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

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ABSTRACT

The correlation between *Streptococcus pneumoniae* serotypes, biofilm production, antibiotic susceptibility and drug efflux in isolates from patients suffering from acute exacerbations of chronic bronchitis (AECB) remains largely unexplored. Using 101 isolates collected from AECB patients for whom partial ($n = 51$) or full ($n = 50$) medical details were available, we determined serotypes (ST)/serogroups (SG) (Quellung reaction), antibiotic susceptibility patterns [MIC (microdilution) using EUCAST and CLSI criteria] and ability to produce biofilm in vitro (10-day model; crystal violet staining). The majority of patients were 55–75 years old and <5% were vaccinated against *S. pneumoniae*. Moreover, 54% showed high severity scores (GOLD 3–4), and comorbidities were frequent including hypertension (60%), cancer (24%) and diabetes (20%). Alcohol and/or tobacco dependence was >30%. Isolates of SG6-11-15-23, known for large biofilm production and causing chronic infections, were the most prevalent (>15% each), but other isolates also produced biofilm (SG9-18-22-27 and ST8-20 being most productive), except SG7, SG29 and ST5 (<2% of isolates each). Resistance (EUCAST breakpoints) was 8–13% for amoxicillin and cefuroxime, 35–39% for macrolides, 2–8% for fluoroquinolones and 2% for telithromycin. ST19A isolates showed resistance to all antibiotics, ST14 to all except moxifloxacin, and SG9 and SG19 to all except telithromycin, moxifloxacin and ceftriaxone (SG19 only). Solithromycin and telithromycin MICs were similar. No correlation was observed between biofilm production and MIC or efflux (macrolides, fluoroquinolones). *S. pneumoniae* serotyping may improve AECB treatment by avoiding antibiotics with predictable low activity, but it is not predictive of biofilm production.

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1. Introduction

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

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

2. Materials and methods

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

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

2.2. Bacterial strains and growth conditions

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

2.3. Susceptibility testing

Minimum inhibitory concentrations (MICs) were determined by microdilution following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [19]. MICs were read after 18–24 h of incubation at 37 °C. To improve accuracy, concentrations at half a value of each standard geometric progression were used as previously described [14] over the whole concentration range investigated. MICs were categorised as susceptible, intermediate or resistant according to the CLSI and European Committee on Antimicrobial Susceptibility Testing (EUCAST) interpretive breakpoints [19,20].

2.4. Assessment of efflux phenotypes

The efflux resistance phenotype to macrolides was determined by examining the dissociation of susceptibilities between clarithromycin and clindamycin (substrate and non-substrate of the macrolide efflux transporters, respectively [14]). Efflux of fluoroquinolones was detected by a decrease in the MIC upon addition of reserpine (10 mg/L), an inhibitor of both PatA/B and PmrA fluoroquinolone efflux transporters in *S. pneumoniae* [14].

2.5. In vitro development of biofilms and determination of biofilm mass

Ninety-six well plates (European cat. no. 734-2327; VWR, Radnor, PA) were used as the support for biofilm growth. In each well, 25 μL of bacterial culture [optical density at 620 nm (OD_{620}) = 0.1] were added to 175 μL of cation-adjusted Mueller–Hinton broth supplemented with 5% lysed horse blood (Oxoid Ltd.) and 2% glucose as previously described [18]. Biofilm development was obtained by incubation for 2–10 days at 37 °C in a 5% CO_2 atmosphere with medium replacement every 48 h. Biofilms examined after 2 days or 10 days are referred to as young and mature biofilms, respectively. Biofilm mass was evaluated by staining with crystal violet followed by measuring the absorbance exactly as previously described [18]. Each isolate was tested twice at different dates, with each assay using three to six measures. The mean coefficient of