Nontypeable *Haemophilus influenzae*: colonization, infection and biofilm formation

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Barcelona, April 2015

A Raül

Als meus pares i germà

A tota la gent que estima la ciència i que gràcies al seu esforç i implicació
fa possible un món millor

La realització d'aquesta Tesis Doctoral ha estat possible gràcies a les concessions de les beques FI-AGAUR otorgada per la Generalitat de Catalunya (Febrer 2010-Gener 2012), a la beca "Formación de Profesorado Universitario, FPU", otorgada pel Ministerio de Educacción (Febrer 2012- Gener 2014) i per un contracte de recerca realitzat pel grup liderat per la Dra. Josefina Liñares (Febrer 2014-Gener 2015).

Els estudis realitzats en aquest treball s'han finançat pel:

Fondo de Investigaciones Sanitarias, Ministerio de Sanidad; Beca FIS 09/1904.

CIBERES (Centro de Investigación Biomédica en Red de Enfermedades Respiratorias); B06/06/0037.

Després de cinc anys aquesta etapa arriba al seu final. Després de molt d'esforç i sacrifici he finalitzat un dels objectius més importants que m'havia plantejat, la tesis doctoral. Però aquest treball no haguera segut possible sense l'ajuda i col·laboració d'un bon grapat de persones.

En primer lloc, agrair molt especialment a Josefina Liñares tota l'ajuda durant aquests anys de tesis. Fina, gracias por brindarme la oportunidad de realizar la tesis en el grupo de investigación que diriges. Agradecerte sinceramente todas las horas que me has dedicado, corrigiendo trabajos y contestando pacientemente a las infinitas preguntas que me han ido surgiendo durante estos años. Gracias por preocuparte siempre por mí y por todo el conocimiento que me has enseñado durante este tiempo.

M'agradaria ampliar aquest agraïment a les co-directores d'aquesta tesis, Carmen Ardanuy i Sara Martí, per introduir-me en el món de la recerca científica, per la confiança dipositada en mi i per encoratjar-me a seguir endavant en els moments més difícils. Gràcies per tot el temps i esforç que li heu dedicat a aquest treball i tots els coneixements que m'heu transmès durant aquest període.

A tots els membres del grup 19 del CIBERES (Centro de Investigación Biomédica en Red de Enfermedades Respiratorias) i del grup d'Epidemiologia de les Infeccions Bacterianes de l'IDIBELL (Institut d'Investigació Biomèdica de Bellvitge).

A la Dra. Teresa Vinuesa i al Prof. Miquel Viñas, del Departament de Patologia i Terapèutica Experimental de la Facultat de Medicina del Campus de Bellvitge pel recolçament en la realització de tasques docents associades a la beca pre-doctoral.

A la Dra. Mª Angeles Domínguez i al Dr. Rogelio Martín i a tot el personal del Servei de Microbiologia de l'Hospital Universitari de Bellvitge. Moltes gràcies per brindar-me la vostra ajuda sempre que l'he necessitada i per congelar tots els *Haemophilus influenzae*, gràcies a això he pogut realitzar part d'aquesta tesis doctoral.

A Fe Tubau, per estar sempre disposta a ajudar-me i per tot el que m'has ensenyat sobre antibiòtics i la resistència antibiòtica.

A la Dra. Imma Grau i al Dr. Roman Pallarés, del Servei de Malalties Infeccioses de l'Hospital Universitari de Bellvitge, per tot el temps i esforç dedicat a l'estudi de les malalties invasives.

A la Dra. Carol Garcia-Vidal i al Dr. Jordi Carratalà, del Servei de Malalties Infeccioses de l'Hospital Universitari de Bellvitge, per la seva col·laboració en el treball de les pneumònies no bacterièmiques.

A la Dra. Salud Santos i al Dr. Jordi Dorca, del Servei de Pneumologia de l'Hospital Universitari de Bellvitge, per la seva implicació en el treball de la Malaltia Pulmonar Obstructiva Crònica.

A la Dra. Adela G. de la Campa y al Dr. José Manuel Tirado-Vélez, del Laboratorio de Genética Bacteriana del Instituto Carlos III, por todo lo enseñado en el mundo de la resistencia a fluoroquinolonas.

A la Dra. Junkal Garmendia del Instituto de Agrobiotecnología CSIC-Universidad Pública de Navarra, por la colaboración en estos trabajos y por las fructíferas charlas sobre *H. influenzae* que han ampliado mi conocimiento sobre este microorganismo.

A la Dra. Ana Fleites, del Hospital de Oviedo, por permitirnos utilizar su colección de muestras de frotis orofaríngeos de niños portadores de *S. pneumoniae*. Gracias a ello pudimos realizar el estudio de colonización por *H. influenzae*.

To Dr Peter HM Hermans and Dr Marien de Jonge from the Laboratory of Pediatric Infectious Diseases, Department of Pediatrics, Radboud University Medical Centre, Nijmegen (The Netherlands) for giving me the opportunity to do part of my thesis in their laboratory. Specially, I want to say thanks to Jeroen Langereis, for all your help inside and outside of the lab during my seven months in Nijmegen and for all the knowledge about *Haemophilus influenzae* you taught me. And to all the LKI and LKO people for receiving me and making me feel so welcomed. Dank you wel!

Com no, agrair als companys de recerca que han estat amb mi durant tot aquest projecte. Molta gent ha anat passant per el grup en el temps que jo he estat, pre-docs, algun post-doc, tècnics i estudiants de pràctiques. Gràcies pel vostre suport en els

moments difícils, per la vostra paciència i per la vostra ajuda sempre que l'he necessitada. M' agradaria agrair especialment a la gent que heu estat fins al final d' aquest projecte. A Sara, per tot el teu suport, per ensenyar-me a pensar per mi mateixa i a ser molt crítica en el lab, per "obligar-me" a fer la llibreta i per fer molt més divertides les infinites hores de sembra de frotis i de biofilm. Com no, per totes les vivències viscudes fora de Bellvitge. A Mariana, por ser siempre un punto de apoyo en los momentos difíciles, una muy buena consejera y por todas las risas que nos hemos echado juntas. A Arnau, per fer més agradables totes les hores dedicades a la sembra d' esputs, per ser un punt de suport dins del lab i pels moments viscuts en congressos i fora de la feina. A Meri, por tu ayuda en el lab siempre que la he necesitado. A Javi, per la teva ajuda al lab en els moments que l'he necessitada i per amenitzar les hores de dinar amb interessants converses.

Als amics de Benassal, per la vostra ajuda i suport en tots aquests anys.

A la meva família, especialment als meus abuelos, que sense entendre massa bé el meu treball sempre m'heu donat suport i m'heu animat a seguir endavant.

Als meus pares i germà, per creure sempre en mi i pel vostre suport i confiança en totes les decisions que he anat prenent al llarg de la vida. Gràcies per ensenyar-me que tot esforç té la seva recompensa, a no abandonar en els moments difícils i a seguir sempre endavant. Sense el vostre sacrifici però sobretot sense la vostra estima no haguera arribat tant lluny.

A Raül, agrair-te que sempre estas al meu costat, en els bons moments però sobretot en els moments difícils, animant-me a no defallir i transmetent-me l'energia que necessito per a seguir endavant. Moltes gràcies per la teva comprensió i paciència durant aquestos anys, sobretot en aquesta recta final del projecte. Però sobretot gràcies per estimar-me com m'estimes i per compartir la vida en mi. Sense tu tot açò no haguera segut possible.

ABSTRACT

Haemophilus influenzae is an opportunistic pathogen that forms part of the human nasopharyngeal microbiota. This microorganism is classified into encapsulated and nonencapsulated or nontypeable (NTHi) isolates, depending on the presence of a polysaccharide capsule. Although *H. influenzae* is a common respiratory commensal, it is also able to cause several infections, especially in patients with comorbidities. The most common respiratory infections in which *H. influenzae* can be identified as the main etiological agent are exacerbations in patients with Chronic Obstructive Pulmonary Disease (COPD), community-acquired pneumonia (CAP), cystic fibrosis, and otitis media. In addition, this pathogen is also a common cause of invasive infections such as bacteraemia and meningitis. Before the introduction of the conjugate vaccine, *H. influenzae* serotype b (Hib) was the main cause of meningitis in children under five years. However, effective childhood vaccination has caused a dramatic reduction in Hib and allowed the expansion of NTHi, which is becoming more relevant in both respiratory and invasive infections.

In this thesis, we studied three different aspects of the epidemiology of NTHi since the introduction of the vaccine. Our study focused on molecular genotyping, antimicrobial resistance and adhesion and biofilm formation of NTHi isolates from healthy children and from adult patients with CAP, COPD and invasive diseases.

Epidemiological relevance of NTHi

In the first part of this thesis, we set out to characterize the NTHi populations that are involved in adult infections in Bellvitge hospital. Furthermore, as humans are the only reservoir of NTHi, we aimed to identify the oropharyngeal carriage rate in healthy children attending day care centres in Oviedo.

In a two-year retrospective study the carriage rate found in healthy children was 40%, although it varied notably from centre to centre. Epidemiologically, NTHi isolates displayed a great genetic variability in both years without any long-term carriage of the same strain.

In the adult population, acute exacerbations of COPD (AECOPD) are the infections in which NTHi is most relevant as an etiological agent. According to the World Health Organization, COPD is one of the most common chronic diseases in the

world; it is the fourth leading cause of mortality worldwide and is expected to rise to third place by 2030. In order to establish the role of NTHi in acute exacerbations of COPD, a total of 188 sputum samples obtained from AECOPD episodes in severe COPD patients were quantitatively cultured for one year. NTHi was the second most frequently isolated pathogen (n=37, 19.7%) after *Pseudomonas aeruginosa* and it was associated with patients with the fewest annual exacerbation episodes.

Pneumonia is also a common infection caused by NTHi, especially in the elderly in whom NTHi is the second or third cause of CAP. We studied the molecular epidemiology of NTHi in this infection in isolates from patients with bacteraemic and non-bacteraemic CAP. We identified differences in the comorbidities between patients with bacteraemic and non-bacteraemic pneumonia and also in the 30-day mortality rate. Nevertheless, 26% of the strains which caused both types of pneumonia presented a high genetic homology, suggesting the dissemination of minor clones that caused small outbreaks.

The majority of invasive infections by *H. influenzae* were caused by NTHi strains with pneumonia as the main focus. Nevertheless, it was also able to cause other types of invasive diseases such as meningitis and biliary infection. On the other hand, invasive infections caused by encapsulated strains were rare and mainly caused by serotype f (Hif) isolates. Unlike NTHi, encapsulated strains belonged to a small number of clones that have been detected worldwide.

The identification in the laboratory of true NTHi is sometimes a challenging process due to its similarity to other species such as *Haemophilus haemolyticus*. However, the implementation and regular updates of the MALDI-TOF-MS technology in microbial identification has provided a useful tool for differentiating between these species. In order to determine the frequency of *H. haemolyticus* in clinical samples, we re-identified all the *Haemophilus* spp, using the latest MALDI-TOF-MS update that differentiates both species, finding a 4% of *H. haemolyticus* previously misidentified as *H. influenzae*. Interestingly, we found a higher presence of *H. haemolyticus* in genitourinary samples (10.5%) than in respiratory samples (4%). Genetically, *H. haemolyticus* were very diverse.

Overall, we can affirm that clinical isolates of NTHi are genetically diverse, although small groups of genetically related strains were observed in isolates causing bacteraemic and non-bacteraemic CAP.

Antimicrobial resistance

The aim of the second part of this thesis was to determine the antimicrobial susceptibility profile of clinical NTHi isolates, placing emphasis on the molecular characterization of β-lactam and fluoroquinolone resistance, the main antimicrobials used in the treatment of NTHi infections.

NTHi presents two mechanisms of resistance to β-lactam antimicrobials: β-lactamase enzyme production and/or alterations in PBP3. In our studies, the prevalence of β-lactamase was 18.5% in healthy carriers, 10.5% in isolates from CAP, 10.2% in invasive isolates and 5.4% in isolates from COPD. We observed a higher frequency of β-lactamase-producing NTHi isolates in children, probably due to differences in the antimicrobial treatment between age groups; amoxicillin is most commonly used in children whereas other antimicrobials such as cephalosporins or fluoroquinolones tend to be used to treat infections in adults. However, the most frequent mechanism of β-lactam resistance in NTHi isolates from adults was altered PBP3, ranging from 39% in invasive diseases to 28.4% in non-bacteraemic pneumonia. On the other hand, only 12% of NTHi isolated from children presented this resistance mechanism.

NTHi isolates were mainly classified as low-BLNAR − β-lactamase negative isolates which showed a low-level ampicillin resistance (MIC between 0.5-2 mg/L) despite having altered PBP3. The high percentage of ampicillin non-susceptible isolates found in respiratory and invasive infection in adults could be attributed to the fact that the majority of NTHi were isolated from elderly patients receiving multiple antibiotic courses for their underlying conditions, and also to the increase in the consumption of aminopenicillins in Catalonia.

In *H. haemolyticus*, β-lactamase producer isolates accounted for 8.7% whereas isolates with altered PBP3 for 26%. However, little is known about resistance due to alterations in PBP3.

Fluoroquinolones are the second most commonly used antimicrobial group in the treatment of NTHi infections in adult patients. Although resistant isolates have been detected worldwide, the prevalence of fluoroquinolone resistance remains low. In our study, 0.39% of isolates were fluoroquinolone-resistant. However, clinically is very important to detect those isolates which presented a first mutation in the quinolone resistance-determining regions (QRDR) because after a treatment with fluoroquinolones these isolates can easily become resistant and cause a therapeutic failure. Using nalidixic acid as an indicator of reduced fluoroquinolone susceptibility; we found five isolates susceptible to ciprofloxacin but resistant to nalidixic acid which already presented changes in GyrA and/or ParC.

Regarding fluoroquinolone resistance in *H. haemolyticus*, we found four isolates that presented alterations in GyrA and/or ParC associated with an increased ciprofloxacin MIC. These modifications in the QRDR were identical to the mutations previously identified in *H. influenzae*.

Adhesion and biofilm formation

The last part of this thesis focused on adhesion and biofilm formation. Biofilm is one of the mechanisms that microorganisms have developed in order to protect themselves and survive in hostile environments. Once the biofilm structure is formed, it is difficult to eliminate and, as a consequence, biofilm-associated infections commonly show recurrent symptoms. Although biofilm formation by NTHi remains controversial, biofilm-like structures have been observed in middle-ear mucosa in experimental chinchilla models of otitis media.

As all the previous studies of biofilm formation have been performed using a small number of NTHi strains, we decided to perform a comparative study of initial adhesion to solid surface and biofilm formation in a large collection of NTHi isolates from patients with respiratory infections, patients with invasive diseases, and healthy children carriers. Interestingly, our results showed clear differences in initial attachment and biofilm formation depending on the pathology associated with NTHi isolation, with significant increases in biofilm formation for NTHi isolates collected from patients with invasive disease and patients with otitis media compared with isolates from patients with CAP, COPD or healthy colonization.

The incorporation of phosphorylcholine (PCho) into the lipooligosaccharide has previously been associated with biofilm formation in experiments using a low number of NTHi strains. Consequently, we wanted to determine the role of PCho in biofilm formation in a large collection of clinical NTHi isolates. We observed that there was no correlation between biofilm formation and presence of PCho in the lipooligosaccharide of clinical NTHi isolates, as this was a strain-dependent relationship. Additional experiments to establish the molecular nature of biofilm formation in NTHi suggested an important role of proteins in the initial adhesion and stability of biofilms.

Taken together, all the studies discussed in this thesis can improve our understanding of the clinical epidemiology of NTHi populations since the introduction of vaccination and of the mechanism of biofilm formation in clinical isolates of this microorganism.

RESUM

Haemophilus influenzae és un patogen oportunista que forma part de la microbiota nasofaríngia humana. Aquest microorganisme es classifica en soques capsulades i no capsulades o no tipables (HiNT) depenent de la presència d'una càpsula polisacarídica. Tot i que *H. influenzae* és un comensal respiratori comú, posseeix la capacitat de causar diferents infeccions, especialment en pacients amb malalties de base. Les infeccions respiratòries més freqüents causades per *H. influenzae* són les exacerbacions agudes en pacients amb Malaltia Pulmonar Obstructiva Crònica (MPOC), pneumònia adquirida en la comunitat (PAC), exacerbacions en pacients amb fibrosis quística i otitis mitjana. A més, aquest patogen és també una causa freqüent de malalties invasives com bacterièmia i meningitis. Abans de la introducció de la vacuna conjugada, *H. influenzae* serotipus b (Hib) fou la causa principal de meningitis en nens/es menors de cinc anys d'edat. No obstant, l'efectiva vacunació ha causat un dramàtic descens del Hib permetent l'expansió dels HiNT, que s'estan convertint en un patogen més rellevant tant en infeccions respiratòries com en infeccions invasives.

Els objectius plantejats en aquesta tesis, foren l'estudi de tres aspectes de la epidemiologia dels HiNT en l'etapa posterior a la introducció de la vacuna en Barcelona: la genotipificació molecular, la resistència antibiòtica i la formació de biofilm en soques d'HiNT aïllades de nens/es sans i de pacients adults amb PAC, MPOC i malalties invasives.

Rellevància epidemiològica d'HiNT

L'objectiu de la primera part d'aquesta tesis fou caracteritzar les poblacions d'HiNT involucrades en les infeccions en pacients adults de l'hospital de Bellvitge així com la determinació de la freqüència de colonització orofaríngia d'HiNT en nens/es sans que van a llars d'infants en Oviedo.

En un estudi retrospectiu de dos anys, la freqüència de colonització trobada en portadors sans fou del 40%, amb una gran variabilitat entre els diferents centres estudiats. Epidemiològicament, els aïllaments d'HiNT presentaren una gran diversitat genètica amb una persistència de la mateixa soca curta en el temps.

En adults, les exacerbacions agudes de la MPOC són les infeccions on HiNT té més rellevància com a agent etiològic. D'acord amb l'Organització Mundial de la Salut,

la MPOC és una de les infeccions cròniques més comunes al món, sent la quarta causa de mort al món i amb la predicció de convertir-se en la tercera causa de mortalitat l'any 2030. Amb l'objectiu d'establir el paper d'HiNT en les exacerbacions agudes del MPOC, van ser cultivats quantitativament durant un any un total de 188 esputs obtinguts d'episodis d'exacerbacions agudes de pacients amb MPOC greu. HiNT fou el segon patogen aïllat en freqüència (n=37, 19.7%) després de *Pseudomonas aeruginosa* i s' associà amb els pacients que presentaren una única exacerbació anual.

