Dynamics of Streptococcus pneumoniae in patients with Chronic Obstructive Pulmonary Disease

Arnau Domenech Pena

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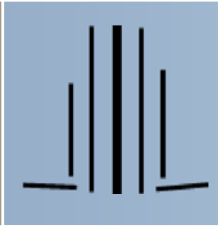

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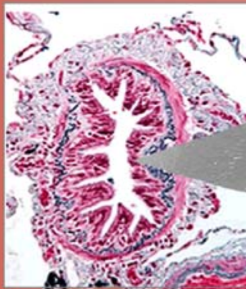

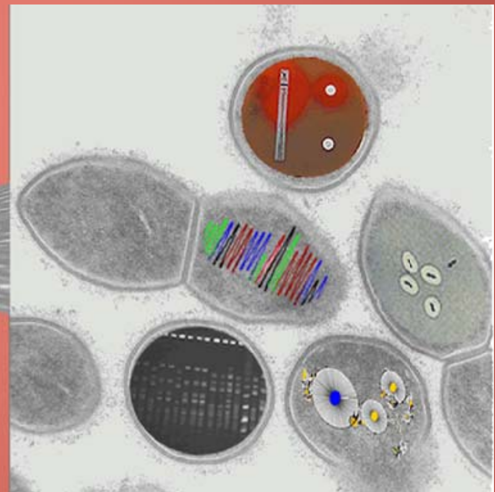

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A Fabi

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ABSTRACT

It is estimated that within a few years chronic obstructive pulmonary disease (COPD) will be the third leading cause of death worldwide. The morbidity and mortality associated with COPD are due, in part to acute exacerbation episodes (AECOPD), mainly caused by microbial pathogens such as *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa.* Moreover, COPD is the main underlying disease associated with pneumococcal pneumonia episodes.

This thesis describes four studies performed to gain insights into the role of pneumococci and their closely-related species *S. pseudopneumoniae* in causing acute exacerbation and pneumonia episodes in COPD patients.

In the first study, a total of 188 sputum samples were obtained from AECOPD episodes occurring in severe COPD patients during a 1-year period. Samples were quantitatively cultured; of them, *S. pneumoniae* was isolated in 31 (16.5%) episodes and *S. pseudopneumoniae* in 9 (4.8%) episodes. *S. pneumoniae* was the third most frequent cause after *Pseudomonas aeruginosa* (28.8%) and *Haemophilus influenzae* (19.7%).

There are major differences in the invasiveness potential of pneumococci, depending on their serotype and genotype. Indeed, in our second study (from 2001 to 2008) we found an association of certain serotypes, and their related genotypes, with different pneumococcal infections. Serotypes 4 (ST247^{4}), 5 (Colombia^{5}-ST289) and 8 (Netherlands^{8}-ST53) were associated with bacteraemic pneumonia, serotypes 1 (Sweden^{1}-ST306) and 3 (Netherlands^{3}-ST180 and ST260^{3}) with bacteraemic and non-bacteraemic pneumonia, and serotypes 16F (ST30^{16F}), 11A and non-typeable pneumococci with AECOPD episodes ($P<0.05$).

Finally, in our experience, serotype 3 pneumococcus was the most frequent cause of pneumonia and acute exacerbations in COPD patients.

Moreover, the implementation of pneumococcal conjugate vaccine PCV7 for children in 2001 in Spain has been shown to be highly effective in reducing invasive pneumococcal disease in children, and in adults as well due to the phenomenon of herd protection. This effect was also observed among pneumococci causing acute exacerbations in adults: PCV7 serotypes decreased from 39.4% in the 2001-04 period to 11.2% in the 2009-12 period. In parallel, the prevalence of multi-drug resistant serotypes 15A and 6C has dramatically increased in recent years. For this reason, although the resistance rates of β-lactams decreased over time, macrolides and multi-drug resistance remained stable throughout the study period.

The presence of bacteria colonizing the lower airways of most severe COPD patients results in bronchial epithelial injury and increases morbidity among these patients. In the third study (1995-2010 period), it was found that a third of recurrent pneumococcal acute exacerbations were relapses (caused by a pre-existing strain), mainly associated with serotypes 9V and 19F ($P<0.02$). This suggests an important role for capsular type in pneumococcal persistence. As was expected, these persistent strains were more resistant to antimicrobials than those causing reinfections. In view of these results, we analysed the impact of antimicrobial consumption in the development of pneumococcal resistance to β-lactams and fluoroquinolones in 13 patients with a long-time persistence of pneumococci (average time: 582 days, SD ±362). Changes in quinolone-resistant determining regions (QRDR)

involved in fluoroquinolone resistance were frequently observed in persistent strains after fluoroquinolone treatment; however, the penicillin-binding protein (PBP) sequences were stable over time, even though all but two patients received multiple courses of β-lactam treatment. These results suggest that an optimal combination of *pbp* genes is maintained to compensate for the fitness cost imposed by additional changes in these genes.

Despite the genetic stability of these persistent strains, *S. pneumoniae* is naturally transformable and is able to acquire exogenous DNA, resulting in a dynamic and complex epidemiology of pneumococcal diseases. This genetic diversity was also observed among the 36 *S. pseudopneumoniae* strains analysed. These strains were isolated from COPD patients, mainly with advanced airflow obstruction (83.3%) and other comorbidities (systemic arterial hypertension, dyslipidemia or diabetes). Despite the genetic variability of the strains, all patients had a successful outcome. However, two-thirds required hospitalization, suggesting an etiological role for *S. pseudopneumoniae* in acute exacerbations of patients with severe COPD.

Altogether, our studies can help to improve the understanding of the dynamics of *S. pneumoniae* and *S. pseudopneumoniae* populations causing disease in COPD patients.

RESUM

L' Organització Mundial de la Salud, estima que la malaltia pulmonar obstructiva crònica (MPOC) serà la tercera causa de mort arreu del món en pocs anys. La morbi-mortalitat associada a la MPOC es deu als episodis d'exacerbació aguda (EAMPOC), principalment causades per patògens microbians com *Streptococcus pneumoniae.* A més a més, la MPOC és la principal malaltia de base associada amb episodis de pneumònia pneumocòccica.

En aquesta tesis, es van dur a terme quatre estudis amb l'objectiu d'aprofundir en el paper de *S. pneumoniae* i de l'espècie *S. pseudopneumoniae*, estretament relacionades, com a causants d'exacerbacions agudes i pneumònia en pacients amb MPOC.

En el primer estudi, es van sembrar quantitativament un total de 188 mostres d'esput obtingudes durant episodis d'EAMPOC en pacients amb MPOC avançat, durant un any d'estudi (febrer 2010 - febrer 2011). *S. pneumoniae* es va aïllar en 31 (16.5%) episodis i *S. pseudopneumoniae* en 9 (4.8%) episodis. *S. pneumoniae* fou la tercera causa d'exacerbació, després de *Pseudomonas aeruginosa* (28.8%) i *Haemophilus influenzae* (19.7%).

Existeixen diferències importants en el potencial invasor dels pneumococs, depenent del seu serotipus i genotipus. D'acord amb aquest fet, en el segon estudi es va trobar una diferent associació d'alguns serotipus i del seus genotipus relacionats, en pacients amb MPOC amb diferents infeccions pneumocòcciques (període 2001-2008). En la nostra experiència, serotipus 3 va ser la causa més freqüent de pneumònia i d'EAMPOC en pacients amb MPOC. Però, els serotipus 4 (ST247[4]), 5 (Colombia[5]-ST289) i 8 (Netherlands[8]-ST53) es varen associar amb pneumònia

bacterièmica; serotipus 1 (Sweden^{1}-ST306) i 3 (Netherlands^{3}-ST180 i ST260^{3}) es varen associar amb pneumònia tant bacterièmica com no bacterièmica; mentre serotipus 16F (ST30^{16F}), 11A i els pneumococs no-tipificables es varen associar amb EAMPOC ($P<0.05$).

Per altra banda, s'ha demostrat que la implementació de la vacuna conjugada PCV7 per nens el 2001 a Espanya, ha sigut efectiva reduint la incidència de malaltia invasora en nens però també en adults, a causa de la protecció de grup. Aquest efecte també s'ha observat en les poblacions de *S. pneumoniae* causants de EAMPOC, ja que els serotipus inclosos en la vacuna han disminuït del 39.4% en el període 2001-2004 a 11.2% en el període 2008-2012. Paral·lelament a aquest descens, els serotipus 15A i 6C, associats amb multiresistència antibiòtica, han augmentat dramàticament en els últims anys. Per aquesta raó, tot i el descens de la resistència a β-lactàmics al llarg dels anys, la resistència a macròlids i la multiresistència s'han mantingut estables durant tot el període d'estudi.

La presència de bacteris colonitzant el tracte respiratori inferior de la majoria de pacients amb MPOC sever, provoca un dany de l'epiteli bronquial, fet que incrementa la morbiditat d'aquests pacients. En el tercer estudi (període 1995-2010), es va observar que un terç dels episodis d'EAMPOC recurrents, varen ser causats per una soca preexistent, principalment serotipus 9V i 19F ($P<0.05$), considerant-se recaigudes. Aquest fet suggereix un paper important del tipus capsular en la persistència de soques de pneumococc en el mateix pacient.

Com a conseqüència d'aquests resultats, es va analitzar l'impacte del consum d'antimicrobians en el desenvolupament de

resistència a β-lactàmics i fluoroquinolones en 13 pacients colonitzats per pneumococc al llarg dels anys (temps mitjà: 582 dies, DS ±362). Es van observar canvis amb freqüència en les "*quinolone-resistant determining regions* (QRDR)", implicades en la resistència a fluoroquinolones, en soques persistents de pacients que van rebre tractament amb fluoroquinolones. En canvi, les "*penicillin-binding protein*s (PBP)" de les soques persistents van romandre estables tot i els múltiples tractaments amb β-lactàmics que van rebre els pacients. Aquests resultats suggereixen que una configuració òptima de les *pbps* és important per a compensar el cost energètic imposat per canvis addicionals en aquests gens.

Tot i l'estabilitat genètica d'aquestes soques persistents, *S. pneumoniae* és naturalment transformable i és capaç d'adquirir ADN exogen, resultant en una complexa però dinàmica epidemiologia de la malaltia pneumocòccica. Aquesta diversitat genètica també es va observar entre les 36 soques de *S. pseudopneumoniae* analitzades. Aquestes soques es varen aïllar de pacients amb MPOC, principalment amb una avançada obstrucció del tracte respiratori (83.3%) i amb altres comorbiditats (hipertensió arterial sistèmica, dislipèmia i/o diabetis mellitus). Tot i la variabilitat genètica d'aquestes soques, tots els pacients van tindre una bona evolució clínica. Tanmateix, dos terços dels pacients van ser hospitalitzats durant un període 4 o més dies, suggerint el paper etiològic de *S. pseudopneumoniae* en pacients amb MPOC avançada.

En total, els estudis presentats han millorat el coneixement de la dinàmica de les poblacions de *S. pneumoniae* i *S. pseudopneumoniae* en pacients amb MPOC.

SCIENTIFIC PRODUCTION

Publications in international peer-reviewed journals

1. Domenech A, Ardanuy C, Calatayud L, Santos S, Tubau F, Grau I, Verdaguer R, Dorca J, Pallares R, Martin R, Liñares J (2011) Serotypes and genotypes of *Streptococcus pneumoniae* causing pneumonia and acute exacerbations in patients with chronic obstructive pulmonary disease. J Antimicrob Chemother. 66:487-93. **The Impact Factor is 5.330 according to 2012 Journal Citation Reports released by Thomson Reuters (ISI) in 2013.**

2. Domenech A, Ardanuy C, Balsalobre L, Marti S, Calatayud L, De la Campa AG, Brueggemann AB, Liñares J (2012) Pneumococci can persistently colonize adult patients with chronic respiratory disease. J Clin Microbiol 50:4047-53. **The Impact Factor is 4.068 according to 2012 Journal Citation Reports released by Thomson Reuters (ISI) in 2013.**

3. Rolo D*, Simões A*, Domenech A* Fenoll A, Liñares J, de Lencastre H, Ardanuy C, Sá-Leão R (2013) Disease Isolates of *Streptococcus pseudopneumoniae* and Non-Typeable S. pneumoniae. Presumptively Identified as Atypical *S. pneumoniae* in Spain. PloS One 8:e57047. * These authors contributed equally to this work. **The Impact Factor is 3.730 according to 2012 Journal Citation Reports released by Thomson Reuters (ISI) in 2013.**

4. Domenech A, Ardanuy C, Pallares R, Grau I, Santos S, De la Campa AG, Liñares J (2013) Some pneumococcal serotypes are more frequently associated with relapses of acute exacerbations in COPD patients. PLoS ONE 8: e59027. **The Impact Factor is 3.730 according to 2012 Journal Citation Reports released by Thomson Reuters (ISI) in 2013.**

5. Domenech A, Ardanuy C, Tercero A, García-Somoza D, Santos S, Liñares J (2013) Dynamics of the pneumococcal population causing acute exacerbations in patients with Chronic Obstructive Pulmonary Disease over a twelve year period. J Antimicrob Chemother (accepted). **The Impact Factor is 5.330 according to 2012 Journal Citation Reports released by Thomson Reuters (ISI) in 2013.**

6. Domenech A*, Puig C*, Martí S, Santos S, Fernández A, Calatayud C, Dorca J, Ardanuy C, Liñares J (2013) Infectious Etiology of Acute Exacerbations in COPD Patients with Severe Airflow Obstruction. * These authors contributed equally to this work. J Infection (accepted; 10.1016/j.jinf.2013.09.003). **The Impact Factor is 5.330 according to 2012 Journal Citation Reports released by Thomson Reuters (ISI) in 2013.**

Poster presentations

a) International meetings

1. A. Domenech, C. Ardanuy, L. Calatayud, S. Santos, F. Tubau, I. Grau, R. Verdaguer, J. Dorca, R. Pallares, R. Martin, J. Liñares. Clonal structure of *Streptococcus pneumoniae* strains isolated from blood and sputum in patients with Chronic Obstructive Pulmonary Diasease. 7th International Symposium on Pneumococci and Pneumococcal Diseases (ISPPD), 14-18 March 2010, Tel Aviv, Israel.
2. A. Domenech, C. Ardanuy, L. Calatayud, F. Tubau, J. Liñares. Bacteriemic *vs*. non-bacteriemic pneumococcal pneumonia in patients with Chronic Obstructive Pulmonary Disease: serotypes and genotypes. 10th European meeting on the molecular biology of the pneumococcus, 23-26 June 2011, Amsterdam, The Netherlands.
3. A. Domenech, C. Ardanuy, R. Pallares, L. Calatayud, S. Santos, J. Liñares. Pneumococcal serotypes causing recurrent episodes of Acute Exacerbations of Chronic Obstructive Pulmonary Disease (AECOPD). 51st Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), 17-20 September 2011, Chicago, United States.
4. A. Domenech, C. Ardanuy, L. Balsalobre, L. Calatayud, AG. De la Campa, A. Brueggemann, J. Liñares. Pneumococci causing multiple episodes of acute

exacerbations in patients with chronic respiratory disease: genetic characterisation of persistent strains. 8th International Symposium on Pneumococci and Pneumococcal Diseases (ISPPD), 11-13 March 2012, Foz do Iguazu, Brasil.

5. D. Rolo, A. S. Simões, A. Domenech, A. Fenoll, J. Liñares, H. de Lencastre, C. Ardanuy, R. Sá-Leão. Identification of clinical *S. pseudopneumoniae* isolates in Spain. 52nd Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), 09-12 September 2012, San Francisco, United States.
6. C. Puig, A. Domenech, S. Martí, A. Fernández, S. Santos, L. Calatayud, C. Ardanuy, J. Liñares. Etiology of acute exacerbations in COPD patients with severe airflow obstruction. 23rd European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), 27-30 May 2013, Berlin, Germany.
7. A. Domenech, C. Puig, S. Santos, S. Marti, C. Ardanuy, J. Liñares. Clinical Characteristics of Patients with Acute Exacerbations of Chronic Obstructive Pulmonary Disease (COPD) Caused by *Streptococcus pseudopneumoniae*. 53rd Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), 09-13 September 2013, Denver, United States.

b) National meetings

1. A. Domenech. Estructura poblacional de cepas de *Streptococcus pneumoniae* invasivas y no invasivas

aisladas en pacientes con Enfermedad Pulmonar Obstructiva Crónica (EPOC). II Jornadas de Formación del CIBERES, Ministerio de Ciencia e Innovación, 15-16 October 2009, Palma de Mallorca, Spain.

2. A. Domenech, C. Ardanuy, C. Puig, L. Calatayud, M. Alegre, F. Tubau, R. Verdaguer, J. Liñares. Enfermedad Pulmonar Obstructiva Crónica (EPOC): Diferentes asociaciones de serotipos y genotipos de *Streptococcus pneumoniae* aislados en pacientes con neumonía o con exacerbación aguda. XIV Congreso de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC), May 2010, Barcelona, Spain.

3. S. Marti, C. Puig, A. Domenech, S. Santos, F. Tubau, C. Ardanuy, J. Liñares. Caracterización de *Pseudomonas aeruginosa* aisladas en exacerbaciones agudas de la Enfermedad Pulmonar Obstructiva Crónica (EAEPOC). IV Jornadas de Formación del CIBERES, Ministerio de Ciencia e Innovación, October 2011, Palma de Mallorca, Spain.

4. A. Domenech, C. Ardanuy, L. Balsalobre, S. Martí, L. Calatayud, A. G. De La Campa, A. B. Brueggemann, J. Liñares. Estabilidad genética de las proteínas fijadoras de penicilina (PBP) en neumococos que persisten durante años en pacientes con enfermedad respiratoria crónica. XVI congreso de la SEIMC, 09-11 May 2012, Bilbao, Spain.

5. C. Puig, A. Domenech, S. Martí, A. Fernández, S. Santos, L. Calatayud, C. Ardanuy, J. Liñares. Etiología bacteriana

de las exacerbaciones agudas de la enfermedad pulmonar obstructiva crónica (EPOC) en pacientes graves. XVI congreso de la SEIMC, 09-11 May 2012, Bilbao, Spain.

Oral communications

A Domenech, A Tercero, D García-Somoza, *et al.* "Impact of heptavalent conjugate vaccine (PCV7) on pneumococci population causing acute exacerbations in patients with Chronic Obstructive Pulmonary Disease (COPD)" 11th European meeting on the molecular biology of the pneumococcus 2013, Madrid

A. Domenech, A. Fenoll, C. Ardanuy, J. Yuste, J. Liñares, AG. De la Campa. Trends in serotypes and genotypes of fluoroquinolone-resistant pneumococci in Spain. 53rd Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), 09-13 September 2013, Denver, United States.

Invited lectures

A. Domenech. Dinámica de las poblaciones de *Streptococcus pneumoniae* en la Enfermedad Pulmonar Obtructiva Crónica (EPOC). XXIV Congreso de Microbiología. Sociedad Española de Microbiología, July 2013, Hospitalet de Llobregat, Barcelona, Spain.

THESIS OUTLINE

The study aims to gain insights into the role of *Streptococcus pneumoniae* and closely-related *S. pseudopneumoniae* populations in patients with COPD. This general objective was achieved firstly by assessing the role of *Streptococcus pneumoniae* and *S. pseudopneumoniae* populations causing pneumonia and acute exacerbations in COPD patients, and secondly by analysing the ability of *S. pneumoniae* to persist in a set of patients, causing multiple acute exacerbation episodes.

Chapter I – General introduction. This chapter provides an outline of *Streptococcus pneumoniae* and Chronic Obstructive Pulmonary Disease in the context of the results described in this thesis. Topics such as epidemiology, antimicrobial resistance and prevention of pneumococcal diseases are discussed.

Chapter II – Infectious aetiology of acute exacerbations in patients with severe COPD. This chapter presents a one-year prospective study in which the quantitative distribution of bacteria and fungi causing acute exacerbations was analysed. Patients were divided into two groups based on the number of annual episodes (one acute exacerbation during the study period, or more than one episode).

This study has been recently published in Journal of Infection:

Domenech A[#], Puig C[#], Marti C, Santos S, Fernandez A, Calatayud C, Dorca J, Ardanuy C, Liñares J. Infectious Etiology of Acute Exacerbations in COPD Patients with Severe

Airflow Obstruction. #These authors contributed equally to this work.

Chapter III – Dynamics of pneumococcal populations causing disease in COPD patients. This chapter combines two different studies. In the first, a comparison of pneumococcal populations causing acute exacerbations, bacteraemic pneumonia and non-bacteraemic pneumonia in COPD patients were analysed (2001-2008 period). In the second, the study period was extended (2009-2012), and the impact of conjugate vaccines in the antimicrobial resistance and serotype and genotype distributions of pneumococci causing acute exacerbations was analysed, comparing the 2009-2012 period with the 2001-2004 and 2005-2008 periods.

The first study was published in the Journal of Antimicrobial Chemotherapy, and the second, was accepted in the same journal:

Domenech A, Ardanuy C, Calatayud L, Santos S, Tubau F, Grau I, Verdaguer R, Dorca J, Pallares R, Martin R, Liñares J (2011) Serotypes and genotypes of *Streptococcus pneumoniae* causing pneumonia and acute exacerbations in patients with chronic obstructive pulmonary disease. J Antimicrob Chemother. 66:487-93.

Domenech A, Ardanuy C, Tercero A, García-Somoza D, Santos S, Liñares J (2013) Dynamics of the pneumococcal population causing acute exacerbations in patients with Chronic Obstructive Pulmonary Disease over a twelve year period. J Antimicrob Chemother (accepted).

Chapter IV – Recurrence and persistence of pneumococcal strains. This chapter presents two studies related to the persistence of pneumococcal strains over time. In the first one, differences on the pneumococcal distribution among relapse and reinfection episodes were described. Based on these data, a new study analysing the impact of antimicrobials on the persistence of pneumococci and the development of antimicrobial resistance was performed.

Two studies have been published based on results obtained in this chapter:

> **Domenech A, Ardanuy C, Pallares R, Grau I, Santos S, De la Campa AG, Liñares J** (2013) Some pneumococcal serotypes are more frequently associated with relapses of acute exacerbations in COPD patients. PLoS ONE 8: e59027

> **Domenech A, Ardanuy C, Balsalobre L, Marti S, Calatayud L, De la Campa AG, Brueggemann AB, Liñares J** (2012) Pneumococci can persistently colonize adult patients with chronic respiratory disease. J Clin Microbiol 50:4047-53.

Chapter V – *S. pseudopneumoniae* populations causing acute exacerbations. In this chapter, we analysed the clinical characteristics of COPD patients infected by the recently described *S. pseudopneumoniae* species. First, a detailed analysis of

phenotypic and genotypic assays was performed in a collection of pneumococcal-like strains isolated from COPD patients, in order to differenciate *S. pseudopneumoniae* from S. pseumoniae and other species of the viridans group. These data, together with an analysis of invasive pneumococcal-like strains has been published in the following study:

> **Rolo D[#], S Simões A[#], Domenech A[#], Fenoll A, Liñares J, de Lencastre H, Ardanuy C, Sá-Leão R** (2013) Disease Isolates of *Streptococcus pseudopneumoniae* and Non-Typeable *S. pneumoniae* Presumptively Identified as Atypical *S. pneumoniae* in Spain. PLoS One 8:e57047 ([#]These authors contributed equally to this study).

Second, we analysed the clinical and demographic data of those COPD patients infected by *S. pseudopneumoniae* strains. A draft of the brief report with the results obtained has been presented in this section.

> **Domenech A, Puig C, Santos S, Marti S, Ardanuy C, Liñares J.** Clinical characteristics of patients with chronic obstructive pulmonary disease (COPD) infected by *Streptococcus pseudopneumoniae.*

Chapter VI – Synopsis of the results and discussion. The results obtained in chapters II to V are discussed as a whole.

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CHAPTER I

1. Chronic obstructive pulmonary disease (COPD)

Today, chronic respiratory diseases such as chronic obstructive pulmonary disease (COPD), bronchiectasis or asthma, are among the most common chronic diseases in the world (World Health Organization, 2005); according with the World Health Organization (WHO) 64 million people have chronic obstructive pulmonary disease (COPD). The burden of preventable chronic respiratory diseases has major adverse effects on the quality of life of the affected individuals, and also has underappreciated adverse economic effects on families, communities and societies (Bousquet *et al.,* 2003).

COPD is a preventable inflammatory disease, characterised by an airflow progressive limitation that is not totally reversible and associated with an inflammatory response of the lungs to noxious particles or gases, mainly cigarette smoke (Rabe *et al.,* 2007).

The BOLD international study (BOLD: Burden of Obstructive Lung Disease), estimated a mean prevalence of 10.1% of COPD for stage GOLD II or higher, with significant differences between countries (Buist *et al.,* 2007). In Spain, the prevalence of COPD between the ages of 40 and 80 is 10.2%, reaching 23% in those older than 60 years (Miravitlles *et al.,* 2009). After vascular diseases and cancer, COPD in the third leading cause of death in the developed countries. However, the global prevalence of COPD is expected to increase over the next two decades as longer life expectancy and the exposure to cigarettes and environmental pollutants increase; the WHO predicts that by 2030 COPD will have

become the third leading cause of death worldwide (Palm *et al.,* 2003; WHO report, 2005).

Long-term exposure to cigarette smoke and other noxious particles can cause chronic lung inflammation, which can result in a permanent enlargement of the airspaces distal accompanied by destruction of parenchymal tissue (emphysema). These pathological changes turn to breathless and other characteristic symptoms of COPD (American Journal of Respiratory and Critical Care Medicine*,* 1995). Many times, definitions of COPD have emphasized the terms “emphysema” and “chronic bronchitis”; however, emphysema is a pathological term that describes only one of several structural abnormalities present in COPD patients, and should not use for describing the COPD (Global Initiative Obstructive Lung Disease report, 2013). In this way, it is important to recognize that chronic bronchitis (defined as a persistent cough for at least three months per year in two consecutive years), is an independent disease entity that may be associated with fixed airflow limitation. In fact, chronic bronchitis also exists in patients with a normal spirometry test (Global Initiative Obstructive Lung Disease report, 2013).

Moreover, it is important to differentiate the COPD from other chronic respiratory diseases such bronchiectasis and asthma, since their optimal management must be based on distinctively different approaches. For example, while asthma usually has a good reversibility from inhaled bronchodilators, COPD has a limited reversibility with pharmacological bronchodilators and steroids (Reddel *et al.,* 2001).

Diagnosis of COPD should be considered in patients who have symptoms of a chronic cough, sputum production, dyspnea (difficult or laboured breathing) and a history of exposure to risk factors for the disease, especially tobacco smoke (Pauwels *et al.,* 2001). The diagnosis is confirmed by spirometry, which measures the post-bronchodilator forced expiratory volume in one second (FEV_1) and its ratio to the forced vital capacity (FVC). Traditionally, the main criterion for COPD classification was a FEV_1/FVC ratio $<70\%$, and the Global Initiative for Obstructive Lung Diseases (GOLD) defined a classification in four stages of disease severity based on the spirometric criteria, as shown in table 1 (Pauwels *et al.,* 2001).

Table 1. Classification of airflow limitation severity in COPD (source: www.goldcopd.org).

In patients with $FEV_1/FVC < 0.70$:	
GOLD I: mild	$FEV_1 \geq 80\%$ predicted
GOLD II: moderate	$50\% \leq FEV_1 < 80\%$ predicted
GOLD III: severe	$30\% \leq FEV_1 < 50\%$ predicted
GOLD IV: very severe	$FEV_1 < 30\%$ predicted

This classification is used for purposes of simplicity; however the correlation between FEV_1, patient's quality of life, and the symptomatology is weak (Jones et al., 2009). For this reason, in 2011 the GOLD guidelines proposed a new assessment method

based on the individual patient's history of exacerbations and the severity of their symptoms (Global Initiative for Chronic Obstructive Lung Disease). Four groups, named A to D, were established to assess the risk of poor outcomes (regarding the GOLD classification of airflow limitation and the number of AECOPD per year) and the symptomatology (dyspnea degree and the COPD Assessment Test, CAT). Briefly, patients in group A typically have mild or moderate airflow obstruction (GOLD I or II), with low risk of suffering acute exacerbations (0 or 1 per year), and fewer symptoms (dyspnea only with strenuous exercise, and CAT score <10). Group B includes patients with the same airway obstruction and risk of suffering exacerbations as those patients of group A, but more clinical symptoms (basal dyspnea and CAT score >10). In this way, groups C and D are associated with a higher airflow obstruction (GOLD III or IV), and a higher risk of suffering acute exacerbations (≥2 per year). However, group C patients had less symptomatology than group D patients (figure 1).

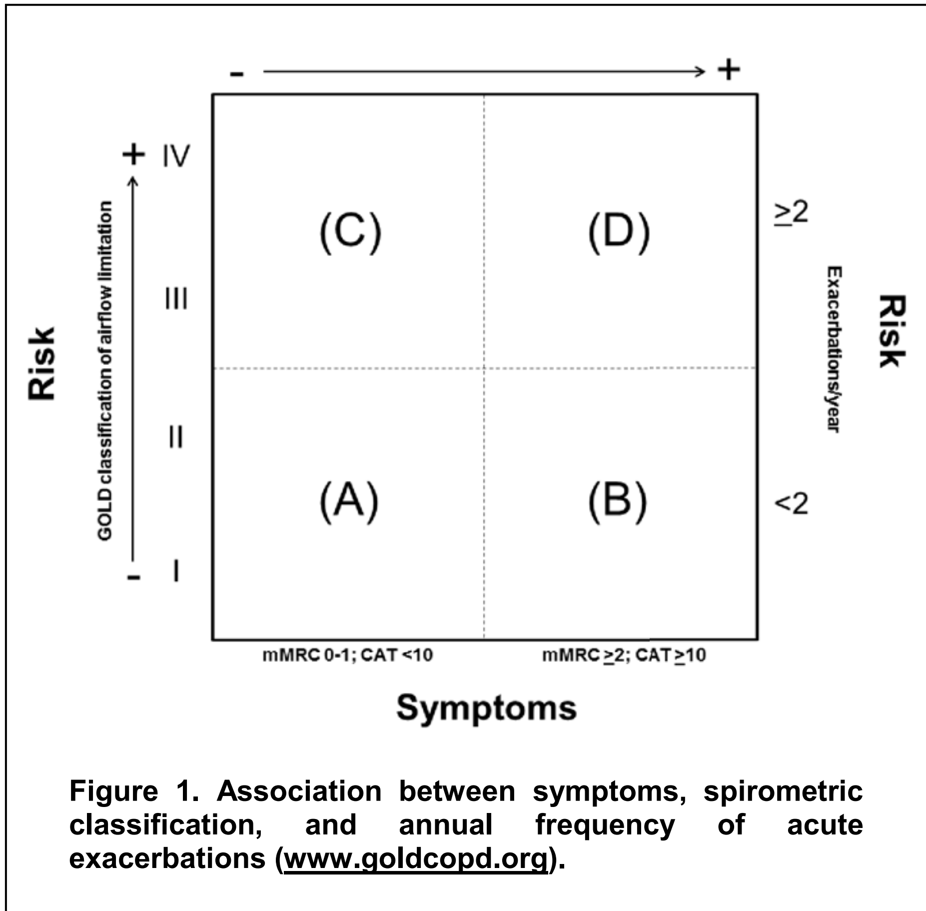


Figure 1. Association between symptoms, spirometric classification, and annual frequency of acute exacerbations (www.goldcopd.org).

1.1. Acute exacerbations of COPD (AECOPD)

An acute exacerbation of COPD is defined as any sustained increase in respiratory symptomatology, such as dyspnea, cough, and/or expectoration, compared with the baseline situation of the patient, which requires a change in regular medication (Anzueto *et al.,* 2007). Patient management differs according to the severity of the episode. Mild acute exacerbations need increased doses of bronchodilators, moderate exacerbations require treatment with

systemic corticosteroids, antibiotics or both, while severe exacerbations are frequently associated with hospital admission (Decramer *et al.,* 2012).

The acute exacerbations are important events which contribute to the progress of the disease, speeding up the decline of the lung function. For this reason, they are associated with an increase in morbidity and mortality of the patients with COPD, and are indicators of poor prognosis (Anzueto *et al.,* 2007).

The cause of an acute exacerbation may be multifactorial, although up to 80% are due to bacteria, viruses, or atypical pathogens. Levels of air pollution and other environmental conditions, probably account for the remaining 20% (Anthonisen *et al.,* 1987). The main bacterial pathogen causing AECOPD is *Haemophilus influenzae* (20-30%), followed by *Streptococcus pneumoniae* and *Moraxella catarrhalis* (10-15% either). In addition, it has been suggested that *Pseudomonas aeruginosa* plays an important role in patients with very severe COPD (table 2).

Table 2: Microbial pathogens in COPD
(source: Sethi and Murphy, 2008)

Microbe	Role in Exacerbations	Role in Stable Disease
Bacteria		
Haemophilus influenzae	20–30% of exacerbations	Major role
Streptococcus pneumoniae	10–15% of exacerbations	Minor role
Moraxella catarrhalis	10–15% of exacerbations	Minor role
Pseudomonas aeruginosa	5–10% of exacerbations, prevalent in advanced disease	Probably important in advanced disease
Enterobacteriaceae	Isolated in advanced disease, pathogenic significance undefined	Undefined
H. haemolyticus	Isolated frequently, unlikely cause	Unlikely
H. parainfluenzae	Isolated frequently, unlikely cause	Unlikely
Staphylococcus aureus	Isolated infrequently, unlikely cause	Unlikely
Viruses		
Rhinovirus	20–25% of exacerbations	Unlikely
Parainfluenza virus	5–10% of exacerbations	Unlikely
Influenza virus	5–10% of exacerbations	Unlikely
Respiratory syncytial virus	5–10% of exacerbations	Controversial
Coronavirus	5–10% of exacerbations	Unlikely
Adenovirus	3–5% of exacerbations	Latent infection seen, pathogenic significance undefined
Human metapneumovirus	3–5% of exacerbations	Unlikely
Atypical bacteria		
Chlamydophila pneumoniae	3–5% of exacerbations	Commonly detected, pathogenic significance undefined
Mycoplasma pneumoniae	1–2% of exacerbations	Unlikely
Fungi		
Pneumocystis jiroveci	Undefined	Commonly detected, pathogenic significance undefined

The bacterial etiology of the acute exacerbations depends partly on the severity degree of the underlying COPD. *S. pneumoniae* predominates in patients with mild COPD (GOLD I-II), while *P. aeruginosa* and *Enterobacteriaceae* are frequently isolated during exacerbations in patients with lower FEV_1; GOLD III-IV (Eller *et al.,* 1998). With reference to *H. influenzae* and *M. catarrhalis*, no

association was found between their frequency and patient severity (Eller *et al.,* 1998).

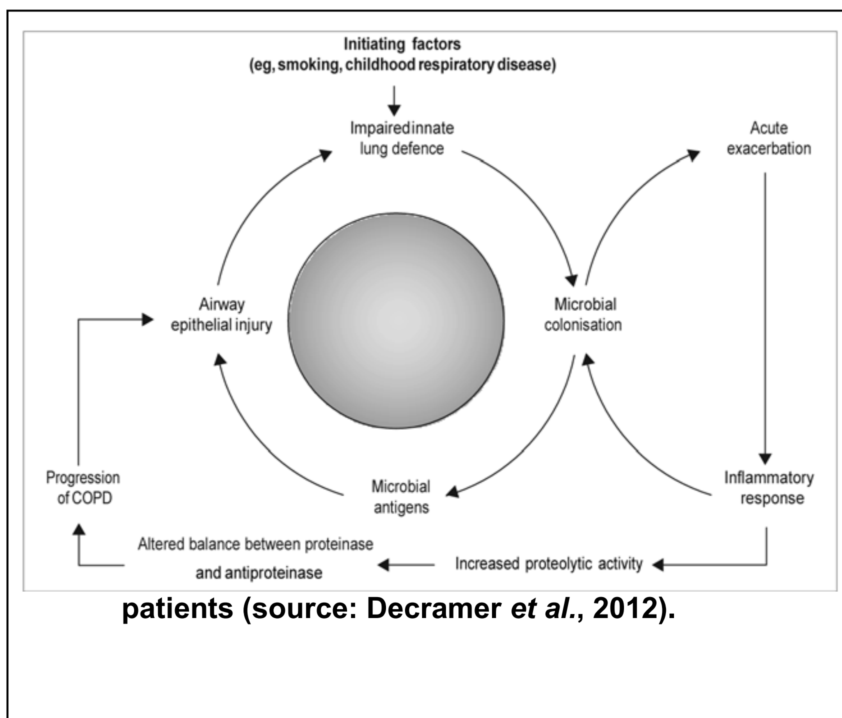
It has been reported that viruses account for 15-25% of all infective exacerbations; however, their precise pathogenic role is difficult to define and the frequency varies within the studies depending on the technique used for their detection (Sethi and Murphy, 2008). Their frequency may be underestimated by using only the viral culture, or overestimated by using current molecular techniques (DNA or RNA amplification). Nevertheless, rhinoviruses were the viruses most frequently detected as causative agents of acute exacerbations, followed by parainfluenza viruses and influenza viruses (Decramer *et al.,* 2012). In addition, co-infection of viruses and bacteria increases the severity of the acute exacerbations, and is detected in 25% of exacerbations requiring hospitalization (Papi *et al.,* 2006).

Acute exacerbations may also be caused by the atypical bacteria *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae,* although their pathogenic role is controversial and difficult to define. Several studies have analysed the role of *C. pneumoniae* but the results vary considerably, from 0% to 34%, depending on the techniques used for their detection (culture detection of respiratory samples, PCR detection of microbial DNA, or serology) (Beaty *et al.,* 1991; Papaetis *et al.,* 2009). Nevertheless, *M. pneumoniae* has rarely been found to cause exacerbations (Lieberman *et al.,* 2002; Smith *et al.,* 1980).

In healthy patients, the lower respiratory tract is sterile, and the natural niche of these potential pathogens is the colonization of the upper respiratory tract. However, in 25-50% of COPD patients

these potential pathogens colonize both upper and lower respiratory tracts, even in stable COPD (Monso *et al.,* 1995; Murphy, 2006). The presence of bacteria colonizing the lower airways of these patients promotes the airway and systemic inflammation, which results in bronchial epithelial injury. This damage, together with impaired mucociliary clearance, facilitates the acquisition of new microbial strains, and thus, increases the risk of developing a new acute exacerbation episode (Patel *et al.,* 2002; Sethi and Murphy, 2008). This is a vicious circle, since the acute exacerbation episodes speed up the progression of COPD through direct injury to the lung tissue, facilitating microbial colonization (figure 2). Hence, a higher frequency of acute exacerbations may be related with a higher incidence of bacterial colonization.

Figure 2. Cycle of infection and colonization in COPD



patients (source: Decramer *et al.*, 2012).

The acquisition of a new strain of *H. influenzae* and *M. catarrhalis* has been associated with a new episode of acute exacerbation. However, this association is not clear for other pathogens such as *S. pneumoniae* or *P. aeruginosa,* since the information available is scarce (Sethi *et al.,* 2002).

1.2. Role of bronchiectasis in COPD patients

Bronchiectasis is an obstructive lung disease characterised by a permanent dilation of the bronchi and bronchioles as a result of destruction of the muscles and elastic connective tissues, which results in chronic cough, sputum production, and recurrent infections (Barker, 2002). Depending on the pathological or radiographic appearance of airways, bronchiectasis can be classified as: cylindrical or tubular, characterised by dilated airways alone; varicose, characterised by focal constrictive areas along the dilated airways that result from defects in the bronchial wall; and cystic bronchiectasis, the most severe form of bronchiectasis mainly associated with Cystic Fibrosis, is characterised by a progressive dilatation of the airways, which ends in large cysts (Reid, 1986).

Different mechanisms lead to the development of bronchiectasis, such as congenital defects, post-infectious cases, or COPD; however, the pathophysiological end stage is similar (Goeminne and Dupont*,* 2010). Among COPD patients, a high prevalence of non-cystic bronchiectasis has been observed, mainly associated with moderate-to-severe COPD (Patel *et al.,* 2004). In fact, 30-50% of patients with very severe COPD show evidence of

bronchiectasis, and have more severe AECOPD exacerbations, lower airway bacterial colonization, and increased levels of sputum inflammatory markers (Patel, *et al.,* 2004). In addition, an association between moderate-to-severe COPD patients with bronchiectasis and an independent increased risk of all-cause mortality was suggested in these patients (Martínez-García *et al.,* 2013).

2. *Streptococcus pneumoniae*

2.1. Historical background

Streptococcus pneumoniae, also known "the pneumococcus", was discovered by Sternberg in September 1880, when he inoculated rabbits with his own saliva. Shortly afterwards, in an independent study in December of the same year, Louis Pasteur was able to isolate the pneumococcus from mice inoculated with saliva from a dead child. Pasteur was the first to report his findings at a meeting of the Medicine Academy of France in January 1881 (Pasteur, 1881). In 1884, Gram developed the Gram stain in order to facilitate the visualization of the pneumococcus in the pulmonary tissue. Today, the Gram is the most frequently used stain for the differentiation of bacteria (Gram, 1884).

The name of the pneumococcus has changed several times since its discovery. It was originally called "*Microbe septicémique du salive"* by Pasteur, and *Micrococcus pneumoniae by* Klein (Klein, 1884). Also known as *Diplococcus pneumoniae* (Weichselbaum, 1886), its present name, *Streptococcus pneumoniae* was officially accepted in 1980 (Skerman *et al.,* 1980).

The pneumococcus has been involved in many historic findings. In 1928, Griffith described the first evidence of bacterial transformation and demonstrated that a mouse co-inoculated with a heat-killed encapsulated pneumococcus together with another avirulent non-encapsulated pneumococcus died due to the

infection by capsulated pneumococci (Griffith, 1928). The substance responsible for this change was named the "transforming principle" (later named DNA) and was identified by Avery and co-workers from the Rockefeller Institute (Avery *et al.,* 1944). Other important discoveries resulting from investigations on pneumococci were the therapeutic efficacy of penicillin, the role of the bacterial capsule in resistance to phagocytosis, and the ability of polysaccharides to induce antibodies (Felton *et al.,* 1955). Another important date in the history of the pneumococcus is 2001, when the first overall genome of a pneumococcus was sequenced (Hoskins *et al.,* 2001; Tettelin, *et al.,* 2001).

2.2. Microbial characteristics

Streptococcus pneumoniae is a Gram-positive diplococcus, sometimes in short chains, from 1-2μm in diameter with an oval shape, immobile and non-sporulated bacteria.

