in Belgium (average European prices) [c]. Only oral forms were considered as these represent the majority of prescriptions in the community (syrups, normally intended to children only, were excluded). The defined daily dose (DDD) of each antibiotic was retrieved from the anatomic therapeutical chemical (ATC) index with DDDs on the World Health Organization (WHO) website (http://www.whocc.no/atcddd/) to calculate a lowest (usually a generic form) and highest (usually the branded product) acquisition cost per DDD (as a first basis for comparison). We then calculated the lowest and highest acquisition cost of the recommended daily doses (RDD; based on the guidelines) of the same antibiotic, as a second, more practical basis for cost comparisons. We then introduced the treatment duration (lowest and highest, also based on the guidelines), providing the final basis for cost comparison as could be experienced when treating a patient according to guidelines. For antibiotic combinations, the acquisition prices of the corresponding individual antibiotics were summed.

[c] Acquisition costs were monitored between 2009 and 2012 to check for price consistency and avoid reporting values directly influenced by introduction or withdrawal of a given drug. As no undue variation was noted (there was actually a small price decline for all branded drugs and most generics), only data of the last survey are shown. We provide in the Supplementary Material a spreadsheet file in which the reader may freely introduce its local prices and, after unlocking, modify dosages and treatment durations as needed to calculate the final price of a treatment fitting her/his choices.

Results

Treatment guidelines: general overview

Table 2 (together with Figure 1) and Table 3 show an overview of the selected guidelines for adults and children. A detailed account of all guidelines, with identification of each individual antibiotic recommended, its position as first line (patient otherwise healthy [<60 y, no specific risk factors]) or as alternative treatment (second line for patient otherwise healthy; first line for patient with risk factors; second line for patient with risk factors), and the most common dosages and duration of treatment, is presented in the Supplementary Material (Tables SP1 and SP2). β-lactams, with amoxicillin most often cited, are the most commonly recommended antibiotics as first line therapy in European countries, with, however, Denmark and Norway limiting themselves to penicillin V and Switzerland adding systematically clavulanic acid. In contrast, macrolides are recommended as first line in Italy, Scotland, Portugal, Canada, North America, Latin America, Brazil, and Saudi Arabia. In France and Spain, telithromycin (active against erythromycin-resistant *S. pneumoniae* by design [47–49] and usually referred to as a ketolide; restricted in most countries because of a higher risk of hepatotoxicity [50]), is specifically recommended. Tetracyclines are recommended as first line antibiotics in Europe (as a whole), Austria, Scotland, Sweden, Switzerland, the Netherlands and Uited States. For combination therapy, Scotland recommends adding a macrolide to the β-lactam.

The guidelines show still more variations when considering alternative antibiotics. For patients otherwise healthy (second line), the recommendations are (i) for the European and Austrian guidelines, an amoxicillin-clavulanic acid combination (co-amoxiclav); (ii) for Brazil, a β-lactam, (iii) for Finland, Germany, Great Britain, the Netherlands, Norway, Portugal, Canada, Saudi Arabia, a tetracycline (Russia lists also a tetracycline as second line antibiotic but for "atypical pathogens" only); (iv) for Europe (as a whole), Austria, Denmark (if penicillin allergy), Finland, Germany, Great Britain, the Netherlands (with restrictions), Norway, Russia and Switzerland, a macrolide; (v) for Spain, a combination of a β-lactam and a macrolide. Fluoroquinolones are also included as second line antibiotics in European guidelines but appear as such in Belgium (with

restrictions), Italy, Russia, Spain and Switzerland only. Considering patients with risk factors (not mentioned as such in the European guidelines), co-amoxiclav is recommended in Belgium (with a high dose of amoxicillin [3 g]), France (also recommending ceftriaxone), Germany, and Russia. Tetracyclines are only recommended in South Africa. Macrolides alone are recommended in Sweden and Latin America, whereas the combination of a β-lactam and a macrolide is recommended in Finland, Germany, Portugal, United States, Brazil, Saudi Arabia and South Africa. Fluoroquinolones are recommended in France, Italy, Norway (only ciprofloxacin), Spain, United States, Latin America and Brazil. As second line antibiotics for patients at risk, recommended drugs include tetracyclines in Russia and fluoroquinolones in Belgium, Germany and Saudi Arabia. Other recommended antibiotics or approaches for these patients are co-amoxiclav (Germany, Spain), the addition of a macrolide to the initial treatment (Belgium), a β-lactam / macrolide combination (Italy, Russia, United States, Saudi Arabia) and a β-lactam / tetracycline combination (Finland, Portugal and United States). Outside France and Spain, the position of telithromycin remains largely ill-defined. A few molecules appear only in the recommendations of specific countries, such as cefuroxime axetil (an orally absorbable prodrug of cefuroxime) in Belgium, pristinamycin (a streptogramin) in France, or pheneticillin (sometimes spelled phenethicillin or feneticillin; an oral, acid-resistant penicillin analogous to penicillin V) in the Netherlands.

Dosages show great variability for β-lactams (from 1.5 g to 3 g/day for amoxicillin) and, to some extent, for levofloxacin (0.5 to 1 g/day). In many cases, however, the dosages of the recommended antibiotic(s) are not specified. The duration of therapy is most often between 7 to 10 days (5 days only for the United States guidelines), except for azithromycin (3-day-therapy due to its specific pharmacokinetics), but remains unspecified in several guidelines.

For children (Table 3), amoxicillin and macrolides are the most often recommended antibiotics for 1st line treatments with third generation cephalosporins as alternatives (telithromycin and fluoroquinolones have no approved indication for children). Co-trimoxazole is only recommended by the World Health Organisation (WHO). As for adults, recommended dosages of amoxicillin are highly variable (most

often from 45 to 100 mg/kg per day, but higher in some countries) with durations of therapy spanning from 5 to 10 days.

Susceptibility patterns of *S. pneumoniae*

The resistance of *S. pneumoniae* to common antibiotics may represent the most important limitation to a wide applicability of guidelines, as its level varies to a large extent between countries and regions, in contrast to the other bacterial organisms commonly encountered in CAP. The most recent data from the resistance programmes surveyed are shown in a synoptic fashion in Figure 2, with two limits of epidemiological and clinical significance (10 and 20 % of the isolates). Considering penicillin first, we see (i) that a number of countries have > 10 % of isolates with the so-called "intermediate" phenotype (decreased susceptibility calling for an increased dosage), with France, Greece and Turkey reporting values exceeding 20 % ; (ii) that several countries have also > 10 % of full-resistant isolates (considered as creating a high likelihood of clinical failure, based on the definition of clinical breakpoints of EUCAST), with Greece and Asia reaching values > 20 %. Conversely, a few countries have very low levels of resistant isolates (Sweden, the Netherlands, and Germany). Thus, while the prevalence of penicillin-resistant and penicillin-intermediate *S. pneumoniae* is around a reasonably optimistic value of 10-15 % in Europe taken globally, this figure cannot be taken as a guide for therapy and local data are essential. Moving to macrolides (with erythromycin taken most often as an indicator of resistance to all macrolides [except telithromycin]), we see that resistance levels are higher than 20 % in a very large number of countries or regions, and reaches > 70 % in Asia, Canada, Japan and Taiwan. Countries reporting < 10 % resistance are rare (The Netherlands, Sweden, Germany). Tetracycline resistance levels range from < 10 % in Germany and Slovenia to > 20 % in Spain, Slovaquia, Turkey, Greece, Italy and France (> 40 % for the latter). Cotrimoxazole resistance affects most countries (with only United Kingdom, Germany, and Canada having figures < 20 %). In sharp contrast to all other antibiotics, levofloxacin (used as a reporter for antistreptococcal fluoroquinolones [d]) shows very low resistance levels

[d] The levels of resistance for moxifloxacin are lower than those of levofloxacin in surveys were both drugs are examined simultaneously [51–53]; ciprofloxacin was not included in the present

(≤ 2 %) throughout all countries or regions surveyed. Three countries (Germany, Turkey, and Greece) report no resistance to either levofloxacin or moxifloxacin.

Safety of recommended antibiotics

Table 4 shows the frequent and/or serious side effects, and the corresponding populations at risk, for the main antibiotics proposed in the guidelines (see Methods for data collection). Focusing on the most frequent and potentially harmful ones, one sees that (i) anaphylactic reactions are more frequent for β-lactams [54,55] (ii) *Clostridium difficile*-associated colitis is seen for all drugs (as a consequence of intestinal flora alteration) [56] and that clavulanic acid significantly increases intestinal discomfort and, more importantly, is associated with a well-known risk of hepatotoxicity (> 1/1,000) [50,57]. A major limitation of macrolides, and of erythromycin in particular, is their potential of inhibiting cytochrome P450-related metabolism, with a risk of drug interactions [58–60]. Macrolides carry also risks of hepatotoxicity [60,61] (exacerbated for telithromycin [62] and having led to its severe restriction in the US and limitations in Europe, making estimation of true incidences quite difficult today [50]), and of cardiac toxicity [63] (mostly related to QTc prolongation [64,65]; less important for azithromycin). Tetracyclines cause oesophagitis and oesophageal ulcerations [59,66,67], phototoxicity [68] and hepatotoxicity [60], and are contra-indicated in pregnant women (as well as in children). Cotrimoxazole adverse events profile is a summation of that of sulphonamide and of trimethoprim [69] causing a wide range of side effects, including haematological toxicity (related to its mode of action) and hepatic toxicity [50]. Fluoroquinolones are well known for their risk of tendonitis [70] especially in elderly patients taking corticosteroids [71] and have received warnings for cardiac and hepatic toxicity. Large surveys show, however that incidences of liver effects are considerably lower than those reported for amoxicillin-clavulanic acid or even macrolides [50,72].

Treatment guidelines: analysis according to the AGREE instrument

analysis as it is usually not recommended for treatment of streptococcal infections; gatifloxacin and gemifloxacin were not included because the former has been withdrawn in many countries and was never introduced in Europe, and the latter is only sparingly used in the countries included in our survey.

The results of our analysis are presented graphically in Figure 3. The upper panel shows that there was a large scatter in the agreement (kappa test) between the two investigators, suggesting that several guidelines could not be unambiguously understood. The same panel shows also a large scatter in the global score given to each guideline. There was no correlation between the results of the kappa test and the overall score (R^2 = 0.072; P = 0.15), ruling out the possibility that disagreements between evaluators could be linked to the low or high quality of specific guidelines. The lower panel of Figure 3 shows the results of the analysis for by 6 domains covered by the AGREE instrument. We see that while "scope & purpose" and "clarity of presentation" were globally satisfactory (median score about 0.75 on a maximum of 1), quite lower median scores were obtained for the 4 other domains. More specifically, (i) the editorial independence of the experts in charge of writing the guideline from the supporting organizations was rarely verifiable (often no or little information was available on the level of the financial support, whether from public of private sources, which, according to the instrument, should be considered a weakness as it undermines the confidence the reader may have in the guideline); (ii) the method of collection of the data and of synthesizing evidence for the decision process, and the definition of plan and frequency of update was often missing (none of the guidelines had been updated more frequently than once every 2 years); (iii) the stakeholder involvement (i.e. active participation of GP's, whom the guidelines are primarily intended to, of representatives of other relevant professional groups, and of patients) was often minimal; (iv) the rigour of development, namely an anticipation of potential risks of the proposed guideline, was highly variable.

Direct antibiotic acquisition costs

These costs are shown in Table 5, for all antibiotics for which a specific dosage and duration of treatment were mentioned in the same guideline. The lowest treatment acquisition costs at the lowest recommended dosage and duration of treatment are for doxycycline, amoxicillin and macrolides. However, the acquisition cost of amoxicillin and doxycycline can increase up to 6-fold if used at their maximal doses and durations of treatment. While among second line antibiotics, levofloxacin (if given at its higher dose [1 g/day]) is the most expensive as well as amoxicillin if using a branded product at its

maximal dose. Co-amoxiclav or the association of amoxicillin and a macrolide also reach a fair price (50 to 60 €) slightly higher than that of amoxicillin alone (branded product at its maximal dose and treatment duration). Globally, however, acquisition costs are quite low (compared to other drugs and taking into account the short duration of treatment) and decreased (of about 20 %) between our fist (2009) and final (2012) surveys.

Discussion

The present overview documents that CAP guidelines (i) show a wide level of divergence (although dealing with a well-characterized disease with quite common features in the various regions examined), and (ii) are of highly variable quality when examined with the AGREE instrument. Actually, guidelines are also from very different origins and intended for implementation in very variable and sometimes overlapping geographical areas (supranational, national, or local). While this makes comparisons difficult, it is also what the primary care physician is confronted with (which guideline should I follow and why?) and is therefore a key point in our analysis. For practical reasons, we could not include more guidelines than what is presented here. This, as well as the fact that the choice of guidelines to be analyzed was ours (we priviledged guidelines from Europe and North America for obvious reasons of availability and correct appreciation of the local medical needs) should be viewed as an intrinsic limitation, especially if considering specific populations (e.g., children or Asian and African patients).

Divergences between guidelines for antimicrobials should, a priori, be related to significant differences in (i) susceptibility of the target organisms (which here must be examined in two respects, namely the resistance of *S. pneumoniae* [73]) to the recommended antibiotics and the appropriate coverage of the so-called "atypical organisms"; (ii) local population characteristics (including environmental factors); (iii) safety issues of critical importance for the target population; (iv) drug availability and / or cost of therapy. Target organism susceptibility data are obviously not systematically taken into full consideration in the final recommendation. Considering the resistance of *S. pneumoniae* first, the same "1st line" antibiotics are recommended in countries where resistance is rare as well as where it is important (often > 10 %) (see e.g. tetracyclines and macrolides). Cotrimoxazole is recommended without specific limit in children even though resistance is high. For β-lactams, where a number of countries show a large proportion of strains with decreased susceptibility (based on EUCAST breakpoints [43]), there is often a lack of recommendation to use the larger dosages that are actually needed in this set-up (see the "rational document" associated with the EUCAST breakpoint; adopting higher breakpoints as proposed by CLSI [44] will not change that

while creating, perhaps, an unjustified feeling of safety) [e]. Moreover, since increased MICs of β-lactams are observed and resistance to macrolides is becoming widespread in Europe (probably because of their large use [74–76]), we may raise the question as to whether maintaining them in 1st line will not further worsen the situation, specifically for β-lactams if optimal (i.e. larger than the original ones) dosages are not systematically recommended. With respect to fluoroquinolones, most guidelines cite the risk of resistance spreading as the main reason to avoid their wide use. Yet, the present analysis shows that resistance of *S. pneumoniae* to antistreptococcal fluoroquinolones (levofloxacin, moxifloxacin) remains marginal (even though there was a marked increase of their consumption in the 1997-2002 period [77]), as also repeatedly observed by other investigators [78–82]. Actually, a modeling study suggests that introducing a molecule active against resistant strains may actually decrease the risk of multiresistant-strain dissemination [83]. Examining next the coverage of atypical organisms, we see wide variations amongst guidelines. The most striking divergence is between US guidelines *vs.* several European guidelines, with the former including fluoroquinolones almost as quasi-first-line therapy partly because of a greater emphasis on the role of "atypical pathogens" [25] while the latter recommend β-lactams only and limit the association of a β-lactam and a macrolide (necessary to cover these "atypical pathogens") to situations of non-response (see also [84] for a comment about differences between the British Thoracic Society and the Infectious Diseases Society of America/American Thoracic Society guidelines).

Safety issues are often a main reason for favouring or excluding whole pharmacological classes of drugs in the process of guideline setting. Yet, and although diverse, all antibiotics, including those recommended as 1st line, have clinically-significant side effects. These can severely affect specific patients (e.g., anaphylactic reactions for β-lactams [85], hepatotoxicity for clavulanic acid and co-trimoxazole [50,86],

[e] Although difficult to prove, the steadily decrease in susceptibility of *S. pneumoniae* to β-lactams may have resulted not only from their large use but also from underdosage. Over-the-counter sales, often considered as a main cause of resistance in community isolates, are known to be important in some countries but are not allowed in many others (such as United States/Canada or France/Belgium/Germany/Austria/Switzerland) where marked increase in MICs of β-lactams has nevertheless been observed.

phototoxicity for tetracyclines, drug interactions and cardiac adverse events [65] for macrolides, especially in patients receiving other potentially cardiotoxic drugs [63], tendinopathies and specific risks for patients with cardiac pathologies, or epilepsia for fluoroquinolones [72,87]), which can make general recommendations somewhat difficult to accept. Yet, defining the true risk-benefit profiles of drugs for specific patients should be part of the process of guideline setting [88]. A major reason for skipping this issue in guidelines could be that the authors actually have no or only limited access to numerical data about side effects of the antibiotics they analyse. Actually, crude incidence rates as observed from registration studies, pharmacovigilance data (with unambiguous assessment of causality), or data from prospective and retrospective cohort studies made by Registration Holders and Regulatory Authorities (which are all essential in this context), rarely appear in public, peer-reviewed and non-Industry-sponsored literature. Direct analysis of drug safety databases such as those maintained by the World Health Organization (The Uppsala Monitoring Center [http://www.who-umc.org]) is difficult for non-specialists and never mentioned as a main source of information in the guidelines analyzed. Yet, and although infrequent, true incidences of side effects need to be more carefully taken into account, especially to minimize the well described toxicities of some older agents in specific populations.

Drug availability is no real reason for divergence as most of the drugs included in the recommendations are commercialized in all the countries surveyed. Drug acquisition costs should also not be a real deterrent. Although prices are in a 1 to 10 range, they remain quite modest in absolute value (and even with a decreasing trend over the last years), especially if considering that treatments yield a high percentage of fast and long-term success with globally minimal side effects. This contrasts sharply with other therapies, such as those used for cancer (much higher acquisition costs although showing much more limited long-lasting effects on mortality and morbidity and much worse and costlier side effects). Moreover, CAP treatments are short, making the financial burden considerably less than that of many other infections. Lastly, 1st line "cheap" antibiotics such as β-lactams may actually cost as much as 2^{d} line antibiotics when considering increased dosages and their frequent association with macrolides.

Examining all evidence, we see that several guidelines may be suboptimal in providing a standard of care optimising outcomes for the majority of patients while

protecting the individual patients as well as the community from unacceptable adverse effects. This leads us to address the issue that guidelines, generally speaking, raise often low awareness [89] and are poorly respected [90–92]. Armitage and Woodhead [93] pointed out a lack of robust evidence behind several aspects of guideline recommendations. Although not providing insurance that methodological rigour will lead to validity of recommendations, the AGREE instrument helps in suggesting where and how guidelines could be improved [94] leading to increased adherence and avoiding mistrust [95]. More specifically, guidelines are supposed to provide benefits that outweigh risks for typical patients [96] but CAP patients are rarely "non-risk, otherwise healthy individuals", which means that 1st line antibiotics may often not be the real antibiotics of choice. For instance, Aujeski *et al*. [97] showed that emergency services often hospitalize many low-risk patients with CAP but with comorbid illnesses that make actually inappropriate the blind application of guidelines based on severity score only. More broadly speaking, guidelines in infectious diseases are indeed difficult not only to write but also to implement due to the multiplicity of target groups and the necessity to develop interventions with optimal effect [98,99]. Guidelines, sometimes, also include recommendations that simply cannot be followed by GP's (for instance, recommending an antibiotic that is no longer commercially available in the corresponding country, or proposing very high doses of intravenous penicillin given every 4 to 6 h to keep on with the decreased susceptibility of the isolates while not moving to another class of antibiotics), illustrating the lack of involvement of stakeholders. Insufficient demonstration of editorial independence (for both scientific societies- and government-sponsored guidelines) or lack of unambiguous information in this context can also be a reason for distrust.

In a broader context, guidelines also tend to be primarily based on published clinical trials that are often Industry-supported with emphasis on hospitalized patients as this is necessary for insurance quality [100]. There are actually very few if any high level studies of treatment of outpatients with CAP, and a recent Cochrane report concluded that currently available evidence from randomized controlled studies is insufficient to make evidence-based recommendations for specific antibiotic(s) in the treatment of CAP in ambulatory patients [13]. In this respect, and although a series of publications have reported beneficial effects of following guidelines [101–106] it remains difficult to directly

attribute any change in patient outcomes to any specific guideline [107] as most recommendations rely on data that are not outcome-based [108] and direct, objective data demonstrating the superiority of guideline-compliant treatments over other treatments in outpatients are lacking [109]. This is particularly important in the specific situation of CAP as mortality is low and other outcomes, such as time to deffervescence, duration of convalescence, loss of labour days at the individual level, and selection of antibiotic resistance at the societal level must be taken into account to obtain a really meaningful picture. These are difficult to document and are, therefore, rarely used to assess the value of the strategies proposed.

Lastly, a frequent weakness of guidelines is the lack of a predefined plan for regular update and the insufficient integration of the potential side effects of the recommended antibiotics. Guidelines need revision every 3 years [110], a condition fulfilled by only a few of those examined here, which is most unfortunate given the fast-occurring changes in resistance epidemiology. Although we only examined the recommendations for non-severe CAP, for which moratlity is low, this concerns the largest number of patients and correspond to situations where sophisticated diagnostic tools are often unavailable or unpractical to use, making, therefore, efficient, directly usable and up-to-date recommendations most important.

Our conclusion is that, while setting up guidelines for CAP is a useful exercise, several of those we analyzed (focusing on treatment of non-hospitalized patients) suffer from limitations making them ineligible, in our opinion, as a "gold standard". Their growing number and heterogeneity (for which the logic is not always obvious) create a disturbing situation for physicians and may, in part explain why efforts to implement them is rarely successful [111]. As we consider that guidelines are important in promoting better standards of care, we hope that the present review may help to improve their design by pointing to areas where efforts should be made so that they better fit the epidemiological realities and clinical needs.

Reference List

1. Woodhead MA, Macfarlane JT, McCracken JS, Rose DH, Finch RG (1987) Prospective study of the aetiology and outcome of pneumonia in the community. Lancet 1: 671-674.

2. Almirall J, Morato I, Riera F, Verdaguer A, Priu R et al. (1993) Incidence of community-acquired pneumonia and Chlamydia pneumoniae infection: a prospective multicentre study. Eur Respir J 6: 14-18.

3. Jokinen C, Heiskanen L, Juvonen H, Kallinen S, Karkola K et al. (1993) Incidence of community-acquired pneumonia in the population of four municipalities in eastern Finland. Am J Epidemiol 137: 977-988.

4. Almirall J, Bolibar I, Vidal J, Sauca G, Coll P et al. (2000) Epidemiology of community-acquired pneumonia in adults: a population-based study. Eur Respir J 15: 757-763.

5. Fine MJ, Orloff JJ, Arisumi D, Fang GD, Arena VC et al. (1990) Prognosis of patients hospitalized with community-acquired pneumonia. Am J Med 88: 1N-8N.

6. Fine MJ, Smith MA, Carson CA, Mutha SS, Sankey SS et al. (1996) Prognosis and outcomes of patients with community-acquired pneumonia. A meta-analysis. JAMA 275: 134-141.

7. Aujesky D, Fine MJ (2008) The pneumonia severity index: a decade after the initial derivation and validation. Clin Infect Dis 47 Suppl 3: S133-S139.

8. Renaud B, Coma E, Hayon J, Gurgui M, Longo C et al. (2007) Investigation of the ability of the Pneumonia Severity Index to accurately predict clinically relevant outcomes: a European study. Clin Microbiol Infect 13: 923-931.

9. Welte T, Kohnlein T (2009) Global and local epidemiology of community-acquired pneumonia: the experience of the CAPNETZ Network. Semin Respir Crit Care Med 30: 127-135.

10. Diaz LA, Mortensen EM, Anzueto A, Restrepo MI (2008) Novel targets in the management of pneumonia. Ther Adv Respir Dis 2: 387-400.

11. Heron M (2007) Deaths: leading causes for 2004. Natl Vital Stat Rep 56: 1-95.

12. Restrepo MI, Anzueto A (2009) Severe community-acquired pneumonia. Infect Dis Clin North Am 23: 503-520.

13. Bjerre LM, Verheij TJ, Kochen MM (2009) Antibiotics for community acquired pneumonia in adult outpatients. Cochrane Database Syst Rev CD002109.

14. Atkinson M, Yanney M, Stephenson T, Smyth A (2007) Effective treatment strategies for paediatric community-acquired pneumonia. Expert Opin Pharmacother 8: 1091-1101.

15. Falguera M, Sacristan O, Nogues A, Ruiz-Gonzalez A, Garcia M et al. (2001) Nonsevere community-acquired pneumonia: correlation between cause and severity or comorbidity. Arch Intern Med 161: 1866-1872.

16. Bochud PY, Moser F, Erard P, Verdon F, Studer JP et al. (2001) Community-acquired pneumonia. A prospective outpatient study. Medicine (Baltimore) 80: 75-87.

17. Woodhead M (2002) Community-acquired pneumonia in Europe: causative pathogens and resistance patterns. Eur Respir J Suppl 36: 20s-27s.

18. Woodhead M, Blasi F, Ewig S, Garau J, Huchon G et al. (2011) Guidelines for the management of adult lower respiratory tract infections--full version. Clin Microbiol Infect 17 Suppl 6: E1-59.

19. Mandell LA, Wunderink RG, Anzueto A, Bartlett JG, Campbell GD et al. (2007) Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults. Clin Infect Dis 44 Suppl 2: S27-S72.

20. Mandell LA, Bartlett JG, Dowell SF, File TM, Jr., Musher DM et al. (2003) Update of practice guidelines for the management of community-acquired pneumonia in immunocompetent adults. Clin Infect Dis 37: 1405-1433.

21. Niederman MS (2007) Recent advances in community-acquired pneumonia: inpatient and outpatient. Chest 131: 1205-1215.

22. Austrian R, Gold J (1964) Pneumococcal bacteriemia with special reference to bacteremic pneumococcal pneumonia. Ann Intern Med 60: 759-776.

23. Kramer MR, Rudensky B, Hadas-Halperin I, Isacsohn M, Melzer E (1987) Pneumococcal bacteremia--no change in mortality in 30 years: analysis of 104 cases and review of the literature. Isr J Med Sci 23: 174-180.

24. Feikin DR, Schuchat A, Kolczak M, Barrett NL, Harrison LH et al. (2000) Mortality from invasive pneumococcal pneumonia in the era of antibiotic resistance, 1995-1997. Am J Public Health 90: 223-229.

25. Niederman MS (2009) Community-acquired pneumonia: the U.S. perspective. Semin Respir Crit Care Med 30: 179-188.

26. Nowak D, Berger K, Lippert B, Kilgert K, Caeser M et al. (2005) Epidemiology and health economics of COPD across Europe: a critical analysis. Treat Respir Med 4: 381-395.

27. Fretheim A, Aaserud M, Oxman AD (2006) Rational prescribing in primary care (RaPP): economic evaluation of an intervention to improve professional practice. PLoS Med 3: e216.

28. Opmeer BC, El Moussaoui R, Bossuyt PM, Speelman P, Prins JM et al. (2007) Costs associated with shorter duration of antibiotic therapy in hospitalized patients with mild-to-moderate severe community-acquired pneumonia. J Antimicrob Chemother 60: 1131-1136.

29. Garcia-Altes A, Navas E, Soriano MJ (2011) [Economic evaluation of public health interventions]. Gac Sanit 25 Suppl 1: 25-31.

30. Hava DL, LeMieux J, Camilli A (2003) From nose to lung: the regulation behind Streptococcus pneumoniae virulence factors. Mol Microbiol 50: 1103-1110.

31. Mufson MA (1999) Pneumococcal Pneumonia. Curr Infect Dis Rep 1: 57-64.

32. Lynch JP, III, Zhanel GG (2009) Streptococcus pneumoniae: epidemiology, risk factors, and strategies for prevention. Semin Respir Crit Care Med 30: 189-209.

33. Klugman KP, Low DE, Metlay J, Pechere JC, Weiss K (2004) Community-acquired pneumonia: new management strategies for evolving pathogens and antimicrobial susceptibilities. Int J Antimicrob Agents 24: 411-422.

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

35. Farrell DJ, Couturier C, Hryniewicz W (2008) Distribution and antibacterial susceptibility of macrolide resistance genotypes in Streptococcus pneumoniae: PROTEKT Year 5 (2003-2004). Int J Antimicrob Agents 31: 245-249.

36. Jenkins SG, Farrell DJ (2009) Increase in pneumococcus macrolide resistance, United States. Emerg Infect Dis 15: 1260-1264.

37. Fritsche TR, Sader HS, Jones RN (2008) Antimicrobial activity of ceftobiprole, a novel anti-methicillin-resistant Staphylococcus aureus cephalosporin, tested against contemporary pathogens: results from the SENTRY Antimicrobial Surveillance Program (2005-2006). Diagn Microbiol Infect Dis 61: 86-95.

38. Rhomberg PR, Jones RN (2009) Summary trends for the Meropenem Yearly Susceptibility Test Information Collection Program: a 10-year experience in the United States (1999-2008). Diagn Microbiol Infect Dis 65: 414-426.

39. Norskov-Lauritsen N, Marchandin H, Dowzicky MJ (2009) Antimicrobial susceptibility of tigecycline and comparators against bacterial isolates collected as part of the TEST study in Europe (2004-2007). Int J Antimicrob Agents 34: 121-130.

40. Fritsche TR, Sader HS, Stillwell MG, Jones RN (2009) Antimicrobial activity of doripenem tested against prevalent Gram-positive pathogens: results from a global surveillance study (2003-2007). Diagn Microbiol Infect Dis 63: 440-446.

41. Jones RN, Ross JE, Bell JM, Utsuki U, Fumiaki I et al. (2009) Zyvox Annual Appraisal of Potency and Spectrum program: linezolid surveillance program results for 2008. Diagn Microbiol Infect Dis 65: 404-413.

42. Farrell DJ, Mendes RE, Ross JE, Jones RN (2009) Linezolid surveillance program results for 2008 (LEADER Program for 2008). Diagn Microbiol Infect Dis 65: 392-403.

43. Anonymous. EUCAST clinical breakpoints. Available: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/EUCAST_breakpoints_v1.3_pdf.pdf. Accessed 10 July 2011.

44. Anonymous (2010) Performance standards for antimicrobial susceptibility testing 20th informational supplement (MS100-S20), Clinical and Laboratory Standard Institute, Wayne, PA, pp 160

45. Grol R, Cluzeau FA, Burgers JS (2003) Clinical practice guidelines: towards better quality guidelines and increased international collaboration. Br J Cancer 89 Suppl 1: S4-S8.

46. Brouwers MC, Kho ME, Browman GP, Burgers JS, Cluzeau F et al. (2010) AGREE II: advancing guideline development, reporting and evaluation in health care. CMAJ 182: E839-E842.

47. Agouridas C, Denis A, Auger JM, Benedetti Y, Bonnefoy A et al. (1998) Synthesis and antibacterial activity of ketolides (6-O-methyl-3-oxoerythromycin derivatives): a new class of antibacterials highly potent against macrolide-resistant and -susceptible respiratory pathogens. J Med Chem 41: 4080-4100.

48. Reinert RR (2009) The antimicrobial resistance profile of Streptococcus pneumoniae. Clin Microbiol Infect 15 Suppl 3: 7-11.

49. Felmingham D, Canton R, Jenkins SG (2007) Regional trends in beta-lactam, macrolide, fluoroquinolone and telithromycin resistance among Streptococcus pneumoniae isolates 2001-2004. J Infect 55: 111-118.

50. Andrade RJ, Tulkens PM (2011) Hepatic safety of antibiotics used in primary care. J Antimicrob Chemother 66: 1431-1446.

51. Boswell FJ, Andrews JM, Jevons G, Wise R (2002) Comparison of the in vitro activities of several new fluoroquinolones against respiratory pathogens and their abilities to select fluoroquinolone resistance. J Antimicrob Chemother 50: 495-502.

52. Morrissey I, Colclough A, Northwood J (2007) TARGETed surveillance: susceptibility of Streptococcus pneumoniae isolated from community-acquired respiratory tract infections in 2003 to fluoroquinolones and other agents. Int J Antimicrob Agents 30: 345-351.

53. Lismond A, Van Bambeke F, Carbonnelle S, Jacobs F, Struelens MJ et al. (2008) Epidemiological survey of resistance to b-lactams (AMX, CFX, CRO), macrolides (CLR, TEL), and fluoroquinolones (LVX, MXF) in a Belgian collection of community-acquired pneumonia isolates of Streptococcus pneumoniae. Clinical Microbiology and Infection 14: S508 (poster no. 1747).