La pneumònia és també una infecció freqüent causada per HiNT, especialment en gent gran en la qual HiNT és la segona o tercera causa de pneumònia. S'estudià la epidemiologia molecular d'aïllaments d'HiNT de pacients amb pneumònia bacterièmica i no bacterièmica, observant-se que un quart de les soques causants d'ambdós tipus de pneumònia presentaven una gran homologia genètica, fet que suggereix una disseminació clonal que causà petits brots.

Els HiNT foren la causa més freqüent de malaltia invasiva causada per *H. influenzae*, sent la pneumònia el principal focus. Per altra banda, les infeccions invasives causades per soques capsulades foren rares i foren causades principalment per soques del serotipus f (Hif). A diferència de les soques HiNT, les soques capsulades pertanyeren a pocs clons, específics de cada serotipus, que ja s'han publicat en altres països.

La identificació en el laboratori de soques d'HiNT pot ser un procés complicat degut a la gran similitud que presenta amb *Haemophilus haemolyticus*. No obstant, la implementació i les regulars actualitzacions de la tecnologia MALDI-TOF-MS en la identificació microbiana ha proporcionat una ferramenta molt útil en la diferenciació d'aquestes especies. Amb l'objectiu de determinar la freqüència d'*Haemophilus haemolyticus* en mostres clíniques, es re-identificaren tots els aïllaments del gènere *Haemophilus* prèviament identificats utilitzant l'actualització del MALDI-TOF-MS que permet la diferenciació d'aquestes dues espècies. Es va trobar un 4% d'*H. haemolyticus* identificats erròniament com HiNT. Genèticament, les soques d'*H. haemolyticus* presentaren una gran diversitat.

En conjunt, es pot afirmar que els aïllaments clínics d'HiNT són genèticament diversos tot i l'observació de petits grups de soques relacionades genèticament en aïllaments de pneumònia bacterièmica i no bacterièmica.

Resistència antibiòtica

L'objectiu de la segona part de la tesis fou determinar els perfils de susceptibilitat antibiòtica dels aïllats clínics d'HiNT, emfatitzant en la caracterització molecular de la resistència a β-lactàmics i fluoroquinolones, ja que són els antibiòtics més utilitzats en el tractament de les infeccions per HiNT.

HiNT presenta dos mecanismes de resistència als β-lactàmics: la producció de β-lactamases i/o alteracions en la PBP3. Els nostres estudis mostren una fluctuació de la freqüència d'aïllaments productors de β-lactamasa entre 18.5% en portadors a un 5.4% en aïllaments de MPOC. Tot i això, el mecanisme més freqüent de resistència antibiòtica identificat en HiNT aïllats d'adults fou l'alteració de la PBP3 (39% en malalties infeccioses i 28.4% en pneumònia no bacterièmica).

La majoria dels aïllaments d'HiNT foren classificats com a *low*-BLNAR, ja que aquests aïllaments β-lactamasa negatius, a pesar de tindre alteracions en la PBP3, presentaren un baix nivell de resistència a l'ampicil·lina (CMI entre 0.5-2 mg/L). L'elevat percentatge d'aïllaments no sensibles a ampicil·lina trobat en les infeccions respiratòries i invasives en adults es pot explicar perquè la majoria dels HiNT foren aïllats de pacients amb edat avançada que han rebut múltiples tractaments antibiòtics degut a les seves malalties de base i perquè el consum d'aminopenicil·lines a Catalunya ha augmentat en els últims anys.

Respecte a *H. haemolyticus*, s'observà un 8.7% de productors de β-lactamasa i un 26% d'aïllaments amb PBP3 alterades. No obstant, el coneixement sobre la resistència a β-lactàmics degut a alteracions en la PBP3 en aquesta espècie bacteriana és escàs.

Les fluoroquinolones són el segon grup d'antibiòtics més comunment utilitzat en el tractament de les infeccions per HiNT en adults. Tot i això, la resistència a fluoroquinolones en aquest microorganisme es manté baixa. En el nostre estudi, es trobà

un 0.39% d'aïllaments resistents a fluoroquinolones. No obstant, des del punt de vista clínic, és molt important detectar els aïllaments que presentaren una primera mutació en les regions determinant de resistència a quinolones (QRDRs), perquè després d'un tractament amb fluoroquinolones poden transformar-se en aïllaments resistents i conduir a un fracàs terapèutic, degut a l'adquisició gradual de mutacions baix una pressió selectiva amb aquest grup d'antibiòtics. Utilitzant l'àcid nalidíxic com a indicador de sensibilitat reduïda a les fluoroquinolones, es trobaren cinc aïllaments sensibles a ciprofloxacina però resistents a l'àcid nalidíxic que ja presentaven canvis en GyrA i/o ParC.

Pel que fa a la resistència a fluoroquinolones en *H. haemolyticus*, s'identificaren quatre aïllaments que presentaren alteracions en GyrA i/o ParC, associades amb un increment de la CMI de ciprofloxacina. Aquestes modificacions en les QRDR foren idèntiques a les mutacions identificades prèviament en *H. influenzae*.

Adhesió i formació de biofilm

L'última part de la tesis està enfocada a l'estudi de l'adhesió i la formació de biofilm. El biofilm és un dels mecanismes que els microorganismes han desenvolupat per a la protecció i supervivència en ambients hostils. Una vegada l'estructura del biofilm està formada és molt difícil d'eliminar i, com a conseqüència, les infeccions associades a biofilm presenten símptomes recurrents. Tot i que la formació de biofilm per HiNT roman controvertida, estructures tipus biofilm s'han observat en la mucosa de l'oïda mitjana en models experimentals d'otitis mitjana en xinxilla.

Com tots els estudis prèviament publicats sobre formació de biofilm es realitzaren utilitzant un nombre reduït de soques d'HiNT, s'ha elaborat un estudi comparatiu d'adhesió inicial a superfície sòlida i formació de biofilm en una amplia col·lecció de soques d'HiNT aïllades de pacients amb infeccions respiratòries, pacients amb malalties invasives i portadors infantils sans. Els resultats mostren una clara diferència en l'adhesió i la formació de biofilm depenent de la patologia associada a l'aïllament d'HiNT, amb un significant augment en la formació de biofilm en soques d'HiNT aïllades de pacients amb malalties invasives i en pacients amb otitis mitjana en

comparació amb els HiNT aïllats de pacients amb pneumònia no bacteriémica, MPOC o en portadors sans.

La incorporació de fosforilcolina (PCho) en el lipooligosacàrid s'ha associat prèviament amb la formació de biofilm en experiments on s'utilitzà un nombre reduït de soques d'HiNT. Conseqüentment,s'ha determinat el paper de la PCho en la formació de biofilm en una gran col·lecció d'aïllaments clínics d'HiNT, no observant cap correlació entre formació de biofilm i presència de PCho en el lipooligosacàrid. Tanmateix, experiments addicionals per a establir la naturalesa molecular de la formació de biofilm en HiNT suggereixen que les proteïnes juguen un important paper en l'adhesió inicial i en l'estabilitat del biofilm.

En conjunt, tots els estudis discutits en aquesta tesis contribueixen a ampliar el coneixement de la epidemiologia clínica, la resistència antibiòtica i la formació de biofilm de les poblacions d'HiNT en un període posterior a la introducció de la vacuna.

SCIENTIFIC PRODUCTION

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1. Domenech A*, Puig C*, Marti S, Santos S, Fernández A, Calatayud L, Ardanuy C, Liñares J (2013). Infectious etiology of acute exacerbations in severe COPD patients. Journal of Infection 67(6):516-23. *These authors equally contributed to this work. **Impact Factor** according to 2013 Journal Citation Reports released by Thomson Reuters (ISI) is **4.017**.

2. Puig C, Calatayud L, Marti S, Tubau F, García-Vidal C, Carratalà-Fernández J, Liñares J, Ardanuy C (2013). Molecular epidemiology of nontypeable *Haemophilus influenzae* causing community acquired pneumonia in adults. PLoS One 8(12):e82515. **Impact Factor** according to 2013 Journal Citation Reports released by Thomson Reuters (ISI) is **3.534**.

3. Puig C, Marti S, Hermans PWM, de Jonge MI, Ardanuy C, Liñares J, Langereis JD (2014). Incorporation of phosphorylcholine into the lipooligosaccharide of nontypeable *Haemophilus influenzae* does not correlate with the level of biofilm formation *in vitro*. Infection & Immunity 82(4):1591-9. **Impact Factor** according to 2013 Journal Citation Reports released by Thomson Reuters (ISI) is **4.156**.

4. Puig C*, Marti S*, Fleites A, Trabazo R, Calatayud L, Liñares J, Ardanuy C (2014). Oropharyngeal colonization by nontypeable *Haemophilus influenzae* among healthy children attending day care centers. Microbial Drug Resistance 20(5):450-5. *These authors equally contributed to this work. **Impact Factor** according to 2013 Journal Citation Reports released by Thomson Reuters (ISI) is **2.524**.

5. Puig C, Grau I, Marti S, Tubau F, Calatayud L, Pallares R, Liñares J, Ardanuy C (2014). Clinical and molecular epidemiology of *Haemophilus influenzae* causing invasive disease in adult patients. PLoS One 9(11):e112711. **Impact Factor** according to 2013 Journal Citation Reports released by Thomson Reuters (ISI) is **3.534**.

6. Puig C, Domenech A, Garmendia J, Langereis JD, Mayer P, Calatayud L, Ardanuy C, Liñares J, Marti S (2014). Increased biofilm formation by nontypeable *Haemophilus influenzae* isolates from patients with invasive disease or otitis media *versus* strains recovered from cases of respiratory infections. Applied Environmental

Microbiology 80(22):7088-95. **Impact Factor** according to 2013 Journal Citation Reports released by Thomson Reuters (ISI) is **3.952**.

7. Puig C, Tirado-Vélez JM, Calatayud L, Tubau F, Garmendia J, Ardanuy C, Marti S, de la Campa AG, Liñares J (2015). Molecular characterization of fluoroquinolone resistance in nontypeable *Haemophilus influenzae* clinical isolates. Antimicrobial Agents and Chemotherapy 59(1):461-6. **Impact Factor** according to 2013 Journal Citation Reports released by Thomson Reuters (ISI) is **4.451**.

8. Marti S, Puig C, de la Campa AG, Tubau F, Domenech A, Calatayud L, Garcia-Somoza D, Ayats J, Liñares J, Ardanuy C. Identification of *Haemophilus haemolyticus* in clinical samples and characterization of their mechanisms of antimicrobial resistance. Submitted to Journal Antimicrobial Chemotherapy.

Collaborations

9. Garmendia J, Martí-Lliteras P, Moleres J, Puig C, Bengoechea JA (2012). Genotypic and phenotypic diversity in the noncapsulated *Haemophilus influenzae*: adaptation and pathogenesis in the human airways. International Microbiology 15(4):157-170. **Impact Factor** according to 2013 Journal Citation Reports released by Thomson Reuters (ISI) is **1.341**.

10. Marti S, Puig C, Domenech A, Liñares J, Ardanuy C (2013). Comparison of restriction enzymes for Pulse-Field Gel Electrophoresis (PFGE) typing of *Moraxella catarrhalis*. Journal Clinical Microbiology 51(7):2448-52. **Impact Factor** according to 2013 Journal Citation Reports released by Thomson Reuters (ISI) is **4.232**.

11. Marti S, Calatayud L, Gilabert-Porres J, Díez-Ferrer M, Puig C, Cubero N, López-Lisbona RM, Borros S, Ardanuy C, Liñares J, Rosell A. Colonization of central airways after tracheobronchial stenting. Could silver-coated silicone stents be a solution? Submitted to Plos One.

Poster presentations

International meetings

1. Fluoroquinolone resistance among nontypable *Haemophilus influenzae* isolated from adults remains stable (Barcelona, 2000-2009). L. Calatayud, AG. de la Campa, F. Tubau, C. Puig, L. Balsalobre, D. García-Somoza, C. Ardanuy, R. Martín, J. Liñares. 50th ICAAC. Boston, USA. 12th-15th September 2010.

2. Community acquired pneumonia (CAP) due to non-typeable *Haemophilus influenzae*. Antimicrobial susceptibility, molecular typing and virulence patterns. C. Puig, L. Calatayud, S. Martí, C. Ardanuy, F. Tubau, C. Garcia-Vidal, J. Garmendia, J. Liñares. 51st ICAAC. Chicago, USA. 16th-20th September 2011.

3. Oropharyngeal colonization by non-typeable *Haemophilus influenzae* (NTHi) among healthy children attending day care centres. S. Martí, C. Puig, A. Fleites, L. Calatayud, C. Ardanuy, J. Liñares. 52nd ICAAC. San Francisco, USA. 9th-12th September 2012.

4. Non-typeable *Haemophilus influenzae* biofilm formation is not correlated to phosphorylcholine incorporation into the lipooligosaccharide structure of clinical isolates. C. Puig, S. Marti, PWM. Hermans, J. Liñares, JD. Langereis. NVVM, Spring meeting 2013. Papendal, the Netherlands. 16th April 2013.

5. Etiology of acute exacerbations in patients with severe chronic obstructive pulmonary disease. C. Puig, A. Domenech, S. Martí, A. Fernández, S. Santos, L. Calatayud, C. Ardanuy, J. Liñares. 23rd ECCMID. Berlin, Germany. 23th-27th April 2013.

6. Adhesion and biofilm formation by non-typeable *Haemophilus influenzae* (NTHi) isolated from patients with Community Acquired Pneumonia, Chronic Obstructive Pulmonary Disease and healthy carriers. C. Puig, A. Domenech, P. Mayer, C. Ardanuy, J, Liñares, S, Martí. 3rd European Congress on Microbial Biofilms (Eurobiofilms). Ghent, Belgium. 9th-12th September 2013.

7. Biofilm formation is not correlated to phosphorylcholine content in clinical isolates of non-typeable *Haemophilus influenzae*. C. Puig, S. Marti, PWM. Hermans, C. Ardanuy, J. Liñares, JD. Langereis. 3rd European Congress on Microbial Biofilms (Eurobiofilms). Gent, Belgium. 9th-12th September 2013.

8. Clinical characteristics of patients with Acute Exacerbations of Chronic Obstructive Pulmonary Disease (COPD) caused by *Streptococcus pseudopneumoniae*. A. Domenech, C. Puig, S. Santos, S. Marti, C. Ardanuy, J. Liñares; 53rd ICAAC. Denver, USA. 10th-13th September 2013.

9. Clinical and molecular epidemiology of *Haemophilus influenzae* causing invasive disease in adult patients. C. Puig, I. Grau, S. Martí, R. Pallarés, C. Ardanuy, J. Liñares. 24th ECCMID. Barcelona, Spain. 10th-13th May 2014.

10. Molecular characterization of fluoroquinolone resistance in nontypeable *Haemophilus influenzae*. C. Puig, JM. Tirado-Velez, L. Calatayud, F. Tubau, J. Garmendia, C. Ardanuy, S. Marti, AG. de la Campa, J. Liñares. 54th ICAAC. Washington, USA. 5th-9th September 2014.

11. Increased biofilm formation by non-typeable *Haemophilus influenzae* (NTHi) isolated from invasive disease and otitis media compared to other respiratory infections. C. Puig, A. Domenech, J. Garmendia, P. Mayer, JD. Langereis, L. Calatayud, J. Liñares, C. Ardanuy, S. Marti. 54th ICAAC. Washington, USA. 5th-9th September 2014.

12. Low prevalence of *Haemophilus haemolyticus* in non-sterile respiratory track samples determined by MALDI identification. S. Martí, C. Puig, A. Domenech, F. Tubau, L. Calatayud, J. Ayats, J. Liñares, C. Ardanuy. 25th ECCMID. Copenhagen, Denmark. 25th-28th April 2015.

National meetings

1. Estructura poblacional de cepas de *Streptococcus pneumoniae* invasivas y no invasivas aisladas en pacientes con Enfermedad Pulmonar Obstructiva Crónica. A. Domenech, C. Puig. II Jornadas de Formación de CIBERES. Mallorca, Spain. 15th-16th October 2009.

2. Estructura poblacional de cepas de *Haemophilus influenzae* aisladas de pacientes con neumonía adquirida en la comunidad (NAC). C. Puig. III Jornadas de Formación de CIBERES. Mallorca, Spain. 28th-29th October 2010.

3. Caracterización genotípica de *H. influenzae* no tipables (NT-Hi) aislados en pacientes adultos con Neumonía Adquirida en la Comunidad (NAC). C. Puig, L. Calatayud, S. Martí, C. Ardanuy, F. Tubau, C. García-Vidal, J. Garmendia, J. Liñares. IV Jornadas de Formación de CIBERES. Mallorca, Spain. 27th-28th October 2011.

4. Caracterización de *Pseudomonas aeruginosa* aisladas en exacerbaciones agudas de la Enfermedad Pulmonar Obstructiva Crónica (EAEPOC). S. Martí, C. Puig, A. Domenech, S. Santos, F. Tubau, C. Ardanuy, J. Liñares. IV Jornadas de Formación de CIBERES Mallorca, Spain. 27th-28th October 2011.

5. Etiología bacteriana de las exacerbaciones agudas de la Enfermedad Pulmonar Obstructiva Crónica (EPOC) en pacientes graves. C. Puig, A. Domenech, S. Marti, A. Fernández, S. Santos, L. Calatayud, C. Ardanuy, J. Liñares. XVI Congreso Nacional de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). Bilbao, Spain. 9th-11th May 2012.

6. Bases genéticas de la resistencia a los β-lactámicos y estructura poblacional de *Haemophilus influenzae* no tipable causante de neumonía no bacteriémica adquirida en la comunidad en pacientes adultos. C. Puig, L. Calatayud, S. Martí, F. Tubau, C. García-Vidal, J. Carratalà, J. Liñares, C. Ardanuy. XVII Congreso Nacional de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). Zaragoza, Spain. 29th-31st May 2013.

7. La formación de biofilm en cepas clínicas de *Haemophilus influenzae* no tipable no está relacionada con la presencia de fosforilcolina en el lipooligosacárido. C. Puig, S. Marti, PWM. Hermans, C. Ardanuy, J. Liñares, JD. Langereis. XXIV Congreso de Microbiología SEM. Hospitalet de Llobregat, Spain. 10th-13th July 2013.

8. Mecanismes de resistència antibiòtica de *Streptococcus pseudopneumoniae* aïllats de pacients amb Malaltia Pulmonar Obstructiva Crònica. J. Moreno, A. Domenech, C. Puig, S. Marti, S. Santos, C. Ardanuy, J. Liñares. XXIII Jornades Societat Catalana de Malalties Infeccioses i Microbiologia Clínica. Tarragona, Spain. 24th-25th October 2014.