It is an exclusively human pathogen, facultatively anaerobic, and its metabolism is fermentative (acid lactic fermentation), although it can grow aerobically (Bergey, *et al.,* 1974). However, the pneumococcus is considered a nutritionally fastidious bacterium; it needs blood or serum to grow, because it is unable to synthesise the catalase enzyme.

On blood agar plates, the pneumococcus produces an α-hemolysis when it is incubated in a 5% CO_2 atmosphere, as a result of the oxidation of the haemoglobin to green methaemoglobin due to the hydrogen peroxide it produces

(Barnard and Stinson, 1996). However, in anaerobic conditions the pneumococcus produces ß-hemolysis due to the pneumolysin (Ply) action (Brzin, 1969).

The colony morphology depends on the capsule and cell wall composition (Watson *et al.,* 1993). Thus, the “typical” shape of the colonies is ruffled and smooth, with a sunken centre (White, 1938). However, non-encapsulated strains have a small and dry shape, while strains of serotypes 3, 8 and 37 showed large mucoid colonies.

Besides the colony morphology, *S. pneumoniae* could be identified by the optochin susceptibility and bile solubility. Both characteristics are useful for discriminating the pneumococcus and other species of the *Streptococcus* genus (i. e. *S. mitis*: bile negative and optochin resistant, or *S. pseudopneumoniae*: bile negative and optochin resistant when it is incubated in a 5% CO_2 enriched atmosphere).

2.3. Taxonomy and pneumococcal identification

The *Streptococcus* genus taxonomy has changed over the years (Lancefield, 1933; Colman and Williams, 1972; Kawamura *et al*., 1995; Facklam, 2002). The current classification is based on molecular methods, whereas earlier classifications were based on phenotypic, physiological or biochemical characteristics.

In the *Bergey’s Manual of Systemic Bacteriology*, the genus *Streptococcus* was listed together with six other genera (*Aerococcus, Leuconostoc, Micrococcus, Pediococcus,*

Staphylococcus, and *Stomatococcus*) as facultatively anaerobic Gram-positive cocci (Bergey *et al.,* 1974).

Lancefield (1933) attempted to classify the streptococcal species based on the polysaccharide antigens present in the cell walls, and shortly afterwards Sherman (1937), divided the genus into four types: pyogenic, enterococci, lactic, and viridans (Lancefield, 1933; Sherman, 1937). The viridans division included the *S. pneumoniae* species plus the other non- β-haemolytic species.

With the application of 16S rRNA gene sequencing, a new classification based on six groups was created: *pyogenic, bovis*, *anginosus*, *salivarius*, *mutans* and finally *mitis*, which includes the most common commensal species together with *S. pneumoniae* (Kawamura *et al.*, 1995). Currently, this is the classification used in most publications, with the addition of a seventh group named *S. sanguis* (Facklam, 2002). However, the phylogenetic analyses based on the 16S rRNA gene sequence did not differentiate *S. pneumoniae* from its closely related species *S. mitis* and *S. oralis*, since they exhibit >99% homology (Whatmore *et al.*, 2000). In addition, the frequent exchange of genetic material by horizontal gene transfer (HGT) between these species and the appearance of new *Streptococcal* species such as *S. pseudopneumoniae* complicate their classification (Jado *et al.*, 2001; Arbique *et al.,* 2004; Chi *et al.,* 2007).

Recently, the matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) fingerprinting has been used for rapid identification of different bacteria. This technique is unable to differentiate the closely related Streptococci

species, using the whole-cell mass spectra from identification (Steensels *et al.,* 2011). Nevertheless, a close analysis of the presence/absence of some peaks seems to be able to differentiate *S. pneumoniae* isolates from isolates of the related species *S. pseudopneumoniae*, *S. oralis* or *S. mitis* group (Werno *et al.,* 2012; Ikryannikova *et al.,* 2012).

Serotyping is universally used to characterise pneumococci, and several methods are available. Traditionally, the most frequently used was the Quellung reaction, based on the biochemical reaction between capsular antigens and specific antibodies. Currently, several multiplex PCRs and also real-time PCR assays have been developed for the identification of the most frequent capsular types detected among pneumococci causing human diseases (http://www.cdc.gov/ncidod/biotech/strep).
However, serotyping is not sufficient to investigate whether isolates of the same serotype are genetically uniform, which limits our understanding of the frequency of serotype exchange (Coffey *et al.,*1998a).

In 1997, the Pneumococcal Molecular Epidemiology Network (PMEN) was established with the aim of performing global surveillance of antibiotic-resistant *Streptococcus pneumoniae*. A nomenclature of the 16 most frequent multi-drug resistant clones was standardized (McGee *et al.,* 2001) and classified according to their Pulse Field Gel Electrophoresis (PFGE) pattern, Multi Locus Sequence Typing (MLST) analysis, and their Penicillin-Binding Proteins (PBPs) fingerprinting (http://www.sph.emory.edu/PMEN). In 2004, PMEN decided to include major invasive antibiotic-

susceptible clones that have a wide geographic spread. Currently 43 PMEN clones are classified.

The Multi Locus Sequence Typing (MLST) is a molecular method developed in 1998 (www.mlst.net), based on the analysis of the internal sequences (around 450pb) of seven housekeeping loci (*aroE, gdk, gki, recP, spi, xpt* and *ddl*), present in all pneumococci, but also in closely-related Streptococci (Enright and Spratt, 1998).

Initially, MLST was developed and validated using *Neisseria meningitis.* However, it is currently used for the identification of clones of several bacterial species. With reference to *S. pneumoniae*, apart from the identification of the major clones circulating in a geographical area or even worldwide, is also used for the differentiation between the closely related species *S. pneumoniae*, *S. pseudopneumoniae*, *S. mitis* and S. oralis (Do, *et al.,* 2009). This new phylogenetic analysis (Multi Locus Sequence Analysis; MLSA, is based on the concatenation of six MLST genes (all but *ddl*).

2.4. Pneumococcal virulence factors

S. pneumoniae produces several virulence factors in order to facilitate the colonization of the nasopharynx in children and/or respiratory tract in adults, especially those over 65 years old. These virulence factors allow the adherence to the host and the acquisition of nutrients required for pneumococcal survival and multiplication. Moreover, several virulence factors are also involved

in pathogenesis, and for this reason are located on the bacterial surface (Jedrzejas, 2001). In this section, the most important virulence factors are described.

2.4.1. Pneumococcal capsule

Pneumococcal strains are usually capsulated. The capsule completely envelops the *S. pneumoniae* bacteria, and acts as a protective layer, isolating the bacterial cell from its environment (figure 3). Although the capsule does not play a role in a adherence, invasion or inflammation in the host (McCullers and Tuomanen, 2001), it increases the pneumococcal virulence because it protects the bacteria against phagocytic killing by reducing the complement binding to the bacterial cell wall and by shielding the bound complement from phagocytes. For this reason, the capsule is considered the main virulence factor.

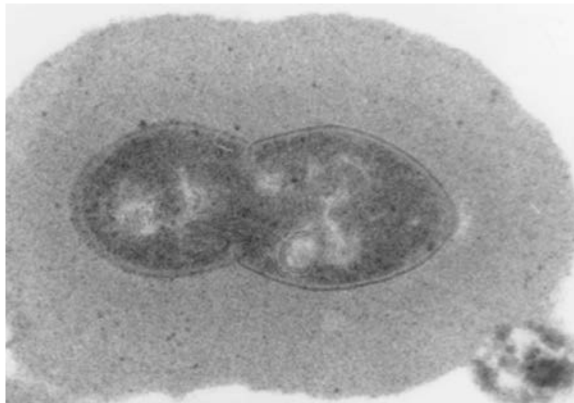


Figure 3. Electron micrograph of a pneumococcal strain, showing the capsular polysaccharide type 3 (source: Kim *et al.,* 1999)

Capsule expression is essential for the persistence of *S. pneumoniae* in normally sterile sites, where it can cause disease. In fact, it has been reported that non-encapsulated pneumococcal variants are virtually avirulent (Griffith, 1928). However, non-capsulated strains have also been isolated from sterile samples in patients with IPD or causing outbreaks of epidemic conjunctivitis (Carvalho *et al.,* 2003).

The US nomenclature for the capsule classification was replaced by the Danish system, based on their reactivity with monoclonal or polyclonal antibodies (Lund, 1970). To date, 94 distinct capsular types (serotypes) have been identified (Calix *et al.,* 2012), and some of them are grouped into serogroups, due to the similar agglutination patterns and the cross reaction with the antibodies. A total of 46 serogroups have been described; some of them included only one serotype (serotypes 1 and 3), and other serogroups included up to six serotypes (serogroup 11; serotypes 11A, 11B, 11C, 11D, 11E and 11F).

The capsular polysaccharide is a linear or ramified polymer constituted by repeating units of two (e.g. serotype 3) or more monosaccharides, such as serotype 17A (8 monosaccharides). Despite the capsular variability, all but two serotypes (3 and 37) share similar biosynthetic pathways and are synthesised by the Wzy-dependent pathway (Llull, *et al.,* 2001; Bentley *et al.*, 2006). The genes encoding the capsule are mapped in a capsular operon named *cps*, and are flanked by the genes *dexB* and *aliA* (Garcia and Lopez, 1997). This operon is similar to the one found for the genes involved in polysaccharide biosynthesis in some lactic acid bacteria (De Vuyst and Degeest,

1999). The capsule is synthesised by successive transferring of monosaccharide to a lipid carrier associated to the membrane. Once these repeat units are transferred to the outer membrane (by a repeat-unit transporter or flippase), are polymerized forming the mature CPS, which is attached to the cell wall (peptidoglycan) by a covalent linkage (Sorensen *et al.,* 1990). For their part, types 3 and 37 use the synthase dependent pathway, requiring only a glycosyltransferase anchored to the membrane (Arrecubieta *et al.*, 1996).

Although all but two serotypes share the similar synthesis pathway, pneumococci expressing different capsular types have been associated with a high invasive potential (such as serotypes 1, 5 and 7F) or with serotypes with low ability to cause disease but commonly found colonizing the nasopharynx in children, such as 6B, 19F or 23F (Brueggemann *et al.*, 2003a).

It has been suggested that the high diversity of the capsular types is due to the selective pressure of the human immune system (Bentley *et al.,* 2006). In fact, several molecular mechanisms may explain this variability: the horizontal gene transfer (HGT) from other pneumococci or from other *Streptococcal* species, the accumulation of point mutations or the movement of mobile genetic elements (Bentley *et al.,* 2006).

In addition, it has been reported that isolates of some serotypes such as serotype 1 are associated with low genetic diversity (Brueggemann and Spratt, 2003b) while other serotypes such as serotype 19A are expressed by several genotypes, showing geographical and temporal variations (Tarrago *et al.,* 2011). Moreover, isolates of several clones (genotypes) expressed

different capsular types due to the exchange of capsular genes by HGT; this is known as “capsular switching”, and was first described two decades ago (Coffey TJ *et al.,* 1991). However, since the introduction of the 7-valent pneumococcal conjugate vaccine (PCV7), serotype switching is a cause of concern because it allows pre-existing clones expressing PCV7 serotypes to escape the vaccine-induced immunity by acquitting capsular genes of a non-PCV7 serotype. This has recently been reported in an isolate with a genotype only associated with vaccine serotype 4 (ST695), which now expresses a non-PCV7 serotype 19A capsule (Brueggemann *et al.,* 2007). The rate of capsular switching among pneumococcal isolates in nature is unknown; however, it may be relatively common, since the expression of a new serotype by isolates of the same clone could arise from different recombination events (Coffey *et al.,* 1998a; Coffey *et al,,*1998b).

2.4.2. Pneumococcal cell wall

The cell wall, common to all pneumococcal serotypes, is located below the capsule and consists of a peptidoglycan layer, alternating N-acetylglucosamine and N-acetylmuramic acid residues, linked by ß-1→4 bonds (AlonsoDeVelasco *et al.,* 1995). It carries covalently attached anionic polymers such as wall teichoic acid (WTA) (Denapaite *et al.,* 2012). Cell wall and the attached carbohydrates (CWPS) are important virulence factors. In contrast to the capsular polysaccharide, this complex induces a similar inflammation to that seen after infection with whole pneumococci. In this way, the injection of purified cell wall in mice mimics otitis media, meningitis

or pneumonia disease (Tuomanen *et al.,* 1985a; Tuomanen *et al.,* 1985b; Carlsen *et al.*, 1992). Attached to the cell wall there are two major groups of pneumococcal cell-surface proteins: the choline-binding proteins and the group of LPxTG-anchored surface proteins (figure 4).

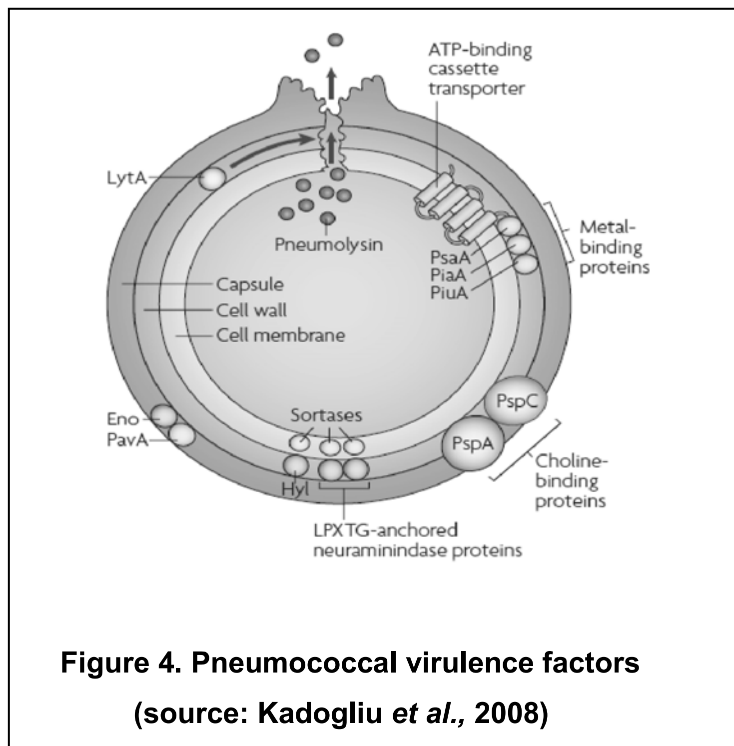


Figure 4. Pneumococcal virulence factors (source: Kadogliu *et al.,* 2008)

2.4.3. Choline-Binding Proteins (CBPs)

Phosphorylcholine is an important component of the pneumococcal cell wall, recognized by components of the host innate immune system (Bergmann and Hammerschmidt, 2006). It is important because it anchors a family of proteins named Choline-

Binding Proteins. This family is also found in other related species such as *S. pseudopneumoniae* (Llull *et al.,* 2006), *S. mitis* (Hakenbeck *et al*., 2009) and *S. oralis* (Reichmann *et al.,* 2011). Some CBPs are highly conserved between *S. pneumoniae* and *S. mitis*, whereas the virulence-associated PspA and PspC are specific to pneumococci and are thus relevant for the characteristic properties of this species. CBPs contribute significantly to the hydrophobic and electrostatic surface characteristics of pneumococci and they may facilitate the adherence to host cells partially through nonspecific, physicochemical interactions (Swiatlo *et al.,* 2002).

Pneumococci can produce up to 15 different CBPs, depending on the strain. The choline-binding domain allows binding to the phosphorylcholine of the cell wall non-covalently through a choline-binding domain (Lopez and Garcia, 2004; Hakenbeck et al., 2009). The amino (N)-terminal sequences vary widely, and are the sites of the specific activities of the different proteins (Jedrzejas MJ, 2001).

Autolysins. This group of murein hydrolases participates in a high variety of fundamental biological functions, such as cell wall synthesis, separation of the daughter, and genetic transformation (Paton *et al.,* 1993). The LytA protein, a N-acetylmuramoyl-L-alanine amidase, is considered the major pneumococcal autolysin, and is required for the stationary-phase autolysis typical of pneumococci. LytA gene has been found in closely related streptococci (Llull *et al.,* 2006) and there is evidence of polymorphic regions arising from recombination events with homologous genes of pneumococcal temperate bacteriophages

(Morales *et al.,* 2010). The physiological role of LytA is unclear, but it may mediate the release of pneumolysin, one of the major virulence factors of pneumococci, into the extracellular environment (Lock *et al.*, 1992).

Two other autolysins, LytB and LytC are also present in pneumococci; LytB is most probably a N-acetylglucosaminidase capable of degrading choline-containing cell walls, and could participate in cell separation (Lopez and García, 2004). LytC is a pneumococcal lysozyme but its physiological role remains to be elucidated.

Pneumococcal surface protein A (PspA). This is a 65kDa protein, which interferes with the complement system, by binding to lactoferrin (Hammerschmidt *et al.,* 1999). Lactoferrin plays an important role in innate immunity, and their interaction decreases the complement component C3 on the pneumococcal surface and inhibits opsonisation (Tu *et al.,* 1999). In addition, PspA may help pneumococci on mucosal surfaces to overcome the iron limitation, which may represent another potential virulence mechanism (Hammerschmidt *et al.,* 1999). PspA mutants lacking *pspA* gene have reduced virulence in a sepsis model (McDaniel *et al.,* 1987) However, the exact mechanisms by which PspA confers virulence are not fully understood.

PspA structure differs from that of autolysins. It has three structural domains: a N-terminal functional helical charged module, separated by a proline-rich linker, which confers flexibility, from the C terminal choline-binding domain, which binds to the phosphorylcholine of the cell wall (Jedrzejas, 2001). PspA is expressed by most of the clinical isolates of *S. pneumoniae* (Rolo

et al., 2009); however it is highly variable, even among isolates belonging to the same clone (Crain *et al.,* 1990; Hollingshead *et al.,* 2000). Based on its N-terminal sequence, PspA can be grouped into three families which, in turn, can be divided into six different clades (Hollingshead *et al.,* 2000).

Pneumococcal surface protein C (PspC). PspC protein is a multifunctional CBP of pneumococci, also known as CbpA, SpsA or PbcA. It acts as an adhesin (Rosenow et al., 1997), by promoting adherence and invasion of epithelial cells (Zhang et al., 2000). In fact, PspC mutants showed a reduced ability for nasopharyngeal colonization (Rosenow et al., 1997). An additional property of PspC is its ability to binds soluble host factors such as the IgA secretory fragment, C3 and complement factor H, avoiding the complement activation (Hammerschmidt et al., 1997). Similar to PspA, comparison of PspC sequences showed a high variability due to mosaic structures, and can be divided into two structural groups (Iannelli F *et al.,* 2002).

Several types of choline-biding surface proteins, especially PspC and PspA, are candidates for a universal pneumococcal vaccine, and are currently under investigation. Recently, invasive disease and nasal colonization were prevented after nanogel-based PspA intranasal vaccination in murines (Kong *et al.,* 2013).

At present, none of the proteins are believed to elicit species-wide pneumococcal protection. A combination of proteins should be considered in future protein vaccine strategies, since there is evidence that some of these combinations provide additive or even synergistic protection (Lebon *et al.,* 2011).

2.4.4. LPxTG-anchored pneumococcal proteins

Several pneumococcal cell-surface proteins are covalently linked to the bacterial cell wall through a sortase transpeptidase, which recognizes the amino-acid sequence LPxTG (where “x” denotes any amino acid residue). It is estimated that up to 20 *S. pneumoniae* proteins are anchored by an LPxTG motif; nearly all of them have been associated with adhesion, but also with the ability to produce biofilm (Löfling *et al.,* 2011). This group includes the neuraminidases, pneumococcal pilus and serine-rich repeat (SRR) proteins.

Neuraminidases. Neuraminidases, also known as sialidases, are enzymes that cleave sialic acid residues from competing pathogens and from host proteins (Bergmann and Hammerschmidt, 2006). *S. pneumoniae* encodes at least three neuraminidase genes: *nanA*, *nanB* and *nanC*, although only NanA contains the LPxTG motive and is expressed by all pneumococcal strains (it is part of the core genome). Both NanB and NanC are part of the accessory genome and seem to be secreted (Pettigrew *et al.,* 2006). A role of the neuraminidases NanA and NanB has been suggested in septicaemia (mutant isolates showed a major decrease in survival in the respiratory tract and bloodstream (Manco *et al.,* 2006). They are also involved in biofilm formation (Trappetti *et al.,* 2009). It has been suggested that NanC has a tissue-specific role, since it was more common in isolates from cerebrospinal fluid than in carriage isolates (Pettigrew *et al.,* 2006).

Pneumococcal Pilus. The number of pilus gene island variants differs within each streptococcal species (Kreikemeyer *et al.,* 2011). In pneumococci, two different types were detected, the

first one described in 2006 (Barocchi *et al.,* 2006). The pilus I (PI-1) structure is codified by a pathogenicity islet of 12 kb (named *rlrA*), which includes seven genes: *rlrA* encoding a transcriptional regulator, *rrgA*, *rrgB* and *rrgC* encoding subunits for a multimeric pilus structure as well as *srtB*, *srtC* and *srtD* encoding three pilin-specific sortases which anchors the pili to the cell wall (Barocchi *et al.,* 2006; Löfling *et al.,* 2011). The *rlrA* islet is present in fewer than 30% of all the clinical pneumococcal isolates.

The PI-1 pilus, and specifically the RrgA protein, promoted adhesion to lung epithelial cells *in vitro,* as well as in colonization, in a murine model of infection (Barocchi *et al.,* 2006; Nelson *et al.,* 2007). However, recent data demonstrate that the pilus-associated adhesin RrgA promotes phagocytosis by bone marrow derived macrophages, in a process requiring complement receptor 3. This process is likely to enhance bacterial clearance, suggesting a dual role of pneumococcal pili in its interaction with the host (Orrskog *et al.,* 2012).

Additionally, a second pilus type (PI-2) has been described, which encodes five genes: two structural proteins (PitA and PitB), a signal peptidase-like protein SipA and two sortases SrtG1 and SrtG2 (Bagnoli *et al.,* 2008). This pilus is present in emergent pneumococcal serotypes and in some cases some strains are capable of expressing both PI-1 and PI2 (Bagnoli *et al.,* 2008).

Glycosylated serine-rich repeat (SRR) proteins. The most important SRR protein is called PsrP, a putative glycosyl transferase (Shivshankar *et al*., 2009). PsrP has a tissue tropism and plays a role in adhesion to host cells, biofilm formation and persistence in a pneumonia murine model (Rose L *et al.,* 2008;

Sanchez *et al.,* 2010); however, several clonal lineages of different serotypes lack this protein (Muñoz-Almagro *et al.,* 2010).

2.4.5. Pneumolysin

Pneumolysin belongs to the family of cholesterol-dependent cytolysins, found in virtually all pneumococcal isolates with high amino acid sequence conservation between isolates (Kadiogliu *et al.,* 2008). Pneumolysin is produced as a 52kDa soluble protein that oligomerizes in the membrane of target cells to form a large ring-shaped transmembrane pore. The pore is 260Å in diameter and is composed of approximately 40 monomer subunits (Tilley *et al.,* 2005). Pneumolysin is a wide-ranging virulence factor which can lyse any eukaryotic cell with cholesterol in its membrane, inhibit the ciliary beating on respiratory epithelium, inhibit the phagocyte respiratory burst and induce cytokine synthesis (Kadioglu *et al.,* 2004).

3. Colonization and pneumococcal diseases

S. pneumoniae is part of the commensal microbiota of the upper respiratory tract, and shared the same ecological niche as other several bacterial species, such as *H. influenzae*, *S. aureus, M. catarrhalis,* and various haemolytic *Streptococci*. The pneumococcal carrier status is transient although the same strain may persist for several months asymptomatically (Gray, *et al.,* 1980). However, pneumococcus is considered one of the leading bacterial causes of morbidity and mortality worldwide, being responsible for a wide variety of invasive and non-invasive diseases. The risk groups for pneumococcal disease include young children, the elderly, and patients with underlying conditions or immunodeficiencies.

3.1. Colonization

Pneumococcal colonization occurs shortly after birth in non-developed countries, although in industrialized countries it occurs later in life (Austrian, 1986). A single strain can persist in the nasopharynx for weeks or months, and is then replaced by other strains. The colonization rates increases above 50% among children under 5 years, and multiple serotypes are often present in the nasopharynx of the same child (Rodrigues *et al.,* 2009, Wroe *et al.,* 2012). In fact, by the age of two, more than 90% of children have been colonized with up to six different serotypes at any time (Gray *et al.,* 1980). For this reason, the nasopharynx of children is considered the major reservoir of *S. pneumoniae*.

In adults, the frequency of colonization found ranges from 3% to 20%, being higher in elderly people or patients with chronic respiratory diseases (Lloyd-Evans *et al.,* 1996).

Pneumococcus is clearly transmissible by direct contact or through aerosols, and family exposure plays an important role up two years of age, regardless of family size (Hoshino *et al.,* 2002). Exposure of children in day care centres (DCC) also plays a role in pneumococcal transmission, but its effect varies depending on the time spent in day care or the size of the centre (Dagan, *et al.* 1996; Leino *et al.,* 2001).

Nasopharyngeal colonisation provides an important key to the burden of pneumococcal disease and its prevention. Colonization by *S. pneumoniae* requires adherence to the epithelial lining of the respiratory tract. However, little information is available about the interaction of pneumococci with the host and the conversion of colonization to disease.

3.2. Pneumococcal disease

According to the World Health Organization, in 2000 about 15 million episodes of serious pneumococcal disease occurred worldwide, resulting in about 800,000 annual deaths in children aged up to 5 years. Pneumococcal diseases are associated with significant morbidity and mortality, causing a high burden and raising costs for health care systems worldwide. In developed countries, mortality associated with *S. pneumoniae*, remains substantial (ranging from <1 to 30%) despite appropriate

antimicrobial therapy. The outcome depends mainly on the age and the underlying conditions of the patients. Thus, the incidence of pneumococcal disease is higher in children up to the age of two years and in adults over 65 years old (Parsons and Dockrell, 2002).

S. pneumoniae usually colonizes the nasopharynx of young children, but it may occasionally invade the lungs, bloodstream or brain, causing severe infections (Hausdorff *et al.*, 2005; De Lencastre and Tomasz, 2002). Sinusitis, conjunctivitis, acute otitis media, acute exacerbations of COPD and non-bacteraemic pneumonia are considered non-invasive diseases, since they occur outside the blood (and can be isolated from mucosal excretions), and usually cause less serious illness (Bogaert *et al.,* 2004). Invasive pneumococcal disease includes meningitis, bacteraemic pneumonia and other bloodstream infections, in which pneumococci is isolated from blood or other normally sterile body fluids such as cephalospinal fluid.

3.2.1. Acute otitis media (AOM)

The development of AOM is a complex process starting in the nasopharynx, which is connected to the middle ear by the Eustachian tube. AOM is a middle ear inflammation that results in collection of fluid in the middle ear and associated local and systemic symptoms (Lieberthal *et al.*, 2013). It is one of the most common diseases in the first five years of life (Teele *et al.,* 1989). The main clinical findings are irritability, fever, earache, and in many cases vertigo, or decreased hearing.

A large percentage of AOM cases is viral; however *S. pneumoniae* is the most common bacterial pathogen responsible for otitis media, which accounts for 30-40% of cases: Non-typeable *H. influenzae* (21%) and *M. catarrhalis* (12%) are also frequent causes of AOM (Kilpi *et al.,* 2001). Most children are treated with antibiotics, predominantly amoxicillin, with or without clavulanic acid. As a consequence of antibiotic use and the introduction of pneumococcal conjugate vaccines (PCV), the aetiology of AOM continues to change over time (Pichichero, 2013).

Capsular types causing pneumococcal AOM are usually the same serotypes with high rates of nasopharynx colonization, mainly 6A, 6B, 14, 19A, 19F, and 23F (Somech *et al.,* 2011).

3.2.2. Pneumonia

The diagnosis of pneumonia is complicated since a large number of diseases can mimic the condition, and distinguishing an infectious from a non-infectious aetiology for pulmonary infiltrates may be difficult. Moreover, the clinical presentation of symptoms depends on the microbiologic agent and host factors such as age, immune status, or underlying conditions, but in general, a pneumonia episode is considered when fever, leucocytosis and radiological findings (new infiltrates on chest radiography) are detected.

Community-acquired pneumonia (CAP) is defined as pneumonia acquired in a nonhospital environment. It is important to

differentiate it from nosocomial and aspiration pneumonia, since the etiological agents are different.

In children with pneumonia, *S. pneumoniae* also plays an important role, causing 37% of overall episodes, while mixed infection (bacterial and viruses) was observed in 30% of patients (Juven *et al.,* 2000). In adult patients with CAP, *S. pneumoniae* is the most commonly identified bacterial pathogen leading to hospitalization (Kalin and Linberg, 1983); about 30% of pneumococcal pneumonia episodes are related to bacteraemia.

COPD is the most frequent underlying condition among adult patients with CAP, especially in patients with recurrent pneumococcal pneumonia, of whom 38.4% had COPD (Garcia-Vidal *et al.,* 2009). In addition, it has been suggested that among COPD patients with advanced airflow obstruction, BPP may be associated with a lower propensity to develop shock and, hence, with a lower risk of death than patients with BPP and no COPD (Calbo *et al.,* 2009).

3.2.3. Meningitis

Clinical symptoms of pneumococcal meningitis are similar to those with other bacterial causes, including fever, headache and neck stiffness, due to the inflammation of the membranes covering the brain and the spinal cord, the meninges. Meningitis may be a complication of an acute otitis media, a pericranial fistula, or a pneumonia episode.

Meningitis is mainly caused by bacteria that have capsules, such as *S. pneumoniae* (47%) and *Neisseria meningitidis* (14%). The introduction of the vaccine against *H. influenzae* type b drastically decreased meningitis caused by this pathogen (Swartz, 2004).

The majority of patients with pneumococcal meningitis have also related bacteraemia, and up to 50% of survivors suffer sequelae due to the meningitis per se, or due to complications associated with the bacteraemia (Schuchat *et al.,* 1997).

3.2.4. Bacteraemia

Around 80% of adult pneumococcal bacteraemia is associated with a pneumococcal pneumonia episode. However, in children, primary pneumococcal bacteraemia (no anatomical focus identified) is frequent, reaching up to 45% of cases in those under the age of two.

Next to meningitis, bacteraemia reaches the highest pneumococcus-related mortality rates due to its severity. In adults, mortality has been associated with patients over 65 years old, immunosuppression, nosocomial acquired infections, polimicrobial pneumonia and other more serious underlying conditions (Pallares *et al.,* 1995).

Pneumococci may cause other less frequent but no less important invasive diseases. Several examples are: otomastoiditis, peritonitis, septic arthritis, endophthalmitis, endocarditis or pericarditis.

4. Prevention of pneumococcal infection

It is well known that the polysaccharide capsule is the major virulence factor of pneumococci, protecting bacteria from phagocytosis. For this reason, all pneumococcal vaccines available so far have included purified capsular polysaccharides. These antigens are highly immunogenic and the antibodies generated protect against infection with the homologous serotype (Bogaert *et al.,* 2004).

The first description of attempts of pneumococcal prevention dates from 1911, even before the demonstration of the immunogenicity of the bacterial capsular polysaccharide. Sir Almroth E. Wright and his colleagues developed a vaccine consisting of whole killed pneumococci to immunize South African gold miners, a population group with high incidence of pneumococcal infections (Wright *et al.,* 1914). Unfortunately, this vaccine failed because of inadequate dosage and the inclusion of only two pneumococcal serotypes.

In 1926, Felton and Bailey reported the isolation of capsular polysaccharides, naming the resulting material a “soluble specific substance”. It speeded up the research into new vaccines, and in 1937 Felton’s capsular material of types I, II, V and VI was used successfully to abort an outbreak of pneumonia at a state hospital in Worcester (Smillie *et al.,* 1938).

After World War II, two hexavalent polysaccharide vaccines were licensed in the United States. However, with the introduction of penicillin and sulfonamides for the treatment of pneumococcal

disease at practically the same time, the vaccines were withdrawn from the market in 1954 due to lack of demand (Austrian, 1981).

However, in the 1960s, Austrian and colleagues noted that, despite the apparent effectiveness of penicillin treatment *in vitro*, pneumococcal pneumonia caused more deaths in America than any other infectious disease. They concluded that penicillin was unable to reduce the prevalence of pneumococcal bacteraemia (Austrian and Gold, 1964). In consequence, in 1967 they developed a multivalent vaccine containing the polysaccharide components of each of the 14 most common pneumococcal serotypes, which caused some 80% of cases of pneumococcal disease (1, 2, 3, 4, 6A, 7F, 8, 9N, 12F, 14, 18C, 19F, 23F and 25). It was licensed in 1977 in United States. This vaccine was expanded in 1983 to a 23-valent vaccine, and had a theoretical coverage of ≥80% of the pneumococci causing infections in adults.

Today, several commercialized pneumococcal vaccines are available, which we discuss below (figure 5).

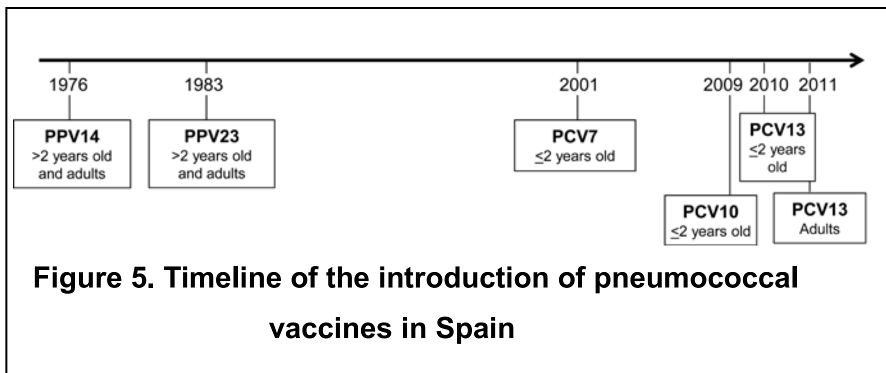


Figure 5. Timeline of the introduction of pneumococcal vaccines in Spain

4.1. Polysaccharide vaccines

At present, only one polysaccharide vaccine is available, the 23-valent vaccine (PPV23) which is licensed for adults and children aged >2 years old (Robbins and Schneerson, 1983). This pneumococcal polysaccharide vaccine (Pneumovax®, Merck & Co. INC) includes 23 purified capsular polysaccharide antigens of the pneumococcal 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.

The vaccine has been shown to be safe and is recommended for populations at risk of serious pneumococcal disease such as the immunocompromised, elderly patients suffering chronic pulmonary diseases as COPD, and patients with cardiac or renal disease (Shapiro *et al.,* 1991).

The immune response elicited by the capsular polysaccharide is a T-cell independent response, and anti-pneumococcal antibody production by mature B-lymphocytes is required. Hence, the period of protection is limited because of the absence of memory B cells. For this reason, together with the poor immunogenicity of many of the polysaccharides included in the 23-valent vaccine, this vaccine is not effective in children <2 yrs (Finn, 2004). Therefore, this vaccine does not reduce mucosal carriage of pneumococcus, and fails to prevent the spread of pneumococcal strains resistant to antimicrobial drugs (Bogaert *et al.,* 2004).

In children over 2 years of age and in adults over the age of 65, the 23-valent vaccine has proved effective in preventing bacteraemia and reduces the risk of IPD. However, its effectiveness in patients with COPD is still a matter of debate:

COPD adults respond differently from the general adult population, due to their impaired antibody response to the vaccine, the colonization of the lower respiratory tract, and the frequent use of inhaled corticosteroids (Schenkein *et al.,* 2008). In fact, two recent reviews on the efficacy of PPV23 in patients with chronic respiratory diseases such as COPD did not provide clear results (Walters *et al.,* 2010; Vila-Corcoles and Ochoa-Gondar, 2012). Although they suggest a possible protection of PPV23 against morbidity in COPD patients, no significant reductions in any outcome were shown, including the annual number of acute exacerbations and hospital admissions (Walters *et al.,* 2010)

Nevertheless, the range of serotypes for which protection is elicited and its efficacy in protection against IPD has led to its continued use. The Advisory Committee of Immunization Practices (ASIP) recommends vaccination with PPV23 in adults with chronic lung diseases such as COPD (Advisory Committee of Immunization Practices, 2013).

4.2. Conjugate vaccines

Conjugate vaccines are based on the covalent binding of capsular polysaccharides to carrier proteins, which increases the immunogenicity of the antigens. This makes the vaccine more complex and costly to produce, and for this reason the number of capsular polysaccharides included is lower. However, unlike PPV23, these vaccines are effective in infants and children because they are able to stimulate the T cell-dependent response

and to develop the polysaccharide-specific B cells into memory cells (Eskola and Anttila, 1999; Finn, 2004).

The **7-valent pneumococcal conjugate vaccine** (PCV7; Prevenar® 7, Pfizer) was the first paediatric conjugate vaccine licensed in the United States in February 2000 and June 2001 in Spain. It includes the seven most common serotypes isolated from invasive pneumococcal disease in children under 6 years old (4, 6B, 9V, 14, 18C, 19F and 23F) in the United States. The carrier protein used was a non-toxic variant of diphtheria toxoid, CRM197), and the recommended primary vaccination schedule is three doses, plus a booster (at 2, 4, 6 and 12-15 months of age). PCV7 has also been introduced into the childhood vaccination program of many other countries such as Canada, UK and Gernany. However, in Spain the coverage of PCV7 serotypes was estimated to be around 70% and was not included in the vaccination program, with exception of the *Comunidad Autonoma de Madrid,* which included universal vaccination for children aged <2 years between November 2006 and June 2012.

PCV7 has proved highly effective in the protection of young children against IPD. In fact, in the United States, the Centers for Disease Control and Prevention’s Active Bacterial Core Surveillance (ABCs) reported a dramatic decline of vaccine serotypes causing IPD in children aged <5 years as early as 2001 (World Health Organization, 2005). In addition, a reduction of PCV7 serotypes was also observed in the adult population among invasive and non-invasive pneumococci (Whitney *et al.,* 2003; Kyaw *et al.,* 2006; Fenoll *et al.,* 2012). This decrease suggests a

level of herd protection which protects the unvaccinated population, as a result of a reduction in the carriage state of vaccine serotypes (VC) in vaccinated children (O'Brien *et al.,* 2007).

Shortly after the introduction of PCV7, an increase in carriage of pneumococcal non-vaccine serotypes (NVS) in children was observed, and an increase in IPD as well (Hicks *et al.,* 2007). There are two possible reasons for these changes in the serotype distributions. The first is the expansion of pre-existing NVS clones, such as the emergence of multi-drug resistant pneumococci of serotype 19A in many regions. In fact, serotype 19A has now been isolated as one of the most frequent cause of IPD (Moore *et al.,* 2008; Ardanuy *et al.,* 2009b; Van der Linden *et al.,* 2013). The other phenomenon is the capsular switching of genotypes related to VS through acquisition of NVS *cps* genes, described in section 2.4.1.

In August 2009, GlaxoSmithKline commercialized a **10-valent pneumococcal conjugate vaccine** (PCV10) in Spain (Synflorix™, GlaxoSmithKline). This vaccine extends the PCV7 coverage, including three additional pneumococcal serotypes (1, 5 and 7F). In contrast to the other conjugate vaccines, most of the capsular polysaccharides included in PCV10 are conjugated to protein D from non-typeable *Haemophilus influenzae*. Hence, it protects against IPD and probably against acute otitis media, in which NTHi plays an important role as a causative agent (Gladstone *et al.,* 2011). However, a recent study showed no differential effect of PCV10 immunization on nasopharyngeal NTHi colonization in healthy children up to 2 years of age compared with PCV7 vaccinated children (Van den Bergh *et al.,* 2013).

In Spain, the PCV7 vaccine was replaced by the **13-valent pneumococcal conjugate vaccine** (PCV13; Prevenar 13®, Pfizer) in June 2010. This vaccine offers coverage against PCV7 serotypes plus six new serotypes (1, 3, 5, 6A, 7F and 19A). It is expected to provide greater protection against pneumococcal infection; however, there is a potential risk that non-PCV13 serotypes may increase. In addition, this vaccine was also approved for adult consumption in 2011, and so hardly any data are available on its efficacy. A clinical trial entitled CAPITA (Community Acquired Pneumonia Immunization Trial in Adults) is under way to establish the efficacy of PCV13 in adult patients (Hak *et al.,* 2008). Nevertheless, it has been predicted that PCV13 is more effective and cost saving than PCV7, preventing 106.000 IPD cases in United States, saving $11.6 billion over a 10-year period (Strutton *et al.,* 2012).

ASIP recommends PCV13 vaccination for adults aged 19 years or older with immunocompromised conditions. In addition, ASIP also recommends a dose of PCV13 in adult immunocompromised patients, who have received one or more doses of PPV23 at least a year before (Advisory Committee of Immunization Practices, 2013). PCV13 was licensed for adults in November 2011.

5. Antimicrobial resistance in pneumococci

In the pre-antibiotic era, the mortality of bacteraemic pneumococcal pneumonia infection was around 77% (Tilghman, *et al.*, 1937). However, after the introduction of sulfonamides and penicillin in the first half of the twentieth century, mortality in patients with pneumococcal pneumonia decreased drastically (Austrian and Gold, 1964). For 40 years after the introduction of antibiotics in the 1940s pneumococcal strains were considered susceptible to antimicrobials. However, since the first description of pneumococci with non-susceptibitly penicillin in Austria (Hansman and Bullen, 1967), resistace to β-lactams and other antimicrobial groups were spread all over the world (Jacobs *et al.,* 1978; Watson *et al.,* 1993). The 1980s marked the beginning of the era of pneumococcal multidrug resistance (resistance to more than two classes of antimicrobials). Multidrug resistance continued rising, and by the year 2000 nearly half of all cases of invasive pneumococcal disease (IPD) in the United were resistant to at least one antimicrobial agent, and isolates that were resistant to penicillin were likely to be resistant to multiple other agents (Whitney *et al.,* 2000).

Nowadays, empirical treatment of the pneumococcal infections depends on the prevalence of resistant strains in each geographical area and the kind of infection. β-lactams are the treatment of choice; however, in cases of allergy or in countries with a high prevalence of β-lactam resistance, the use of macrolides (azithromycin or clarithromycin) or new quinolones (levofloxacin or moxifloxacin) is indicated.

5.1. β-lactams

The β-lactams are the most numerous and most important class of antimicrobial drugs in the treatment of infectious diseases. β-lactams are active against a wide variety of bacteria including Gram-negative bacteria, such as *Escherichia coli* or *Neisseria meningitidis,* and Gram-positive bacteria such as *Staphylococcus aureus,* and *S. pneumoniae*. They all have in common a β-lactam ring in their core structure.