54. Johannes CB, Ziyadeh N, Seeger JD, Tucker E, Reiter C et al. (2007) Incidence of allergic reactions associated with antibacterial use in a large, managed care organisation. Drug Saf 30: 705-713.

55. Hamzaoui A (2006) Allergie aux antiinfectieux. Rev Mal Respir 23: 10S70-10S72.

56. Hull MW, Beck PL (2004) Clostridium difficile-associated colitis. Can Fam Physician 50: 1536-5.

57. Garcia Rodriguez LA, Stricker BH, Zimmerman HJ (1996) Risk of acute liver injury associated with the combination of amoxicillin and clavulanic acid. Arch Intern Med 156: 1327-1332.

58. von Rosensteil NA, Adam D (1995) Macrolide antibacterials. Drug interactions of clinical significance. Drug Saf 13: 105-122.

59. Abu-Gharbieh E, Vasina V, Poluzzi E, De Ponti F (2004) Antibacterial macrolides: a drug class with a complex pharmacological profile. Pharmacol Res 50: 211-222.

60. Polson JE (2007) Hepatotoxicity due to antibiotics. Clin Liver Dis 11: 549-61, vi.

61. Hautekeete ML (1995) Hepatotoxicity of antibiotics. Acta Gastroenterol Belg 58: 290-296.

62. Brinker AD, Wassel RT, Lyndly J, Serrano J, Avigan M et al. (2009) Telithromycin-associated hepatotoxicity: Clinical spectrum and causality assessment of 42 cases. Hepatology 49: 250-257.

63. Guo D, Cai Y, Chai D, Liang B, Bai N et al. (2010) The cardiotoxicity of macrolides: a systematic review. Pharmazie 65: 631-640.

64. Simko J, Csilek A, Karaszi J, Lorincz I (2008) Proarrhythmic potential of antimicrobial agents. Infection 36: 194-206.

65. Owens RC, Jr., Nolin TD (2006) Antimicrobial-associated QT interval prolongation: pointes of interest. Clin Infect Dis 43: 1603-1611.

66. Ollyo JB, Fontolliet C, Monnier P, Wellinger J, Restellini A et al. (1990) [Drug-induced esophagitis and its complications. Retrospective study of 30 case reports and review of 650 published cases (1970-1987)]. Schweiz Rundsch Med Prax 79: 394-397.

67. Zografos GN, Georgiadou D, Thomas D, Kaltsas G, Digalakis M (2009) Drug-induced esophagitis. Dis Esophagus .

68. Wainwright NJ, Collins P, Ferguson J (1993) Photosensitivity associated with antibacterial agents. Drug Saf 9: 437-440.

69. Brumfitt W, Hamilton-Miller JM (1994) Limitations of and indications for the use of co-trimoxazole. J Chemother 6: 3-11.

70. Melhus A (2005) Fluoroquinolones and tendon disorders. Expert Opin Drug Saf 4: 299-309.

71. van der Linden PD, Sturkenboom MC, Herings RM, Leufkens HM, Rowlands S et al. (2003) Increased risk of achilles tendon rupture with quinolone antibacterial use, especially in elderly patients taking oral corticosteroids. Arch Intern Med 163: 1801-1807.

72. Van Bambeke F, Tulkens PM (2009) Safety profile of the respiratory fluoroquinolone moxifloxacin: comparison with other fluoroquinolones and other antibacterial classes. Drug Saf 32: 359-378.

73. van der Poll T., Opal SM (2009) Pathogenesis, treatment, and prevention of pneumococcal pneumonia. Lancet 374: 1543-1556.

74. Baquero F, Baquero-Artigao G, Canton R, Garcia-Rey C (2002) Antibiotic consumption and resistance selection in Streptococcus pneumoniae. J Antimicrob Chemother 50 Suppl S2: 27-37.

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

76. Sande-Bruinsma N, Grundmann H, Verloo D, Tiemersma E, Monen J et al. (2008) Antimicrobial drug use and resistance in Europe. Emerg Infect Dis 14: 1722-1730.

77. Elseviers MM, Ferech M, Vander Stichele RH, Goossens H (2007) Antibiotic use in ambulatory care in Europe (ESAC data 1997-2002): trends, regional differences and seasonal fluctuations. Pharmacoepidemiol Drug Saf 16: 115-123.

78. Garcia-Rey C, Martin-Herrero JE, Baquero F (2006) Antibiotic consumption and generation of resistance in Streptococcus pneumoniae: the paradoxical impact of quinolones in a complex selective landscape. Clin Microbiol Infect 12 Suppl 3: 55-66.

79. Gonullu N, Catal F, Kucukbasmaci O, Ozdemir S, Torun MM et al. (2009) Comparison of in vitro activities of tigecycline with other antimicrobial agents against Streptococcus pneumoniae, Haemophilus influenzae and Moraxella catarrhalis in two university hospitals in Istanbul, Turkey. Chemotherapy 55: 161-167.

80. Jacobs E, Dalhoff A, Korfmann G (2009) Susceptibility patterns of bacterial isolates from hospitalised patients with respiratory tract infections (MOXIAKTIV Study). Int J Antimicrob Agents 33: 52-57.

81. Pletz MW, van der Linden M, von Baum H, Duesberg CB, Klugman KP et al. (2011) Low prevalence of fluoroquinolone resistant strains and resistance precursor strains in Streptococcus pneumoniae from patients with community-acquired pneumonia despite high fluoroquinolone usage. Int J Med Microbiol 301: 53-57.

82. Vanhoof R, Camps K, Carpentier M, De Craeye S, Frans J et al. (2010) 10th survey of antimicrobial resistance in noninvasive clinical isolates of Streptococcus pneumoniae collected in Belgium during winter 2007-2008. Pathol Biol (Paris) 58: 147-151.

83. Opatowski L, Temime L, Varon E, Leclercq R, Drugeon H et al. (2008) Antibiotic innovation may contribute to slowing the dissemination of multiresistant Streptococcus pneumoniae: the example of ketolides. PLoS One 3: e2089.

84. Mandell L (2010) Community acquired pneumonia. BMJ 341: c2916.

85. Chaabane A, Aouam K, Boughattas NA, Chakroun M (2009) [Allergy to betalactams: myth and realities.]. Med Mal Infect 39: 278-287.

86. Chang CY, Schiano TD (2007) Review article: drug hepatotoxicity. Aliment Pharmacol Ther 25: 1135-1151.

87. Liu HH (2010) Safety profile of the fluoroquinolones: focus on levofloxacin. Drug Saf 33: 353-369.

88. Psaty BM (2008) Clinical trial design and selected drug safety issues for antibiotics used to treat community-acquired pneumonia. Clin Infect Dis 47 Suppl 3: S176-S179.

89. El Solh AA, Alhajhusain A, Saliba RG, Drinka P (2011) Physicians' attitudes toward guidelines for the treatment of hospitalized nursing home-acquired pneumonia. J Am Med Dir Assoc 12: 270-276.

90. Heselmans A, Donceel P, Aertgeerts B, Van de Velde S, Ramaekers D (2009) The attitude of Belgian social insurance physicians towards evidence-based practice and clinical practice guidelines. BMC Fam Pract 10: 64.

91. Peetermans WE, Ramaekers D (2002) Clinical practice guidelines in infectious diseases. Neth J Med 60: 343-348.

92. Lim WS, Woodhead M (2011) British Thoracic Society adult community acquired pneumonia audit 2009/10. Thorax 66: 548-549.

93. Armitage K, Woodhead M (2007) New guidelines for the management of adult community-acquired pneumonia. Curr Opin Infect Dis 20: 170-176.

94. Burls A (2010) AGREE II-improving the quality of clinical care. Lancet 376: 1128-1129.

95. Vlayen J, Aertgeerts B, Hannes K, Sermeus W, Ramaekers D (2005) A systematic review of appraisal tools for clinical practice guidelines: multiple similarities and one common deficit. Int J Qual Health Care 17: 235-242.

96. Guyatt G, Gutterman D, Baumann MH, Addrizzo-Harris D, Hylek EM et al. (2006) Grading strength of recommendations and quality of evidence in clinical guidelines: report from an american college of chest physicians task force. Chest 129: 174-181.

97. Aujesky D, McCausland JB, Whittle J, Obrosky DS, Yealy DM et al. (2009) Reasons why emergency department providers do not rely on the pneumonia severity index to determine the initial site of treatment for patients with pneumonia. Clin Infect Dis 49: e100-e108.

98. De Souza V, MacFarlane A, Murphy AW, Hanahoe B, Barber A et al. (2006) A qualitative study of factors influencing antimicrobial prescribing by non-consultant hospital doctors. J Antimicrob Chemother 58: 840-843.

99. Cortoos PJ, De Witte K, Peetermans WE, Simoens S, Laekeman G (2008) Opposing expectations and suboptimal use of a local antibiotic hospital guideline: a qualitative study. J Antimicrob Chemother 62: 189-195.

100. Guyatt GH, Oxman AD, Kunz R, Vist GE, Falck-Ytter Y et al. (2008) What is "quality of evidence" and why is it important to clinicians? BMJ 336: 995-998.

101. Nathwani D, Rubinstein E, Barlow G, Davey P (2001) Do guidelines for community-acquired pneumonia improve the cost-effectiveness of hospital care? Clin Infect Dis 32: 728-741.

102. Menendez R, Ferrando D, Valles JM, Vallterra J (2002) Influence of deviation from guidelines on the outcome of community-acquired pneumonia. Chest 122: 612-617.

103. Dean NC, Bateman KA (2004) Local guidelines for community-acquired pneumonia: development, implementation, and outcome studies. Infect Dis Clin North Am 18: 975-991.

104. Dean NC, Bateman KA, Donnelly SM, Silver MP, Snow GL et al. (2006) Improved clinical outcomes with utilization of a community-acquired pneumonia guideline. Chest 130: 794-799.

105. Dambrava PG, Torres A, Valles X, Mensa J, Marcos MA et al. (2008) Adherence to guidelines' empirical antibiotic recommendations and community-acquired pneumonia outcome. Eur Respir J 32: 892-901.

106. Fung HB, Monteagudo-Chu MO (2010) Community-acquired pneumonia in the elderly. Am J Geriatr Pharmacother 8: 47-62.

107. Levin A (2008) Practice guidelines do improve patient outcomes: association or causation? Blood Purif 26: 67-72.

108. Wunderink RG (1998) Clinical practice guidelines for the management of pneumonia--do they work? New Horiz 6: 75-83.

109. Amerling R, Winchester JF, Ronco C (2008) Guidelines have done more harm than good. Blood Purif 26: 73-76.

110. Shekelle PG, Ortiz E, Rhodes S, Morton SC, Eccles MP et al. (2001) Validity of the Agency for Healthcare Research and Quality clinical practice guidelines: how quickly do guidelines become outdated? JAMA 286: 1461-1467.

111. Grol R (2001) Successes and failures in the implementation of evidence-based guidelines for clinical practice. Med Care 39: II46-II54.

112. Donowitz GR. Acute pneumonia (Chapter 64) - Pneumonia syndromes (In: Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases, 7th Edition -on-line version). Available: http://www.expertconsultbook.com. Accessed 20 January 2012.

113. Fang GD, Fine M, Orloff J, Arisumi D, Yu VL et al. (1990) New and emerging etiologies for community-acquired pneumonia with implications for therapy. A prospective multicenter study of 359 cases. Medicine (Baltimore) 69: 307-316.

114. Everett KD, Bush RM, Andersen AA (1999) Emended description of the order Chlamydiales, proposal of Parachlamydiaceae fam. nov. and Simkaniaceae fam. nov., each containing one monotypic genus, revised taxonomy of the family

Chlamydiaceae, including a new genus and five new species, and standards for the identification of organisms. Int J Syst Bacteriol 49 Pt 2: 415-440.

115. Woodhead M, Blasi F, Ewig S, Huchon G, Ieven M et al. (2005) Guidelines for the management of adult lower respiratory tract infections. Eur Respir J 26: 1138-1180.

116. Agence française sanitaire des produits de santé. Antibiothérapie par voie générale dans les infections respiratoires basses de l'adulte et de l'enfant. Available: http://www.afssaps.fr/var/afssaps_site/storage/original/application/b45044683f79b86c2bafe9f3b2ac7f2c.pdf. Accessed 2 February 2010.

117. Agence française sanitaire des produits de santé. Antibiothérapie par voie générale dans les infections respiratoires basses de l'adulte - Pneumonie aiguë communautaire - Exacerbations de Bronchopneumopathie Chronique Obstructive (Mise au point). Available: http://www.afssaps.fr/content/download/26334/348020/version/7/file/map-infections-respiratoires-basses-adultes.pdf. Accessed 7 January 2012.

118. Helsebiblioteket. Helsebiblioteket. Available: http://www.helsebiblioteket.no. Accessed 10 January 2010.

119. Service Public Fédéral Belge Santé Publique. Guide Belge des traitements anti-infectieux en pratique ambulatoire. Available: https://portal.health.fgov.be/pls/portal/url/ITEM/5B8EF73EFFAF6E11E04400144F3EAABC. Accessed 27 February 2012.

120. Lim WS, Baudouin SV, George RC, Hill AT, Jamieson C et al. (2009) BTS guidelines for the management of community acquired pneumonia in adults: update 2009. Thorax 64 Suppl 3: iii1-55.

121. Suomalainen Lääkäriseura Duodecim / Käypä hoito (Finnish Medical Society Duodecim / Current Care). Keuhkokuume Käypä hoito. Available: http://www.kaypahoito.fi/web/english/summaries/naytaartikkeli/tunnus/hoi50073. Accessed 2 January 2011.

122. Gruppo Operativo Controllo Infezioni Ospedaliere. Linee guida per il trattamento della polmonite acqusita in communita' (CAP). Available: http://www.sanfilipponeri.roma.it/file_allegati/protocollo_cap.pdf. Accessed 12 January 2012.

123. Institut for Rational Farmacoterapi. Antibiotika (systemisk brug). Available: http://www.irf.dk/dk/rekommandationsliste/baggrundsnotater/infektionssygdomme/antibiotika_systemisk_brug.htm. Accessed 15 January 2012.

124. KEEL - Κεντρο Ελεγχου και Προληψης Νοηματων (ΚΕ.ΕΛ.Π.ΝΟ) (2007) Κατευθυντηριες Οδηγιες για τη Διαγνωση και την Εμπειρικη Θεραπεια των Λοιμωξεων. Αθηνα: Focus on Health Ltd. 313 p.

125. Österreichische Gesellschaft für Infektionskrankheiten. Ambulant erworbene Pneumonie (CAP). Available: http://www.oeginfekt.at/download/experten_statement_ambulant_erworbene_pneumonie_(cap)_2008.pdf. Accessed 15 January 2012.

126. Höffken G, Lorenz J, Kern W, Welte T, Bauer T et al. (2009) Epidemiologie, Diagnostik, antimikrobielle Therapie und Management von erwachsenen Patienten mit ambulant erworbenen unteren Atemwegsinfektionen sowie ambulant erworbener Pneumonie - Update 2009. Pneumologie 63: e1-68.

127. Sinopalnikov AI, Strachunsky LS. [Community-acquired pneumonia in adults:diagnosis, treatment and prevention]. Available: http://www.antibiotic.ru/rus/all/metod/pneumonia/index.shtml. Accessed 12 December 2011.

128. Alfageme I, Aspa J, Bello S, Blanquer J, Blanquer R et al. (2005) Guidelines for the diagnosis and management of community-acquired pneumonia. Spanish Society of Pulmonology and Thoracic Surgery (SEPAR). Arch Bronconeumol 41: 272-289.

129. Svenska Infektionsläkarföreningen. Vårdprogram för samhällsförvärvad pneumoni 2011. Available: http://www.infektion.net/sites/default/files/pdf/Vardprogram_pneumoni_2011-02-15.pdf. Accessed 2 January 2012.

130. Scottish Intercollegiate Guidelines Network. Community management of lower respiratory tract infection in adults. Available: http://www.sign.ac.uk/pdf/sign59.pdf. Accessed 5 January 2012.

131. Société de Pathologie Infectieuse de Langue Française. Antibiothérapie par voie générale dans les infections respiratoires basses de l'adulte - Mise au point (21/07/2010). Available: http://www.infectiologie.com/site/medias/_documents/consensus/2010-infVRB-spilf-afssaps.pdf. Accessed 12 January 2012.

132. Sociedade Portuguesa de Pneumologia. Recomendações de abordagem diagnóstica e terapêutica da pneumonia da comunidade em adultos imunocompetentes. Available: http://www.sppneumologia.pt/sites/sppneumologia.pt/files/pdfs/RPP_2003_5_435_recomendacoes.pdf. Accessed 2 January 2012.

133. Swiss Society for Infectious Diseases. Swiss Society for Infectious Diseases. Available: www.sginf.ch. Accessed 15 December 2010.

134. Schouten JA, Prins JM, Bonten MJ, Degener J, Janknegt RE et al. (2005) Revised SWAB guidelines for antimicrobial therapy of community-acquired pneumonia. Neth J Med 63: 323-335.

135. Mandell LA, Marrie TJ, Grossman RF, Chow AW, Hyland RH (2000) Canadian guidelines for the initial management of community-acquired pneumonia: an evidence-based update by the Canadian Infectious Diseases Society and the Canadian Thoracic Society. The Canadian Community-Acquired Pneumonia Working Group. Clin Infect Dis 31: 383-421.

136. Grupo de trabajo de la Asociación Latinoamericana del Tórax (ALAT) (2004) [Update to the Latin American Thoracic Association (ALAT) recommendations on community acquired pneumonia]. Arch Bronconeumol 40: 364-374.

137. Correa RA, Lundgren FL, Pereira-Silva JL, Frare e Silva RL, Cardoso AP et al. (2009) Brazilian guidelines for community-acquired pneumonia in immunocompetent adults - 2009. J Bras Pneumol 35: 574-601.

138. Memish ZA, Shibl AM, Ahmed QA (2002) Guidelines for the management of community-acquired pneumonia in Saudi Arabia: a model for the Middle East region. Int J Antimicrob Agents 20 Suppl 1: S1-12.

139. Feldman C, Brink AJ, Richards GA, Maarten G, Bateman ED (2007) Management of Community-Acquired Pneumonia in Adult. S Afr Med J 97: 1295-1306.

140. World Health Organization. Pocket book of hospital care for children - Guidelines for the management of common illnesses with limited resources. Available: http://whqlibdoc.who.int/publications/2005/9241546700.pdf. Accessed 5 December 2011.

141. British Thoracic Society Standards of Care Committee (2002) British Thoracic Society Guidelines for the Management of Community Acquired Pneumonia in Childhood. Thorax 57 Suppl 1: i1-24.

142. Secção de Pneumologia da Sociedade Portuguesa de Pediatria (2007) Pneumonia adquirida na comunidade. Orientações para actuação em Pediatria. Acta Pediátrica Portuguesa 38: 90-92.

143. Cincinnati Children's Hospital Community Acquired Pneumonia Guideline Team. Evidence-based care guideline for medical management of Community Acquired Pneumonia in children 60 days to 17 years of age. Available: http://www.cincinnatichildrens.org/WorkArea/linkit.aspx?LinkIdentifier=id&ItemID=87957&libID=87645. Accessed 2 December 2011.

144. Brazilian guidelines in community-acquired pneumonia in pediatrics (2007) [Brazilian guidelines in community-acquired pneumonia in pediatrics- 2007]. J Bras Pneumol 33 Suppl 1: S31-S50.

145. Lee PI, Chiu CH, Chen PY, Lee CY, Lin TY (2007) Guidelines for the management of community-acquired pneumonia in children. Acta Paediatr Taiwan 48: 167-180.

146. Pediatric Society of New Zealand. Pediatric Society of New Zealand. Available: www.paediatrics.org.nz. Accessed 17 January 2010.

147. Zar HJ, Jeena P, Argent A, Gie R, Madhi SA (2009) Diagnosis and management of community-acquired pneumonia in childhood--South African Thoracic Society Guidelines. S Afr J Emidemiol Infect 24: 977-990.

148. Farrell DJ, Turnidge JD, Bell J, Sader HS, Jones RN (2010) The in vitro evaluation of tigecycline tested against pathogens isolated in eight countries in the Asia-Western Pacific region (2008). J Infect 60: 440-451.

149. Isturiz RE, Luna CM, Ramirez J (2010) Clinical and economic burden of pneumonia among adults in Latin America. Int J Infect Dis 14: e852-e856.

150. Jones RN, Sader HS, Moet GJ, Farrell DJ (2010) Declining antimicrobial susceptibility of Streptococcus pneumoniae in the United States: report from the SENTRY Antimicrobial Surveillance Program (1998-2009). Diagn Microbiol Infect Dis 68: 334-336.

151. The AGREE Next Steps Consortium. Appraisal of Guidelines for Research & Evaluation (AGREE II). Available: http://www.agreetrust.org/index.aspx?o=1397. Accessed 15 August 2011.

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Author Contributions

SC, AL and FVB searched for and selected the source materials, SC and FVB performed the analysis of the guidelines and AL that of the resistance patterns. D.P. provided critical reading and suggestions based on his perception as a General Practitioner. PMT initiated the work, reviewed the data, wrote the Discussion and coordinated the submission. All authors approved the final version of the paper.

Competing Interests

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Table 1. Frequency of isolated pathogens in community-acquired pneumonia in the community setting

Pathogen	**Frequency (%)**	
	from Woodhead (2002) [17] [a]	from Woodhead *et al.* (2011) [18] [b]
No pathogen identified	49.8	22.2 – 63.8
Streptococcus pneumoniae	19.3	0 - 36
Viruses (incl. Influenza)	11.7	2 - 33
Mycoplasma pneumoniae [c]	11.1	0 - 3
Chlamydia pneumoniae [c,d]	8.0	7 - 37
Haemophilus influenzae	3.3	0 - 14
Legionella spp [c]	1.9	0 - 13
Other organisms	1.6	
Chlamydia psittaci [c,d]	1.5	0 - 9
Coxiella burnetii [c]	0.9	0 - 3
Moraxella catarrhalis	0.5	0 - 3
Gram-negative enteric bacteria	0.4	0 - 1
Staphylococcus aureus	0.2	0 - 1

[a] means of 41 studies
[b] range from 17 studies
[c] bacterial agents causing the so-called "atypical pneumonia syndrome" [112] (those agents are often but mistakenly referred to as "atypical organisms"; they are noteworthily not susceptible to β-lactams). Atypical pneumonia syndrome may also be caused by viruses. Differentiation of causative agents in community acquired pneumonia based on clinical examination only is, however, imprecise [113].
[d] Taxonomic analysis [114] has suggested to split the *Chlamydia* genus into *Chlamydia* and *Chlamydophila* genera and to move *C. pneumoniae* and *C. psittac*i into the latter genera, renaming them as *Chlamydophila pneumoniae* and *Chlamydophila psittaci*, respectively. However, most of the current literature (and all guidelines) sill continue to refer to *Chlamydophila* as *Chlamydia*.

Table 2. Classes of antibiotics recommended as first-line or alternative/second line treatment according to current guidelines for initial oral (unless otherwise specified) empiric antibiotic therapy for adult outpatients with community-acquired pneumonia. Key: **+** : first line recommendation; (+) : alternative/second line.

	Class (level 3 [J01] of the ATC classification: http://www.whocc.no)										
Organization [a] (country [b] or region)	β-lactam [c] (J01C or D)	macrolide (J01F)	tetracycl. (J01A)	quinolone [d] (J01M)	strepto-gramin [e] (J01F)	lincosam-ide [f] (J01F)	β-lactam + macrolide	β-lactam + tetracycl.	β-lactam + quinolone	quinolone + macrolide	quinolone+ lincosam.
ERS/ESCMID Europe (EUR)	**+** (+)	(+)	**+**	(+)							
AFSSAPS France (FR)	**+** (+)	**+** (+)		(+)	**+** (+)						
ASP Norway (NO)	**+** (+)	(+)	(+)								
BAPCOC Belgium (BE)	**+** (+)			(+)			(+)			(+)	
BTS Great Britain (GB)	**+**	(+)	(+)								
DSMF/SLD/SYY Finland (FI)	**+**	(+)	(+)	(+)			(+)	(+)			
CIO Italy (IT)		**+**		(+)			(+)				
IRF Denmark (DK)	**+**	(+)									
KEEL Greece (GR)	**+**						(+)		(+)		

OEGI Austria (AT)	**+** (+)	(+)	**+**								
PESC/GRS/GSI/CAP NETZ Germany (DE)	**+** (+)	(+)	(+)	(+)							
RRS/IACMAC [g] Russia (RU)	**+** (+)	(+)	(+)	(+)			(+)				
SEPAR Spain (ES)	(+)	**+**		(+)			(+)				
SILF Sweden (SE)	**+**	(+)	**+**			**+**					
SIGN (Scotland)	**+**	**+**	**+**				**+**				
SPILF French-speaking countries	**+** (+)	**+** (+)		(+)	**+** (+)						
SPP Portugal (PT)		**+**	(+)	(+)			(+)	(+)			
SSI Switzerland (CH)	**+**	(+)	**+**	(+)							
SWAB The Netherlands (NL)	**+** (+)	(+)	**+** (+)								
CIDS/CTS Canada (CA)		**+** (+)	(+)	(+)			(+)				(+)
IDSA/ATS United States (US)	(+)	**+**	**+**	(+)			(+)	(+)			
ALAT Latin America		**+** (+)		(+)							
BTA Brazil (BR)	(+)	**+**		(+)			(+)				
SACAPWG Saudi Arabia (SA)		**+**	(+)	(+)			(+)				

SATS South Africa (ZA)	**+** (+)	(+)		(+)

[a] organizations and source of the corresponding guidelines; each web site or publication was analyzed in 2010. Updates were looked for 2011-2012 and, if found, compared to the previous version for significant changes [see comments]).

ERS/ESCMID: European Respiratory Society/European Society of Clinical Microbiology and Infectious Diseases [115]

Updated in November 2011 [18] with the following significant points: (i) no major change in causative pathogens; (ii) the prevalence of resistance to penicillin and other drugs has considerably complicated the empirical treatment with additional special concern for multi-resistant organsims; (iii) the daily dose of penicillin can be up to 12 g (in 6 administrations) for organisms with an MIC ≤ 8 mg/L; (iv) other recommendations essentially unchanged.

AFSSAPS: Agence Française de Sécurité Sanitaire des Produits de Santé (France) [116]

Updated in July 2010 [117] with the following significant points: (i) telithromycin is maintained (even as first choice in in case of uncertainty about the presence of so-called "atypical" organisms) with a warning of side effects; levofloxacin is the fluroquinoloine of choice whereas moxifloxacin should only be used when no other antibiotic can be used).

ASP: Antibiotikasenteret for primærmedisin (Norway) [118]

BAPCOC: Belgian Antibiotic Policy Coordination Committee (Belgium) [119]

BTS: British Thoracic Society (United Kingdom) [120]

Subject to an audit in 2011 [92] concluding that "efforts should be directed at improving adherence to local CAP guidelines and specific processes of care".

DSMF/SLD/SYY: Duodecim Societas Medicorum Fennica/Suomalaisen Lääkäriseuran Duodecimin/Suomen Lastenlääkäriyhdistyksen/Suomen Yleislääketieteen Yhdistys (Finland) [121]

CIO (SFN): Commissione Controllo Infezioni Ospedaliere (San Filippo Neri) (Italy) [122]

IRF: Institut for Rationel Farmakoterapi (Denmark) [123]

KEEL: Κέντρο Ελέγχου και Πρόληψης Νοσημάτων (Greece) [124]

OEGI: Österreichische Gesellschaft für Infektionskrankheiten und Tropenmedizin (Austria) [125]

PESC/GRS/GSI/CAPNETZ: Paul-Ehrlich Society for Chemotherapy/German Respiratory Society/German Society for Infectiology/Competence Network Community-Acquired Pneumonia KompetenzNETZwerk (Germany) [126]

Updated in 2009 (cefuxroxime axetil has been removed from recommended β-lactams; association of a β-lactam with a macrolide is no longer recommended in outpatients; outpatients with risk factors may receive a fluoroquinolone).

RRS/IACMAC: Russian Respiratory Society/Interregional Association of Clinical Microbiology and Antimicrobial Chemotherapy (Russia) [127]

SEPAR: Sociedad Española de Neumología y Cirugía Torácica (Spain) [128]

SILF: Svenska Infektionsläkarföreningen (Sweden) [129]

Updated in 2011 (with a change of URL [the link to the version used in the analysis is broken; the reference points to the new version]; fluroquinolones are an alternative in patients with 2 points at CURb65 and and with severe [type 1] penicillin allergy)

SIGN: Scottish Intercollegiate Guidelines Network (Scotland) [130]

A consultation for update is ongoing (http://www.sign.ac.uk/pdf/SIGN59_LRTI_review.pdf)

SPILF: Société de Pathologie Infectieuse de Langue Française (France and other French-speaking countries) [131]

Updated in 2010 in a joint guideline with AFSSAPS (see above); the link to the 2005 version used in our analysis is broken; the reference points to the new version)

SPP: Sociedade Portugesa de Pneumologia (Portugal) [132]

SSI: Swiss Society for Infectious Diseases (Switzerland) [133]

SWAB: Stichting Werkgroep AntibioticaBeleid (The Netherlands) [134]

CIDS/CTS: Canadian Infectious Disease Society/Canadian Thoracic Society (Canada) [135]

IDSA/ATS: American Thoracic Society Infectious Diseases Society of America (United States of America) [19]

ALAT: Asociación Latinoamericana del Tórax (Latin America) [136]

BTA: Brazilian Thoracic Association (Brazil) [137]

SACAPWG: Saudi Arabian Community Acquired Pneumonia Working Group (Saudi Arabia) [138]

SATS: South African Thoracic Society [139]

[b] with country ISO 3166-1-alpha-2 code (http://www.iso.org/iso/english_country_names_and_code_elements)

[c] amoxicillin most often cited

[d] levofloxacine or moxifloxacin (ciprofloxacin only in Norway; gemifloxacin is not mentioned in guidelines and is available only in a few countries)

[f] pristinamycin

[g] not included in the quality assessment study

Table 3. Classes of antibiotics recommended as first-line or alternative/second line treatment according to current guidelines for initial oral (unless otherwise specified) empiric antibiotic therapy for paediatric outpatients with community-acquired pneumonia.

Key: **+** : first line recommendation; (+) : alternative/second line.

	Class (level 3 [J01] of the ATC classification: http://www.whocc.no)										
Organization [a] (country [b] or region)	β-lactam [c] (J01C or D)	macrolide (J01F)	quinolone [d] (J01M)	streptogramin [f] (J01F)	cotrimoxazole (J01E)	β-lactam + macrolide	β-lactam + aminoglyc.	β-lactam + / - cotrimox. + / - macrolide + / - aminoglycoside	macrolide + / - cotrimox. + / - aminoglycoside		
WHO World	**+**				**+**						
OEGI Austria (AT)	**+** (+)	**+** (+)	(+)								
BAPCOC Belgium (BE)	**+**	**+**				(+)					
AFSSAPS France (FR)	**+**	**+**		**+**							
BTS Great-Britain (GB)	**+** (+)	**+** (+)									
ASP Norway (NO)	**+**	(+)									
SPP Portugal (PT)	**+**	**+**									

CCHMC United States (US)	**+** (+)		**+** (+)								(+)									
BTA Brazil (BR)	**+**		(+)																	
TPA Taiwan (TW)	**+** (+)										**+** (+)		**+**							
PSNZ New Zealand (NZ)	**+**		**+**																	
SATS South Africa (ZA)																	**+**		(+)	

[a] organizations

WHO: World Health Organization [140]

AFSSAPS: Agence française de sécurité sanitaire et des produits de santé (France) [116]

The link has been broken and the document is no longer available (January 2012)

ASP : Antibiotic Center for Primary Care (Norway) [118]

BAPCOC: Belgian Antibiotic Policy Coordination Committee (Belgium) [119]

BTS: British Thoracic Society (United Kingdom) [141]

KEEL: Κέντρο Ελέγχου και Πρόληψης Νοσημάτων (Greece) [124]

OEGI: Österreichische Gesellschaft für Infektionskrankheiten und Tropenmedizin (Austria) [125]

SPP: Sociedade Portuguesa de Pediatria (Portugal) [142]

CCHMC: Cincinnati Children's Hospital Medical Center (United States) [143]

BTA: Brazilian Thoracic Association (Brazil) [144]

TPA: Taiwan Pediatric Association (Taiwan) [145]

PSNZ: Pediatric Society of New-Zealand (New Zealand) [146]

SATS: South African Thoracic Society (South Africa) [147]

[b] with country ISO 3166-1-alpha-2 code (http://www.iso.org/iso/english_country_names_and_code_elements)

[c] amoxicillin most often cited

[d] this recommendation applies mainly to cystic fibrosis patients and is limited to ciprofloxacin (quiinolones are not registered nor recommended for children).