Oral communications

1. Etiología bacteriana de las exacerbaciones agudas de la Enfermedad Pulmonar Obstructiva Crónica (EPOC) en pacientes graves. C. Puig, A. Domenech, S. Marti, A. Fernández, S. Santos, L. Calatayud, C. Ardanuy, J. Liñares. XVI Congreso Nacional de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). Bilbao, Spain. 9th-11th May 2012.

2. Estudio de los mecanismos de resistencia antibiótica, genotipos y virulencia de *H. influenzae* no tipable causante de neumonía adquirida en la comunidad. C. Puig, L. Calatayud, S. Martí, C. Ardanuy, F. Tubau, C. García-Vidal, J. Garmendia, AG. de la Campa, J. Liñares. IV Congreso Grupo Especializado Biología de Microorganismos Patógenos. Badajoz, Spain. 5th-7th July 2012.

3. Colonización orofaríngea por *Haemophilus influenzae* no-tipable (Hi-NT) en niños sanos. C. Puig, A. Fleites, R. Trabazo, L. Calatayud, C. Ardanuy, J. Liñares, S. Marti. XVII Congreso Nacional de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). Zaragoza, Spain. 29th-31st May 2013.

4. Estudio comparativo de enzimas de restricción para el tipado de *Moraxella catarrhalis* mediante campo pulsado (PFGE). C. Puig, A. Domenech, J. Liñares, C. Ardanuy, S. Marti. XVII Congreso Nacional de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). Zaragoza, Spain. 29th-31st May 2013.

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INTRODUCTION

I. INTRODUCTION

Haemophilus influenzae was described for the first time by the German physician Richard Pfeiffer, who observed tiny bacilli in sputum samples from patients with epidemic influenza during the pandemic in 1889-1892 (Pfeiffer, 1892). The microorganism, named *Bacillus influenzae* or Pfeiffer bacillus in his honour, was considered the etiological agent of this epidemic influenza. For this reason, research into *B. influenzae* intensified during the influenza pandemic in 1918-1920, which caused devastating mortality worldwide. The studies reported contradictory results and questioned the etiological significance of *B. influenzae* or other microorganisms cultivated from the respiratory tract as causative agents of influenza (Dochez *et al*., 1936; Olitsky *et al*., 1921; Wollstein, 1919). However, in 1918, further research suggested a connection between a filterable virus and the influenza pandemic, but the small number of experiments, together with the lack of reproducibility, did not support the hypothesis of a viral etiological cause of influenza (Dochez *et al*., 1936). In 1917, *B. influenzae* took its current name, *H. influenzae*, the blood lover (*haema*-blood and *philus*-loving) (Winslow *et al*., 1920) although the new name was not used immediately and the microorganism continued to be called *B. influenzae* or Pfeiffer bacillus.

In the early 20th century, the most important difficulties encountered by researchers were the isolation and growth of *H. influenzae* without contamination by other upper airway bacteria. In 1929, Alexander Fleming proposed the addition of a few drops of penicillin over the agar plate to allow the separation of *H. influenzae* from Gram-positive microorganisms. These experiments were published in a paper entitled "*On the antibacterial action of cultures of a Penicillium, with a special reference to their use in the isolation of B. influenzae*"(Fleming, 1929).

In 1931, Margaret Pittman described two different morphologies on agar plates for *H. influenzae* strains and named them smooth (S) and rough (R). In addition, she observed that S strains precipitated with antisera while R strains did not, and defined a relationship between encapsulated strains and severe cases of disease (Pittman, 1931). In 1933, the influenza virus was isolated by Smith *et al.* (Smith *et al*., 1933). Little research into *H. influenzae* was then undertaken until the early 1970s due to an

increasing incidence of invasive diseases, in particular meningitis due to *H. influenzae* serotype b (Peltola, 2000).

More recently, in 1995, *H. influenzae* became the first organism to have its whole genome sequenced (Fleischmann *et al*., 1995). The sequenced strain was the non-pathogenic *H. influenzae* Rd strain, a nonencapsulated variant of an encapsulated serotype d isolate that lost its capsule in a recombination event (Martin *et al*., 1998).

1. CHARACTERISTICS OF *H. influenzae*

1.1 Taxonomic characteristics

The taxonomic classification of the genus *Haemophilus* has been reorganized over the last 60 years. In Bergey's Manual of Determinative Bacteriology, 7th edition, the genus *Haemophilus* was classified in the family *Brucellaceae*, together with the genera *Pasteurella*, *Bordetella*, *Brucella*, *Actinobacillus*, *Calymmatobacterium*, *Moraxella* and *Noguchia* (Breed *et al*., 1957).

In 1984, the genus *Haemophilus* was classified as a member of the family *Pasteurellaceae*, together with the genera *Actinobacillus*, *Pasteurella*, *Mannheimia*, *Phocoenobacter* and *Lonepinella*. Excluding *Haemophilus* and some species of *Actinobacillus*, the other genera comprise environmental species or animal pathogens. Although some species within the genera *Haemophilus* and *Actinobacillus* are also associated with animal pathology, both genera include species associated with human diseases (Holt, 1994).

Particularly, the genus *Haemophilus* is formed by twelve species, eight of which are human pathogens and the remaining four are associated with pathology in animals (Table 1) (Holt, 1994; Norskov-Lauritsen, 2014; Winn *et al*., 2008).

Table 1. Species which form the genus *Haemophilus* and their hosts.

Haemophilus spp.

Human pathogen	**Animal pathogen**	
H. influenzae	*H. parasuis*	Swine
H. haemolyticus	*H. paracuniculus*	Rabbits
H. parainfluenzae	*H. haemoglobinophilus*	Dogs
H. parahaemolyticus	*H. felis*	Cats
H. paraphrohaemolyticus		
H. sputorum		
H. pittmaniae		
H. ducreyi		

With the application of new techniques for identification, some species initially classified in the genus *Haemophilus* have been transferred to other genera such as *H.aprophilus, H. paraphrophilus* (a growth form of *H. aprophilus*) and *H. segnis* have been transferred to the genus *Aggregatibacter*; *H. pleuropneumonia*e has been transferred to the genus *Actinobacillus*, and *H. avium* and *H. paragallinarum* to the genus *Avibacterium* (Norskov-Lauritsen *et al*., 2006).

1.2 Laboratory identification

Accurate identification of the causative agent is essential for patient management, antimicrobial treatment selection and hospital infection control. Identification processes have changed over time in parallel with technological advances. For a long time, microbial identification was achieved using conventional techniques based on culture, morphology and biochemical features. The main drawback of these methodologies is that they are time-consuming and microbial identification is delayed. The development of new diagnostic techniques allowing more rapid and accurate identification has initiated a revolution in clinical microbiology (Nomura, 2015).

1.2.1 Phenotypical identification

Species of the genus *Haemophilus* are Gram-negative pleomorphic coccobacilli, which range from small to filamentous rods, non-acid fast, and non-spore-forming. These species are facultative anaerobes, chemo-organotrophic, with both respiratory and fermentative types of metabolism, nitrate reducers, and their optimal growth temperature is 35-37ºC in an atmosphere supplemented with 5% to 10% of CO_2. Oxidase and catalase reactions vary among species. With respect to *in vitro* growth, they require supplementary factors from the blood, particularly X factor (haem) and/or V factor (β-nicotinamide adenine dinucleotide [NAD] or NAD phosphate [NADP]). Basically, the identification of the different species is performed depending on the factors they need for growth (Table 2). Although growth factors are commonly used to identify *H. influenzae* in clinical laboratories, it is associated with misidentifications in 20% of cases. Consequently, this identification must be confirmed by biochemical tests or by using a molecular-based methodology (Murray *et al*., 2007).

Table 2. Principal differential characteristics of *Haemophilus* species with human clinical relevance. Table adapted from Norskov-Lauritsen *et al*. 2014 (Norskov-Lauritsen, 2014).

	H. influenzae	*H. haemolyticus*	*H. parainfluenzae*	*H. parahaemolyticus*	*H. paraphrohaemolyticus*	*H. sputorum*	*H. pittmaniae*	*H. ducreyi*
Porphyrin synthesis (Haem not required)	-	-	+	+	+	+	+	-
NAD synthesis (NAD not required)	-	-	-	-	-	-	-	+
Catalase	+	+	d	d	d	d	d	-
Haemolysis	-	+	d	+	+	+	+	d
β-Galactosidase	-	-	d	-	+	+	+	-
Tryptophanase	d	d	d	-	-	-	-	-
Urease	d	+	d	+	+	+	-	-
Ornitine descarboxilase	d	-	d	-	-	-	-	-
Fermentation								
Sucrose	-	-	+	+	+	+	+	-
Mannose	-	-	+	-	-	-	+	-
Lactose	-	-	-	-	-	-	-	-
IgA1 protease	+	-	-	+	-	-	-	-

Abbreviations: +: positive; -: negative; d: variable.

H. influenzae can be isolated in specific laboratory media such as chocolate agar or Levinthal's medium. Growth on conventional agar media can be achieved by supplementation with haem and NAD (adding the growth factors in the liquid medium or adding a filter paper disk to its surface). Owing to the small size of the *Haemophilus* colonies, their presence in cultures with other microorganisms may be overlooked. This problem can be resolved by including selective agents or a special incubation procedure. For instance, the overgrowth of *H. influenzae* by *Pseudomonas aeruginosa* in sputum samples from patients with cystic fibrosis has been solved using media with bacitracin (Murray *et al*., 2007). There are several commercial kits for identification of *Haemophilus* species such as API NH (bioMérieux Inc.), Vitek NHI Card V1308 (bioMérieux), the Haemophilus ID Test Kit (Remel), the RIM-H system (Austin Biological Laboratories), and RapidID NF (Innovative Diagnostics). However, most of

these kits do not provide sufficient information for an accurate identification at the species level (Murray *et al*., 2007).

The colonies that *H. influenzae* form on chocolate agar are greyish, semiopaque, smooth, flat and convex with a diameter of 1 to 2 mm after 24h of incubation. Growth on agar media gives the bacterium a characteristic smell defined as a "mouse nest" (Murray *et al*., 2007). Regarding bacterial morphology, *H. influenzae* is commonly a small coccobacilli with a size of 1 μm by 0.8 μm, but it may have various degrees of polymorphism (Smith, 1931), with an "L-form" morphology induced, for example, by antibiotics such as β-lactams (Fig. 1) (Klein *et al*., 1977; Want *et al*., 1975).

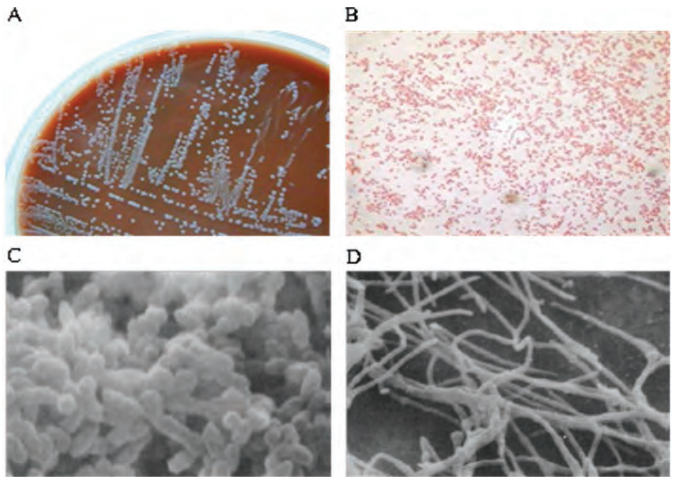

Figure 1: (A) Bacterial growth of *H. influenzae* on chocolate agar plates. (B) Gram stain of *H. influenzae* (Images A & B obtained from the Servei de Microbiologia de l'Hospital Universitari de Bellvitge). (C) Scanning electron microscopy of untreated *H. influenzae* cells. (D) *H. influenzae* cells after 4h incubation with 10 times the ampicillin MIC (Images C & D obtained from Klein *et al*.) (Klein *et al*., 1977).

1.2.2 Identification based on genomic fingerprinting

Due to the difficulty in identifying certain species of the genus *Haemophilus* using conventional phenotypic methods, genomics-based approaches have become the main methodology used to identify and/or confirm the classical identification of *H. influenzae* (Pickering *et al*., 2014). The most common methodologies are the study of 16S rRNA, DNA hybridization and detection of marker genes (Binks *et al*., 2012;

McCrea *et al*., 2008; Norskov-Lauritsen *et al*., 2009; Pickering *et al*., 2014). In recent years, whole genome sequencing has become one of the most widely used techniques in phylogenetic studies (De Chiara *et al*., 2014).

The application of molecular techniques has allowed significant progress in the identification of *H. influenzae* and other closely related groups such as *H. aegyptius* and *H. haemolyticus*. None of the phenotypic tests proposed to separate *H. aegyptius* from *H. influenzae* (distinct rod shape, susceptibility to troleandomycin, inability to grow on tryptic soy agar with haem and NAD, ability to agglutinate human erythrocytes and inability to ferment D-xylose) were successful. In addition, based on interspecific DNA transformation and DNA hybridization, the two organisms have been shown to be the same species. The designation of *H. influenzae* biotype aegyptius has been proposed to include traditional *H. aegyptius* and the related clones responsible for Brazilian purpuric fever (Brenner *et al*., 1988).

H. haemolyticus is closely related to *H. influenzae*. Phenotypically, both species require haem and NAD for growth, cannot ferment sucrose, and show a similar colony and cellular morphology. One characteristic that allows the identification of *H. haemolyticus* is the β-haemolysis on horse blood agar. However, some authors have reported that β-haemolysis is a poor indicator for distinguishing the two species because this haemolytic activity may be lost over the course of several subcultures, and furthermore, non-haemolytic *H. haemolyticus* strains have also been identified (Binks *et al*., 2012). Therefore, molecular methodologies are essential for the identification of *H. haemolyticus*. Whole genome sequencing and multilocus sequence analysis provide the most accurate identification of true *H. influenzae* (Binks *et al*., 2012; Norskov-Lauritsen *et al*., 2009). Nevertheless, these methodologies are laborious and expensive and are not sustainable for routine clinical screening (Binks *et al*., 2012). For this reason, many genes have been proposed as unique targets to differentiate the two species, among them the lipooligosaccharide gene *lgtC*, the IgA protease gene *iga*, the fuculose kinase gene *fucK*, the pilus gene *pilA*, the 16S rRNA, and the gene *hdp* encoding for the protein D (Pickering *et al*., 2014). However, no single gene tested was able to fully discriminate between *H. influenzae* and *H. haemolyticus* (Binks *et al*., 2012). In fact, the possibility of recombination by transformation between these two species has been suggested; thus,

despite the powerful molecular tools used for their differentiation, the genetic diversity of strains associated with *H. influenzae* makes it difficult to define the borders between species (McCrea *et al*., 2008; Norskov-Lauritsen *et al*., 2009; Norskov-Lauritsen, 2014).

1.2.3 Identification based on proteomic fingerprinting

Recently, proteomic profiling by matrix-assisted laser-desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has been successfully used in the identification of several microorganisms (Randell, 2014). This methodology consists in the ionisation of proteins and allows the generation of a mass spectrum based on the time-of-flight analysis of the ionized particles. The comparison between the generated spectrum with the spectra in the reference database permits identification (Randell, 2014).

In 1998, Haag and co-workers demonstrated that this technique was able to identify *H. influenzae* (Haag *et al*., 1998). Since then, the incorporation of new algorithms and improved databases has facilitated the differentiation between nontypeable *H. influenzae* (NTHi) and *H. haemolyticus* using mass spectometry, and it has been proposed as a reliable method for the identification of these species (Bruin *et al*., 2014; Zhu *et al*., 2013).

1.3 Typing of *H. influenzae*

1.3.1 Capsular identification by serotyping

H. influenzae strains are divided into two differentiated groups, encapsulated and nonencapsulated or nontypeable, depending on the presence or absence of a polysaccharide capsule. Margaret Pittman, in 1931, described both groups, S and R, based on the appearance of the colonies (Pittman, 1931). The S strains (encapsulated strains) had smooth surface, large size, opaqueness, and iridescence in oblique transmitted light. On the other hand, R strains (nonencapsulated strains) were rough and irregular in outline, were less opaque than the S colonies, smaller in size, and were not iridescent. In the same study, she described distinct immunological types in the S

strains, called type a and type b (Pittman, 1931). Later, in 1934, the number of serotypes was extended to six (a-f), based on distinct capsular polysaccharides (Platt, 1937).

Two methodologies are used in the laboratory identification of encapsulated *H. influenzae*: latex agglutination and molecular identification by PCR. Latex agglutination is based on the clumping of bacterial cells in the presence of the right antibody which facilitates the visible expression of the aggregation of antigens and antibodies. In this test, antibody molecules are bound to latex beads. In the presence of a specific antigen, the antibody will bind to the bacteria to form visible aggregates, or clumps. There are commercial kits of antisera for serotyping of encapsulated *H. influenzae* strains (Murray *et al*., 2007). PCR identification is based on the detection of a specific region of the capsular locus in the encapsulated isolates. The detection of *bexA* gene is used to discriminate between encapsulated and nontypeable isolates. This gene is common in all the capsular types because it is essential for capsular expression. Identification of the six serotypes is performed by amplification of the serotype specific regions in the capsular locus. Furthermore, there are capsule-deficient mutants of type b strains (b^-) that have lost the gene *bexA* but conserve the specific genes for type b strains. The PCR can recognize these mutants, whereas with agglutination they would be identified as nontypeable (Falla *et al*., 1994).

1.3.2 Biotyping

On the basis of three biochemical reactions based on indole production (detection of the tryptophanase activity), urease activity (capacity to hydrolyse urea) and ornithine decarboxylase activity (ability to use the amino acid ornithine as a source of carbon), *H. influenzae* can be divided into eight biotypes or biovars (Table 3) (Murray *et al*., 2007).

Table 3. Classification of the eight biotypes described for *H. influenzae*. Table adapted from Murray (Murray *et al*., 2007).

Biotype	Enzyme test		
	Indole	Urease	Ornitine Descarboxilase
I	+	+	+
II	+	+	-
III	-	+	-
IV	-	+	+
V	+	-	+
VI	-	-	+
VII	+	-	-
VIII	-	-	-

1.3.3 Molecular typing

H. influenzae strains (especially nontypeable strains) present a high level of diversity, and many methods have been proposed for typing these strains in epidemiological studies. With the development of genotyping methodologies, the classical typing techniques such as biotyping and others based on membrane proteins, metabolic enzymes, or lipooligosaccharide analysis have been replaced and are not currently used in epidemiological studies (Barenkamp *et al*., 1982; Campagnari *et al*., 1987; Porras *et al*., 1986).