The β-lactam family comprises the penicillins, the amino-penicillins (e.g. meticillin, amoxicillin and ampicillin), the carbapenems (e.g. imipenem, meropenem, ertapenem), monobactams (aztreonam), the cephalosporin group, usually divided into first- (e.g. cephalotin), second- (e.g. cefuroxime), third- (e.g. cefotaxime, ceftriaxone, ceftazidime) and fourth-generation (e.g. cefepime), and combinations of penicillins with β-lactamase inhibitors (e.g. amoxicillin/clavulanic acid, piperacillin/tazobactam and ampicillin/sulbactam).

β-lactams are classically bactericidal, since they inhibit the peptidoglycan synthesis of the bacterial cell wall by binding irreversibly to the active site of the penicillin-binding proteins (PBP), leading to bacterial cell hydrolysis (Waxman, *et al.,* 1983). The pneumococcus has six types of PBP (PBP1A, PBP1B, PBP2A, PBP2B, PBP2X and PBP3), which catalyse the latter steps of murein biosynthesis, cross-linking peptidoglycan molecules in the bacterial cell wall (Hakenbeck, *et al.*, 1999a). The PBPs conserved three active site domains, Ser-X-X-Lys, Ser-X-Asn and Lys-Thr/Ser-Gly, spaced at similar distances but differing in their amino acid positions between the proteins.

In pneumococci, ß-lactam resistance arises as a consequence of the expression of modified PBPs, which reduce the affinity for the ß-lactams, preventing the binding to the active site of the proteins (Coffey, *et al.,* 1995). The presence of ß-lactamases has not been reported in *S. pneumoniae* isolates.

In general, the sequences of the PBPs of the ß-lactam susceptible isolates are highly conserved. In contrast, the pbp genes of resistant strains revealed the presence of mosaic structures composed by DNA blocks acquired by homologous recombination with other pneumococci or with related *Streptococci* (Dowson, *et al.* 1990). In fact, a correlation between the degree of divergence of the pbp genes and the minimum inhibitory concentration (MIC) of ß-lactams has been reported (Granger, *et al.* 2006). Alterations in particular PBPs with high molecular weight are related to resistance of different ß-lactams. For instance, mutations in PBP2B or PBP2X confer low levels of penicillin resistance, and are prerequisites for high level ß-lactam resistance, mediated by additional alterations in PBP1A (Hakenbeck, *et al.,* 1999b; Smith and Klugman, 1998). In this way, alterations in PBP2X and PBP1A also confer resistance to third-generation cephalosporines.

Both *pbp1a* and *pbp2x* genes are located flanking the *cps* locus (approximately 8kb upstream and 7kb downstream of the *cps* locus respectively). In some cases, the recombination fragment also includes part or all of these genes, leading to the acquisition of a new capsular type and the acquisition of new PBP genes (Brueggemann *et al*, 2009; Moore *et al.* 2007). It is likely that the implementation of new conjugate vaccines will favour capsular switch variants with acquisition of new *pbp* genes that conferred

reduced susceptibility on ß-lactams (Wyres *et al.,* 2013) However, it has been reported that changes in the *pbp* genes are related to an increase in the fitness cost, suggesting that an optimal combination of these genes in necessary to maintain a compensated fitness (Albarracin Orio, *et al.,* 2011).

Traditionally, the breakpoint of penicillin non-susceptibility was MIC ≥0.12 µg/mL, since pneumococcal isolates with a higher MIC have been associated with treatment failure in patients with pneumococcal meningitis (Friedland and Klugman, 1992). This is explained by the low permeability to penicillin of the blood-brain barrier, preventing the achievement of high levels of antibiotic in the cerebrospinal fluid (CSF), which are insufficient to eradicate the infecting organism. The same occurs with the third-generation cephalosporin, whose non-susceptible breakpoints were MIC ≥1mg/L (Pallares et al., 1995). However, the traditional breakpoints of susceptibility were modified in 2008, considering the source of infection and route of administration (Clinical Laboratory Standards Institute, 2008). Traditional penicillin non-susceptible breakpoint were considered for meningeal infections (MIC ≥0.12 µg/mL), and a new breakpoint (MIC ≥4 µg/mL) was created by non-meningeal infections such as pneumonia, since high doses of intravenous penicillin might be effective (Pallares *et al.,* 1995; Pallares *et al.,* 1997).

Pneumococcal strains with decreased susceptibility to penicillin were first identified in Australia in the 1960s (Hansman and Bullen, 1967), with an increase in prevalence in many countries during the 1980s and 1990s (Klugman, 1990; Liñares *et al.,* 1992; Jacobs *et al.,* 1997; Fenoll *et al.,* 1998; Felmingham *et*

al., 2005). In Spain, rates of penicillin non-susceptibility increased from 24.9% to 30.2% in 1992 and 2001, respectively (Liñares *et al.,* 2010). However, a marked decrease in the penicillin non-susceptible rates (21.3% in 2001 to 16.3% in 2008) was detected after the implementation of the pneumococcal conjugate vaccine PCV7 for children, as described in other countries (Kyaw *et al.,* 2006; Liñares *et al.,* 2010).

Similar rates were found by the Alexander Project in France, Greece and USA, while in Germany and UK, resistance rates remained below 5% during this period (Felmingham *et al.,* 2005; Jenkins *et al.,* 2008). In contrast, the PROTEKT study showed higher rates in South Africa (75%), Far East (63%) and 54% in the Middle East (Jenkins *et al.,* 2008).

However, in a recent study performed in 43 US hospitals, the authors found a statistically significant increase in the percentage of penicillin-nonsusceptible pneumococci (MIC ≥0.12 mg/L)/mL: from 33% in 1999–2000 to 39% in 2010–2011; $p<0.001$ (Richter *et al.*, 2013). This increase has been associated with multi-drug resistant serotypes 19A and 6C.

5.2. Macrolides

Macrolides are a broad spectrum class of antimicrobial drugs most active against Gram-positive bacteria. For several years they have been considered the best alternative to β-lactams, since they are relatively non-toxic antibiotics; however, the spread

of pneumococcal strains with resistance to macrolides has limited their use (Daneman, *et al.,* 2006).

Macrolides are characterised by the presence of a macrocyclic lactone ring structure, made up of 14 atoms (erythromycin or clarithromycin), 15 atoms (azithromycin) or 16 atoms (josamycin). The first macrolide introduced into clinical use was erythromycin, in the early 1950s.

Macrolides inhibit protein synthesis by binding to the 50S ribosomal subunit of susceptible pneumococcus, preventing either the translocation of the growing peptide or petidyl transferase activity. Their effect is generally bacteriostatic; however, depending on the bacterial inoculum, the bacterial species, or the antimicrobial concentration reached in the infection site, the macrolide effect could be bactericidal.

Macrolide resistance in *S. pneumoniae* is mediated mainly by two mechanisms, associated with different phenotypes. The MLS_B phenotype results from the presence of a methylase which leads to cross resistance to 14-, 15- and 16-membered macrolides, as well as lincosamides (such as clindamycin) and streptogramin B (Weisblum, 1995). The methylases are enzymes that catalyze the mono- or di-methylation of adenine residues in 23S rRNA, encoded by the family of *erm* genes. More than 20 different methylases have been described; however in *S. pneumoniae* the prevailing methylase gene is *erm*(B) (Weisblum, 1995). Another less common methylase gene found in *S. pneumoniae* is *erm*(A) (Seppälä *et al.,* 1998). Resistance to MLS_B antimicrobials may be constitutive or inducible in isolates harbouring the *erm* gene (Weisblum, 1995; Calatayud *et al.*, 2007).

The pneumococcal M phenotype, resulting usually from the possession of the *mef*(E) gene (and more rarely the *mef*(A) subtype), confers resistance to 14- and 15-membered macrolides, due to the presence of an efflux pump which removes the antibiotic from within the cell (Leclercq and Courvalin, 2002). However, these strains are susceptible to 16-membered macrolides, lincosamides and streptogramin B.

Another less frequent mechanism among pneumococci is the presence of mutations in L4 or L22 ribosomal proteins and 23S rRNA, which prevent the antimicrobial from binding to its target site (Weisblum, 1995).

Both pneumococcal *erm* and *mef* genes are usually located in a wide variety of conjugative transposons. These transposons are integrated DNA elements with inverted repeat (IRs) sequences at each end, with the presence of genes coding for transposases, which allows the reintegration in the same bacterium or can be transferred by conjugation to another bacterium (Salyers *et al.*, 1995).

The most frequent transposons carrying the *erm*(B) gene are related to the *Tn916* family. All the transposons of this family, derive from the *Tn916* transposon, which carries the *tet*(M) gene, conferring tetracycline-resistance [figure 6] (Franke and Clewell, 1981). For its part, the *mef*(E) gene is usually carried by a genetic element named mega, with multiple insertion sites in the pneumococcal genome (Del Grosso *et al.,* 2002; Gay and Stephens, 2001).

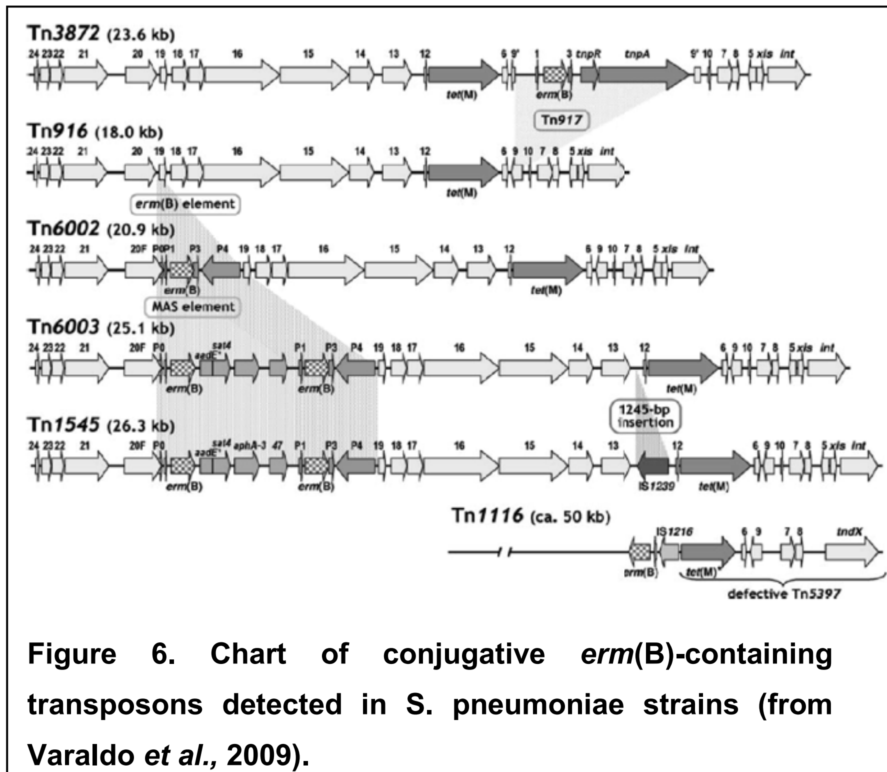


Figure 6. Chart of conjugative *erm*(B)-containing transposons detected in S. pneumoniae strains (from Varaldo *et al.,* 2009).

Macrolide resistance was described in Canada in 1967; however, until the late 1980s resistance rates remained low (<3%). After the introduction of long half-life macrolides such as azithromycin and clarithromycin, a stepwise increase of macrolide-resistant rates was detected worldwide (Klugman, 1990; Liñares, 1992).

Nowadays, macrolide resistance in pneumococcal strains is a global concern, although their prevalence varies according to geographic region and antimicrobial consumption. Rates vary from <5% in Czech Republic and Bulgaria, to 13% in Latin America and over 30% in France and US (Castanheira *et al.,* 2004; Felmingham *et al.,* 2005; Jenkins *et al.,* 2008). Due to the high macrolide

consumption in Asia, resistance rates are higher than 80% (Song *et al.,* 2004). In Spain, overall macrolide resistance rates among invasive pneumococci remained below 5% until 1986, but the introduction of PCV7 for children in June 2001 had a major impact on macrolide resistance, due to the serotype replacement. Thus, among invasive paediatric pneumococci, a significant decline in erythromycin resistance was observed, from 42.9% in 2003 to 20.8% in 2006 (Fenoll *et al.,* 2009). In contrast, macrolide resistance rates among adult invasive pneumococci remained stable over time, at around 25% (Fenoll *et al.,* 2009). Another interesting finding was the increase in rates of co-resistance to penicillin and macrolides from 3.7 to 17% during the 2000s (Felmingham *et al.,* 2005).

5.3. Fluoroquinolones.

The quinolones are a broad-spectrum family of synthetic antimicrobials. The majority of quinolones used for clinical treatment, such as ciprofloxacin, levofloxacin or moxifloxacin, have in common the presence of a fluorine atom attached typically at the C6 or C7 position. This subset is known as the fluoroquinolones. Their activity is bactericidal, since they prevent DNA synthesis by inhibition of DNA gyrase and topoisomerase IV (Wolfson and Hooper, 1989).

In pneumococci, resistance to fluoroquinolones is mainly mediated by alteration of their intracellular drug targets, the DNA topoisomerase IV (primary target) and DNA gyrase (secondary target). Resistance is acquired by point mutations as well as by

intraspecific or interspecific recombinations with other *Streptococcal* species (Ferrandiz *et al.,* 2000; Stanhope *et al.,* 2005).

Alterations in either ParC or ParE, subunits of topoisomerase IV, confer decreased susceptibility on ciprofloxacin. This is known as low-level resistance, and is often difficult to detect clinically because the strains are susceptible to levofloxacin using the current breakpoints. This is a cause of concern, since strains with first-step mutations are able to acquire additional amino acid changes in the GyrA subunit of the DNA gyrase, conferring a high level of fluoroquinolone resistance which is related to treatment failures. Another less frequent mechanism of resistance is the over-expression of efflux pump, encoded by the *PmrA* gene (Gill *et al.,* 1999).

New generations of fluoroquinolones (levofloxacin and moxifloxacin) have become therapeutic alternatives when there is suspicion of resistance to other antimicrobials, especially during pneumococcal pneumonia episodes. Currently, rates of pneumococcal fluoroquinolone resistance remain low in many regions, with the exception of Finland (6.6%), Italy (7.2%) and certain Asian countries (Fuller and Low, 2005; Riedel *et al.,* 2007). However, the increased use of these antimicrobials has been associated with the emergence of resistance in *S. pneumoniae* (Chen *et al.,* 1999). In Spain, resistance rates of pneumococci to ciprofloxacin remained stable over the last decade (2.6% in 2002 and 2.3% in 2006), despite the introduction of PCV7 (De la Campa *et al.,* 2004; De la Campa *et al.,* 2009). Moreover, pneumococci isolated from patients aged >65 years showed a higher prevalence

of ciprofloxacin resistance, possibly reflecting increased fluoroquinolone use in this group of patients. For instance, pneumococci isolates from non-invasive sources were higher than those from invasive pneumococci (Liñares *et al*., 1999).

Resistance to fluoroquinolones can evolve rapidly during treatment, and there are numerous reports of treatment failures with the use of fluoroquinolones in pneumococcal infections caused by strains with first-step mutations (Fuller and Low, 2005). These reports stress that treatment is more likely to fail in COPD patients or hospitalized patients. This could be explained by the fluoroquinolone pressure on a high pneumococcal inoculum ($>10^6$ CFU/ml) observed in the lower airways of patients with COPD or bronchiectasis, which may select for spontaneous mutants at the QRDRs of *S. pneumoniae* strains (Davidson *et al.,* 2002).

6. Epidemiology of pneumococcal disease: impact of PCV7

Invasive pneumococcal infections have a major global impact on healthcare, and are considered a leading cause of death in children. As a consequence of the implementation of PCV7 in national immunization programs in several countries, a change in the epidemiology of pneumococcal disease has been observed. This vaccine had a drastic impact on the distribution of serotype and their associated genotypes, as well as on pneumococcal drug-resistance (Whitney *et al.,* 2003; Kyaw *et al.,* 2006).

Seven years after the introduction of PCV7 in the United States, the incidence of IPD remained lower than the pre-vaccine period, with a 45% decrease for all age groups and a 76% decrease for children aged <5 years (Pilishvili *et al.,* 2010). However, this reduction was partially offset by the increase in IPD caused by non-PCV7 serotypes and by the maintenance of antimicrobial use. The increase of multi-drug resistant serotype 19A worldwide is particulary notable, frequently associated with failed medical treatment (Beall *et al*., 2006; Hicks *et al.,* 2007).

The introduction of PCV7 also had an impact on the ecology of pneumococci carried by healthy children. Thus, while less invasive PCV7 serotypes such as 6B, 19F and 23F were able to persist in the nasopharynx in children for long periods of time before the introduction of PCV7 (Brueggemann *et al.,* 2003a), several studies reported a drastic decrease of PCV7 carriage rates, currently lower than 5% (Hanage *et al.,* 2010; Rodrigues *et al.,* 2012; Wroe *et al.,* 2013). However, the persistence of serotype 19F in healthy children from Portugal, Greece and Norway, nearly a decade after PCV7 introduction, is interesting (Vestrheim *et al.,*

2010; Grivea *et al.,* 2011; Rodrigues *et al.,* 2012). The newly vacant ecological niche was rapidly filled by non-vaccine serotypes, mainly 19A, 6C, 15B/C, 3, 23A, 23B and 35B (Hanage *et al.,* 2010; Rodrigues *et al.,* 2012; Wroe *et al.,* 2013). In contrast to IPD incidence in children, overall carriage rates changed little in the United States and the United Kingdom (Hanage *et al.,* 2010; Tocheva *et al.,* 2011); however, in a recent study performed in Coimbra, Portugal, an overall decrease from 61% in 2007 to 51% in 2009 was observed (Rodrigues *et al.,* 2012). On the other hand, despite the serotype replacement, the antimicrobial resistance rates of pneumococci colonizing the nasopharynx of healthy children remained unchanged after PCV7 introduction (Simoes *et al.,* 2011).

As we noted above, in Spain PCV7 was not introduced in the public health vaccination program; however, it has been used in private practice, and by 2006 the vaccine uptake had reached 45%-50% of children <2 years old (Grupo de Trabajo de la Ponencia de Registro y Programa de Vacunas, 2006). Though this vaccine uptake remained low, the decrease of PCV7 serotypes and the serotype replacement phenomenon was also observed in our country among children with IPD (Muñoz-Almagro *et al.,* 2008). This is explained by the herd protection, a form of indirect protection for individuals who have not received the vaccine and have not developed immunity provided by PCV7 use.

Herd protection may also reduce the transmission in general populations. For instance, an impact of PCV7 on pneumococci populations causing IPD and non-IPD in adults mainly over 65 years old was also observed (Ardanuy *et al.,* 2009a; Fenoll *et al.,*

2012) even though this vaccine was not licensed for the adult population.

The capsule plays an important role in pneumococcal disease, and for this reason, the differences in distribution of serotypes causing IPD and non-invasive pneumococcal infections in adults imply a different level of herd protection by PCV7 (Ardanuy *et al.,* 2009a; Fenoll *et al.,* 2012). For example, differences in the decline of PCV7 serotypes causing IPD were also observed among young adults and adults over 64 years old (Ardanuy *et al.,* 2009a; Grau *et al.,* 2012). The immunological status of the patients is also important. Differences in the distribution of serotypes causing IPD were observed in young adults (18-64 years old): when healthy adults were compared with adults with comorbidities, a high frequency of serotypes 1, 7F and 5 (not included in PCV7) was observed among healthy adults, whereas, serotypes 19F and 23F were more frequently found among adults with comorbidities (Grau *et al.,* 2012).

Serotype 19A has also increased dramatically as a cause of non-IPD in adult patients after PCV7 introduction (Ardanuy *et al.,* 2009b; Fenoll *et al.,* 2012), as table 3 shows. Furthermore, other serotypes such as 15A, 10A and 6C also show a significant increase among non-invasive infections in the adult population (Fenoll *et al.,* 2012).

Table 3. Variation in percentages of pneumococcal serotypes causing non-IPD in adult patients, between the pre- and post-PCV7 periods (source: Fenoll *et al.,* 2012)

Serotype	Pre PCV7 (n = 650)	Post PCV7 (n = 624)	% Variation
PCV7 serotypes (n = 368)	281 (43.2)	87 (13.9)*	−67.8
19F (n = 89)	68 (10.5)	21 (3.4)*	−67.6
23F (n = 70)	60 (9.2)	10 (1.6)*	−82.6
9V (n = 67)	51 (7.8)	16 (2.6)*	−66.7
6B (n = 66)	51 (7.8)	15 (2.4)*	−69.2
14 (n = 55)	36 (5.5)	19 (3.0)*	−45.5
Other (4 and 18C) (n = 21)	15 (2.3)	6 (1.0)	−56.5
Non-PCV7 serotypes (n = 906)[a]	369 (56.8)	537 (86.1)*	+51.6
3 (n = 170)	88 (13.5)	82 (13.1)	−3.0
19A (n = 53)	11 (1.7)	42 (6.7)*	+294.1
6A (n = 45)	26 (4.0)	19 (3.0)	−25.0
35B (n = 39)	19 (2.9)	20 (3.2)	+10.3
15A (n = 36)	10 (1.5)	26 (4.2)*	+180.0
6C (n = 34)	0 (0.0)	34 (5.4)*	–
10A (n = 34)	12 (1.8)	22 (3.5)	+94.4
11A (n = 33)	0 (0.0)	33 (5.3)*	–
Other (36 serotypes) (n = 352)	144 (22.2)	208 (33.3)*	+50.0
Non-typeable (n = 110)	59 (9.1)	51 (8.2)	−9.9

PCV7, 7-valent pneumococcal conjugate vaccine.

[a] Those serotypes with >30 isolates in total are shown individually.

* $P < 0.05$ vs. pre-PCV7.

Between the 1970s and 1990s, a high prevalence of antimicrobial resistance among pneumococci was reported worldwide, as a result of increased antimicrobial consumption (Jacobs *et al.,* 1978; Klugman KP, 1990; Michel J *et al.,* 1983; Fenoll A *et al.,* 1991; Appelbaum *et al.,* 1992; Low DE *et al.,* 2005).

The emergence of antimicrobial resistance was associated with a small number of highly successful clones, which dominated the population of drug-resistant pneumococci before the introduction of PCV7. These clones, mainly associated with PCV7 serotypes 9V, 6B, 19F and 23F, were standardized by the PMEN in 1997 (described in chapter I, section 2.3), the most important clones being Spain^{23F}-ST81 (PMEN1), Spain^{6B}-ST90 (PMEN2), Spain^{9V}-ST156 (PMEN3), England^{14}-ST9 (PMEN9), Taiwan^{19F}-ST236 (PMEN14), Taiwan^{23F}-ST242 (PMEN15) or Poland^{6B}-ST315 (PMEN20) (McGee *et al.,* 2001). These clones often showed a combined resistance to more than one antimicrobial: β-lactam resistance (due to changes in their PBPs), macrolide-, tetracycline- and chloramphenicol-resistance (due to the presence of transposons carrying determinants of resistance) and/or intrinsic resistance to co-trimoxazole (McGee *et al.,* 2001; Liñares *et al.,* 2010). However, the prevalence of macrolide resistance mechanisms differs considerably among countries (Liñares *et al.,* 2010).

Three of these successful clones (Spain^{23F}-ST81, Spain^{6B}-ST90 and Spain^{9V}-ST156) were first identified in Spain (Muñoz *et al.,* 1991; Coffey *et al.,* 1996). However, they were widely disseminated throughout the world, and were isolated in United States (Muñoz *et al.,* 1991; Versalovic *et al.,* 1993), South America

(Castañeda *et al.,* 1998), and several European countries including France, United Kingdom, Italy and Germany (Lefèvre *et al.,* 1995; Marchese *et al.,* 1998; Reinert *et al.,* 2005). In the United States, Spain^{23F}-ST81 caused 40% of all highly penicillin-resistant pneumococci (Corso *et al.,* 1998), while approximately 75% of multi-drug resistant pneumococci from children of Iceland belonged to England^{14}-ST9 (Kristinsson KG, 1995). This clone, harbouring the *mefA* gene of macrolide resistance, spread in the United States, and European countries such as the UK, Germany, Greece and Italy (Hall *et al.,* 1996; Monaco *et al.,* 2005; McEllistrem *et al.,* 2005; van der Linden *et al., 2007;* Liñares *et al.* 2010). In contrast, in Spain, serotype 14 was strongly associated with the Spain^{9V}-ST156 clone.

In the United States and elsewhere, the introduction of PCV7 together with a reduction in antimicrobial use were associated with a dramatic reduction in invasive pneumococcal infections caused by serotypes targeted by this vaccine (Whitney *et al.,* 2003). In addition, a significant decline in multi-drug resistance in IPD was also observed, as a consequence of the decline in multi-resistant clones with serotypes included in PCV7 (Kyaw *et al.,* 2006; Dagan and Klugman, 2008; Liñares *et al.,* 2010). This decrease has been especially marked for clones Spain^{23F}-ST81, Spain^{6B}-ST90 and England^{14}-ST9; in contrast, Spain^{9V}-ST156 (serotypes 9V and 14) has been major cause of IPD in many countries, both before and after the introduction of PCV7 (van der Linden *et al.,* 2007; Ardanuy *et al.,* 2009a; Zhou *et al.,* 2009).

However, the long-term effectiveness of PCV7 is not clear, in part due to the increase in the incidence of non-PCV7 serotypes.

In fact, new emerging serotypes have been detected in the late PCV7 period in the United States (Hicks *et al.*, 2007). Changes observed in serotype and genotype distributions have been related to the capsular switching phenomenon and/or the serotype replacement phenomenon by expansion of pre-existing clones or the emergence of new clones (Byington *et al.,* 2005; Brueggemann *et al.,* 2007).

This is a global cause of concern because some of the emerging serotypes, such as 19A, 6C and 15A, are usually associated with multi-drug resistance. In addition, serotypes 6C and 15A are not targeted by any of the current pneumococcal conjugate vaccines. One example is the worldwide increasing prevalence of multi-drug resistant serotype 19A, observed in the United States, Europe and Asia (Moore *et al.,* 2008; Ardanuy *et al.,* 2009b; Dagan *et al.,* 2009; Mahjoub-Messai F *et al.,* 2009; Aguiar *et al.,* 2010; Ho *et al.,* 2011). This serotype has been associated with the worldwide spread of CC320, a double-locus variant of Taiwan^{19F}-ST236 (Moore *et al.,* 2008; Ardanuy *et al.,* 2009b; Ho *et al.,* 2011). This clone is the main cause of the emergence of dual resistance to macrolides, carrying both *ermB* and *mefE* genes, as well as the tetracycline resistance determinant (*tetM*); these genes have been related to the composite element Tn2010, which is present in most multidrug-resistant isolates of serotype 19A of clonal complex 320 (Del Grosso *et al.,* 2007). Although an increase in this clone has been observed in some European countries, the major serotype 19A clone in Europe is ST276, related to Denmark^{14}-ST230 (Mahjoub-Messai F *et al.,* 2009, Aguiar *et al.,* 2010; Gherardi *et al.,* 2012).

Another serotype which has emerged during last decade is the recently described serotype 6C. This serotype is more frequent among adult than paediatric isolates (Jacobs *et al.,* 2009). In Spain, two major clones were found associated with serotypes 6C (Rolo *et al.,* 2011). The first one, CC224, initially only showed reduced susceptibility to penicillin, but recently some isolates have acquired macrolide-resistant determinants. This clone was the most frequently detected in Spain, and has also been found as a cause of IPD in other countries of Europe and United States (www.mlst.net; Gertz *et al.,* 2010; Rolo *et al.,* 2011). The second clone, CC386, is a double locus variant of Poland^{6B}-ST315, and emerged in Spain in 2007, associated with resistance to penicillin, macrolide, and tetracycline. Few data are available about the distribution of this clone in other countries as a cause of pneumococcal disease; however, it has been found colonizing healthy children in Portugal (Nunes *et al.,* 2009).

Sweden^{15A}-ST63 was included in the PMEN (PMEN25) collection as a consequence of its worldwide spread and its association with macrolide and tetracycline resistance and reduced susceptibility to penicillin. However, its frequency as a cause of IPD is low and it is more usually isolated the nasopharynx of healthy children and from sputum samples in adult patients with non-invasive disease (Fenoll *et al.,* 2009; Simoes *et al.,* 2011). Although the Sweden^{15A}-ST63 clone was already in circulation in the pre-PCV7 era, it remains a frequent clone worldwide.

OBJECTIVES

1. To study the microbial aetiology of acute exacerbations in patients with severe COPD with different frequency of annual episodes (2010-2011).

2. To analyse the dynamics and population structures of *S. pneumoniae* strains causing acute exacerbations and pneumonia (bacteraemic and non-bacteraemic) in patients with COPD, from 2001 to 2008.

3. To evaluate the impact of PCV7 in the pneumococcal populations causing acute exacerbations, from 2001 to 2012.

4. To establish the frequency and distribution of pneumococci causing recurrent episodes: relapse *vs*. reinfection episodes (1995-2010).

5. To investigate the pneumococcal persistence in the respiratory tract of COPD patients, as well as the impact of antimicrobial consumption in the development of antimicrobial resistance (1995-2010).

6. To characterise *S. pseudopneumoniae* strains causing acute exacerbations by phenotypic and genotypic methods (2001-2009).

7. To analyse the clinical and demographic characteristics of COPD patients infected by *S. pseudopneumoniae* (2001-2012).

RESULTS

CHAPTER II: Infectious aetiology of acute exacerbations in patients with severe COPD.

Objective 1: To study the microbial aetiology of acute exacerbations in patients with severe COPD with different frequency of annual episodes (2010-2011).

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Infectious etiology of acute exacerbations in severe COPD patients

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KEYWORDS
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Summary *Objectives:* Since the new GOLD guidelines were implemented no data have been published about the etiology of acute exacerbations (AECOPD) in severe COPD patients with a different frequency of annual episodes.
Methods: One hundred and eleven COPD patients ($FEV_1 < 50\%$) were prospectively followed up for a year. Good-quality sputum samples recovered during AECOPD were processed, including quantitative culture and PCR detection of atypical bacteria.
Results: A total of 188 sputum samples were obtained from AECOPD episodes. Forty patients had a single episode, and 71 patients had ≥2.

In 128 episodes a single pathogen was isolated, while 42 episodes were polymicrobial (≥2 pathogens). Overall, the most frequent pathogen isolated was *Pseudomonas aeruginosa* ($n = 54$), followed by *Haemophilus influenzae* ($n = 37$), *Streptococcus pneumoniae* ($n = 31$), *Moraxella catarrhalis* ($n = 29$) and *Staphylococcus aureus* ($n = 12$). *P. aeruginosa* was the most frequent in both groups of patients (35% and 27% in those with 1 and ≥2 AECOPD, respectively). *H. influenzae* was associated with patients with a single annual AECOPD (33% *vs.* 16%; $P = 0.006$), while *Enterobacteriaceae* were associated with frequent exacerbators (0% *vs.* 12%; $P < 0.044$).
Conclusion: Overall, *P. aeruginosa* was the most frequent pathogen isolated from exacerbations. However, different bacterial etiology was observed depending on the number of annual episodes.
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Introduction

Chronic obstructive pulmonary disease (COPD) is a cause of high morbidity and mortality in developed countries.[1] According to the latest World Health Organization (WHO) report, 64 million people had COPD in 2004, and 3 million of them died (http://www.goldcopd.org). In Spain, the prevalence of COPD among people aged between 40 and 80 years is 10.2%, reaching 23% in those older than 60.[2]

Acute exacerbations of COPD (AECOPD) contribute to the progress of the disease, are indicators of poor prognosis, and are associated with enormous health care costs.[2] Up to 80% of AECOPD are caused by microbial pathogens, including bacteria, viruses, atypical bacteria, and fungi. Air pollution and other environmental conditions probably account for the remaining 20%.[3,4]

AECOPD exacerbations are mainly caused by bacteria, with *Haemophilus influenzae* being the most frequently isolated, followed by *Streptococcus pneumoniae* and *Moraxella catarrhalis*. However, the bacterial pathogen also varies according to the severity of the illness, with *Pseudomonas aeruginosa* being particularly common in patients with advanced disease.[4–6] Notably, little information is available about AECOPD caused by more than one potentially pathogenic bacterium.[6]

AECOPD can also be caused by viruses, fungi, and atypical bacteria such as *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae*, although their pathogenic role is controversial.[7,8] Several studies have analyzed the role of *C. pneumoniae* in exacerbations, with conflicting results and considerable variability (from 0% to 34%) depending on the detection techniques used.[8]

Patients with severe and very severe COPD, classified by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) as degrees III and IV, usually have several AECOPD per year.[9,10] However, a recent method based on the individual patient's history of exacerbations assesses the risk of symptomatology and poor outcomes, classifying patients into four groups, A—D, with groups C and D being those with the highest risk of suffering AECOPD (www.goldcopd.org).

It should also be noted that patients with frequent exacerbations receive multiple courses of antimicrobial therapy which select resistant microorganisms. The optimal therapy is a multidisciplinary choice that remains controversial, with variations among different guidelines.[9] Choosing the most suitable antimicrobial is therefore important in order to avoid the acquisition of resistance, especially in polymicrobial exacerbations. In this context, the acquisition of fluoroquinolone resistance in isolates of *P. aeruginosa*, *H. influenzae*, and *S. pneumoniae* has been widely described.[11–14]

Since the new GOLD guidelines were implemented there have been no reports based on data gleaned from sputum cultures of COPD patients with a low or high frequency of annual AECOPD. Therefore, the present study aimed to determine the microbial etiology of AECOPD in 111 patients with advanced airway obstruction and who suffered moderate or severe AECOPD episodes. In addition, and with the aim of contributing more specific knowledge for patient management, we also analyzed the microbial etiology according to whether patients had a single episode or frequent exacerbations during the study period.

Methods

Ethical statement

This study and publication of the results were approved by the "Comité Ètic d'Investigació Clínica de l'Hospital Universitari de Bellvitge (HUB)". Sputum samples and bacterial strains were recorded in an anonymized database.

Patient selection

Sputum samples were prospectively collected from all patients with severe COPD ($FEV_1 < 50\%$ and baseline dyspnea CFIII-IV according to Medical Research Council criteria) who were seen in the specialist COPD consulting room of the Respiratory Medicine Department at HUB between February 2010 and February 2011.

COPD was assessed with chest radiography and CT scan at recruitment to ensure the absence of other significant respiratory disease. Patients with high comorbidity (Charlson index $\geq$5), immunodeficiency, terminal malignancy, or other chronic respiratory diseases (evidence of bronchiectasis not associated with COPD, asthma, or bronchial interstitial lung disease) were excluded.[15] In addition, AECOPD episodes related to cardiac failure of the patient or other non-infectious causes were also excluded from the study.

Following the criteria set out in the new GOLD guidelines, patients were assigned to one of two groups based on the number of acute exacerbations suffered during the study period. Thus, those with fewer than two AECOPD episodes were classified as patients with infrequent exacerbations, while those with two or more episodes during the study period were considered as frequent exacerbators.[2,10]

An acute exacerbation episode was defined as any sustained increase in respiratory symptomatology compared with the baseline situation that required a modification of regular medication and, possibly, hospital treatment. Hence, acute exacerbations were considered as either moderate (not requiring hospitalization) or severe (requiring hospitalization). In those patients with more than one AECOPD a new episode was only considered when the interval between episodes was more than four weeks and the second episode occurred after a successful outcome.

Sputum collection and bacterial load detection

Sputum samples were recovered during the AECOPD episodes, before the antimicrobial treatment, if it was necessary. Only good-quality sputum samples were considered ($<$10 squamous cells and $>$25 leukocytes per low-power field),[16] and all samples were cultured within 4 h of being collected. Briefly, samples were homogenized with dithiothreitol (Sputolysin), and after performing serial dilutions ($1:10^{-1}$, $1:10^{-2}$, and $1:10^{-3}$) they were plated onto blood agar, chocolate agar, and MacConkey agar before being incubated overnight at 37 °C in a 5%-CO_2 atmosphere (blood and chocolate agar) and ambient air atmosphere (MacConkey agar). After incubation, colony-forming units (cfu/ml) were calculated and sub-cultured for bacterial identification by

standard methods.[17] Only isolates with a count $\geq 10^6$ cfu/ml were considered. If *P. aeruginosa*, *H. influenzae*, *M. catarrhalis*, or *S. pneumoniae* was present, up to 8 individual colonies of each bacterial species were isolated and saved as frozen stocks at −80 °C.

Mass spectrometry analysis

Isolates classified as *Corynebacteriaceae*, as well as the isolates of the genus *Candida*, were further identified by mass spectrometry analysis in order to identify the species. Briefly, a single bacterial colony was placed on a polished steel MSP 96-target plate (Bruker Daltonics GmbH, Bremen, Germany), overlaid with 1 μl of formic acid, and dried at room temperature. The samples were covered with 1 μl of matrix solution (α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile-2.5% trifluoroacetic acid) and were dried again at room temperature. Identification was performed using the MALDI Biotyper version 3.0 software (Bruker). Correct identification to the species level was accepted when the score was ≥2.

Atypical bacteria detection

The possible presence of atypical bacteria *C. pneumoniae* and *M. pneumoniae* was analyzed in all the sputum samples by real-time PCR. DNA was extracted using a magnetic particles protocol (Sample Preparation Systems RNA and DNA, Promega, Abbott, USA). Upon DNA extraction from the sputum samples, two different monoplex real-time PCR were performed, as described previously.[18] Commercially available DNA controls were used in each run (Vircell, Granada, Spain).

Antimicrobial susceptibility, serotyping, and molecular typing

The antimicrobial susceptibility to the frequent antibiotics used for the treatment of each bacterial pathogen was tested by microdilution and/or the disk diffusion method, following the Clinical Laboratory Standard Institute (CLSI) recommendations.[19]

Serotyping of *H. influenzae* strains was performed using the latex agglutination kit Phadebact® Haemophilus Test (Bactus AB, Huddinge, Sweden), while *S. pneumoniae* isolates were determined by means of a multiplex PCR protocol using previously described methodology.[20]

Molecular typing of *H. influenzae*, *S. pneumoniae*, *P. aeruginosa*, and *M. catarrhalis* was performed by pulsed-field gel electrophoresis (PFGE). Genomic DNA embedded in agarose plugs was restricted with *Sma*I (*S. pneumoniae* and *H. influenzae*) or *Spe*I (*P. aeruginosa* and *M. catarrhalis*), and fragments were separated in a CHEF-DRIII apparatus (Bio-Rad), as previously described.[21]

Statistical analysis

Statistical analyses were carried out using SPSS version 18.0, using Chi-square or Fisher's exact tests to compare proportions. Two-sided *P* values less than 0.05 were considered statistically significant.

Results

During the study period a total of 224 AECOPD episodes occurred in 111 COPD patients seen at the Monographic COPD consulting room. A sputum sample from each was sent to the laboratory. Of these, 36 low quality sputum samples (≥10 epithelial cells per low-power field) were excluded from the analysis.

Table 1 shows the clinical characteristics of patients. The mean age was 70 years, and the majority of them (95.5%) were men. Clinical data of patients were compared based on the frequency of acute exacerbations suffered during the study period. This revealed no differences between the two patient groups (infrequent *vs.* frequent exacerbators) as regards lung functional and analytical characteristics. The presence of bronchiectasis not associated with COPD was an exclusion criterion. However, as a result of the severity of the patients included in the present study, nearly a half of them developed bronchiectasis, as it is shown in Table 1. This comorbidity was associated with patients with more than one AECOPD episode ($P = 0.007$).

All patients were continuously treated with inhaled corticosteroids, long-acting beta-agonists, and anticholinergics for COPD management.

One half of AECOPD episodes ($n = 94$) required hospitalization of the patient. However, the presence of more than one potential pathogen in the sputum sample was not associated with higher rates of hospitalization ($P = 0.642$). Regarding the bacterial distribution, no pathogen was related to the need for hospitalization, although *Streptococcus pseudopneumoniae* was associated with those moderate acute exacerbations that did not require hospitalization, a finding that could explain their limited pathogenic role ($P < 0.02$).

Isolation of potentially pathogenic bacteria

Among the 188 good-quality sputum samples that were processed and obtained from 111 patients, significant bacterial counts were observed in 170 (90.4%) episodes (Table 2). Of these, 42 (22.3%) showed more than one potential pathogen (Table S1). In the remaining 18 episodes (9.6%) no microorganisms were detected with $>10^6$ cfu/ml, and they were therefore considered episodes with normal oral microbiota.

The most frequent pathogen isolated was *P. aeruginosa* (28.7%), followed by *H. influenzae* (19.7%), *S. pneumoniae* (16.5%), and *M. catarrhalis* (15.4%). Notably, at least one of these four pathogens was isolated in 125 (66.5%) of the overall episodes studied. Other less widely reported pathogens were also frequently recovered in our study (Table 2): *Staphylococcus aureus* ($n = 12$, 6.4%), *S. pseudopneumoniae* ($n = 9$, 4.8%), and some species of the *Enterobacteriaceae* ($n = 19$, 10.1%) and *Corynebacteriaceae* ($n = 10$, 5.3%) families.

In all but one of the polymicrobial episodes ($n = 42$), at least one of the following pathogens was recovered: *P. aeruginosa*, *H. influenzae*, *S. pneumoniae*, or *M. catarrhalis*. The most frequent combination was *S. pneumoniae* plus *H. influenzae* (11.9%).

Fig. 1 shows the distribution of the main bacteria isolated from patients with a single AECOPD episode ($n = 40$ patients/episodes) and the remainder ($n = 71$)

INFECTIOUS AETIOLOGY OF AECOPD IN SEVERE COPD PATIENTS

CHAPTER II

Table 1 Clinical and demographic characteristics of the 111 COPD patients included.