[f] pristinamycin

Table 4: Frequent or serious side affects associated with the use of antibiotics most frequently cited in the guidelines for non-hospitalized CAP

Class	Drugs within the class	Frequent or serious side effects	Populations at higher risk of side effects
β-lactams	amoxicillin	• Infrequent anaphylactic reactions • *Clostridium difficile*-associated colitis • Digestive tract: diarrhoea, nausea • Hepatic toxicity (infrequent) • CNS: agitation, anxiety, insomnia, confusion, convulsions, behavioural changes, and/or dizziness.	• Allergic patients
	amoxicillin/ clavulanic acid	• Infrequent anaphylactic reactions • *Clostridium difficile*-associated colitis • Hepatic toxicity, including hepatitis and cholestatic jaundice • Digestive tract: diarrhoea, nausea • CNS : agitation, anxiety, insomnia, confusion, convulsions, behavioural changes, and/or dizziness • Vaginitis	• Allergic patients • Erythematous skin rash : patients with mononucleosis • Hepatic toxicity: Patients with hepatic dysfunction • Nephrotoxicity: elderly patients
macrolides	clarithromycin	• Infrequent anaphylactic reactions • *Clostridium difficile*-associated colitis • Drug interactions (CYP450) • Hepatic toxicity, including hepatitis and cholestatic jaundice	• Cardiac effects: patients taking other drugs with effects on QTc or class 1A or III antiarrythmics • Pregnancy • Patients with severe renal impairment with or without coexisting hepatic

	• Palpitations, arrhythmias including prolonged QTc • Digestive tract: diarrhoea, nausea, vomiting, abnormal taste • CNS: headache, confusion, …	impairment • Patients taking drugs metabolized by CYP450
azithromycin	• Infrequent anaphylactic reactions • *Clostridium difficile*-associated colitis • Drug interactions (CYP450), less frequent than with other macrolides • Hepatic toxicity, including hepatitis and cholestatic jaundice • Digestive tract: diarrhoea, nausea, abdominal pain • CNS: dizziness, fatigue, vertigo, … • Genitourinary: nephritis, vaginitis	• Hepatotoxicity: patients with liver failure
telithromycin	• Infrequent anaphylactic reactions and allergic skin reactions • *Clostridium difficile*-associated colitis • Hepatotoxicity • Visual disturbance • Loss of consciousness • Respiratory failure in patients with myastenia gravis • QTc prolongation • Drug interactions (CYP450) • Digestive tract: diarrhoea, nausea, vomiting, dysgueusia	• Cardiac effects: elderly patients taking other drugs with effects on QTc or class 1A or III antiarrythmics, or with known QT prolongation or hypokaliemia • Myopathies : co-administration of statins • Patients with severe renal impairment • Pregnancy • Children (no studies so far)

		• CNS: headache, dizziness	
tetracyclines	doxycycline	• Infrequent anaphylactic reactions and allergic skin reactions • *Clostridium difficile*-associated colitis • Digestive tract: anorexia, glossitis, dysphagia, nausea, vomiting, diarrhoea • esophagitis and esophageal ulcerations • Blood cells: hemolytic anemia, neutropenia, thrombocytopenia, eosinophilia • Hepatotoxicity • Photosensitivity	• Pregnancy, lactation, infants
sulfamides	cotrimoxazole	• Infrequent anaphylactic reactions and allergic skin reactions • *Clostridium difficile*-associated colitis • Blood cells: agranulocytosis, anemia, thrombocytopenia, leukopenia, neutropenia, hypoprothrombinemia, methemoglobinemia, eosinophilia • Hepatitis (including cholestatic jaundice and hepatic necrosis) • Gastrointestinal: pancreatitis, stomatitis, glossitis, nausea, emesis, abdominal pain, diarrhoea, anorexia. • Genitourinary: renal failure, interstitial nephritis • Metabolic and Nutritional: hyperkalemia • CNS: aseptic meningitis, convulsions, peripheral neuritis, ataxia, vertigo, tinnitus, headache. Hallucinations, depression, apathy, nervousness.	• Hypoglycemia : patients with renal dysfunction, liver disease, malnutrition or those receiving high doses of cotrimoxazole • Pregnancy • Hematological changes : elderly patients or in patients with preexisting folic acid deficiency or kidney failure.

		• Musculoskeletal: arthralgia and myalgia. • Respiratory: cough, shortness of breath and pulmonary infiltrates	
fluoroquinolones	levofloxacin	• Infrequent anaphylactic reactions and allergic skin reactions • *Clostridium difficile*-associated colitis • Hematologic toxicity • Hepatotoxicity • Central nervous system effects: headache, insomnia, dizziness, convulsions • Musculoskeletal: tendinopathies • Peripheral neuropathy • Prolongation of the QTc interval and isolated cases of torsade de pointes • Digestive tract: nausea, diarrhoea	• Tendon disorders: elderly, patients taking corticoids, or with kidney, heart or lung transplants • Cardiac effects: elderly patients taking other drugs with effects on QTc or class 1A or III antiarrythmics, or with known QT prolongation or hypokaliemia • CNS effects: patients at risk of epilepsy • Dysglycemia: diabetic patients • Pregnancy, lactation, infants
	moxifloxacin	• Infrequent anaphylactic reactions and allergic skin reactions • *Clostridium difficile*-associated colitis • Musculoskeletal: Tendinopathies • Peripheral neuropathy • Prolongation of the QT interval • Central nervous system effects: headache, insomnia, dizziness, convulsions • Digestive tract: nausea, diarrhoea	• Tendon disorders: elderly, patients taking corticoids, or with kidney, heart or lung transplants • Cardiac effects: elderly patients taking other drugs with effects on QTc or class 1A or III antiarrythmics, or with known QT prolongation or hypokaliemia • CNS effects: patients at risk of epilepsy • Pregnancy, lactation, infants

Table 5: Mean European drug acquisition costs for treatments most frequently cited in the guidelines for non-hospitalized CAP [1]

Treatment	DDD (g) [a]	DDD acquisition cost (€)		Recommended daily dose (RDD) in g [d]		RDD acquisition cost (€) [e]		Treatment duration (days) [b]		Treatment acquisition cost (€)	
		min. [b]	max. [c]	min.	max.	min.	max.	min.	max.	min. [f]	max. [g]
1st line given alone											
amoxicillin	1	0.75	1.14	1.5	3	1.01	3.18	7	14	7.04	44.52
doxycycline	0.1	0.29	1.02	0.2	0.3	0.84	2.59	5	10	4.19	25.92
erythromycin	1	1.33	1.33	1	4	1.37	5.88	7	7	9.59	41.16
clarithromycin	0.5	1.05	2.85	1	1	1.72	3.03	7	10	12.04	30.30
roxithromycin	3	1.94	3.16	0.3	0.6	1.81	5.21	7	10	12.64	52.08
azithromycin	3	1.96	3.36	0.5	0.5	2.59	3.72	3	3	7.76	11.15
clindamycin	1.2	5.12	6.00	0.9	0.9	1.89	2.57	7	7	13.23	17.93
2nd line or combinations											
co-amoxiclav	1	1.08	1.43	1.75	4.0	1.14	5.56	5	7	5.69	38.92
amoxicillin +azithromycin	1/0.3	2.71	4.50	3/0.5	3/0.5	4.60	6.90	10 / 3	10 / 5	27.86	50.38
amoxicillin +clarithromycin	1/0.5	1.80	3.99	3/1	3/1	3.73	6.21	10	10	37.30	62.10
telithromycin	0.8	3.30	3.65	0.8	0.8	2.98	3.49	7	10	20.89	34.88
levofloxacin	0.5	4.41	6.38	0.5	1	2.22	7.54	7	10	15.54	75.40
moxifloxacin	0.4	4.40	5.50	0.4	0.4	4.20	4.84	7	10	29.40	48.44

[1] based on prices observed in Belgium (average European prices) in January 2012; see calculator in Supplementary Material for values used and for imputing other values as needed.

[a] Defined Daily dose, as from the current values published by the WHO Collaborating Centre for Drug Statistics Methodology (http://www.whocc.no/)

[b] usually a generic form

[c] usually the branded product

[d] from the analyzed guidelines (see Table 2)

[e] calculated from the lowest highest retail prices for the corresponding antibiotic

[f] lowest RDD and shortest duration of treatment

[g] highest RDD and longest duration of treatment

* 0.1 g on days 2-5 according to German guidelines

Figure 1

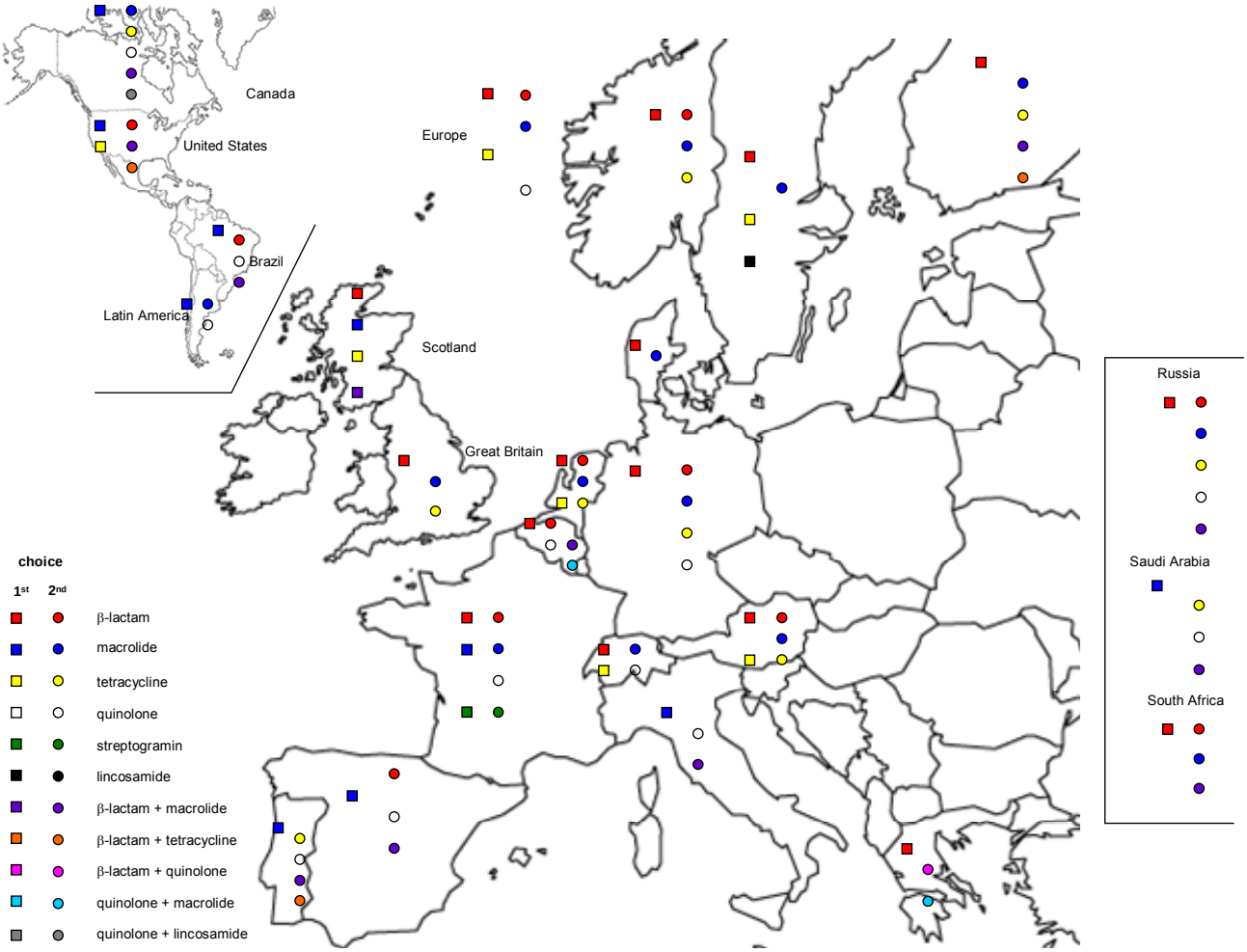

Figure 1: Pictorial representation of the diversity of guidelines for outpatients with CAP in Europe, North and Latin America and in 2 selected countries in Middle-East and Africa.

Figure 2

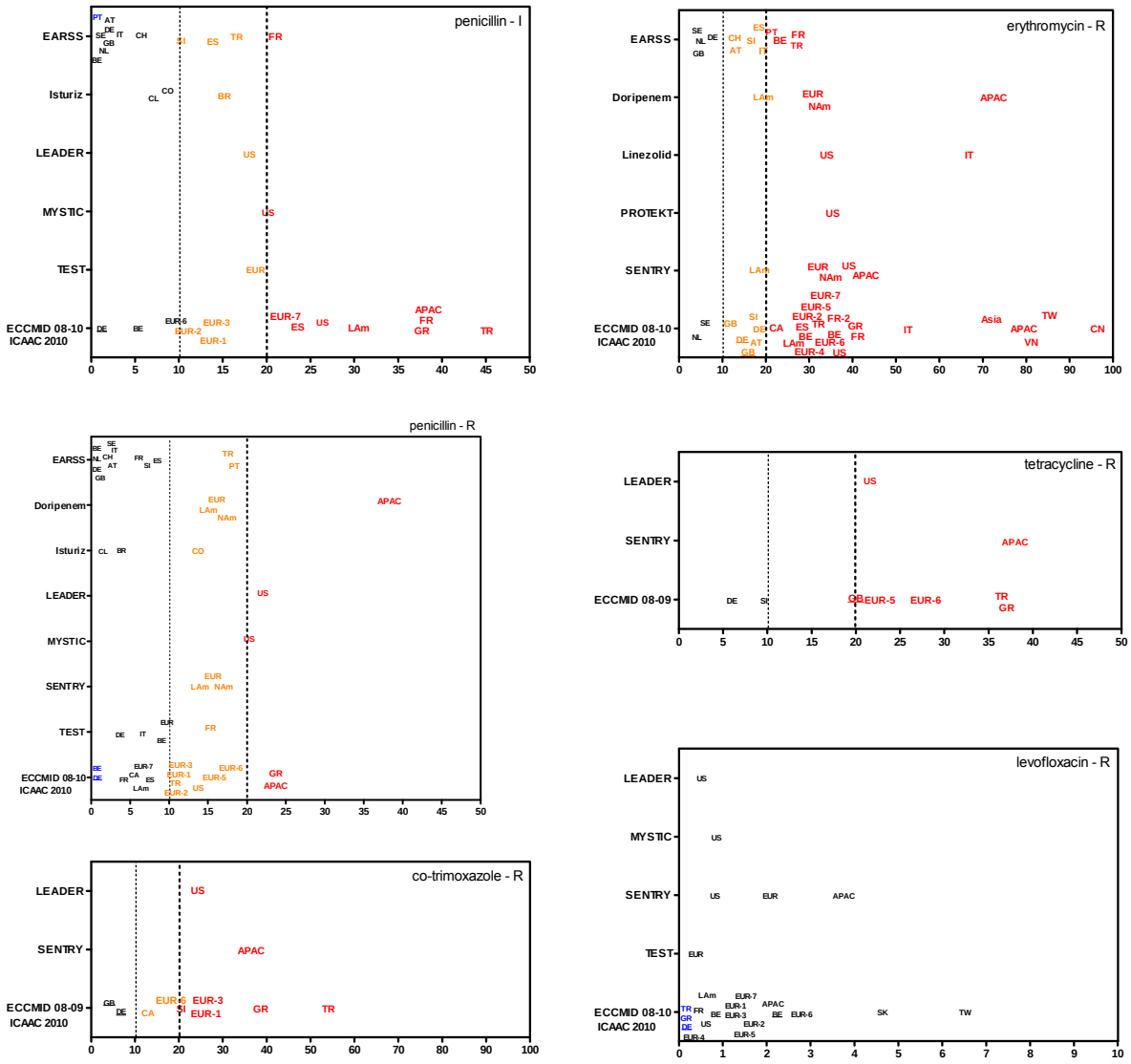

Figure 2: Graphic representation of resistance patterns of *S. pneumoniae* to the antibiotics proposed in the guidelines, based on references [36–38,40–42,148–150] and on CLSI or EUCAST susceptibility breakpoints (see text for further discussion about breakpoints) . The ordinate shows the data base used (EARSS [European Antimicrobial Surveillance system - http://www.ecdc.europa.eu/en/activities/surveillance/EARS-Net/Pages/index.aspx]); PROTEKT [Prospective Resistant Organism Tracking and Epidemiology for the

Ketolide Telithromycin]) [36]; SENTRY [Antimicrobial Surveillance Program] [37,148,150]; MYSTIC [Meropenem Yearly Susceptibility Test Information Collection] [38]; TEST [Tigecyline Evaluation Surveillance Trial] [39], Doripenem [Doripenem surveillance program] [40]; ZAAPS [Zyvox Annual Appraisal of Potency and Spectrum] [41]; LEADER [Linezolid Surveillance Program] [42](Linezolid in the graph refers to data from these two studies); ECCMID08-10: abstracts of the 18th [2008], 19th [2009] and 20th [2010] European Congress of Clinical Microbiology and Infectious Diseases [ECCMID]; ICAAC2010: abstracts from the 50th [2010] Interscience Conference on Antimicrobial Agents and Chemotherapy [ICAAC]); one study by isturiz et al. in South America [149]). The criteria for susceptibility are those used by the authors with specific reference to the breakpoints of either the Clinical and Laboratory Scientific Institute (CLSI - http://www.clsi.org [formerly NCCLS]) or the European Committee for Antibiotic Susceptibility Testing (EUCAST - http://www.eucast.org). For penicillin, data were stratified for intermediate (I) and resistant (R) isolates. Countries are shown by their ISO 3166-1-alpha-2 code (http://www.iso.org/iso/english_country_names_and_code_elements; see Tables 2 and 3). Regions are Europe (EUR; with a number if more than one data base), Latin America (Lam), Asia (Asia), or Asia-Pacific (APAC). Colour code: blue: no resistance reported; black: < 10 % of resistant isolates; orange: 10 to < 20 of resistant isolates; red: ≤ 20 % resistant isolates.

Figure 3

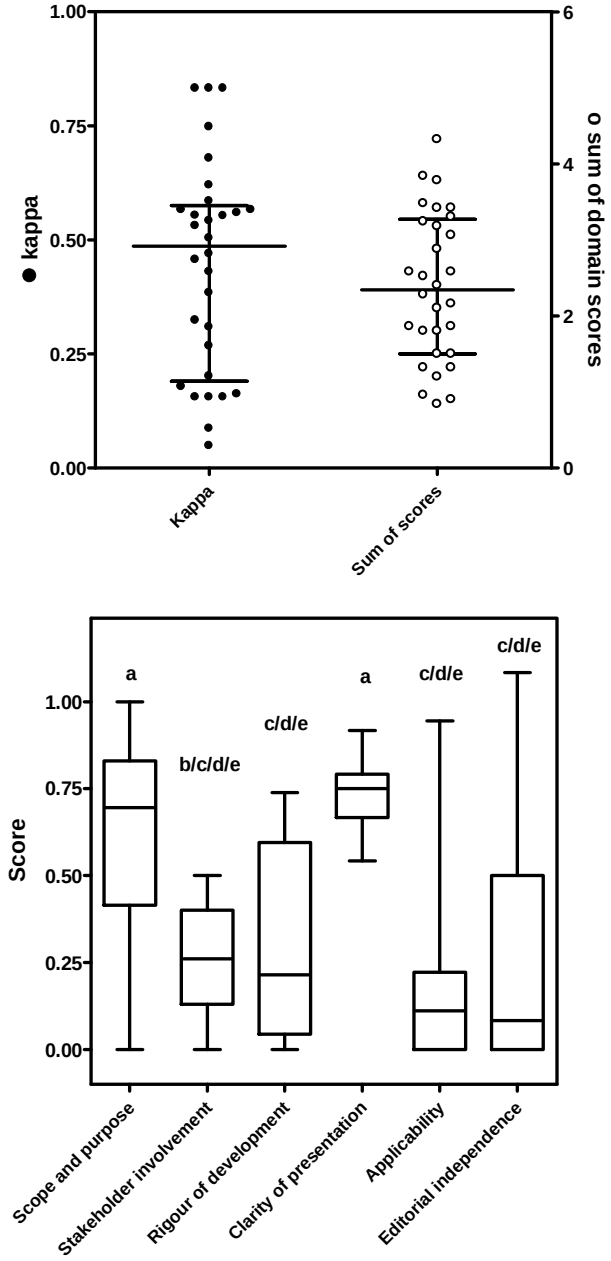

Figure 3: Analysis guidelines (Tables 2 and 3) using the AGREE instrument. Top: agreement 2 independent investigators and sum of mean scores; bottom: score distribution in each domain (extremes, 25-75 percentile, median; statistical analysis

[Kruskal-Wallis test using Dunn's Multiple Comparison]: domains with different letters are significantly different from each other [$p \leq 0.05$]).

Table S1. Current guidelines for initial oral (otherwise specified) empiric antibiotic therapy for adult outpatients with community-acquired pneumonia, issued by international and national organizations, and ordered by main regions (with the corresponding countries by alphabetical order)

Region Organization (Country)	**Year**	**First line treatment** (patient otherwise healthy [<60 y, no specific risk factors])			**Alternative treatment** **(A)** second line for patient otherwise healthy **(B)** first line for patient with risk factors **(C)** second line for patient with risk factors		
		antibiotic class antibiotic	**dosage** (/day)	**days**	**antibiotic class** antibiotic	**dosage** (/day)	**days**
Europe							
ERS/ ESCMID (Europe)	2005	**beta-lactam**[a]			**beta-lactam (A)**		
		amoxicillin	3x875 mg		co-amoxiclav	2x2 g	
		tetracycline[a]			**macrolide (A)**		
		tetracycline	4x250-500 mg		erythromycin	4x500 mg-1 g	
		doxycycline	1x200 mg		clarithromycin	2x500 mg	
					roxithromycin		
					azithromycin	3x500 mg	
					telithromycin[b]	800 mg	
					fluoroquinolone[c] **(A)**		
					levofloxacin	1-2x500 mg	
					moxifloxacin	400 mg	

[a] if no clinically relevant bacterial resistance; [b] for record only (insufficient evidence to make specific recommendations); [c] if clinically relevant pneumococcal resistance against first line antibiotic or hypersensitivity

AFSSAPS (France)	2005	**beta-lactam**			**fluoroquinolone (A[b,e,f], B)**		
		amoxicillin[a,b]	3 g	7-14[c]	levofloxacin (preferred)		
		macrolide[d]			moxifloxacin (only if no other antibiotic)		
		not specified, but not azithromycin			**beta-lactam**		
		telithromycin[b]			amoxicillin[g] **(A)**	3 g	7-14[c]
		streptogramin			co-amoxiclav **(B)**	3 g	
		pristinamycin[b]			ceftriaxone **(B)**		
					macrolide[h] (A)		
					not specified, but not azithromycin		
					telithromycin[f] **(A)**		
					streptogramin		
					pristinamycin[f]		
ASP (Norway)	2008	**beta-lactam**			**tetracycline**		
		penicillin V[a]	4x1300 mg	7-10	doxycycline **(A)** [b]	1x100 mg (200 mg first day)	7-10
					macrolide (A)		

[a] suspicion of *S. pneumoniae*; [b] uncertain etiology; [c] mean:10 days; [d] suspicion of "atypical" pathogens; [e] not recommended unless intolerance or contraindication; [f] if no improvement >48-72 h of initial treatment; [g] instead of a macrolide if no improvement >48-72 h; [h] instead of amoxicillin if no improvement >48-72 h

					erythromycin [b]	(2x500 or 4x250 mg)[c] (2x1000 or 4x500 mg)	7-10
					beta-lactam		
					amoxicillin **(A)**[d]	3x500 mg	7-10

[a] grade of recommendation D; [b] if penicillin allergy or high probability of mycoplasmal or chlamydial pneumonia; [c] enterocapsules; [d] in patients with impaired immune system

BAPCOC (Belgium)	2008	**beta-lactam**			**beta-lactam**		
		amoxicillin	3x1 g	8	amoxicillin + co-amoxiclav **(B)**	3x[500 mg+500 mg]	8
					co-amoxiclav **(C)**	3x875 mg	8
					cefuroxime-axetil[a] **(A,C)**	3x500 mg	
					fluoroquinolone[b] (A,C)		8
					moxifloxacin	1x400 mg	
					initial treatment + macrolide[c] (A,C)		8
					initial treatment + azithromycin	1x500 mg	
					initial treatment + clarithromycin	2x500 mg	3
					initial treatment + roxithromycin	2x150 mg	8

[a] in case of non-IgE mediated allergy to penicillin; [b] in case of IgE mediated allergy to penicillin; [c] in combination with initial antibiotic in case of no improvement within 48 h

BTS (Great Britain)	2009	**beta-lactam**		**macrolide (A)**	
		amoxicillin	3x500 mg	clarithromycin	2x500 mg
				erythromycin	
				tetracycline (A)	
				doxycycline[a]	200 mg (loading dose, then 100 mg)

[a] if intolerance or hypersensitivity to amoxicillin

CIO (Italy)	2004	**macrolide**		**fluoroquinolone (A, B)**	
		clarithromycin	2x500 mg	levofloxacine	1x500-700 mg[a]
		azithromycin	500 mg	moxifloxacine	400 mg
		roxithromycin	2x150 mg	**macrolide+ beta-lactam (C)**	
				clarithromycin+co-amoxiclav	2x500 mg+2x1 g
				azithromycin+co-amoxiclav	500 mg+2x1 g
				roxithromycin+co-amoxiclav	2x150 mg+2x1 g

[a] patient without risk factors

DSMF/ SLD/ SYY (Finland)	2009	**beta-lactam**			**macrolide (A)**		
		amoxicillin[a]	3x1 g		telithromycin[b,c,e]	1x800 mg	
					tetracycline (A)		
					doxycycline[b,d,e]	2x100 mg	
					beta-lactam+macrolide		
					amoxicillin+macrolide[b]		
					beta-lactam+tetracycline		
					amoxicillin+doxycycline[b]		
					fluoroquinolone[f] (A)		
					levofloxacin	1-2x500 mg or 1x750 mg	
					moxifloxacin	1x400 mg	

[a] grade of recommendation A; [b] if mycoplasm or Chlamydia; [c] grade of recommendation B; [d] grade of recommendation C; [e] if penicillin allergy; [f] if the patient has been abroad during the last three months or has already received anti-microbial agents, should be avoided in order to secure performance of fluoroquinolones in other uses

IRF (Denmark)	2003	**beta-lactam**			**macrolide[a] (A)**		
		penicillin V	3x2x10^6	7	roxithromycin	2x150 mg	7

[a] if penicillin allergy

KEEL	2007	**beta-lactam**	4x1 g	7-10	**beta-lactam±macrolide[a]**		

(Greece)					**(A, B)**		
		amoxicillin			amoxicillin±azithromycin		
					amoxicillin±clarithromycin		
					amoxicillin±telithromycin[b]		
					beta-lactam+fluoroquinolone[a] (A, B)		
					amoxicillin±levofloxacin		
					amoxicillin±moxifloxacin		

[a]if prior use of antibiotics in the last trimester (patients without comorbidity); [b]The risk of hepatotoxicity must be weighed against the benefits; not if prior use of antibiotics in the last trimester (patients with comorbidity)

OEGI (Austria)	2008	**beta-lactam**		7-10	**beta-lactam (A)**		
		amoxicillin	3x1 g		co-amoxiclav	3x1 g	
		tetracycline		7-10	cefalexin	3x1 g	
		doxycycline	1x200-300 mg		**macrolide (A)**		
					azithromycin	1x500 mg	3
					clarithromycin	2x500 mg	6-10
					josamycin	2x750 mg	6-10
					roxithromycin	2x300 mg	6-10
PESC/ GRS/ GSI/	2009	**beta-lactam**			**macrolide [b] (B)**		
		amoxicillin	3x1g[a]	5-7	azithromycin	1x500 mg	3
					clarithromycin	2x500 mg	5-7

CAPNETZ (Germany)				roxithromycin	1x300 mg 5-7
				tetracycline	
				doxycycline	1x200 mg[c] 5-7
				beta-lactam (B, C)	
				co-amoxiclav	2x875 mg[d] 5-7
				sultamicillin	2x750 mg 5-7
				fluoroquinolone (B, C)	
				levofloxacin	1x500 mg [e] 5-7
				moxifloxacin	1x400 mg 5-7
				beta-lactam+macrolide[b] (B)	

[a] <70 kg: 3x750 mg; [b] if suspicion of Mycoplasma, Chlamydia or Legionella; [c] <70 kg: 1x100 mg the 2nd and next days; [d] <70 kg: 2x1 g; [e] dosage of 1x750 mg/day (duration: 5 days) exists

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RRS/ IACMAC (Russia)	2006	**beta-lactam**	3x500 mg-1 g	**macrolide (A)**	
		amoxicillin	3x500 mg-1 g	clarithromycin	2x500 mg
		co-amoxiclav	3x625 mg or 2x1 g	azithromycin	1 x 250-500 mg
		amoxicillin/sulbactam	3x1 g	spiramycin	$2x3x10^6$
				fluoroquinolone (A)	
				levofloxacin	
				moxifloxacin	
				gemifloxacin	
				beta-lactam (B)	
				amoxicillin	

					co-amoxiclav		
					amoxicillin/sulbactam		
					tetracycline[a] (A, C)		
					doxycycline		
					beta-lactam+macrolide (C)		

[a] if atypical pathogen

SEPAR (Spain)	2005	**macrolide**			**beta-lactam+macrolide (A)**		
		telithromycin	800 mg	7-10	amoxicillin+azithromycin	3x1 g+500 mg	10[a]
					amoxicillin+clarithromycin	3x1 g+2 x 500 mg	10
					fluoroquinolone[b] (B)		
					moxifloxacin	400 mg	7-10
					levofloxacin	500 mg	7-10
					beta-lactam (C)		
					co-amoxiclav[c]		

[a] 3-5 days for azithromycin ; [b] with comorbidities or recent antibiotherapy; [c] with comorbidities or *H. influenzae*

SILF (Sweden)	2008	**beta-lactam**			**macrolide[b](B)**		
		penicillin V	3x1 g	7	erythromycin[c]	2x500 mg	7

	amoxicillin	3x500 mg[a]	7
	tetracycline		
	doxycycline	1x200 mg (first day, then 1x100 mg)	7
	lincosamide		
	clindamycin	3x300 mg	7

[a] 1 g if reduced penicillin susceptibility; [b] other macrolides possible; [c] if penicillin allergy or atypical agents suspected

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SIGN (Scotland)	2007	**beta-lactam**	
		aminopenicillin	
		macrolide[b,c]	
		tetracycline[b]	
		(beta-lactam+macrolide)[a]	
		aminopenicillin+macrolide	

[a] patients who might normally be referred to hospital, but for various reasons are managed in the community; [b] if consideration of *M. pneumoniae* or diagnosis of Chlamydial pneumonia; [c] also without consideration of *M. pneumoniae* or diagnosis of Chlamydial pneumonia

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SPILF (France and some French-speaking countries)	2006	**beta-lactam**		**beta-lactam(B)**	
		amoxicillin	3x1 g	co-amoxiclav	3x1 g
		streptogramin		ceftriaxone[c]	1 g
		pristinamycin[a]	3x1 g	**fluoroquinolone**[d] **(A, B)**	
		macrolide		levofloxacin	500 mg

		telithromycin[a]	800 mg	moxifloxacin	400 mg
				macrolide (A)	
				macrolide[b], not specified but not erythromycin nor azithromycin)	
				telithromycin[d]	800 mg
				streptogramin (A)	
				pristinamycin[d]	3x1 g

[a] if suspicion of an "atypical" pathogen; [b] if amoxicillin failure; [c] im, iv or sc; [d] also if beta-lactams contra-indicated

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SPP (Portugal)	2003	**macrolide[a]**		**tetracycline (A)**	
		erythromycin		doxycycline	
		clarithromycin		**fluoroquinolone[b] (A, B)**	
		azithromycin		levofloxacin	
				moxifloxacin	
				beta-lactam+macrolide (B)	
				amoxicillin	3x1 g
				co-amoxiclav	3x(875/125) mg
				ceftriaxone	
				+	
				erythromycin	
				clarithromycin	
				azithromycin	

					beta-lactam+tetracycline (C)	
					beta-lactam+doxycycline	

[a] azithromycin and clarithromycin favored on erythromycin due to convenient dosage and fewer side effects ; [b] to use with caution, if recent therapy with a new quinolone