Several genotyping methodologies have been proposed for the epidemiological characterization of *H. influenzae* such as randomly amplified polymorphic DNA profiles (RAPD) (Jordens *et al*., 1993), intergenic dyad sequence-PCR (Bruant *et al*., 2003), 16S rRNA (Sacchi *et al*., 2005), and multiple-locus variable number tandem repeat analysis (MLVA) (Schouls *et al*., 2005). However, the methodologies most frequently used in *H. influenzae* genotyping are pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST).

- Pulsed-field gel electrophoresis (PFGE): This methodology involves the chromosomal DNA digestion with restriction endonucleases and subsequent separation of the DNA fragments in a pattern of discrete bands by pulsed-field electrophoresis. The level of relatedness depends on the number of different bands in the patterns (Tenover *et al*., 1995). This technique presents high reproducibility and discriminative power (Aparicio *et al*., 1996).

- Multi-locus sequence typing (MLST): This methodology is based on the assignation of allele numbers to different sequences of internal fragments of seven housekeeping genes. The sequence type (ST) or allelic profile is determined by the combination of these seven gene loci. In 2003, Meats *et al*. described the technique for *H. influenzae*, using *adk*, *atpG*, *frdB*, *fucK*, *mdh*, *pgi*, and *recA* as the housekeeping genes (Meats *et al*., 2003). The advantages of MLST are that it provides data that can be compared between laboratories, and that all the data can be stored in an online database (http://haemophilus.mlst.net/).

Recently, with the development of sequencing technology that allows sequencing of whole genomes from multiple strains in a short period of time at a low cost, whole genome sequencing has become a powerful tool to study the diversity within *H. influenzae* (Power *et al*., 2012).

1.4 Population structure

H. influenzae is a microorganism that presents a high genetic diversity. Encapsulated isolates present a clonal structure, whereas NTHi are genetically diverse and distant from encapsulated strains (Fig. 2) (LaCross *et al*., 2008; Meats *et al*., 2003; Musser *et al*., 1986; Musser *et al*., 1988).

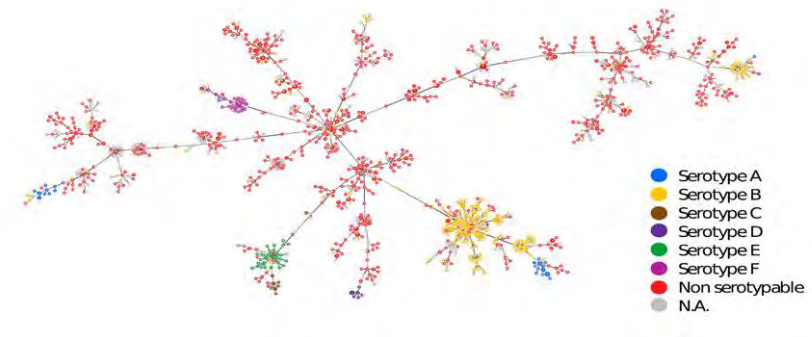

Figure 2: Minimum spanning tree based on the allelic profile of all isolates present in the MLST database. Each ST is coloured according to the serotype. Most capsular isolates were grouped in a small number of clonal complexes whereas NTHi were dispersed in a high number of clonal complexes. Figure obtained from De Chiara *et al*. (De Chiara *et al*., 2014).

In the 1980s, Musser and co-workers studied the population structure of a vast and representative collection of encapsulated strains recovered worldwide over a long period of time and from different medical conditions, mostly invasive diseases. Using multilocus enzymatic electrophoresis, outer membrane protein profiles and *cap* region restriction fragment length polymorphism, they demonstrated that encapsulated strains had a clonal structure formed by two phylogenetic divisions (Fig. 3): division I grouped the majority of serotype a (Hia) and serotype b (Hib) isolates, and all the serotype c (Hic) and serotype d (Hid) isolates; division II grouped all serotype f (Hif) isolates and a group of Hia and Hib isolates (Musser *et al*., 1988). Moreover, they also found that although *H. influenzae* is a competent microorganism, the recombination frequency between particular clones of encapsulated isolates was low. Consequently, the hypotheses suggested to explain this observation were that the capsule acts as a physical barrier to taking exogenous DNA, that various restriction modification systems limit chromosomal recombination, or that different capsular strains were infrequently carried in the same host (Musser *et al*., 1988). Years later, this division was corroborated using molecular methodologies such as MLST (Erwin *et al*., 2008; Meats *et al*., 2003).

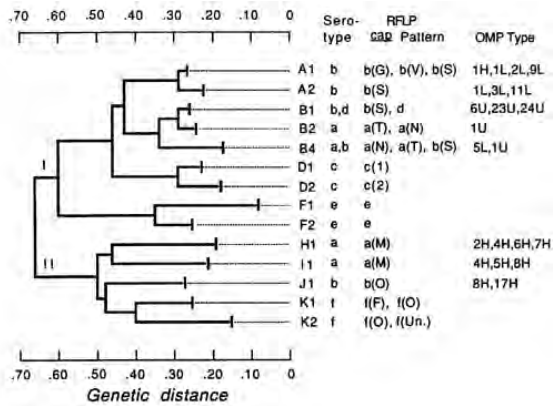

Figure 3: Schematic dendrogram representing the genetic relationship between encapsulated *H. influenzae*, formed by two divisions (I and II). Figure obtained from Musser *et al*. (Musser *et al*., 1988).

On the other hand, NTHi presents more genetic heterogeneity than encapsulated strains (LaCross *et al*., 2008; LaCross *et al*., 2013; Meats *et al*., 2003; Musser *et al*., 1986). Several studies using MLST showed that despite the diversity, NTHi isolates were grouped in clusters (Erwin *et al*., 2008; LaCross *et al*., 2008; LaCross *et al*., 2013).

The maintenance of these groups was not affected by geographical location or clinical origin (Erwin *et al*., 2008; LaCross *et al*., 2013). However, Erwin *et al*. suggested that these phylogenetic groups might be maintained due to the presence of different adhesins denoting the occupancy of niches within the nasopharynx for strains from different clusters, and also by a limited exchange of DNA caused by the diversity of the restriction systems (Erwin *et al*., 2008). Moreover, the evolution of recombination within NTHi may have had more impact than in the encapsulated strains because NTHi had a higher capacity to transform, or because they had more opportunities to meet other NTHi strains in the nasopharynx of the same host, since co-colonization with more than one strain has been described (Meats *et al*., 2003).

Recently, whole genome sequencing has been used to characterize the population structure of NTHi (De Chiara *et al*., 2014). With this approach, the NTHi isolates studied were classified into six different clades (Fig. 4), and although there was some correlation between some of the clades and some of the MLST groups, they could not be totally predicted using MLST. As described above, no association between geography and disease was observed among strains from the same clade. However, a certain correlation has been identified between population structure and the presence of genes encoding for virulence factors such as LOS or surface proteins (De Chiara *et al*., 2014).

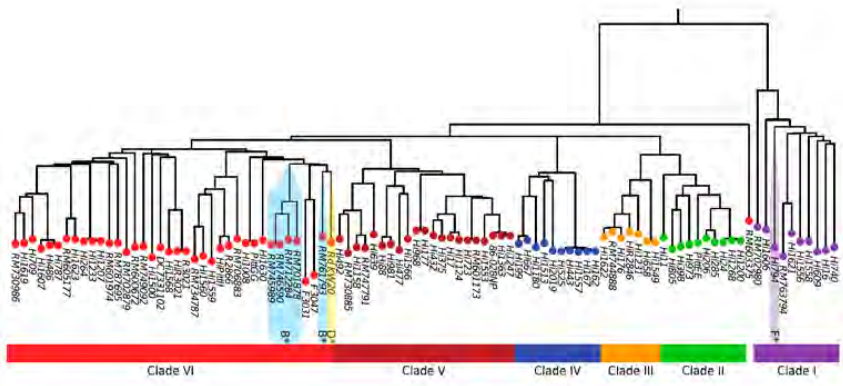

Figure 4: Phylogenetic tree based on genome sequenced NTHi strains showing the six clades. Strains marked with B*, D* and F* are strains that have most of the capsular locus but they do not express the polysaccharide capsule. Figure obtained from De Chiara *et al*. (De Chiara *et al*., 2014).

1.5 Reservoir and colonization

H. influenzae is a human restricted microorganism that forms part of the normal nasopharyngeal microbiota (Erwin *et al*., 2007). Pharyngeal carriers are the unique reservoir of this bacterium and the transmission vector of disease (Moxon, 1986). Exposure to *H. influenzae* occurs early, within the first three days of life according to Gratten *et al.* and before 10 days according to Leach *et al.* (Gratten *et al*., 1986; Leach *et al*., 1994). In any case, most children are colonized by *H. influenzae* before the age of three (Principi *et al*., 1999). The colonization rate is high among children and decreases with age (Kuklinska *et al*., 1984).

Nasopharyngeal colonization by *H. influenzae* is a dynamic process that starts with the acquisition of a strain, which is kept for some time, and is followed by either a colonization-free period or the acquisition of a new strain (Raymond *et al*., 2001; Trottier *et al*., 1989). Colonization by more than one strain of *H. influenzae* at the same time is also common (Farjo *et al*., 2004). Certain factors have been associated with colonization, including prolonged full-time exposure in day-care centres, large family size, socioeconomic factors linked to the size of the living area and recent antibiotic treatment (Raymond *et al*., 2001). By contrast, other proposed factors such as seasonal colonization are still controversial. Some authors have not found significant differences between seasons (Dabernat *et al*., 2003; Sulikowska *et al*., 2004) though others have reported an increased rate of colonization in spring or in winter time (Hashida *et al*., 2008; Marchisio *et al*., 2001).

Most studies of colonization in children have been conducted at day-care centres, with a highly variable colonization rate ranging from 32.1% to 87%. The highest colonization rate was due to NTHi strains (5% to 64%). By contrast, colonization by encapsulated strains was lower than by NTHi, ranging between 0.6% and 10%. The colonization for the other serotypes was low (0.4% to 4.6%) (Dabernat *et al*., 2003; de Carvalho *et al*., 2011; Farjo *et al*., 2004; Fontanals *et al*., 2000; Hashida *et al*., 2008; Munsawaengsub *et al*., 2010; Murphy *et al*., 1985; Oh *et al*., 2008; Sa-Leao *et al*., 2008; Trottier *et al*., 1989; Wang *et al*., 2008).

The few colonization studies performed in healthy adults have reported lower rates than in children. Data published by Kuklinska *et al*. showed colonization rates for NTHi of 40% in adults and 80% in children (Kuklinska *et al*., 1984). The same behaviour was shown in colonization by non-type b capsular strains (0.8% in adults *vs*. 1.9% in children), as well as in serotype b, with no Hib strains identified in adults *vs*. 4.2% found in children (Oh *et al*., 2008).

2. INFECTIONS CAUSED BY *H. influenzae*

H. influenzae can cause a wide spectrum of diseases. The most important invasive diseases such as meningitis, bacteraemia, and epiglottitis have frequently been associated with encapsulated strains, mainly Hib, the most virulent serotype (Zwahlen *et al*., 1989). These severe diseases were common worldwide prior to the introduction of the conjugate Hib vaccines, and are still frequent in countries where the vaccine has not been implemented (Peltola, 2000). The other serotypes (mainly Hia and Hif) account for a small proportion of these severe invasive diseases (Ulanova *et al*., 2009).

NTHi is generally associated with respiratory tract infections such as community-acquired pneumonia or otitis media, and causes exacerbations in patients with chronic obstructive pulmonary disease (COPD). It is an important cause of morbidity and mortality in developed and developing countries (Foxwell *et al*., 1998). Despite the fact that severe invasive diseases have mostly been associated with encapsulated strains, since the introduction of the vaccine NTHi has become an important cause of invasive infections (Ulanova *et al*., 2009).

2.1 Meningitis

Clinical symptoms of meningitis due to *H. influenzae* are similar to those produced by other bacteria, including fever, decreased mental status, and stiff neck due to the inflammation of the meninges. A third of survivors present deafness or other neurological sequelae as a result of the infection (Saez-Llorens *et al*., 2003). Meningitis can occur through direct spread from the middle ear or through a haematogenous phase (Moxon, 1992).

Hib was one of the most common pathogens causing meningitis in infants and small children between three months and three years of life (Saez-Llorens *et al*., 2003). However, since the introduction of the conjugate vaccine against Hib, NTHi has become a frequent cause of meningitis in children (van Wessel K. *et al*., 2011).

In adults, meningitis due to *H. influenzae* is rare, mainly affecting people with risk factors such as immunological disorders or underlying diseases (van de Beek *et al*., 2006). After the introduction of Hib vaccination, NTHi became the most common type

of *H. influenzae* causing meningitis and other invasive diseases (Brouwer *et al*., 2007; Dworkin *et al*., 2007; Perdue *et al*., 2000).

2.2 Bloodstream infections

The presence of viable bacteria in the blood is known as bacteraemia and it can be classified as transient, intermittent, or persistent depending on the period of bacterial persistence in the bloodstream. Consequently, bloodstream infections (BSI) are defined as the presence of viable bacteria in the blood documented by a positive bacterial detection in a blood culture. In this case, bloodstream infections can be classified as primary BSI when the source of infection is not identified, or as secondary BSI when the bacterial entrance is a complication of other infections such as pneumonia, meningitis, biliary tract infection, skin or soft-tissue infection, and wound infection (Seifert, 2009).

Since the introduction of the Hib vaccine, bacteraemia due to NTHi has become one of the most common clinical manifestations in invasive diseases, both in children and in adults (O'Neill *et al*., 2003; Sarangi *et al*., 2000). In adults, the most commonly reported clinical manifestations of bloodstream infections due to NTHi are bacteraemic pneumonia followed by primary bacteraemia (Macneil *et al*., 2011; Sarangi *et al*., 2000).

2.3 Pneumonia

Pneumonia is an infection of the parenchymal part of the lung that can be defined as community-acquired pneumonia (CAP), hospital-acquired pneumonia (HAP), ventilator-associated pneumonia (VAP), or health care-associated pneumonia (HCAP) (Niederman, 2010). Generally, CAP is defined as pneumonia acquired outside the hospital environment (Chacon *et al*., 2010). An episode of CAP is diagnosed when new pulmonary infiltrates are detected on chest radiography, together with the presence of other clinical symptoms such as fever, new cough, pleuritic chest pain, dyspnoea or altered breath sounds on auscultation (Garcia-Vidal *et al*., 2009). This infection is called HAP when it develops after at least 48h of hospitalization, and VAP if the patient has previously received mechanical ventilation (Niederman, 2010). The differentiation in

the type of the acquisition, nosocomial or community, is important because they are caused by different etiological agents (Seong *et al*., 2014), and require different kinds of management (American Thoracic Society, 2005; Mandell *et al*., 2007).

CAP is one of the most common respiratory infections and a major cause of mortality and morbidity. The incidence of CAP is more prevalent in children and the elderly than in other age groups (Spoorenberg *et al*., 2014). Due to the high percentage of patients with CAP who require hospitalization, the economic burden of this infection is particularly high. In Spain, the estimated cost per patient in a tertiary hospital is 5,500 dollars (Ostermann *et al*., 2014).

In children, pneumonia is the main cause of death worldwide with an estimated mortality rate of between one and two million deaths each year (UNICEF *et al*., 2006). In adults, the incidence of CAP increases with age, along with the hospitalization and mortality rates which are higher in the elderly (Simonetti *et al*., 2014). In Europe, the most common cause of CAP is *S. pneumoniae*. The prevalence of *H. influenzae* fluctuates according to the study, but it has been identified as a frequent pathogen along with *Mycoplasma pneumoniae*, Gram-negative enteric bacilli, and respiratory viruses (Torres *et al*., 2014). Furthermore, *H. influenzae* is also a common cause of CAP in patients with Chronic Obstructive Pulmonary Disease (Gomez-Junyent *et al*., 2014).

2.4 Acute exacerbations in COPD

COPD is one of the most common chronic diseases in the world and a major cause of mortality and morbidity. Its global prevalence has been estimated at around 10% in the over-40s (Halbert *et al*., 2006), but with variations between countries, being higher in developing regions (Decramer *et al*., 2012). According to the World Health Organization (WHO), COPD is currently the seventh cause of disability and the fourth cause of death worldwide (Mathers C *et al*., 2008), and is predicted to become the third cause of death by 2030. In addition, COPD is a high economic burden in health care systems and will remain so in the near future (Sullivan *et al*., 2000).

COPD is a preventable disease characterized by a progressive and non-reversible airflow limitation, associated with an abnormal chronic inflammatory response in the airways and the lungs to noxious particles and gases, mainly cigarette smoke (Rabe *et*

al., 2007). Although the main risk factor is tobacco, other factors can increase the risk such as maternal smoking, childhood respiratory diseases, air pollution, and biomass smoke exposure. Furthermore, in a small percentage of patients, COPD is due to a genetic cause, α_1 antitrypsin deficiency. Typically, COPD has been diagnosed in men, but due to the increase in the number of female smokers, the detection of COPD is increasing in this population (Decramer *et al*., 2012).

During the natural progression of the disease, acute exacerbations are manifested. These acute events are characterized by an increase in the patient's baseline symptomatology in terms of dyspnoea, cough, and/or expectoration, which may require a change in the regular medication and admission to hospital (Anzueto *et al*., 2007). Acute exacerbations have a negative effect on patient's quality of life, accelerate disease progression, and increase the risk of mortality. Despite the high impact of exacerbations on patients, there are no biomarkers that can predict them and diagnosis is based only on clinical symptomatology (Decramer *et al*., 2012). The cause of acute exacerbations may be multifactorial, but most are caused by viral or bacterial infection. The most common bacterial pathogens involved in acute exacerbations are *H. influenzae* (20-30%), followed by *S. pneumoniae* and *Moraxella catarrhalis* (10-15%). Furthermore, it has been suggested that *P. aeruginosa* can play an important role in severe COPD (Sethi *et al*., 2008). The acquisition of a new strain of NTHi, *S. pneumoniae* or *M. catarrhalis* has been associated with a new episode of exacerbation (Sethi *et al*., 2002), and recently, this association has also been described for *P. aeruginosa* (Murphy *et al*., 2008).