	Total (n = 111)[a]	Patients with a single episode (n = 40)	Patients with ≥2 acute exacerbations (n = 71)	p-Value
Gender, men	106 (95.5%)	40 (100%)	66 (93.0%)	0.198
Age, years	70.1 ± 6.7	67.7 ± 5.9	70.5 ± 6.9	0.055
BMI, kg/m^2	26.6 ± 5.1	27.1 ± 6.3	26.4 ± 4.6	0.527
Current smoker, n (%)	20 (18.0%)	12 (30.0%)	8 (11.3%)	**0.020**
Number of exacerbations	188	40	148	–
AECOPD requiring hospitalization	94 (50.0%)	22 (55.0%)	72 (48.6%)	0.674
Long-term oxygen therapy	59 (53.2%)	16 (40.0%)	43 (60.6%)	0.076
Lung functional and analytical characteristics (average % ± SD):				
FEV$_1$, L	0.94 ± 0.3	0.98 ± 0.3	0.92 ± 0.3	0.283
FEV$_1$, %	35.8 ± 11.1	34.7 ± 10.3	36.5 ± 11.6	0.441
FVC, L	2.34 ± 0.7	2.35 ± 0.6	2.31 ± 0.7	0.774
FVC, %	68.9 ± 20.5	66.6 ± 18.0	70.0 ± 21.9	0.431
FEV$_1$/FVC, %	41.7 ± 11.4	43.5 ± 12.6	41.0 ± 10.8	0.316
Underlying conditions (number of patients, %):				
Bronchiectasis[b]	46 (41.4%)	10 (25.0%)	36 (50.7%)	**0.007**
Systemic arterial hypertension	56 (50.5%)	22 (55.0%)	34 (47.9%)	0.569
Obesity	11 (9.9%)	4 (10.0%)	7 (9.9%)	1.000
Alcohol abusers	22 (19.8%)	11 (27.5%)	11 (15.5%)	0.349
Cirrhosis	4 (3.6%)	0 (0.0%)	4 (5.6%)	0.129
Cardiovascular disease	37 (33.3%)	13 (32.5%)	24 (33.8%)	0.890
Pulmonary cancer development	7 (6.3)	5 (12.5%)	2 (2.8%)	0.248
Diabetes mellitus	28 (25.2%)	13 (32.5%)	15 (21.1%)	0.203

Definition of abbreviations: BMI = body mass index; FEV$_1$ = forced expiratory volume in 1 s; FCV = forced vital capacity. Bold values mean statistically significant differences ($P < 0.05$) between both groups of patients.

[a] The overall 111 patients were divided in patients with low frequency of AECOPD (a single episode) and high frequency of AECOPD (≥2 episodes).

[b] Bronchiectasis associated with COPD, observed in high-resolution computed tomography scan.

Table 2 Microbial pathogens isolated among 188 acute exacerbation episodes analyzed, with >10^6 cfu/ml of sputum sample.[a]

	Total number of episodes (n = 188)	No. of episodes with a single pathogen (n = 128)	No. of episodes with ≥2 pathogen (n = 42)
Potential pathogen bacteria			
Pseudomonas aeruginosa	54 (28.7%)	33 (25.8%)	21 (50.0%)
Haemophilus influenzae	37 (19.7%)	24 (18.8%)	13 (31.0%)
Streptococcus pneumoniae	31 (16.5%)	13 (10.2%)	18 (42.9%)
Moraxella catarrhalis	29 (15.4%)	14 (10.9%)	15 (35.7%)
Staphylococcus aureus[b]	12 (6.4%)	10 (7.8%)	2 (4.8%)
Enterobacteriaceae	19 (10.1%)	15 (11.7%)	4 (9.5%)
Escherichia coli	8 (4.3%)	6 (4.7%)	2 (4.8%)
Proteus mirabilis	7 (3.7%)	7 (5.5%)	0
Corynebacteriaceae	10 (5.3%)	3 (2.3%)	7 (17.1%)
C. striatum	5 (2.7%)	1 (0.8%)	4 (9.5%)
C. pseudodiphteriticum	2 (1.1%)	1 (0.8%)	1 (2.4%)
C. propinquum	2 (1.1%)	1 (0.8%)	1 (2.4%)
Other bacteria			
Streptococcus pseudopneumoniae	9 (4.8%)	4 (3.1%)	5 (11.9%)
Stenotrophomonas maltophilia	2 (1.1%)	1 (0.8%)	1 (2.4%)
Alcaligenes xylosoxidans	2 (1.1%)	1 (0.8%)	1 (2.4%)
Fungi			
Candida albicans	4 (2.1%)	2 (1.6%)	2 (4.8%)
Aspergillus fumigatus	5 (2.7%)	5 (3.9%)	0
Normal oral microbiota	18 (9.6%)		

[a] Only pathogens detected in more than two sputum samples were detailed in this table.

[b] A half of the *S. aureus* isolated were caused by a meticillin-resistant isolate (MRSA).

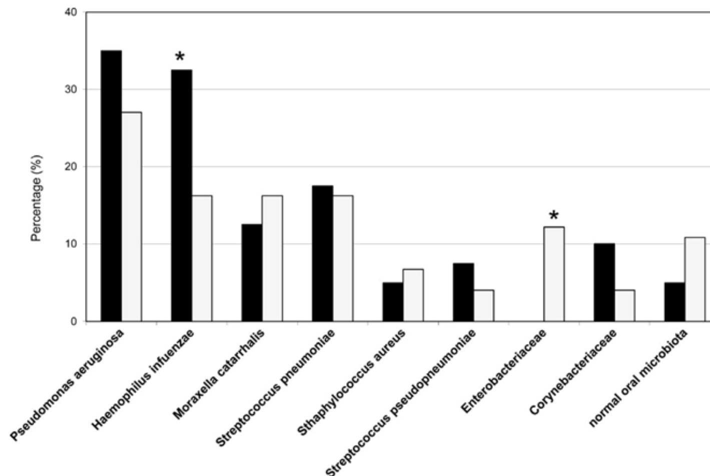

Figure 1 Distribution of the main bacteria isolated from patients with a low frequency (black bars) and high frequency (white bars) of acute exacerbations. *Significant differences between groups ($P < 0.05$).

who presented frequent exacerbations ($n = 148$ episodes). *P. aeruginosa* was the most frequent pathogen isolated in both groups. The presence of *H. influenzae* was associated with patients with a single AECOPD ($P = 0.006$), while *Enterobacteriaceae* species were only isolated from patients with a high frequency of exacerbations ($P < 0.05$).

Antimicrobial susceptibility of the main bacterial pathogens

Table 3 shows the *in vitro* antimicrobial susceptibility of the four main pathogens isolated. *P. aeruginosa* strains showed high susceptibility to carbapenems (around 90%) and anti-pseudomonal cephalosporins (80–90%). However, susceptibility rates were lower with respect to ciprofloxacin (50%) and aminoglycosides (42.6% for gentamicin, 74.1% for tobramycin, and 66.7% for amikacin).

H. influenzae isolates were highly susceptible to all the antimicrobials tested, and only two isolates harbored a beta-lactamase. By contrast, all *M. catarrhalis* isolates carried a beta-lactamase that conferred penicillin and ampicillin resistance but which were 100% susceptible to the remaining antimicrobials studied.

All *S. pneumoniae* isolates were susceptible to fluoroquinolones and beta-lactams (according to non-meningeal breakpoints of CLSI), but susceptibility rates were low for macrolides, tetracycline, and co-trimoxazole (61.3%, 67.7, and 74.2%, respectively).

Serotyping and molecular typing of the main bacterial pathogens

The most frequent serotypes of *S. pneumoniae* isolates were 6C ($n = 5$), 15A ($n = 4$), 3 ($n = 3$), and 9V ($n = 3$). All 37 *H. influenzae* isolates were non-capsulated (non-typables by latex agglutination).

Seventeen of 111 patients had two or more consecutive AECOPD episodes caused by the same bacterial species: nine patients with *P. aeruginosa*, four with *S. pneumoniae*, two with *H. influenzae*, and two with *M. catarrhalis*.

The molecular typing analysis of the *P. aeruginosa* studied ($n = 54$ from 37 patients) revealed different PFGE patterns (unique PFGE pattern per patient).

In order to detect persistent strains the molecular typing of these isolates was compared, showing differences by species. For instance, all *P. aeruginosa* strains were persistent, as illustrated by the identical PFGE profile observed among all isolates collected from the same patient in consecutive AECOPD episodes. One of two patients with *H. influenzae* had an identical PFGE pattern in all episodes (persistence), as did one of four patients with *S. pneumoniae*. No persistence was detected among *M. catarrhalis* isolates.

Atypical bacteria and fungi detection

DNA detection of *C. pneumoniae* was positive in 84 (44.7%) samples, 77 of which were detected in samples in which at least one other pathogen was isolated. In the remaining seven samples, *C. pneumoniae* was the only potential pathogen detected. When patients with one annual AECOPD episode were compared with patients with $\geq$2 episodes, rates of *C. pneumoniae* positive samples were similar (42.5% *vs.* 45.3%).

M. pneumoniae was only detected in two AECOPD episodes of two patients. In both cases, $\geq$2 pathogenic bacteria were isolated from the same sputum sample.

Regarding fungi isolation, *Aspergillus fumigatus* growth was observed in five samples, all with negative bacteria growth, while *Candida albicans* was isolated in four cases, two of them as a single pathogen.

Discussion

The microbial etiology of COPD patients has been analyzed in several studies, often including patients with different degrees of severity.[5,6,22] However, few data have been

Table 3 **A:** *In vitro* activity of eleven antimicrobials against *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* isolated from sputum samples during acute exacerbation episodes of patients with severe COPD. **B:** *In vitro* activity of eleven antimicrobials against *P. aeruginosa* isolates.

A

Antimicrobial	Susceptibility (%)		
	S. pneumoniae (n = 31)[a]	*H. influenzae* (n = 37)	*M. catarrhalis* (n = 29)
Penicillin	100%	NT	0%
Ampicillin[b]	100%	94.6%	0%
Amoxicillin/clavulanic acid	100%	100%	100%
Cefuroxime	NT	97.3%	100%
Cefotaxime/Ceftriaxone	100%	100%	100%
Tetracycline	67.7%	97.3%	100%
Erythromycin	61.3%	NT	100%
Clindamycin	71.0%	NT	NT
Co-trimoxazole	74.2%	83.7%	100%
Ciprofloxacin	100%	100%	100%
Chloramphenicol	96.8%	100%	100%

B

Antimicrobial	Susceptibility (%)
	P. aeruginosa (n = 54)
Ticarcillin	88.9%
Piperacillin/tazobactam	88.9%
Ceftazidime	83.3%
Cefepime	81.5%
Aztreonam	81.5%
Imipenem	87.0%
Meropenem	90.7%
Ciprofloxacin	50.0%
Gentamicin	42.6%
Tobramycin	74.1%
Amikacin	66.7%

NT = Not tested.

[a] For *S. pneumoniae* non-meningeal CLSI breakpoints for parenteral drugs were used: penicillin (susceptible ≤2 mg/L), ampicillin (susceptible ≤2 mg/L) and cefotaxime/ceftriaxone (susceptible ≤1 mg/L).

[b] A beta-lactamase was detected in 2 *H. influenzae* isolates (5.4%) and all 29 *M. catarrhalis* isolates (100%).

reported since publication of the GOLD guidelines for better patient management, and this lack of information is especially notable as regards patients with advanced disease (http://www.goldcopd.org). Our study, based on sputum culture and real-time PCR, evaluates the etiology and microbial load of 188 AECOPD that occurred in 111 COPD patients with advanced airway obstruction, and who suffered moderate or severe AECOPD. During the study period (one year) we also analyzed microbiological differences between patients who suffered just one AECOPD episode and those with a high frequency of AECOPD. The clinical and demographic data showed that these two groups of patients were very similar, since all patients were elderly and had similar lung functionality and underlying conditions (Charlson index <5).

Overall, our study identified potential pathogens in 90.4% of AECOPD episodes, with 22.3% of episodes being caused by more than one pathogen. These rates are higher than previously described.[6,22,23] The fact that AECOPD episodes related to cardiac failure of the patient or other non-infectious causes were excluded from our study may account for the increased proportion of samples with potential pathogens.

In contrast to all previously published data, *P. aeruginosa* was the most frequent pathogen isolated from AECOPD in our series.[3–6,22,23] This confirms the important role played by *P. aeruginosa* as a cause of AECOPD in patients with advanced disease. In fact, a third of our patients suffered an AECOPD caused by this pathogen at any time. The molecular typing analysis ruled out the possibility of cross-infection between patients attended in our Consulting Room, because all *P. aeruginosa* isolates studied had different PFGE patterns (unique PFGE pattern per patient).

The presence of *P. aeruginosa* has been associated with the presence of bronchiectasis.[3–5] In the present study, we excluded patients with evident bronchiectasis not associated with COPD (bronchiectasis found in CT previous to development of COPD); however, nearly a half of patients developed bronchiectasis associated with the severity of the COPD. Nevertheless, among the 37 patients with

AECOPD caused by *P. aeruginosa*, only a half *of them* showed evidence of bronchiectasis ($P = 1.000$). In this way, bronchiectasis were only found in two of the nine patients who were persistently colonized by *P. aeruginosa*.

The high frequency of *P. aeruginosa* found in this study is important because GOLD guidelines recommend an initial empirical treatment with an aminopenicillin with or without clavulanic acid, macrolide or tetracycline, which are not active against *P. aeruginosa*. For this reason, in severe COPD patients an empirical anti-pseudomonal treatment should be taken into account, irrespective of the number of annual exacerbation episodes. The antimicrobial choice should be based on the local bacterial resistance pattern. In this way, the high rates of resistance to ciprofloxacin among *P. aeruginosa* isolates found preclude the empirical use of fluoroquinolones in our geographical area.

The frequencies of *H. influenzae*, *S. pneumoniae*, and *M. catarrhalis* as etiological agents of AECOPD were similar to previous reports,[3–6,22,23] and their antimicrobial susceptibility was fairly consistent with published findings.[24–26] In our series, only 5.4% of *H. influenzae* isolates harbored a beta-lactamase, which coincides with the important decrease observed in Spain over the last decade.[25] However, all *M. catarrhalis* produced beta-lactamase, this being similar to what was found among isolates recovered from the general population in the USA, although it is much higher than the rate reported (54.5%) in a study performed in Hong Kong among isolates recovered from AECOPD.[6]

In the present study the frequency of *S. aureus* and *Enterobacteriaceae* species was also similar to previous reports.[6] However, few data are available about the pathogenic role of *S. pseudopneumoniae* and *Corynebacteriaceae* species. Indeed, the clinical relevance of *S. pseudopneumoniae* has not been clearly established, although some authors have shown a possible association with COPD.[27] Among our patients, in a half of AECOPD with presence of *S. pseudopneumoniae*, it was isolated as a single pathogen, suggesting it may have a role as a causative agent of moderate episodes that do not require the patient's hospitalization. *Corynebacteriaceae* species have been recognized as opportunistic pathogens, although under specific circumstances they can cause disease.[28] In our series, a high bacterial load of *Corynebacteriaceae* species was detected in 10 episodes of AECOPD, and in 3 of them it was a single potential pathogen. The most frequent species found, *Corynebacterium striatum* and *Corynebacterium pseudodiphteriticum*, have been previously reported as etiological agents of respiratory infections.[28]

A correlation between deterioration of lung function and the distribution of microbial etiology has been reported.[6,22] However, our study revealed that even among patients with identical airflow obstruction, *H. influenzae* was associated with patients with a low frequency of AECOPD episodes, while *Enterobacteriaceae* species were only detected in patients with frequent exacerbations. It could be explained by the frequent treatment with amoxicillin-clavulanic acid and fluoroquinolones due to the multiple AECOPD episodes (data not shown), but also by the presence of bronchiectasis associated with COPD in two thirds of these patients. No differences were observed among the distributions of the remaining pathogens.

Notably, *C. pneumoniae* was detected in almost 50% of AECOPD episodes, a higher frequency than previously reported.[8] The fact that we used PCR to detect this species in sputum samples could have led to an overestimate of its frequency, although one previous study showed a high correlation between PCR detection in respiratory samples and serological methods.[29] In addition, a study performed in the chinchilla model of otitis media demonstrated that purified DNA was quickly cleared from the respiratory tract, suggesting that bacterial DNA present in respiratory samples such as sputum indicates the presence of viable bacteria.[30] Further studies using both serological and molecular methods are needed in order to elucidate the pathogenic role of this species in patients with an advanced airway obstruction.

To conclude, the present study confirms that *P. aeruginosa* plays an important role in causing AECOPD in patients with an advanced airflow obstruction. It should also be noted that a fifth of the exacerbations in our patients with severe COPD were polymicrobial. Although the frequency of bacteria causing exacerbations is known to depend in part on the severity of airflow obstruction, our results also suggest that the bacterial etiological agents also depend on the number of annual episodes in patients with identical airflow obstruction. This fact, together with the high number of polymicrobial infections, should be taken into account when assessing how best to manage these patients, not least so as to prevent symptom progression and improve their quality of life.

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Transparency declarations

None to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.jinf.2013.09.003.

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Table S1. Distribution according to aetiology of 42 AECOPD episodes caused by two or more potential pathogens.

Microbe	No. of episodes (n=42)
S. pneumoniae + H. infuenzae	5
S. pneumoniae + P. aeruginosa	4
H. influenzae + M. catarrhalis	3
S. pneumoniae + P. aeruginosa + M. catarrhalis	3
P. aeruginosa + M. catarrhalis	3
P. aeruginosa + C. striatum	3
S. pseudopneumoniae + P. aeruginosa	3
S. pneumoniae + M. catarrhalis	2
S. pneumoniae + H. infuenzae + M. catarrhalis	1
S. pneumoniae + M. catarrhalis + S. aureus	1
S. pneumoniae + K. pneumoniae + C. albicans	1
S. pneumoniae + E. coli	1
H. influenzae + C. pseudodiphteriticum	1
H. influenzae + P. fluorescens	1
H. influenzae + B. bronchiseptica	1
P. aeruginosa + S. aureus + K. pneumoniae	1
P. aeruginosa + S. maltophilia	1
P. aeruginosa + M. non-liquefaciens	1
P. aeruginosa + C. albicans	1
P. aeruginosa + C. propinquum	1
M. catarrhalis + E. coli	1
A. xylosoxidans + C. argentoratense	1
S. pseudopneumoniae + H. influenzae	1
S. pseudopneumoniae + M. catarrhalis	1

CHAPTER III: Dynamics of pneumococcal populations causing disease in COPD patients.

Objective 2: To analyse the dynamics and population structures of *S. pneumoniae* strains causing acute exacerbations and pneumonia (bacteraemic and non-bacteraemic) in patients with COPD, from 2001 to 2008.

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Serotypes and genotypes of *Streptococcus pneumoniae* causing pneumonia and acute exacerbations in patients with chronic obstructive pulmonary disease

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Objectives: This study aimed to compare the antibiotic susceptibilities, serotypes and genotypes of pneumococci causing pneumonia or acute exacerbations of chronic obstructive pulmonary disease (AECOPD) in patients with COPD.

Methods: A total of 611 pneumococci collected from 487 COPD patients with pneumonia (n=255, 94 bacteraemic pneumonia) or AECOPD episodes (n=356), from 2001 to 2008, were analysed. Antibiotic susceptibility was studied by microdilution. Serotypes (PCR or Quellung) and genotypes (PFGE and multilocus sequence typing) were determined.

Results: Pneumococci isolated from AECOPD episodes were significantly more resistant to co-trimoxazole and chloramphenicol than those isolated from pneumonia episodes (39.0% versus 29.7% and 13.8% versus 8.2%, respectively, $P<0.05$). Comparing serotypes of isolates causing bacteraemic pneumonia, non-bacteraemic pneumonia and AECOPD, serotypes 4, 5 and 8 were associated with bacteraemic pneumonia ($P<0.05$), serotypes 1 and 3 were associated with bacteraemic and non-bacteraemic pneumonia ($P<0.05$) and serotypes 16F and 11A and non-typeable pneumococci were associated with AECOPD episodes ($P<0.05$). The genotypes related to serotypes 3 (Netherlands^{3}-ST180 and ST260^{3}), 1 (Sweden^{1}-ST306), 5 (Colombia^{5}-ST289) and 8 (Netherlands^{8}-ST53) were isolated more frequently in pneumonia episodes ($P<0.05$), whereas genotype ST30^{16F} (serotype 16F) was more frequently recovered from AECOPD episodes.

Conclusions: In our experience, serotype 3 pneumococci (Netherlands^{3}-ST180 and ST260^{3} genotypes) commonly cause pneumonia and acute exacerbations in COPD patients. Pneumococci of serotypes 1 (Sweden^{1}-ST306), 4 (ST247^{4}), 5 (Colombia^{5}-ST289) and 8 (Netherlands^{8}-ST53) were more often associated with pneumonia. Non-typeable pneumococci may play an important role in acute exacerbations.

Keywords: *S. pneumoniae*, COPD, *Streptococcus pseudopneumoniae*

Introduction

Chronic obstructive pulmonary disease (COPD) is a cause of high morbidity and mortality in developed countries. The BOLD international study (where BOLD stands for Burden of Obstructive Lung Disease) estimated a mean prevalence of 10.1% of COPD for stage GOLD II or higher (where GOLD stands for Global Initiative for Chronic Obstructive Lung Disease), with significant differences between countries.[1] In Spain, the prevalence of COPD between 40 and 80 years old is 10.2%.[2] Acute exacerbations of COPD (AECOPD) contribute to the progress of the disease; they are indicators of poor prognosis and are associated with high healthcare costs.[3,4]

In patients with COPD, *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis* are the main pathogens causing AECOPD episodes.[3] Furthermore, a recent study showed that 30% of patients with recurrent community-acquired pneumonia had COPD as the main underlying disease, *S. pneumoniae* being the most frequent causative microorganism.[5]

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The worldwide spread of penicillin- and multidrug-resistant *S. pneumoniae* is a cause for concern. In Spain, the global rates of invasive penicillin non-susceptible isolates (MIC $\geq$ 0.12 mg/L) have fallen in the last decade, from 32.1% (1999) to 21.1% (2008) in adults, and from 48.4% to 27.4% in children, especially since the implementation of the paediatric 7-valent pneumococcal conjugate vaccine (PCV-7).[6,7] Rates of macrolide resistance among invasive pneumococci remained stable in adults (21.9% in 1999 and 20.7% in 2008), but fell significantly in children from 39.6% to 26.6%.[6,7] Antibiotic-resistant pneumococci have been associated with patients with underlying diseases including COPD.[8]

The capsular polysaccharide is the most important known virulence factor of *S. pneumoniae* and has been related to the invasive potential of pneumococci. Currently, 93 different serotypes have been identified, which are defined by differences in their polysaccharide capsule.[9] Although serotypes causing invasive pneumococcal disease or colonizing healthy children have been extensively analysed, information on serotypes and genotypes causing pneumonia or acute exacerbations in patients with COPD is scarce.

The aim of the study was to compare the antibiotic susceptibilities, serotypes and genotypes of *S. pneumoniae* isolates causing pneumonia (including bacteraemic and non-bacteraemic isolates) or AECOPD in patients with COPD.

Methods

Hospital setting and study period

This laboratory-based study was carried out between 2001 and 2008 at the Hospital Universitari de Bellvitge in the south of Barcelona. Pneumococci isolated from clinical samples (invasive and non-invasive) were prospectively collected in our laboratory. Pneumococcal susceptibility and clinical data of patients were prospectively recorded in a database. Only sputum samples of good quality were considered (<10 squamous cells and >25 leucocytes per low-power field).[10]

Computerized medical records of patients with pneumococci isolated from blood and/or respiratory specimens were reviewed in order to determine the COPD status according to the international GOLD criteria.[11] Patients with high co-morbidity (Charlson index $\geq$5), immunodeficiency, terminal malignancy and other chronic respiratory diseases (bronchiectasis, asthma or bronchial interstitial lung disease) were excluded.[12] Moreover, clinical charts were reviewed to define each episode as pneumonia or AECOPD. An acute exacerbation of COPD was defined as any sustained increase in respiratory symptomatology compared with the baseline situation requiring an increase in regular medication and hospital treatment. An episode of pneumonia was considered when fever, leucocytosis and radiological findings (new infiltrates on chest radiography) were detected.

Bacterial isolates and antimicrobial susceptibility

Pneumococcal isolates were identified by optochin susceptibility and latex agglutination with specific antisera (Phadebact®). Isolates showing a negative PCR for *cps* loci genes were tested for susceptibility to optochin in ambient and CO_2 atmospheres and for bile solubility (2% sodium desoxycholate solution).

Antimicrobial susceptibility to penicillin, cefotaxime, erythromycin, clindamycin, tetracycline, chloramphenicol, co-trimoxazole, ciprofloxacin and levofloxacin was tested by microdilution (Sensititre™) following the CLSI methods and criteria.[13] *S. pneumoniae* ATCC 6303 and *S. pneumoniae* ATCC 49619 were used as control isolates.

Serotyping and molecular typing

Serotyping of blood isolates was performed by Quellung reaction at the Spanish Reference Laboratory for Pneumococci (SRLP) in Majadahonda, Madrid. Respiratory isolates were first serotyped by a multiplex PCR protocol using methodology previously described.[14] Respiratory isolates non-serotyped by PCR were typed by Quellung reaction at SRLP.

Molecular typing was performed by PFGE. Genomic DNA embedded in agarose plugs was restricted with SmaI or ApaI (New England BioLabs) and fragments were separated by PFGE in a CHEF-DRIII apparatus (Bio-Rad) as described previously.[15] PFGE patterns were compared with representative international pneumococcal clones of the Pneumococcal Molecular Epidemiology Network.[15] Band patterns were visually compared following the criteria described by Tenover *et al.*[16] Major clusters were defined as those that included three or more pneumococcal isolates. In order to assess the identity with global pneumococcal clones, at least one representative isolate of each major cluster was analysed by multilocus sequence typing (MLST) as described previously.[17] Allele numbers and sequence types (STs) were assigned using the pneumococcal MLST web site.[18]

Statistical analysis

Statistical analyses were carried out using SPSS for Windows (version 18.0) and EpiInfo (version 6.0, CDC). We used χ^2 or Fisher's exact test to compare proportions of serotypes and genotype distribution in COPD patients with pneumonia and acute exacerbations. The odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. Two-sided P values <0.05 were considered statistically significant.

Results

Bacterial isolates and antimicrobial susceptibility

From 2001 to 2008, 3364 pneumococci (one per episode) were isolated from blood and/or respiratory specimens in 2942 adult patients. Computerized medical records were available in 3115 (92.6%) episodes in order to review the COPD status. Seven hundred and ten pneumococci (22.8%) were recovered from 551 COPD patients (18.7% of the total). The COPD status according to the GOLD criteria was available in 336 patients (61%); 13 patients were identified as GOLD I for COPD disease, 73 as GOLD II, 89 as GOLD III and 161 as GOLD IV. The remaining 215 patients were classified as COPD patients according to the clinical diagnosis without a spirometric evaluation.

Eighty-one pneumococci were excluded from the analysis because they were isolated from patients without clinical symptoms suggestive of respiratory disease, in whom the situation of COPD disease was considered to be stable. Eighteen isolates showed a negative PCR for *cps* loci genes; they were susceptible to optochin in an ambient atmosphere and resistant in CO_2, and they were bile insoluble. These 18 isolates probably belong to the recently described species *Streptococcus pseudopneumoniae* and were excluded from the analysis.

Six hundred and eleven pneumococci were finally analysed; 356 pneumococci (all of them from sputum samples) were isolated from AECOPD episodes and 255 were isolated from pneumonia episodes (149 from sputum, 94 from blood, 7 from pleural fluid, 4 from bronchoalveolar lavage and 1 from transthoracic needle aspiration). Among patients with pneumonia, the mortality rate was 10.4%. Two hundred and thirty-seven (92.9%) of the 255 pneumonia episodes occurred in men, and the mean age was 70 $\pm$ 9.8.

Among AECOPD episodes (336/356, 94.4% men; mean age 71±9.3), pneumococcus was the single isolated pathogen in 215 episodes (60.4%), whereas a second pathogen was recovered from the sputum samples in 39.6% of cases; *H. influenzae* (*n*=74, 20.8%), *M. catarrhalis* (*n*=28, 7.9%), *Pseudomonas aeruginosa* (*n*=24, 6.7%) *Haemophilus parainfluenzae* (*n*=4), *Stenotrophomonas maltophilia* (*n*=4) *Corynebacterium pseudodiphtheriticum* (*n*=2), *Staphylococcus aureus* (*n*=2), *Acinetobacter baumannii* (*n*=1) and Enterobacteriaceae (*n*=2).

In the respiratory specimens collected from 161 pneumonia episodes, pneumococcus was the single isolated pathogenic microorganism in 112 (69.6%) episodes and was recovered with a second pathogen in the remaining episodes, mainly *H. influenzae* (*n*=18, 11.2%), *M. catarrhalis* (*n*=12, 7.5%) and *P. aeruginosa* (*n*=7, 4.3%). In contrast, only in four of 94 episodes (4.3%) of bacteraemic pneumonia was a co-infection observed and in all four episodes the pneumococcus was isolated together with *H. influenzae*.

Table 1 shows the activity of nine antimicrobials against pneumococci isolated from pneumonia or acute exacerbation episodes of patients with COPD. Pneumococci isolated in AECOPD episodes had higher antimicrobial resistance rates than those isolated from pneumonia episodes, but this difference reached statistical significance only for co-trimoxazole (39% versus 29.7%, $P<0.05$) and chloramphenicol (13.8% versus 8.2%, $P<0.05$).

Serotyping and molecular typing

Figure 1 shows the serotype distribution of pneumococci isolated from bacteraemic pneumonia, non-bacteraemic pneumonia or AECOPD episodes.

The most frequent serotypes among bacteraemic pneumonia episodes were 3 (*n*=17, 18.1%), 1 (*n*=8, 8.5%), 5 (*n*=8, 8.5%), 19A (*n*=7, 7.4%), 4 (*n*=6, 6.4%) and 8 (*n*=6, 6.4%), which accounted for 55.3% of the total.

The most frequent serotypes among non-bacteraemic pneumonia episodes were 3 (*n*=24, 14.9%), 19F (*n*=19, 11.8%), 23F (*n*=10, 6.2%), 11A (*n*=10, 6.2%), 6B (*n*=9, 5.6%), 1 (*n*=8, 5.0%), 19A (*n*=7, 4.3%) and 14 (*n*=7, 4.3%), which accounted for 58.4% of the total. Among AECOPD isolates the most frequent serotypes were 3 (*n*=30, 8.4%), 19A (*n*=23, 6.5%), 19F (*n*=21, 5.9%), 23F (*n*=19, 5.3%), 11A (*n*=19, 5.3%), 14 (*n*=18, 5.1%), 16F (*n*=14, 3.9%), 23A (*n*=13, 3.7%) and 31 (*n*=12, 3.4%) and non-typeable isolates (*n*=20, 5.6%), which accounted for 53.1% of the total.

Serotypes of bacteraemic pneumonia isolates were compared with those causing non-bacteraemic pneumonia and with those causing AECOPD. The ORs and 95% CIs were calculated and are shown in Table S1 (available as Supplementary data at *JAC* Online). Comparing serotypes of isolates causing bacteraemic pneumonia, non-bacteraemic pneumonia and AECOPD, serotypes 4, 5 and 8 were associated with bacteraemic pneumonia ($P<0.05$), whereas serotypes 1 and 3 were associated with bacteraemic and non-bacteraemic pneumonia ($P<0.05$) and serotypes 16F and 11A and non-typeable pneumococci were associated with AECOPD episodes ($P<0.05$). Serotypes 19F and 6B were associated with pneumonia, especially non-bacteraemic pneumonia ($P<0.05$).

Table 1. *In vitro* activity of nine antimicrobials against pneumococci isolated from pneumonia or acute exacerbation episodes of patients with COPD

	Pneumonia (*n*=255)						Acute exacerbations (*n*=356)					
Antibiotic	MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC range (mg/L)	% S	% I	% R	MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC range (mg/L)	% S	% I	% R
Penicillin	≤0.03	1	≤0.03 to 4	73.0[a]	19.2	7.8	≤0.03	2	≤0.03 to 4	65.4[a]	22.8	11.8
				98.8[b]	1.2	0				97.5[b]	2.5	0
Cefotaxime	≤0.03	0.5	≤0.03 to 2	91.8[c]	7.1	1.1	≤0.03	1	≤0.03 to 2	87.4[c]	11.2	1.4
				98.8[d]	1.2	0				98.7[d]	1.3	0
Ciprofloxacin[e]	≤0.5	2	≤0.5 to >32	96.5	—	3.5	≤0.5	2	≤0.5 to ≥32	93.8	—	6.2
Levofloxacin	≤0.5	1	≤0.5 to >32	98.1	0.5	1.4	≤0.5	1	≤0.5 to ≥32	96.2	0.3	3.5
Tetracycline	≤2	>32	≤2 to >32	71.8	2.0	26.2	≤2	>4	≤2 to >32	69.4	3.1	27.5
Erythromycin	≤0.25	≥32	≤0.25 to >32	71.0	0	29.0	≤0.25	≥32	≤0.25 to >32	68.3	0	31.7
Clindamycin	≤0.25	≥32	≤0.25 to >32	74.5	0	25.5	≤0.25	≥32	≤0.25 to ≥0.5	69.7	0.6	29.7
Chloramphenicol	≤2	≤2	≤2 to ≥16	91.8	—	8.2	≤2	8	≤2 to ≥16	86.2	—	13.8
Co-trimoxazole	≤0.5/9.5	4/76	≤0.5/9.5 to >4/76	70.3	4.9	24.8	≤0.5/9.5	≥2/38	≤0.5/9.5 to ≥2/38	61.0	5.3	33.7

[a] Penicillin oral breakpoints: susceptible (S), ≤0.06 mg/L; intermediate (I), 0.12–1 mg/L; and resistant (R), ≥2 mg/L.
[b] Penicillin parenteral (non-meningitis) breakpoints: susceptible, ≤2 mg/L; intermediate, 4 mg/L; and resistant, ≥8 mg/L.
[c] Cefotaxime (meningitis) breakpoints: susceptible, ≤0.5 mg/L; intermediate, 1 mg/L; and resistant, ≥2 mg/L.
[d] Cefotaxime parenteral (non-meningitis) breakpoints: susceptible, ≤1 mg/L; intermediate, 2 mg/L; and resistant, ≥4 mg/L.
[e] Non-susceptibility to ciprofloxacin breakpoint, ≥4 mg/L; and susceptible breakpoint, ≤2 mg/L.[19]

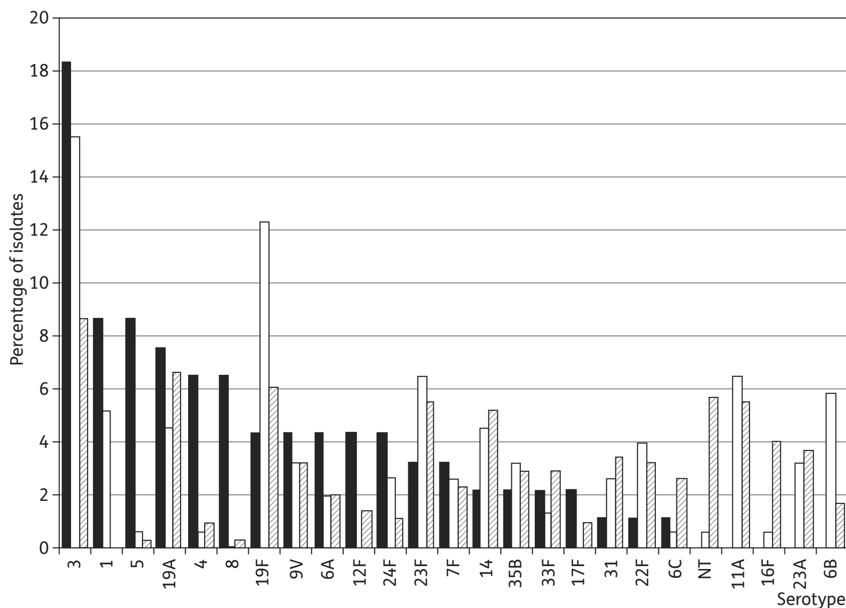

Figure 1. Distribution of serotypes of pneumonia and AECOPD *S. pneumoniae* isolates isolated from adult patients with COPD (2001–08). Black bars, blood isolates from pneumonia episodes; white bars, respiratory isolates from pneumonia episodes; diagonally striped bars, AECOPD isolates.

The serotypes included in the pneumococcal 23-valent polysaccharide vaccine (PPS23v) accounted for 79.6% of all pneumonia isolates (blood and respiratory isolates) and for 61.0% of AECOPD isolates. The serotypes included in PCV-7 accounted for 28.2% of all pneumonia isolates and for 23.6% of AECOPD isolates. The coverage of the new 10-valent (PCV-10) and 13-valent (PCV-13) conjugate vaccines in all pneumonia isolates is 40.8% and 65.1%, respectively, whereas coverage in AECOPD isolates is 26.1% and 43.0%, respectively.

The GOLD status was available in 336 patients—too few for a comparison of the groups according to GOLD status to have sufficient statistical power. Interestingly, however, in patients with GOLD I and II 48.6% of pneumococci were isolated from pneumonia episodes and belonged mostly to serotypes 1, 3, 5 and 24F; in patients with GOLD III and IV pneumococci were isolated mainly from AECOPD episodes (67.2%) and the predominant serotypes were 7F, 14, 16F and 19A and non-typeable pneumococci.

Six hundred (98.2%) pneumococci were available for PFGE study (246 pneumococci from pneumonia and 354 from AECOPD). These isolates were classified into 246 PFGE patterns. Thirty-four PFGE patterns accounted for ≥3 isolates and were considered major lineages. These major lineages were related to 28 different STs.

Table 2 shows the distribution of major lineages among pneumococci causing pneumonia (bacteraemic and non-bacteraemic) or AECOPD episodes. The genotypes related to serotypes 3 (Netherlands^{3}-ST180 and ST260^{3}), 1 (Sweden^{1}-ST306), 5 (Colombia^{5}-ST289) and 8 (Netherlands^{8}-ST53) were isolated more frequently in pneumonia episodes ($P<0.05$), whereas genotype ST30^{16F} (related to serotype 16F) was isolated significantly more frequently in AECOPD episodes. All non-typeable isolates had different PFGE patterns, showing a high genetic diversity.

Nine genotypes showed more than one serotype: Spain^{9V}-ST156 (serotypes 9V and 14); Spain^{23F}-ST81 (serotypes 23F, 19A and 19F); ST42 (serotypes 23A and 23F); Sweden^{15A}-ST63 (serotypes 15A, 15F, 19A and 19F); Denmark^{14}-ST230 (serotypes 24F, 19A and 7F); ST1201 (serotypes 19A and 19F); ST558 (serotypes 35B and 35F); Tennessee^{14}-ST67 (serotypes 14, 9N and 9V); and Columbia^{23F}-ST338 (serotypes 23F and 23B).

Discussion

S. pneumoniae plays an important role in both pneumonia and acute exacerbations of patients with COPD. Moreover, in patients with recurrent pneumonia, COPD has been shown to be the main underlying disease.[5,20] However, little information is available on capsular types and genotypes in pneumonia and acute exacerbations in COPD patients.

The present study analyses the differences in antibiotic susceptibility rates, serotypes and genotypes of pneumococci isolated from pneumonia episodes and acute exacerbations.

It is well known that invasive isolates are more susceptible to antibiotics than those isolated from non-invasive sites, such as sputum samples.[21] In this study, isolates causing pneumonia were isolated from invasive and non-invasive sources, whereas those causing AECOPD were all from sputum. The difference in the sources of pneumococci may explain the higher susceptibility rates of pneumonia episodes.

The new fluoroquinolones are widely used to treat respiratory tract infections, especially in patients with COPD. Although our

Table 2. Major lineages of pneumococci isolated from pneumonia and AECOPD episodes

ST	Related PMEN clone	No. of isolates	Serotypes isolated from pneumonia or AECOPD	
			pneumonia (no. of isolates)[a]	AECOPD (no. of isolates)
ST180	Netherlands[3]-31	36	3 (21)[b]	3 (15)
ST156	Spain[9V]-3	32	14 (5), 9V (5)	14 (13), 9V (9)
ST81	Spain[23F]-1	28	23F (5), 19F (2), 19A (1)	23F (12), 19F (4), 19A (4)
ST62		26	11A (10)	11A (16)
ST260		26	3 (17)[b]	3 (9)
ST42		19	23A (4), 23F (2)	23A (12), 23F (1)
ST63	Sweden[15A]-25	19	15A (3), 19A (1), 19F (5)	15A (7), 19F (3), 15F (1)
ST88		15	19F (7)	19F (8)
ST30		14	16F (1)	16F (13)[b]
ST433		14	22F (7)	22F (7)
ST306	Sweden[1]-28	13	1 (13)[b]	
ST191	Netherlands[7F]-39	12	7F (6)	7F (6)
ST230	Denmark[14]-32	11	24F (4), 19A (3)	24F (2), 19A (1), 7F (1)
ST90	Spain[6B]-2	10	6B (6)	6B (4)
ST289	Colombia[5]-19	9	5 (8)[b]	5 (1)
ST1201		9	19A (4), 19F (1)	19A (4)
ST558		8	35B (2)	35B (3), 35F (3)
ST989		8	12F (4)	12F (4)
ST247		7	4 (5)	4 (2)
ST53	Netherlands[8]-33	7	8 (6)[b]	8 (1)
ST97		7	10A (1)	10A (6)
ST717		7	33 (3)	33 (4)
ST67	Tennessee[14]-18	6	9N (2), 9V (1)	9N (2), 14 (1)
ST110		6		18C (6)
ST338	Colombia[23F]-26	6	23F (2)	23B (4)
ST202		4	19A (1)	19A (3)
ST224		3		6C (3)
ST1046		3	34 (1)	34 (2)

PMEN, Pneumococcal Molecular Epidemiology Network.
[a]Overall pneumonia isolates were considered (isolated from blood or respiratory samples).
[b]Statistically significant differences between lineages of pneumonia and AECOPD isolates.

study shows a low rate of ciprofloxacin resistance (3.5% in pneumonia and 6.2% in AECOPD isolates), it was higher than rates found in a recent study also performed in Spain (3.3% among non-invasive pneumococci and 1.3% among invasive isolates).[22] The high consumption of quinolones in this group of patients could explain the higher resistance rate. The differences observed in the rates of susceptibility to ciprofloxacin and to levofloxacin are due to isolates with first-step mutations in the quinolone resistance-determining regions (data not shown). These isolates (ciprofloxacin resistant and levofloxacin susceptible) may become highly resistant under selective fluoroquinolone pressure and are associated with treatment failure when quinolones are used.[23]

Using classical criteria, the rates of penicillin and cefotaxime resistance of pneumococci isolated from patients with COPD are high.[13] However, pneumococci with penicillin or cefotaxime/ceftriaxone MICs $\geq$4 mg/L have rarely been described in our area. Using the revised non-meningeal CLSI breakpoints, more than 98% of pneumococci isolated from COPD patients were penicillin and cefotaxime susceptible, so β-lactam antibiotics should continue to be first-line therapy for pneumococcal pneumonia.[8]

The second main finding of our study is the differences observed between serotypes detected in pneumonia and AECOPD episodes. Serotypes 1, 3, 4, 5 and 8 were more frequently associated with pneumonia (mainly among bacteraemic pneumonia) than with AECOPD. Some serotypes, such as 1, 5 and 7F, were considered primary pathogens when they were analysed in nasopharyngeal and invasive pneumococci isolated from children.[24]

Although the invasive potential of serotype 3 is reported to be low,[24] it was the most frequent cause of bacteraemic and non-bacteraemic pneumococcal pneumonia in COPD patients in our study and the second most frequent cause of AECOPD. Among pneumonia isolates, serotype 3 accounted for 18.1% (n=17/94) of bacteraemic pneumonia episodes and 14.9% (n=24/161) of non-bacteraemic episodes, suggesting that the patient's underlying condition plays a key role in the development of bacteraemia, as other authors have proposed previously.[20,25,26]

It was striking that non-typeable pneumococci, a common cause of AECOPD, were only rarely found causing pneumonia. The absence of a capsule in these isolates may reduce their

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invasiveness. Some non-capsulated and atypical pneumococci have recently been identified as a new species of *Streptococcus*, *S. pseudopneumoniae*, whose role in COPD is unknown.[27,28]

Although several pneumococcal vaccines are available, only PPS23v is currently licensed for use in adults. The proportion of serotypes included in this vaccine was high in our COPD population. Unfortunately, although this vaccine can protect against invasive disease in healthy adults, its protective efficacy in COPD populations is controversial.[29,30] Pneumococcal conjugate vaccines with enhanced immunity potential may play an important role in the prevention of pneumonia and AECOPD in COPD patients. PCV-7, which includes 4, 6B, 9V, 14, 18C, 19F and 23F, has been shown to increase the immune response in COPD patients.[30] A new formulation of this vaccine that adds serotypes 1, 5, 7F, 3, 6A and 19A (PCV-13) has recently been commercialized for children and it will be licensed for adults in the next 2 years. Our study suggests that this vaccine may play an important role in preventing pneumococcal pneumonia in COPD patients, with coverage of 65.1% of pneumonia isolates.