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SSI (Switzerland)	2006	**beta-lactam**			**macrolide (A)**	
		co-amoxiclav	3x625 mg	[a]	clarithromycin	2x500 mg
					azithromycin	1x500 mg
		tetracycline			**fluoroquinolone (A)**	
		doxycycline	2x100 mg		levofloxacin	1-2x500 mg
					moxifloxacin	400 mg

[a] until patient is afebrile for 3-5 days

SSI (Switzerland)	2006	**beta-lactam**			**macrolide (A)**	
		co-amoxiclav	3x625 mg	[a]	clarithromycin	2x500 mg
					azithromycin	1x500 mg
		tetracycline			**fluoroquinolone (A)**	
		doxycycline	2x100 mg		levofloxacin	1-2x500 mg
					moxifloxacin	400 mg

[a] until patient is afebrile for 3-5 days

SWAB (the Netherlands)	2005	**beta-lactam**		**macrolide[a,b] (A)**	
		amoxicillin	3-4x500-750 mg	**tetracycline (A)**	
		tetracycline		doxycycline[b]	100 mg (loading dose of 200 mg)
		doxycycline	100 mg (loading dose of 200 mg)	**beta-lactam**	
				feneticillin	4x500 mg

[a] if penicillin allergy or if use of doxycycline not possible due to pregnancy or lactation; [b] if no improvement with amoxicillin within 48 h

Americas

CIDS/CTS (Canada)	2000	**macrolide**	**tetracycline (A, C[a])**
		erythromycin	doxycycline
		clarithromycin	**macrolide (B)[a]**
		azithromycin	clarithromycin
			azithromycin
			fluoroquinolone(B)[b]
			levofloxacin
			gatifloxacin
			moxifloxacin
			trovafloxacin[c]

				beta-lactam+macrolide[b,d]		
				co-amoxiclav+macrolide (B, C)		
				2nd generation cephalosporin+macrolide (C)		
				fluoroquinolone+lincosamide or metronidazole (C)[d]		
				levofloxacin+clindamycin or metronidazole		
				other fluoroquinolone, notspecified+clindamycin or metronidazole		

[a]COLD with no recent antibiotics nor po steroids within past 3 mo; [b] COLD with recent antibiotics or po steroids within past 3 mo, H. influenzae and enteric gram- rods implicated; [c] restricted; [d] suspected macroaspiration (oral anaerobes)

IDSA/ATS (North America)	2007	**macrolide**[a]	5[c]	**fluoroquinolone**[d,e] **(B)**		5[c]
		azithromycin		moxifloxacin		
		clarithromycin		gemifloxacin		
		erythromycin		levofloxacin	750 mg	
		tetracycline[b]	5[c]	**beta-lactam+macrolide**[d,e]		5[c]
		doxycycline		amoxicillin+macrolide **(B)**	3x1 g - nd	
				co-amoxiclav+macrolide **(B)**	2x2 g - nd	
				ceftriaxone+macrolide **(C)**		
				cefpodoxime+macrolide **(C)**		

					cefuroxime+macrolide **(C)**	2x500 mg - nd	
					beta-lactam+tetracycline (C)		
					amoxicillin+doxycycline	3x1 g - nd	
					co-amoxiclav+doxycycline		
					ceftriaxone+doxycycline		
					other class of antibiotics, nd[f] (C)		
					antibiotic, nd[g]		

[a] no use of antimicrobials within the previous 3 months; [b] weak recommendation; [c] minimum duration and if afebrile for 48-72 h and no CAP-associated sign of clinical instability; [d] if comorbidities and/or use of antimicrobials within the previous 3 months; [e] in any patient, including those without comorbidities, in regions with a high rate of infection with high-level macrolide-resistant *S. pneumoniae* (but moderately recommended); [f] if use of antimicrobials within the previous 3 months; [g] antimicrobial other than that used within the previous 3 months

ALAT (Latin America)	2004	**macrolide**			**fluoroquinolone (B)**		
		azithromycin	500 mg first day, then 250 mg	5	moxifloxacin	400 mg	7-10
		clarithromycin	2x500 mg	7-14	gatifloxacin	400 mg	7-10
					levofloxacin	2x500 mg	7-10
					macrolide (B)		
					telithromycin	800 mg	7-10

BTA (Brazil)	2007	**macrolide**			**beta-lactam (A)**		
		azithromycin	1x500 mg[a]	3	amoxicillin	3x500 mg	7
		clarithromycin	1-2x500 mg	7	**fluoroquinolone (B)**		
					levofloxacin	500 mg	
					moxifloxacin	400 mg	
					beta-lactam+macrolide (B)		
					beta-lactam+azithromycin	1x500 mg[b]	3

[a] or 500 mg first day, then 250 mg for 4 days, [b] dose for azithromycin

Middle East

SACAPWG (Saudi Arabia)	2002	**macrolide**			**tetracycline (A)**		
		clarithromycin			doxycycline		
		azithromycin			**beta-lactam±macrolide**		
		roxithromycin			cefuroxime±macrolide **(B)**		
					cefaclor±macrolide **(B)**		
					cefprozil±macrolide **(B)**		
					co-amoxiclav+macrolide **(C)**		
					ampicillin/sulbactam+macrolide **(C)**		
					fluoroquinolone (C)		
					moxifloxacin		

					levofloxacin		
					gatifloxacin		
					gemifloxacin		
Africa							
SATS (South Africa)	2007	**beta-lactam combination**			**beta-lactam+macrolide (A)**		-10
					amoxicillin+erythromycin[b]	4x500 mg	5
		penicillin G[a] im+amoxicillin	2x10^6 U/3x1 g	10	**macrolide (A)**		
					erythromycin[c]	4x500 mg	5
				7-10	**beta-lactam (B)** [d]		
					co-amoxiclav+amoxicillin	3x(375/500) mg	5-10

[a] loading dose; [b] if no response to treatment after 48 h; [c] in penicillin-allergic patients; [d] if comorbidities or >65 y

ERS/ESCMID: European Respiratory Society/European Society of Clinical Microbiology and Infectious Diseases;

AFSSAPS: Agence Française de Sécurité Sanitaire des Produits de Santé (France);

ALAT: Asociación Latinoamericana del Tórax (Latin America);

ASP: Antibiotikasenteret for primærmedisin (Norway);

BAPCOC: Belgian Antibiotic Policy Coordination Committee (Belgium);

BTA: Brazilian Thoracic Association (Brazil);

BTS: British Thoracic Society (United Kingdom);

CIDS/CTS: Canadian Infectious Disease Society/Canadian Thoracic Society (Canada);

DSMF/SLD/SYY: Duodecim Societas Medicorum Fennica/Suomalaisen Lääkäriseuran Duodecimin/Suomen Lastenlääkäriyhdistyksen/Suomen Yleislääketieteen Yhdistys (Finland);

CIO (SFN): Commissione Controllo Infezioni Ospedaliere (San Filippo Neri) (Italy);

IDSA/ATS: American Thoracic Society Infectious Diseases Society of America (North America);

IRF: Institut for Rationel Farmakoterapi (Denmark);

KEEL: Κέντρο Ελέγχου και Πρόληψης Νοσημάτων (Greece);

OEGI: Österreichische Gesellschaft für Infektionskrankheiten (Austria);

PESC/GRS/GSI/CAPNETZ: Paul-Ehrlich Society for Chemotherapy/German Respiratory Society/German Society for Infectiology/Competence Network Community-Acquired Pneumonia KompetenzNETZwerk (Germany);

RRS/IACMAC: Russian Respiratory Society/Interregional Association of Clinical Microbiology and Antimicrobial Chemotherapy (Russia);

SACAPWG: Saudi Arabian Community Acquired Pneumonia Working Group (Saudi Arabia);

SATS: South African Thoracic Society (South Africa);

SEPAR: Sociedad Española de Neumología y Cirugía Torácica (Spain);

SPILF: Société de Pathologie Infectieuse de Langue Française (France and other French-speaking countries);

SIGN: Scottish Intercollegiate Guidelines Network (Scotland);

SPP: Sociedade Portugesa de Pneumologia (Portugal) ;

SSI: Swiss Society for Infectious Diseases (Switzerland);

SILF: Svenska Infektionsläkarföreningen (Sweden);

SWAB: Stichting Werkgroep AntibioticaBeleid (The Netherlands);

Table SP2. Current guidelines for initial empiric antibiotic therapy for pediatric outpatients with community-acquired pneumonia, issued by international and national organizations

Organization	Year	First line treatment			Alternative treatment		
		antibiotic class antibiotic	**dosage** (/day)	**days**	**antibiotic class** antibiotic	**dosage** (/day)	**days**
WHO	2005	**sulfonamide/trimethoprim**					
		co-trimoxazole	2x4 mg/kg / 2x20 mg/kg	3			
		beta-lactam					
		amoxicillin	2x25 mg/kg	3			
AFSSAPS (France)	2005	**beta-lactam**					
		amoxicillin[a]	80-100 mg/kg in 3 times[b]	10			
		3rd generation cephalosporin[c]	nd[d]				
		macrolide		14			
		streptogramin					
		pristinamycin[e]	50mg/kg in 2-3 times				

[a]>3 yrs; [b]>10 yrs: not more than 3 g; [c]<3 yrs if allergy to beta-lactam; [d] im injection; [e]>6 yrs if allergy to beta-lactam

ASP (Norway)	2008	**beta-lactam**			**macrolide**		
		penicillin V[a]	15 mg/kg	7-10	erythromycin[b]	<25 kg: 2x20 or 4x10 mg/kg 25-35 kg: 2x 250 mg[c]	7-10

[a] grade of recommendation D; [b] if penicillin allergy or high probability of mycoplasmal or chlamydial pneumonia; [c] enterocapsules

BAPCOC (Belgium)	2008	**beta-lactam**			**beta-lactam+macrolide**[c]		
		amoxicillin	75-100 mg/kg in 3-4 times	5-7	amoxicillin+azithromycin		
		cefuroxime-axetil[a]	30-50 mg/kg in 3 times	5-7	amoxicillin+clarithromycin		
		macrolide[b]					
		azithromycin	10 mg/kg (first day), then 5 mg/kg	5			
		clarithromycin	15 mg/kg in 2 times	5-7			

[a]if non IgE-mediated allergy ; [b] if >5 yrs with high probability of atypic pneumonia; [c] if treatment with amoxicillin and no improvement>48 h and no pleural effusion

BTS	2002	**beta-lactam**			**beta-lactam**		

(Great Britain)	amoxicillin	1 m-2 y: 3x125 mg or 3x8 mg/kg[c]	7-10[a]	co-amoxiclav	0-1 y: 3x0.266 ml/kg[c]	7-10[a]
		2-12 y: 3x125-250 mg or 3x8 mg/kg[c]	7-10[a]		1-6 y: 3x5ml (125/31 suspension)[c]	7-10[a]
		12-18 y: 3x500 mg[c]	7-10[a]		7-12 y: 3x5 ml (250/62 suspension)[c]	7-10[a]
					12-18 y: 1 tablet (250/125)[c]	7-10[a]
	macrolide[b]			cefaclor		
	erythromycin	0-1 m: 3x10-15 mg/kg[c]	7-10[a]	**macrolide**		
		1 m-2 y: 4x125 mg[c]		erythromycin	[e]	
		2-8 y: 4x250 mg[c]		clarithromycin	[e]	
		9-18 y: 4x500 mg[c]		azithromycin	[e]	
	clarithromycin	0-1 y: 2x7.5 mg/kg	7-10[a]			
		1-2 y: 2x62.5 mg	7-10[a]			
		3-6 y: 2x125 mg/kg	7-10[a]			
		7-9 y: 2x187.5 mg	7-10[a]			
		10-18 y: 2x250 mg	7-10[a]			

	azithromycin	6 m-2 y: 1x10 mg/kg	5
		3-7 y: 1x200 mg	5
		8-11 y: 1x300 mg	5
		12-14 y: 1x400 mg	5
		>14 y: 1x500 mg	5
	beta-lactam combination		
	amoxicillin+flucloxacillin[b]	[d]	

[a] may need up to 14 days depending on clinical response; [b] if suspicion of *M. pneumoniae*, *C. pneumoniae* or *S. aureus*;[c] doses may be doubled in severe infections; [d] amoxicillin: same doses as for amoxicillin alone, flucloxacillin: not specified; [e] same dose as for first line therapy

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KEEL (Greece)	2007	< 5 y:		
		beta-lactam		7-10
		amoxicillin	90-100 mg/kg	
		co-amoxiclav	90 mg/kg	
		cefuroxime	30 mg/kg	
		> 5 y:		
		macrolide		
		azithromycin	10 mg/kg (1 dose)	3-5
		clarithromycin	15-30 mg/kg	7-10
		beta-lactam		7-10
		penicillin	100 000 IU/kg	
		amoxicillin		
		beta-lactam±macrolide		7-10
		2nd generation		

		cephalosporin±macrolide		
OEGI (Austria)	2008	**beta-lactam** amoxicillin[b] **macrolide**[c]		**beta-lactam** cephalosporin[d] **macrolide**[d] **fluoroquinolone**[e]
SPP (Portugal)	2007	**macrolide**		7-10
		erythromycin[a]	40 mg/kg in 4 times	
		clarithromycin	15 mg/kg in 2 times	
		azithromycin	1x10 mg 1st day, 1x5 mg next 4 days	
		beta-lactam		7-10
		amoxicillin[b]	80-100 mg/kg in 2 times	
		ampicillin[b]	150 – 200 mg/kg in 4 times	
		flucloxacillin[b]	50 mg/kg in 3 times	

[a]This guideline concerns children<or=14 yrs; [b] until 5 yrs; [c] from 5 yrs; [d] if 3 mo to 5 yrs; [e] reserve antibiotics with specific indications until 8 yrs

Children <3 mo require hospitalization. [a] suspicion of *M. pneumoniae* in child up to 5 y; [b] suspicion of *S. pneumoniae* if child>5 y

Cincinnati Children's Hospital Medical Center (US)	2006	**beta-lactam**			**beta-lactam**		
		amoxicillin[a]	2x40-45 mg/kg or 3x25-30 mg/kg	7-10	cefprozil	2x15 mg/kg	
					ceftriaxone[c]	1x50 mg/kg	
					cefuroxime	2x15 mg/kg	
		macrolide[b]			**macrolide**		
		azithromycin	1x10 mg/kg (1 d, then 1x5 mg/kg)	5	clarithromycin	2x7.5 mg/kg	
					macrolide+beta-lactam		7-10[d]
					macrolide+amoxicillin[e,f]		
					macrolide+ceftriaxone[f]		

This guideline concerns children aged 60 d-17 y.[a] <5 y, likely bacterial cause; [b] 5+ y or <5 y if allergy to penicillin; [c] im single initial dose to be considered prior to starting oral antibiotics if child unable to tolerate liquids; [d] 5 d if azithromycin; [e] if *M. pneumoniae* or *C. pneumoniae* is suspected >24-48 h; [f] if more severe disease

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BTA (Brazil)	2007	**beta-lactam**			**macrolide**		
		amoxicillin	50 mg/kg in 3 times	nd[a]	erythromycin	30-40 mg/kg	nd[a]
		penicillin G/procaine[b]	50 000 U/kg in 1-2 times	nd[a]			

[a] 3-5 d treatment after symptom resolution necessary

Taiwan Pediatric Association (Taiwan Working Group for "Guideline on the management of CAP in children")	2008	<1 m:		7-10	7-10
		beta-lactam+aminoglycoside			**beta-lactam combination**
		ampicillin+aminoglycoside			ampicillin+cefotaxime
					ampicillin+ceftriaxone
					beta-lactam combination (+macrolide[a])
					ampicillin+cefotaxime (+macrolide)
					ampicillin+ceftriaxone (+macrolide)
		2m-1y:		7-10	7-10
		beta-lactam			**beta-lactam (+macrolide[a])**
		penicillin	4-6x300 000-400 000 U/kg		2nd generation cephalosporin (+macrolide)
		ampicillin	4x150-200 mg/kg		cefotaxime (+macrolide)
		beta-lactam/beta-lactamase inhibitor			ceftriaxone (+macrolide)
		co-amoxiclav	2-3x80-90 mg(AMX)/kg		
		ampicillin/sulbactam	3-4x150-200 mg(AMP)/kg		
		2-5y:		7-10	7-10
		beta-lactam±macrolide			**beta-lactam**
		penicillin±macrolide			2nd generation cephalosporin

		ampicillin±macrolide			cefotaxime	4x150-200 mg/kg
		beta-lactam/beta-lactamase inhibitor±macrolide			ceftriaxone	1-2x100 mg/kg
		co-amoxiclav±macrolide				
		ampicillin/sulbactam±macrolide				
		6-18y:		7-10		7-10
		beta-lactam±macrolide			**beta-lactam/beta-lactamase inhibitor**	
		penicillin±macrolide			co-amoxiclav	2-3x80-90 mg(AMX)/kg
					ampicillin/sulbactam	3-4x150-200 mg(AMP)/kg
					beta-lactam	
					2nd or 3rd generation cephalosporin	
PSNZ (New Zealand)	2005	**beta-lactam**				
		amoxicillin	3x15-30 mg/kg/dose	3-5		
		macrolide				

[a] when *C. trachomatis* infection is considered

		erythromycin[a]	4x10 mg/kg/dose	10-14[b]			

This guideline concerns children >1 mo and <1 y; [a] if suspicion of Chlamydia or pertussis, or allergy to penicillin; [b] 5-7 if allergy to penicillin

SATS (South Africa)	2009	3 m-5 y					
		beta-lactam (±cotrimoxazole [a,e] ±macrolide [c] ±aminoglycoside [d])					
		amoxicillin	3x(15-)30mg/kg	5			
		5-12 y					
		beta-lactam (±cotrimoxazole [a,b] ±macrolide [c] ±aminoglycoside [d])			**macrolide [e] (A) (±cotrimoxazole [a,b] ±aminoglycoside [d]) (B)**		
		amoxicillin	3x(15-)30mg/kg	5	erythromycin	4x10mg/kg	10
					clarithromycin	2x15mg/kg	3-5
					azithromycin	15mg/kg 1st d, then 7.5mg/kg	3-5

[a] if *P. jiroveci* pneumonia suspected in HIV child or HIV-exposed child <1y, **[b]** to be considered in addition to amoxicillin and an aminoglycoside for older HIV-infected children with features of AIDS who are not on co-trimoxazole prophylaxis; **[c]** if *C .trachomatis* suspected; **[d]** if high risk of being HIV-infected or with symptomatic HIV disease or severely malnourished (or can be covered with an alternative regimen that provides adequate effective treatment against gram-); **[e]** if *M. pneumoniae* or *C. spp.* suspected

WHO: World Health Organization;

BTA: Brazilian Thoracic Association (Brazil);

AFSSAPS: Agence Française de Sécurité Sanitaire des Produits de Santé (France);

BAPCOC: Belgian Antibiotic Policy Coordination Committee (Belgium);

BTS: British Thoracic Society (Great Britain);

PSNZ: Paediatric Society of New Zealand (New Zealand)

OEGI: Österreichische Gesellschaft für Infektionskrankheiten (Austria);

SATS: South African Thoracic Society (South Africa);

SPP: Sociedade Portugesa de Pediatria (Portugal);

Figure SP1: AGREE evaluation schemes (from ref. [151])

Original AGREE Item	AGREE II Item
Domain 1. Scope and Purpose	
1. The overall objective(s) of the guideline is (are) specifically described.	No change
2. The clinical question(s) covered by the guideline is (are) specifically described.	The health question(s) covered by the guideline is (are) specifically described.
3. The patients to whom the guideline is meant to apply are specifically described.	The population (patients, public, etc.) to whom the guideline is meant to apply is specifically described.
Domain 2. Stakeholder Involvement	
4. The guideline development group includes individuals from all the relevant professional groups.	No change
5. The patients' views and preferences have been sought.	The views and preferences of the target population (patients, public, etc.) have been sought.
6. The target users of the guideline are clearly defined.	No change
7. The guideline has been piloted among end users.	Delete item. Incorporated into user guide description of item 19.
Domain 3. Rigour of Development	
8. Systematic methods were used to search for evidence.	No change in item. Renumber to 7.
9. The criteria for selecting the evidence are clearly described.	No change in item. Renumber to 8.
	NEW Item 9. The strengths and limitations of the body of evidence are clearly described.
10. The methods for formulating the recommendations are clearly described.	No change
11. The health benefits, side effects, and risks have been considered in formulating the recommendations.	No change

see continuation on next page

Original AGREE Item	AGREE II Item
12. There is an explicit link between the recommendations and the supporting evidence.	No change
13. The guideline has been externally reviewed by experts prior to its publication.	No change
14. A procedure for updating the guideline is provided.	No change
Domain 4. Clarity of Presentation	
15. The recommendations are specific and unambiguous.	No change
16. The different options for management of the condition are clearly presented.	The different options for management of the condition or health issue are clearly presented.
17. Key recommendations are easily identifiable.	No change
Domain 5. Applicability	
18. The guideline is supported with tools for application.	The guideline provides advice and/or tools on how the recommendations can be put into practice. AND Change in domain (from Clarity of Presentation) AND renumber to 19
19. The potential organizational barriers in applying the recommendations have been discussed.	The guideline describes facilitators and barriers to its application. AND change in order – renumber to 18
20. The potential cost implications of applying the recommendations have been considered.	The potential resource implications of applying the recommendations have been considered.
21. The guideline presents key review criteria for monitoring and/ or audit purposes.	The guideline presents monitoring and/ or auditing criteria.
Domain 6. Editorial Independence	
22. The guideline is editorially independent from the funding body.	The views of the funding body have not influenced the content of the guideline.
23. Conflicts of interest of guideline development members have been recorded.	Competing interests of guideline development group members have been recorded and addressed.

The original AGREE instrument was used in the first steps of our analysis. Changes introduced with the updated version (AGREE II [46]) only slightly affected our evaluation grid since (i) many items [1-6, 8-16, 17 and 20] were either unchanged or with changes that were not of primary concern for our analysis), (ii) the new formulations of items 19, 20, 21 and 23 essentially adressed similar aspects in the guidelines than the original ones. The most important change (see comment in [94]) was the introduction of the additional item 9 in domain 3 ("*The strengths and limitations of the body of evidence are clearly defined*") but this concept was fully taken into account by the evaluators when scoring the other items of this domain (and especially the item "*There is an explicit link between the recommendations and the supporting evidence*").

3.2. Focus on fluoroquinolone resistance by efflux in *Streptococcus pneumoniae*

In *Streptococcus pneumoniae*, two main types of transporters for fluoroquinolones were described at the time of this study, namely PmrA and PatA-PatB. Yet, their impact on susceptibility to different fluoroquinolones, was unclear, as well as their implication in resistance in clinical isolates, and the regulation of their expression.

3.2.a. Efflux and resistance in clinical isolates

We examined the prevalence of efflux in part of our clinical collection by phenotypic approaches (determination of MICs in the absence or in the presence of the efflux pump inhibitor reserpine) and compared the impact of this resistance mechanisms on different fluoroquinolones used either as markers of efflux (norfloxacin, ciprofloxacin), or because they are or have been used in pneumococcal infections (at least in some countries).

> Article: Efflux of novel quinolones in contemporary *Streptococcus pneumoniae* isolates from community-acquired pneumonia.
> Ann Lismond, Sylviane Carbonnelle, Paul M. Tulkens and Françoise Van Bambeke

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Efflux of novel quinolones in contemporary *Streptococcus pneumoniae* isolates from community-acquired pneumonia

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Keywords: gemifloxacin, ciprofloxacin, levofloxacin, moxifloxacin, garenoxacin, reserpine

Sir,

Quinolones with enhanced activity against *Streptococcus pneumoniae* are included as a treatment option for community-acquired pneumonia in therapeutic guidelines from both North America and Europe,[1,2] and epidemiological surveys show that resistance to levofloxacin or moxifloxacin remains low even with large usage of these antibiotics.[3] Yet, *S. pneumoniae* harbours efflux transporters for quinolones[4,5] that may reduce the susceptibility of clinical isolates in a manner that will remain undetected if reporting is based only on the interpretative criteria proposed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) or the US CLSI. While efflux in *S. pneumoniae* seems to primarily affect ciprofloxacin and norfloxacin (which are not recommended for treating infections caused by *S. pneumoniae*), much less is known about the susceptibility of novel quinolones to these transporters in current clinical isolates.

In the present study, we collected 183 non-duplicate isolates from patients with confirmed clinical and radiological diagnosis of community-acquired pneumonia during the 2007–09 period. We measured the MICs of ciprofloxacin, levofloxacin, moxifloxacin and the two new quinolones garenoxacin and gemifloxacin for these isolates. We followed exactly the CLSI methodology except that we used 0.5 $\log_2$ concentration increments to reduce the intrinsic 1 $\log_2$ dilution error associated with the conventional methods of MIC determinations, and performed the determinations in the presence or absence of reserpine (10 mg/L; commonly used to detect the efflux-mediated decrease in susceptibility of *S. pneumoniae* to quinolones).[6] The results are shown in the left-hand panels of Figure 1. In the absence of reserpine, median MICs were 1 mg/L of ciprofloxacin, 0.75 mg/L of levofloxacin, 0.125 mg/L of moxifloxacin, 0.047 mg/L of garenoxacin and 0.012 mg/L of gemifloxacin [see Table S1, available as Supplementary data at *JAC* Online, for more numerical data (MIC range, MIC_{50} and MIC_{90})]. All strains should be considered as susceptible to levofloxacin and moxifloxacin (using either the EUCAST or CLSI breakpoints) and also to gemifloxacin for 181/183 strains (using the CLSI breakpoint; no EUCAST breakpoint defined). In the presence of reserpine, the MIC distributions of ciprofloxacin, garenoxacin and gemifloxacin were markedly shifted towards lower values, with median values lowered by 1 $\log_2$ dilution for ciprofloxacin and gemifloxacin, and 0.5 $\log_2$ dilution for garenoxacin. In contrast, only minor shifts in distribution were seen for levofloxacin and moxifloxacin. To get further insight into the impact of efflux on the decrease in bacterial susceptibility to each quinolone, we calculated the MIC change for each isolate (by decrements of 0.5 $\log_2$ dilutions) and present the results as a function of the original MIC (without reserpine) in the right-hand panels of Figure 1. For ciprofloxacin, 93.4% of the strains had an MIC $\geq$ 0.75 mg/L, with 29.2% of these showing a difference of more than 1 $\log_2$ dilution upon exposure to reserpine. For gemifloxacin, reserpine caused an increase in susceptibility of $\geq$1 $\log_2$ dilution in 65% of the isolates with a basal MIC (in the absence of reserpine) $\geq$0.006 mg/L. For garenoxacin, the susceptibility of 60% of the isolates was increased in the presence of reserpine (this was seen whatever the basal MIC), but the effect rarely exceeded 1 $\log_2$ dilution. For moxifloxacin and levofloxacin, increases in susceptibility were seen for 39% and 45% of the isolates, respectively, but affecting mainly the strains with a corresponding basal MIC $\geq$0.188 mg/L (moxifloxacin) or $\geq$0.75 mg/L (levofloxacin). The shift was $<$1 $\log_2$ dilution in 59% of the isolates for moxifloxacin and in 86% for levofloxacin.

The data strongly suggest that gemifloxacin and ciprofloxacin are both subject to efflux in *S. pneumoniae*. Of interest is the fact that gemifloxacin has so far not been used in Europe and could, therefore, not have triggered its own efflux. Ciprofloxacin has never been included in therapeutic recommendations for treatment of streptococcal infections in Belgium. We may suspect that it is its wide use for other indications that has triggered the emergence of *S. pneumoniae* strains capable of developing efflux-mediated resistance to ciprofloxacin through repeated exposure to subinhibitory concentrations of this antibiotic.[6] It is ironic that this affects gemifloxacin, a not-yet-used but potentially very active antibiotic, even though not all isolates were positive in our assay. Since efflux is known to facilitate the selection of first-step mutants amongst fluoroquinolone-susceptible organisms, our data must be taken as a warning should gemifloxacin be introduced on a wide scale in therapeutics. In a more general context, and based on the observation that strains with efflux may be quite frequent, surveillance studies for the detection of new variants of efflux transporters affecting levofloxacin and moxifloxacin may be warranted. This could have a direct clinical significance if those strains, as recently suggested,[5] were also to show mutations or other low-level mechanism(s) of resistance.

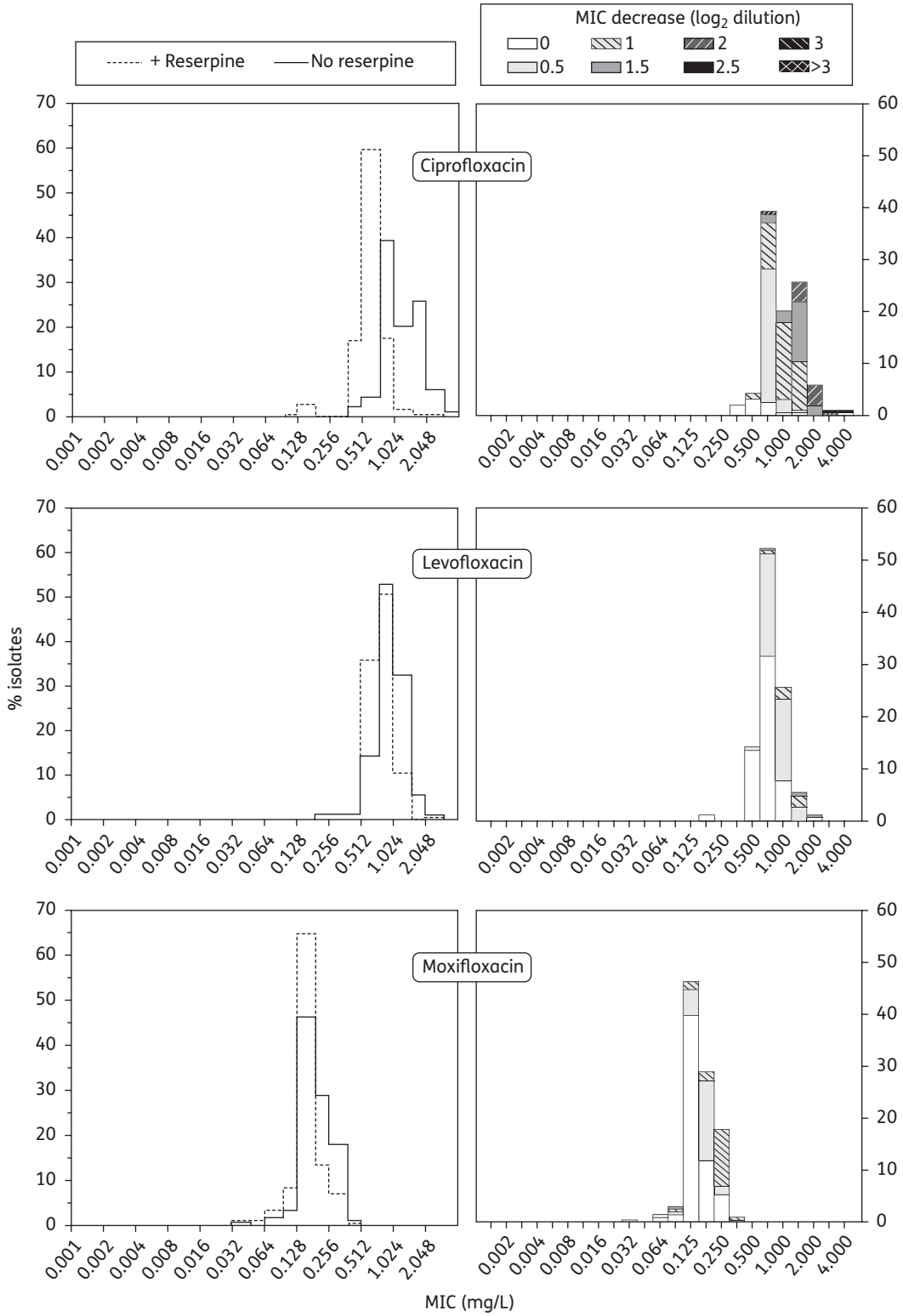

Figure 1. MIC distribution of five quinolones for 183 non-duplicate isolates of *S. pneumoniae* obtained from clinically confirmed cases of community-acquired pneumonia collected in Belgium during the 2007–09 period. Left-hand panels: MIC distributions determined in the absence (control; continuous line) or presence (broken line) of 10 mg/L reserpine {statistical analysis: $P<0.0001$ for each quinolone when comparing distributions in the absence and presence of reserpine by two-tailed paired tests [Wilcoxon signed rank test (non-parametric) and by *t*-test (parametric)]. Right-hand panels: reduction of MIC (in blocks of 0.5 $\log_2$ dilutions from 0 to 3 $\log_2$ 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.