2.5 Acute otitis media

Acute otitis media (AOM) is one of the most common infections in children requiring consultation, and it is the most frequent reason for surgery and antibiotic prescription in the paediatric population (Rovers *et al*., 2004). AOM is an inflammation of the middle ear as a consequence of a bacterial migration via the Eustachian tube from the nasopharynx. Frequently, viral infections can trigger this migration (Leibovitz *et al*., 2004; Murphy *et al*., 2009). AOM is characterized by the presence of middle ear effusion and a pool of clinical manifestations such as fever, persistent ear pain, nauseas, vomiting, conductive hearing loss, and diarrhoea. *H. influenzae* is one of the most

common causes of AOM, accounting for 25 to 35% of clinical episodes (Leibovitz *et al*., 2004; Murphy *et al*., 2009). Recurrent otitis media is defined as four or more episodes of AOM in a year, or eight months of middle ear effusion in a year. More than 10% of children suffer from recurrent otitis media, and NTHi is the most common bacterial cause (Murphy *et al*., 2009).

2.6 Other infections

- Epiglottitis is an acute infection of the supraglottic structures, the tissue in the throat that covers and protects the larynx during swallowing, which may cause fatal airway obstruction (Winn *et al*., 2008). Hib used to be the main cause of epiglottitis but with the introduction of the conjugate Hib vaccine, the number of cases in the paediatric population has fallen dramatically. Consequently, the disease is now more common in the adult population (Berger *et al*., 2003; Guldfred *et al*., 2008; Mathoera *et al*., 2008; Shah *et al*., 2010)

- Cellulitis is an infection of the dermal and subcutaneous layers of the skin (Gunderson, 2011). Cellulitis caused by *H. influenzae* is manifested as orbital cellulitis, with blue swellings over the cheeks and around the eyes, and it is more associated with the paediatric population (Ambati *et al*., 2000). Hib was a common cause of orbital cellulitis before the vaccination, with high rates of bacteraemia due to periorbital cellulites (Smith *et al*., 1978). Recently, cases of cellulitis associated with non-b strains have been described in adults (Lev *et al*., 1999).

- Conjunctivitis is any inflammation of the conjunctiva. Generally, it is characterised by irritation, itching, foreign body sensation, and discharge (Epling, 2012). Acute conjunctivitis is a common infection in childhood and NTHi is the causal agent in 50% of the cases (Chawla *et al*., 2001). By contrast, in the adult population *Staphylococcus* species are the most common pathogens for bacterial conjunctivitis, followed by *S. pneumoniae* and *H. influenzae* (Epling, 2012).

- Brazilian purpuric fever (BPF), a fulminant paediatric disease caused by *H. influenzae* biogroup *aegypticus*, is characteristically preceded by a purulent conjunctivitis that has resolved before the onset of fever. This infection starts with acute fever and haemorrhagic skin lesions, followed by vascular collapse, hypotensive shock, and death,

usually within 48h of onset. BPF was first recognized in 1984 during an outbreak in Sao Paulo (Brenner *et al*., 1988), and remains a rare paediatric disease with only 69 cases described between the first identification in 1984 and 2007 (Santana-Porto *et al*., 2009).

- Acute sinusitis is an inflammation of the mucosal lining of the nasal passage and paranasal sinuses which can be caused by a variety of factors, such as environmental irritants, allergy, and infection by pathogenic microorganisms. *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* are important pathogens in acute and chronic sinusitis (Brook, 2006; Brook, 2013).

H. influenzae is a rare cause of other infections such as endocarditis (Frayha *et al*., 1996; Georgilis *et al*., 1998; Malik, 1995), urogenital infections (Quentin *et al*., 1990), peritonitis (Dimopoulou *et al*., 2013; Saadi *et al*., 2013), osteomyelitis (Sarria *et al*., 2001), and septic arthritis (Howard *et al*., 1999; Le *et al*., 2013; Shoaib *et al*., 2007).

3. EPIDEMIOLOGY OF *H. influenzae* INFECTIONS

The introduction of the Hib conjugate vaccine in the 1990s represented a turning point in the epidemiology of *H. influenzae* infections. Child vaccination had a high impact in the epidemiology of this bacterium (Peltola, 2000).

3.1 Epidemiology before vaccination against Hib

Before the introduction of the vaccination, most *H. influenzae* infections were caused by Hib and were associated with invasive diseases, mainly meningitis. Although Hib was the most common cause of meningitis in children less than five years old, variability between different geographical areas and ethnic groups was reported. In European countries, the incidence of invasive Hib disease in children less than five years old in the 1980s and 1990s ranged between 21 cases per 100,000 population in France and 60 cases per 100,000 in Switzerland (Levine *et al*., 1998). In the US, the incidence was higher, with 60-100 cases per 100,000 children. In Asian countries there was a high variability between geographical areas, with a very low incidence in places as Hong-Kong and Malaysia (three and eight cases per 100,000, respectively) in contrast with high incidence regions such as Vanuatu (163 cases per 100,000). In specific ethnic groups, the incidence of invasive Hib disease was high, for example, in native children from Australia (450 cases per 100,000) and from the native population in the US, such as Apache (254 cases per 100,000) and Navajo (152 cases per 100,000) (Levine *et al*., 1998). However, in Alaskan Eskimos, the incidence was lower (84.4 cases per 100,000) (Ward *et al*., 1981).

In Spain, the incidence of invasive disease by *H. influenzae* in children under five years prior to Hib vaccination showed a high variability between regions, ranging from 8.4 cases per 100,000 population in Navarre to 26.3 per 100,000 in the Basque Country (Guallar-Castillon *et al*., 1997). In Catalonia, the incidence of invasive diseases due to *H. influenzae* in paediatric population was 8.4 per 100,000, the same rate as in Navarre (Dominguez *et al*., 2004). However, in Catalan adult population, the incidence rate was lower: 1.2 per 100,000 inhabitants (Deulofeu *et al*., 1994).

The estimated worldwide incidence for the different invasive diseases in young children presents variability between developed and developing regions (Peltola, 2000).

Table 4 summarizes the incidence and mortality rate of the most important invasive diseases in both types of country.

Table 4. Incidence and mortality of the main *H. influenzae* invasive diseases in developing and developed countries before the introduction of conjugate vaccination.

	Developing countries		Developed countries	
	Cases/ 100,000 population	Mortality (%)	Cases/ 100,000 population	Mortality (%)
Meningitis	60	30	32	5
Epiglottitis	<1	20	13	2
Pneumonia	300	13-24	6	5
Other infections	12	10	12	2

3.2 The introduction of vaccination

The initial development of vaccines against Hib was promoted by the high mortality rate of children with invasive infection, the high incidence of sequelae in the central nervous system in children who survived meningitis infection, and the gradual emergence of strains with antimicrobial resistance (Barbour, 1996). The type b polysaccharide capsule, a polymer of ribose ribitol phosphate (PRP), is the most important virulent factor in Hib and is highly immunogenic, characteristics that made the capsule an ideal candidate for vaccine development. In the 1970s, a polysaccharide vaccine directed against Hib was tested and presented good results in children older than 18-20 months of age (Peltola *et al*., 1984). However, this vaccine had three important limitations: poor immunogenicity in children younger than 18 months, lack of a booster effect, and no clear effect in nasopharyngeal carriage (Kayhty *et al*., 1984; Peltola *et al*., 1984; Takala *et al*., 1989). For these reasons, in the 1980s, conjugated peptides were added to the polysaccharide vaccine in order to enhance the immunogenicity (Schneerson *et al*., 1980). The immunogenic principle of the conjugates is the transformation of a T-cell independent antigen into a T-cell dependent one using a carrier protein, which allows this transformation (Stein, 1992). Peptide conjugation allows increased production of protective antibodies, even in children under 18 months of age, and an improvement in protection with booster doses compared with the polysaccharide vaccine (Schneerson *et al*., 1980). The first conjugate vaccine introduced was the diphtheria toxoid conjugate (PRP-D; ProHIBiT), followed by the

mutant diphtheria toxin conjugate (PRP-CRM or HbOC), the meningococcal outer membrane protein conjugate (PRP-OMP), and the tetanus toxoid conjugate (PRP-T). These conjugate vaccines have an excellent safety record (Peltola, 2000).

Since the 1980s, the large-scale implementation of conjugate Hib vaccines has been extraordinarily successful. With considerable efforts, the vaccines had been implemented in 184 countries by the end of 2012, representing a worldwide coverage of around 45%. However, in some regions of south-east Asia and the western Pacific the vaccine coverage is still low, between 11% and 14% (WHO, 2014). The conjugate Hib vaccine was commercialized in Spain in 1993 and was included in the Spanish vaccination program for children in 1998. The vaccine was given at two, four, six months with a booster dose at 18 months of age. In 1998, vaccine coverage was 81.6%, and has been more than 95% since 2000 (Ministerio de Sanidad, 2014).

3.3 Epidemiology after vaccination against Hib

3.3.1 Impact on invasive Hib disease

The introduction of conjugate vaccines brought down the incidence of the infections caused by Hib rapidly and significantly in the countries that implemented the vaccination in their immunization programs (Adams *et al*., 1993; Murphy *et al*., 1993; Slack *et al*., 1998). This successful decrease could be partially explained by the prevention of nasopharyngeal Hib colonization due to vaccination. The reduction of circulating strains in the population has a beneficial effect in non-vaccinated subjects and contributes significantly to the effectiveness of the vaccine (Agrawal *et al*., 2011; Peltola, 2000). In the post-vaccine era, invasive Hib has affected adults more often than children, especially the elderly, immunocompromised patients and those with underlying conditions (Collins *et al*., 2013; Nix *et al*., 2012; Rubach *et al*., 2011).

In Europe, the incidence of Hib diseases ranged from <1 per 100,000 in Scandinavia, the UK and the Netherlands to 10 per 100,000 in Switzerland. Similar decreases have been occurred in countries in America, Asia and Oceania. Specifically, in Spain, in the Basque region, in 1997 the incidence rate for invasive Hib disease was 2 per 100,000 (Peltola, 2000). In a study performed in Madrid during 1999-2000, the

overall incidence rate was 1 per 100,000, with a high incidence in children under 1 year old (16.3 per 100,000) and adults over 70 (2.6 per 100,000) (Campos *et al*., 2004).

Since the 1990s, some failures have been reported in children who received the conjugate vaccine. These failures are rare and were sometimes associated with an underlying condition and/or immunoglobulin deficiency (Heath *et al*., 2000; Holmes *et al*., 1992; Lee *et al*., 2008). Prematurity, Down's syndrome, malignancy, cyclical neutropenia, and IgG2 deficiency are risk factors and immunological deficiencies associated with vaccine failure. These factors account for less than half of all vaccine failure cases, while other predisposing conditions, including defects of the vaccines, may explain the rest of the cases (Heath *et al*., 2000; Lee *et al*., 2008). In Spain, in a 5-year period (1997-2002), five cases of vaccine failure were described: among them, one child had HIV and the rest did not have an appropriate immune response although they completed the vaccination program (Campos *et al*., 2003). Furthermore, in order to explain vaccine failures not caused by immunological deficiencies, it has been suggested that the presence of multiple copies of the capsular locus in Hib strains increasing capsular production may be the reason for the vaccine failure in immunized patients because these strains would be more resistant to host defences (Cerquetti *et al*., 2005).

Although a substantial reduction in Hib diseases has been observed since the introduction of widespread vaccination, the WHO estimated that in 2007 Hib still caused at least three million cases of serious illness and about 386,000 deaths worldwide (WHO, 2006).

3.3.2 Impact on invasive non-Hib disease

Since the introduction of vaccination, the epidemiology of invasive diseases has changed in terms of the age of patients who suffer from invasive diseases, the clinical manifestations, and the strains producing the infection (Ulanova *et al*., 2009). Instead of children, the groups who suffer from an invasive *H. influenzae* infection have been currently identified as neonates (first weeks of life), the elderly (≥ 65 years) and individuals with underlying diseases (Dworkin *et al*., 2007; Laupland *et al*., 2011; O'Neill *et al*., 2003). Regarding the clinical manifestation, bacteraemia rather than meningitis is the most common clinical infection in children (O'Neill *et al*., 2003), and

bacteraemia and pneumonia in adults (Dworkin *et al*., 2007; Rubach *et al*., 2011; Sarangi *et al*., 2000).

Since vaccination became available, there has been a shift in the serotypes causing invasive infections. NTHi is the most frequent cause of invasive disease in children and in adults (Dworkin *et al*., 2007; Kalies *et al*., 2009; Sarangi *et al*., 2000; Tsang *et al*., 2007). Infections caused by encapsulated non-type b strains, especially Hia and Hif, have been described in several locations (Adam *et al*., 2010; Dworkin *et al*., 2007; Ladhani *et al*., 2010; Ladhani *et al*., 2012; Mathers C *et al*., 2008; Millar *et al*., 2005; Ulanova *et al*., 2014).

The increase in the incidence of invasive diseases caused by non-Hib *H. influenzae* is controversial. Some reports published in the US, Canada and Europe have suggested an increase in incidence (Dworkin *et al*., 2007; Ladhani *et al*., 2010; Rubach *et al*., 2011; Tsang *et al*., 2007; Bajanca *et al*., 2004), but others do not (Campos *et al*., 2004; Kalies *et al*., 2009; Ladhani *et al*., 2008b).

4. VIRULENCE FACTORS

H. influenzae produces several virulence factors which allow the bacterium to colonize the respiratory human epithelium and are responsible for the pathogenesis (Rao *et al*., 1999).

4.1 Polysaccharide capsule

The capsule is a well-characterized virulence factor that makes the strains more resistant to the phagocyte-mediated killing and enhances the ability of *H. influenzae* to cause invasive disease (Noel *et al*., 1992; Weller *et al*., 1977). Based on animal models of infection using isogenic mutants transformed with serotype-specific capsular DNA, it has been demonstrated that the most virulent serotype is Hib, followed by Hia. The remaining four serotypes (c to f) have a reduced capacity for virulence (Zwahlen *et al*., 1983; Zwahlen *et al*., 1989). The genes encoding the capsular polysaccharide are located in the *cap* locus which is formed by three functional regions (Fig. 5).

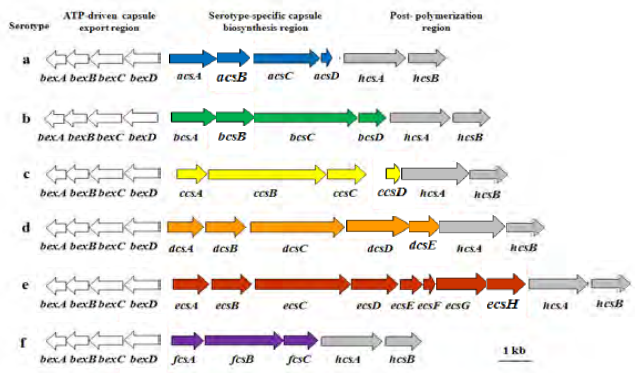

Figure 5: Capsular loci for the six *H. influenzae* serotypes. Figure from the Centers for Disease Control and Prevention (http://www.cdc.gov/meningitis/lab-manual/chpt10-pcr.html) (Centers For Disease Control and Prevention (CDC), 2014).

Region I includes the components of an ATP-driven polysaccharide export apparatus (*bexDCBA*). Region III, formed by genes *hcsA* and *hcsB*, is involved in modification and export of the capsular polysaccharides. These regions are common to all six serotypes. The serotype-specific region (Region II) contains genes for capsular synthesis and is unique to each serotype. This region is named *acs* to *fcs*, depending on

the capsular type, and is defined as “a-capsule synthesis” to the last type “f-capsule synthesis” (Kroll *et al*., 1989; Satola *et al*., 2003a).

Encapsulated strains from the two previously described phylogenetic divisions present a different chromosomal location of their capsular locus and genetic differences in the flanking regions of this locus (Satola *et al*., 2003a; Satola *et al*., 2003b). The division I strains present a *cap* locus flanked by repeats of insertion element *IS1016*, which do not appear in the division II strains (Kroll *et al*., 1991; Satola *et al*., 2003a; Satola *et al*., 2003b).

Most of the Hib strains present a partial duplication of the *cap* locus, with two complete copies of regions II and III, one complete copy of region I, and a truncated copy of region I with a deletion between the *IS1016* and *bexA* gene (Fig. 6A) (Kroll *et al*., 1988b). If a complete copy of the *cap* locus was lost in a recombination event, the truncated copy would remain in the genome forming a capsule-negative phenotype known as Hib$^-$ or b$^-$ because, although they cannot express the capsule due to the *bexA* gene deletion, they preserve the rest of the capsular locus (Fig. 6 B) (Hoiseth *et al*., 1985; Kroll *et al*., 1988a). Furthermore, this partial duplication of the *cap* locus has also been described in Hia strains in which, as in the Hib strains, it has been associated with virulence (Kapogiannis *et al*., 2005).

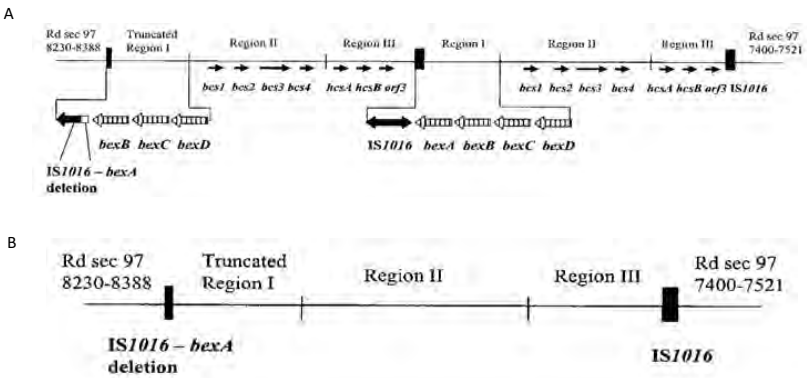

Figure 6: (A) Duplicated *cap* locus in a Hib strain showing the truncated region I with the deletion between *IS1016* and *bexA*. (B) Representation of the *cap* locus of Hib$^-$ strain. Figure from the Satola *et al*. 2003 (Satola *et al*., 2003a).