Our study shows that genotypes Netherlands[3]-ST180 and ST260[3] (both related to serotype 3), Sweden[1]-ST306 (serotype 1), Colombia[5]-ST289 (serotype 5) and Netherlands[8]-ST53 (serotype 8), were isolated significantly more frequently in pneumonia episodes. These results reflect the clonal composition of the major genotypes among pneumonia isolates. In contrast, all non-typeable isolates had different PFGE patterns and showed a high genetic diversity, as reported previously.[28]

The main limitation of our study is that it is retrospective and that some data were not recorded (for instance the GOLD status of COPD patients). These missing data hampered a powerful analysis of serotypes by GOLD status. Another limitation of our study is that it was conducted at a single medical centre. However, to our knowledge it is the largest study of pneumococcal serotypes and genotypes in pneumonia and AECOPD episodes of recently recovered patients with COPD. The differences we have found should be evaluated in a prospective, multicentre study that also includes the vaccination status of the patient. The third limitation is the difficulty of analysing the role of *S. pneumoniae* in episodes with samples yielding more than one pathogen. Further studies are needed in order to establish the role of each pneumococcal serotype in AECOPD and pneumonia episodes of patients with COPD and to assess the impact of the new conjugate vaccines in preventing pneumococcal disease in these patients.

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Transparency declarations

None to declare.

Supplementary data

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

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CHAPTER III: Dynamics of pneumococcal populations causing disease in COPD patients.

Objective 3: To evaluate the impact of PCV7 on the pneumococcal populations causing acute exacerbations, from 2001 to 2012.

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1 **Dynamics of the pneumococcal population causing acute exacerbations in**
2 **patients with Chronic Obstructive Pulmonary Disease (2009-2012).**
3 **Comparison with 2001-2004 and 2005-2008 periods.**

4

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21 **Key words:** *Streptococcus pneumoniae,* chronic obstructive pulmonary disease,
22 acute exacerbation, conjugate vaccines, serotype, genotype.

"This study was partially presented at the XI European Meeting on the Molecular Biology of the Pneumococcus. May 2013, Madrid, Spain"

Abstract.

Background. Pneumococci are an important cause of acute exacerbations in patients with chronic obstructive pulmonary disease (COPD). In the last decade, the pneumococcal population has changed, mainly due to the introduction of the 7-valent conjugate vaccine (PCV7).

Methods: We analysed the antimicrobial susceptibility (microdilution), serotype (PCR) and genotype (PFGE/MLST) of pneumococci causing acute exacerbations during the period 2009-12. Results were compared with two previously published historic periods (2001-04 and 2005-08).

Results: A total of 206 pneumococci were collected from 162 COPD patients with acute exacerbations. Compared with previous periods, no significant changes in the rate of multi-drug resistance were observed (36.5% in 2001-04 to 33.5% in 2009-12 periods; *P*=0.644).

The most frequent serotypes in the 2009-12 period were: 15A (9.2%), 3 (7.8%), 19F (6.3%), 11A (5.8%) and 6C (5.3%), which accounted for 34.5%. A drastic decrease in PCV7 serotypes was observed throughout the study period (from 39.4% in 2001-04 to 11.2% in 2009-12; *P*<0.001); non-PCV13 serotypes increased from 45.3% to 72.3%, especially 15A (from 2.2% to 9.2%) and 6C (0% to 5.3%) [*P*<0.05].

The most frequent genotypes (Clonal Complexes, CCs) in the 2009-12 period were: $CC63^{15A,19F,15F}$ (8.7%), $CC180^{3}$ (4.4%), $CC62^{11A}$ (3.9%), $CC97^{10A}$ (3.9%), $CC386^{6C}$ (3.4%), $CC260^{3}$ (3.4%), and $CC30^{16F}$ (3.4%). Serotypes 19F, 19A, 6A and 6C were genetically diverse.

Conclusions: PCV7-serotypes have decreased dramatically. In parallel, two non-PCV7 serotypes (15A and 6C), and their related genotypes ($CC63^{15A}$ and $ST386^{6C}$) showed a significant increase. Although resistance rates to betalactams decreased over time, multi-drug resistance remained stable.

54 **Introduction.**

55 Chronic obstructive pulmonary disease (COPD) is an important cause of morbidity
56 and mortality in developed countries,[1] mainly due to the recurrence of acute
57 exacerbation episodes which contribute to the progress of the disease. Moreover,
58 acute exacerbations are indicators of poor prognosis and have been associated
59 with high healthcare costs.[2]

60 It is estimated that *Streptococcus pneumoniae* is responsible for around 15% of
61 acute exacerbations caused by bacterial pathogens.[3] Its capsule is the most
62 important known virulence factor, and has been related to the invasive potential of
63 pneumococci; to date, 94 different capsular polysaccharides have been identified.[4]

64 Until the introduction of conjugate vaccines in 2000, the prevention of
65 pneumococcal diseases was based on the use of the 23-valent pneumococcal
66 polysaccharide vaccine (PPSV23), which targeted 23 common infecting serotypes
67 of *S. pneumoniae* (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F,
68 18C, 19A, 19F, 20, 22F, 23F and 33F). This vaccine was introduced in 1983 and
69 currently is recommended for children over 2 years of age who are at a
70 substantially increased risk of developing pneumococcal infection:
71 Immunocompetent children with chronic heart disease; chronic lung disease,
72 diabetes mellitus; cerebrospinal fluid leaks; or cochlear implant; children with
73 anatomic or functional asplenia; children with immunocompromising conditions
74 such as HIV infection, chronic renal failure and nephrotic syndrome or diseases
75 associated with treatment with immunosuppressive drugs.[5] In addition, PPSV23 is
76 also recommended for all adults aged 65 years and older or adults younger than
77 age 65 years with chronic lung disease (including COPD, emphysema, and
78 asthma); chronic cardiovascular diseases; and other underlying conditions with a
79 substantially increased risk of developing pneumococcal infection.[5] The Health

80 Department of the Catalonia government also recommends vaccination with
81 PPSV23 in adults with chronic lung diseases such as COPD
82 (www.gencat.cat/salut/pir.html). However, its effectiveness in preventing morbidity
83 and mortality associated with pneumococcal infection is unclear.[6] In Spain, no data
84 is available about the national rates of PPSV23 vaccination in adult patients;
85 however, studies performed by the *Societat Catalana de Medicina Familiar i*
86 *Comunitària (*CAMFIC) in our geographical area showed vaccination rates around
87 70-80% of COPD patients (http://butlleti.camfic.cat/pdf/butlleti21_5.pdf).

88 Since 2000, widespread use of the 7-valent pneumococcal conjugate vaccine
89 (PCV7) for children has reduced the incidence of pneumococcal disease in all age
90 groups. [7,8] In children, this effect is the direct result of vaccination; protection of
91 non-vaccinated children and adults, known as the "herd effect", results from
92 reduced nasopharyngeal carriage of PCV7 serotypes (4, 6B, 9V, 14, 18C, 19F and
93 23F) in the vaccinated population.[7] The use of PCV7 has also substantially
94 reduced the incidence of antibiotic resistance in invasive diseases among children
95 and adults over 65 years of age.[9]

96 Although PCV-7 was introduced into the childhood vaccination programme of
97 many countries such as the United States, Canada or the United Kingdom, in
98 Spain (June, 2001) it was not included in the vaccination programme. However,
99 the Vaccine Advisory Committee of the Spanish Paediatric Association
100 recommended its use in children aged <2 years, with doses at 2, 4, 6 and 12-15
101 months, and children aged 2-5 years with risk medical conditions
102 (http://www.aeped.es).

103 In our geographical area (Catalonia), many private and public paediatric practices
104 recommended PCV7, which is paid for by parents. In a cross-sectional multicenter
105 study performed in the South of Catalonia, 45.1% of children received at least one

106 dose of PCV7, during the 2002-2011 period.[10] Vaccination uptake increased
107 progressively after PCV7 introduction. The percentage of children vaccinated was
108 18.5% born during 2002-03 period, 38.6% in 2004-05 and 62.5% in 2006-07.
109 However, this uptake decreased to 50.8% in the 2008-09 period.[10]

110 Two new conjugate vaccines, PCV10 and PCV13, have been introduced in Spain
111 recently for children aged <2 years; PCV10, which targets PCV7 serotypes plus 1,
112 5 and 7F was introduced in November 2009; and PCV13, including PCV10
113 serotypes plus 3, 6A and 19A, was introduced in June 2010, replacing PCV7.
114 PCV13 was also licensed for adults in October 2012 in Spain. There are still no
115 data available on the uptake of both vaccines in our geographical area.

116 In order to analyse the impact of conjugate vaccines in patients with COPD
117 suffering pneumococcal acute exacerbations, we studied the antibiotic
118 susceptibility and the dynamics of serotypes and genotypes of *S. pneumoniae*
119 isolates causing acute exacerbation episodes in patients with COPD during the
120 period 2001-12.
121

122 **Materials and methods.**

123 **Ethical statement.**

124 This study and publication of the results were approved by the "Comité Ètic
125 d'Investigació Clínica del Hospital Universitari de Bellvitge" and the written or oral
126 informed consent was considered not necessary, because the source of bacterial
127 isolates was anonymised.

128 **Hospital setting and study period**

129 This laboratory-based study was carried out between 2009 and 2012 at the
130 Hospital Universitari de Bellvitge. Our tertiary adult hospital is located in the
131 southern area of Barcelona and serves a population of around 600,000 people,
132 and is also a centre of reference to more that 2 million people of the southern
133 metropolitan area when their cases require the use of the latest technology.

134 In order to explore the impact of paediatric vaccination with conjugate vaccines on
135 antimicrobial susceptibility and serotype and genotype distribution, the period
136 2009-12 was compared with 2001-04 and 2005-08 periods. Serotypes and
137 genotypes performed from 2001 to 2008 were published previously in a study
138 comparing pneumococcal serotypes and genotypes recovered from acute
139 exacerbations with those isolated from bacteremic and non-bacteremic pneumonia
140 episodes in COPD patients.[11]

141 The inclusion criteria of the patients have been described previously,[11] and the
142 GOLD status of the patients (where GOLD stands for Global Initiative for Chronic
143 Obstructive Lung Disease) was determined according to the international GOLD
144 criteria.[12] An acute exacerbation of COPD was defined as any sustained increase
145 in respiratory symptomatology compared with the baseline situation which

146 required an increase in regular medication and hospital treatment. Only good
147 quality sputum samples were considered (<10 squamous cells and >25 leukocytes
148 per low-power field).[13] In those patients with more than one acute exacerbation
149 episode, only those consecutive acute exacerbations which lasted for between
150 four weeks and one year were included in the study.

151 **Bacterial strains and antimicrobial susceptibility**

152 Pneumococcal strains isolated from 2009 to 2012 were identified by optochin
153 susceptibility, bile solubility and latex agglutination with specific antisera
154 (Phadebact®).

155 Antimicrobial susceptibility was tested by disk diffusion and microdilution methods,
156 in accordance with European Committee on Antimicrobial Susceptibility Testing
157 (EUCAST) recommendations of 2013 (www.eucast.org). The following
158 antimicrobials were tested: penicillin, cefotaxime, erythromycin, clindamycin,
159 tetracycline, chloramphenicol, co-trimoxazole, ciprofloxacin and levofloxacin. *S.*
160 *pneumoniae* ATCC 6303 and *S. pneumoniae* ATCC 49619 were used as control
161 strains.

162 **Serotyping and molecular typing**

163 A total of 197 (95.6%) of 206 pneumococci isolated during 2009-12 period were
164 available for serotyping and molecular typing. Serotyping was performed by
165 multiplex PCR, using a protocol described elsewhere.[14] Pneumococci with
166 serotypes not serotypable by multiplex PCR were serotyped by Quellung reaction
167 at the Spanish Reference Laboratory for Pneumococci (SRLP) in Majadahonda,
168 Madrid.[15] Non-capsulated isolates with a susceptibility to optochin in ambient
169 atmosphere and resistance in CO_2 atmosphere, together with a negative test for
170 bile solubility (2% sodium deoxycholate solution) were excluded.

171 Genotyping was performed by Pulse Field Gel Electrophoresis (PFGE) as
172 described previously.[11] PFGE patterns were compared with representative
173 international pneumococcal clones (clones 1–26) of the Pneumococcal Molecular
174 Epidemiology Network (http://www.sph.emory.edu/PMEN). 84 representative
175 isolates were studied by multilocus sequence typing,[16] according to the following
176 criteria: 1) a minimum of one isolate from each PFGE cluster containing three to
177 nine isolates, 2) at least three isolates from each PFGE cluster containing ≥ 10
178 isolates, 3) for certain serotypes (6A, 6C, 19A and 19F) associated with a high
179 genetic diversity (with several minor clusters), at least one isolate from each PFGE
180 cluster was analysed. Allele number and sequence types (STs) were assigned
181 using the pneumococcal MLST web site (http://www.mlst.net).

182 **Statistical Analysis**

183 Statistical analyses were carried out using SPSS for Windows (version 18.0) and
184 R (The R Foundation for Statistical Computing) version 2.15.0. We used Chi-
185 square or Fisher's exact tests to compare proportions of antimicrobial
186 susceptibility and serotype and genotype distribution in COPD patients with acute
187 exacerbation episodes. Two-sided P-values less than 0.05 were considered
188 statistically significant.

189

190 **Results.**

191 A total of 206 pneumococci were recovered from 2009 to 2012 causing acute
192 exacerbations in COPD patients. The mean age (Standard Deviation) of the
193 patients was 70.2 (SD± 8.7), and most of them (96.1%) were men. The degree of
194 airflow obstruction was available in 178 (86.4%) of patients, who according to the
195 GOLD criteria were classified as: GOLD I (n=2 patients), GOLD II (n=33), GOLD
196 III (n=41) and GOLD IV (n=102). The remaining 28 patients were classified as
197 COPD patients according to clinical diagnosis without a spirometric evaluation.
198 Most of the patients (n=135/206) had a single pneumococcal acute exacerbation
199 episode during the study period; 18 patients had 2 episodes and the remaining 9
200 patients had 3 or more episodes.

201 Three periods of time were defined for the analysis: 2001-04 period (early-PCV7
202 period), including 138 (24.6%) episodes; 2005-08 period (late PCV7 period),
203 including 218 (38.8%) episodes; and 2009-12 period, including 206 (36.6%)
204 episodes. There were no statistically significant differences in the proportion of
205 GOLD status classifications in the three periods (data not shown).

206 **Serotypes.** During the 2009-12 period, a total of 38 different serotypes were
207 detected; however, eleven of them accounted for 58.7% of the pneumococcal
208 acute exacerbations: serotype 15A (n=19, 9.2%), 3 (n= 16, 7.8%), 19F (n= 13,
209 6.3%), 11A (n=12, 5.8%), 6C (n=11, 5.3%), 19A (n=10, 4.9%), 23B (n=9, 4.4%),
210 23A (n=9, 4.4%), 10A (n=8, 3.9%), 35B (n=7, 3.4%) and 16F (n=7, 3.4%).

211 Table 1 shows the dynamics of serotypes over the study period, and the coverage
212 of pneumococcal conjugate vaccines in each period of time. A statistically
213 significant decrease in PCV7 serotypes was observed throughout the study
214 period, from 39.4% in the 2001-04 period to 17.0% in the 2001-05 period
215 (*P*<0.001) and 10.7% in the 2009-12 period (*P*<0.01). In this respect, PCV10
216 showed a similar decrease to PCV7 (from 39.9% to 20.6% (*P*<0.001) and to

217 13.1%; *P*<0.01), because the three extra serotypes not included in PCV7 (1, 5 and
218 7F) were detected in a low frequency. PCV13 showed a higher coverage in the
219 first period (54.3%); however, this coverage decreased in the periods 2005-08
220 (39.0%) and 2009-12 (27.7%), *P*=0.006 and *P*<0.005, respectively.
221 On the other hand, serotypes not included in PCV13 increased dramatically
222 throughout the study period (28.3% in 2001-04, 41.7% in 2005-08 and 50.9% in
223 2009-12). The increase in serotypes 15A and 6C was particularly marked,
224 especially in the final period (from 2.2% to 9.2%, and from 0.0% to 5.3%, *P*=0.011
225 and *P*=0.004, respectively). Finally, serotypes included in PPSV23 showed a
226 statistically significant decline from 71.8% in 2001-04 to 58.3% in 2005-08
227 (*P*<0.001) and to 49.0% in 2009-12 (*P*<0.001).
228 **Pneumococcal genotypes.**
229 A total of 84 PFGE clusters were identified during the 2009-12 period, of which
230 nineteen comprised 3 or more isolates. Among the 84 of 206 isolates
231 characterized by MLST, a total of 43 sequence types (STs) were found. The most
232 frequent genotypes (defined as clonal complexes, CC) were: $CC63^{15A,19F,15F}$ (n=18,
233 8.7%), $CC180^{3}$ (n=9, 4.4%), $CC62^{11A}$ (n=8, 3.9%), $CC97^{10A}$ (n=8, 3.9%), $CC260^{3}$
234 (n=7, 3.4%), $CC30^{16F}$ (n=7, 3.4%), $CC386^{6C}$ (n=7, 3.4%), $CC156^{9V,14,11A}$ (n=6,
235 2.9%), $CC320^{19A}$ (n=6, 2.9%), $CC338^{23B}$ (n=5, 2.4%), $CC42^{23A}$ (n=5, 2.4%) and
236 $CC717^{33F}$ (n=5, 2.4%).
237 Table 2 shows the distribution of the 32 genotypes which had 3 ore more isolates
238 by periods of time, and their related serotypes. Three genotypes decreased
239 throughout the study period: $CC156^{9V,14,11A}$ (from 12.3% to 2.9% in the 2009-12
240 period, *P*<0.001), $CC81^{23F,19A,19F}$ (from 8.7% in 2001-04 period to 0.5% in the last
241 period, *P*<0.001) and $CC110^{18C}$ (from 2.9% to 0.0%, *P*=0.025). On the other hand,
242 genotypes $CC63^{15A,19F,15F}$ and $CC386^{6C}$ increased in the final period (from 3.2% to
243 8.7%, *P*=0.022; and from 0.0% to 3.4%, *P*=0.006).

244 **Antibiotic susceptibility.** Table 3 shows the *in vitro* activity of 9 antimicrobials
245 against pneumococci isolated during the 3 study periods. A statistically significant
246 increase of susceptibility to cefotaxime/ceftriaxone was observed throughout the
247 study period (from 79.6% in 2001-04 period, to 90.8% in 2005-08 period, and
248 93.2% in 2009-12 period). In contrast, susceptibility to chloramphenicol and co-
249 trimoxazole decreased throughout the study period. On the other hand, a stepwise
250 increase in fluoroquinolone resistance was observed, but these differences were
251 not statistically significant (P>0.05). In this respect, non-susceptibility to
252 erythromycin, clindamycin and tetracycline decreased during the 2005-08 period,
253 coinciding with the introduction of PCV7; however, a significant increase in
254 erythromycin resistance was detected in the 2009-12 period, associated with an
255 increase in pneumococci resistant to erythromycin and susceptible to clindamycin
256 (M phenotype).

257 Overall, 226 (40.2%) isolates were susceptible to all the antimicrobials tested, 93
258 (16.6%) were resistant to one antimicrobial class and 71 (12.6%) isolates were
259 resistant to two antimicrobial classes. Lastly, 172 pneumococci (30.6%) were
260 multi-drug resistant, defined as non-susceptible to at least three antimicrobial
261 classes (table 4). Comparing the frequency of multi-drug resistant pneumococci by
262 periods, a statistically significant decrease was observed from 2001-04 to 2005-08
263 (36.5% to 24.3%; P=0.017); However multi-drug resistance increased in the final
264 period, reaching 33.5% (P=0.042). For this reason, differences on multi-drug
265 resistance observed between the first and last periods were not statistically
266 significant (P= 0.644). Multi-resistance to $\geq$5 antimicrobials decreased drastically
267 throughout the study period, from 22.6% in the 2001-04 period to 9.2% in the
268 2009-12 period (P<0.001). However, multi-drug resistance to 3 and 4
269 antimicrobials showed a stepwise increase throughout the study period, but these
270 differences were not statistically significant (P=0.359 and P=0.057, respectively).

271 These changes were associated with the dynamics of the genotypes, especially
272 those genotypes expressing PCV7 serotypes. Thus, four genotypes
273 (CC81^{23F,19A,19F}, CC156^{9V,14,11A}, CC90^{6B} and CC88^{19F}) were the main contributors
274 to multi-resistance in the 2001-04 period (n= 23 of 50 multi-drug resistant
275 pneumococci). Whereas, in the final period a replacement by multi-drug resistant
276 serotypes 15A, 19A, 6C, and 23A was observed, especially associated with
277 genotypes CC63^{15A,19F,15F}, CC386^{6C}, CC320^{19A}, CC42^{23A}, respectively (n= 35/69).

279 **Discussion**

280 Since the implementation of PCV7, an important decrease in IPD caused by PCV7
281 serotypes has been observed not only in children, but also in adults.[7-9,17-18]
282 However, few data are available about trends in pneumococcal serotypes causing
283 non-invasive disease in adult patients in the conjugate vaccine era.[19] The present
284 study describes changes in the antimicrobial susceptibility rates and serotypes
285 and genotypes of pneumococci causing acute exacerbations in COPD patients,
286 over the twelve year period which has elapsed since the implementation in Spain
287 of PCV7.

288 PPSV23 was introduced in Spain in 1983, and had an uptake up to 80% of COPD
289 patients. During the study period, the serotype coverage of PPSV23 decreased
290 over time (from 71.7% to 49.5%); however, this decrease is consequence of the
291 PCV7 herd immunity, since all PCV7 serotypes are also covered by PPSV23. In
292 fact, the remaining serotypes included in PPSV23 remained nearly stable during
293 all three periods, or even increased, such as serotype 17F (*P*<0.001). In addition,
294 41 of 75 patients vaccinated with PPSV23 were infected by serotypes included in
295 this vaccine (data not shown). These facts supports the idea that their
296 effectiveness avoiding pneumococcal infections in COPD patients in
297 controversial.[20] However, beyond this hypothetical coverage, it has been
298 suggested a possible protection of this vaccine against the morbidity of these
299 patients, and for this reason vaccination with PPSV23 is still recommended.[5] In
300 our series, PPSV23 vaccination data was only available from 2010 to 2012, and
301 the vaccination rate was lower (n=75 of 149 patients, 50.3%) than other studies
302 performed in our area (http://butlleti.camfic.cat/pdf/butlleti21_5.pdf).

303 On the other hand, PCV7 was introduced in Spain for children in 2001, reaching a
304 higher uptake in 2006-07 (62.5%).[10] As a consequence of PCV7 herd immunity, a
305 dramatic decrease of serotypes 14, 9V, 6B and 18C was observed, causing acute

exacerbations of COPD. In parallel, genotypes expressing these serotypes: CC156 (expressing serotypes 14 and 9V) CC110 (serotype 18C) and CC90 (serotype 6B), also drastically decreased. In contrast, although serotypes 19F and 23F showed a progressive decrease, both serotypes caused an 8.7% of overall episodes during 2009-12 period. Serotype 19F was associated with a high genetic diversity: while during 2001-04 period it was mainly related to genotypes Spain^{23F}-ST81 and CC88^{19F}, a drastic decrease of Spain^{23F}-ST81 (also associated with the decrease of serotype 23F) was observed, in parallel to an increase of CC177^{19F} clone in the last period. In our area, the maintenance of serotype 19F has been reported among pneumococcal carriage in children, as it has in Portugal.[21,22]

Shortly after PCV7 introduction, an increase of non-PCV7 serotypes was observed worldwide.[7,8,17] It is remarkable the emergence of the multi-drug resistant serotype 19A reported worldwide (mainly associated with CC276 in Europe and CC320 worlwide), as cause of invasive and non-invasive infections among children and adults.[23-25] Surprisingly, in our study the frequency of serotype 19A remained stable during the 2005-08 period, and decreased during the last period. This serotype was mainly associated with CC81^{23F,19A,19F} in 2001-04 period; however, a remarkable increase of multidrug-resistant CC320^{19A} clone was observed during the 2009-12 period, ranking first among 19A serotype isolates. Nevertheless, serotype 19A is included in PCV13, which was introduced in Spain in June 2010 for children and in 2012 for adults.

Although it is too early to evaluate the impact of new conjugate vaccines introduced for children in Spain (PCV10 in 2009 and PCV13 in 2010) a non-significant decrease in PCV13 serotypes not included in PCV7 (1, 3, 5, 6A, 7F and 19A) was observed in the 2009-12 period. Regarding PCV10, its coverage during the study period was similar than PCV7, because the three extra serotypes included in PCV10 (1, 7F and 5) are strongly associated with pneumococcal

333 invasive disease[26], and were detected in a low frequency causing acute
334 exacerbations.
335 In agreement with our results, a recent multi-centric study performed in United
336 States (where PCV13 was licensed in March 2010 as part of routine childhood
337 immunization) found a slight decrease in the relative number of serotype 19A
338 causing invasive and non-invasive disease, since PCV13 introduction (2010-11
339 period).[27] Whereas, authors found a fairly constant prevalence of serotype 3, with
340 no evidence of change after PCV13 introduction, as cause of invasive and non-
341 invasive disease.[27] In our series, serotype 3 decreased 1.4% in the last period.
342 However, when this period was divided in 2009-10 and 2011-12 periods an
343 identical frequency of serotype 3 was observed (n=10/126, 7.9% *vs.* n=6/80, 7.5%;
344 respectively). Hence, trends of this serotype in the 2013-2014 respiratory season
345 would we needed to asses the effectiveness of PCV13 reducing pneumococcal
346 infections caused by serotype, including acute exacerbations of COPD where
347 serotype 3 is the leading cause.
348 Overall PCV13 coverage of pneumococcal acute exacerbations during the 2009-
349 12 period was low (27.2%). However, COPD is the main underlying disease
350 associated with pneumococcal invasive diseases; vaccination of these patients
351 could improve their quality of life, avoiding several pneumococcal invasive and
352 non-invasive diseases. In this scenario, a study performed in Spain analysing the
353 coverage of PCV13 for adults over 65, found a good coverage of this vaccine
354 (59.3% for adults over 65 years, or 64.7% over 50).[28]
355 The introduction of PCV7 has also had an impact on rates of antimicrobial-
356 resistant pneumococci causing acute exacerbations. Thus, we observed a
357 significant increase in the proportion of isolates with antimicrobial susceptibility to
358 betalactams during 2005-08 period, in spite of the gradual increase in betalactam
359 prescriptions in Catalonia, from 6.56 daily doses per 1000 inhabitants and day

360 (DID) in 2001 to 8.87 DID in 2008.[29,30] During the last period, penicillin
361 susceptibility slightly decreased, while betalactam prescriptions remained stable
362 (8.52 DID in 2011).[29,30]
363 In contrast, a significant increase in macrolide resistance was observed in the
364 2009-2012 period, in spite of macrolide consumption in our geographical area
365 decreased from 1.84 DID in 2009 to 1.68 DID in 2011.[29,30] The increase in co-
366 resistance to penicillin and macrolides in the last period, could be explained in part
367 by the expansion of the multi-drug resistant CC63^{15A,19F,15F} and the emergence of
368 new multi-drug resistant genotypes CC320^{19A} and CC386^{6C}, which are with
369 resistance to both antimicrobial classes.
370 Although the present study was carried out in a single hospital, we described a
371 change in pneumococcal serotype and genotype trends, as well as in multi-drug
372 resistance trends among pneumococci causing acute exacerbations in the era of
373 conjugate vaccines. In parallel to a drastic decrease in PCV7 serotypes and their
374 related genotypes, two multi-drug resistant clones, CC63^{15A,19F,15F} and CC386^{6C},
375 increased in the final period studied.
376 Further surveillance studies are needed to assess the impact of PCV13 and future
377 conjugate vaccines on the prevention of pneumococcal diseases in adult patients.
378

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392

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Table 1. Frequency of pneumococcal serotypes isolated from acute exacerbations throughout the 2001-2012 perio d[a].

serotypes	No. of isolates (n=562)	periods of time (%)			2001-04 vs. 2005-08 periods		2005-08 *vs.* 2009-12 periods		2001-04 *vs.* 2009-12 periods	
		2001-04 (n=138)	2005-08 (n=218	2009-12 (n=206)	change between periods, %	P value	change between periods, %	P value	change between periods, %	P value
PCV7 serotypes	114 (20.3)	39.1	17.0	11.2	**-22.1**	**<0.00**	-5.8	0.010	**-27.9**	**<0.00**
19F*	34 (6.1)	10.9	2.8	6.3	**-8.2**	[1]**0.002**	3.6	0.100	-4.6	[1]0.159
23F*	25 (4.5)	6.6	5,0	2.4	-1.6	0.544	-2.6	0.204	-4.2	0.092
14*	21 (3.7)	6.6	4.6	1.0	-2.0	0.419	**-3.6**	**0.037**	**-5.6**	**0.009**
9V*	18 (3.2)	8,0	2.3	1.0	**-5.7**	**0.011**	-1.3	0.451	**-7,0**	**0.001**
6B*	6 (1.1)	3.6	0.5	0.0	**-3.2**	**0.034**	-0.5	1.000	**-3.6**	**0.010**
18C*	6 (1.1)	2.9	0.9	0.0	-2.0	0.689	-0.9	1.000	**-2.9**	**0.025**
4*	4 (0.7)	0.7	0.9	0.5	0.2	1.000	-0.4	1.000	-0.2	1.000
PCV10 serotypes	127 (22.6)	39,9	20,6	13,1	**-19.3**	**<0.00**	-7.5	0.052	**-26.8**	**<0.00**
7F*	11 (2.0)	0.7	3.2	1.5	2.5	[1]0.159	-1.8	0.340	0.8	[1]0.652
1*	1 (0.2)	0.0	0,0	0.5	0.0	1.000	0.5	0.486	0.5	1.000
5*	1 (0.2)	0.0	0.5	0.0	0.5	1.000	-0.5	1.000	0,0	1.000
PCV13 serotypes	217 (38.6)	54,3	39,0	27,7	**-15.3**	**0.006**	**-11.3**	**0.018**	**-26.6**	**<0.001**
3*	46 (8.2)	7.3	9.2	7.8	1.9	0.696	-1.4	0.728	0.5	1.000
19A*	33 (5.9)	6.6	6.4	4.9	-0.1	0.956	-1.6	0.533	-1.7	0.631
6A	11 (2.0)	0.7	2.8	1.9	2.0	0.256	-0.8	0.752	1.2	0.652
non-PCV13 serotypes	345 (61.6)	39,1	44,5	56,8	5.4	0.324	**12.3**	**0.012**	**17.7**	**0.001**
11A*	34 (6.1)	7.3	5.5	5.8	-1.8	0.505	0.3	1.000	-1.5	0.656
15A	28 (5.1)	2.2	2.8	9.2	0.6	1.000	**6.5**	**0.006**	**7,0**	**0.011**
23A	22 (3.9)	2.9	4.1	4.4	1.2	0.555	0.2	1.000	1.5	0.574
16F	22 (3.9)	5.1	3.7	3.4	-1.4	0.512	-0.3	1.000	-1.7	0.579
6C	20 (3.6)	0.0	4.1	5.3	**4.1**	**0.014**	1.2	0.649	**5.3**	**0.004**
23B	19 (3.4)	3.6	2.3	4.4	-1.4	0.518	2.1	0.283	0.8	0.789
31	18 (3.2)	2.9	4.1	2.4	1.2	0.555	-1.7	0.419	-0.5	1.000
35B	18 (3.2)	2.2	3.7	3.4	1.5	0.540	-0.3	1.000	1.2	0.746
22F*	18 (3.2)	2.2	4.1	2.9	1.9	0.383	-1.2	0.603	0.7	0.745
10A*	17 (3.0)	1.5	3.2	3.9	1.8	0.491	0.7	0.796	2.4	0.326
33F*	17 (3.0)	4.4	2.3	2.9	-2.1	0.348	0.6	0.766	-1.5	0.554
35F	12 (2.1)	1.5	3.2	1.5	1.8	0.491	-1.8	0.340	0,0	1.000
21	10 (1.8)	1.5	1.8	1.9	0.4	1.000	0.1	1.000	0.4	1.000
17F*	9 (1.6)	0,0	1.4	2.9	1.4	0.287	1.5	0.327	**2.9**	**0.085**
24F	9 (1.6)	0.7	1.4	2.4	0.6	1.000	1.1	0.493	1.7	0.408
9N*	7 (1.2)	0.7	1.4	1.5	0.6	1.000	0.1	1.000	0.8	0.652

Table 1 (CONT)

12F*	7 (1.2)	0.7	1.8	1.0	1.1	0.653	-0.9	0.686	0.3	1.000
15B*	7 (1.2)	0.7	0.9	1.9	0.2	1.000	1.0	0.438	1.2	0.652
34	6 (1.1)	0,0	1.8	1.0	1.8	0.303	-0.9	0.686	1,0	1.000
38	5 (0.9)	0,0	2.3	0.0	2.3	0.161	-2.3	0.062	0,0	1.000
20*	5 (0.9)	0.7	0.9	1.0	0.2	1.000	0.1	1.000	0.3	1.000
non-typeable	4 (0.7)	0	0	1.9	0.0		1.9	1.000	1.9	0.518
15C	2 (0.4)	0	0	1.0	0.0	1.000	1.0	0.236	1.0	1.000
37	2 (0.4)	0	0.5	0.5	0.5	1.000	0.0	1.000	0.5	1.000
15F	1 (0.2)	0.7	0	0	-0.7	0.388	0.0	1.000	-0.7	0.401
27	1 (0.2)	0.7	0	0	-0.7	0.388	0.0	1.000	-0.7	0.401
42	1 (0.2)	0.7	0	0	-0.7	0.388	0.0	1.000	-0.7	0.401
10F	1 (0.2)	0	0	0.5	0	1.000	0.5	1.000	0.5	1.000
13F	1 (0.2)	0	0	0.5	0	1.000	0.5	1.000	0.5	1.000
35F	1 (0.2)	0	0	0.5	0	1.000	0.5	1.000	0.5	1.000
8*	1 (0.2)	0	0.5	0	0.5	1.000	-0.5	1.000	0.0	1.000
*PPSV23 serotypes	328 (58.4)	71.7	58.3	49.5	**-13.4**	**0.012**	**-8.8**	0.080	**22.2**	**<0.001**

[a]Seventeen isolates were not available for serotype analysis (3 pneumococci from 2001-04, 7 from 2005-08 and 9 from 2009-12).

Table 2. Genotypes detected in 3 or more pneumococci isolated from acute exacerbations during 2001-2012 period.

Clonal Complex, CC	STs found	No. of isolates (%)	serotypes related (No. of isolates)	2001-04 (n=138)	2005-08 (n=218)	2009-12 (n=206)	p-value of periods 1 *vs.* 2	p-value of periods 2 *vs.* 3	p-value of periods 1 *vs.* 3
CC156	ST156, ST838, ST6521	34 (6.0)	9V (16), 14 (15), 11A (3)	17 (12.3)	11 (5.0)	6 (2.9)	**0.013**	0.323	**<0.001**
CC63	ST63, ST2313, ST2100	31 (5.5)	15A (26), 19F (4), 15F (1)	6 (4.3)	7 (3.2)	18 (8.7)	0.573	**0.022**	0.135
CC62	ST62, ST4305	27 (4.8)	11A (27)	9 (6.5)	10 (4.6)	8 (3.9)	0.473	0.812	0.314
CC180	ST180, ST2314	25 (4.4)	3 (25)	7 (5.1)	9 (4.1)	9 (4.4)	0.794	1.000	0.798
CC81	ST81	21 (3.7)	23F (12), 19A (5), 19F (4)	12 (8.7)	8 (3.7)	1 (0.5)	0.058	**0.038**	**<0.001**
CC30	ST30, ST570	21 (3.7)	16F (21)	6 (4.3)	8 (3.7)	7 (3.4)	0.784	1.000	0.775
CC42	ST42, ST7916	20 (3.6)	23A (17), 14 (1), 19A (1), 23F (1)	4 (2.9)	11 (5.0)	5 (2.4)	0.422	0.204	1.000
CC260	ST260, ST1220	16 (2.8)	3 (16)	2 (1.4)	7 (3.2)	7 (3.4)	0.491	1.000	0.324
CC97	ST97	15 (2.7)	10A (15)	2 (1.4)	5 (2.3)	8 (3.9)	0.711	0.406	0.326
CC88	ST88, ST87	11 (2.0)	19F (11)	5 (3.6)	3 (1.4)	3 (1.5)	0.270	1.000	0.275
CC558	ST558	10 (1.8)	35B (7), 35F (3)	2 (1.4)	4 (1.8)	4 (1.9)	1.000	1.000	1.000
CC433	ST433	10 (1.8)	22F (10)	1 (0.7)	7 (3.2)	2 (1.0)	0.158	0.177	1.000
CC3584	ST1766, ST7800	10 (1.8)	31 (10)	1 (0.7)	5 (2.3)	4 (1.9)	0.411	1.000	0.652
CC338	ST338	10 (1.8)	23B (8), 23F (1)	2 (1.4)	2 (0.9)	5 (2.4)	0.643	0.273	0.706
CC191	ST191	9 (1.6)	7F (9)	1 (0.7)	5 (2.3)	3 (1.5)	0.412	0.725	0.652
CC717	ST717, ST1012	9 (1.6)	33F (8)	3 (2.2)	2 (0.9)	3 (1.5)	0.380	0.273	1.000
CC432	ST432	8 (1.4)	21 (8)	2 (1.4)	4 (1.8)	2 (1.0)	1.000	0.686	1.000
CC989	ST989	7 (1.2)	12F (7)	1 (0.7)	4 (1.8)	2 (1.0)	0.653	0.686	1.000
CC230	ST230	7 (1.2)	24F (4), 15A (1), 19A (1), 7F (1)	0 (0.0)	4 (1.8)	3 (1.5)	0.161	1.000	0.277
CC320	ST320, ST2383	3 (0.5)	19A (7)	0 (0.0)	1 (0.5)	6 (2.9)	1.000	0.061	0.085
CC386	ST386, ST4825	7 (1.2)	6C (6), 6A (1)	0 (0.0)	0 (0.0)	7 (3.4)	1.000	**0.006**	**0.045**
CC110	ST110	6 (1.1)	18C (6)	4 (2.9)	2 (0.9)	0 (0.0)	0.212	0.500	**0.025**
CC1201	ST1201	6 (1.1)	19A (6)	1 (0.7)	3 (1.4)	2 (1.0)	1.000	1.000	1.000
CC67	ST67	5 (0.9)	9N/L (4), 14 (1)	2 (1.4)	1 (0.5)	2 (1.0)	0.562	0.614	1.000
CC177	ST177, ST51	5 (0.9)	19F (5)	1 (0.7)	0 (0.0)	4 (1.9)	1.000	0.055	0.652
CC392	ST392	5 (0.9)	15F (5)	0 (0.0)	1 (0.5)	4 (1.9)	1.000	0,204	0.152
CC90	ST90	4 (0.7)	6B (4)	3 (2,2)	1 (0,5)	0 (0,0)	0.303	1.000	0.064
CC6988	ST6988	3 (0.5)	23B (3)	2 (1,4)	0 (0,0)	1 (0,5)	0,150	1.000	0.567
CC247	ST247	3 (0.5)	4 (3)	1 (0,7)	1 (0,5)	1 (0,5)	1.000	1.000	1.000
CC5135	ST5135	3 (0.5)	35B (3)	1 (0,7)	1 (0,5)	1 (0,5)	1.000	1.000	1.000
CC224	ST224	3 (0.5)	23B (3)	0 (0,0)	3 (1,4)	0 (0,0)	0,286	0.249	1.000
CC439	ST439, ST2372	3 (0.5)	23B (3)	0 (0,0)	1 (0,5)	2 (1,0)	1.000	0.614	0.518

Table 3. Comparison of antimicrobial susceptibility of pneumococci isolated in three periods from acute exacerbations in COPD patients.