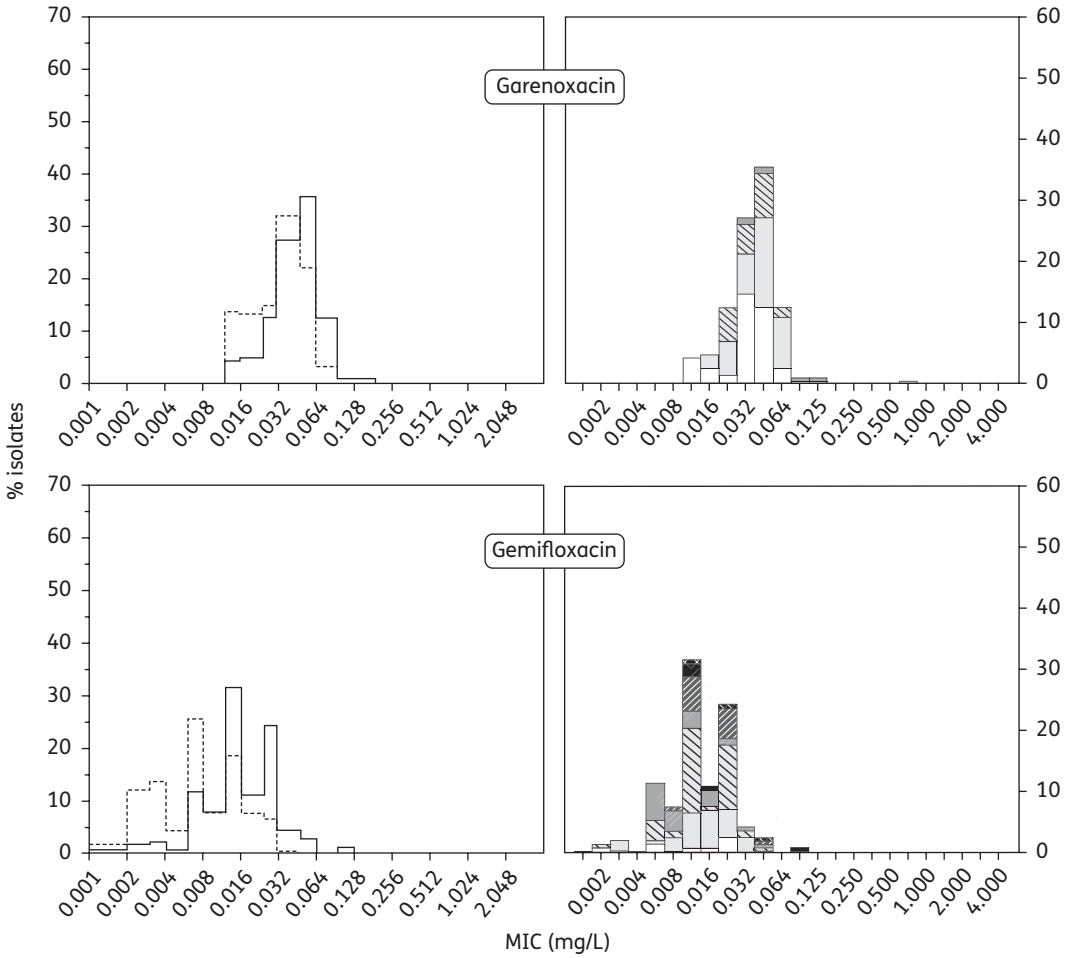

Figure 1. (Continued)

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Transparency declarations

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Supplementary data

Table S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

References

1 Woodhead M, Blasi F, Ewig S *et al*. Guidelines for the management of adult lower respiratory tract infections. *Eur Respir J* 2005; **26**: 1138–80.

2 Mandell LA, Wunderink RG, Anzueto A *et al*. Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults. *Clin Infect Dis* 2007; **44** Suppl 2: S27–72.

3 Pletz MW, van der Linden M, von Baum H *et al*. Low prevalence of fluoroquinolone resistant strains and resistance precursor strains in *Streptococcus pneumoniae* from patients with community-acquired pneumonia despite high fluoroquinolone usage. *Int J Med Microbiol* 2011; **301**: 53–7.

4 Piddock LJ. Mechanisms of fluoroquinolone resistance: an update 1994–1998. *Drugs* 1999 Suppl 2; **58**: 11–8.

5 Garvey MI, Baylay AJ, Wong RL *et al*. Overexpression of *patA* and *patB*, which encode ABC transporters, is associated with fluoroquinolone resistance in clinical isolates of *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 2011; **55**: 190–6.

6 Avrain L, Garvey M, Mesaros N *et al*. Selection of quinolone resistance in *Streptococcus pneumoniae* exposed *in vitro* to subinhibitory drug concentrations. *J Antimicrob Chemother* 2007; **60**: 965–72.

Supplementary data

Table S1. MIC distribution of quinolones for *S. pneumoniae* from clinically confirmed community-acquired pneumonia in the absence (−) or in the presence (+) of 10 mg/L reserpine

MIC (mg/L)	Ciprofloxacin		Levofloxacin		Moxifloxacin		Garenoxacin		Gemifloxacin	
	−	+	−	+	−	+	−	+	−	+
Lowest	0.375	0.094	0.188	0.188	0.032	0.032	0.012	0.012	0.001	<0.001
MIC_{50}	1	0.5	0.75	0.75	0.125	0.125	0.047	0.031	0.012	0.006
MIC_{90}	1.5	0.75	1	1	0.25	0.188	0.064	0.05	0.024	0.016
Highest	4	2	2	2	0.375	0.375	0.75	0.75	0.094	0.032

CLSI breakpoints (susceptible ≤/resistant ≥): levofloxacin, 2/8; moxifloxacin, 1/4; gemifloxacin, 0.12/0.5.

EUCAST breakpoints (susceptible ≤/resistant >): ciprofloxacin, 0.12/2; levofloxacin, 2/2; moxifloxacin, 0.5/0.5.

This study was phenotypic only, and did not allow therefore identifying the transporters involved in efflux. As a complement to this study, we selected a few strains showing a clear phenotype of efflux, in which we inactivated the genes coding for PmrA, PatA, or PatB and examined the consequences of this disruption on susceptibility to fluoroquinolones in order to identify the transporter causing resistance. We used as control laboratory strains for which efflux transporters involved in resistance had been identified previously (Avrain *et al.*, 2007).

Poster: Respective contribution of PatA/PatB and PmrA in fluoroquinolone resistance in clinical isolates of *Streptococcus pneumoniae*

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P922

Respective contribution of PatA/PatB and PmrA in fluoroquinolone resistance in clinical isolates of *Streptococcus pneumoniae*

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Background

Two efflux systems have been identified so far for fluoroquinolones (FQ) in *Streptococcus pneumoniae* (SP), namely PmrA (MFS superfamily),[1] and PatA / PatB (ABC transporters superfamily).[2] Previous studies have suggested a predominant role of PatA/PatB in FQ resistance of laboratory strains.[3,4] The aim of the present study was to determine which of these two systems could be primarily involved in the resistance of clinical SP isolates to FQ.

Methods

Clinical strains showing a phenotype suggestive of efflux were selected from a large collection of SP isolates obtained from CAP patients, and compared to ATCC49619 and to PatA/PatB-positive controls. MICs were measured in Mueller Hinton II agar supplemented with 5% defibrinated horse blood (± 20mg/L reserpine [efflux inhibitor]).

Expression levels of *patA, patB,* and *pmrA* were evaluated by real-time PCR. Gene inactivation was obtained by transformation using genomic DNA of ATCC49619 disrupted in one of the genes under study (spectinomycin resistance cassette inserted in the middle of the gene of interest).

Statistical analysis was made using Friedman test (non-parametric paired one-way ANOVA) and Dunn's post-test of selected pairs on strains showing efflux (control & clinical).

A copy of this poster will be made available after the meeting at *http://www.facm.ucl.ac.be/posters.htm*

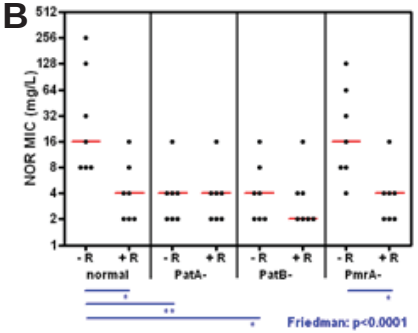
A MICs of CIP and NOR for each strain measured without or with reserpine and in disruptants for *patA*, *patB*, or *pmrA*

strains	gene expression[a]			CIP MIC (mg/L)[b,c]					NOR MIC (mg/L)[b,c]				
	patA	*patB*	*pmrA*	w/o R	with R	*patA*-	*patB*-	*pmrA*-	w/o R	with R	*patA*-	*patB*-	*pmrA*-
ATCC 49619	basal	basal	basal	0.5	0.5	**0.5**	**0.5**	**0.5**	4	2	**4**	**2**	**4**
SP13	+	+	+	16	4	**2**	**2**	16	256	16	**16**	**16**	128
SP207	+	+	+	4	0.5	1	**0.5**	-[d]	32	2	**4**	**2**	no[d]
SP295	+	+	basal	2	0.5	**0.5**	**0.5**	1	16	2	**2**	**2**	8
SP257	basal	basal	+	1	0.5	**0.5**	**0.5**	1	8	2	**2**	**2**	**4**
SP298	basal	basal	+	2	0.5	**0.5**	**1**	2	8	2	**4**	**4**	16

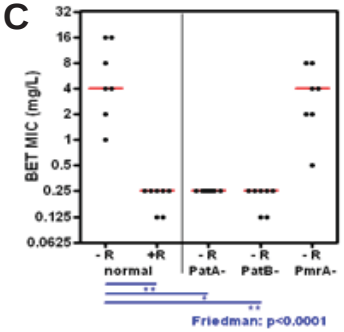
[a] + denotes a value > 2-fold that measured in ATCC49619 (basal level)
[b] in control conditions (without reserpine ([w/o R]); + reserpine 20 mg/L [with R]; in strain disrupted for *patA* [*patA*-], *patB* [*patB*-], or *pmrA* ([*pmrA*-])
[c] figures in bold denote MICs brought back to those measured with reserpine (+/- 1 dilution)
[d] disruptant non obtained so far

NOR (B) and BET (C) MICs for strains showing efflux (3 ctrl + 4 clinical isolates) and their respective disruptants (for *patA*, *patB* or *pmrA*) measured without (- R) or with (+ R) reserpine

B



C



Legend:

red line: median value

Dunn's post-test performed only for normal condition w/o reserpine versus +R, *patA*, *patB* or *pmrA* disruption (C,D) and with or without reserpine (B).

D

	Friedman	Dunn's post-test			
		+R	patA-	patB-	prmA-
CIP	p<0.0001	p<0.01	p<0.01	p<0.01	NS
NOR	p<0.0001	p<0.01	p<0.01	p<0.01	NS
LVX	p=0.0163	p<0.05	NS	NS	NS
MXF	p=0.0724				
GEM	p=0.0032	NS	NS	NS	NS
ACR	p<0.0001	p<0.05	p<0.01	p<0.01	NS
BET	p<0.0001	p<0.01	p<0.05	p<0.01	NS

Statistical analysis: Friedman's test & Dunn's post-test (only comparison to normal MICs w/o reserpine) for graph C (BET tested) and other FQ or substrate of the pumps.

CIP= ciprofloxacin, NOR= norfloxacin, LVX= levofloxacin, MXF= moxifloxacin, GEM= gemifloxacin, ACR= acriflavin, BET= ethidium bromide, NS= non significant.

Results

A. - Reserpine or gene disruption had no effect in ATCC49619.
- Disruption of *patA* or *patB* was as effective as reserpine to decrease CIP and NOR MICs in clinical isolates, irrespective of the gene(s) overexpressed (SP13 did not revert to wild-type MIC because of the presence of target mutations).
- Disruption of *pmrA* had only a modest effect on NOR MIC in SP257.

B. For strains showing efflux of CIP and NOR (discounting SP207 [no *pmrA* disruptant]) and disrupted for *patA* or *patB*, addition of reserpine had no effect on NOR or CIP [not shown] MICs (in contrast with what was observed for *pmrA* disruptants).

C. For BET, *pmrA* disruption did not cause MIC decrease while reserpine was effective.

D. Similar experiments made with CIP, NOR, LVX, MXF or GEM showed that only CIP and NOR are affected by disruption of *patA*/*patB* (change in MIC for LVX in the presence of reserpine is 1 $\log_2$ dilution only).

Conclusions

- Disruption of *patA* or *patB* is sufficient to reduce NOR and CIP MIC to the value measured in the presence of reserpine.
- PatA/PatB, even when expressed at a basal level, contribute to resistance to these two FQ in the clinical isolates analyzed.
- PmrA seems to have almost no impact on resistance of the clinical isolates analyzed.

References

1. Gill *et al*. Identification of an Efflux Pump Gene, pmrA, Associated with Fluoroquinolone Resistance in Streptococcus pneumoniae. Antimicrob Agents Chemother. 1999; 43:187-189.
2. Marrer *et al*. Involvement of the Putative ATP-Dependent Efflux Proteins PatA and PatB in Fluoroquinolone Resistance of a Multidrug-Resistant Mutant of *Streptococcus pneumoniae*. Antimicrob Agents Chemother. 2006; 50: 685–693
3. Avrain *et al*. Selection of quinolone resistance in *Streptococcus pneumoniae* exposed in vitro to subinhibitory drug concentrations. Journal of Antimicrobial Chemotherapy. 2007; 60:, 965–972.
4. Garvey and Piddock. The Efflux Pump Inhibitor Reserpine Selects Multidrug-Resistant *Streptococcus pneumoniae* Strains that Overexpress the ABC Transporters PatA and PatB. Antimicrob Agents Chemother. 2008; 52: 1677–1685.

3.2.b. Fluoroquinolones as inducers of the expression of PatA/PatB

Previous work of our laboratory had shown that fluoroquinolones that were substrates for PatA/PatB were able to select for resistance by overexpression of this efflux system (Avrain *et al.*, 2007). We wonder whether fluoroquinolones that are not affected by the transporter could have the same effect, and if yes, what could be the underlying mechanism.

Article Fluoroquinolones induce the expression of *patA* and *patB* which encode ABC efflux pumps in Streptococcus pneumoniae

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Journal of Antimicrobial Chemotherapy

Fluoroquinolones induce the expression of *patA* and *patB*, which encode ABC efflux pumps in *Streptococcus pneumoniae*

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Background: Active efflux is a common mechanism of resistance to fluoroquinolones in *Streptococcus pneumoniae*. Two efflux systems have been described so far in this species: PmrA, a member of the major facilitator superfamily; and the two ABC transporters PatA and PatB. We studied the inducibility of expression of *pmrA*, *patA* and *patB* by using subinhibitory concentrations of fluoroquinolones.

Methods: A wild-type susceptible strain, two clinical isolates resistant to fluoroquinolones and two efflux mutants selected *in vitro* after exposure to ciprofloxacin were studied. MICs were determined for these strains and their mutants in which *pmrA*, *patA* or *patB* had been disrupted. Gene expression was determined after exposure to half the MIC of norfloxacin, ciprofloxacin, levofloxacin, moxifloxacin or gemifloxacin and quantified by real-time PCR.

Results: Increased MICs of norfloxacin, ciprofloxacin and levofloxacin (to a lesser extent) and increased expression of *patA* and *patB* were seen for all resistant strains; these were reduced in *patA* or *patB* disruptants or in the presence of reserpine. Exposure to any of the five fluoroquinolones caused a reversible increase in expression of *patA* and *patB*, but not of *pmrA*. Mitomycin C, an inducer of the competence system in *S. pneumoniae*, also induced *patA* and *patB* expression in the two strains tested.

Conclusion: The ABC efflux system PatA/PatB is induced upon exposure to subinhibitory concentrations of fluoroquinolones, whether substrates of the transporter or not. This effect, possibly resulting from the activation of the competence pathway, may contribute to resistance.

Keywords: resistance, induction, ABC transporters, DNA damaging agents

Introduction

Streptococcus pneumoniae is a leading cause of respiratory tract infections, including community-acquired pneumonia (CAP).[1,2] The so-called respiratory fluoroquinolones (levofloxacin, moxifloxacin and gemifloxacin)[3] are active in the treatment of CAP.[1,2] However, the use of levofloxacin has been associated with a decrease in bacterial susceptibility and subsequent clinical failures.[4] High-level fluoroquinolone resistance is mainly due to mutations in structural genes for the GyrA subunit of DNA gyrase and for the ParC subunit of topoisomerase IV.[5] However, there is increasing evidence that active efflux can play an important role in decreasing the susceptibility of the isolates,[6–8] with ciprofloxacin and norfloxacin often being used as reporter antibiotics in this context.

PmrA, a member of the major facilitator superfamily (MFS), was the first efflux pump shown to confer resistance to norfloxacin and ciprofloxacin.[9] More recently, an efflux system belonging to the ATP binding cassette (ABC) superfamily and composed of two transporters encoded by *patA* (SP2075) and *patB* (SP2073) was identified.[10,11] Expression of these genes was increased in strains with decreased susceptibility to fluoroquinolones,[7,12,13] and induced by ciprofloxacin or norfloxacin.[10,14] These studies, carried out with a reference strain and derivative mutants, were limited to fluoroquinolones that are substrates for this efflux system. In the present study, we have

compared induction of expression of *pmrA*, *patA* and *patB* by five fluoroquinolones (putative good or poor substrates) in various strains including clinical isolates. Because fluoroquinolones and the DNA-damaging agent mitomycin C can induce a competence pathway and chromosomal transformation in *S. pneumoniae*,[15,16] we examined whether mitomycin C was able to induce *patA* and *patB* expression as part of a global stress response. In a nutshell, we report that: (i) all fluoroquinolones can induce *patA* and *patB* expression in a concentration-dependent manner; (ii) the extent of overexpression depends on the strain rather than on the inducer; and (iii) mitomycin C is able to trigger overexpression of *patA* and *patB*, confirming that this efflux system is part of a general stress response related to DNA damage.[14]

Materials and methods

Bacterial strains and growth conditions

The five strains studied were: (i) the reference *S. pneumoniae* ATCC 49619; (ii) two laboratory mutants (SP334, derived from *S. pneumoniae* ATCC 49619, and SP335, derived from the clinical isolate SP32, selected after 13 days of exposure to ciprofloxacin[12]); and (iii) two clinical isolates (SP295 and SP13) (see Table 1). Cultures were performed at 37°C in a 5% CO_2 atmosphere using Todd-Hewitt broth supplemented with 1% yeast extract (THY; BD, Franklin Lakes, NJ, USA) or Mueller-Hinton agar supplemented with 5% defibrinated sheep blood (International Medical Products, Brussels, Belgium).

Determination of MICs

MICs of fluoroquinolones and of ethidium bromide and acriflavine (two well-known substrates for efflux pumps) were determined by the serial 2-fold macrodilution method in Mueller-Hinton agar supplemented with 5% defibrinated horse blood, with an inoculum of $\sim 10^5$ bacteria per spot.[12,17] The efflux inhibitor reserpine was used at a final concentration of 20 mg/L.[18]

DNA techniques

Chromosomal DNA was purified with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Plasmid DNA was prepared using the Plasmid Midi Preps Kit (Qiagen) and transformed into *Escherichia coli*.[19] Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs (Ipswich, MA, USA). Blunt-ending of restricted plasmid DNA was performed by the addition of 1 U of Klenow enzyme (New England Biolabs) and 33 μM deoxynucleoside triphosphates to the reaction mixture at the end of enzymatic digestion. Restriction fragments were purified from agarose gels with the QIAquick Gel Extraction Kit (Qiagen). PCR amplifications were performed according to the manufacturer's protocol for BIOTAQ Red DNA polymerase (Gentaur, Kampenhout, Belgium). The sequences of the primers used are shown in Table S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

Quantitative real-time PCR

S. pneumoniae was grown overnight at 37°C in a 5% CO_2 atmosphere on Mueller-Hinton agar supplemented with 5% defibrinated sheep blood. Bacteria were resuspended in 15 mL of THY medium supplemented or not with inducers at an optical density (620 nm) of 0.2–0.4. For induction, bacteria were grown for up to 6 h at 37°C in 5% CO_2. For experiments examining the reversal of induction, bacteria were harvested by centrifugation (3000 **g** for 10 min) after 4 h of culture in the presence of antibiotic at half the MIC, washed once at room temperature in drug-free medium and centrifuged, and the pellet was then resuspended in THY drug-free medium and cultured for up to 5 h. Bacteria were harvested by centrifugation (5000 **g** for 5 min at 4°C) and the pellets were frozen and kept at −80°C for at least 30 min before nucleic acid extraction. Total RNA extraction and reverse transcription were performed as previously described.[12] Real-time PCR was performed in an iQ cycler (Bio-Rad Laboratories, Hercules, CA, USA) in 25 μL reaction mixtures containing 12.5 μL of iQ SYBR Green Supermix (2×), 400 nM of forward and reverse primers and 5 μL of cDNA in RNase/DNase-free water. The *rpoD* and *proC* genes were used as references to normalize transcript levels, as specified by PrimerDesign (Southampton, UK).

Inactivation of patA, patB and pmrA genes

To inactivate *patA* or *patB*, the strains were transformed with genomic DNA of M246 or M240 strains, which have a *magellan2* minitransposon inserted in either *patA* or *patB*.[13] Transformants were selected on Mueller-Hinton agar containing 5% defibrinated sheep blood supplemented with 100 mg/L spectinomycin. Gene inactivation was verified by PCR.[13] To inactivate *pmrA*, a 1 kb BamHI-KpnI PCR fragment, amplified with the PmrARec-F and PmrARec-R primers (Table S1) and carrying the *pmrA* gene, was cloned in BamHI-KpnI-restricted pUC18.[20] The plasmid was then cleaved in the insert by ClaI and blunt-ended with Klenow enzyme. The *aad9* gene of *magellan2* conferring resistance to spectinomycin[21] was amplified by PCR with the Spec-1 and Spec-2 primers (Table S1), ligated with the linearized plasmid to generate pUC18Ω*pmrA*::*spt* and transformed into *S. pneumoniae* strain R6. Inactivation of *pmrA* was confirmed by PCR using the PmrA-Delta-F and PmrA-Delta-R primers.

Quinolone resistance-determining region (QRDR) sequencing

The QRDRs of *gyrA*, *parC* and *parE* were amplified and sequenced using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and a Genetic Analyzer 3100 (Applied Biosystems) as previously described.[12]

Antibiotics, other substrates and pump inhibitor

Levofloxacin, moxifloxacin and gemifloxacin were obtained as microbiological standards from Aventis Pharma (Antony, France), Bayer HealthCare (Leverkusen, Germany) and LG Life Sciences (Seoul, Korea), respectively. Other antibiotics, substrates or inducers were obtained as pure substances from Sigma–Aldrich (St Louis, MO, USA).

Results

Antibiotic susceptibility of the strains

Table 1 summarizes the MICs of the five fluoroquinolones and of acriflavine and ethidium bromide, determined in the absence or presence of reserpine. The MICs of norfloxacin, ciprofloxacin, acriflavine and ethidium bromide were significantly (≥2 dilutions; 1 dilution for acriflavin and SP334) higher for SP334, SP335, SP295 and SP13 than for *S. pneumoniae* ATCC 49619. The MICs of levofloxacin and gemifloxacin were significantly higher in SP334, SP335 and SP13 [reaching or exceeding the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical resistance breakpoint for levofloxacin]. Moxifloxacin MIC was increased by two dilutions only against SP335 (but remained below the EUCAST resistance breakpoint).

Table 1. Susceptibility of *S. pneumoniae* to fluoroquinolones and substrates of efflux pumps in the absence (−R) or presence (+R) of reserpine (20 mg/L)

			MIC (mg/L) of:													
			norfloxacin		ciprofloxacin		levofloxacin		moxifloxacin		gemifloxacin		acriflavine		ethidium bromide		
Strains	Relevant characteristics[a]	Mutations in QRDR	−R	+R	−R	+R	−R	+R	−R	+R	−R	+R	−R	+R	−R	+R	Reference or source
ATCC 49619	wild-type	none	4	2	0.5	0.5	0.5	0.5	0.125	0.125	0.031	0.031	2	1	0.5	0.125	LGC Standards
ATCC 49619*patA*	ATCC 49619 *patA*::*magellan2*, SPT^R	none	4	2	0.5	0.5	0.5	0.5	0.125	0.125	0.031	0.016	1	0.5	0.25	0.125	this study
ATCC 49619*patB*	ATCC 49619 *patB*::*magellan2*, SPT^R	none	2	2	0.5	0.5	0.5	0.5	0.125	0.125	0.031	0.031	1	1	0.25	0.125	this study
ATCC 49619*pmrA*	ATCC 49619 *pmrA*::*magellan2*, SPT^R	none	4	2	1	0.5	0.5	0.5	0.125	0.125	0.031	0.031	4	1	1	0.125	this study
SP334	ATCC 49619 after 13 days of exposure to ciprofloxacin, CIP^R	none	**32**	4	**4**	0.5	**2**	1	0.25	0.25	**0.125**	0.031	4	1	**4**	0.125	[12]
SP334*patA*	SP334 *patA*::*magellan2*, SPT^R	none	4	4	1	0.5	1	1	0.125	0.125	0.063	0.031	1	1	0.25	0.125	this study
SP334*patB*	SP334 *patB*::*magellan2*, SPT^R	none	8	4	1	1	1	1	0.25	0.25	0.063	0.063	1	1	0.125	0.125	this study
SP334*pmrA*	SP334 *pmrA*::*aad9*, SPT^R	none	**32**	4	**4**	0.5	1	0.5	0.25	0.25	**0.125**	0.063	4	0.5	**2**	0.125	this study
SP335	clinical strain SP32 after 13 days of exposure to ciprofloxacin, CIP^R	ParE (Ile460Val)	**64**	8	**32**	2	**4**	2	**0.5**	0.25	**0.5**	0.125	**16**	1	**8**	0.25	[12]
SP335*patA*	SP335 *patA*::*magellan2*, SPT^R	ParE (Ile460Val)	4	4	1	0.5	1	1	0.125	0.125	0.031	0.031	1	1	0.25	0.125	this study
SP335*patB*	SP335 *patB*::*magellan2*, SPT^R	ParE (Ile460Val)	4	4	1	0.5	1	1	0.125	0.125	0.031	0.031	1	1	0.25	0.25	this study
SP335*pmrA*	SP335 *pmrA*::*aad9*, SPT^R	ParE (Ile460Val)	**64**	4	**8**	0.5	1	0.5	0.25	0.125	0.063	0.016	**8**	0.5	**8**	0.125	this study
SP295	clinical isolate[b]	none	**16**	2	**2**	0.5	1	0.5	0.125	0.125	0.063	0.031	**16**	1	**16**	0.25	this study
SP295*patA*	SP295 *patA*::*magellan2*, SPT^R	none	2	2	0.5	0.5	0.5	0.5	0.125	0.125	0.031	0.031	1	1	0.25	0.25	this study
SP295*patB*	SP295 *patB*::*magellan2*, SPT^R	none	2	2	0.5	0.5	0.5	0.5	0.125	0.125	0.016	0.016	1	1	0.25	0.25	this study
SP295*pmrA*	SP295 *pmrA*::*aad9*, SPT^R	none	8	4	1	0.5	1	0.5	0.25	0.125	0.063	0.031	**8**	1	**4**	0.25	this study
SP13	clinical isolate[c]	ParC (Ser79Phe, Lys137Asn); ParE (Ile460Val)	**256**	16	**16**	4	**2**	1	0.25	0.25	**0.25**	0.063	**16**	1	**16**	0.25	this study
SP13*patA*	SP13 *patA*::*magellan2*, SPT^R	ParC (Ser79Phe, Lys137Asn); ParE (Ile460Val)	**16**	16	**2**	1	1	1	0.25	0.25	0.063	0.063	1	0.5	0.25	0.125	this study
SP13*patB*	SP13 *patB*::*magellan2*, SPT^R	ParC (Ser79Phe, Lys137Asn); ParE (Ile460Val)	**16**	16	**2**	2	**2**	1	0.25	0.25	0.063	0.063	2	1	0.25	0.25	this study
SP13*pmrA*	SP13 *pmrA*::*aad9*, SPT^R	ParC (Ser79Phe, Lys137Asn); ParE (Ile460Val)	**128**	16	**16**	2	**2**	1	**0.5**	0.25	**0.125**	0.063	**8**	0.5	**8**	0.25	this study

EUCAST breakpoints for resistance: ciprofloxacin and levofloxacin, >2 mg/L; and moxifloxacin, >0.5 mg/L (no values for norfloxacin and gemifloxacin). Figures in bold indicate MICs at least two dilutions higher than those of wild-type *S. pneumoniae* ATCC 49619.

[a]CIP^R, resistance to ciprofloxacin; SPT^R, resistance to spectinomycin.

[b]Isolated from blood culture (Cliniques Universitaires St Luc, Brussels).

[c]Isolated from expectoration (Universitair Ziekenhuis Brussel, Brussels).

In the presence of reserpine, the MICs of acriflavine and ethidium bromide were similar to those for the wild-type strain, suggesting an efflux mechanism in the four strains. For fluoroquinolones, restoration of wild-type MICs by reserpine was complete for SP334 and SP295, but only partial for SP335 and SP13, which have mutations in the QRDR (Table 1).

Role of PmrA, PatA and PatB in antibiotic resistance

The expression of *pmrA*, *patA* and *patB* was quantified by real-time PCR in all strains (Figure 1). As compared with *S. pneumoniae* ATCC 49619, the four resistant strains overexpressed *patA* and *patB* to levels ranging from 4.4-fold for *patA* in SP334 to 13.6-fold for *patB* in SP13. In contrast, only SP335 and SP13 showed modest overexpression of *pmrA*.

Every gene was inactivated in each of the five strains, and the MICs for the disruptants were determined (Table 1). For all strains, inactivation of either *patA* or *patB* reduced the MIC of acriflavine and ethidium bromide to a value similar to that for *S. pneumoniae* ATCC 49619 in the presence of reserpine. Likewise, the MICs of fluoroquinolones for *patA*- or *patB*-inactivated strains were reduced (±1 dilution) to those measured for the corresponding parental strain in the presence of reserpine (or even lower for SP335). In contrast, inactivation of *pmrA* did not cause a marked decrease in MICs (0 to 1 dilution).

Induction of patA, patB or pmrA expression by fluoroquinolones

The expression of these genes was then measured in bacteria grown for 4 h in the presence of fluoroquinolones at half their MIC (preliminary experiments with ciprofloxacin showed that this concentration caused the maximal effect; see Figure S1, available as Supplementary data at *JAC* Online http://jac.oxfordjournals.org). All fluoroquinolones were potent inducers of *patA* and *patB* in strains SP335 and SP295, but showed a lower effect in strains ATCC 49619 and SP334 and no effect for norfloxacin and levofloxacin in strain SP13 (Figure 2). In contrast, the expression of *pmrA* remained unaffected or even decreased upon exposure to fluoroquinolones. Specificity of induction was tested with tetracycline and chloramphenicol under the same conditions, but no change in the expression of *patA*, *patB* or *pmrA* was observed (data not shown).

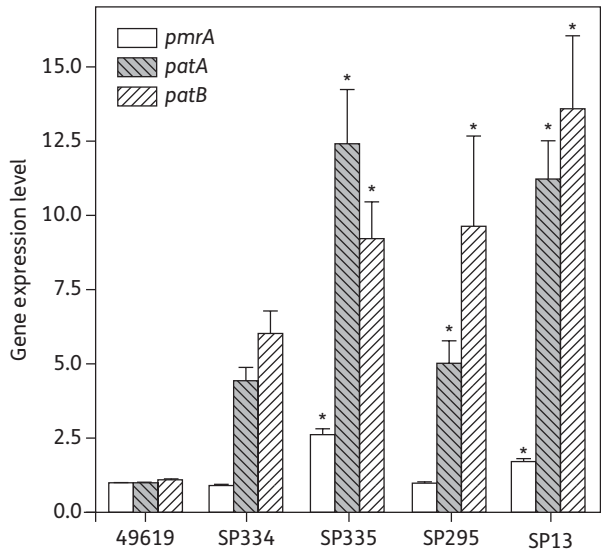

Figure 1. Expression levels of *patA*, *patB* and *pmrA* in non-induced *S. pneumoniae*. Data are expressed as the ratio to the value in *S. pneumoniae* ATCC 49619. Values are the means±SEM of duplicates from five independent experiments. *$P<0.05$ (one-way ANOVA with Dunnett's *post hoc* test for comparison with *S. pneumoniae* ATCC 49619).