4.2 Immunoglobulin A1 proteases

Immunoglobulin A1 (IgA1) proteases, which degrade the IgA produced by mucosal tissues, have been described in different human mucosal pathogens such as *H. influenzae*, *S. pneumoniae*, *Neisseria meningitidis*, and *Neisseria gonorrhoeae* (Rao *et al*., 1999). These extracellular enzymes are able to cleave human IgA1 within the hinge region of the α-heavy chain, releasing the antigen binding domains (Fab) from the constant region (Fc), and resulting in the elimination of the agglutinating activity of both free and antigen-bound IgA (Plaut *et al*., 1975). Depending on the cleavage in the hinge region of the molecule, IgA1 proteases are classified as type 1 or 2. In *H. influenzae*, IgA1 proteases are encoded by two genes, *iga* and *igaB* (Fernaays *et al*., 2006) and they present four different patterns depending on the presence of protease activity and type: production of type 1, production of type 2, production of both types, and no protease activity. Differential IgA1 protease activity has been shown between strains isolated from infections and isolated from carriers, with a higher activity observed for the clinical isolates (Vitovski *et al*., 2002).

4.3 Lipooligosaccharide

The lipooligosaccharide (LOS) of *H. influenzae* is analogous to the lipopolysaccharide (LPS) of many Gram-negative bacteria. This molecule consists of an outer membrane-associated glycolipid formed by lipid A joined via 2-keto-3-deoxyoctulosonic acid (kdo) to an oligosaccharide core composed mainly of neutral heptose (Hep) and hexose (Hex) (Fig. 7A). The conserved heptose trisaccharide inner core provides a point where oligosaccharide chains and noncarbohydrate substituents can be added to form the outer core (Fig. 7B and Table 5) (Schweda *et al*., 2007). A characteristic of the *H. influenzae* LOS is its heterogeneity, both intra- and inter-strain. The intra-strain variability is a result of the variable length in the oligosaccharide chains due to incomplete biosynthesis and a phase-variable expression of some genes. Genetic differences are the cause of the inter-strain variability (Schweda *et al*., 2007).

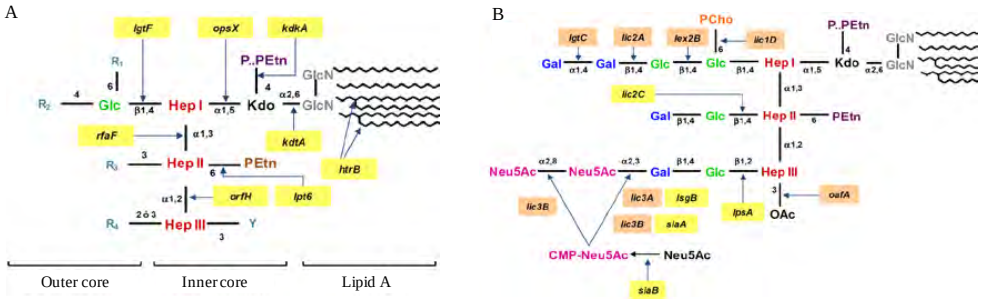

Figure 7: LOS structure of *H. influenzae*. (A) Conserved LOS structure of *H. influenzae* formed by lipid A, inner and outer core. In yellow, genes encoding enzymes involved in biosynthesis of the inner core. (B) Schematic representation of the LOS of *H. influenzae*. In yellow and orange, genes encoding enzymes involved in biosynthesis of the outer core. Genes regulated by phase variation are coloured in orange. Figure from the thesis of Martí-Lliteras, JP (Marti-Lliteras, 2012). **Abbreviations:** Hep: heptose; Glc: glucose; Gal: galactose; GlcN: glucosamine; PEtn: phosphoethanolamine; PCho: phosphorylcholine; Neu5Ac: sialic acid; OAc: O-acetyl group; P: phosphate group.

H. influenzae incorporates molecules into the LOS such as digalactoside, phosphorylcholine (PCho), and sialic acid which have been identified as host mimicking structures, helping in the bacterial camouflage from the immune system, and giving them an advantage during adhesion and invasion (Mandrell *et al*., 1993; Schweda *et al*., 2007). The presence of digalactoside confers resistance to human serum by antibody-dependent and complement-mediated killing, and by decreasing the C4b deposition on the bacterial surface due to the molecular mimicry with the antigen p^k present in host cells (Erwin *et al*., 2006; Ho *et al*., 2007; Weiser *et al*., 1998a). Another molecule that has been associated with virulence is PCho (Schweda *et al*., 2007). Although PCho confers susceptibility to complement-mediated killing by the C-reactive protein (CRP), the incorporation of PCho into the LOS plays an important role in persistence in the respiratory tract. Several mechanisms have been reported to decrease CRP binding, among them the position of PCho in the LOS, the selection of $PCho^-$ variants by phase-variable expression of *lic1*, and the presence of abundant pulmonary surfactant (Gould *et al*., 2002; Lysenko *et al*., 2000a; Weiser *et al*., 1998b). The adhesion to and invasion of the epithelial cells mediated by PCho has been linked to the receptor for platelet activating factor (rPAF), whose natural ligand also contains PCho, increasing the bacterial ability to colonize and persist within the respiratory tract

(Swords *et al*., 2000; Swords *et al*., 2001). Furthermore, PCho also confers resistance to LL-37/hCAP18 antimicrobial peptide killing (Lysenko *et al*., 2000b).

Sialylation of LOS is a major factor in the virulence of NTHi causing otitis media (Bouchet *et al*., 2003). Sialic acid acts as a mask of the LOS epitopes that are targets for the immune system (Harvey *et al*., 2001), confers complement resistance by inhibiting the C3 and C4 deposition (Figueira *et al*., 2007), and is involved in biofilm formation (Swords *et al*., 2004). In addition, O-acetylation of the LOS also plays a role in the virulence, increasing the resistance to human serum (Fox *et al*., 2005).

Table 5. Genes that encode proteins involved in the LOS biosynthesis.

Gene	Enzyme	Function	References
lgtF	Glucosyltransferase	Extension of HepI adding Glu	(Hood *et al*., 2004)
lic2C	Glucosyltransferase	Extension of HepII adding Glu	(Hood *et al*., 2004)
lpsA	Glucosyltransferase	Extension of HepIII adding Glu or Gal	(Hood *et al*., 2004)
lex2	Glucosyltransferase	Addition of Glu to the Glu in the inner core of HepI	(Griffin *et al*., 2003)
lic2A	Galactosyltransferase	Addition UDP-Gal to the Glu of HepI forming lactose	(Hood *et al*., 2001b)
lic2B	Glucosyltransferase	Addition Glu/Gal to the Glu of HepII	(Twelkmeyer *et al*., 2011; Wong *et al*., 2011)
lgtC	Galactosyltransferase	Addition UDP-Gal to lactose forming globotriose	(Hood *et al*., 2001b)
lgtD	Galactosyltransferase	Addition GalNAc to globotriose forming globotetraose	(Hood *et al*., 2001b)
lic1A	Choline kinase	Addition of PCho	(Weiser *et al*., 1997)
lic1B	Choline transporter	Addition of PCho	(Weiser *et al*., 1997)
lic1C	PCho pirophosphorilase	Addition of PCho	(Weiser *et al*., 1997)
lic1D	PCho transferase	Addition of PCho	(Weiser *et al*., 1997)
lic3A	Sialyltransferase	Addition Neu5Ac to lactose	(Hood *et al*., 2001a)
lic3B	Sialyltransferase	Addition Neu5Ac to lactose or sialyllactose	(Fox *et al*., 2006)
lsgB	Sialyltransferase	Addition Neu5Ac to lactose or N-acetyllactosamine	(Jones *et al*., 2002)
siaA	Sialyltransferase	Addition Neu5Ac to a terminal N-acetyllactosamine	(Jones *et al*., 2002)
oafA	Acetilase	Addition of OAc groups to the distal Hep	(Fox *et al*., 2005)
lpt6	PEtn transferase	Addition PEtn to HepII	(Wright *et al*., 2004)
losA	Glucosyltransferase	Extension HepIV (some strains)	(Hood *et al*., 2010)
losB	Heptosyltransferase	Extension HepIV (some strains)	(Hood *et al*., 2010)

Abbreviations: Hep: heptose; Glu: glucose; Gal: galactose; GlcN: glucosamine; PEtn: phosphoethanolamine; PCho: phosphorylcholine; Neu5Ac: sialic acid; GalNAc: N-acetylgalactosamine; OAc: O-acetyl group; P: phosphate group; UDP: Uridine diphosphate.

4.4 Adhesins

Specific adhesion to the human cells is an important virulence factor in bacterial pathogens. This attachment is commonly mediated by adhesins, proteins that are able to recognize and bind to a specific host receptor, or to soluble macromolecules. This specific binding activates different signalling processes that can facilitate the bacterial colonization and pathogenesis. Adhesins present a variety of structures, usually classified as hair-like appendages (pili or fimbriae) or nonpilus adhesins, located directly on the bacterial surface (Kline *et al*., 2009; Soto *et al*., 1999).

H. influenzae can express different types of adhesins which can be classified as piliated and non-piliated.

4.4.1 Piliated adhesins

- Haemagglutinating pili are helical polymeric structures encoded by a chromosomal gen cluster formed by five genes (*hifA-hifE*) (Geluk *et al*., 1998; Stull *et al*., 1984). This adhesin is expressed in Hib and NTHi strains, where it has been linked with adherence to respiratory mucus and epithelial cells, and with agglutination of human erythrocytes to a sialyl ganglioside receptor (Kubiet *et al*., 2000; van Alphen L. *et al*., 1991). Although *hif* pili have an important role in the first steps of colonization, their expression is reduced in later stages of the infection process. Expression of pili is reversibly regulated by phase variation, converting a piliated variant that is more equipped for colonization into a nonpiliated variant capable of invasion (Mhlanga-Mutangadura *et al*., 1998).

- Type IV pili are filamentous polymers composed of helically arranged pilin subunits that are encoded by the operons *pilABCD* and *comABCDEF* (Bakaletz *et al*., 2005). This structure is involved in several processes such as adhesion to respiratory epithelial cells, formation of bacterial aggregates, colonization of upper respiratory tract (Jurcisek *et al*., 2007), acquisition of exogenous DNA (Dougherty *et al*., 1999), and twitching (Bakaletz *et al*., 2005).

- Fimbriae are thin, non-hemagglutinating and flexible filaments that are evenly distributed along the bacterial surface. This structure is encoded by a homologous gene

to the one encoding OmpA in other Gram-negative bacteria (Sirakova *et al*., 1994). In a chinchilla model it was reported that non-fimbriated strains have lower levels of adherence to respiratory tract mucus, human oropharyngeal cells, and reduced induction of otitis media (Barsum *et al*., 1995; Sirakova *et al*., 1994).

4.4.2 Nonpiliated adhesins

- High molecular weight proteins (HMW1 and 2): Approximately 80% of NTHi strains express high molecular weight adhesins (HMW1 and HMW2) which are generally not present in encapsulated strains. These proteins, encoded by two different chromosomal loci (*hmw1* and *hmw2*), mediate the attachment to human epithelial cells and are target of human opsonophagocytic antibodies (Giufre *et al*., 2008; St Geme *et al*., 2009).

- *Haemophilus influenzae* adhesin (Hia): Around 20-25% of NTHi strains that lack the HMW1/HMW2 proteins express the Hia non-pilus adhesin. This protein is a trimeric autotransporter which remains cell linked, and has been associated with adherence to epithelial cells (Meng *et al*., 2006; St Geme *et al*., 2000). The cell receptor for Hia remains unknown (Laarmann *et al*., 2002).

- *Haemophilus* surface fibril (Hsf): Hsf is the main non-pilus adhesin in Hib strains. The *hsf* gene, highly conserved among encapsulated strains, encodes a trimeric autotransporter protein (240-KDa) made up of three repetitive domains (Cotter *et al*., 2005; St Geme *et al*., 1996). Hsf is a short and thin surface fibril (highly homologous to the Hia adhesin from NTHi) which is associated with adherence to epithelial cells. Both genes, *hsf* and *hia*, are alleles of the same locus, with a similarity of 81% and an identity of 72% (St Geme *et al*., 1996). Besides adherence to epithelial cells, Hsf contributes to serum resistance inhibiting MAC formation in the complement system by vitronectin binding (Hallstrom *et al*., 2006).

- *Haemophilus* adherence and penetration (Hap): The *H. influenzae* Hap is an autotransporter expressed in encapsulated and NTHi strains, which promotes adherence to epithelial cells and selected extracellular matrix proteins such as fibronectin, laminin, and collagen IV, and also mediates bacterial aggregation and microcolony formation. Hap is a 155-KDa protein formed by an internal passenger domain called Hap_s and a C-terminal translocator called Hap_β (Fink *et al*., 2003). The adhesion to epithelial cells and

to fibronectin, laminin, and collagen IV resides in different residues of Hap_s. Bacterial aggregation is also mediated by Hap_s and occurs via a Hap_s-Hap_s interaction between neighbouring bacteria (Meng *et al*., 2011). The secretory leukocyte protease inhibitor, found in the respiratory secretions, can block the autoproteolysis of Hap, resulting in the retention of Hap_s on the bacterial surface and producing an increased adherence to epithelial cells and extracellular matrix proteins, together with an increased bacterial aggregation and microcolony formation (Hendrixson *et al*., 1998).

- Opacity-associated protein A (OapA): OapA, a 47-KDa adhesin found in all *H. influenzae* strains, is responsible for an opaque colonial phenotype and has been associated with attachment to epithelial cells (Prasadarao *et al*., 1999; Weiser *et al*., 1995).

4.4.3 Other proteins that function as adhesins

- Outer membrane proteins (OMPs): The OMPs P2 and P5 function as adhesins for the human mucin and carcinoembryonic antigen cell adhesion molecule 1 (CEACAM1) respectively, having a role in adherence and colonization (Hill *et al*., 2001; Reddy *et al*., 1996). Due to their high immunogenicity and heterogeneity, these OMPs play important roles in the evasion of the immune system (Duim *et al*., 1996; Duim *et al*., 1997).

- Other surface proteins such as Proteins E and D are also associated with bacterial adhesion. Protein E is a lipoprotein present in all NTHi strains with a role in adhesion to epithelial cells which contributes to a proinflammatory response (Ronander *et al*., 2009). Protein D is a surface lipoprotein found in all *H. influenzae* strains which is involved in pathogenesis by enhancing damage to ciliated epithelial cells, promoting the adherence and internalization to human monocytes and facilitating the acquisition of choline from the host epithelial cells (Forsgren *et al*., 2008).

4.5 Transferrin-binding proteins (Tbp 1 and 2)

In *H. influenzae*, iron acquisition is accomplished by a siderophore-independent mechanism mediated by specific transferrin receptors called transferrin-binding proteins which are encoded by *tbpA* and *tbpB* (Gray-Owen *et al*., 1995; Morton *et al*., 1990). Incorporation of iron into the cytoplasm is encoded by the operon *hitABC* (Adhikari *et*

al., 1995; Sanders *et al*., 1994). In 2010, Morton *et al*. described in NTHi the *fhu* operon, an iron/haem-repressible siderophore utilization locus, although these strains do not have genes encoding proteins associated with siderophore synthesis. They speculated that the siderophore utilization locus could use the siderophores produced by other microorganisms that colonise the human nasopharynx (Morton *et al*., 2010).

4.6 Haem utilization proteins

H. influenzae is totally dependent on haem or haem-derivatives for growth in aerobic conditions because it cannot synthesize the precursor protoporphyrin IX. Consequently, *H. influenzae* expresses different mechanisms for binding free haem and haemoglobin or extracting haem from haemopexin and haptoglobin. Utilization of the free haem and haem-haemopexin complex is mediated by the proteins encoded by the *huxABC* operon (Cope *et al*., 1995).

Moreover, *H. influenzae* is also capable of binding free haemoglobin and haemoglobin-haptoglobin by haemoglobin-haptoglobin binding proteins (HgpA, HgpB, and HgpC) (Morton *et al*., 1999). The Hgp proteins have different affinities to the three human haptoglobin phenotypes; HgpB is the most efficient in using all of them, and HgpC the least efficient (Morton *et al*., 2006).

Haem-binding lipoprotein (HbpA) is also involved in haem acquisition, although different sources of haem are used in NTHi and Hib (Morton *et al*., 2009a). In addition, a periplasmic haem/porphyrin transport encoded by the locus *dppBCDF* and the lipoprotein *e* (P4), involved in the utilization of NAD, are also components in the haem acquisition although their roles are still to be clarified (Morton *et al*., 2007; Morton *et al*., 2009b).

5. PATHOGENESIS and HOST DEFENCES

The pathogenesis of *H. influenzae* can be divided in three phases: i) establishment on the mucus surface and adherence to the epithelial cells, ii) persistence in the respiratory tract and evasion of the immune system, and iii) invasion of the epithelial cells or endothelial cells. Figure 8 shows a schematic representation of the various steps involved in NTHi pathogenesis.

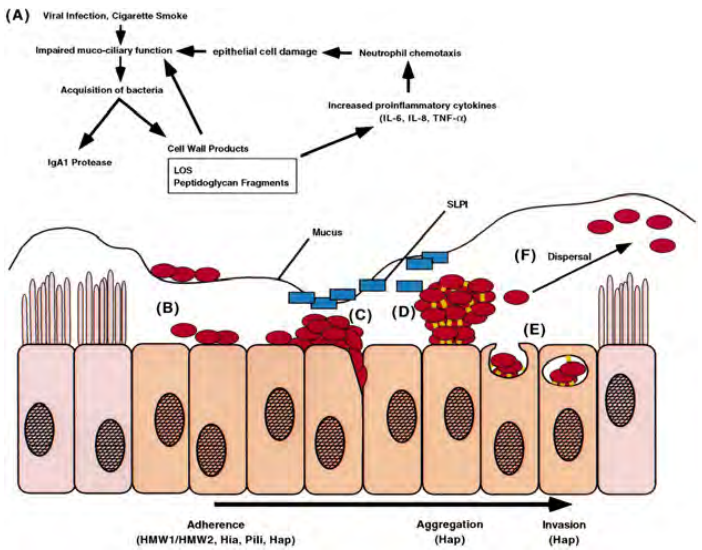

Figure 8: (A) Acquisition of the bacteria and impairment of the mucociliary function by direct damage caused by LOS, peptidoglycan fragments, and because these bacterial products stimulate proinflammatory cytokines that also disrupt mucociliary function. The IgA protease inactivates IgA facilitating the access to the mucosal surface. (B, C) Bacterial adhesion on non-ciliated and damaged epithelial cells. (D) Bacterial aggregation forming microcolonies that facilitate the persistence. (E) Invasion of the epithelial cells. (F) Dispersal of the bacteria within the respiratory tract. Figure from Rao *et al*. 1999 (Rao *et al*., 1999).

5.1 Establishment on the mucosal surface

The initial step in NTHi pathogenesis involves the establishment of the bacteria on the mucosal surface. The host defence mechanisms that bacteria have to overcome to their establishment are clearance by mucociliary function and secretory IgA. NTHi has developed mechanisms to inactivate the cilia and the IgA1 (Rao *et al*., 1999).