	2001-04 period (n=138)				2005-08 period (n=218)					2009-12 period (n=206)					
	MIC_{50} mg/L	MIC_{90} mg/L	S, %	R, %	MIC_{50} mg/L	MIC_{90} mg/L	S, %	R, %	P^b	MIC_{50} mg/L	MIC_{90} mg/L	S, %	R, %	P^c	P^d
Penicillin (non-meningitis)	≤0.03	2	54.7	8.0	≤0.03	1	72.5	0.5	**<0.001**	≤0.03	1	65.0	1.5	0.116	0.070
Cefotaxime / Ceftriaxone	≤0.03	1	79.6	0.0	≤0.03	0.5	90.8	0.0	**0.004**	≤0.03	0.5	93.2	1.5	0.379	**<0.001**
Ciprofloxacin	≤0.5	2	-[a]	5.1	≤0.5	2	-[a]	7.8	0.390	≤0.5	1	-[a]	8.3	1.000	0.289
Levofloxacin	≤0.5	<1	96.4	3.6	≤0.5	1	95.9	4.1	1.000	≤0.5	1	92.2	7.8	0.148	0.167
Tetracycline	≤2	>4	63.5	36.5	≤2	>4	75.7	24.3	**0.016**	≤2	>4	67.5	32.5	0.067	0.486
Erythromycin	≤0.25	>32	65.0	35.0	≤0.25	>32	73.4	26.6	0.097	≤0.25	>32	60.7	39.3	**0.007**	0.429
Clindamycin	≤0.25	>32	65.7	34.3	≤0.25	>32	76.1	23.9	**0.038**	≤0.25	>32	69.4	30.6	0.127	0.481
Chloramphenicol	≤2	8	81.2	18.3	≤2	8	91.7	8.3	**0.007**	≤2	4	94.2	5.8	0.350	**<0.001**
Co-trimoxazole	≤0.5/9.5	>4/76	62.0	38.0	≤0.5/9.5	>4/76	69.6	28.5	0.164	≤0.5/9.5	>4/76	76.7	21.4	0.123	**0.005**

S, antimicrobial susceptibility; R, drug resistance. [a]Norfloxacin disk was used as screening of resistance to fluoroquinolones. Those isolates with a ciprofloxacin MIC< 2mg/L were categorised as intermediate. bP-value comparing the 2001-04 period with the 2005-08 period. [c]P-value comparing 2005-08 period with the 2009-12 period. [d]P-value comparing the 2001-04 period with the 2009-12 period.

Table 4. Frequency of multi-drug resistant pneumococci isolated from acute exacerbations, by period of time.[a]

	2001-04 (36.2%, 50/138)	2005-08 (24.3%, 53/218)	2009-12 (33.5%, 69/206)	serotypes (No. of isolates)	ST (No. of isolates)[b]
Resistan e to 3 antimicrobial classes (n=32)	6 (4.3)	11 (5.0)	15 (7.3)		
PEN, ERI ,TET (n=2)	0 (0.0)	0 (0.0)	2 (1.0)	15A (1), NT (1)	CC63, unrelated
PEN, ERI, SXT (n=1)	0 (0.0)	1 (0.5)	0 (0.0)	14 (1)	unrelated
PEN, TET, SXT (n=2)	0 (0.0)	2 (0.9)	0 (0.0)	19A (2)	CC81, unrelated
PEN, SXT, CIP (n=1)	1 (0.7)	0 (0.0)	0 (0.0)	9V (1)	CC156
ERY, CLI, TET (n=16)	2 (1.4)	4 (1.8)	10 (4.9)	6A (3), 33F (2), 15B (2), 19F (2), 15A (1), 23A (1), 19A (1), 6C (1), 14 (1), 15C (1), 1 (1)	CC386, CC63, CC717, CC42, CC177, unrelated
ERY, CLI, CIP (n=4)	2 (1.4)	1 (0.5)	1 (0.5)	33F (2), 19A (1), 16F (1)	CC717, CC42, CC30
ERY, CLI, SXT (n=2)	0 (0.0)	2 (0.9)	0 (0.0)	16F (2)	CC30
ERI, TET, SXT (n=1)	0 (0.0)	0 (0.0)	1 (0.5)	23F (1)	CC242
TET, CHL, SXT (n=3)	1 (0.7)	1 (0.5)	1 (0.5)	23A (1), 12F (1), 3 (1)	CC42, CC989, CC180/2314
Resistance to 4 antimicrobial classes (n=65)	13 (9.5)	17 (7.8)	35 (17.0)		
PEN, ERI, CLI, TET (n= 40)	7 (5.1)	8 (3.7)	25 (12.1)	15A (19), 24F (5), 6C (4), 19A (4), 6B (2), 11A (1), 6A (1), 15F (1), 19F (1), NT (1)	CC63, CC386, CC230, CC1201, CC320, CC42, unrelated
PEN, ERI, CLI, CIP (n= 1)	0 (0.0)	0 (0.0)	1 (0.5)	19F (1)	CC63/2100/2313
PEN, ERI, CLI, SXT (n= 3)	1 (0.7)	1 (0.5)	1 (0.5)	14 (1), 6A (1), NT (1)	CC67, unrelated
PEN, ERI, TET, SXT (n= 3)	0 (0.0)	0 (0.0)	3 (1.5)	19A (3)	CC320
PEN, TET, CHL, SXT (n= 1)	0 (0.0)	1 (0.5)	0 (0.0)	23F (1)	CC81
ERI, CLI, TET, CHL (n= 9)	4 (2.8%)	4 (1.8)	1 (0.5)	19A (4), 16F (3), 23A (2)	CC1201, CC30, CC42, CC202, unrelated
ERI, CLI, TET, SXT (n= 5)	1 (0.7)	3 (1.4)	1 (0.5)	11A (1), 14 (1), 24F (1), 6A (1), 19A (1)	CC62, CC230, CC320, CC327, CC42
ERI, CLI, TET, CIP (n= 3)	0 (0.0)	0 (0.0)	3 (1.5)	20 (1), 33F (1), NT (1)	CC717, unrelated
Resistance to 5 antimicrobial classes (n=75)	31 (22,6)	25 (11,5)	19 (9,2)		
PEN, ERI, CLI, TET, CIP (n= 7)	1 (0.7)	1 (0.5)	5 (2.4)	15A (4), 19F (1), 33F (1), 31 (1)	CC63, CC717, unrelated
PEN, ERI, CLI, TET, CHL, SXT, CIP (n= 4)	2 (1.4)	2 (0.9)	0 (0.0)	19F (2), 23F (2)	CC81, CC88
PEN, ERI, CLI, TET, CHL, SXT (n= 21)	15 (10.9)	4 (1.8)	2 (1.0)	23F (8), 19A (6), 19F (5), 14 (1), 6B (1)	CC81, CC88, CC156, CC320, CC90, unrelated
PEN, ERI, CLI, TET, SXT (n= 24)	11 (8.0)	9 (4.1)	4 (1.9)	19F (11), 6B (3), 15A (2), 14 (2), 24F (2), 7F (1), 6C (1), 19A (1), 23F (1)	CC88/87, CC90, CC63, CC230, CC156/838, CC320, CC386, CC81, unrelated
PEN, ERI, TET, CHL, SXT (n= 1)	1 (0.7)	0 (0.0)	0 (0.0)	19F (1)	CC81
PEN, ERI, CLI, TET, SXT, CIP (n= 2)	0 (0.0)	1 (0.5)	1 (0.5)	33F (2)	unrelated
ERY, CLI, CHL, SXT, CIP (n= 1)	0 (0.0)	0 (0.0)	1 (0.5)	16F (1)	CC30
ERI, CLI, TET, SXT, CIP (n= 1)	0 (0.0)	1 (0.5)	0 (0.0)	23A (1)	CC42
ERI, CLI, TET, CHL, SXT (n= 13)	1 (0.7)	6 (2.8)	6 (2.9)	23A (6), 16F (4), 19A (1), 23F (1), NT (1)	CC42, CC30, CC202
ERI, CLI, TET, CHL, SXT, CIP (n= 1)	0 (0.0)	1 (0.5)	0 (0.0)	16F (1)	CC30

[a]Defined as non-susceptibility to at least three antimicrobial classes. Cefotaxime/Ceftriaxone was not considered because it belongs to the same antimicrobial class as penicillin (betalactams). For the same reason, only ciprofloxacin was considered as fluroquinolones class. [b]Strains belonging to PFGE clusters with 3 or more isolates were not analysed by MLST and were considered unrelated.

Abbreviations: PEN: penicillin, ERI: erythromycin, CLI: clindamycin, TET: tetracycline, CHL: chloramphenicol, SXT: co-trimoxazole, CIP: ciprofloxacin.

CHAPTER IV: Recurrence and persistence of pneumococcal strains.

Objective 4: To establish the frequency and distribution of pneumococci causing recurrent episodes: relapse *vs.* reinfection episodes (1995-2010).

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Some Pneumococcal Serotypes Are More Frequently Associated with Relapses of Acute Exacerbations in COPD Patients

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Abstract

Objectives: To analyze the role of the capsular type in pneumococci causing relapse and reinfection episodes of acute exacerbation in COPD patients.

Methods: A total of 79 patients with 116 recurrent episodes of acute exacerbations caused by *S. pneumoniae* were included into this study (1995–2010). A relapse episode was considered when two consecutive episodes were caused by the same strain (identical serotype and genotype); otherwise it was considered reinfection. Antimicrobial susceptibility testing (microdilution), serotyping (PCR, Quellung) and molecular typing (PFGE/MLST) were performed.

Results: Among 116 recurrent episodes, 81 (69.8%) were reinfections, caused by the acquisition of a new pneumococcus, and 35 (30.2%) were relapses, caused by a pre-existing strain. Four serotypes (9V, 19F, 15A and 11A) caused the majority (60.0%) of relapses. When serotypes causing relapses and reinfection were compared, only two serotypes were associated with relapses: 9V (OR 8.0; 95% CI, 1.34–85.59) and 19F (OR 16.1; 95% CI, 1.84–767.20). Pneumococci isolated from relapses were more resistant to antimicrobials than those isolated from the reinfection episodes: penicillin (74.3% *vs.* 34.6%, $p<0.001$), ciprofloxacin (25.7% *vs.* 9.9%, $p<0.027$), levofloxacin (22.9% *vs.* 7.4%, $p=0.029$), and co-trimoxazole (54.3% *vs.* 25.9%, $p<0.001$).

Conclusions: Although the acquisition of a new *S. pneumoniae* strain was the most frequent cause of recurrences, a third of the recurrent episodes were caused by a pre-existing strain. These relapse episodes were mainly caused by serotypes 9V and 19F, suggesting an important role for capsular type.

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Introduction

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality in developed countries [1]. Approximately 50% of acute exacerbation episodes of COPD are caused by bacterial pathogens, mainly *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* [2]. The development of an acute exacerbation episode caused by *S. pneumoniae* is thought to be associated with the acquisition of a new strain, although scarce information is available [3].

Capsular type, the principal pneumococcal virulence factor, had been related to the ability of pneumococci to cause invasive disease or colonization [4]. However, the aetiological role of pneumococcal serotypes in relapse episodes of COPD patients remains to be determined.

The aims of this study were to analyse the relationship between serotype and genotype and the ability to cause relapse or reinfection episodes in patients with COPD. In addition, we have explored the influence of previous antimicrobial therapy in this occurrence.

Results

Epidemiological and clinical data

A total of 79 COPD patients were included in the study. Their mean age was 69 (SD ±6) years, and 77 (97.5%) of them were males. In terms of COPD severity there was 1 patient with mild

(1.3%), 14 patients with moderate (17.7%), 18 patients with severe (22.8%) and 39 patients with very severe (49.4%) airflow obstruction. The GOLD status of the remaining 7 patients was not available.

Fifty-two patients had ≥1 reinfections (caused by pneumococci with different serotype and genotype), 16 had ≥1 relapses (caused by the same pneumococcus), and the remaining 11 patients had both relapses and reinfections. No differences were observed as regards the age of patients with relapse or reinfection (69.9±9 *vs.* 70.5±9, respectively), nor in terms of COPD severity (GOLD II: 71.7±13 *vs.* 69.2±8, respectively, GOLD III: 66.4±7 *vs.* 70.6±11, respectively; and GOLD IV: 69.1±9 *vs.* 69.4±8, respectively).

Finally, a total of 116 recurrent episodes from the 79 patients were studied; of these, 35 (30.2%) were relapses and 81 (69.8%) reinfections. The mean time between episodes was 166±96 days, being shorter among relapses (133±89 days) than among reinfections (181±96 days; $P = 0.020$). Table 1 shows the distribution of the episodes based on the mean time between episodes. When the consecutive episodes occurred during a period ≤120 days, significantly higher frequency of relapse episodes was observed (57.1% *vs.* 35.8%, $P = 0.033$). Whereas, when the period of time between episodes was higher than 240 days the frequency of reinfection episodes was higher (14.3% *vs.* 30.9%, $P = 0.061$).

In 13 of the 35 (37.1%) relapses and in 31 of the 81 (38.3%) reinfections, pneumococcal strains were isolated together with other potential pathogens.

However, *P. aeruginosa* was more frequently isolated from relapses than from reinfections (17.1% *vs.* 7.4%, respectively; $P = 0.108$), whereas *H. influenzae* was more closely associated with reinfections (2.9% *vs.* 23.5%; $P = 0.006$).

Serotyping and genotyping

Four of the 31 serotypes detected caused 60.0% of relapses. These serotypes were 9V (17.1%), 19F (17.1%), 15A (14.3%) and 11A (11.4%). The most frequent serotypes isolated from reinfections were 15A (8.6%), 16F (7.4%), 3 (6.2%) and 33F (6.2%). Statistically significant differences were only observed in two serotypes associated with relapses when compared with reinfections: 9V (OR 8.0; 95% CI, 1.34–85.59) and 19F (OR 16.1; 95% CI, 1.84–767.20) [Table 2].

Serotypes included in the polysaccharide pneumococcal 23-valent (23vPPV) accounted for 74.3% (n = 26) of relapses, and 56.8% (n = 46) of reinfections. Whereas, the coverage of the 10-valent (PCV-10) and 13-valent (PCV-13) pneumococcal conjugate vaccines in all relapses were 40.0% (n = 16) and 54.3% (n = 19), respectively; and the coverage of reinfections were 23.5% (n = 19) and 34.6% (n = 28), respectively.

Seventeen PFGE patterns (related with 14 sequence types) were observed among the relapses, with the most frequent clonal complexes (CC) being $CC156^{9V}$ (22.9%), $CC63^{15A,19F}$ (17.1%), $CC88^{19F}$ (11.4%), $CC81^{19A,19F}$ (8.6%) and $CC260^{3}$ (5.7%). Among reinfections, 56 different PFGE patterns were observed, and the most frequent clones were $CC63^{15A}$ (7.4%), $CC30^{16F}$ (7.4%), $CC717^{33F}$ (4.9%), $CC156^{9V}$ (4.9%), $CC42^{23A,23F}$ (3.7%), $CC62^{11A}$ (3.7%), $CC97^{10A}$ (3.7%), $CC191^{7F}$ (3.7%) and $CC260^{3}$ (3.7%).

Only the $CC156^{9V}$ genotype was associated with relapses (OR 5.8; 95% CI, 1.61–20.73). Serotype 19F was genetically heterogeneous [$CC88^{19F}$ (6.2%), $CC81^{19F}$ (2.9%), and $CC63^{19F}$ (2.9%)].

Antimicrobial consumption and susceptibility

Table 3 shows the activity of nine antimicrobials against pneumococci isolated from relapses and reinfections. Resistance to betalactams, fluoroquinolones and co-trimoxazole was higher among the strains that caused relapses than among those causing reinfections (P<0.01).

Table 2. Serotypes causing relapse and reinfection episodes of acute exacerbations in COPD patients.

serotype	relapses (n = 35)	reinfections (n = 81)	OR	95%CI
9V	6 (17,1%)	2 (2.5%)	**8.00**	1.34–85.59
19F	6 (17,1%)	1 (1.2%)	**16.11**	1.84–767.20
15A	5 (14,3)%	7 (8.6%)	1.75	0.41–7.01
11A	4 (11.4%)	3 (3.7%)	3.32	0.53–23.95
3	3 (8.6%)	5 (6.2%)	1.42	0.21–7.82
6C	2 (5.7%)	0	infinite	0.44–infinite
22F	2 (5.7%)	3 (3.7%)	1.57	0.13–14.36
19A	2 (5.7%)	4 (4.9%)	1.17	0.10–8.59
35B	1 (2.9%)	1 (1.2%)	2.33	0.03–186.68
33F	1 (2.9%)	5 (6.2%)	0.45	0.01–4.24
23F	1 (2.9%)	2 (2.5%)	1.16	0.02–22.99
23A	1 (2.9%)	2 (2.5%)	1.16	0.02–22.99
14	1 (2.9%)	4 (4.9%)	0.57	0.01–6.03
16F	0	6 (7.4%)	0	0.00–1.93
7F	0	4 (4.9%)	0	0.00–3.51
38	0	4 (4.9%)	0	0.00–3.51
31	0	4 (4.9%)	0	0.00–3.51
Non-typeable	0	4 (4.9%)	0	0.00–3.51
6B	0	3 (3.7%)	0	0.00–5.63
10A	0	3 (3.7%)	0	0.00–5.63
35F	0	2 (2.5%)	0	0.00–12.39
23B	0	2 (2.5%)	0	0.00–12.39
Other serotypes	0	10[a]		

[a]Serotypes 1, 5, 9N/L, 12F, 15B/C, 17F, 18C, 24F, 29 and 34 were detected in only one reinfection episode.

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Table 1. Distribution of the number of relapses and reinfections based on the time between episodes.

Time between episodes (days)	Relapses (n = 35)	Reinfections (n = 81)	P-value
≤120	20 (57.1%)	29 (35.8%)	**0.033**
121–240	10 (28.6%)	27 (33.3%)	0.613
>240	5 (14.3%)	25 (30.9%)	0.061

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Table 3. *In vitro* activity of nine antimicrobials against pneumococci isolated from relapses and reinfections

	Relapses (n = 35)						Reinfections (n = 81)						P-value[f]
Antibiotic	MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC range (mg/L)	% S	% I	% R	MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC range (mg/L)	% S	% I	% R	
Penicillin	0.5	4	≤0.03–4	25.7[a]	45.7	28.6	0.06	0.5	≤0.03–2	65.4[a]	28.4	6.2	0.000
				82.9[b]	17.1	0				98.8[b]	1.2	0	0.003
Cefotaxime	0.12	1	≤0.03–2	68.6[c]	28.6	2.9	0.05	0.5	≤0.03–1	92.6[c]	7.4	0	0.001
				97.1[d]	2.9	0				100[d]	0	0	0.302
Ciprofloxacin[e]	1	>32	≤0.5–>32	74.3	–	25.7	≤0.5	1	≤0.5–>32	90.1	–	9.9	0.027
Levofloxacin	≤0.5	>32	≤0.5–>32	77.1	2.9	20.0	≤0.5	1	≤0.5–>32	92.6	0	7.4	0.029
Tetracycline	≤2	>32	≤2–>32	54.3	2.9	42.8	≤2	>32	≤2–>32	70.4	0	29.6	0.205
Erythromycin	≤0.25	>32	≤0.25–>32	57.1	0	42.9	≤0.25	>32	≤0.25–>32	67.9	0	32.1	0.266
Clindamycin	≤0.25	>32	≤0.25–>32	57.1	0	42.9	≤0.25	>32	≤0.25–>32	65.4	0	34.6	0.396
Chloramphenicol	≤2	>8	≤2–>8	88.6	–	11.4	≤2	8	≤2–>8	93.8	–	6.2	0.081
Co-trimoxazole	2/38	>4/76	≤0.5/9.5–>4/76	45.7	5.7	48.6	≤0.5/9.5	>4/76	≤0.5/9.5–>4/76	74.1	1.2	24.7	0.000

Clinical Laboratory Standard Institute (CLSI) breakpoints: [a]Penicillin oral breakpoints: susceptible ≤0.06 mg/L, intermediate 0.12–1 mg/L and resistant ≥2 mg/L. [b]Penicillin parenteral (non-meningitis) breakpoints: susceptible ≤2 mg/L, intermediate 4 mg/L and resistant ≥8 mg/L. [c]Cefotaxime (meningitis) breakpoints: susceptible ≤0.5 mg/L, intermediate 1 mg/L and resistant ≥2 mg/L. [d]Cefotaxime parenteral (non-meningitis) breakpoints: susceptible ≤1 mg/L, intermediate 2 mg/L and resistant ≥4 mg/L. [e]Non-susceptibility to ciprofloxacin breakpoint MIC ≥4 mg/L and susceptibility breakpoint ≤2 mg/L. [f]*P*-value comparing susceptible strains.
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The most frequent antimicrobials consumed by these patients during the episode prior to reinfection or relapse (n = 116) were beta-lactams (49.2%), fluoroquinolones (25.9%) or both (5.2%). Consumption of fluoroquinolones during the previous episode was higher in relapses than in reinfections (40.0% *vs.* 19.8%, respectively; $P = 0.02$), whereas, no differences in the betalactams consumption was observed (25.7% of relapses and 27.1% of reinfections; $P = 0.872$).

Discussion

Capsular type is known to play an important role in the invasiveness of pneumococcal strains [4]. Thus, some serotypes have been associated with invasive pneumococcal disease or with acute exacerbations of COPD [5]. Although the isolation of a new pneumococcal strain has been associated with a significantly increased risk of a new acute exacerbation [2], little information is available about the persistence of *S. pneumoniae* isolates.

Our results agree with other reports in which reinfection through acquisition of a new strain was the most frequent cause of acute exacerbation episodes [3]. However, our study shows that a third of these recurrences were caused by a persistent pneumococcal strain, suggesting that in COPD patients the persistence of the same strain could be underestimated.

Although in 12 of 35 relapses *S. pneumoniae* was isolated together with another pathogen (*P. aeruginosa*, *H. influenzae*, *M. catarrhalis* or *Staphylococcus aureus*) the role of *S. pneumoniae* in causing the acute exacerbation episodes is supported by the high predominance of Gram positive diplococci in the Gram stain of a good quality sputum sample.

Our results show that serotypes 9V and 19F were associated with relapses, suggesting that serotype could play an important role in the persistence of pneumococcal isolates. In addition, differences in genotype distribution were also detected. All isolates that expressed the serotype 9V belonged to the Spain^{9V}-CC156; hence, this clone was associated with the relapse episodes. In contrast, several genotypes expressed the serotype 19F and none of them was significantly associated with relapses. These results suggest that capsular type, rather than genetic background, may play an important role in the persistence of pneumococci among COPD patients.

Most of the patients included in the study had severe or very severe COPD, suffering frequent episodes of acute exacerbation and they received multiple antibiotic courses [6]. Although there were no differences in the betalactam consumption among groups, the betalactam resistance rates were higher among relapse episodes. This finding could be explained because relapse episodes were caused by few multi-resistant clones (mainly CC156 and CC88). Whereas, strains causing reinfection episodes showed a higher genetic diversity including penicillin-susceptible and -resistant clones. However, we found an association between fluorquinolone consumption and development of resistance. In fact, the development of fluorquinolone resistance during or after an antimicrobial course has been largely described in the literature [7–8].

The proportion of serotypes covered by the 23vPPV vaccine was high, especially those causing relapses. Unfortunately, vaccination data of patients included in the present study was not available; however, its protective efficacy in COPD populations is controversial since COPD adults respond differently than the general adult population, due to their impaired antibody response to the vaccine, the colonization of the lower respiratory tract, or the frequent use of inhaled corticosteroids [9]. In the other hand, conjugate vaccines (PV10 and PCV13) vaccine, which have an enhanced immunity potential, could prevent the 40% and a half of the overall relapse episodes, respectively.

The major limitations of our study are the low number of relapse episodes, and also that it is a retrospective study. Nonetheless, our study provides new data about the association of certain serotypes with the persistence of pneumococci and the ability to some clones, especially Spain^{9V}-CC156, to cause relapse episodes. In addition, our study suggests that new episodes that occurred within the first 3 months after a previous one, had higher probability to be caused by the same pneumococcal strain and this fact could help to give an adequate empirical therapy.

Further studies with a high number of recurrent episodes are now needed to investigate not only the role of capsular type in relapses of acute exacerbations, but also whether the pneumococcal conjugate vaccine 13 could be beneficial for COPD patients.

Methods

Study design

Pneumococci and other potential pathogens isolated from sputum samples were prospectively collected into our laboratory between 1995 and 2010, and were frozen at −80°C for further analysis. Only pneumococci isolated from good quality sputum were considered (<10 squamous cells and >25 leucocytes per low-power field), with a predominance of Gram positive diplococci.

All COPD patients (n = 79) with two or more acute exacerbation episodes and seen at the Bellvitge University Hospital during the study period were included, after retrospective review of their computerized medical charts. Only those consecutive acute exacerbations which lasted for between four weeks and one year were included in the study.

The severity of airflow obstruction was categorized according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria [10].

An acute exacerbation of COPD was defined as any sustained increase in respiratory symptomatology compared with the baseline situation requiring an increase in regular medication and hospital treatment. A 'relapse' episode was defined as two or more consecutive acute exacerbations caused by the same pneumococcus (identical serotype and genotype). When the consecutive episodes were caused by pneumococci with different serotype and Pulsed Field Gel Electrophoresis (PFGE) pattern they were defined as 'reinfection'.

Ethical statement

This study and publication of the results were approved by the 'Comité Ètic d'Investigació Clínica del Hospital Universitari de Bellvitge' and the written or oral informed consent was considered not necessary, because the source of bacterial isolates was anonymized and the study was retrospective.

Serotyping and genotyping

Serotyping was performed by multiplex PCR, using a previously described methodology [11]. All isolates were genotyped by PFGE. Multi Locus Sequence Typing (MLST) was performed on all relapse isolates in order to confirm the identity of the isolates [12–13].

Antimicrobial susceptibility

Antimicrobial susceptibility was tested by microdilution (STRHAE, Sensititre^{TM}), following the Clinical Laboratory Standards Institute (CLSI) criteria [14]. The ciprofloxacin MIC of resistant strains (MIC ≥4 mg/L) was confirmed by E-test. *S. pneumoniae* ATCC49619 was used as the control strain.

Statistical analysis

Statistical analyses were carried out using SPSS 18 for Windows. The odds ratios (OR) and 95% confidence intervals (CI) were calculated, and Fisher's exact test was used when appropriate. Two-sided *P* values <0.05 were considered statistically significant.

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Author Contributions

Conceived and designed the experiments: AD JL RP CA. Performed the experiments: AD. Analyzed the data: AD SS IG RP AC. Contributed reagents/materials/analysis tools: JL. Wrote the paper: AD CA JL.

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CHAPTER IV: Recurrence and persistence of pneumococcal strains.

Objective 5: To investigate the pneumococcal persistence in the respiratory tract of COPD patients, as well as the impact of antimicrobial consumption in the development of antimicrobial resistance (1995-2010).

Domenech A, Ardanuy C, Balsalobre L, Marti S, Calatayud L, De la Campa AG, Brueggemann AB, Liñares J (2012) Pneumococci can persistently colonize adult patients with chronic respiratory disease. J Clin Microbiol 50:4047-53.

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Pneumococci Can Persistently Colonize Adult Patients with Chronic Respiratory Disease

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***Streptococcus pneumoniae* plays an important role in causing acute exacerbations in patients with chronic respiratory disease. However, few data are available regarding pneumococcal persistence in adult patients with chronic respiratory diseases. Fifty pneumococci recovered from sputum samples (1995 to 2010) from 13 adult patients with ≥3 episodes of acute exacerbation or pneumonia, with the same serotype and pulsed-field gel electrophoresis (PFGE) pattern, were studied. Multilocus sequence typing (MLST) loci, penicillin-binding protein (PBP) genes (*pbp2x*, *pbp1a*, *pbp2b*), and the quinolone-resistant determining regions (QRDRs) of *parC*, *parE*, and *gyrA* were PCR amplified and sequenced. The average time between the first and last episode was 582 days (standard deviation [SD], ±362). All but two patients received multiple courses of β-lactam treatment, and all persistent strains were resistant to penicillin; however, the PBP sequences were stable over time apart from one variable nucleotide in *pbp2x*, observed among pneumococci isolated from three patients. In contrast, 7/11 patients treated with fluoroquinolones had fluoroquinolone-resistant pneumococci. In three patients, the initially fluoroquinolone-susceptible strain developed resistance after fluoroquinolone therapy, and in the remaining four patients, the persistent strain was fluoroquinolone resistant from the first episode. QRDR changes involved in fluoroquinolone resistance were frequently observed in persistent strains after fluoroquinolone treatment; however, the PBP sequences and MLST genotypes of these strains were stable over time.**

Patients with chronic respiratory disease, such as chronic obstructive pulmonary disease (COPD) and bronchiectasis, are often persistently colonized by respiratory pathogens (27, 32). Airway colonization, mainly by *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Streptococcus pneumoniae*, contributes to progressive pulmonary damage, increasing the morbidity and the risk of death of these patients due to frequent and recurrent episodes of acute exacerbations (27).

Most of the acute exacerbations caused by *P. aeruginosa* are due to a preexisting strain which colonizes the lower airway. Often, these strains are hypermutable (strains with defects in genes involved in DNA repair) and have been related to an increase of antimicrobial resistance due to a stepwise accumulation of point mutations (28). In contrast, when *S. pneumoniae* has been recovered during an acute exacerbation, this has generally been associated with the acquisition of a new strain, and the high prevalence of multidrug-resistant pneumococci associated with acute exacerbations has been related to the consumption of antimicrobials that these patients receive as empirical treatment (18, 26). The role of pneumococcal hypermutable strains is unclear and could be related to the persistent strains that colonize among 15 to 17% of COPD patients at any time (12, 32).

Antimicrobial treatment for acute exacerbations includes β-lactams, macrolides, and fluoroquinolones, and the high rates of antimicrobial resistance to these classes of antimicrobials in patients with respiratory diseases are a cause of concern (18). Among pneumococci, resistance to β-lactams is the result of alterations in the penicillin-binding proteins (PBPs), most importantly PBP1A, PBP2B, and PBP2X (7). Macrolide resistance is mediated by two main mechanisms, target site modification by methylases encoded by the *erm*(B) or *erm*(TR) genes (referred to as the MLS_B phenotype) and an efflux pump encoded by the *mef*(A) gene (referred to as the M phenotype) (5). In *S. pneumoniae*, macrolide resistance is frequently associated with tetracycline resistance due to the presence of the Tn*916* family of transposons, which can result in the spread of resistance to both antimicrobials (29). Fluoroquinolone resistance is caused mainly by changes in the quinolone-resistant determining regions (QRDRs) of DNA topoisomerase IV subunits (ParC and ParE) and the DNA gyrase (GyrA) subunit (10).

Data describing the antimicrobial susceptibility, serotype, and pulsed-field gel electrophoresis (PFGE) pattern of a large collection of over 600 pneumococci isolated from COPD patients were recently reported (13). However, little information is available about the evolution of pneumococci associated with multiple acute exacerbation episodes over a long period of time in a patient with chronic respiratory disease. In the present work, we characterized 50 pneumococci isolated from 13 patients with chronic respiratory disease who had 3 or more episodes of acute exacerbations caused by the same pneumococcal strain (as defined by the same serotype and PFGE pattern). Isolates were genotyped by multilocus sequence typing (MLST); *pbp2x*, *pbp1a*, *pbp2b*, *parC*, *parE*, and *gyrA* were PCR amplified and sequenced; and *erm*(B), *erm*(TR), *mef*(A/E), and *tet*(M) were detected by PCR. We hypothesized that the pneumococcal strains that persistently colo-

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nize and are associated with multiple episodes of acute exacerbation in these patients would acquire changes in the resistance determinants of β-lactams, macrolides, and fluoroquinolones over time: if so, this has important relevance for the clinical management of these patients.

(This study was presented at the 8th International Symposium on Pneumococci and Pneumococcal Diseases, Foz do Iguazu, Brazil, 2012 [13a].)

MATERIALS AND METHODS

This study and publication of the results were approved by the "Comité Ètic d'Investigació Clínica del Hospital Universitari de Bellvitge."

Study setting, bacterial strains, and antimicrobial susceptibility. Pneumococci isolated from clinical samples (invasive and noninvasive) were prospectively collected in our laboratory. All patients with 3 or more pneumococcal episodes of acute exacerbations detected between 1995 and 2010 were analyzed to identify persistent colonization, defined as the same serotype and PFGE pattern. A new episode was considered when the range of time between episodes was more than 4 weeks, which occurred after a successful outcome. Only sputum samples of good quality (<10 squamous cells and >25 leukocytes per low-power field) in which the diplococcus Gram-positive bacteria were the most frequently detected were cultured (24). Pneumococci were identified by optochin susceptibility and bile solubility. Serotyping was performed by Quellung reaction at the Spanish Reference Laboratory.

An acute exacerbation of COPD or bronchiectasis was defined as any sustained increase in respiratory symptomatology, compared with the baseline situation that required an increase in regular medication and hospital treatment. An episode of pneumonia was considered when fever, leukocytosis, and radiological findings (new infiltrates on chest radiography) were detected. The COPD status was defined according to the international Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria (23).

Antimicrobial susceptibility, serotype, and PFGE pattern of six pneumococci isolated from two patients (patients 7 and 10) have been published previously among 611 pneumococci isolated from pneumonia and acute exacerbation episodes of COPD patients (13).

Susceptibility to 22 antimicrobials (MIC) was tested by the microdilution method (STRHAE1; Sensititre, West Sussex, United Kingdom), following the Clinical and Laboratory Standards Institute (CLSI) recommendations (6). The ciprofloxacin MIC of resistant strains (MIC ≥ 4 μg/ml) was confirmed by Etest. *S. pneumoniae* ATCC 49619 was used as the control strain.

PBP detection and sequence analysis. DNA was extracted from pneumococcal strains using the DNeasy tissue kit (Qiagen). *pbp1a*, *pbp2x*, and *pbp2b* were amplified and sequenced, using primer sets and conditions described previously (4). Sequences were assembled and edited using Pregap4 and Gap4 (Staden Package, http://staden.sourceforge.net/). Once assembled, sequences of PBPs were compared between strains of the same patient.

Gene detection of macrolide and tetracycline resistance. Macrolide resistance genes [*erm*(B), *erm*(TR), and *mef*(A/E)] and the tetracycline resistance determinant *tet*(M) were studied by PCR as described previously (5).

Characterization of quinolone resistance. The *parC*, *parE*, and *gyrA* genes were amplified as described previously (22). Restriction fragment length polymorphism assay (RFLP) of PCR products was performed to detect point mutations at the main QRDR positions involved in quinolone resistance: S79 and S83 of *parC*, D435 of *parE*, and S81 and E85 of *gyrA*. Briefly, S79 and D83 mutations in the *parC* gene (using HinfI and *Sfa*NI enzymes, respectively), a D435 mutation in the *parE* gene (using HinfI enzyme), and S81 and E85 mutations in the *gyrA* gene (using HinfI and MboII enzymes, respectively) were detected (2, 22).

Point mutations were confirmed by sequencing. The oligonucleotide pairs *parE*398/*parE*483, *parC*50/*parC*152, *gyrA*44/*gyrA*170, and *gyrB*376/*gyrB*512 were used to amplify and sequence *parE*, *parC*, *gyrA*, and *gyrB* QRDRs, respectively (11).

Molecular typing. Genotyping was performed by MLST, as described previously (15). Allele numbers and sequence types (ST) were assigned using the pneumococcal MLST website (http://spneumoniae.mlst.net/).

RESULTS

Patients, pneumococcal strains, and antimicrobial resistance. During the study period (1995 to 2010), 231 adult patients were identified who had 3 or more episodes of acute exacerbation, and *S. pneumoniae* was isolated. A total of 218 of these 231 patients had *S. pneumoniae* strains that differed by serotype and/or genotype. Thirteen (6.1%) patients had at least 3 different episodes during which the same strain was isolated (i.e., with the same serotype and PFGE pattern) and were selected for this study. Eleven of the thirteen patients had chronic respiratory diseases: 8 had COPD (1 patient with GOLD II status, 2 patients with GOLD III status, and 3 patients with GOLD IV status; the GOLD status of two patients was not available), and 3 had bronchiectasis. The remaining two patients had an endotracheal prosthesis implanted due to a post-tracheostomy stenosis.

A total of 50 pneumococci isolated from the 13 patients were analyzed. The average time between episodes was 210 days (range, 30 to 531 days), and the average time between first and last episodes was 582 days (standard deviation [SD], ±362). All 50 pneumococci analyzed were nonsusceptible to penicillin using oral breakpoints and also showed resistance to at least one other antimicrobial class. Strains from 11 patients were multidrug resistant (≥3 antimicrobial classes; Table 1). All pneumococci examined from the same patient had the identical ST as defined by MLST genotyping, with the exception of patient 7, who had 8 pneumococcal isolates, 5 of which expressed serotype 15A (ST63) and 3 of which expressed serotype 35B (ST558). Moreover, antimicrobial MICs for all strains were conserved over time apart from those of the fluoroquinolones. All macrolide-resistant strains possessed *erm*(B) and *tet*(M) genes, and no acquisition or loss of macrolide or tetracycline resistance determinants was observed. Additionally, we analyzed six transient pneumococcal strains collected from patients 5 (Sp^{10A} and Sp^{NT}), 6 (Sp^{NT}), 7 (Sp^{35F}), and 10 (Sp^{23F}), whose serotypes were different from the persistent one (Fig. 1). These transient isolates also were fully susceptible to all antibiotics tested and had a different PFGE type from that of the persistent strain.

Finally, the same *pbp1a* and *pbp2b* DNA sequences were maintained in each persistent strain over time (Table 1). The sequence of *pbp2*x was maintained in all but 3 patients (3, 6, and 11) whose strains had a single nucleotide polymorphism (SNP) that was not involved in an increase of the β-lactam MICs. These results strengthen the suggestion that these were persistent strains in all 13 patients (Fig. 1).

All but two patients (8 and 9) received multiple courses of β-lactam therapy (amoxicillin-clavulanic acid and ceftriaxone). All persistent strains from these 11 patients were susceptible to ceftriaxone and cefotaxime, whereas 5 persistent strains (patients 3, 4, 5, 6, and 13) were amoxicillin-clavulanic acid resistant. Seven patients had macrolide-resistant strains, but previous macrolide consumption could be documented in only one of them (patient 7). Furthermore, all but 3 patients (2, 6, and 13) received at least one course of fluoroquinolone treatment between acute exacerbation episodes. In three cases (patients 3, 10, and 12), the persistent

TABLE 1 Antimicrobial MICs, characteristics of the 13 patients, and molecular characterization of the persistent pneumococcal strains[a]

Patient no. (age, gender)	MLST genotype	Episode no. (day/mo/yr)	β-lactam MIC (μg/ml)			PBP1A/2B/2X allele[b]	Fluoroquinolone MIC (μg/ml)			Aa substitutions in ParC/GyrA	MIC (μg/ml)[d]	
			PEN	A/C	CTX-CRO		CIP	LEV	MOX		ERY	TET
1 (58, male)	ST156^{9V}	1st (19/10/1995)	**2**	2/1	1	A/A.1/A.1	**8**	2	<0.25	S79F/none	<0.25	≤2
		2nd (29/11/1995)	**2**	2/1	1	A/A.1/A.1	**8**	2	<0.25	S79F/none	<0.25	≤2
		3rd (28/04/1996)	**2**	2/1	1	A/A.1/A.1	**8**	2	<0.25	S79F/none	<0.25	≤2
2 (46, male)	ST156^{9V}	1st (24/07/1995)	**2**	1/0.5	1	A/A.1/A.1	2	1	<0.25	None	<0.25	≤2
		2nd (07/11/1995)	**2**	1/0.5	1	A/A.1/A.1	2	1	<0.25	None	<0.25	≤2
		3rd (17/03/1996)	**2**	1/0.5	1	A/A.1/A.1	2	1	<0.25	None	<0.25	≤2
3 (76, male)	ST838^{9V}	1st (06/11/2002)	**2**	**8/4**	1	A/B.1/A.2	2	1	<0.25	None	<0.25	≤2
		2nd (08/05/2003)	**2**	**8/4**	1	A/B.1/A.2	2	1	<0.25	None	<0.25	≤2
		3rd (24/09/2003)	**2**	**8/4**	1	A/B.1/A.2[c]	2	1	<0.25	None	<0.25	≤2
		4th (17/04/2004)	**2**	**8/4**	1	A/B.1/A.2	2	1	<0.25	None	<0.25	≤2
4 (77, female)	ST838^{9V}	1st (11/08/2007)	**2**	**8/4**	1	A/B.1/A.2	**>32**	**32**	**4**	S79F/S81F	<0.25	≤2
		2nd (24/01/2008)	**2**	**8/4**	1	A/B.1/A.2	**>32**	**32**	**4**	S79F/S81F	<0.25	≤2
		3rd (16/06/2008)	**2**	**8/4**	1	A/B.1/A.2	**>32**	**32**	**4**	S79F/S81F	<0.25	≤2
5 (62, male)	ST838^{9V}	1st (29/10/1997)	**2**	**8/4**	1	A/B.1/A.2	**>32**	**32**	**4**	S79Y/S81F	<0.25	≤2
		2nd (20/10/1998)	**2**	**8/4**	1	A/B.1/A.2	**8**	2	<0.25	S79F/none	<0.25	≤2
		3rd (03/04/2000)	**2**	**8/4**	1	A/B.1/A.2	**>32**	**32**	**4**	S79F/S81F	<0.25	≤2
6 (60, male)	ST6521^{11A}	1st (21/05/2009)	**2**	**8/4**	1	A/B.2/A.2	2	1	<0.25	None	<0.25	≤2
		2nd (25/10/2009)	**2**	**8/4**	1	A/B.2/A.2[c]	2	1	<0.25	None	<0.25	≤2
		3rd (14/03/2010)	**2**	**8/4**	1	A/B.2/A.2[c]	2	1	<0.25	None	<0.25	≤2
7 (75, male)	ST63^{15A}	1st (09/11/2008)	**0.25**	<0.5/0.25	0.12	B/C/B	2	2	<0.25	None	**>32**	**>4**
		2nd (28/12/2008)	**0.25**	<0.5/0.25	0.12	B/C/B	2	1	<0.25	None	**>32**	**>4**
		3rd (29/01/2009)	**0.25**	<0.5/0.25	0.12	B/C/B	**>32**	**32**	**4**	S79Y/S81F	**>32**	**>4**
		4th (15/04/2009)	**0.25**	<0.5/0.25	0.12	B/C/B	**>32**	**32**	**4**	S79Y/S81F	**>32**	**>4**
		6th (16/06/2009)	**0.25**	<0.5/0.25	0.12	B/C/B	**>32**	**32**	**4**	S79Y/S81F	**>32**	**>4**
	ST558^{35B}	5th (10/05/2009)	**1**	2/1	1	C/D/C	1	1	<0.25	None	<0.25	≤2
		7th (16/03/2010)	**1**	2/1	1	C/D/C	1	1	<0.25	None	<0.25	≤2
		8th (05/06/2010)	**1**	2/1	1	C/D/C	1	1	<0.25	None	<0.25	≤2
8 (59, female)	ST63^{15A}	1st (25/10/2005)	**0.25**	<0.5/0.25	0.12	B/C/B	2	1	<0.25	None	**>32**	**>4**
		2nd (02/05/2006)	**0.25**	<0.5/0.25	0.12	B/C/B	**4**	2	<0.25	D78N/none	**>32**	**>4**
		3rd (07/12/2006)	**0.25**	<0.5/0.25	0.12	B/C/B	**4**	2	<0.25	D78N/none	**>32**	**>4**
		4th (06/11/2007)	**0.25**	<0.5/0.25	0.12	B/C/B	**4**	2	<0.25	D78N/none	**>32**	**>4**
		5th (02/06/2008)	**0.25**	<0.5/0.25	0.12	B/C/B	**4**	2	<0.25	D78N/none	**>32**	**>4**
9 (78, male)	ST88^{19F}	1st (24/12/1999)	**0.25**	<0.5/0.25	0.5	D/E/D.1	1	1	<0.25	None	**>32**	**>4**
		2nd (24/12/2000)	**0.25**	<0.5/0.25	0.5	D/E/D.1	**>32**	**32**	**4**	S79F/S81Y	**>32**	**>4**
		3rd (28/04/2002)	**0.25**	<0.5/0.25	0.5	D/E/D.1	**>32**	**32**	**4**	S79F/S81Y	**>32**	**>4**
10 (64, male)	ST87^{19F}	1st (26/10/2007)	**1**	2/1	0.5	E/F/D.1	1	1	<0.25	None	**>32**	**>4**
		2nd (23/01/2008)	**1**	2/1	0.5	E/F/D.1	1	1	<0.25	None	**>32**	**>4**
		3rd (19/09/2008)	**1**	2/1	0.5	E/F/D.1	1	1	<0.25	None	**>32**	**>4**
		4th (09/12/2008)	**1**	2/1	0.5	E/F/D.1	1	1	<0.25	None	**>32**	**>4**
		5th (25/12/2010)	**1**	2/1	0.5	E/F/D.1	1	1	<0.25	None	**>32**	**>4**
11 (73, male)	ST2100^{19F}	1st (08/01/2008)	**2**	2/1	1	E/A.2/D.2	**>32**	**32**	**4**	S79Y/S81Y	**>32**	≤2
		2nd (28/04/2009)	**2**	2/1	1	E/A.2/D.2[c]	**>32**	**32**	**4**	S79Y/S81Y	**>32**	≤2
		3rd (19/10/2009)	**2**	2/1	1	E/A.2/D.2[c]	**>32**	**32**	**4**	S79Y/S81Y	**>32**	≤2
12 (38, male)	ST276^{19A}	1st (12/12/2007)	**2**	2/1	1	F/G/A.1	2	1	<0.25	None	**>32**	**>4**
		2nd (22/12/2008)	**2**	2/1	1	F/G/A.1	2	1	<0.25	None	**>32**	**>4**
		3rd (18/03/2009)	**2**	2/1	1	F/G/A.1	2	1	<0.25	None	**>32**	**>4**
13 (65, male)	ST1624^{6B}	1st (19/06/2006)	**4**	**8/4**	1	G/B/E	1	0.5	<0.25	None	**>32**	**>4**
		2nd (20/03/2007)	**4**	**8/4**	1	G/B/E	1	0.5	<0.25	None	**>32**	**>4**
		3rd (22/08/2008)	**4**	**8/4**	1	G/B/E	1	0.5	<0.25	None	**>32**	**>4**
		4th (06/10/2009)	**4**	**8/4**	1	G/B/E	1	0.5	<0.25	None	**>32**	**>4**

[a] PEN, penicillin (susceptible: MIC < 0.12 μg/ml); CTX-CRO, cefotaxime-ceftriaxone (susceptible: MIC ≤ 1 μg/ml); A/C, amoxicillin-clavulanic acid (susceptible: MIC < 4/2 μg/ml); CIP, ciprofloxacin (susceptible: MIC < 4 μg/ml); LEV, levofloxacin (susceptible: ≤ 2 μg/ml); MOX, moxifloxacin (susceptible: ≤ 1 μg/ml); ERY, erythromycin (susceptible: ≤ 0.25 μg/ml); and TET, tetracycline (susceptible: ≤ 2 μg/ml). Boldface font indicates resistant isolates.