Kinetics of induction

To follow the kinetics of induction of *patA* and *patB* and the time needed to revert to basal level, strains ATCC 49619 and SP335 were used as they showed a low and high basal level of *patA*/*patB* expression, respectively. Ciprofloxacin and moxifloxacin were selected as substrate and non-substrate (Figure 3). In both strains, a lag phase of ~30–40 min was observed during which no change in expression level was observed, followed by increased expression levels over time. Reduction in expression was detected as soon as the drugs were removed and reversal to original pre-exposure levels was obtained after 3–4 h. To test if changes in *patA* and *patB* expression over time did not result from growth variations, expression of the genes under non-inducing conditions in bacteria from the exponential to the stationary phase was measured and no differences were seen. Conversely, there was no change in optical density over the 6 h of induction, indicating absence of significant growth over the time frame of the experiment (see Figure S2, available as Supplementary data at *JAC* Online http://jac.oxfordjournals.org/).

Induction of patA/patB and of the competence regulon by mitomycin C

DNA-damaging agents or antibiotics are capable of inducing the SOS response,[22] or a competence pathway in bacteria devoid of an SOS system, such as *S. pneumoniae*.[15,16,23] We therefore examined whether mitomycin C, a DNA-damaging agent known to induce competence in *S. pneumoniae*,[15,16] was also able to induce expression of *patA* and *patB*. In parallel, we quantified the expression levels of two genes involved in competence via the *com* regulon,[16] namely *recA*[24] and *ssbB*.[25,26] The expression of these genes upon induction by ciprofloxacin or mitomycin C was largely parallel to that of *patA* and *patB*, with a correlation coefficient of 0.879 and 0.897 for *ssbB* and *recA* versus *patA* and *patB*, respectively (see Figure S3, available as Supplementary data at *JAC* Online http://jac.oxfordjournals.org/).

Discussion

Two important observations were made. Our study shows that PatA and PatB play a major role in fluoroquinolone resistance in the two clinical isolates and the two *in vitro* mutants examined here, while PmrA does not, confirming the data of Piddock *et al.*[27] and Garvey and Piddock.[13] Inactivation of either *patA* or *patB* restored full susceptibility to ethidium bromide or acriflavine in the four strains, or to fluoroquinolones in those strains that did not harbour mutations in the genes encoding the target proteins.

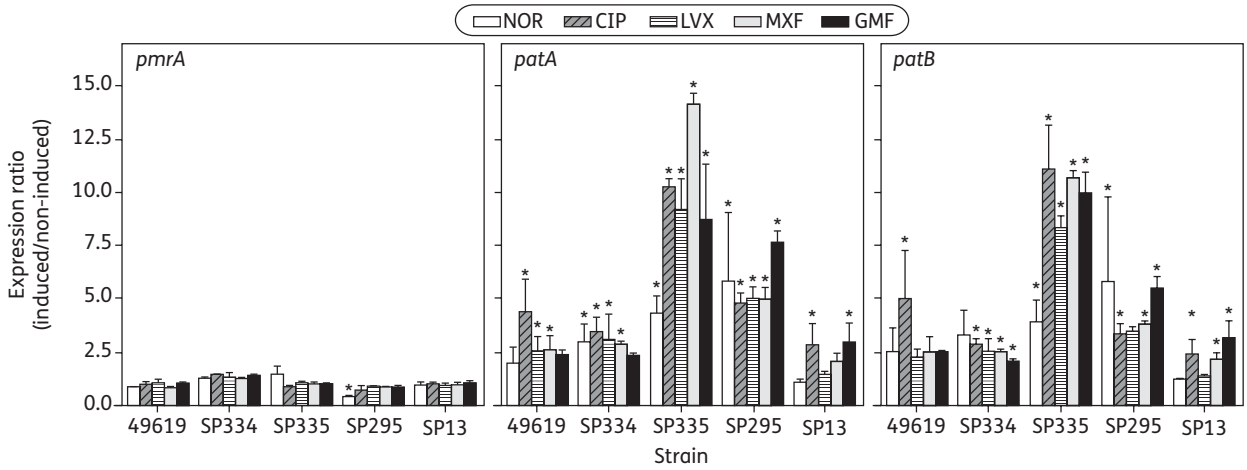

Figure 2. Induction of *pmrA*, *patA* and *patB* expression in *S. pneumoniae* exposed for 4 h to half the MIC of various fluoroquinolones. Data are presented as ratios of expression measured under induced and non-induced conditions. Values are the means ± SEM of duplicates from two independent experiments. *$P < 0.05$ (one-way ANOVA with Dunnett's *post hoc* test for comparison with the non-induced condition). NOR, norfloxacin; CIP, ciprofloxacin; LVX, levofloxacin; MXF, moxifloxacin; GMF, gemifloxacin.

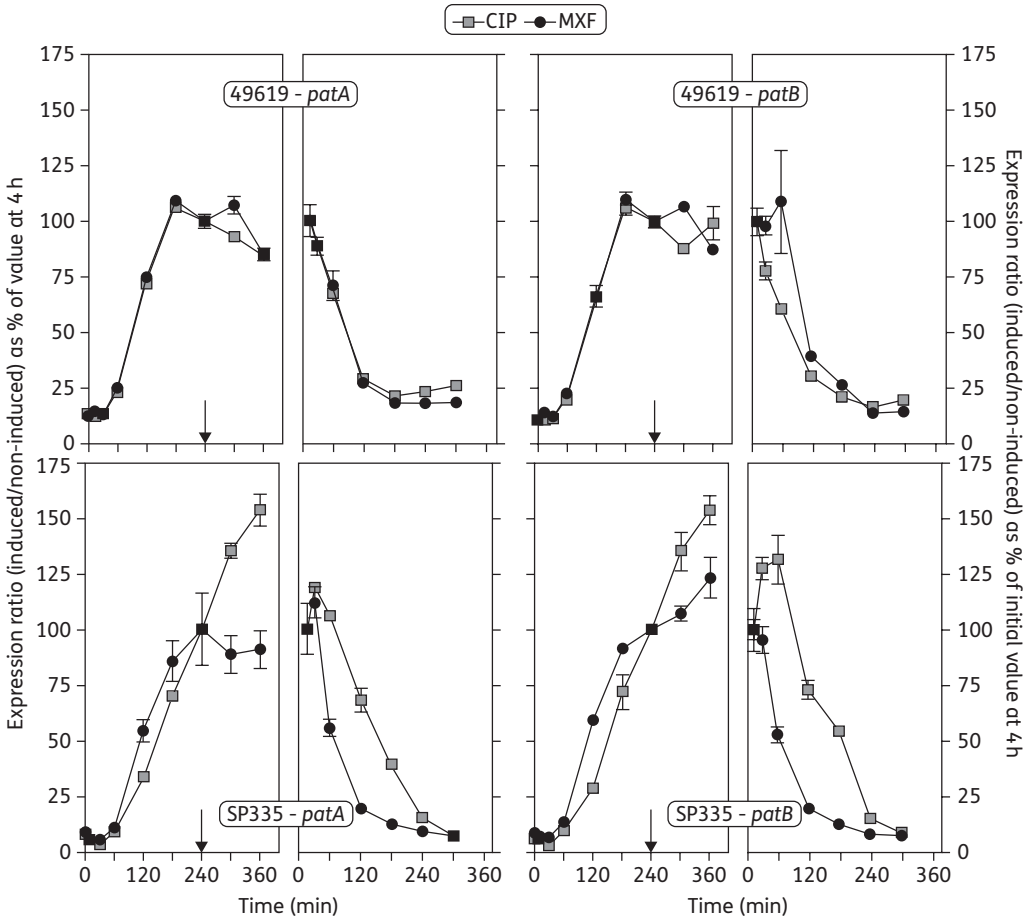

Figure 3. Kinetics of induction and deinduction of *patA* and *patB* expression by ciprofloxacin and moxifloxacin in *S. pneumoniae* ATCC 49619 (top) and SP335 (bottom). Bacteria were induced by exposure to half the MIC of ciprofloxacin or moxifloxacin over 6 h. For reversion, bacteria induced for 4 h were harvested and regrown in broth without antibiotic for 5 h. Data are presented as ratios of *patA* (left) or *patB* (right) expression measured under induced and non-induced conditions for each strain as a percentage of the value at 4 h (staring point of reversion, as indicated by the arrows). Values are the means ± SEM of duplicates from two independent experiments. CIP, ciprofloxacin; MXF, moxifloxacin.

In contrast, *pmrA* inactivation had only a marginal effect, in agreement with other studies.[7,11,28]

First, resistance mediated by PatA/PatB did not affect all fluoroquinolones to the same extent, with norfloxacin being the most affected, followed by ciprofloxacin, gemifloxacin, levofloxacin and finally moxifloxacin. This is in agreement with a previous ranking established for fluoroquinolone susceptibility to efflux in *S. pneumoniae*[6,29,30] or in *S. aureus*,[31] suggesting that hydrophilic molecules are better substrates. We extend here this observation to other fluoroquinolones, but show that moxifloxacin, the most lipophilic molecule among those tested here, was little affected by the overexpression of PatA/PatB. Interestingly this ranking seems to apply to efflux pumps of the ABC superfamily (like PatA/PatB) as well as to those of the MFS superfamily (such as NorA in *S. aureus*). This observation may suggest that common molecular or physicochemical determinants in substrates are recognized by non-phylogenetically related transporters.

Second, inactivation of either *patA* or *patB* is sufficient to restore full susceptibility to fluoroquinolones and no phenotypic discrepancies are observed between *patA* and *patB* knockouts with respect to fluoroquinolone, acriflavine or ethidium susceptibility. Together with the facts that (i) homologues of PatA and PatB appear as pairs of proteins working together[13] and (ii) predictions of topologies for PatA and PatB propose four to seven transmembrane segments for each of these proteins [using either TMPRED (http://www.ch.embnet.org/software/TMPRED_form.html) or SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui)], these observations suggest that the two proteins may constitute a heterodimeric ABC-type multidrug transporter[13,32] or, at least, a need to interact to confer fluoroquinolone resistance.[10]

When bacteria are exposed to antibiotics, dyes, solvents or detergents, they can adapt by inducing the expression of efflux systems.[33,34] It has been shown that *patA* and *patB* expression of a wild-type *S. pneumoniae* and of an *in vitro* resistant mutant thereof is inducible upon exposure to norfloxacin or ciprofloxacin.[10,14] This observation is extended here by showing that induction is obtained (i) for all fluoroquinolones tested, whether substrates of PatA/PatB or not, and (ii) not only in a wild-type strain, but also in *in vitro* mutants and in clinical isolates overexpressing *patA* and *patB* under non-inducing conditions. Increase in expression develops rapidly, irrespective of the fluoroquinolone used, and is fully reversible. Because induction seems specific to fluoroquinolones, is observed even in strains with pre-existing high basal efflux expression and is observed with inducers that are or are not substrates, it is tempting to speculate that overexpression is the consequence of a change in global regulatory responses induced by fluoroquinolones.

Regulation of ABC-type efflux transporters involves local regulators, repressors or activators, as well as global transcriptional regulators.[32–34] Yet the regulators of *patA* and *patB* expression are unknown. A microarray analysis showed that exposure of *S. pneumoniae* to ciprofloxacin induces the expression of genes involved in the competence pathway, mismatch repair system or replication.[14] We found here a coexpression of *patA* and *patB* and of two genes of the competence pathway[15,16,23] upon exposure to ciprofloxacin or the DNA-damaging agent mitomycin C. This strongly suggests that the overexpression of *patA* and *patB* observed upon induction by fluoroquinolones is not only dependent upon local regulators, but is also part of a global response related to the stress imposed by their interaction with DNA.[22,35]

The data presented here may have important implications for the clinical use of fluoroquinolones. Induction of *patA* and *patB* expression by subinhibitory concentrations of any fluoroquinolone may contribute to increased levels of resistance to the molecules of the class that are substrates for efflux. As MICs may remain below or at the limit of the susceptibility breakpoint for the more potent fluoroquinolones, this highlights the usefulness of antibiotics like norfloxacin or ciprofloxacin in laboratory screens and/or for identifying resistance mechanisms at the molecular level. This inducible character also compromises the potential importance of efflux inhibitors that would act as competitive substrates, as illustrated by the cross-resistance to reserpine observed in a strain overexpressing *patA*.[13]

Acknowledgements

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Transparency declarations

None to declare.

Supplementary data

Table S1 and Figures S1–S3 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

References

1 Segreti J, House HR, Siegel RE. Principles of antibiotic treatment of community-acquired pneumonia in the outpatient setting. *Am J Med* 2005; **118** Suppl 7A: 21S–8S.

2 Lujan M, Gallego M, Rello J. Optimal therapy for severe pneumococcal community-acquired pneumonia. *Intensive Care Med* 2006; **32**: 971–80.

3 Van Bambeke F, Michot JM, Van Eldere J *et al*. Quinolones in 2005: an update. *Clin Microbiol Infect* 2005; **11**: 256–80.

4 Endimiani A, Brigante G, Bettaccini AA *et al*. Failure of levofloxacin treatment in community-acquired pneumococcal pneumonia. *BMC Infect Dis* 2005; **5**: 106.

5 Canton R, Morosini M, Enright MC *et al*. Worldwide incidence, molecular epidemiology and mutations implicated in fluoroquinolone-resistant *Streptococcus pneumoniae*: data from the global PROTEKT surveillance programme. *J Antimicrob Chemother* 2003; **52**: 944–52.

6 Piddock LJ, Johnson M, Ricci V *et al*. Activities of new fluoroquinolones against fluoroquinolone-resistant pathogens of the lower respiratory tract. *Antimicrob Agents Chemother* 1998; **42**: 2956–60.

7 Martinez-Garriga B, Vinuesa T, Hernandez-Borrell J *et al*. The contribution of efflux pumps to quinolone resistance in *Streptococcus pneumoniae* clinical isolates. *Int J Med Microbiol* 2007; **297**: 187–95.

8 Brenwald NP, Gill MJ, Wise R. Prevalence of a putative efflux mechanism among fluoroquinolone-resistant clinical isolates of *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 1998; **42**: 2032–5.

9 Gill MJ, Brenwald NP, Wise R. Identification of an efflux pump gene, *pmrA*, associated with fluoroquinolone resistance in *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 1999; **43**: 187–9.

10 Marrer E, Schad K, Satoh AT *et al*. Involvement of the putative ATP-dependent efflux proteins PatA and PatB in fluoroquinolone resistance of a multidrug-resistant mutant of *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 2006; **50**: 685–93.

11 Robertson GT, Doyle TB, Lynch AS. Use of an efflux-deficient *Streptococcus pneumoniae* strain panel to identify ABC-class multidrug transporters involved in intrinsic resistance to antimicrobial agents. *Antimicrob Agents Chemother* 2005; **49**: 4781–3.

12 Avrain L, Garvey M, Mesaros N *et al*. Selection of quinolone resistance in *Streptococcus pneumoniae* exposed *in vitro* to subinhibitory drug concentrations. *J Antimicrob Chemother* 2007; **60**: 965–72.

13 Garvey MI, Piddock LJ. The efflux pump inhibitor reserpine selects multidrug-resistant *Streptococcus pneumoniae* strains that overexpress the ABC transporters PatA and PatB. *Antimicrob Agents Chemother* 2008; **52**: 1677–85.

14 Marrer E, Satoh AT, Johnson MM *et al*. Global transcriptome analysis of the responses of a fluoroquinolone-resistant *Streptococcus pneumoniae* mutant and its parent to ciprofloxacin. *Antimicrob Agents Chemother* 2006; **50**: 269–78.

15 Claverys JP, Prudhomme M, Martin B. Induction of competence regulons as a general response to stress in Gram-positive bacteria. *Annu Rev Microbiol* 2006; **60**: 451–75.

16 Prudhomme M, Attaiech L, Sanchez G *et al*. Antibiotic stress induces genetic transformability in the human pathogen *Streptococcus pneumoniae*. *Science* 2006; **313**: 89–92.

17 Andrews JM. Determination of minimum inhibitory concentrations. *J Antimicrob Chemother* 2001; **48** Suppl 1: 5–16.

18 Brenwald NP, Gill MJ, Wise R. The effect of reserpine, an inhibitor of multi-drug efflux pumps, on the in-vitro susceptibilities of fluoroquinolone-resistant strains of *Streptococcus pneumoniae* to norfloxacin. *J Antimicrob Chemother* 1997; **40**: 458–60.

19 Hanahan D, Jessee J, Bloom FR. Plasmid transformation of *Escherichia coli* and other bacteria. *Methods Enzymol* 1991; **204**: 63–113.

20 Yanisch-Perron C, Vieira J, Messing J. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 1985; **33**: 103–19.

21 Martin B, Prudhomme M, Alloing G *et al*. Cross-regulation of competence pheromone production and export in the early control of transformation in *Streptococcus pneumoniae*. *Mol Microbiol* 2000; **38**: 867–78.

22 Janion C. Inducible SOS response system of DNA repair and mutagenesis in *Escherichia coli*. *Int J Biol Sci* 2008; **4**: 338–44.

23 Gasc AM, Sicard N, Claverys JP *et al*. Lack of SOS repair in *Streptococcus pneumoniae*. *Mutat Res* 1980; **70**: 157–65.

24 Mortier-Barriere I, de Saizieu A, Claverys JP *et al*. Competence-specific induction of *recA* is required for full recombination proficiency during transformation in *Streptococcus pneumoniae*. *Mol Microbiol* 1998; **27**: 159–70.

25 Peterson SN, Sung CK, Cline R *et al*. Identification of competence pheromone responsive genes in *Streptococcus pneumoniae* by use of DNA microarrays. *Mol Microbiol* 2004; **51**: 1051–70.

26 Dagkessamanskaia A, Moscoso M, Henard V *et al*. Interconnection of competence, stress and CiaR regulons in *Streptococcus pneumoniae*: competence triggers stationary phase autolysis of *ciaR* mutant cells. *Mol Microbiol* 2004; **51**: 1071–86.

27 Piddock LJ, Johnson MM, Simjee S *et al*. Expression of efflux pump gene *pmrA* in fluoroquinolone-resistant and -susceptible clinical isolates of *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 2002; **46**: 808–12.

28 Brenwald NP, Appelbaum P, Davies T *et al*. Evidence for efflux pumps, other than PmrA, associated with fluoroquinolone resistance in *Streptococcus pneumoniae*. *Clin Microbiol Infect* 2003; **9**: 140–3.

29 Piddock LJ, Johnson MM. Accumulation of 10 fluoroquinolones by wild-type or efflux mutant *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 2002; **46**: 813–20.

30 Zhanel GG, Walkty A, Nichol K *et al*. Molecular characterization of fluoroquinolone resistant *Streptococcus pneumoniae* clinical isolates obtained from across Canada. *Diagn Microbiol Infect Dis* 2003; **45**: 63–7.

31 Kaatz GW, Seo SM. Inducible NorA-mediated multidrug resistance in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 1995; **39**: 2650–5.

32 Lubelski J, Konings WN, Driessen AJ. Distribution and physiology of ABC-type transporters contributing to multidrug resistance in bacteria. *Microbiol Mol Biol Rev* 2007; **71**: 463–76.

33 Grkovic S, Brown MH, Skurray RA. Regulation of bacterial drug export systems. *Microbiol Mol Biol Rev* 2002; **66**: 671–701.

34 Grkovic S, Brown MH, Skurray RA. Transcriptional regulation of multidrug efflux pumps in bacteria. *Semin Cell Dev Biol* 2001; **12**: 225–37.

35 Drlica K, Zhao X. DNA gyrase, topoisomerase IV, and the 4-quinolones. *Microbiol Mol Biol Rev* 1997; **61**: 377–92.

Supplementary data

Table S1. Primers used in this study

Primers	Sequence (5'-3') (endonuclease)[a]	Source or reference
Gene expression		
rpoD-F	CAGGTAGCAGAATTTATCCGTAATC	PrimerDesign Ltd[b]
rpoD-R	CCCATCAGCGTCCAAGGTA	PrimerDesign Ltd[b]
proC-F	TTATCCCAAGTCAACACCGAAT	PrimerDesign Ltd[b]
proC-R	GCAATTAGGAGACAAGGCATAAC	PrimerDesign Ltd[b]
pmrA-S	TCCAGTATGGGCTTTTCCAG	[1]
pmrA-AS	CCAATCCAAAGAGGAAACGA	[1]
patA-F	TCCTGATGACAGGCTTGATG	This study
patA-R	TGCGAGGACAACATTGAGTC	This study
patB-F	ATGGCAAAGCCTATCAGGAA	This study
patB-R	AGGATATCGCCATCTTGTCG	This study
recA-2-F	CTCATCATACGAGCCTGCAA	This study
recA-2-R	GTCTTGAGATTGCGGGAAAA	This study
ssbB-2-F	AAAGACCAAAACGGTGAACG	This study
ssbB-2-R	TACGCAATTCTCCATCAACG	This study
Sequencing		
PNC10	TGGGTTGAAGCCGGTTCA	[2]
PNC11	CAAGACCGTTGGTTCTTTC	[2]
SPPARE7	CCAATCTAAGAATCCTG	[3]
SPPARE8	GCAATATAGACATGACC	[3]
gyrA-S	CCTGTTCACCGTCGCATTCT	[1]
gyrA-AS	AGTTGCTCCATTAACCA	[1]
Gene inactivation		
PmrARec-F	CTCGGATCCGCATTGCCTGGTTTGGTAAT (BamHI)	This study
PmrARec-R	CTCGGTACCCACAAAGGCTTGTCGCATAA (KpnI)	This study
Spec-1	CTCGCGGCCGCCCCCGGTCTGACACATAGAT (NotI)	This study
Spec-2	CTCAGATCTTCCCCGGATCTAACAAAGAA (BglII)	This study
PmrA-Delta-F	CCTTCTTGAGGGAGGTAGGC	This study
PmrA-Delta-R	TGGATTGGTTTTTGGTTGGT	This study

[a] Restriction sites introduced in primers are underlined and the corresponding endonuclease indicated in parentheses. Amplification reactions were conducted at 61°C, 50°C, 54°C for gene inactivation, sequencing experiments and gene expression experiments, respectively.

[b] primers designed by this company (http://www.primerdesign.co.uk/research_with_integrity.html)

[1] Avrain L, Garvey M, Mesaros N *et al*. Selection of quinolone resistance in Streptococcus pneumoniae exposed in vitro to subinhibitory drug concentrations. *J. Antimicrobial Chemother.* 2007; **60**, 965-72.

[2] Janoir C, Zeller V, Kitzis MD *et al*. High-level fluoroquinolone resistance in Streptococcus pneumoniae requires mutations in parC and gyrA. *Antimicrob.Agents Chemother.* 1996; **40**, 2760-4.

[3] Perichon B, Tankovic J, Courvalin P . Characterization of a mutation in the parE gene that confers fluoroquinolone resistance in Streptococcus pneumoniae. *Antimicrob.Agents Chemother.* 1997; **41**, 1166-7.

Figure S1. Induction of *pmrA*, *patA,* and *patB* expression in *S. pneumoniae* exposed for 4 h to increasing concentrations of ciprofloxacin. Data are presented as the ratios of gene expression in every strain grown with and without inducer. Values are the mean ± SEM of duplicates from 2 independent experiments. Statistical analysis for the global effect of concentration on gene expression levels (Friedman test, one-way paired ANOVA, with Dunnett's post-hoc test for comparison with non-induced condition): p-value = 0.012 for *patA* and 0.001 p*atB*, and 0.2096 (NS) for *pmrA*, with $p<0.05$ for 1/4 and 1/2 MIC for *patA* and for 1/2 MIC for *patB* vs. non induced conditions.

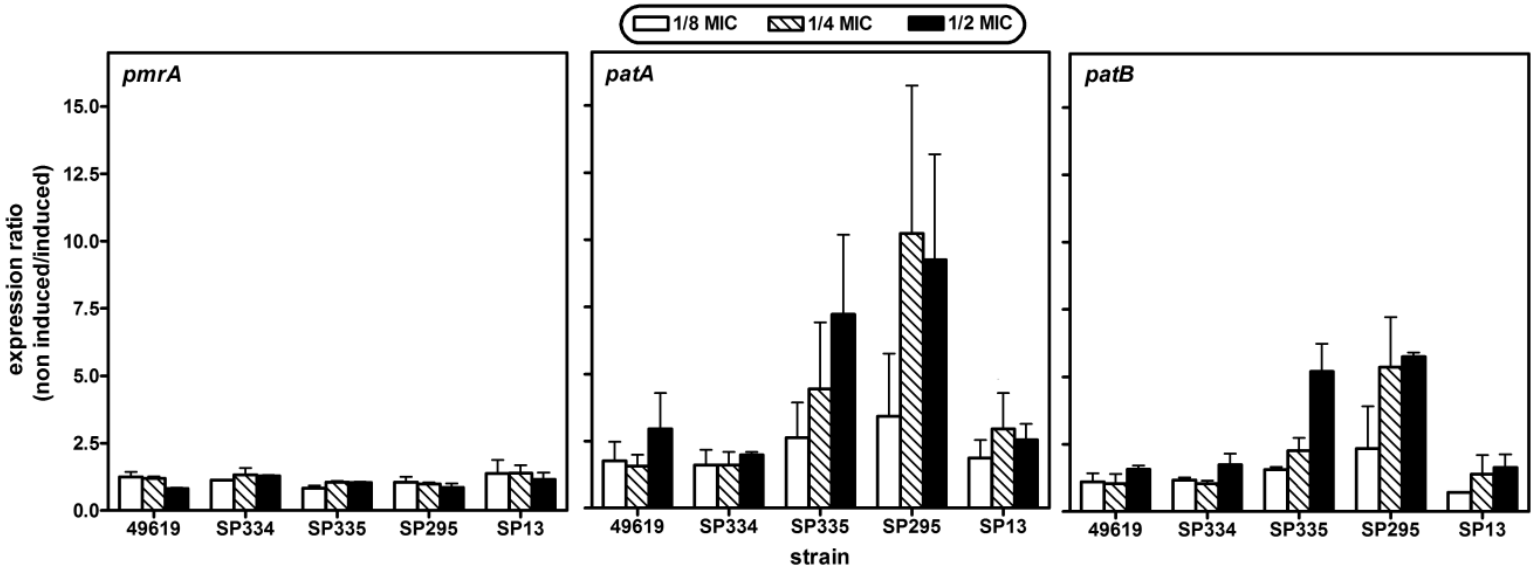


Figure S2. Evolution over time of $OD_{620\ nm}$ (left axes) and of *patA* and *patB* expression (right axes) in non induced (left panels) and induced (right panels; 1/2 x MIC of ciprofloxacin) *S. pneumoniae* ATCC49619 (top) and SP335 (bottom). Values are the means ± SEM of duplicates from 2 independent experiments.

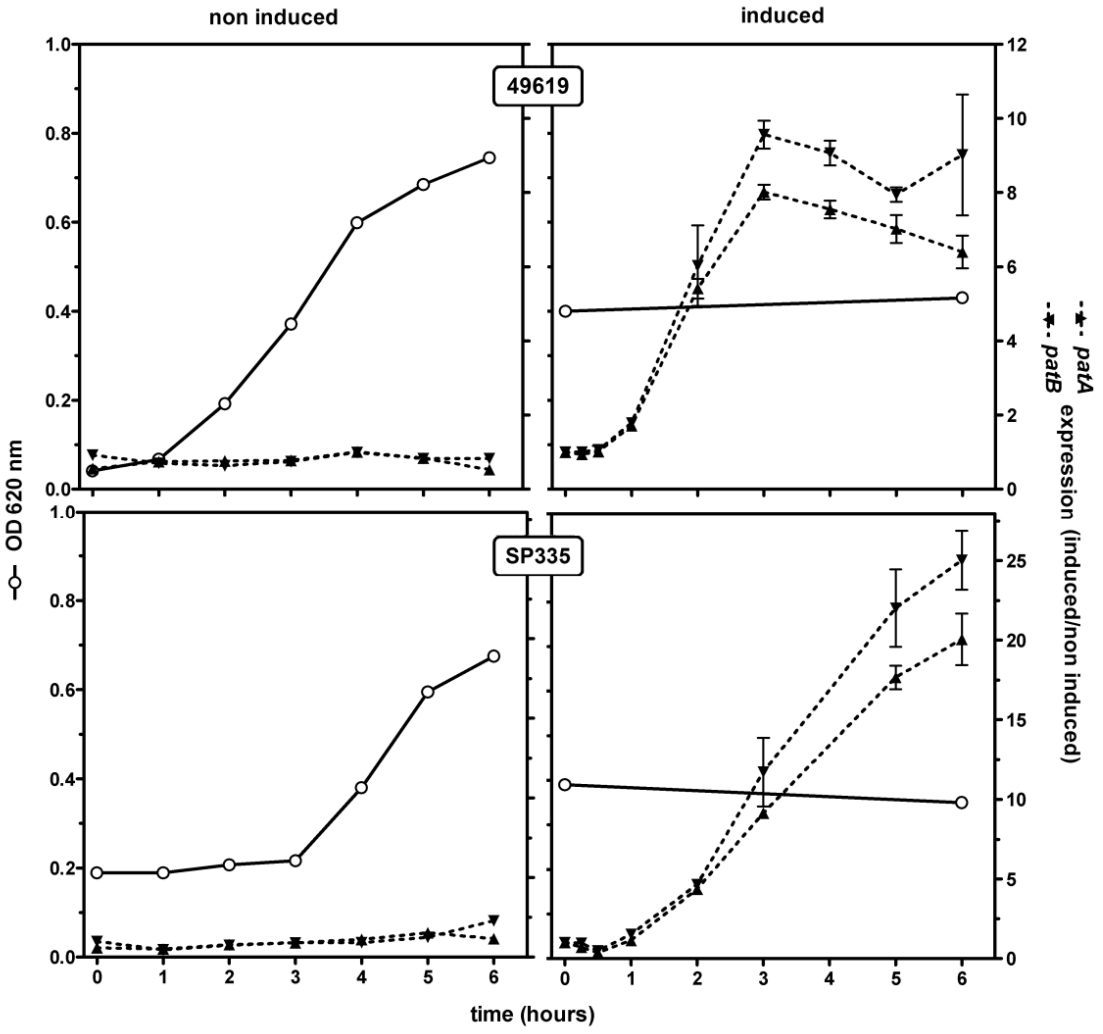
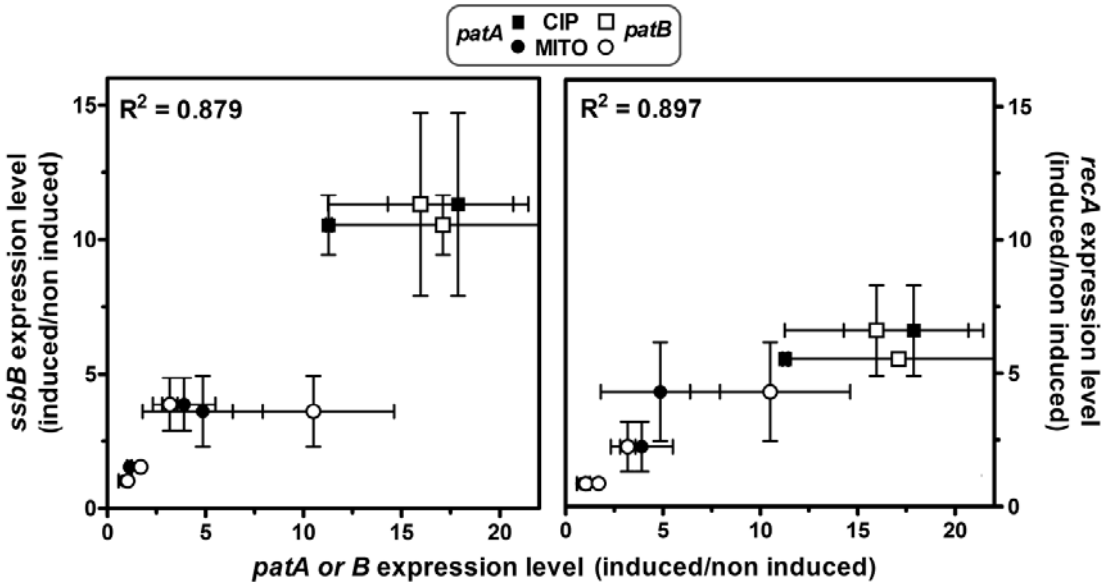


Figure S3. Relation between induction of *patA* and *patB* and of *ssbB* (left panel) and *recA* (right panel) in *S. pneumoniae* SP335 exposed for 4 h to ciprofloxacin (0.5 or 1x MIC ; higher concentrations could not be tested because of an intense bactericidal activity) or mitomycin C (0.5, 1, 10, or 100 x MIC). The data are presented as the ratios of expression measured for each strain grown in induced and non-induced conditions. Values are the means ± SEM of duplicates from 2 independent experiments. No change in the expression of the housekeeping genes was noticed, excluding a non specific effect. Correlation coefficients are calculated from linear regressions of the data.