5.1.1 Disruption of the mucociliary escalator

The first interaction of NTHi in the respiratory tract is with the mucus layer, which eliminates the vast majority of inhaled bacteria. However, NTHi has developed various strategies to counteract the mucociliary escalator, such as attachment to non-ciliated or damaged epithelial cells (Read *et al*., 1991), microcolony formation within the mucus layer, alteration of cilia movement, and even loss of cilia (Rao *et al*., 1999). The effect on the movement and detachment of ciliated cells has been linked to substances such as LOS, peptidoglycan fragments liberated during replication (Johnson *et al*., 1986), and some other non-cytotoxic and temperature labile substances in the supernatant of NTHi cultures that induce the Protein Kinase C epsilon (PKCε) (Bailey *et al*., 2012). Besides these strategies, the ability of NTHi to circumvent mucociliary clearance is increased when the respiratory tract is damaged due to exposure to cigarette smoke and other environmental agents, respiratory viral infection, or by diseases such as cystic fibrosis (Rao *et al*., 1999). Furthermore, the OMPs P2 and P5 have the ability to bind mucin, facilitating the adherence to nasopharyngeal epithelial cells (Reddy *et al*., 1996).

5.1.2 Inactivation of IgA

IgA1 is the predominant immunoglobulin produced by human mucosal tissues in the respiratory tract and is the first mechanism of the host defence, inhibiting bacterial adherence and invasion, and inactivating bacterial toxins. IgA1 proteases expressed by *H. influenzae* are responsible for degrading the immunoglobulin molecule, inhibiting agglutination and mucociliary clearance. Moreover, the released Fab fragments are able to bind to the antigen, which results in a masking of the epitopes on the bacterial surface and prevention of their recognition by intact antibodies (Mansa *et al*., 1986).

5.1.3 Adherence to host epithelium

The next step towards disease is effective attachment to the epithelium. *H. influenzae* attaches to epithelial cells in the upper airway, and preferably to damaged epithelium (Read *et al*., 1991). A range of adhesins that attach to different components of the epithelium have been identified. Figure 9 illustrates the major NTHi adhesins that mediate adhesion to the epithelial cells.

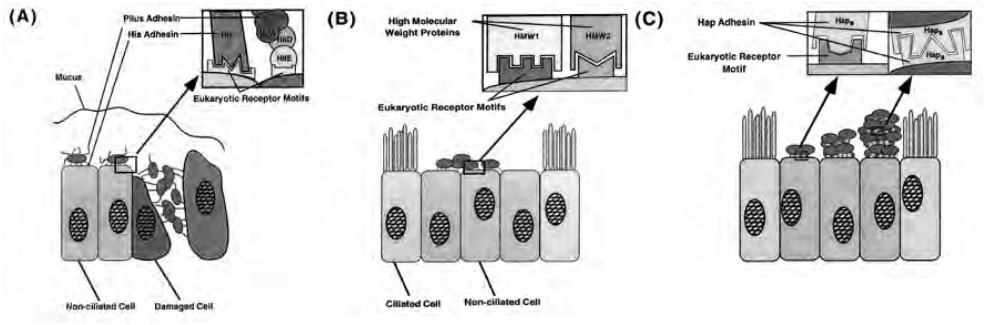

Figure 9: (A) Pili promote low affinity adherence to damaged cells whereas Hia interacts preferentially with non-ciliated cells and mediates a higher adherence. (B) HMW1 and HMW2 facilitate the adhesion by recognition of different eukaryotic receptors. (C) Complementing the action of the other adhesins, Hap protein mediates low-level adherence to components of the extracellular matrix, and in addition facilitates formation of bacterial aggregates and microcolonies on the epithelial surface. Figure from Rao *et al*. 1999 (Rao *et al*., 1999).

5.2 Persistence and evasion of the immune system

Once established on the mucosal surface, bacteria have to persist in it. This persistence requires a permanent supply of nutrients and a continued evasion of the immune system attack. In order to persist in this hostile environment, *H. influenzae* has developed a battery of strategies such as complex iron and haem acquisition systems and mechanisms of antigenic variation (Rao *et al*., 1999).

5.2.1 Iron and haem acquisition

H. influenzae has to capture iron from the host to survive and grow. In humans, the level of free iron is very low because most of it is located inside the cells, bound to ferritine or present in haem-containing compounds, or outside the cells, bound to transferrin in the serum or to lactoferrin in mucosal secretions. In order to acquire this essential nutrient, *H. influenzae* expresses a siderophore-independent mechanism mediated by Tbp1 and Tbp2, which is capable of incorporating iron bound to transferrin (Rao *et al*., 1999). Another essential nutrient that *H. influenzae* has to acquire from the host is haem because the bacterium cannot synthesize the haem precursor protoporphyrin IX. For this task, it expresses the battery of haem-binding proteins described in point 4.6 of this introduction.

5.2.2 High genetic diversity

H. influenzae, principally NTHi strains, presents a high genetic diversity, due to a variety of phenomena such as transformation, horizontal gene transfer, high frequency of genetic polymorphisms, hypermutability, and phase variable regulation (Garmendia *et al*., 2012). This high genetic variation is known as a 'supragenome', in which non-essential loci are frequently exchanged between organisms, enabling rapid adaptation of the bacterial population to fluctuating conditions (Hogg *et al*., 2007). The human respiratory tract is colonized by several microorganisms. Among them, polyclonal NTHi populations have been described in the airways of patients with chronic respiratory diseases and nasopharyngeal carriers (Moller *et al*., 1995; Mukundan *et al*., 2007; Murphy *et al*., 1999). This bacterial mixture stimulates the uptake of exogenous DNA by transformation or by horizontal gene transfer (Hiltke *et al*., 2003; Mell *et al*., 2011). Moreover, *H. influenzae* presents a high frequency of genetic polymorphisms due to point mutations, insertions, deletions, and duplications, mainly in genes that encode surface proteins, increasing their antigenic variability (Gilsdorf, 1998). Hypermutable strains with defects in the methyl-directed mismatch repair (MMR) system are detected at high frequencies, mainly in cystic fibrosis patients (Watson, Jr. *et al*., 2004).

The phase variable gene regulation system is one of the most important mechanisms for increasing genetic variability that are present in *H. influenzae*. Phase variation is a stochastic, reversible, and high frequency mechanism that facilitates the adaptation of the bacterial population to changes in the host environment (Moxon *et al*., 2006). Phase variation is mediated by simple sequence repeats (SSRs), which are small DNA repeats located in promoter regions or within open reading frames. Changes in their length can result in a random, high frequency, reversible loss, gain or modulation of gene expression (Power *et al*., 2009). In NTHi, three different mechanisms of phase variation have been described and regulate the expression of genes involved in LOS, hemagglutinating pili, HMW1, HMW2, and haem receptor biosynthesis.

(A) Genes involved in the LOS biosynthesis or haemoglobin-binding proteins contain tetranucleotide tandem repeats in the 5'-coding region. The number of the repeats are

the cause of translational frame shifts resulting in production of the protein with different N-terminal or no protein production at all (Weiser *et al*., 1990).

(B) The *hifA* and *hifB* genes (involved in pilus biosynthesis) have overlapping promoters with a variable number of tandems of AT repeats. Depending on the number of repeats, the pili are synthesized or not, resulting in a fully piliated, intermediately level of piliated or nonpiliated cell (van Ham *et al*., 1993).

(C) The *hmw1A* and *hmw2A* genes, which encode the adhesins HMW1/HMW2, contain tandem 7-base pair repeats upstream of the start codon. There is a correlation between the number of repeats and the level of protein expression (Dawid *et al*., 1999).

5.2.3 Resistance to innate immunity

H. influenzae can interact with elements of the innate immunity system to confer resistance to complement-mediated killing or to antimicrobial peptides. Encapsulated strains are especially resistant to serum because the capsule protects them from the complement system and from the opsonophagocytosis, but NTHi strains also present serum resistance without the benefit of the capsule (Nakamura *et al*., 2011).

NTHi has the ability to delay the C3 binding and subsequent membrane attack complex (MAC). Blocking the complement cascade at that step, the bacteriolysis and opsonisation processes are reduced (Williams *et al*., 2001). Furthermore, NTHi can bind to C4b-binding protein (C4BP), inhibiting the classical and lectin pathways (Hallstrom *et al*., 2007), while the expression of the *lgtC* gene may play a role in the inhibition of the C4b deposition on the bacterial surface (Ho *et al*., 2007). NTHi can also bind factor H, the major regulator of the alternative pathway of complement activation (Fleury *et al*., 2014; Hallstrom *et al*., 2008), and the interaction between Protein E and vitronectin also contributes to serum resistance inhibiting the MAC (Hallstrom *et al*., 2009). Additionally, the control in the expression of the genes *vacJ* and *yrb* is another mechanism described that confers serum resistance (Nakamura *et al.*, 2011). The last structure involved in serum resistance is the OMP P5. Its role consists in binding human complement regulatory factor H, preventing C3 deposition on the bacterial surface, and decreasing IgM binding, which is a potent activator of the classical pathway (Langereis *et al*., 2014; Rosadini *et al*., 2014).

Regarding resistance to antimicrobial peptides, besides the acylation of lipid A and the incorporation of the PCho into the LOS structure, NTHi has the transport system Sap, an important mechanism that provides resistance to antimicrobial peptides by binding these peptides to the periplasm, with posterior translocation to the cytosol where they are degraded (Mason *et al*., 2006; Shelton *et al*., 2011).

5.2.4 Alteration of the adaptive immune response

OMPs such as P2 are highly immunogenic and show an antigenic drift that causes non-bactericidal activity in the original antibodies due to the high variability of the epitopes (Duim *et al*., 1996). In healthy people, the response produced against NTHi is mediated by Th1 cytokines; however, people who suffer chronic respiratory diseases present a different immune response, mediated by Th2 cytokines, with a reduced production of CD40 ligand, a lower activation of macrophages, and higher levels of IgG1 and IgG3 (King *et al*., 2003). As a consequence, chronic inflammation ensues and the bacteria are less well cleared (Berenson *et al*., 2006; Marti-Lliteras *et al*., 2009).

5.2.5 Biofilm formation

Bacterial aggregation and biofilm formation are involved in bacterial population survival (Costerton *et al*., 1999). This structure confers protection to bacteria against clearance by host defences or antimicrobial therapy, facilitating bacterial persistence in the host (Costerton *et al*., 1999).

Despite the controversy about the capacity of *H. influenzae* to form biofilm (Moxon *et al*., 2008), a variety of mechanisms and factors have been implicated in biofilm formation in this bacterium, such as adhesins, incorporation of PCho and sialic acid into the LOS structure, and quorum sensing (Hong *et al*., 2007b; Murphy *et al*., 2002; Swords *et al*., 2004; Swords, 2012a).

5.3 Invasion

With the invasion or internalization by respiratory cells, *H. influenzae* can evade host immune effectors and may acquire a protective reservoir for persistence and recurrent infections. *In vitro* studies have shown that *H. influenzae* can pass through epithelial cell layers, and persist embedded in or below the epithelium (Rao *et al*.,

1999). Moreover, NTHi can invade several cellular types such as epithelial cells and monocytes (Ahren *et al*., 2001), Chang epithelial cells and nonciliated epithelial cells (Ketterer *et al*., 1999), human bronchial epithelial cells via an interaction of LOS with platelet activating factor receptor (PAFR)(Swords *et al*., 2000), and macrophage-like cells found in human adenoid tissue (Forsgren *et al*., 1994). By contrast, the presence of capsule decreases the efficiency of bacterial internalization into human eukaryotic cells (St Geme *et al*., 1991).

Several mechanisms for NTHi uptake and internalization by respiratory cells have been reviewed by Clementi and Murphy although all the process remains poorly understood (Fig. 10) (Clementi *et al*., 2011).

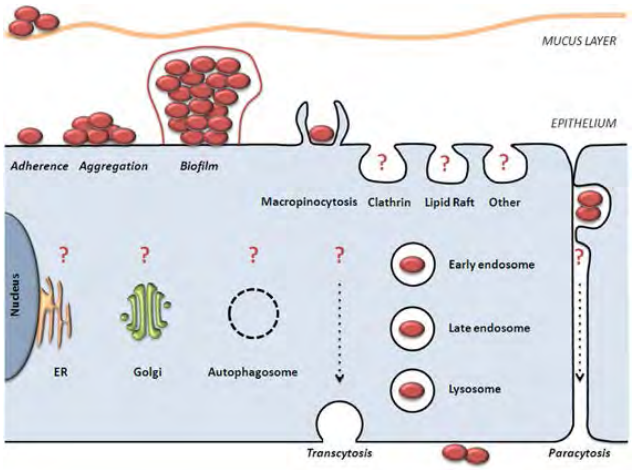

Figure 10: Model of NTHi invasion of respiratory epithelial cells. NTHi are internalized by macropinocytosis and are trafficked to vesicles. It is not clear the role of other internalization mechanisms and trafficking pathways (represented by question marks). Figure from Clementi *et al*. 2011 (Clementi *et al*., 2011).

Two mechanisms have been implicated in NTHi invasion. Phagocytosis may be used by NTHi in the invasion of monocytes and macrophages because bacterial cells have been observed within these eukaryotic cells. Alternatively, macropinocytosis has been proposed as a mechanism of invasion of epithelial cells because the extension of lamellipodia from the host cell to engulf the bacteria has been observed using electron microscopy. (Fig. 11) (Ketterer *et al*., 1999).

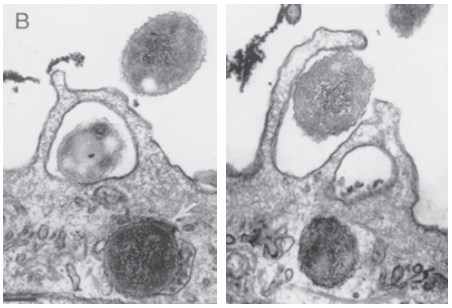

Figure 11: Image from TEM microscopy showing the lamellipodia formed by an airway cell surrounding an NTHi bacterium. Figure from Ketterer *et al*. 1999 (Ketterer *et al*., 1999).

Other suggested mechanisms of invasion to epithelial cells include the lipid raft-mediated endocytosis, clathrin or receptor-mediated endocytosis, transcytosis and paracytosis although the role of these mechanisms remains unknown (Clementi *et al*., 2011; Morey *et al*., 2011; van Schilfgaarde M. *et al*., 1995). Once inside the respiratory epithelial cell, NTHi resides in a vacuole in a non-replicative state (Morey *et al*., 2011). It is not known how NTHi can survive the endolysosomal trafficking, nor have the roles of autophagy and the eukaryotic secretion pathway in the intracellular stage been determined (Clementi *et al*., 2011).

In order to cause bloodstream and meningeal infections, *H. influenzae* has to interact with endothelial cells. These interactions have been studied *in vitro*, and it has been shown that the invasion of endothelial cells is associated with bacterial concentration and is inversely related to the presence of the capsule (Virji *et al*., 1992). Previous studies have demonstrated that *H. influenzae* can pass through a monolayer of endothelial cells by disrupting the tight junctions and can cause cytotoxic effects in blood-brain barriers and blood-pulmonary barriers (Patrick *et al*., 1992; Tunkel *et al*., 1992).

6. ANTIMICROBIAL TREATMENT AND RESISTANCE

Since the first description of penicillin by Fleming in 1929, a large number of antimicrobial agents have been described and/or synthesized. There are three proven targets for the main antimicrobial groups: bacterial cell wall biosynthesis, bacterial protein synthesis, and bacterial DNA replication (Fig. 12A). However, microorganisms have acquired various mechanisms of antimicrobial resistance which are based on enzymatic drug inactivation, modification of the drug targets, active expulsion, and reduction of the bacterial cell wall permeability (Fig. 12B) (Walsh, 2000).

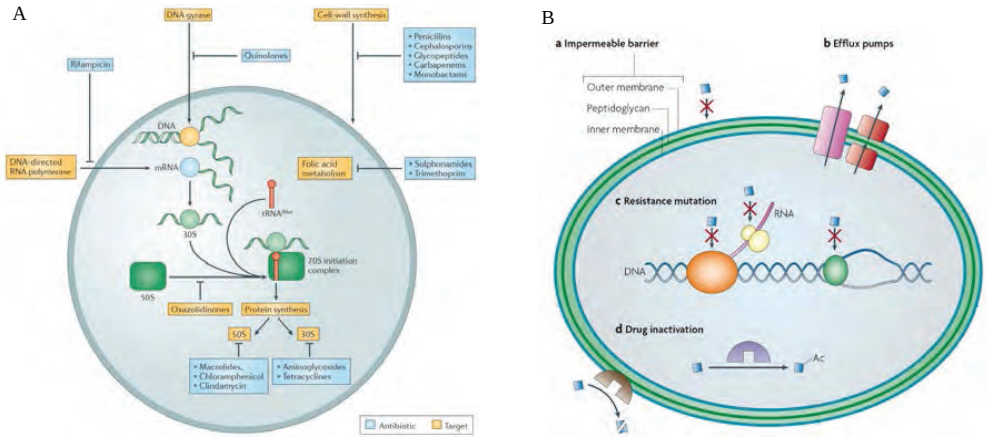

Figure 12: (A) Schematic representation of the basic targets of the main antimicrobial agents. Figure from Lewis 2013 (Lewis, 2013). (B) Representation of the main antimicrobial resistance mechanisms in bacteria. Figure from Allen *et al*. 2010 (Allen *et al*., 2010).

Susceptibility breakpoints for several agents with activity against *H. influenzae* have been established based on their intrinsic activity in the wild-type population (Turnidge *et al*., 2006). Currently, two major institutions have set the susceptibility breakpoints: the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI).

6.1 Antimicrobial agents and mechanisms of resistance

6.1.1 β-lactams

β-lactams are the most commonly prescribed antimicrobial agents for the treatment of *H. influenzae* infections. The basic characteristic of this antimicrobial group is the presence of a four-membered β-lactam ring. The addition of a different secondary ring and side chains has made possible the development of a large collection of agents with antimicrobial activity. Currently, the β-lactam antibiotics include the penicillins, cephalosporins, carbapenems, monobactams, and β-lactamase inhibitors (Marin *et al*., 2003).

β-lactam antibiotics are bactericidal agents that inhibit the biosynthesis of the peptidoglycan layer and thus, bacterial cell wall synthesis. The target of this group of antimicrobials are the Penicillin Binding-Proteins (PBPs), transpeptidase enzymes which crosslink the peptidoglycan. Without this peptidoglycan layer the bacterial membranes would burst as a consequence of the high osmotic pressure inside the cell (Marin and Gudiol, 2003; Walsh, 2000).