[b] Capital letters were used to define different alleles of each PBP, and numbers were used to differentiate small differences among sequences with identical capital letter (for details of the amino acid substitutions, see Fig. 3).

[c] Acquisition of an SNP in the PBP2X with respect to the first episode of the same patient.

[d] Gene *erm*(B) was detected only in ERY-resistant strains; gene *tet*(M) was detected only in TET-resistant strains.

FIG 1 Number of acute exacerbation and pneumonia episodes of each patient, bacterial pathogen isolated, and courses of antimicrobial treatment. Horizontal lines are proportional time lines between the first and the last episode analyzed. Vertical lines mean infection episodes (acute exacerbation, continuous lines; pneumonia, dotted lines). Pathogens isolated were Sp, *Streptococcus pneumoniae*; Hi, *Haemophilus influenzae*; Mc, *Moraxella catarrhalis*; Pa, *Pseudomonas aeruginosa*; Sa, *Staphylococcus aureus*. Pneumococcal serotypes are superscripted (NS means nonserotyped isolate, and NT means nontypeable isolate). Those pneumococci examined in detail are in boldface font. Colored dots indicate the course of antimicrobial therapy: fluoroquinolone, black; amoxicillin-clavulanic acid, green; ceftriaxone, yellow; and macrolide, white. Abbreviations of the QRDR substitutions are showed in red color (no abbreviation means no changes in the QRDRs).

strains remained fluoroquinolone susceptible over time, whereas strains of 4 patients (1, 4, 5, and 11) were ciprofloxacin resistant from the first episode, and pneumococci isolated from another 3 patients (7, 8, and 9) became ciprofloxacin resistant after fluoroquinolone therapy.

Sequence type, serotype, and mechanisms of resistance. The *pbp2x*, *pbp1a*, and *pbp2b* sequences of the persistent strains were compared to those of the susceptible pneumococcal R6 strain (Fig. 2). The *pbp2b* gene was the most conserved; this gene has been described to be involved in amoxicillin-clavulanic acid resistance. The *pbp1a* and *pbp2x* genes possessed mosaic blocks that conferred amino acid substitutions in their transpeptidase domains, which have been associated with a reduction in the affinity for penicillin and cefotaxime (16). Those amino acid substitutions in PBP1A, PBP2B, and PBP2X that were related to β-lactam resistance are shown in Fig. 3.

The most frequent serotype was 9V, expressed by pneumococci isolated from 5 patients, and all 16 pneumococci were either ST156 (Spain^{9V}-ST156 Pneumococcal Molecular Epidemiology Network [PMEN] clone) or ST838, a single locus variant (SLV) of ST156 (Table 1). These 16 pneumococci had the same *pbp1a* allele, one of two *pbp2b* alleles, and the same *pbp2x* allele, albeit with some minor nucleotide changes in a few isolates. Pneumococci of patient 6 expressed serotype 11A and were ST6521, which is an SLV of ST838, and shared the *pbp1a*, *pbp2b*, and *pbp2x* sequences with ST838^{9V} isolates, with the exception of an amino acid substitution in the PBP2X (P504L) in the isolates recovered from the 2nd and 3rd episodes.

The persistent strain of patient 1 (ST156^{9V}) was ciprofloxacin resistant (mutation S79F) from the first episode and after a previous ciprofloxacin course (Fig. 1). All three isolates of patient 4 (ST838^{9V}) were ciprofloxacin resistant due to changes in ParC(S79F) and GyrA(S81F). All four isolates (ST838^{9V}) from patient 3 remained fluoroquinolone susceptible throughout the period studied, in spite of one course of ciprofloxacin in the third episode. Finally, an analysis of the QRDRs of isolates collected from patient 5 was previously published, describing the acquisition of low-level and subsequent high-level fluoroquinolone resistance (9).

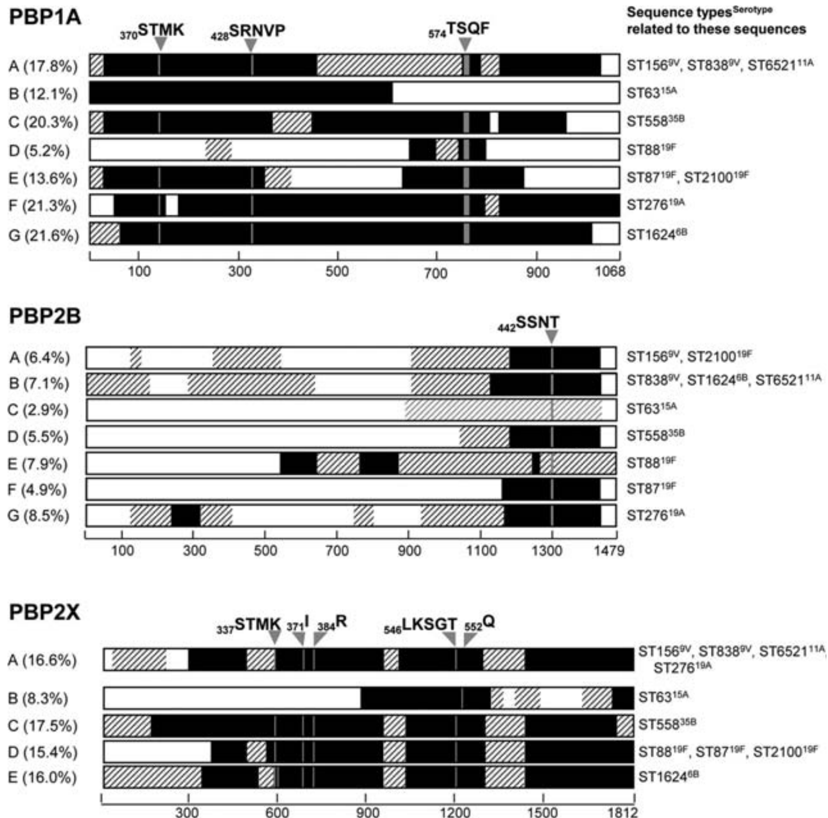

FIG 2 Schematic of the mosaic genes encoding penicillin-binding proteins (PBPs) 1A, 2B, and 2X of the persistent strains. Alleles of each PBP are shown as bars. Mutations associated with resistance to β-lactams are indicated at the top of each PBP. The PBP sequences of the susceptible pneumococcal R6 strain were used as the reference. Blocks showing the percent sequence divergence from the corresponding regions of R6 are indicated. White boxes, regions highly conserved ($<$1.5% divergence); striped boxes, regions that differed by 1.6 to 9.0%; black boxes, regions that differed by $>$9.0%. Percentage value indicates rate of divergence of each allele with respect to the R6-susceptible sequence.

Strains of patients 7 and 8 expressed serotype 15A and were ST63, PMEN clone Sweden^{15A}-ST63. Both persistent strains developed mutations at QRDRs over time after levofloxacin and ciprofloxacin courses, respectively. The persistent strain of patient 8 showed a new ParC change (D78N) in the 2nd episode, and in the case of patient 7, the persistent strain acquired and maintained high-level ciprofloxacin resistance at the third episode, due to changes in ParC (S79F) and GyrA (S81F). Patient 7 also had 3 episodes caused by a serotype 35B, ST558 (SLV of Utah^{35B}-ST377 clone) persistent strain. The first strain of ST558^{35B} was detected between the fourth and the fifth episode of the former ST63^{15A} persistent strain; thereafter, two new episodes were caused by the ST558^{35B} strain. Since these two last episodes occurred in 2010 and the sputum sample was available, the DNA was extracted and an attempt was made to detect serotype 15A by PCR, but the PCR was negative (data not shown).

The persistent strains of patients 9, 10, and 11 all expressed serotype 19F, although the genotypes were different (ST88, ST87, and ST2100). The strain of the 2nd episode of patient 9 (ST88^{19F}) developed high-level ciprofloxacin resistance (ParC-S79F and GyrA-S81Y mutations) after a levofloxacin course, and the strain was also recovered during the 3rd episode. Persistent strains of patient 10 belonged to ST87 (SLV of ST88) and remained stable over time. Finally, the isolates from patient 11 were ST2100^{19F} (SLV of ST63). The strain from the 2nd episode acquired an amino acid substitution in the PBP2X (L600S). After multiple courses of moxifloxacin, the pneumococci isolated from the first acute exacerbation episode was high-level ciprofloxacin resistant (mutations S79F in ParC and S81Y in GyrA) and persisted over time. Finally, all isolates from patient 12 were of the same serotype, ST, and susceptibility pattern over time; the same was true of all isolates from patient 13.

DISCUSSION

Once *S. pneumoniae* causes an acute exacerbation episode in patients with chronic respiratory disease, the isolate is usually replaced by another *S. pneumoniae* strain with a different serotype/genotype or by a different bacterial species, such as *P. aeruginosa*, *H. influenzae*, or *M. catarrhalis* (26, 27, 32). However, in the present study, we showed, based on stability in capsular type, ST, PBPs, and other resistance determinants, that pneumococci can persist over a long period of time, colonizing and causing acute

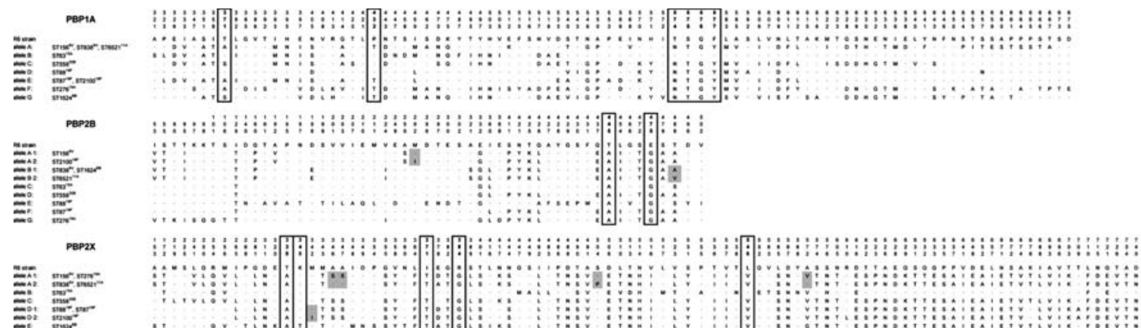

FIG 3 Amino acid substitutions in PBP1A, PBP2B, and PBP2X, compared to those PBPs of the susceptible pneumococcal R6 strain. Highlighted positions indicate changes related to β-lactam resistance. Dots are placed when amino acids are identical to R6. Allele differences between sequence types, i.e., alleles A and B of PBP2B and alleles A and D of PBP2X, are divided into two alleles (e.g., A1 and A2), and differences are shaded. See also Table 1.

exacerbation or pneumonia episodes in patients with chronic respiratory disease.

Persistent bacterial colonization in patients with bronchiectasis and/or severe COPD is frequently associated with *P. aeruginosa* but rarely with pneumococci. In fact, in the present study, the same strain was recovered from only 13 of 213 (6.1%) patients with multiple pneumococcal episodes.

Persistence of *P. aeruginosa* in COPD is often associated with hypermutable strains that dramatically speed up resistance development due to the acquisition of point mutations during exposure to antimicrobial agents (20, 28). In contrast, our study shows that among this collection of isolates, most pneumococcal genes involved in antimicrobial resistance were stable over time, with the exception of *parC* and *gyrA*, which were associated with the development of fluoroquinolone resistance after treatment.

Several factors could explain the persistence of pneumococcal strains in patients with COPD or bronchiectasis. First, it is well known that these patients have several impairments in innate lung defenses, facilitating the permanent colonization by microorganisms (27). Second, the persistence of the strains could be related to their serotype and/or genotype. The capsule is the main virulence factor of *S. pneumoniae*, since it prevents the opsonization by macrophages (19). An association between capsular type polysaccharide, susceptibility to neutrophil-mediated killing, and carriage prevalence has been demonstrated, and the serotypes expressed by several persistent strains of the present study (19F, 6B, 11A, 19A, and 9V) are able to avoid neutrophil-mediated killing (30).

Ten out of 14 strains belonged to three clonal complexes (CC156, CC88, and CC63), suggesting a major role of the genetic background on persistence. In agreement with this finding, a major genotype (related to CC177) was recovered from children attending day care centers, and prolonged colonization was observed in 22% of children (25). Genetic characteristics could favor the colonization over time of these clonal complexes, such as the presence of biofilm or adhesins such as PspC or PspA and/or pilus, which can facilitate the adhesion to the epithelial cells (14, 17). The presence of pilus has been shown to be a clonal property, and when the type I pilus was analyzed, it was detected only in persistent strains belonging to both genotypes CC156 and CC90 (data not shown), as was documented previously (21).

Third, possible biofilm formation (by *S. pneumoniae* or another pathogen) in the respiratory tract of these patients may prevent the appropriate diffusion of antibiotics and therefore may result in a decrease in the bacterial load but not bacterial eradication. This might explain why the persistent, amoxicillin-clavulanic acid-susceptible (MIC ≤ 2 mg/liter) strains of 6 patients could persist over time in spite of multiple courses of this antibiotic.

On the other hand, it is well known that pneumococci can acquire β-lactam resistance by acquisition of exogenous DNA at their PBPs from either β-lactam-resistant pneumococci or commensal streptococci, such as *Streptococcus oralis* or *Streptococcus mitis* (31). Surprisingly, in spite of the amoxicillin-clavulanic acid pressure on the six persistent pneumococci resistant to amoxicillin-clavulanic acid, neither new recombination events nor point mutations in the *pbp2b* of the resistant strains were observed. These results suggest that an optimal combination of *pbp* genes is maintained to compensate for the fitness cost imposed by additional changes in these genes, either by point mutation or recombination, as has previously been shown (1).

In contrast, the development of fluoroquinolone resistance was observed among the persistent strains isolated from 3 of 6 patients after receiving single or multiple courses of fluoroquinolone treatment. The frequency of mutation to ciprofloxacin resistance in *S. pneumoniae* has been shown to be in the range of 10^{-8} to 10^{-9}; hence, it is possible that the fluoroquinolone pressure on the high pneumococcal inoculum (≥10^6 CFU/ml) observed in patients with COPD or bronchiectasis could select for spontaneous mutants at the QRDRs (8). The persistence of fluoroquinolone-resistant isolates could be related both to an inadequate treatment and to the fitness cost of the mutations. Some patients with chronic respiratory disease are colonized by *P. aeruginosa* and *H. influenzae* and sometimes receive multiple courses of fluoroquinolones, usually empirically. Furthermore, the mutations found in these persistent strains are not related to a decrease in bacterial fitness [fitness has been associated only with the amino acid change GyrA(E85K), which is not present in any of the persistent resistant isolates] (3). Overall, our study demonstrates the risk of the development of fluoroquinolone resistance among persistent pneumococci after fluoroquinolone therapy. This fact should be considered before starting a new empirical fluoroquinolone treatment in order to avoid therapeutical failures.

The analyses of the sequences determined in this study (7

housekeeping genes, QRDRs of *parC*, *parE*, and *gyrA*; *pbp1a*, *pbp2b*, *pbp2x*), together with the analyses of macrolide and tetracycline determinants, suggests that a pneumococcal strain can colonize the respiratory airways for an extended period of time. Moreover, the low clonal diversity observed among these persistent strains also suggests that some pneumococci are successfully adapted to persist over a long period of time in patients with chronic respiratory disease and thus potentially cause multiple acute exacerbation episodes.

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CHAPTER V: *S. pseudopneumoniae* populations causing acute exacerbations.

Objective 6: To characterise *S. pseudopneumoniae* strains by phenotypic and genotypic methods (2001-09).

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Disease Isolates of *Streptococcus pseudopneumoniae* and Non-Typeable *S. pneumoniae* Presumptively Identified as Atypical *S. pneumoniae* in Spain

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Abstract

We aimed to obtain insights on the nature of a collection of isolates presumptively identified as atypical *Streptococcus pneumoniae* recovered from invasive and non-invasive infections in Spain. One-hundred and thirty-two isolates were characterized by: optochin susceptibility in ambient and CO_2-enriched atmosphere; bile solubility; PCR-based assays targeting pneumococcal genes *lyt*A, *ply*, *psp*A, *cps*A, *Spn*9802, *ali*B-like ORF2, and a specific 16S rRNA region; multilocus sequence analysis; and antimicrobial susceptibility. By multilocus sequence analysis, 61 isolates were *S. pseudopneumoniae*, 34 were pneumococci, 13 were *S. mitis*, and 24 remained unclassified as non-pneumococci. Among *S. pseudopneumoniae* isolates, 51 (83.6%) were collected from respiratory tract samples; eight isolates were obtained from sterile sources. High frequency of non-susceptibility to penicillin (60.7%) and erythromycin (42.6%) was found. Only 50.8% of the *S. pseudopneumoniae* isolates displayed the typical optochin phenotype originally described for this species. None harbored the *cps*A gene or the pneumococcal typical *lyt*A restriction fragment length polymorphism. The *Spn*9802 and the specific 16S rRNA regions were detected among the majority of the *S. pseudopneumoniae* isolates (n = 59 and n = 49, respectively). The *ply* and *psp*A genes were rarely found. A high genetic diversity was found and 59 profiles were identified. Among the *S. pneumoniae*, 23 were capsulated and 11 were non-typeable. Three non-typeable isolates, associated to international non-capsulated lineages, were recovered from invasive disease sources. In conclusion, half of the atypical pneumococcal clinical isolates were, in fact, *S. pseudopneumoniae* and one-fourth were other streptococci. We identified *S. pseudopneumoniae* and non-typeable pneumococci as cause of disease in Spain including invasive disease.

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Introduction

Streptococcus pneumoniae (pneumococcus) is an important human pathogen worldwide responsible for systemic diseases such as meningitis, pneumonia, and bacteraemia. [1,2] Culture-based identification methods usually rely on colony morphology, optochin susceptibility, bile solubility, and agglutination by the Quellung reaction. [3] However, exceptions have been described and include pneumococci that are optochin-resistant, [4,5] bile-insoluble, [6] and do not have a specific agglutination in the Quellung reaction due to lack of capsule. [7,8] This latter group is generally called non-typeable pneumococci and is often found in colonization. [7,9] Although sporadically, non-typeable pneumococci have also been associated with disease such as conjunctivitis (including large outbreaks), [10,11] acute otitis media, [12] acute exacerbations in patients with chronic obstructive pulmonary disease (COPD), [13] and more recently in invasive disease. [14].

Pneumococcal isolates displaying odd properties in the assays described above have been collectively named atypical pneumococci and are often difficult to identify. On the other hand, sporadic isolates of closely-related species that have one or more properties typically associated with pneumococci have been described. [9,15,16].

In 2004, Arbique and colleagues identified some of these atypical pneumococci as a new species – *Streptococcus pseudopneumoniae*. [17] Although similar to pneumococci, they were character-

S. pseudopneumoniae CAUSING AECOPD

ized by being bile insoluble and optochin-resistant when incubated under a 5% CO_2 atmosphere but optochin–susceptible when incubated under ambient atmosphere. *S. pseudopneumoniae* have been identified among colonizing children and respiratory samples. [15,18] Although, their clinical relevance has not been clearly established, *S. pseudopneumoniae* have been associated with COPD, [19] and its disease potential has been demonstrated in mice models of peritonitis and sepsis. [20].

As biochemical tests are often insufficient to distinguish atypical *S. pneumoniae* from *S. pseudopneumoniae* or other closely related streptococci several molecular assays have been proposed. The construction of phylogenetic trees using six concatenated multilocus sequence typing (MLST) alleles, called Multilocus Sequence Analysis (MLSA), is considered a good approach to differentiate *S. pneumoniae* from closely related species. [15,21] In addition, several other assays have been developed most of which are PCR-based and target specific pneumococcal virulence factors, such as autolysin A (*lyt*A), pneumolysin (*ply*), pneumococcal surface protein A (*psp*A), or the capsular polysaccharide biosynthesis gene A (*cps*A). [6,15] Unknown putative genes, specific intergenic DNA sequences, or specific regions of the 16S rRNA, have also been proposed to be pneumococcal species-specific. [22,23] However, the occurrence of *Streptococcus mitis* isolates harbouring genes encoding *S. pneumoniae* virulence factors has been reported and whether the genetic assays recently proposed universally distinguish pneumococci from the closely related species remains to be seen. [15,24,25,26,27].

In this study, we aimed to characterize a large collection of invasive and non-invasive disease isolates obtained in Spain, which had been identified as atypical pneumococci. We have combined MLSA with a panel of phenotypic and molecular assays in order to gain insights on the nature of such isolates.

Materials and Methods

Ethics Statement

This study and publication of the results were approved by the "Comité Ètic d'Investigació Clínica del Hospital Universitari de Bellvitge" and written or oral informed consent was considered not necessary, because data were analyzed anonymously.

Bacterial Isolates

A total of 132 clinical isolates classified as non-(sero)typeable or atypical pneumococci collected at two Spanish laboratories were included in the study. There were no duplicates within or between the two sets studied.

The first set comprised 56 isolates collected at the Spanish Reference Pneumococcal Laboratory (Centro Nacional de Microbiologia, ISCIII, Madrid, Spain), which receives pneumococcal disease isolates from 190 hospitals throughout the entire country. The isolates were obtained between 2004 and 2009, and were mostly (44 out of 56) from non-sterile sites. This set represented 7.7% (56 out of 728) of the total non-(sero)typeable or atypical pneumococci *S. pneumoniae* isolated during that period which, in turn, corresponded to 4.6% of all pneumococcal isolates identified in the same period. This set included: i) 44 specimens with atypical pneumococcal identification [optochin resistant in CO_2 atmosphere, bile negative, and Accuprobe^{TM} positive (Gen-Probe, San Diego, California)] of which 43 had been isolated from non-sterile sites; and ii) 12 non-typeable pneumococci (optochin susceptible in CO_2 atmosphere, and showing no agglutination in the Quellung reaction), of which eight were invasive isolates.

The second set comprised 76 isolates collected at the tertiary adult Hospital Universitari de Bellvitge (Barcelona, Spain) obtained between 1991 and 2009 and were mostly (63 out of 76) from non-sterile sites. This set represented 43.9% (76 out of 173) of the total non-(sero)typeable or atypical pneumococci *S. pneumoniae* isolated during that period which, in turn, corresponded to 5.1% of all pneumococcal isolates identified in the same period. This collection also include two groups of isolates: i) 35 specimens with atypical pneumococcal identification [reduced optochin susceptibility in CO_2 atmosphere, positive Slidex® pneumo-Kit aglutination test (bioMérieux, Marcy-l'Etoile, France)] of which 30 had been isolated from non-sterile sites; and ii) 41 non-typeable pneumococci (optochin susceptible in CO_2 atmosphere and showing no agglutination in the Quellung reaction), of which eight were invasive isolates.

In the total collection invasive isolates were obtained from blood (n = 11), bronchoalveolar lavage (n = 7), transthoracic needle aspiration (n = 1), cerebrospinal fluid (n = 1), bronchoscopic-protected catheter brush (n = 1) and ascitic fluid (n = 1). Non-invasive isolates were obtained from sputum (n = 75), bronchial aspiration (n = 23), conjunctiva swab (n = 4), and others (n = 8).

Optochin Susceptibility

Optochin susceptibility was tested by disk diffusion, using commercially available optochin disks (5 µg; 6 mm; Oxoid, Hampshire, England) applied onto blood agar plates (trypticase soy agar supplemented with 5% sheep blood), which had been inoculated with a 0.5 McFarland standard suspension of the culture to be tested. Plates (two per isolate) were incubated in parallel overnight at 37°C in a 5% CO_2 and ambient atmosphere as described by Arbique *et al.* to differentiate *S. pneumoniae* from *S. pseudopneumoniae*. [*17*] Isolates were considered to be resistant to optochin if they displayed inhibition zones smaller than 14 mm. [17].

Bile Solubility Test

The bile solubility assay was performed according to standard procedures described by Rouff *et al.* [3].

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility against penicillin, cefotaxime, erythromycin, clindamycin, cotrimoxazole, tetracycline, ciprofloxacin, levofloxacin and chloramphenicol was performed by disk-diffusion and microdilution method, following the recommendations and definitions of the Clinical and Laboratory Standards Institute (CLSI). [28] In particular, for penicillin, pneumococcal oral penicillin V breakpoints were used (S:≤0.06, I:0.12-1, R:≥2); for cefotaxime, pneumococcal meningeal breakpoints were used (S:≤0.5, I:1, R:≥2). For ciprofloxacin, an MIC≥4 mg/L was considered resistant.

Capsular Typing

For pneumocccal capsular detection, isolates were serotyped by the Quellung reaction, and/or by a PCR-based assay following the protocols described by the CDC. [29,30] Isolates for which a capsule could not be assigned were probed against Omniserum (Statens Serum Institute, Copenhagen, Denmark), a serum that contains antibodies to all known pneumococcal types.

Multiplex PCR for Detection of *lytA*, *cpsA* and *ali*B-like ORF2

A multiplex PCR assay was used to distinguish *S. pneumoniae* from closely related species as previously described. [9] This multiplex PCR detects internal fragments of *cps*A (a conserved pneumococcal capsular polysaccharide gene); *lytA* (the major

pneumococcal autolysin); *ali*B-like ORF2 (a gene described as frequently present in the capsular region of non-capsulated pneumococci); [8] and 16S rRNA (positive internal control).

PCR Screening for Additional Putative Specific Pneumococcal Signatures - *psp*A, *Spn*9802 and *16S* rRNA

Screening for the presence of *pspA* (the gene that encodes for the pneumococcal surface protein A), *Spn*9802 (a genetic region which encodes for a protein of unknown function that has initially been described as a specific target for *S. pneumoniae*), and a 16S rRNA allele that has been described as pneumococcal-specific, was done as described. [22,23,31].

lytA RFLP Signatures

The *lytA* gene was amplified by PCR and RFLP signatures characteristic of typical pneumococcal *lytA* or atypical (non-pneumococcal) *lytA* were determined by digesting the amplification product with BsaAI and separating the fragments by agarose gel electrophoresis, as published. [16].

ply and *mly* PCR Detection and RFLP Signatures

The presence of *ply* (encoding pneumolysin, a cholesterol-dependent pneumococcal citolysin) or *mly* (a *ply* homologue identified in some *S. mitis* isolates), [32] was screened by digesting the amplification product with BsaAI and separating the fragments by agarose gel electrophoresis, as published. [15].

Multilocus Sequence Typing (MLST)

The amplification of internal fragments of seven housekeeping genes (*aroE*, *gdh*, *gk*i, *recP*, *spi*, *xpt*, and *ddl*) and allele assignment were carried out essentially as described in the international pneumococcal MLST database. [33] Sequencing was performed at Macrogen, Inc. (Seoul, Korea) and the sequencing analysis was conducted with DNAStar (Lasergene). For non-pneumococcal isolates allele assignment was done internally using arbitrary numbers following the same principles of the published MLST schemes. The eBURST algorithm [34] was used for determining the population structure of the *S. pseudopneumoniae* isolates. Two strains were considered in the same clonal complex when at least four of the six alleles were identical (the *ddl* allele was not systematically determined for these isolates and was thus excluded from the analysis). Nucleotide sequences were submitted to the GenBank database (submission grp 3980184) and are also available from the corresponding author.

Multilocus Sequence Analysis (MLSA)

Phylogenetic analysis using MLST data was done by concatenating the sequences of all MLST loci except *ddl* to obtain one single sequence of 2,758 bp. [21] MLST allele sequences of *S. pneumoniae, S. mitis*, *S. pseudopneumoniae*, and *S. oralis* previously described were used as controls. [15,35,36,37] Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 as previously described. [15,38].

Results and Discussion

To obtain insights on the nature and characteristics of 132 Spanish isolates presumptively identified as atypical pneumococci recovered from invasive and non-invasive disease sources, we performed several phenotypic and genotypic assays.

For species assignment MLSA was performed as described previously using the study isolates as well as the collections previously described by Chi et al. and Simões et al. [15,35] For 22 isolates one or more MLST alleles could not be obtained despite repeated attempts using various primers and several different amplification conditions. For this reason, these isolates were not fully characterized. For the 110 remaining isolates MLSA was performed and identified 61 isolates as *S. pseudopneumoniae*, 34 as *S. pneumoniae*, and 13 as *S. mitis*; within the *S. pneumoniae* branch two outliers closer to the root of the tree were noted and these remained unidentified (Figure 1). Isolates which are clearly closely related to *S. pneumoniae* but for which species assignment is not obvious have also been described by others. [39].

Overall, the 22 invasive isolates were identified as 12 *S. pneumoniae*, 8 *S. pseudopneumoniae*, 1 *S. mitis*, and 1 unidentified isolate. The 110 non-invasive disease isolates were identified as 53 *S. pseudopneumoniae*, 22 *S. pneumoniae*, 12 *S. mitis*, and 23 unidentified isolates. In all groups sporadic alleles associated in the MLST database with typical pneumococci were noted (Table S1). The phenotypic and genotypic characteristics of each group of isolates are summarized in Table 1 and are discussed below.

S. pseudopneumoniae

A total of 61 *S. pseudopneumoniae* were identified by MLSA and were further analyzed. The clinical sources of the *S. pseudopneumoniae* isolates were: sputum (n = 32), bronchial aspirate (n = 17), bronchoalveolar lavage (n = 4), blood (n = 2), conjunctiva (n = 2), nasal swab (n = 1), bronchoscopic-protected catheter brush (n = 1), pharyngeal swab (n = 1), and ascitic fluid (n = 1). The majority (88.5%) of the *S. pseudopneumoniae* were isolated from adults, and the male gender was predominant (68.9%) (data not shown).

Antimicrobial non-susceptibility rates were high against penicillin (60.7%) and erythromycin (42.6%), as shown in Table 2. Among the 26 macrolide-resistant isolates, the MLSB phenotype and the M phenotype were equally distributed. Only nine *S. pseudopneumoniae* isolates were fully susceptible to all antimicrobials tested. High macrolide-resistance rates have been described among isolates recovered from respiratory samples from New Zealand, [40] and France. [18] Fluoroquinolone resistant isolates have also been described. [41] The high antimicrobial resistance rates together with the confirmation of the ability of this microorganism to cause invasive diseases raises this pathogen as a real clinical concern.

The 61 *S. pseudopneumoniae* isolates displayed heterogeneous profiles regarding several of the phenotypic and genotypic characterization assays that were performed (Table 1). In particular, 16.4% of the isolates were susceptible to optochin in a 5%CO_2-enriched atmosphere and 63.9% were susceptible in ambient atmosphere. Only 50.8% of the *S. pseudopneumoniae* isolates displayed the typical phenotype originally described for this species (optochin-resistant in CO_2 but susceptible in O_2 atmosphere). Also, 36.1% of the isolates were bile soluble. Although these biochemical traditional identification tests are the first step for phenotypic identification of *S. pseudopneumoniae*, in the present study we observed that these characteristics were frequently diverse among the isolates of this species, as previously shown. [42].

Screening for genetic markers described by others as species-specific for *S. pneumoniae* – specific 16S-rRNA, *Spn9802, pspA* and *ply* - revealed their presence in some *S. pseudopneumoniae* isolates in contrast with previous publications. [22,23,43] No *S. pseudopneumoniae* isolates harbored the pneumococcal *lytA* nor the *cps*A capsular gene. The *ali*B-like ORF2 was present in all isolates. The lack of *cpsA* was in line with previous observations that suggest *S. pseudopneumoniae* lacks a pneumococcus-like capsule. [44].

A high clonal diversity was found as 59 allelic profiles were detected by MLST (Figure 2 and Table S1). By e-BURST seven clonal groups were identified and each contained only two allelic

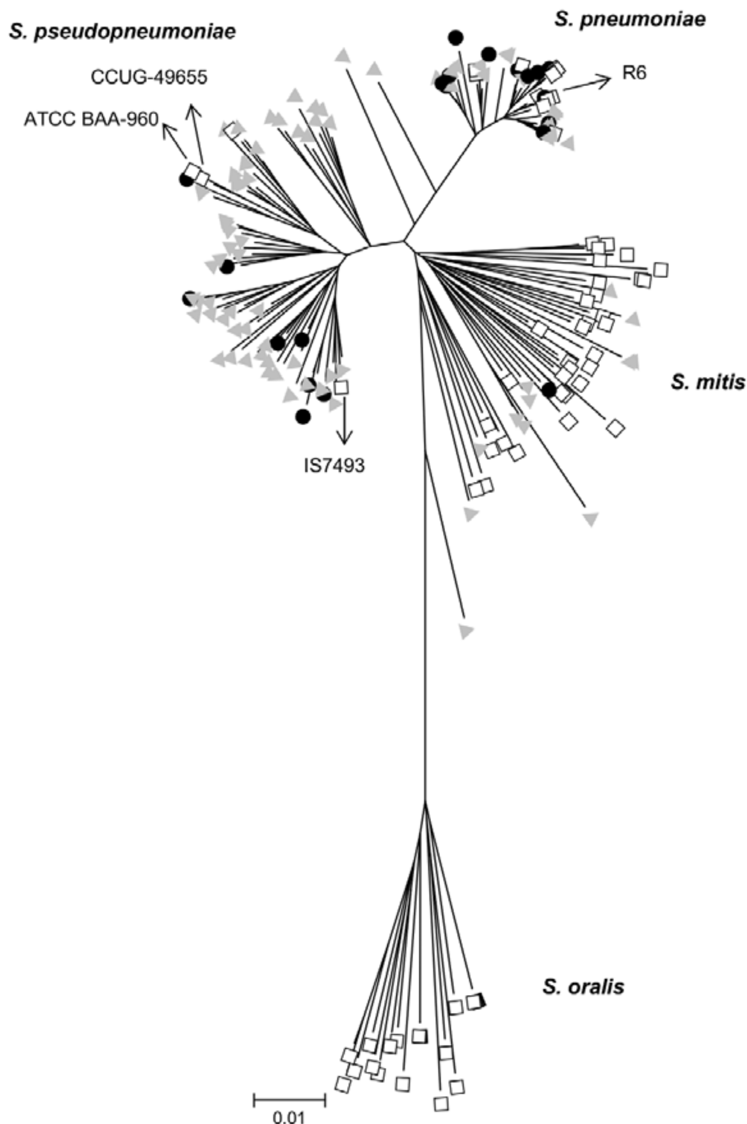

Figure 1. Genetic relationships of the strains determined by MLSA. The symbols indicate: grey triangle, non-invasive disease strains; black circle, invasive disease strains; white square, strains described in other studies. [15,17,35,36,37].
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profiles. On two occasions, pairs of isolates were found to have the same allelic profile. No association between isolates sharing a same allelic profile or being in the same clonal group was obvious.

S. pneumoniae

Out of 34 *S. pneumoniae* identified in this collection, 23 isolates previously identified as non-(sero)typeable pneumococci were in fact capsulated when reanalyzed; the other 11 were confirmed as non-typeable. Several explanations could be put forward to justify why isolates previously identified as atypical pneumococcal were found to be capsulated upon reanalysis. For example, differences in the quality of the antisera, lack of capsular production due to passage of isolates on agar plates, and human error.

The clinical sources of the capsulated isolates were sputum (n = 14), bronchoalveolar lavage (n = 2), blood (n = 5), transthoracic needle aspiration (n = 1), and umbilical swab (n = 1). The majority were isolated from adults (87.5%), and the male gender was predominant (75.0%). The clinical sources of the non-capsulated isolates were blood (n = 4), sputum (n = 4), conjunctival

Table 1. Phenotypic and genotypic characterization of MLSA typeable isolates.

	MLSA classification (%)			
	S. pseudopneumoniae (n = 61)	*S. pneumoniae*		*S. mitis* (n = 13)
		typeable (n = 23)	nontypeable (n = 11)	
Phenotypic characterization				
optochin susceptibility (≥14 mm)				
5% CO_2	10 (16.4)	8 (34.8)	6 (54.6)	4 (30.8)
ambient atmosphere	39[a] (63.9)	21 (91.3)	6[b] (54.6)	8[c] (61.5)
bile solubility	22 (36.1)	22 (95.7)	11 (100)	2 (15.4)
Genotypic characterization				
PCR-based				
pneumococcal *lyt*A	0 (0)	23 (100)	11 (100)	0 (0)
pneumococcal specific 16S-rRNA	49 (80.3)	23 (100)	11 (100)	2 (15.4)
*Spn*9802	59 (96.7)	23 (100)	8 (72.7)	8 (61.5)
*psp*A	1 (1.6)	21 (91.3)	10 (90.9)	7 (53.8)
*cps*A	0 (0.0)	17 (73.9)	2 (18.2)	0 (0)
*ali*B-like ORF2	61 (100.0)	7 (30.4)	9 (81.8)	12 (92.3)
RFLP signatures				
pneumococcal *lyt*A/atypical *lytA*	0 (0)/61 (100)	23 (100)	9 (81.8)/2 (18.2)	0/11(84.6)[d]
ply/mly	7 (11.5)/54 (88.5)	23 (100)/0 (0)	11 (100)/0	2 (15.4)/7 (53.8)[e]

[a]11 strains did not grow in an ambient atmosphere, among the 39 isolates susceptible to optochin in ambient atmosphere, 31 were resistant in CO_2.
[b]3 strains did not grow in ambient atmosphere.
[c]2 strains did not grow in ambient atmosphere.
[d]2 strains were not screened.
[e]2 strains did not amplify, 2 yielded a mixed pattern.
doi:10.1371/journal.pone.0057047.t001

swab (n = 2), and nasal swab (n = 1). The majority (90%) were isolated from adults, and 40% were males (data not shown).

Among the capsulated isolates, the most frequent serotypes were 38 and 6B (3 isolates each, Table 3). Interestingly, these serotypes were also frequently misidentified as atypical pneumococci in a recent study from the USA. [45] This observation may indicate that some representatives of these serotypes may be hard to visualize by the Quellung reaction, leading to misidentification, or that these serotypes may contain unknown different subtypes.

Multiresistance (non-susceptibility to three or more classes of antimicrobials) was found among 11 isolates (3 were from invasive disease) and was associated to NT (n = 4), and serotypes 6B (n = 3), 19F (n = 2), 19A (n = 1), and 33F (n = 1) (Table 3). Three of the eleven NT isolates were multiresistant. A high frequency of multiresistance among non-typeable strains has been observed in other studies. [7,9].

Regarding the classical presumptive identification of pneumococci based on optochin susceptibility in CO_2 atmosphere and bile solubility, many exceptions were found among this group of isolates: 20 were optochin resistant and one was bile insoluble. Although rare, these exceptional phenotypes were previously reported in other studies. [46].

Table 2. Antimicrobial susceptibility of 61 *S. pseudopneumoniae* clinical isolates.