A similar experiment performed with *S. pneumoniae* ATCC49619 (mitomycin C MIC = 0.015 mg/L) produced essentially the same results, but with lower levels of over-expression (data not shown).

4. GENERAL DISCUSSION

4.1. Main findings of this work

We assessed the situation of antibiotic resistance in Belgium in *Streptococcus pneumoniae* isolates due to community-acquired pneumonia in 2007-2009. At that time, Belgium guidelines (Belgian Antibiotic Policy Coordination Committee, 2006) in use for outpatients therapy suggested amoxicillin as first line (combined with clavulanic acid in case of comorbidities), and cefuroxime-axetil or moxifloxacin (adults only) as alternative in case of allergy to penicillin. The same recommendations were confirmed in 2012 (Belgian Antibiotic Policy Coordination Committee, 2012). Based on current MIC distributions, we stressed the fact that amoxicillin and levofloxacin should be used at high doses. This is not a problem for amoxicillin, which shows an excellent safety profile. Yet, the new cephalosporin ceftaroline may offer an interesting alternative in the future for those strains that are poorly susceptible to amoxicillin, as recently demonstrated in our laboratory (Lemaire *et al.*, 2013). This molecule has been registered for use in community acquired pneumonia in the US (2010) and in Europe (2012), based on promising clinical data (Shorr *et al.*, 2013), its positioning should be discussed in next updates of guidelines. With respect to fluoroquinolones, dose-related toxicity is clearly a potential issue, so that moxifloxacin may constitute a better alternative in this respect for this indication (Van Bambeke *et al.*, 2005; Tulkens *et al.*, 2012; Shorr *et al.*, 2013). We also noted the inappropriateness of cefuroxime-axetil in CAP treatment due to the high proportion of strains with a decreased susceptibility in that collection. As literature reports clear link between high MIC and treatment failure Buckingham *et al.*, 1998; Dowell *et al.*, 1999; Klugman, 2002), our data would support removing this antibiotic from treatment guidelines Macrolides such as clarithromycin are proposed in the guidelines when atypical pathogens are suspected but are not usable anymore for pneumococci due to high resistance rates. However, we showed that new compounds such as solithromycin (formerly known as CEM-101) are promising against *S. pneumoniae*. If the safety profile of this type of new ketolides is improved as compared to that of telithromycin (its usage being limited by rare but severe adverse events: hepatotoxicity, respiratory failure in patients with *myasthenia gravis*, QTc interval prolongation) (Van Bambeke *et al.*, 2008), they might be included in future guidelines as an appropriate alternative to amoxicillin.

At the time of the study, children were vaccinated with the PCV7. More than 70% of the serotypes isolated in children were not targeted by the PCV7. Those serotypes, 19A, 7F, 1,

6A, 3 and 5, are now included in the formulation of the PCV13, which is used in Belgium since September 2011. Serotype 6A was not present in PCV7, neither in the PPV23 vaccine used in adults. Its non-inclusion in these vaccines can be explained as a cross-immunological response was expected for this serotype by the presence of 6B (Hausdorff *et al.*, 2000b; Sun *et al.*, 2001; Reinert *et al.*, 2010). This cross-immunity has been proven to occur, but response is smaller than for serotype 6B (~80%) and is not systematic (Lee *et al.*, 2009). The same reasoning applies for the absence of 19A from PCV7, unfortunately there is almost no cross-immunity with 19F (<20%) (Whitney *et al.*, 2006; Lee *et al.*, 2009).

If we check the serotypes of isolates from children and adults of our epidemiology study, we see that the currently recommended PCV13 vaccine would have covered theoretically 72,3% of the isolates. In this study, 129 strains were of serotypes 19A, 1, 3, 5 or 7F. Serogroup 6 was only systematically subtyped for isolates coming from vaccinated patients in order to verify vaccine efficacy. Serotypes 5 and 7F were fully susceptible to all antibiotics tested. Non-susceptibility to clarithromycin was detected in 60 isolates, of which 37 were of serotypes 19A, 1 or 3. Seven strains of these serotypes were non-susceptible to amoxicillin (on a total of 14 non-susceptible isolates). All together, the serotypes newly included in the PCV13 were associated to 61.7 % of resistance to clarithromycin and 50 % of resistance to amoxicillin. Also taking the known virulence and invasive disease potential of serotypes 1, 3, 5 and 7F (Crook *et al.*, 2004; Hausdorff *et al.*, 2005; Sjostrom *et al.*, 2006; Hausdorff, 2007), the introduction of the PCV13 is definitely a big step in the battle against pneumococcal diseases.

Recently this vaccine has also been accepted in Belgium to prevent invasive pneumococcal diseases in adults ≥50 years old. Regarding our data, PCV13 would have cover 55-67% of the serotypes infecting the elderly population (≥60y) which is much lower than what PPV23 covers (58-87%) (Table 2). However, our data indicate an apparent failure of the PPV23 in half of the cases. If the PCV13 triggers a better immune response than PPV23 (Scott and Sanford, 2012), its use in elderly might be of real benefit. The PPV23 is still recommended for adults > 65years old as yet no data supports the replacement of PPV23 by PCV13 in elderly.

Clinical trials are still evaluating PCV13 response in adult population. While the immunogenicity and safety of PCV13 have been proven better or non-inferior compare to PPV23 for the serotypes that are common to both vaccines, the efficacy is still under evaluation (Hak *et al.*, 2008).

The immunogenicity of PCV13 was evaluated in different age categories (50 to >80 years of age), in pneumococcal vaccine-naïve persons as well as in adults previously vaccinated with PPV23, in various schedules of administration or combination (co-administration with

influenza vaccine, before or after PPV23 administration), and in adults with various medical conditions (HIV, transplant receivers,...). Even if many trials are not entirely completed yet, some conclusions can already be drawn.

The immune response to PCV13 is constantly greater in adults 50–59 years of age compared to older adults, which is in favour of a vaccination program starting before the age of 65 years. The immunogenicity of PCV13 is at least similar, but mainly greater, than the one of PPV23 for the 12 serotypes those vaccines have in common across all age groups (≥50y) (Jackson *et al.*, 2013c). While PPV23 is usually given once because a subsequent administration leads to a hyporesponsiveness towards many serotypes, the booster of PCV13 improves the immunogenicity towards the majority of the serotypes. The response to a subsequent administration of PCV13 is diminished when patient had a prior dose of PPV23 (but not for PCV13) (Jackson *et al.*, 2013a). On the other site, an initial vaccination with PCV13 establishes an immune state that results in recall anti-pneumococcal responses upon subsequent immunization with either PCV13 or PPV23 (Jackson *et al.*, 2013b). Therefore it would be very interesting to have an initial vaccination with PCV13, followed by one with PPV23 that would act as a booster for the 12 serotypes in common and also enlarge the serotype coverage from 13 to 24. This is currently under study.

	ST in children <5y (%)		ST (or SG) in ≥60y (%)			
Serotypes	PCV7	PCV13	PPV23	PPV23 SG	PCV13	PCV13 SG
1		13.8	4.6		4.6	
2			0			
3		10.3	16.8		16.8	
4	0	0	1.5		1.5	
5		6.9	3.1		3.1	
6A		3.4			1.5	
6B	10.3	10.3		3.1		3.1
7F		17.2	8.4		8.4	
8			1.5			
9N				3.1		
9V	3.5	3.5				3.1
10A				1.5		
11A				3.1		
12F				6.1		
14	0	0	3.1		3.1	
15B				2.3		
17F				0.8		
18C	0	0		3.1		3.1
19A		24.1	13.7	1.5	13.7	1.5
19F	0	0	1.5		1.5	
20			1.5			
22F			0.8	0.8		
23F	3.5	3.5	0.8	1.5	0.8	1.5
33F			0.8	2.3		
Sum	17.2	93.1	58.0	29.0	55.0	12.2
Theoretical coverage	17.2	93.1	58 - 87 %		55 - 67.2 %	

Table 2: Vaccine-related serotypes (ST) frequency in children under 5 years of age and in elderly (at least 60 years old), and theoretical coverage of corresponding vaccines used at the time of the study (PCV7 for children, PPV23 for elderly) and of new PCV13 in both populations.

For adults, subtyping was not systematic, therefore frequency was splitted into two columns: the “PPV23” column shows the frequency of strains for which the exact serotype is known and included in the PPV23 vaccine, while the “PPV23 SG” column shows the frequency of strains for which serogroup (SG) is known, but not the exact serotype giving an uncertainty about the theoretical vaccine coverage (which is then expressed as a range). Same applies for PCV13 in elderly.

Our studies showed that fluoroquinolone efflux is not contributing to major loss of susceptibility (<2 dilutions decrease in MIC when reserpine is added) against moxifloxacin and levofloxacin, which are used to treat pneumonia in Belgium, but that it is not the case for gemifloxacin which is used for this indication in other countries (Lode *et al.*, 2008) or for

ciprofloxacin which is no more indicated for CAP due to increasing resistance to this drug (Powis *et al.*, 2004; Schurek *et al.*, 2005). However efflux was highly prevalent in our collection: 39 and 45% of the isolates showed a detectable efflux (0.5 to 1.5 dilution) to, respectively, moxifloxacin and levofloxacin, which was largely unanticipated. A significant efflux (>2 dilutions) was observed in 10 and 16% of the isolates towards ciprofloxacin and gemifloxacin respectively.

At the molecular level, we showed that PatA and PatB are the transporters involved in the decreased susceptibility to fluoroquinolones, which was further confirmed by our coworkers on a larger collection (Garvey *et al.*, 2010), and suggested that PatA and PatB worked as an heterodimeric pump, which has been recently confirmed by Boncoeur *et al*. (Boncoeur *et al.*, 2012).

Putting together the molecular and epidemiological aspects of this study, we also showed that the fluoroquinolones tested were differently affected by efflux, but that they were all inducers of *patA* and *patB* overexpression. These data suggest thus that, even though not visible in the routine laboratory, resistance mechanisms to fluoroquinolones do exist in clinical isolates and can be further modulated upon exposure to these drugs, calling for a prudent use thereof.

These concepts will need to be taken into consideration for design of new molecules.

4.2. Limitations of this study

Representativeness of strains collected:

As our main objectives were to challenge the guidelines to treat pneumococcal pneumonia and to have an overview of antibiotic resistance situation in this population, we needed a collection of *Streptococcus pneumoniae* isolates that represent the infectious strains causing the disease. To increase our chances of collecting such isolates rather than carried strains, we restricted the collection to samples coming from blood or lower respiratory tract. As those kinds of sampling are only performed at the hospital, this excluded the possibility to have isolates from mild pneumococcal pneumonia treated only at home. So, by design, we decided to only enrol patients admitted to hospital, knowing those will have more severe pneumonia and, therefore, the collection will be biased from the isolates causing mild pneumonia.

However, more than half of the patients (53 %) came directly to the hospital without a first visit to their general practitioner, including adults with mild pneumonia. Only 10 % of the adults had severe pneumonia requiring hospitalization in ICU. As most cases were of moderate severity, corresponding to situations where the same antibiotics are used for home therapy, we believe the findings of the study are relevant for the whole population.

Relevance of efflux in resistance:

One of our main objectives was to study the effect of a previous antibiotic treatment on antimicrobial resistance, in particular the prevalence of efflux mechanism. Only 36 of the 249 patients had received previous antimicrobials therapy, among them 2 had a fluoroquinolone and 5 had a macrolide alone or in combination. Due to this small number, we failed to accomplish this objective. Community-acquired pneumonia is an acute illness. During this study we discovered that general practitioners direct the majority of their patients to the hospital when the pneumonia seems severe or when patient has risk factors. Therefore, most of the patients included in this study, did not take any antibiotic prior hospitalization, but treatment was initiated directly in hospital. To reach our goal, we should have taken clinical isolates from a recurrent disease that needs frequent antimicrobials treatment (which favors development of resistance), such as the chronic obstructive bronchopneumopathy (COBP). The laboratory has now started collecting strains from COBP to examine prevalence of resistance.

Another limitation is that we could not collect data about antibiotic treatment given at the hospital to treat the pneumonia. Therefore we do not know if guidelines were followed, or if treatment was adjusted for non-susceptible strains, or if the resistance led to complications or clinical failures.

Overexpression of fluoroquinolone transporters:

We had promising results in 3 laboratory strains and 2 clinical isolates regarding the expression of fluoroquinolones transporters. We quickly faced some technical issues while extending the methodology to the rest of the clinical isolates.

For the disruption experiments, two variants of the competence stimulating peptide (CSP) are described (Havarstein *et al.*, 1995; Pozzi *et al.*, 1996), each of them induces competence only when compatible variant of the receptor, ComD, is expressed. As Pozzi *et al*. showed, it is more difficult to induce competence in encapsulated strains: half of his strains remained not competent despite the use of CSP-1 and CSP-2 (Pozzi *et al.*, 1996). This might explain the difficulty to disrupt any of the transporters in some clinical isolates. On the other hand, even after various attempt *pmrA* could not be disrupted in some strains while we could disrupt *patA* and *patB* (such as SP-207), suggesting that PmrA might be crucial for those isolates.

For the quantification of gene expression by real-time PCR, these experiments were set up using two laboratory strains (ATCC 49619, R6) and their respective mutants: culture conditions, choice of control strain for relative quantification and selection of housekeeping genes. Experiments were reproducible for those strains and for the few first clinical isolates

tested, but while extending to a larger number we found that the two housekeeping genes selected, *rpoD* and *proC*, had different level of expression in the various clinical isolates.
In control strains, there were approximately two copies of *rpoD* mRNA for one of *proC*, giving a ratio of 2. In the clinical isolates this ratio can vary from 0.5 to 5. To solve this issue, we looked for a third housekeeping gene. According to Genorm/PrimerDesign analysis, *hexA* is the next best housekeeping gene with a stable expression level between the three strains used for the analysis. However, this gene is located exactly next to *patA* on the pneumococcal chromosome. Co-translation of *hexA*, *patA* and *patB* within same operon cannot be excluded. The three genes might also share the same regulator. Marrer *et al.* showed that *patA* and *patB* expression was correlated with those of genes involved in competence pathway, DNA mismatch repair or replication (Marrer *et al.*, 2006a). HexA protein is involved in DNA mismatch repair. So we cannot use *hexA* as a third housekeeping gene. In order to solve this issue we should send new cDNA from various clinical isolates to Genorm to perform a new selection of housekeeping gene candidates.

4.3. Clinical interest of this study

Our data clearly illustrate that treatment guidelines as well as vaccine development programs should take into account recent epidemiological surveys. Resistance is evolving over time, due to antibiotic usage and spread of specific clones. In its turn, vaccination can cause serotype replacement and we have seen that resistance is often associated to specific serotypes. The regional character of these studies is essential, because resistance rates are highly dependent on local clinical practices.

In Belgium two reference laboratories are collecting *Streptococcus pneumoniae* isolates. In J. Verhaegen's laboratory, antimicrobial susceptibilities are assessed via disc diffusion method. But a comparison of MICs with the collection of R. Vanhoof could be performed (Figure 8). We compared our strains with the ones received in 2008 by R. Vanhoof. MIC distributions are very similar in the two populations. They are even equivalent for the non-susceptible strains, but the MIC_{50} is usually higher in our collection. They remained however in the wild-type range of MICs. Even if some isolates might be common to both collections, this difference may be explained by the fact that our collection consists of isolates coming exclusively from community-acquired pneumonia, that were invasive or not, while R. Vanhoof's collection is composed of invasive isolates coming from various invasive diseases (bacteraemia, sepsis, pneumonia or meningitis).
As some serotypes are known to be more invasive and as antibiotic susceptibilities are linked to serotypes, this could explain the difference in MIC_{50} seen between those two populations.

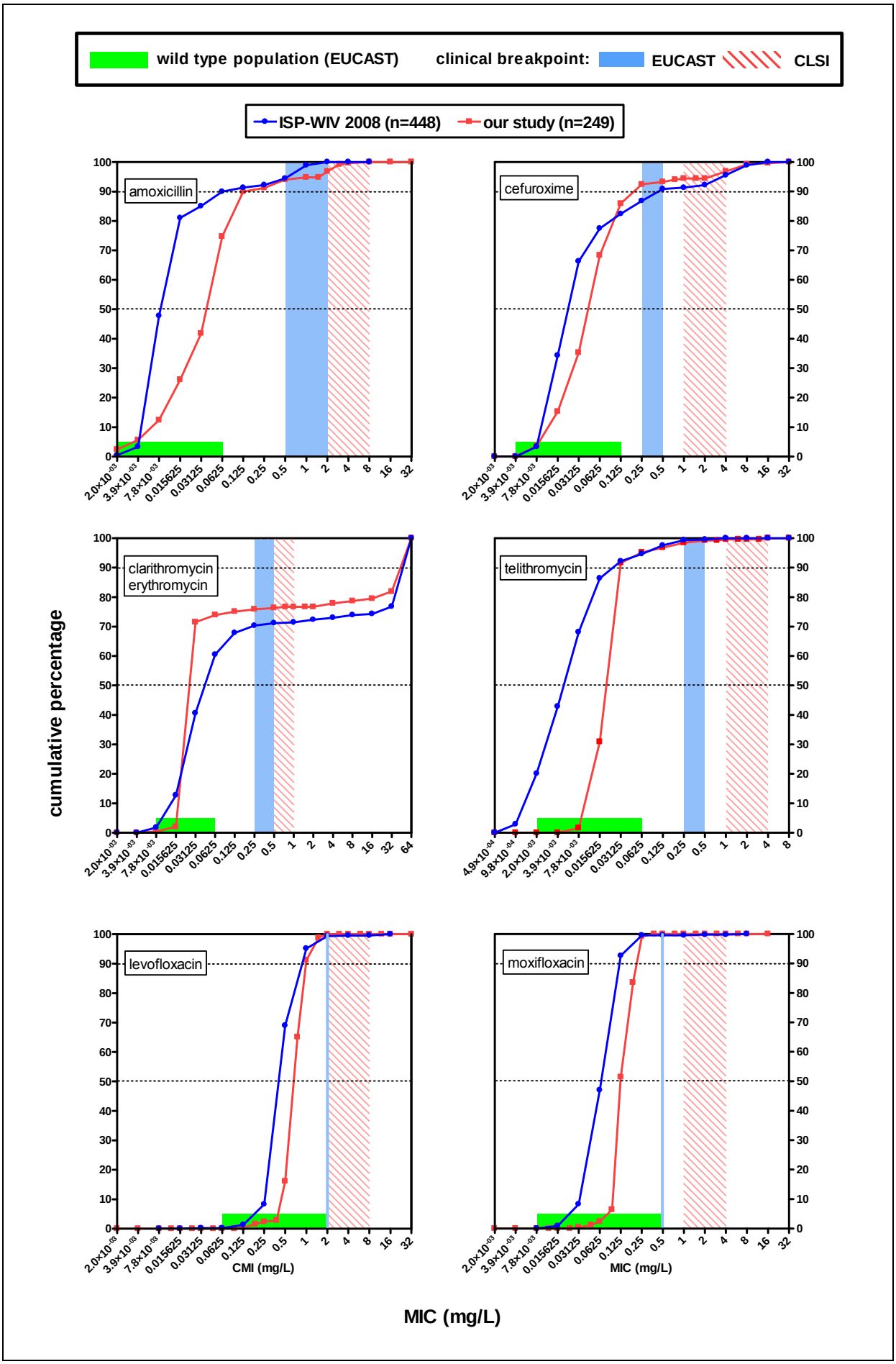

wild type population (EUCAST)
clinical breakpoint:
EUCAST
CLSI
ISP-WIV 2008 (n=448)
our study (n=249)
amoxicillin
cefuroxime
clarithromycin
erythromycin
telithromycin
levofloxacin
moxifloxacin
cumulative percentage
CMI (mg/L)
MIC (mg/L)
MIC (mg/L)

Figure 8: MIC distributions (cumulative percentages) of non-duplicate *S. pneumoniae* isolates for amoxicillin, cefuroxime (breakpoints are for oral form, cefuroxime-axetil)**, erythromycin** (for ISP-WIV strains)**, clarithromycin** (for our collection), **telithromycin, levofloxacin, and moxifloxacin.** The horizontal green zone corresponds to the range of MICs covered by the wild type population as defined by EUCAST. The blue and hatched red vertical zones corresponds to the MIC range of susceptible to resistant clinical breakpoints defined by EUCAST and CLSI respectively.

Our study, together with similar ones performed recently in our laboratory with other drugs in development, may also contribute to help for the positioning of new molecules in the clinics. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) defines clinical breakpoints of susceptibility as the level of antimicrobial activity associated with a high likelihood of therapeutic success (http://www.srga.org/Eucastwt/eucastdefinitions.htm) . The procedure for determining these breakpoints includes the performance of MICs distribution, including strains with known resistance mechanisms (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/EUCAST_SOPs/EUCAST_SOP_1._1_Setting_breakpoints_new_agents_1_June_2013.pdf), which is then combined with pharmacokinetic, pharmacodynamic, and clinical data. A collection like the one used here offers a specific opportunity for testing new drugs, as it offers a unique opportunity to use isolates collected from a single pathology, and for which susceptibility to a large panel of drugs is already known as well as the prevalence of some specific mechanisms of resistance.

4.4. Perspectives

As long as *Streptococcus pneumoniae* remains a major human pathogen, epidemiological surveys should not stop. Each country needs to follow antimicrobial resistance over time to confront the guidelines on a regular basis and to update them or confirm their applicability.

As we rely on vaccines targeting only certain capsular polysaccharides, serotyping is needed as well to update regularly the vaccine formulation to match the clinical and epidemiological situation. With the use of PCV13 in children since September 2011 in Belgium, we can expect a switch of serotypes in the following years. It would be interesting to analyse if the resistance to antibiotics will also be affected and decrease as part of the newly included serotypes (1, 3, 5, 6A, 7F and 19A) which are largely involved in the resistance. Among strains of serogroup 19A, ~73% were non-susceptible to clarithromycin, accounting for 10% of the global resistance to macrolides. All together, those serotypes specific of PCV13 were

responsible of ~65 and ~50% of the reported non-susceptibility to clrarithromycin and amoxicillin, respectively. Some new vaccines targeting other antigens (e.g. the Pneumococcal surface protein A, PspA) are in development.
When these will be used, those antigens will need to be closely monitored as the pneumococcus is an extremely adaptable pathogen and will surely find a way to escape those new vaccines.
In order to understand the pneumococcal evolution and foresee some changes, molecular typing method (such as multilocus sequence typing) might be of great interest. It was demonstrated that the majority of pneumococcal diseases are actually due to a restricted number of clones. This explains the relation between serotypes and susceptibility to antibiotics. Molecular typing also takes the genetic background into account, which is of importance for the expression of some virulence factors (Imai *et al.*, 2011; Donkor *et al.*, 2012).
Strains coming from carriage should not be ignored from surveys. Even if some strains are rarely involved in infections, they are still representing a genetic reservoir.
Ideally further surveillance should thus monitor antimicrobials resistance, serotyping and genotyping of all isolates from infections, whether invasive or not, as well as isolates from carriage.

The PCV13 is now available for adults (≥50 years old) and has a proven better immunogenicity (for the serotypes in common) than PPV23. Its efficacy might be higher than for PPV23 alone (currently under study), a combination of immunization by PCV13 followed by a PPV23 would be very interesting. But, as seen earlier, the vaccination rates in elderly population is very low (20% in our CAP elderly population). This is really surprising as vaccination is the best way to protect against invasive pneumococcal infections and their consequences. It would be interesting to know why that rate is so low. Various reasons can be raised: the vaccine prize (~30 € for PPV23, ~75 € for PCV13), the non-reimbursement (for both), the mediocre reputation of the PPV23, the partial coverage (only 13 or 23 serotypes covered out of >90), the lack of hindsight concerning PCV13 (Is a booster needed? When?), and more general reasons like the fact that people don't feel the need to be vaccinate as long as they are not ill, general mistrust of the public towards vaccines… It would be interesting to identify those reasons in order to improve vaccination rate in adults. A survey conducted among adults ≥50 years of age, general practitioners or pharmacists might help solving this issue.

In this study, we faced the difficulty of correlating resistance with previous antibiotic usage. Repeating the same type of study but focusing on patients that are chronically or recurrently

infected may be helpful in this respect. This could be the case in recurrent otitis media or in acute exacerbations of chronic obstructive pulmonary disease. In the perspective of studying resistance by efflux, acute exacerbations of chronic obstructive pulmonary disease would appear as a better target, because fluoroquinolones are proposed as alternative to amoxicillin-clavulanate in Belgian guidelines for this pathology (Service Public Fédéral Belge Santé Publique, 2008) while their use is not recommended in children who suffer more frequently than adult from otitis. The laboratory has started to collect such strains, study their susceptibility to antibiotic, and their serotype in relation with their ability to form biofilms (Vandevelde *et al.*, 2013).

Concerning the transporters PatA and PatB, it would be useful to continue trying to disrupt the pumps in clinical isolates, to measure the MICs of the strains and their respective mutants to the fluoroquinolones and dyes used previously, and to quantify the expression of the three transporters. All these data could be used to perform multivariate analysis in order to determine if another pump could affect efflux of fluoroquinolones in clinical isolates, as recently suggested for levofloxacin (Tocci *et al.*, 2013). Even if PmrA does not seem to be strongly involved, this should be demonstrated with a larger number of clinical isolates.
Moreover, little is known about those transporters. What is their original function? What are the usual substrates of those transporters? PatA and PatB form a functional efflux pump as heterodimer, but do they have any function as monomers? What are the regulation mechanisms behind the over-expresssion of *patA* and *patB*? What is the cost for a strain over-expressing those genes? All these questions remain open, and would need to be addressed using appropriate molecular biology approaches.

5. REFERENCES

Abdullahi O, Karani A, Tigoi C C, Mugo D, Kungu S, Wanjiru E, Jomo J, Musyimi R, Lipsitch M and Scott J A (2012) Rates of acquisition and clearance of pneumococcal serotypes in the nasopharynges of children in Kilifi District, Kenya. *J Infect Dis* **206**: 1020-1029. PubMed: PM:22829650.

Ackermann G and Rodloff A C (2003) Drugs of the 21st century: telithromycin (HMR 3647)--the first ketolide. *J Antimicrob Chemother* **51**: 497-511. PubMed: PM:12615850.

Akova M (2008) Sulbactam-containing beta-lactamase inhibitor combinations. *Clin Microbiol Infect* **14 Suppl 1**: 185-188. PubMed: PM:18154545.

Alloing G, Martin B, Granadel C and Claverys J P (1998) Development of competence in Streptococcus pneumonaie: pheromone autoinduction and control of quorum sensing by the oligopeptide permease. *Mol Microbiol* **29**: 75-83. PubMed: PM:9701804.

Alonso M, Marimon J M, Ercibengoa M, Perez-Yarza E G and Perez-Trallero E (2013) Dynamics of Streptococcus pneumoniae serotypes causing acute otitis media isolated from children with spontaneous middle-ear drainage over a 12-year period (1999-2010) in a region of northern Spain. *PLoS One* **8**: e54333. PubMed: PM:23349853.

Ambrose K and Stephens D S (2004) chap 22, Macrolide, Quinolone, and Other Non-beta-Lactam Antibiotic Resistance in Streptococcus pneumoniae. In *The Pneumococcus* (Tuomanen E ed) ASM press, Washington, DC.

Avrain L, Garvey M, Mesaros N, Glupczynski Y, Mingeot-Leclercq M P, Piddock L J, Tulkens P M, Vanhoof R and Van Bambeke F (2007) Selection of quinolone resistance in Streptococcus pneumoniae exposed in vitro to subinhibitory drug concentrations. *J Antimicrob Chemother* **60**: 965-972. PubMed: PM:17693451.

Barocchi MA, Ries J, Zogaj X, Hemsley C, Albiger B, Kanth A, Dahlberg S, Fernebro J, Moschioni M, Masignani V, Hultenby K, Taddei A R, Beiter K, Wartha F, von Euler A, Covacci A, Holden D W, Normark S, Rappuoli R and Henriques-Normark B (2006) A pneumococcal pilus influences virulence and host inflammatory responses. *Proc Natl Acad Sci U S A* **103**: 2857-2862. PubMed: PM:16481624.

Belgian Antibiotic Policy Coordination Committee (2006) *Guide Belge Des Traitements Anti-Infectieux En Pratique Ambulatoire (Édition 2006)*. Service Public Fédéral Santé Publique, Sécurité de la Chaîne Alimentaire et Environnement, Bruxelles.

Belgian Antibiotic Policy Coordination Committee (2012) *Guide Belge Des Traitements Anti-Infectieux En Pratique Ambulatoire (Édition 2012)*. Service Public Fédéral Santé Publique, Sécurité de la Chaîne Alimentaire et Environnement, Bruxelles.

Bemer-Melchior P, Juvin M E, Tassin S, Bryskier A, Schito G C and Drugeon H B (2000) In vitro activity of the new ketolide telithromycin compared with those of macrolides against Streptococcus pyogenes: influences of resistance mechanisms and methodological factors. *Antimicrob Agents Chemother* **44**: 2999-3002. PubMed: PM:11036012.

Bergmann C, Chi F, Rachid S and Hakenbeck R (2004) chap 21, Mechanisms for Penicillin Resistance in Streptococcus pneumoniae: Penicillin Binding Proteins, Gene Transfer, and Cell Wall Metabolism. In *The Pneumococcus* (Tuomanen E ed) ASM press, Washington, DC.

Bertrand D, Bertrand S, Neveu E and Fernandes P (2010) Molecular characterization of off-target activities of telithromycin: a potential role for nicotinic acetylcholine receptors. *Antimicrob Agents Chemother* **54**: 5399-5402. PubMed: PM:20855733.

Black S, Shinefield H, Fireman B, Lewis E, Ray P, Hansen J R, Elvin L, Ensor K M, Hackell J, Siber G, Malinoski F, Madore D, Chang I, Kohberger R, Watson W, Austrian R and Edwards K (2000) Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. *Pediatr Infect Dis J* **19**: 187-195. PubMed: PM:10749457.

Blasi F, Mantero M, Santus P and Tarsia P (2012) Understanding the burden of pneumococcal disease in adults. *Clin Microbiol Infect* **18 Suppl 5**: 7-14. PubMed: PM:22882668.

Blondeau JM and Tillotson G (2008) Role of gemifloxacin in the management of community-acquired lower respiratory tract infections. *Int J Antimicrob Agents* **31**: 299-306. PubMed: PM:18276120.

Bolan G, Broome C V, Facklam R R, Plikaytis B D, Fraser D W and Schlech W F, III (1986) Pneumococcal vaccine efficacy in selected populations in the United States. *Ann Intern Med* **104**: 1-6. PubMed: PM:3940476.

Boncoeur E, Durmort C, Bernay B, Ebel C, Di Guilmi A M, Croize J, Vernet T and Jault J M (2012) PatA and PatB form a functional heterodimeric ABC multidrug efflux transporter responsible for the resistance of Streptococcus pneumoniae to fluoroquinolones. *Biochemistry* **51**: 7755-7765. PubMed: PM:22950454.

Brueggemann AB, Griffiths D T, Meats E, Peto T, Crook D W and Spratt B G (2003) Clonal relationships between invasive and carriage Streptococcus pneumoniae and serotype- and clone-specific differences in invasive disease potential. *J Infect Dis* **187**: 1424-1432. PubMed: PM:12717624.

Buckingham SC, Brown S P and Joaquin V H (1998) Breakthrough bacteremia and meningitis during treatment with cephalosporins parenterally for pneumococcal pneumonia. *J Pediatr* **132**: 174-176. PubMed: PM:9470026.

Butler JC (2004) chap 10, Epidemiology of Pneumococcal Disease. In *The Pneumococcus* (Tuomanen E ed) ASM press, Washington,D.C.