Antibiotic	MIC (mg/L)			No. non-susceptible isolates (%)
	Range	MIC_{50}	MIC_{90}	
Penicillin	≤0.03–2	≤0.03	0.5	37 (60.7%)
Cefotaxime	≤0.03–1	≤0.12	0.25	2 (3.3%)
Erythromycin	≤0.12–≥128	≤0.12	≥32	26 (42.6%)
Clindamycin	≤0.12–≥128	≤0.12	≥0.5	13 (21.3%)
Cotrimoxazole	≤0.5/9.5–≥2/38	≤0.5/9.5	≥2/38	24 (39.3%)
Tetracycline	≤0.12–64	≤0.25	4	18 (29.5%)
Ciprofloxacin	≤0.12–32	≤1	≤1	6 (9.8%)
Levofloxacin	≤0.12–≥16	≤1	≤1	3 (4.9%)
Chloramphenicol	≤2–4	≤2	≤2	0 (0%)

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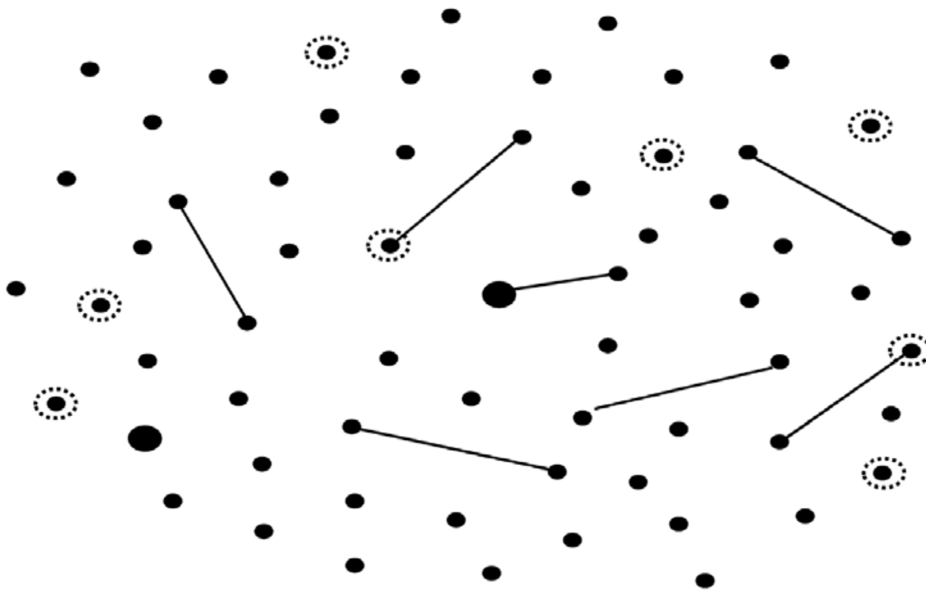

Figure 2. Representation of the *S. pseudopneumoniae* population by eBURST analysis. Each point represents a different allele combination. Solid lines, single-locus variants; dashed circles, invasive disease isolates; larger circles indicate two isolates with the same allele combination.
doi:10.1371/journal.pone.0057047.g002

Genotypic analysis showed the ubiquitous presence of pneumococcal *lytA*, specific16S-rRNA, and *ply*. *Spn9802* was present in all but three non-typeable isolates contrasting with previous publications that suggested that this ORF was ubiquitous in pneumococcus. [22,23].

The *lytA*-typical pneumococcal RFLP signature was identified in all but two isolates. The two exceptions were associated with a novel signature also distinct from the characteristic atypical pattern associated with non-pneumococcal isolates. The molecular basis of this novel signature is currently being investigated.

The capsular gene *cps*A was present in most capsulated isolates with the exception of those of serotypes 25A and 38 in agreement with published literature. [9,47] Instead, isolates of serotype 25A and 38 had *aliB*-likeORF2 as described, [9] which was also detected in single isolates of serotype 35A. Among non-typeable isolates, nine had aliB-like ORF2 and two had *cpsA*. A possible explanation for this latter observation is that the isolates may have lost the capacity to produce a capsule *in vitro* [14] due to alterations in the capsular genes. [48].

MLST analysis of the *S. pneumoniae* isolates showed that close to one-third (32.4%) had novel allelic profiles. Of interest, six of the nine allelic profiles identified among the non-typeable pneumococcal isolates were previously identified in other countries and were also associated to non-serotypeability. The international PMEN lineages USA^{NT}-ST448 and Norway^{NT}-ST344 accounted for five isolates, three having been recovered from invasive disease. Non-typeable pneumococci were previously found not only among colonization, but also as causative agents of acute otitis media and conjunctivitis. [7,9,11,12,14] The association of MLST lineages exclusive of non-capsulated isolates to invasive disease has only been described recently. [14] These observations suggest that, in spite of their sporadic occurrence, non-typeable pneumococci have a higher clinical impact than previously thought as they have been associated with a varied spectrum of infections including invasive disease.

S. mitis

Although the 13 *S. mitis* isolates were phenotypically and genotypically heterogeneous, *lytA* analyses (in addition to MLSA) consistently suggested they were not pneumococci. Of interest, and as observed for some *S. pseudopneumoniae* isolates, a few of the *S. mitis* harboured genetic markers – *Spn9802*, *pspA* and *ply* - previously associated to pneumococci. The occurrence of *S. mitis* isolates harbouring genes encoding *S. pneumoniae* virulence factors has been described, [15,26] and led to the suggestion that identification of this group of bacteria by a single identification marker may not be possible as horizontal gene transfer between them can occur. [24,27].

Regarding antimicrobial susceptibility, 84.6% were non-susceptible to penicilin and 69.2% were multidrug resistant. Most of the isolates (12/13) were recovered from non-invasive disease; however, one isolate was recovered from bronchoalveolar lavage. *S. mitis* isolates have been previously associated with disease, [36,49,50] and high levels of antimicrobial resistance. [15,51].

Non-classified Isolates

Close to one-fifth of the isolates (18.2%) remained non-classified. Although MLSA associated to the MLST *S. pneumoniae* scheme works well to identify atypical isolates, we were unable to apply it to 24 isolates due to lack of amplification of some DNA fragments with the combinations of primers that are routinely used for *S. pneumoniae*. For these isolates, alternative primers, MLSA schemes or assays would have been needed. [52] Of note, only one isolate was recovered from invasive disease.

Table 3. Properties of *S. pneumoniae* clinical isolates.

Serotypes	Sequence type[a] (no. of isolates)	MLST allelic profile	Antimicrobial non-susceptibility pattern[b]	Observations[c]
6B	90 (1)	5-6-1-2-6-3-4	PEN, TET, ERY, CLI, CTX	Spain[6B]-ST90
	94 (1)	5-6-1-2-6-3-54	PEN, TET, CHL, ERY, CLI, SXT, CIP	Spain[6B]-ST90 SLV
	8270 (1)	32-28-1-1-15-52-15	TET, ERY, CLI	
38	393 (2)	10-43-41-18-13-49-6	Susceptible	
	8278 (1)	10-61-41-18-13-49-6	Susceptible	
13	70 (1)	2-13-1-4-6-12-1	Susceptible	
	8271 (1)	7-13-**368**-4-6-1-20	Susceptible	
19F	89 (1)	5-5-7-7-8-5-1	PEN, TET, CHL, SXT	
	8275 (1)	5-5-7-7-8-5-**538**	PEN, TET, CHL, ERY, CLI, CTX, SXT	
25A	393 (1)	10-43-41-18-13-49-6	Susceptible	
	8274 (1)	10-43-41-18-13-37-6	PEN, SXT	
3	180 (1)	7-15-2-10-6-1-22	Susceptible	Netherlands[3]-ST180
4	247 (1)	16-13-4-5-6-10-14	Susceptible	
7F	2178 (1)	10-20-14-1-6-20-29	TET	Denmark[12F]-ST218 SLV
10A	**8272 (1)**	5-13-4-4-6-1-20	Susceptible	
17A	**8277 (1)**	5-**365**-2-16-6-3-245	Susceptible	
18C	191 (1)	8-9-2-1-6-1-17	Susceptible	Netherlands[7F]-ST191
19A	81 (1)	4-4-2-4-4-1-1	PEN, TET, CHL, ERY, CLI, CTX, SXT, CIP, LEV	Spain[23F]-ST81
20	**8269 (1)**	15-**364**-8-18-15-1-31	Susceptible	
22F	2104 (1)	2-16-1-4-6-1-1	Susceptible	
33F	1012 (1)	2-5-29-18-42-3-18	TET, ERY, CLI	
35A	1273 (1)	10-12-4-12-9-28-18	Susceptible	
NT	448 (2)	8-5-2-27-2-11-71	Susceptible	USA[NT]-ST448
	508 (2)	13-8-65-1-60-16-6	Susceptible	
	66 (1)	2-8-2-4-6-1-1	PEN, TET, SXT, CIP, LEV	
	72 (1)	2-13-2-4-9-4-1	Susceptible	
	344 (1)	8-37-9-29-2-12-53	PEN, TET, ERY, SXT	Norway[NT]-ST344
	942 (1)	8-10-15-27-2-28-4	PEN, SXT	
	8268 (1)	8-10-84-1-2-14-4	Susceptible	
	8273 (1)	8-37-2-27-2-11-53	Susceptible	USA[NT]-ST448 DLV
	8276 (1)	8-178-9-29-2-12-15	PEN, TET, ERY, CLI, SXT	Norway[NT]-ST344 DLV

[a]Novel STs and alleles found in this study are represented in bold.
[b]PEN, penicillin; CTX, cefotaxime; ERY, erythromycin; CLI, clindamycin; TET, tetracycline; CHL, chloramphenicol, SXT, trimethoprim-sulfamethoxazole non-susceptible; CIP, ciprofloxacin; LEV, levofloxacin.
[c]International clones of PMEN; SLV, Single Locus Variant; DLV, Double Locus Variant.
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Conclusions

In summary, among disease isolates classified as atypical pneumococci, close to half (46.2%) were *S. pseudopneumoniae*, and only a quarter were pneumococci (17.4% were capsulated and 8.3% were non-typeable). In addition, 9.8% were *S. mitis* and the rest were non-pneumococci that remained unidentified. In agreement with other studies, we found that many of the currently proposed methodologies to distinguish pneumococci from closely-related species are not species-specific. Furthermore, *S. pseudopneumoniae* that failed to have the optochin phenotypes described by Arbique et al. were also identified.

We found that *S. pseudopneumoniae* have low clonality and that antimicrobial resistance is well-disseminated is this species. Our study stresses the clinical role of *S. pseudopneumoniae* and non-typeable pneumococci since they have the capacity to cause invasive disease and the high antimicrobial resistance rates are of concern.

Supporting Information

Table S1 MLST allelic profiles of non-pneumococcal isolates. Invasive strains are indicated in bold. Most alleles are divergent from all the alleles described at the *S. pneumoniae* MLST database as of July 26, 2012. The allele number of the closest match is indicated; similarity (in %) is indicated in parenthesis. ND, not determined.
(DOCX)

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Author Contributions

Revised the manuscript and approved the final version: RSL DR ASS AD AF JL HL CA. Conceived and designed the experiments: DR ASS JL CA RSL. Performed the experiments: DR ASS AD. Analyzed the data: DR ASS AD CA RSL. Contributed reagents/materials/analysis tools: AF JL HL CA RSL. Wrote the paper: DR ASS RSL.

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CHAPTER V: *S. pseudopneumoniae* populations causing acute exacerbations.

Objective 7: To analyse the clinical and demographic characteristics of COPD patients infected by *S. pseudopneumoniae* (2001-2012).

Domenech A, Puig C, Santos S, Marti S, Ardanuy C, Liñares J. Clinical characteristics of patients with chronic obstructive pulmonary disease (COPD) infected by *Streptococcus pseudopneumoniae.* 53rd Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC). September 2013, Denver, United States.

1 **Clinical characteristics of patients with chronic obstructive**
2 **pulmonary disease (COPD) infected by *Streptococcus***
3 ***pseudopneumoniae.***

4

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21 **Running title:** *S. pseudopneumoniae* in COPD

22 **Key words:** *Streptococcus pseudopneumoniae,* chronic obstructive
23 pulmonary disease, acute exacerbation

24

25

26

27 **Abstract.**

28 **Background.** Few data are available about the clinical relevance of *S.*
29 *pseudopneumoniae* in COPD patients. We aimed to analyze the clinical
30 characteristics of COPD patients infected by this potential pathogen.

31 **Methods.** From 2002 to 2012 a collection of 36 *S. pseudopneumoniae*
32 were isolated from good quality sputum samples and high predominance
33 of Gram positive diplococci. They were recovered from 36 patients
34 suffering acute exacerbations (n=35) and pneumonia (n=1). Identification
35 was described previoulsy (Rolo *et al.* PlosOne 2013); antimicrobial
36 susceptibility was performed by microdilution.

37 **Results.** The mean age of the patients was 69.7 years (SD±8.6) and 32
38 (88.9%) of them were men. Their GOLD status was: GOLD II (n= 8),
39 GOLD III (n= 13) and GOLD IV (n= 15). Besides COPD, 25 patients had
40 other co-morbidities, mainly systemic arterial hypertension (n=15),
41 dyslipemia (n=15), diabetes mellitus (n=9) and/or cardiovascular disease
42 (n=5). In 20 episodes (19 acute exacerbations and one pneumonia) *S.*
43 *pseudopneumoniae* was isolated as a single pathogen.

44 Thirteen patients did not required hospitalization, six patients were
45 hospitalized ≤3 days, seven patients between 4 and 9 days, and eight
46 patients suffered severe episodes which required long-term hospitalization
47 (≥10 days); nevertheless, all 36 patients had a successful outcome.

48 Antimicrobial susceptibility rates were: 25.0% for penicillin, 58.3% for
49 erythromycin, 75.0% to clindamycin, 72.2% for tetracycline and 97.2% to
50 levofloxacin. Six isolates were susceptible to all antimicrobials tested.

51 **Conclusions.** *S. pseudopneumoniae,* despite the low frequency has an
52 aetiological role in acute exacerbations of patients with advanced COPD.
53 It is remarkable that two thirds of patients required hospitalization.

54

55

56 **Introduction.**

57 *Streptococcus pseudopneumoniae* is a species which belongs to the
58 viridans group streptococci (VGS), and shares certain characteristics with
59 *Streptococcus pneumoniae.*[1] Both species have 99% 16S rRNA gene
60 identity, but *S. pseudopneumoniae* exhibits DNA-DNA hybridization
61 values of 70%, and is phenotypically distinct.[1]

62 Two phenotypic characteristics are classically used for the presumptive
63 identification of *S. pseudopneumoniae:* deoxycholate (DOC) insolubility
64 (commonly referred to as bile insolubility) and optochin (OPT) resistance
65 when incubate in an atmosphere enriched in CO_2, but OPT susceptible
66 when incubated in ambient atmosphere. However, in a recent study
67 performed in our geographical area, only a half of the *S.*
68 *pseudopneumoniae* isolates displayed this typical phenotype, making
69 proper identification even more difficult.[2]

70 It has been reported that *S. pseudopneumoniae* can be isolated during
71 episodes of invasive disease, such as bacteriemia or pneumonia;
72 however, it has usually been associated with sputum samples, maybe
73 associated with Chronic Obstructive Pulmonary Disease (COPD).[2-4] In
74 fact, it can be difficult to determine whether *S. pseudopneumoniae* acts a
75 respiratory pathogen or whether it is simply a respiratory tract colonizer,
76 such as other species of VGS.[4]

77 It has been suggested that non-typeable *S. pneumoniae* isolates acts as a
78 reservoir for genes conferring antimicrobial resistance. *S.*
79 *pseudopneumoniae* also could act as the same way, being supported by
80 the fact that a recent analysis of the whole genome of 4 *S.*
81 *pseudopneumoniae* isolates revealed the presence of several
82 pneumococcal virulence factors and determinants of antimicrobial
83 resistance.[5]

84 In this study we aimed to analyse the clinical characteristics of COPD
85 patients infected by this potential pathogen, and its role as a reservoir of
86 antimicrobial-resistant determinants.

87 **Material and methods.**

88 **Ethical statement**

89 This study and the publication of the results were approved by the
90 “Comité Ètic d’Investigació Clínica de l’Hospital Universitari de Bellvitge
91 (HUB)”. Sputum samples and bacterial strains were recorded in an
92 anonymised database.

Study design and bacterial isolates

This laboratory-based study was prospectively carried out between 2009 and 2012 at the Hospital Universitari de Bellvitge in the south of Barcelona. Only *S. pseudopneumoniae* isolated from good quality sputum samples were considered (<10 squamous cells and >25 leucocytes per low-power field), with a predominance of Gram-positive diplococci. Clinical and demographic characteristics of the patients were recorded in a database.

Sputum samples were quantitatively cultured as was described previously, with the aim to know the colony forming units per ml of sputum sample (cfu/ml).[(J linf)] When the presence of *S. pseudopneumoniae* was suspected, twelve colonies were picked, and phenotypic methods were performed to all of them.[2] Further molecular analysis (multi locus sequence analysis, MLSA) was performed in order to confirm its identification (www.mlst.net).

In addition, a retrospective analysis was performed, including those COPD patients infected with *S. pseudopneumoniae* during the period 2001-2009, which molecular analysis was previously published.[2] For this second part, computerized medical records of COPD patients were reviewed and COPD status was determined according to the international GOLD criteria (where GOLD stands for Global Initiative for Chronic Obstructive Lung Disease).[6]

Antimicrobial susceptibility

116 The antimicrobial susceptibility to the nine frequent antibiotics used for the
117 treatment of *S. pneumoniae* infections was tested by microdilution and/or
118 the disk diffusion method. The *S. pneumoniae* antimicrobial susceptibility
119 breakpoints defined by Clinical Laboratory Standard Institute (CLSI) were
120 used,[8] since no breakpoints for S. pseudopneumoniae has been defined
121 yet.

122 **Statistical analysis.**

123 Statistical analyses were carried out using SPSS version 18.0, using Chi-
124 square or Fisher's exact tests to compare proportions. Two-sided P values
125 less than 0.05 were considered statistically significant.

126

127

128 **Results.**

129 A total of 36 *S. pseudopneumoniae* strains were isolated from 36 COPD
130 patients. The mean age was 69.7 years (Standard Deviation ±8.6), and 32
131 (88.9%) of them were men. The GOLD status of the patients is shown in
132 table 1.

133 Besides COPD, 25 (69.4%) patients had other co-morbidities: one
134 underlying disease (n=11 patients), two underlying diseases (n=6), three
135 underlying diseases (n=6) and four underlying diseases (n=2). The main
136 co-morbidities of these patients were detailed in table 1.

137 Thirty-five of 36 episodes were diagnosed as acute exacerbation
138 episodes, which required an increase in regular medication; the remaining
139 case was diagnosed as pneumonia episode due to the presence of new
140 infiltrates on chest radiography.

141 In 20 episodes (nineteen acute exacerbations and one pneumonia), *S.*
142 *pseudopneumoniae* was isolated as a single pathogen. Whereas, in the
143 remaining 16 episodes *S. pseudopneumoniae* was isolated together with
144 another potentially pathogenic bacterium: *Pseudomonas aeruginosa*
145 (n=9), *Haemophilus influenzae* (n=3), *Haemophilus parainfluenzae* (n=2),
146 *Moraxella catarrhalis* (n=1) and *Acinetobacter baumannii* (n=1). However,
147 all sputum samples were purulent, with a good quality and had
148 predominance of Gram-positive diplococci, suggesting the pathogenic role
149 of *S. pseudopneumoniae*. In addition, in 7 of the 16 polimicrobial
150 episodes, occurred between 2010 and 2012, and the sputum sample was
151 available for a quantitative culture. In all cases *S. pseudopneumoniae* was
152 detected in $>10^8$ colony-forming units per millilitre (cfu/ml).

153 Regarding the infection severity, 13 patients suffered mild acute
154 exacerbations and did not required hospitalization, whereas the remaining
155 22 patients had severe acute exacerbations and were hospitalized: 6
156 patients less than ≤3 days, 7 patients between 4 and 9 days, and 8
157 patients ≥10 days. The patient diagnosed with pneumonia episode
158 required only one-day hospitalization. Nevertheless, all 36 patients had a
159 successful outcome.

160 Although the number of acute exacerbations caused by *S.*
161 *pseudopneumoniae* is low, no differences were observed among the
162 severity of the episodes and the number of underlying conditions or the
163 FEV_1 value: 10 of 13 patients suffering mild acute exacerbations had at
164 most another underlying disease than COPD and a mean FEV_1= 35.3%
165 ±12.3; while, 7 of 8 patients requiring long-time hospitalization (≥10 days)
166 had less than 2 co-morbidities besides COPD with a mean FEV_1= 36.7%
167 ±12.3 (*P*= 1.000). In this way, no differences among the severity of acute
168 exacerbations and the number of potentially pathogenic bacterium
169 isolated: 6 of 20 (30%) acute exacerbations caused by *S.*
170 *pseudopneumoniae* as a single pathogen required ≥10 days of
171 hospitalization, and 2 of 16 (12.5%) episodes with two pathogens (*P*=
172 0.257).

173 The most frequent antimicrobial treatments were betalactams (n=16)
174 [amoxicillin-clavulanic acid (n=14), cefuroxime (n=1) and piperacillin-
175 tazobactam (n=1)], followed by fluoroquinolones (n=10) [levofloxacin
176 (n=4), moxifloxacin (n=4) and ciprofloxacin (n=1)], and clindamycin (n=1).
177 Eight patients (most of them suffering mild acute exacerbations) were
178 treated with inhaled corticosteroids and did not received antimicrobial
179 treatment.

180 Table 2 shows the antimicrobial susceptibility of the 36 *S.*
181 *pseudopneumoniae* isolates. According to *S. pneumoniae* CLSI
182 breakpoints for *S.pneumoniae*, six isolates were susceptible to all the

183 antimicrobials tested. Susceptibility rates to penicillin were low, 25.0%
184 using oral penicillin V breakpoints for *S. pneumoniae* and 30.6% using
185 *Streptococcus* spp Viridans Group breakpoints. All isolates were
186 susceptible to chloramphenicol and only two isoltates showed resistance
187 to fluoroquionolones. In contrast, nearly a half of isolates were only
188 susceptible to erythromycin (58.3%) and co-trimoxazole (55.6%). Among
189 fifteen *S. pseudopneumoniae* isolates with resistance to erythromycin,
190 nine also showed resistance to clindamycin (MLS_B phenotype), whereas
191 the remaining six isolates were clindamycin-susceptibility (M phenotype).

192

193

194 **Discussion.**

195 The clinical relevance of *S. pseudopneumoniae* has not been established
196 yet, in part due to its phenotypic and genetic heterogeneity, which has
197 made it difficult to identify. Several approaches are reported; however,
198 only multi-locus sequence analysis (MLSA) seems to be able to
199 discriminate *S. pseudopneumoniae* from *S. pneumoniae* and other SGVs
200 (2). For this reason, the prevalence of *S. pseudopneumoniae* as a human
201 pathogen could be underestimated.
202 In the present study, we analysed a prospective collection of *S.*
203 *pseudopneumoniae* isolates recovered from COPD patients during 2010-
204 2012 period, and a retrospective analysis from 2001 to 2009. In our
205 series, a half (n= 16/36) of *S. pseudopneumoniae* isolates were isolated

206 during the 2010-2012 period. It could be explained by two facts: the first
207 one, as consequence of an emergence of this pathogen causing acute
208 exacerbations in the recent years. This species has been described nine
209 years ago and no data are available about its frequency causing infection.
210 In a prospective study performed by our group, we isolated *S.*
211 *pseudopneumoniae* in a 5% (n=9/184) of acute exacerbations which
212 occurred in severe COPD patients during one-year period (2010-2011).
213 The main limitation of this study is that it was performed in a single
214 hospital and no incidence data was available; therefore, further studies
215 are needed in order to determine its frequency and its relevance as a
216 pathogen.
217 The second one, could be explained by the fact that in the prospective
218 part (from 2010 to 2012), the sputum samples were quantitatively cultured
219 and blood agar plates were incubated in 5% CO2 and ambient air
220 atmospheres; in contrast, sputum samples from 2001 to 2009 period were
221 qualitative cultured and only incubated into a CO_2 atmosphere; in this
222 condition, *S. pseudopneumoniae* usually shows optoquin-resistance, as
223 other SGV species). For this reason, in COPD patients, especially those
224 with severe airflow obstruction which has the lower respiratory tract
225 colonized by several microorganisms, the isolation of *S.*
226 *pseudopneumoniae* could be underestimated because the absence of a
227 gold standard technique which difficult its identification.
228 In our series, in all but one episodes (a pneumonia episode), *S.*
229 *pseudopneumoniae* were isolated during acute exacerbation episodes. All

230 patients had a successful outcome; however, it is remarkable that two
231 thirds of patients required hospitalization and eight of them, a long- term
232 hospitalization (more than 10 days). In addition, despite the number of
233 episodes is low, no differences were observed between the number of
234 underlying conditions of the patients and the severity of the episode.
235 These facts confirm the role of *S. pseudopneumoniae* as an etiological
236 agent of acute exacerbations, often requiring the hospitalization of the
237 patient, even though this pathogen has a low fatal outcome.
238 *S. pseudopneumoniae* is a species belonging to Viridans Group, however
239 it is closely related to *S. pneumoniae* and for this reason, we tested the
240 antimicrobial susceptibility using the CLSI breakpoints for *S. pneumoniae*.
241 Globally, non-susceptibility of *S. pseudopneumoniae* isolates was high, as
242 was reported previously in France and New Zeland.[3,9] In fact, only 6
243 isolates were fully susceptible to all the antimicrobials tested, and a third
244 of the 36 isolates was multi-drug resistant (defined as non-susceptibility to
245 at least three antimicrobial classes). Regarding penicillin non-
246 susceptibility, similar rates of reduced susceptibility were found for *S.*
247 *pseudopneumoniae*, using the oral penicillin V breakpoints for *S.*
248 *pneumoniae* and the *Streptococcal* spp. Viridans Group.[8] This high
249 antimicrobial resistance rates is a cause of concern, however, in the
250 present study, all 14 patients which received antimicrobial treatment with
251 betalactams, had a successful outcome, and no treatment failure was
252 observed.

253 This study was performed in a single medical centre, and the
254 number of isolates found may not to be representative of the frequency of
255 *S. pseudopneumoniae* causing acute exacerbations in COPD patients.
256 Although the patients included in this study had several underlying
257 diseases, two thirds of them required hospitalization for the treatment of
258 an acute exacerbation episode caused by *S. pseudopneumoniae*. The
259 absence of a rapid gold standard technique for the identification of *S.*
260 *pseudopneumoniae,* supported by the fact that a half of episodes
261 occurred during the 2010-2012 period (prospective period), suggests that
262 the frequency of *S. pseudopneumoniae* causing disease in COPD
263 patients is underestimated.

264

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301

302 Table 1. Clinical and demographic characteristics of the 36 patients
303 infected by *S. pseudopneumoniae* isolates.
304

Mean age in years (SD; range):	69.7 (± 8.6)
Male	32 (88.9%)
GOLD status:	
I	0
II	8
III	13
IV	15
Clinical syndrome:	
Acute exacerbation	35
Pneumonia	1
Other main underlying diseases:	
systemic arterial hypertension	15 (41.7%)
dyslipemia	15 (41.7%)
Diabetes mellitus	9 (25.0%)
Cardiovascular disease	5 (13.9%)
Obesity	3 (8.3%)
Drink abuser	1 (2.8%)
Cancer	1 (2.8%)

305 Table 2. *In vitro* activity of nine antimicrobials against *S.*
306 *pseudopneumoniae* isolates causing disease in COPD patients

307

Antimicrobial	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	MIC range (µg/mL)	% S	% I	% R
Penicillin	0.5	2	≤0.03 - 4	25.0[a] 97.2[b] 30.6[c]	61.1 2.8 69.4	13.9 0.0 0.0
Cefotaxime	0.25	0.5	≤0.03 - 2	97.2	2.8	0.0
Ciprofloxacin	≤0.5	2	≤0.5 - >32	94.3[d]	-	5.7
Levofloxacin	≤0.5	2	≤0.5 - >32	97.2	0.0	2.8
Tetracycline	≤2	>32	≤2 - >32	72.2	2.8	25.0
Erythromycin	≤0.25	≥32	≤0.25 - >32	58.3	0.0	41.7
Clindamycin	≤0.25	≥32	≤0.25 - >32	75.0	0.0	25.0
Chloramphenicol	≤2	≤2	≤2	100.0	-	0.0
Co-trimoxazole	≤0.5/9.5	≥2/38	≤0.5/9.5 - >4/76	55.6	13.8	30.6

308

309 Clinical Laboratory Standard Institute (CLSI) breakpoints for *S.*
310 *pneumoniae*: [a]Oral penicillin V breakpoints (S: ≤0.06 mg/L, I: 0.12 - 1
311 mg/L and R: ≥2 mg/L). [b]Penicillin parenteral penicillin (non-meningitis)
312 breakpoints (S: ≤2 mg/L, I: 4 mg/L and R: ≥8 mg/L). [c]Penicillin
313 breakpoints for *Streptococcus* spp. Vºiridans Group (S: ≤0.12 mg/L, I: 0.25
314 - 2 mg/L and R: ≥4 mg/L). [d]Non-susceptibility to ciprofloxacin breakpoint
315 MIC >4 mg/L.

CHAPTER VI: synopsis of results and discussion, and conclusions.

Synopsis of results and discussion

S. pneumoniae is an important human pathogen which has been widely studied all over the world. Most clinical studies have focused on invasive disease, and little information is available on the role of *S. pneumoniae* in patients with COPD. However, COPD is the main underlying disease of serious pneumococcal serious illness, and the third cause of non-contagious disease deaths throughout the world, after cardiovascular diseases and cancers (World Health Report, 2012); in fact, the WHO estimates that by 2030 COPD will be the third leading cause of all deaths worldwide (World Health Organization, 2005).

This thesis aimed to assess the role of *S. pneumoniae* and *S. pseudopneumoniae* populations causing disease in COPD patients, examining at the same time the serotype and genotype distribution of pneumococci causing acute exacerbations and pneumonia, analyzing the role of capsular type in the persistence of pneumococcal strains over time, and finally the impact of antimicrobial consumption on these persistent strains.

Several studies have described the frequency of the most important bacterial causes of acute exacerbations, all of them based on the GOLD status of the patients (Eller *et al.,* 1998; Ko *et al.,* 2007; Sethi *et al*., 2008). However, the new GOLD guidelines recommendations have changed, and a recent method based on the GOLD status, but also including the annual acute exacerbation episodes and the symptomatology of the patients has been

proposed. In order to establish the frequency of *S. pneumoniae* and other bacterial and fungal causes of acute exacerbations following the new guidelines, we performed the study described in chapter II.

Our series showed that *P. aeruginosa* was the most frequent pathogen isolated from AECOPD in patients with an advanced GOLD status, confirming the importance of this pathogen as a cause of acute exacerbations in patients with advanced disease; in fact, a third of patients analyzed suffered an AECOPD caused by *P. aeruginosa* at any time. The frequencies of *H. influenzae*, *S. pneumoniae*, and *M. catarrhalis* as etiological agents of AECOPD in our study were similar to those already described (Decramer *et al.,* 2012*;* Ko *et al.,* 2007; Sethi *et al*., 2008), *S. pneumoniae* being the cause of a sixth of the overall episodes.

No differences were observed in the pneumococcal distribution between patients with one annual AECOPD episode and patients with ≥2 episodes. In contrast, *H. influenzae* isolates were associated with patients with a low frequency of AECOPD episodes, and *Enterobacteriaceae* species were only detected in patients with frequent exacerbations. The high frequency of *P. aeruginosa* isolation has not been reported previously, and it should be taken into account in the management of these patients, especially with regard to the empirical antimicrobial treatment.

Several questions remained to be answered regarding *S. pneumoniae* and COPD patients, specifically with reference to the

distribution of pneumococcal populations (serotypes and genotypes), the persistence of pneumococcal strains, and the impact of antimicrobial therapy, which have led to the studies described in chapter III and chapter IV.

It is well known that capsular polysaccharide is the main virulence factor, and has an important role in the invasiveness of pneumococcal strains, since certain serotypes are associated with colonization or with pneumococcal bacteremia (Brueggemann *et al.,* 2003). For this reason, a different serotype distribution among pneumococci causing pneumonia and acute exacerbations in COPD patients was expected. This serotype distribution, together with a different antimicrobial susceptibility was described in chapter III. Thus, serotypes (and their related genotypes) 1 (Sweden^{1}-ST306), 3 (Netherlands^{3}-ST180 and ST260^{3}), 4 (ST247^{4}), 5 (Colombia^{5}-ST289) and 8 (Netherlands^{8}-ST53) were more frequently associated with pneumonia (mainly among bacteraemic pneumonia) than with AECOPD ($P<0.05$). These results reflect the clonal composition of the major genotypes among pneumonia isolates. It is remarkable that in our series, serotype 3 was the most frequent cause of pneumococcal pneumonia in COPD patients, since the invasive potential of this serotype was reported to be low (Brueggemann *et al.,* 2004). This might suggest that the underlying conditions of these COPD patients play a key role in the development of bacteraemia, as has been proposed previously (Alanee *et al,* 2007). On the other hand, serotypes 16F and 11A and non-typeable pneumococci were associated with AECOPD, showing a low invasive potential. Furthermore, in agreement with a

previous study (Liñares *et al.,* 1992), pneumococci isolated from AECOPD episodes were more resistant to antimicrobials than invasive isolates, especially to fluoroquinolones (3.5% in pneumonia and 6.2% in AECOPD isolates). It should be taken into account because the new fluoroquinolones are widely used to treat respiratory tract infections, especially in patients with COPD.

COPD is complicated by frequent and recurrent acute exacerbations, which are indicators of poor prognosis since increased morbidity affects quality of life (Anzueto *et al.,* 2007), and is also associated with high healthcare costs. For these reasons, the prevention of infectious AECOPD is an important issue. Regarding *S. pneumoniae*, until the introduction of conjugate vaccines in 2000, the prevention of pneumococcal diseases was based on the use of the PPV23 (introduced in 1983). It is recommended for persons over the age of 2 years who are at a substantially increased risk of developing pneumococcal infection; however, its effectiveness in preventing morbidity and mortality associated with pneumococcal infection is unclear, especially in COPD patients (Vila-Corcoles and Ochoa-Gondar, 2012). In contrast, as a consequence of PCV7 herd protection, a dramatic decrease in invasive disease caused by PCV7 serotypes and their related genotypes has been observed.

In order to confirm whether the introduction of PCV7 had an impact (by herd protection) on pneumococci causing AECOPD, we performed the second study presented also in <u>chapter III</u>, in which we extended the period of study (from 2001 to 2012) and analysed

the impact of PCV7 on serotypes causing AECOPD and the coverage of PCV13, recently recommended for adults.

As expected, a dramatic decrease in AECOPD caused by PCV7 serotypes and their related genotypes was observed throughout the study period, especially Spain^{9V}-CC156, Spain^{23F}-CC81 and CC88^{19F} clones. In parallel, two multi-drug resistant serotypes, and their related clones, Sweden^{15A}-ST63 and ST386^{6C} also showed significant increases. As a consequence, although resistance rates of β-lactams decreased over time, macrolide resistance and multi-drug resistance remained stable.

The proportion of serotypes covered by PCV13 during the 2009-12 period was low (27.2%); however, vaccination improved patient's quality of life, since COPD is the main underlying disease associated with serious pneumococcal infections. In fact, although it is too early to evaluate the herd protection impact of PCV13 in pneumococci causing acute exacerbations, a non-significant decrease in PCV13 serotypes not included in PCV7 (1, 3, 5, 6A, 7F and 19A) was observed between 2009 and 2012.

Among COPD patients, the isolation of new strains of *H. influenzae* and *M. catarrhalis* was associated with AECOPD (Sethi *et al.,* 2002). However, hardly data are available for *S. pneumoniae*. Our interest in the impact of pneumococcal persistence on COPD patients, together with the lack of information previously published led us to perform the studies described in chapter IV.

Our results, based on the inclusion of patients with recurrent episodes, confirm that reinfection through acquisition of a new strain was the most frequent cause of AECOPD episodes. However, we showed that the persistence of the same strain has been underestimated, since in a third of the recurrences, the same persistent pneumococcal strain caused two consecutive episodes.

In addition, serotypes 9V and 19F were associated with relapses (*P*<0.05). All isolates expressing the serotype 9V belonged to the Spain^{9V}-CC156; hence, this clone was also associated with relapses. In contrast, several genotypes expressed the serotype 19F and none of them was significantly associated with relapses. These results suggest that capsular type, rather than genetic background, may play an important role in the persistence of pneumococci among COPD patients. Both serotypes are covered by the three conjugate vaccines, PCV7, PCV10 and PCV13; however, it is notable that three emerging serotypes not included in the PCV13 (serotypes 15A, 11A and 6C) caused a third of the relapses.

The influence of previous antimicrobial therapy in the recurrent AECOPD episodes was also explored. Most of the patients included in the study had severe or very severe COPD, and received multiple antibiotic courses. Regarding β-lactams, although there were no differences in the β-lactam consumption between groups (relapses and reinfections), β-lactam resistance rates were higher among relapses. This may have been because relapse episodes were caused by few multi-resistant clones (mainly CC156 and CC88), contrasting with a higher genetic diversity including penicillin-susceptible and -resistant clones isolated from

reinfections. However, we found an association between fluoroquinolone consumption and development of resistance, as has been frequently described in the literature (Davidson *et al.,* 2002; De la Campa *et al.*, 2003).

In the second study in chapter IV we focused on the pneumococcal strains that were able to persist over a long time period, causing multiple episodes of acute exacerbation (≥3 episodes). In agreement with the previous study, in eight of 13 patients with largely pneumococcal persistence the strains expressed serotypes 9V and 19F, previously associated with relapses; so, a clear association between fluoroquinolone consumption and development of resistance was also detected among these persistent strains.

S. pneumoniae can achieve β-lactam resistance by acquiring exogenous DNA at its *pbp*s from either β-lactam-resistant pneumococci or commensal streptococci, such as *Streptococcus oralis* or *Streptococcus mitis* (Willems *et al.,* 2011). However, in contrast to fluoroquinolones, neither acquisition of β-lactams nor an increase of the β-lactams MICs was observed, in spite of the β-lactam pressure on the persistent pneumococci. This was associated with the stability of its *pbps*, which did not accumulate point mutations over time. These results, together with the fact that all persistent strains of different patients with the same genotype shared the same *pbps,* suggest that an optimal combination of *pbp* genes is necessary for the viability of the strains (Albarracin *et al.,* 2011).

Since the description of *S. pseudopneumoniae* species by Arbique *et al.* in 2004*,* few data were available on the pool of un-capsulated pneumococcal-like strains usually found in sputum samples of COPD (Arbique *et al.,* 2004; Keith *et al.,* 2006). It has recently been suggested that *S. pseudopneumoniae* strains could act as a reservoir for pneumococcal virulence factor genes and for genes conferring antimicrobial resistance, since the whole genome sequencing has revealed the presence of these genes in clinical strains of *S. pseudopneumoniae* (Johnston C *et al.*, 2010; Shahinas *et al.,* 2011). However, the clinical relevance of *S. pseudopneumoniae* has not been established.

In Chapter V, we combined a panel of phenotypic and molecular assays in order to gain insights into the nature of *S. pseudopneumoniae* clinical strains, as well as their role in causing disease in COPD patients.

Some *S. pseudopneumoniae* isolates presented atypical phenotypes (optochin-susceptible in CO_2 and O_2 atmosphere, bile positive, and harboring typical pneumococcal virulence genes). Hence, the biochemical tests performed were occasionally insufficient to distinguish *S. pseudopneumoniae* from *S. pneumoniae* or other closely-related streptococci, whereas the MLSA analysis was considered a good approach to differentiate the *Streptococcal* species. Moreover, all clinical isolates showed a high genetic variability, and only seven clonal groups were detected among the 59 strains available for this analysis. This finding, together with the high antimicrobial resistance showed by these strains, supports the idea that this species could play an important role in the context of pneumococcal ecology. In fact, a

recent comparison of the genome structure of a *S. pseudopneumoniae* isolate with the genome of R6 pneumococci, showed a closely relationship between both species (Shahinas *et al.,* 2013).

Clinical data of COPD patients infected by *S. pseudopneumoniae* were also analysed. Our results showed that all COPD patients had a successful outcome, suggesting a low attack rate of this pathogen; however, two thirds of patients required hospitalization, which confirms its etiological role causing acute exacerbations in COPD patients. Finally, a half of episodes occurred during the 2010-2012 period, when the sputum samples were quantitatively cultured and biochemical phenotypes were performed in up to 12 colonies per sample. This fact, together with the absence of a rapid gold standard technique for the identification of *S. pseudopneumoniae*, suggests that its frequency causing disease in COPD patients has been underestimated

Overall, the studies reported in this thesis have contributed to a better understanding of the dynamics of *S. pneumoniae* and *S. pseudopneumoniae* populations causing disease in COPD patients. Future surveillance studies are needed to assess the impact of PCV13 and future vaccines on prevention of pneumococcal infection in adult patients, especially those suffering from COPD.

CONCLUDING REMARKS

1. Among severe COPD patients, *Streptococcus pneumoniae* was isolated in a sixth of microbial acute exacerbations; it was the third most common cause after *P. aeruginosa* and *H. influenzae*.

2. In our experience, serotype 3 pneumococci (Netherlands[3]-ST180 and ST260[3] genotypes) commonly caused pneumonia and acute exacerbations in COPD patients.

3. Pneumococci of serotypes 1 (Sweden[1]-ST306), 4 (ST247[4]), 5 (Colombia[5]-ST289) and 8 (Netherlands[8]-ST53) were more often associated with pneumonia, while serotypes 16F and 11A were associated with acute exacerbations.

4. Serotypes included in PCV7 have dramatically decreased among COPD patients. In parallel, serotypes 15A and 6C, and their related genotypes, have shown a significant increase.

5. Macrolide and multi-drug resistance remained stable among pneumococci causing acute exacerbations during the last decade. In contrast, resistance rates of betalactams decreased over time.

6. The acquisition of a new strain was the most frequent cause of acute exacerbations; however, a third of recurrences were caused by a persistent strain, mainly of serotypes 9V and 19F.

7. QRDR changes involved in fluoroquinolone resistance were frequently observed in persistent strains after fluoroquinolone treatment. However, the PBP sequences and MLST genotypes of these strains were stable over time.

8. *Streptococcus pseudopneumoniae* was mainly isolated from acute exacerbations in severe COPD patients. It is remarkable that two thirds of patients required hospitalization.

9. Due to their genetic variability and antimicrobial resistance, *S. pseudopneumoniae* could act as a reservoir of antimicrobial resistance determinants and virulence genes.

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