Canu A, Malbruny B, Coquemont M, Davies T A, Appelbaum P C and Leclercq R (2002) Diversity of ribosomal mutations conferring resistance to macrolides, clindamycin, streptogramin, and telithromycin in Streptococcus pneumoniae. *Antimicrob Agents Chemother* **46**: 125-131. PubMed: PM:11751122.

Chidiac C (2012) Pneumococcal infections and adult with risk factors. *Med Mal Infect* **42**: 517-524. PubMed: PM:23099069.

Ciruela P, Soldevila N, Selva L, Hernandez S, Garcia-Garcia J J, Moraga F, de Sevilla M F, Codina G, Planes A M, Esteva C, Coll F, Cardenosa N, Jordan I, Batalla J, Salleras L, Munoz-Almagro C and Dominguez A (2013) Are risk factors associated with invasive pneumococcal disease according to different serotypes? *Hum Vaccin Immunother* **9**. PubMed: PM:23295982.

Cochetti I, Vecchi M, Mingoia M, Tili E, Catania M R, Manzin A, Varaldo P E and Montanari M P (2005) Molecular characterization of pneumococci with efflux-mediated erythromycin resistance and identification of a novel mef gene subclass, mef(I). *Antimicrob Agents Chemother* **49**: 4999-5006. PubMed: PM:16304164.

Crook DW, Brueggemann A B, Sleeman K and Peto T E A (2004) chap 9, Pneumococcal Carriage. In *The Pneumococcus* (Tuomanen E ed) ASM press, Washington, DC.

Davidson R, Cavalcanti R, Brunton J L, Bast D J, de Azavedo J C, Kibsey P, Fleming C and Low D E (2002) Resistance to levofloxacin and failure of treatment of pneumococcal pneumonia. *N Engl J Med* **346**: 747-750. PubMed: PM:11882730.

Del Grosso M, Iannelli F, Messina C, Santagati M, Petrosillo N, Stefani S, Pozzi G and Pantosti A (2002) Macrolide efflux genes mef(A) and mef(E) are carried by different genetic elements in Streptococcus pneumoniae. *J Clin Microbiol* **40**: 774-778. PubMed: PM:11880392.

Depardieu F and Courvalin P (2001) Mutation in 23S rRNA responsible for resistance to 16-membered macrolides and streptogramins in Streptococcus pneumoniae. *Antimicrob Agents Chemother* **45**: 319-323. PubMed: PM:11120988.

Dockrell DH, Whyte M K and Mitchell T J (2012) Pneumococcal pneumonia: mechanisms of infection and resolution. *Chest* **142**: 482-491. PubMed: PM:22871758.

Donkor ES (2013) Understanding the pneumococcus: transmission and evolution. *Front Cell Infect Microbiol* **3**: 7. PubMed: PM:23471303.

Donkor ES, Stabler R A, Hinds J, Adegbola R A, Antonio M and Wren B W (2012) Comparative phylogenomics of Streptococcus pneumoniae isolated from invasive disease and nasopharyngeal carriage from West Africans. *BMC Genomics* **13**: 569. PubMed: PM:23107513.

Douthwaite S (2001) Structure-activity relationships of ketolides vs. macrolides. *Clin Microbiol Infect* **7 Suppl 3**: 11-17. PubMed: PM:11523556.

Dowell SF, Smith T, Leversedge K and Snitzer J (1999) Failure of treatment of pneumonia associated with highly resistant pneumococci in a child. *Clin Infect Dis* **29**: 462-463. PubMed: PM:10476773.

Edelstein PH (2004) Pneumococcal resistance to macrolides, lincosamides, ketolides, and streptogramin B agents: molecular mechanisms and resistance phenotypes. *Clin Infect Dis* **38 Suppl 4**: S322-S327. PubMed: PM:15127365.

Edwards KM (2004) chap 19, Pneumococcal Infections: Therapeutic Strategies and Pitfalls. (Tuomanen E ed) ASM press, Washington,D.C.

El Garch F, Lismond A, Piddock L J, Courvalin P, Tulkens P M and Van Bambeke F (2010) Fluoroquinolones induce the expression of patA and patB, which encode ABC efflux pumps in Streptococcus pneumoniae. *J Antimicrob Chemother* **65**: 2076-2082. PubMed: PM:20709735.

Eskola J, Kilpi T, Palmu A, Jokinen J, Haapakoski J, Herva E, Takala A, Kayhty H, Karma P, Kohberger R, Siber G and Makela P H (2001) Efficacy of a pneumococcal conjugate vaccine against acute otitis media. *N Engl J Med* **344**: 403-409. PubMed: PM:11172176.

Farrell DJ, Castanheira M, Sader H S and Jones R N (2010) The in vitro evaluation of solithromycin (CEM-101) against pathogens isolated in the United States and Europe (2009). *J Infect* **61**: 476-483. PubMed: PM:20831882.

Farrell DJ, Mendes R E, Ross J E, Sader H S and Jones R N (2011) LEADER Program results for 2009: an activity and spectrum analysis of linezolid using 6,414 clinical isolates from 56 medical centers in the United States. *Antimicrob Agents Chemother* **55**: 3684-3690. PubMed: PM:21670176.

Feikin DR and Klugman K P (2002) Historical changes in pneumococcal serogroup distribution: implications for the era of pneumococcal conjugate vaccines. *Clin Infect Dis* **35**: 547-555. PubMed: PM:12173128.

File TM, Jr., Garau J, Blasi F, Chidiac C, Klugman K, Lode H, Lonks J R, Mandell L, Ramirez J and Yu V (2004) Guidelines for empiric antimicrobial prescribing in community-acquired pneumonia. *Chest* **125**: 1888-1901. PubMed: PM:15136404.

Flamaing J, Verhaegen J, Vandeven J, Verbiest N and Peetermans W E (2008) Pneumococcal bacteraemia in Belgium (1994 2004): the pre-conjugate vaccine era. *J Antimicrob Chemother* **61**: 143-149. PubMed: PM:17999974.

Fuller JD and Low D E (2005) A review of Streptococcus pneumoniae infection treatment failures associated with fluoroquinolone resistance. *Clin Infect Dis* **41**: 118-121. PubMed: PM:15937772.

Garvey MI, Baylay A J, Wong R L and Piddock L J (2010) Over-expression of patA and patB, which encode ABC transporters, is associated with fluoroquinolone resistance in clinical isolates of Streptococcus pneumoniae. *Antimicrob Agents Chemother*. PubMed: PM:20937787.

Garvey MI and Piddock L J (2008) The efflux pump inhibitor reserpine selects multidrug-resistant Streptococcus pneumoniae strains that overexpress the ABC transporters PatA and PatB. *Antimicrob Agents Chemother* **52**: 1677-1685. PubMed: PM:18362193.

Gay K and Stephens D S (2001) Structure and dissemination of a chromosomal insertion element encoding macrolide efflux in Streptococcus pneumoniae. *J Infect Dis* **184**: 56-65. PubMed: PM:11398110.

Gill MJ, Brenwald N P and Wise R (1999) Identification of an efflux pump gene, pmrA, associated with fluoroquinolone resistance in Streptococcus pneumoniae. *Antimicrob Agents Chemother* **43**: 187-189. PubMed: PM:9869592.

Gordon C, Regamey C and Kirby W M (1972) Comparative clinical pharmacology of amoxicillin and ampicillin administered orally. *Antimicrob Agents Chemother* **1**: 504-507. PubMed: PM:4680813.

Grebe T and Hakenbeck R (1996) Penicillin-binding proteins 2b and 2x of Streptococcus pneumoniae are primary resistance determinants for different classes of beta-lactam antibiotics. *Antimicrob Agents Chemother* **40**: 829-834. PubMed: PM:8849235.

Gwaltney JMJ (2005) chap 55, Sinusitis. In *Principles and Practice of Infectious Diseases* (Mandell G, Bennett JE and Dolin R eds) pp 772-783, Elsevier Churchill Livingstone, Philadelphia, PA.

Hak E, Grobbee D E, Sanders E A, Verheij T J, Bolkenbaas M, Huijts S M, Gruber W C, Tansey S, McDonough A, Thoma B, Patterson S, van Alphen A J and Bonten M J (2008) Rationale and design of CAPITA: a RCT of 13-valent conjugated pneumococcal vaccine efficacy among older adults. *Neth J Med* **66**: 378-383. PubMed: PM:18990781.

Harboe ZB, Benfield T L, Valentiner-Branth P, Hjuler T, Lambertsen L, Kaltoft M, Krogfelt K, Slotved H C, Christensen J J and Konradsen H B (2010) Temporal trends in invasive pneumococcal disease and pneumococcal serotypes over 7 decades. *Clin Infect Dis* **50**: 329-337. PubMed: PM:20047478.

Hausdorff WP (2007) The roles of pneumococcal serotypes 1 and 5 in paediatric invasive disease. *Vaccine* **25**: 2406-2412. PubMed: PM:17055620.

Hausdorff WP, Bryant J, Kloek C, Paradiso P R and Siber G R (2000a) The contribution of specific pneumococcal serogroups to different disease manifestations: implications for conjugate vaccine formulation and use, part II. *Clin Infect Dis* **30**: 122-140. PubMed: PM:10619741.

Hausdorff WP, Bryant J, Paradiso P R and Siber G R (2000b) Which pneumococcal serogroups cause the most invasive disease: implications for conjugate vaccine formulation and use, part I. *Clin Infect Dis* **30**: 100-121. PubMed: PM:10619740.

Hausdorff WP, Feikin D R and Klugman K P (2005) Epidemiological differences among pneumococcal serotypes. *Lancet Infect Dis* **5**: 83-93. PubMed: PM:15680778.

Havarstein LS, Coomaraswamy G and Morrison D A (1995) An unmodified heptadecapeptide pheromone induces competence for genetic transformation in Streptococcus pneumoniae. *Proc Natl Acad Sci U S A* **92**: 11140-11144. PubMed: PM:7479953.

Ho PL, Cheng V C and Chu C M (2009) Antibiotic resistance in community-acquired pneumonia caused by Streptococcus pneumoniae, methicillin-resistant Staphylococcus aureus, and Acinetobacter baumannii. *Chest* **136**: 1119-1127. PubMed: PM:19809053.

Iannini PB, Paladino J A, Lavin B, Singer M E and Schentag J J (2007) A case series of macrolide treatment failures in community acquired pneumonia. *J Chemother* **19**: 536-545. PubMed: PM:18073153.

Imai S, Ito Y, Ishida T, Hirai T, Ito I, Yoshimura K, Maekawa K, Takakura S, Niimi A, Iinuma Y, Ichiyama S and Mishima M (2011) Distribution and clonal relationship of cell surface virulence genes among Streptococcus pneumoniae isolates in Japan. *Clin Microbiol Infect* **17**: 1409-1414. PubMed: PM:21143699.

Jackson LA, Gurtman A, Rice K, Pauksens K, Greenberg R N, Jones T R, Scott D A, Emini E A, Gruber W C and Schmoele-Thoma B (2013a) Immunogenicity and safety of a 13-valent pneumococcal conjugate vaccine in adults 70 years of age and older previously vaccinated with 23-valent pneumococcal polysaccharide vaccine. *Vaccine* **31**: 3585-3593. PubMed: PM:23688527.

Jackson LA, Gurtman A, van Cleeff M, Frenck R W, Treanor J, Jansen K U, Scott D A, Emini E A, Gruber W C and Schmoele-Thoma B (2013b) Influence of initial vaccination with 13-valent pneumococcal conjugate vaccine or 23-valent pneumococcal polysaccharide vaccine on anti-pneumococcal responses following subsequent pneumococcal vaccination in adults 50 years and older. *Vaccine* **31**: 3594-3602. PubMed: PM:23688525.

Jackson LA, Gurtman A, van Cleeff M, Jansen K U, Jayawardene D, Devlin C, Scott D A, Emini E A, Gruber W C and Schmoele-Thoma B (2013c) Immunogenicity and safety of a 13-valent pneumococcal conjugate vaccine compared to a 23-valent pneumococcal polysaccharide vaccine in pneumococcal vaccine-naive adults. *Vaccine* **31**: 3577-3584. PubMed: PM:23688526.

Jacobs MR (2007) Clinical significance of antimicrobial resistance in Streptococcus pneumoniae. *S Afr Med J* **97**: 1133-1140. PubMed: PM:18250924.

Johnsborg O and Havarstein L S (2009) Regulation of natural genetic transformation and acquisition of transforming DNA in Streptococcus pneumoniae. *FEMS Microbiol Rev* **33**: 627-642. PubMed: PM:19396959.

Käyhty H and Mäkelä H (2004) chap 25, Vaccine-Induced Immunity. In *The Pneumococcus* (Tuomanen E ed) ASM press, Washington, DC.

Keam SJ, Croom K F and Keating G M (2005) Gatifloxacin: a review of its use in the treatment of bacterial infections in the US. *Drugs* **65**: 695-724. PubMed: PM:15748100.

Klein JO (2005) chap 54, Otitis Externa, Otitis Media, and Mastoiditis. In *Principles and Practice of Infectious Diseasses* (Mandell G, Bennett JE and Dolin R eds) pp 766-772, Elsevier Churchill Livingstone, Philadelphia, PA.

Klugman KP (2002) Bacteriological evidence of antibiotic failure in pneumococcal lower respiratory tract infections. *Eur Respir J Suppl* **36**: 3s-8s. PubMed: PM:12168746.

Klugman KP (2004) chap 20, Clinical Relevance of Antibiotic Resistance in Pneumococcal Infections. In *The Pneumococcus* (Tuomanen E ed) ASM press, Washington, DC.

Klugman KP (2007) Clinical impact of antibiotic resistance in respiratory tract infections. *Int J Antimicrob Agents* **29 Suppl 1**: S6-10. PubMed: PM:17307654.

Lacks SA (2004) chap 7, Transformation. In *The Pneumococcus* (Tuomanen E ed) ASM press, Washington, DC.

Leclercq R and Courvalin P (2002) Resistance to macrolides and related antibiotics in Streptococcus pneumoniae. *Antimicrob Agents Chemother* **46**: 2727-2734. PubMed: PM:12183222.

Lee H, Nahm M H, Burton R and Kim K H (2009) Immune response in infants to the heptavalent pneumococcal conjugate vaccine against vaccine-related serotypes 6A and 19A. *Clin Vaccine Immunol* **16**: 376-381. PubMed: PM:19144787.

Lemaire, S., van der Linden, M., and Tulkens, P. M. (2013) 28th International Congress on Chemotherapy and Infection, Yokoama, Japan , Abstract 105.

Lewis KS (1990) Chronic illness and marriage: endings and beginnings. *Perit Dial Int* **10**: 11-13. PubMed: PM:2085573.

Li XZ and Nikaido H (2009) Efflux-mediated drug resistance in bacteria: an update. *Drugs* **69**: 1555-1623. PubMed: PM:19678712.

Lim WS, Macfarlane J T, Boswell T C, Harrison T G, Rose D, Leinonen M and Saikku P (2001) Study of community acquired pneumonia aetiology (SCAPA) in adults admitted to hospital: implications for management guidelines. *Thorax* **56**: 296-301. PubMed: PM:11254821.

Lode HM, Schmidt-Ionas M and Stahlmann R (2008) Gemifloxacin for community-acquired pneumonia. *Expert Opin Investig Drugs* **17**: 779-786. PubMed: PM:18447602.

Lopez R, Garcia E, Garcia P and Garcia J L (2004) chap 6, Cell Wall Hydrolases. In *The Pneumococcus* (Tuomanen E ed) ASM press, Washington, DC.

Lubelski J, Mazurkiewicz P, van Merkerk R, Konings W N and Driessen A J (2004) ydaG and ydbA of Lactococcus lactis encode a heterodimeric ATP-binding cassette-type multidrug transporter. *J Biol Chem* **279**: 34449-34455. PubMed: PM:15192086.

Luna VA, Coates P, Eady E A, Cove J H, Nguyen T T and Roberts M C (1999) A variety of gram-positive bacteria carry mobile mef genes. *J Antimicrob Chemother* **44**: 19-25. PubMed: PM:10459806.

Luna VA, Cousin S Jr, Whittington W L and Roberts M C (2000) Identification of the conjugative mef gene in clinical Acinetobacter junii and Neisseria gonorrhoeae isolates. *Antimicrob Agents Chemother* **44**: 2503-2506. PubMed: PM:10952602.

Marrer E, Satoh A T, Johnson M M, Piddock L J and Page M G (2006a) Global transcriptome analysis of the responses of a fluoroquinolone-resistant Streptococcus pneumoniae mutant and its parent to ciprofloxacin. *Antimicrob Agents Chemother* **50**: 269-278. PubMed: PM:16377697.

Marrer E, Schad K, Satoh A T, Page M G, Johnson M M and Piddock L J (2006b) Involvement of the putative ATP-dependent efflux proteins PatA and PatB in fluoroquinolone resistance of a multidrug-resistant mutant of Streptococcus pneumoniae. *Antimicrob Agents Chemother* **50**: 685-693. PubMed: PM:16436727.

Martinez-Garriga B, Vinuesa T, Hernandez-Borrell J and Vinas M (2007) The contribution of efflux pumps to quinolone resistance in Streptococcus pneumoniae clinical isolates. *Int J Med Microbiol* **297**: 187-195. PubMed: PM:17350332.

Melegaro A, Gay N J and Medley G F (2004) Estimating the transmission parameters of pneumococcal carriage in households. *Epidemiol Infect* **132**: 433-441. PubMed: PM:15188713.

Mitchell TJ (2004) chap 5, Pneumolysin and Other Virulence Proteins. In *The Pneumococcus* (Tuomanen E ed) ASM press, Washington, DC.

Musher DM (2004) chap 14, A Pathogenic Categorization of Clinical Syndromes Caused by Streptococcus pneumoniae. In *The Pneumococcus* (Tuomanen E ed) ASM press, Washington, DC.

Nagai K, Davies T A, Dewasse B E, Jacobs M R and Appelbaum P C (2001) Single- and multi-step resistance selection study of gemifloxacin compared with trovafloxacin, ciprofloxacin, gatifloxacin and moxifloxacin in Streptococcus pneumoniae. *J Antimicrob Chemother* **48**: 365-374. PubMed: PM:11533001.

Nseir S and Ader F (2008) Prevalence and outcome of severe chronic obstructive pulmonary disease exacerbations caused by multidrug-resistant bacteria. *Curr Opin Pulm Med* **14**: 95-100. PubMed: PM:18303416.

Nuorti JP, Butler J C, Farley M M, Harrison L H, McGeer A, Kolczak M S and Breiman R F (2000) Cigarette smoking and invasive pneumococcal disease. Active Bacterial Core Surveillance Team. *N Engl J Med* **342**: 681-689. PubMed: PM:10706897.

Obaro S and Adegbola R (2002) The pneumococcus: carriage, disease and conjugate vaccines. *J Med Microbiol* **51**: 98-104. PubMed: PM:11863272.

Oldach D, Clark K, Schranz J, Das A, Craft J C, Scott D, Jamieson B D and Fernandes P (2013) Randomized, double-blind, multicenter phase 2 study comparing the efficacy and safety of oral solithromycin (CEM-101) to those of oral levofloxacin in the treatment of patients with community-acquired bacterial pneumonia. *Antimicrob Agents Chemother* **57**: 2526-2534. PubMed: PM:23507282.

Pallares R, Fenoll A and Linares J (2003) The epidemiology of antibiotic resistance in Streptococcus pneumoniae and the clinical relevance of resistance to cephalosporins, macrolides and quinolones. *Int J Antimicrob Agents* **22 Suppl 1**: S15-S24. PubMed: PM:14512221.

Perez-Trallero E, Marimon J M, Iglesias L and Larruskain J (2003) Fluoroquinolone and macrolide treatment failure in pneumococcal pneumonia and selection of multidrug-resistant isolates. *Emerg Infect Dis* **9**: 1159-1162. PubMed: PM:14519256.

Pestova E, Millichap J J, Siddiqui F, Noskin G A and Peterson L R (2002) Non-PmrA-mediated multidrug resistance in Streptococcus pneumoniae. *J Antimicrob Chemother* **49**: 553-556. PubMed: PM:11864959.

Peterson SN, Sung C K, Cline R, Desai B V, Snesrud E C, Luo P, Walling J, Li H, Mintz M, Tsegaye G, Burr P C, Do Y, Ahn S, Gilbert J, Fleischmann R D and Morrison D A (2004) Identification of competence pheromone responsive genes in Streptococcus pneumoniae by use of DNA microarrays. *Mol Microbiol* **51**: 1051-1070. PubMed: PM:14763980.

Pichichero ME (2013) Otitis media. *Pediatr Clin North Am* **60**: 391-407. PubMed: PM:23481107.

Piddock LJ, Johnson M M, Simjee S and Pumbwe L (2002) Expression of efflux pump gene pmrA in fluoroquinolone-resistant and -susceptible clinical isolates of Streptococcus pneumoniae. *Antimicrob Agents Chemother* **46**: 808-812. PubMed: PM:11850265.

Pletz MW, Maus U, Krug N, Welte T and Lode H (2008) Pneumococcal vaccines: mechanism of action, impact on epidemiology and adaption of the species. *Int J Antimicrob Agents* **32**: 199-206. PubMed: PM:18378430.

Powis J, McGeer A, Green K, Vanderkooi O, Weiss K, Zhanel G, Mazzulli T, Kuhn M, Church D, Davidson R, Forward K, Hoban D, Simor A and Low D E (2004) In vitro antimicrobial susceptibilities of Streptococcus

pneumoniae clinical isolates obtained in Canada in 2002. *Antimicrob Agents Chemother* **48**: 3305-3311. PubMed: PM:15328089.

Pozzi G, Masala L, Iannelli F, Manganelli R, Havarstein L S, Piccoli L, Simon D and Morrison D A (1996) Competence for genetic transformation in encapsulated strains of Streptococcus pneumoniae: two allelic variants of the peptide pheromone. *J Bacteriol* **178**: 6087-6090. PubMed: PM:8830714.

Reinert R, Jacobs M R and Kaplan S L (2010) Pneumococcal disease caused by serotype 19A: review of the literature and implications for future vaccine development. *Vaccine* **28**: 4249-4259. PubMed: PM:20416266.

Reinert RR, Wild A, Appelbaum P, Lutticken R, Cil M Y and Al Lahham A (2003) Ribosomal mutations conferring resistance to macrolides in Streptococcus pneumoniae clinical strains isolated in Germany. *Antimicrob Agents Chemother* **47**: 2319-2322. PubMed: PM:12821488.

Riedel S, Beekmann S E, Heilmann K P, Richter S S, Garcia-de-Lomas J, Ferech M, Goosens H and Doern G V (2007) Antimicrobial use in Europe and antimicrobial resistance in Streptococcus pneumoniae. *Eur J Clin Microbiol Infect Dis* **26**: 485-490. PubMed: PM:17551759.

Roberts MC, Sutcliffe J, Courvalin P, Jensen L B, Rood J and Seppala H (1999) Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. *Antimicrob Agents Chemother* **43**: 2823-2830. PubMed: PM:10582867.

Robertson GT, Doyle T B and Lynch A S (2005) Use of an efflux-deficient streptococcus pneumoniae strain panel to identify ABC-class multidrug transporters involved in intrinsic resistance to antimicrobial agents. *Antimicrob Agents Chemother* **49**: 4781-4783. PubMed: PM:16251330.

Romero-Steiner S, Pilishvili T, Sampson J S, Johnson S E, Stinson A, Carlone G M and Ades E W (2003) Inhibition of pneumococcal adherence to human nasopharyngeal epithelial cells by anti-PsaA antibodies. *Clin Diagn Lab Immunol* **10**: 246-251. PubMed: PM:12626450.

Ruoff KL and Bisno A L (2013) chap 197, Classification of Streptococci. In *Principles and Practice of Infectious Diseases (Web Edition)* (Mandell G, Bennet JE and Dolin R eds) Elsevier.

Sakai F, Talekar S J, Klugman K P and Vidal J E (2013) Expression of Virulence-Related Genes in the Nasopharynx of Healthy Children. *PLoS One* **8**: e67147. PubMed: PM:23825636.

Santagati M, Iannelli F, Oggioni M R, Stefani S and Pozzi G (2000) Characterization of a genetic element carrying the macrolide efflux gene mef(A) in Streptococcus pneumoniae. *Antimicrob Agents Chemother* **44**: 2585-2587. PubMed: PM:10952626.

Schito GC, Pesce A and Debbia E A (1994) Stability in the presence of widespread beta-lactamases. A prerequisite for the antibacterial activity of beta-lactam drugs. *Drugs* **47 Suppl 3**: 1-8. PubMed: PM:7518761.

Schurek KN, Adam H J, Siemens C G, Hoban C J, Hoban D J and Zhanel G G (2005) Are fluoroquinolone-susceptible isolates of Streptococcus pneumoniae really susceptible? A comparison of resistance mechanisms in Canadian isolates from 1997 and 2003. *J Antimicrob Chemother* **56**: 769-772. PubMed: PM:16126779.

Scott LJ and Sanford M (2012) Pneumococcal polysaccharide conjugate vaccine (13-valent, adsorbed): a guide to its use in older adults. *Drugs Aging* **29**: 847-855. PubMed: PM:23038610.

Service Public Fédéral Belge Santé Publique. Guide Belge des traitements anti-infectieux en pratique ambulatoire. Service Public Federal Belge "Santé publique". Available from: https://portal.health.fgov.be/pls/portal/url/ITEM/5B8EF73EFFAF6E11E04400144F3EAABC Last updated: 2008; last accessed: 27-2-2012.

Shen CF, Wang S M, Lee K H, Ho T S and Liu C C (2013) Childhood invasive pneumococcal disease caused by non-7-valent pneumococcal vaccine (PCV7) serotypes under partial immunization in Taiwan. *J Formos Med Assoc* **112**: 561-568. PubMed: PM:23916313.

Shorr AF, Kollef M, Eckburg P B, Llorens L and Friedland H D (2013) Assessment of ceftaroline fosamil in the treatment of community-acquired bacterial pneumonia due to Streptococcus pneumoniae: insights from two randomized trials. *Diagn Microbiol Infect Dis* **75**: 298-303. PubMed: PM:23357290.

Sjostrom K, Spindler C, Ortqvist A, Kalin M, Sandgren A, Kuhlmann-Berenzon S and Henriques-Normark B (2006) Clonal and capsular types decide whether pneumococci will act as a primary or opportunistic pathogen. *Clin Infect Dis* **42**: 451-459. PubMed: PM:16421787.

Spratt BG, Hanage W P and Brueggemann A B (2004) chap 8, Evolutionary and Population Biology of Streptococcus pneumoniae. In *The Pneumococcus* (Tuomanen E ed) ASM press, Washington, DC.

Sun Y, Hwang Y and Nahm M H (2001) Avidity, potency, and cross-reactivity of monoclonal antibodies to pneumococcal capsular polysaccharide serotype 6B. *Infect Immun* **69**: 336-344. PubMed: PM:11119522.

Swiatlo S, McDaniel L S and Briles D E (2004) chap 4, Choline-Binding Proteins. In *The Pneumococcus* (Tuomanen E ed) ASM press, Washington, DC.

Tait-Kamradt A, Clancy J, Cronan M, Dib-Hajj F, Wondrack L, Yuan W and Sutcliffe J (1997) mefE is necessary for the erythromycin-resistant M phenotype in Streptococcus pneumoniae. *Antimicrob Agents Chemother* **41**: 2251-2255. PubMed: PM:9333056.

Tait-Kamradt A, Davies T, Appelbaum P C, Depardieu F, Courvalin P, Petitpas J, Wondrack L, Walker A, Jacobs M R and Sutcliffe J (2000) Two new mechanisms of macrolide resistance in clinical strains of Streptococcus pneumoniae from Eastern Europe and North America. *Antimicrob Agents Chemother* **44**: 3395-3401. PubMed: PM:11083646.

Tettelin C and Hollingshead S (2004) chap 2, Comparative Genomics of S. pneumoniae: Intra-Strain Diversity and Genome Plasticity. In *The Pneumococcus* (Tuomanen E ed) ASM press, Washington, DC.

Tocci N, Iannelli F, Bidossi A, Ciusa M L, Decorosi F, Viti C, Pozzi G, Ricci S and Oggioni M R (2013) Functional analysis of pneumococcal drug efflux pumps associates the MATE DinF transporter with quinolone susceptibility. *Antimicrob Agents Chemother* **57**: 248-253. PubMed: PM:23114782.

Trappetti C, Ogunniyi A D, Oggioni M R and Paton J C (2011) Extracellular matrix formation enhances the ability of Streptococcus pneumoniae to cause invasive disease. *PLoS One* **6**: e19844. PubMed: PM:21611130.

Tulkens PM, Arvis P and Kruesmann F (2012) Moxifloxacin safety: an analysis of 14 years of clinical data. *Drugs R D* **12**: 71-100. PubMed: PM:22715866.

Van Bambeke F, Glupczynski Y, Plesiat P, Pechere J C and Tulkens P M (2003) Antibiotic efflux pumps in prokaryotic cells: occurrence, impact on resistance and strategies for the future of antimicrobial therapy. *J Antimicrob Chemother* **51**: 1055-1065. PubMed: PM:12697642.

Van Bambeke F, Harms J M, Van Laethem Y and Tulkens P M (2008) Ketolides: pharmacological profile and rational positioning in the treatment of respiratory tract infections. *Expert Opin Pharmacother* **9**: 267-283. PubMed: PM:18201149.

Van Bambeke F, Lambert D L, Mingeot-Leclercq M P and Tulkens P M (2010) chap 130, Anti-infective therapy: Mechanism of action. In *Infectious Diseases* (Armstrong D and Cohen J eds) pp 1288-1307, Mosby, London, United Kingdom.

Van Bambeke F, Michot J M, Van Eldere J and Tulkens P M (2005) Quinolones in 2005: an update. *Clin Microbiol Infect* **11**: 256-280. PubMed: PM:15760423.

Van Bambeke F, Reinert R R, Appelbaum P C, Tulkens P M and Peetermans W E (2007) Multidrug-resistant Streptococcus pneumoniae infections: current and future therapeutic options. *Drugs* **67**: 2355-2382. PubMed: PM:17983256.

Van Steenkiste M (2013) [Pneumococcal vaccines: different types and their use in practice]. *J Pharm Belg* 4-11. PubMed: PM:23638606.

Vandevelde, N. M., Diaz Iglesias, Y., Tulkens, P. M., Rodriguez-Villalobos, H., Liistro, G., Boel, A., Van Vaerenbergh, K., Jordens, P., Philippart, I., d'Odemont, J. P., Cadrobbi, J. P., Coppens, N., and Van Bambeke, F. (2013) 53d Interscience Conference on Antimicrobial Agents and Chemotherapy, Denver, CO .

Webber MA and Piddock L J (2003) The importance of efflux pumps in bacterial antibiotic resistance. *J Antimicrob Chemother* **51**: 9-11. PubMed: PM:12493781.

Weisblum B (1995a) Erythromycin resistance by ribosome modification. *Antimicrob Agents Chemother* **39**: 577-585. PubMed: PM:7793855.

Weisblum B (1995b) Insights into erythromycin action from studies of its activity as inducer of resistance. *Antimicrob Agents Chemother* **39**: 797-805. PubMed: PM:7785974.

Weiser JN, Austrian R, Sreenivasan P K and Masure H R (1994) Phase variation in pneumococcal opacity: relationship between colonial morphology and nasopharyngeal colonization. *Infect Immun* **62**: 2582-2589. PubMed: PM:8188381.

Welte T, Torres A and Nathwani D (2010) Clinical and economic burden of community-acquired pneumonia among adults in Europe. *Thorax*. PubMed: PM:20729232.

Whitney CG, Farley M M, Hadler J, Harrison L H, Bennett N M, Lynfield R, Reingold A, Cieslak P R, Pilishvili T, Jackson D, Facklam R R, Jorgensen J H and Schuchat A (2003) Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine. *N Engl J Med* **348**: 1737-1746. PubMed: PM:12724479.

Whitney CG, Pilishvili T, Farley M M, Schaffner W, Craig A S, Lynfield R, Nyquist A C, Gershman K A, Vazquez M, Bennett N M, Reingold A, Thomas A, Glode M P, Zell E R, Jorgensen J H, Beall B and Schuchat A (2006) Effectiveness of seven-valent pneumococcal conjugate vaccine against invasive pneumococcal disease: a matched case-control study. *Lancet* **368**: 1495-1502. PubMed: PM:17071283.

Yother J (2004) chap 3, Capsules. In *The Pneumococcus* (Tuomanen E ed) ASM press, Washington, DC.