Several mechanisms of resistance to β-lactams have been described. They are generally due to point mutations on the chromosome or to the acquisition of resistance genes integrated in mobile elements such as plasmids or transposons. The resistance may be due to the expression of a single mechanism of resistance or to the additive effect of several mechanisms (Fig. 12B) (Marin *et al*., 2003).

- Reduced membrane permeability: in contrast to other Gram-negative bacteria, the outer membrane of *H. influenzae* provides very little resistance to the entrance of β-lactam antibiotics through the membrane. So far, no mechanisms of reduced permeability have been demonstrated in *H. influenzae* (Coulton *et al*., 1983).

- Efflux pumps: using an energy-dependent mechanism, efflux pumps expel the antibiotic outside the bacterial cell. In *H. influenzae*, the AcrAB efflux pump has a lesser effect on the efflux of β-lactams due to the rapid entrance of these antibiotics through porin channels. Despite this, AcrAB increases the level of resistance in combination with alterations in the PBPs (Kaczmarek *et al*., 2004; Sanchez *et al*., 1997b).

- β-lactamase production: This is the most common mechanism of resistance to β-lactams. These enzymes, with a structure similar to the PBPs, hydrolyze the β-lactam ring, inactivating the antibiotic. Two types of β-lactamases have been described in *H. influenzae*, TEM and ROB (Medeiros *et al*., 1975; Medeiros *et al*., 1986).

- Target modification: The modification of the PBPs reduces the affinity of the β-lactam antibiotic to the target. In the case of *H. influenzae*, modifications in the PBP3 are involved in β-lactam resistance (Ubukata *et al*., 2001).

Various resistance phenotypes have been described in *H. influenzae* in relation to β-lactamase expression and/or to modifications in the PBP3 (Table 6).

Table 6. Definitions of β-lactam phenotypes of *H. influenzae*.

Acronym	Complete name	Definition
BLNAS	β-lactamase negative ampicillin susceptible	Ampicillin susceptible No production of β-lactamase
BLNAR	β-lactamase negative ampicillin resistant	Ampicillin resistant No production of β-lactamase
gBLNAR	genomic β-lactamase negative ampicillin resistant	Changes in the PBP3 but non-ampicillin resistant according to breakpoints
BLPAR	β-lactamase positive ampicillin resistant	Ampicillin resistant β-lactamase production
BLPACR	β-lactamase positive amoxicillin-clavulanate resistant	Amoxicillin-clavulanate resistant β-lactamase production

- **BLPAR**

Two different types of β-lactamases have been identified in *H. influenzae;* TEM-1 and ROB-1. Both are plasmid-mediated class A β-lactamases which confer resistance to ampicillin and are effectively inhibited by β-lactamase inhibitors such as clavulanic acid (Bush *et al*., 1995; Medeiros *et al*., 1975; Medeiros *et al*., 1986). In clinical isolates, TEM β-lactamases are more common than ROB β-lactamases (Farrell *et al*., 2005). The TEM-1 β-lactamase gene ($bla_{\text{TEM-1}}$) can be carried by two different plasmid types: a small non-conjugative plasmid (<10 Kb) as a unique resistance determinant or a large conjugative plasmid (approximately 40 Kb) together with other resistance genes for chloramphenicol, tetracycline or kanamycin (Elwell *et al*., 1975; Saunders *et al*., 1978). A transfer of large plasmids between *H. influenzae* and *H. parainfluenzae* has been described; hence the latter might be an important reservoir of antimicrobial resistance determinants for *H. influenzae* (Leaves *et al*., 2000). By contrast, the ROB-1

β-lactamase gene (*bla*$_{ROB-1}$), located in small plasmids (approximately 4-5 Kb), encodes an enzyme more closely related to the Gram-positive β-lactamases (Juteau *et al*., 1990).

- **BLNAR**

It is very difficult to define what a BLNAR strain is. Strictly speaking, BLNAR strains are ampicillin-resistant strains that do not carry a β-lactamase gene. However, it is hard to interpret the results because these strains have the MIC of ampicillin close to the defined resistance breakpoints, which vary from 1 to 4 mg/L due to the lack of international consensus (Fig. 13) (Jacobs *et al*., 2002; MacGowan *et al*., 2001).

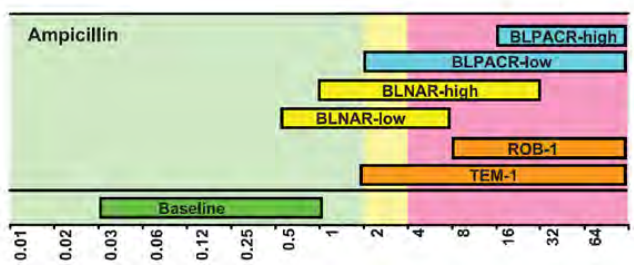

Figure 13: Correlation between mechanisms of resistance to β-lactams and susceptibility to ampicillin. Figure from Tristam *et al*. 2007 (Tristram *et al*., 2007).

With the identification of the alterations in the PBP3 as a mechanism of resistance in β-lactamase negative isolates, the definition of BLNAR has become more complex because strains with changes in the PBP3 but susceptible to ampicillin according to the breakpoints are considered genomic BLNAR (gBLNAR). Resistance phenotypes resulting from PBP3 alterations vary considerably; BLNAR strains are considered "low" when the ampicillin MIC ranges from 0.5 to 2 mg/L, and "high" with an ampicillin MIC ranging from 1 to 16 mg/L. BLNAR isolates are often resistant to early-generation cephalosporins such as loracarbef, cefaclor and cefuroxime (Tristram *et al*., 2007).

Ubukata and colleagues reported that mutations in the *ftsI* gene, which encodes the transpeptidase region of PBP3, are involved in the development of resistance to β-lactam antibiotics in BLNAR strains (Ubukata *et al*., 2001). Based on amino acid substitution patterns, several gBLNAR subdivisions have been proposed. Currently, the most commonly used subdivision is the Dabernat classification, formed by groups I and II, which is subdivided further into IIa, IIb, IIc, and IId (Dabernat *et al*., 2002). Ubukata

also reported a group III, and García-Cobos a group called III-like based on the similarity in the substitutions previously defined in Ubukata's group III (Garcia-Cobos *et al*., 2007; Ubukata *et al*., 2001). Although changes in 24 different positions have been described and associated with the BLNAR phenotype, two changes are considered necessary for a definition of gBLNAR, Arg517His or Asn526Lys (Tristram *et al*., 2007).

- **BLPACR**

BLPACR is a phenotypic term which was described before the characterization of alterations of PBP3 as a mechanism of resistance. The first BLPACR strains reported were β-lactamase producers and presented resistance to amoxicillin-clavulanate, suggesting that this resistance was due to the hyperproduction of TEM1 or ROB1 or to the action of a novel β-lactamase (Tristram *et al*., 2007). Currently, strains that present altered PBP3 and β-lactamase production are categorized as BLPACR, despite the fact that only the ampicillin MIC is higher in BLPACR strains due to the β-lactamase expression: there are no differences in the MICs of amoxicillin-clavulanate or cephalosporins between BLNAR and BLPACR strains (Matic *et al*., 2003; Tristram *et al*., 2007). Tristam *et al*. suggested that the term β-lactamase-enhanced BLNAR might be more appropriate to define these strains (Tristram *et al*., 2007).

6.1.2 Quinolones

Quinolones are synthetic antimicrobials with a broad-spectrum of activity. Between the synthesis of nalidixic acid in 1962 and the development of fluoroquinolones, their spectrum of activity broadened significantly (Emmerson *et al*., 2003).

Quinolones are bactericidal antibiotics that inhibit bacterial replication and transcription by blocking the DNA gyrase and topoisomerase IV, enzymes involved in maintaining the integrity of the supercoiled DNA helix during these processes. The DNA gyrase, formed by two subunits (A_2B_2) encoded by the *gyrA* and *gyrB* genes, is responsible for the negative supercoiling of the DNA. The topoisomerase IV, also an A_2B_2 enzyme, encoded by *parC* and *parE* genes, is responsible for the decatenation of daughter replicons following DNA replication. Fluoroquinolones act by stabilizing the

enzyme-DNA complex, and consequently causing chromosomal disruption and cell death (Peterson, 2001).

The widespread use of quinolones, especially fluoroquinolones, to treat human infection (as well as in veterinary practice) has caused a rapid development of bacterial resistance. The mechanisms of quinolone resistance are caused mainly by target alterations and reduced accumulation of the antimicrobial inside the cell, either due to decreased membrane permeability or overexpression of efflux pumps. Recently, quinolone resistance determinants have been reported in mobile elements (Ruiz, 2003). In *H. influenzae*, the quinolone resistance rate is very low (Biedenbach *et al*., 2003; Perez-Trallero *et al*., 2010).

- Target alteration: in Gram-negative bacteria the main target is the DNA gyrase, and mutations involved in quinolone resistance occur mainly in a region of the *gyrA* gene known as the quinolone-resistance determining region (QRDR). The presence of a single mutation results in high-level resistance to nalidixic acid; however, additional mutations in the *gyrA* or *parC* genes are necessary to obtain high-level fluoroquinolone resistance (Ruiz, 2003). In *H. influenzae*, the most frequently described mutations that confer resistance are in positions 84 and 88 of both GyrA and ParC (Georgiou *et al*., 1996).

- Decreased membrane permeability: the outer membrane of *H. influenzae* confers higher permeability to some antimicrobials (including fluoroquinolones) than other Gram-negative bacteria, and it has not been shown to be a resistance mechanism in this bacterium (Sanchez *et al*., 1997a). By contrast, Pérez-Vázquez and colleagues found that the loss of the P2 porin might be involved in fluoroquinolone resistance in hypermutable *H. influenzae* strains (Perez-Vazquez *et al*., 2007).

- Efflux pumps: several efflux pumps that expel quinolones have been described in Gram-negative bacteria (Ruiz, 2003). In a study published in 1997, Sánchez *et al*. did not find any involvement of the AcrAB efflux pump in fluoroquinolone resistance in *H. influenzae* (Sanchez *et al*., 1997b). However, other studies showed that an efflux pump system might be implicated in fluoroquinolone resistance in *H. influenzae*. Consequently, HmrM was described as a multidrug efflux pump involved in norfloxacin resistance (Xu *et al*., 2003), and recently, studies in hypermutable strains have shown

significant increases in the accumulation of norfloxacin in experiments with efflux pump inhibitors (Perez-Vazquez *et al*., 2007).

- Plasmid-mediated resistance: the *qnr* gene is generally located in an integron and transmitted between bacteria by plasmid transfer. This gene encodes a protein that protects the DNA gyrase from the action of quinolones, conferring low-level resistance to these antimicrobials (Tran *et al*., 2002). This mechanism of resistance has not been described in *H. influenzae* but it has been reported in *H. parasuis* in China (Guo *et al*., 2011). Moreover, a new variant of the aminoglycoside acetyltransferase Aac (6')-Ib also confers reduced susceptibility to ciprofloxacin (Robicsek *et al*., 2006) and has been described in *H. influenzae* and *H. parasuis* (Guo *et al*., 2011; Pfeifer *et al*., 2013).

6.1.3 Macrolides

Macrolides are inhibitors of protein synthesis. They are mainly active against Gram-positive bacteria, Gram-negative cocci, and intracellular bacteria, while Gram-negative bacilli are generally resistant. Macrolides are composed by two or more amino or neutral sugars attached to a lactone ring which varies in size (Euba *et al*., 2015; Leclercq, 2002). Generally, the mechanisms of resistance to macrolides include target modification (ribosomal methylase encoded by *erm* genes or mutations in 23S rRNA), alterations in ribosomal proteins L4 and L22, efflux pumps encoded by *mef* gene, and less commonly, antibiotic inactivation (Leclercq, 2002).

H. influenzae is more susceptible to macrolides than other Gram-negative bacteria, but less susceptible than macrolide-susceptible Gram-positive bacteria. The main mechanism of macrolide resistance in this microorganism is an efflux pump encoded by a gene cluster homologous to the *acrAB* in *E. coli* (Peric *et al*., 2003). Accordingly, strains of *H. influenzae* can be classified in three groups depending on their susceptibility to macrolides: i) susceptible strains (<2%) without any resistance mechanism; ii) baseline strains with an efflux pump as a resistance mechanism; and iii) high-level resistant strains with an efflux pump combined with alterations in the ribosomal proteins and/or mutations in the 23S rRNA (Bogdanovich *et al*., 2006; Peric *et al*., 2003; Peric *et al*., 2004).

6.1.4 Tetracyclines

Tetracyclines are broad-spectrum antibiotics with activity against a large variety of microorganisms, including Gram-positive and Gram-negative bacteria, atypical organisms (chlamydiae, mycoplasmas, rickettsiae), and parasites. In addition to their applications in human therapeutics, they have been widely used in the treatment of animal infections, in plant agriculture and used in subtherapeutic levels as growth promoters in animals (Chopra *et al*., 2001).

Tetracyclines inhibit bacterial protein synthesis by binding to the ribosomal complex, preventing the association of aminoacyl-tRNA with the bacterial ribosome. Tetracycline resistance is mainly due to the acquisition of the tetracycline resistance *tet* genes which generally encode for efflux pumps or altered ribosomal proteins (Chopra *et al*., 2001). In *H. influenzae*, tetracycline resistance is associated with an efflux pump encoded by the *tet* (B) gene, commonly located in a conjugative plasmid (Tristram *et al*., 2007). A few years ago, the *tet* (M) gene, previously described in *H. ducreyi*, was detected in three *H. influenzae* strains together with *tet* (B). In the same study, *tet* (M) was transferred by conjugation into an unrelated Gram-positive *Enterococcus faecalis*, indicating that other tetracycline resistant determinants might be introduced into *H. influenzae* from different bacterial species (Roberts, 1989; Soge *et al*., 2011).

6.1.5 Chloramphenicol

The mode of action of chloramphenicol is the inhibition of the protein synthesis by binding to the 50S ribosomal subunit (Nierhaus *et al*., 1973). In *H. influenzae*, chloramphenicol resistance is generally due to the production of chloramphenicol acetyltransferase (CAT) encoded by the *cat* gene located in a conjugative plasmid or integrated in the chromosome (Powell *et al*., 1988; Roberts *et al*., 1980). In addition, decreased membrane permeability due to a loss of an outer membrane protein has also been described as a mechanism of chloramphenicol resistance (Burns *et al*., 1985).

6.1.6 Folic Acid Metabolism Inhibitors

Folic acid metabolism inhibitors are substrate analogues that exert an antimicrobial effect by interfering with cellular metabolism and replication. Trimethoprim is an analog of the dihydrofolate that blocks the dihydrofolate reductase

(DHFR) whereas sulfamethoxazole is an analog of para-aminobenzoic acid which inhibits the dihydropteroate synthetase enzyme. Thus, the use of both components in combination limits the production of the tetrahydrofolate which is essential for the transformation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), an important precursor in thymine synthesis (Chatterjee *et al*., 2008; Hartman, 1993). In *H. influenzae*, resistance to trimethoprim occurs due to overproduction of an altered DHFR with decreased affinity for trimethoprim, which is encoded by the *folH* gene (de Groot R. *et al*., 1988; de Groot R. *et al*., 1996).

6.2 Treatment of the most common *H. influenzae* infections

Generally, the recommended therapy for non-invasive infections is amoxicillin/clavulanic acid, a second or third-generation cephalosporin. As alternative therapies fluoroquinolones, azithromycin and clarithromycin are suggested. On the other hand, in the treatment of invasive infections including meningitis, ceftriaxone or cefotaxime is the first therapeutic option, with quinolones and carbapenems as alternative antimicrobial therapies (Farreras *et al*., 2012).

7. BIOFILM

Bacterial biofilms are defined as congregations of bacterial cells which are irreversibly associated to a solid surface and enclosed within a self-produced polysaccharide matrix (Fig. 14A). These structures have been observed in living tissues as well as in inert surfaces such as medical devices, industrial water systems, or natural aquatic systems. Although these structures are considered universal, biofilms are very heterogeneous and every microbial biofilm is unique (Donlan, 2002). Biofilms are principally formed by microbial cells and extracellular polymeric substances, mainly polysaccharides. Moreover, these structures can also incorporate other substances such as metal ions, divalent cations, macromolecules (proteins, DNA, and lipids), and particles from the host or the environment (Donlan, 2002).

Biofilms allow bacteria to survive in hostile environments; thus, it is not surprising that they may be involved in a large number of chronic bacterial infections. These biofilms confer protection against the host immune system and also increase antimicrobial resistance. For these reasons, infections in which biofilm structures are involved typically show recurrent symptoms (Fig. 14B) (Costerton *et al*., 1999).

A B

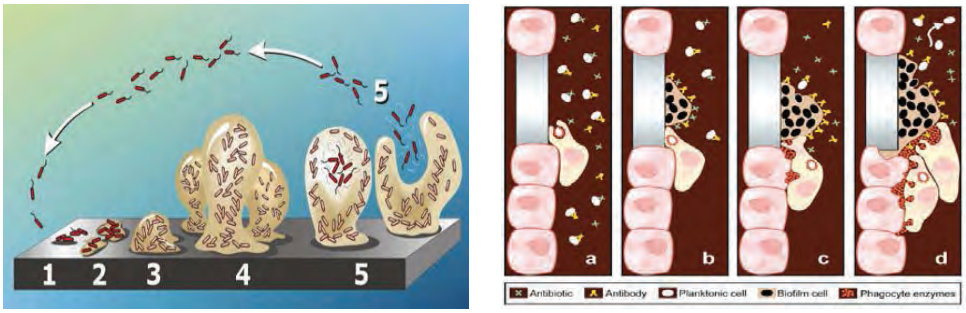

Figure 14: (A) Phases of biofilm formation. 1-2: reversible and irreversible attachment to the surface; 3-4: growth and maturation state; 5: Dispersion state (© Davies, DG). (B) Representation of medical biofilm formation. (a) Planktonic bacteria cleared by antibodies, phagocytes, and susceptible to antibiotics (b) Bacterial biofilm confers resistance to antibodies, phagocytosis, and antimicrobials (c) Release of phagocytic enzymes damaging the tissues (d) Dispersion of planktonic cells from the biofilm causing dissemination. Figure from Costerton *et al*. 1999 (Costerton *et al*., 1999).

In the specific case of *H. influenzae*, various studies have revealed the presence of biofilm communities in the lower and upper respiratory airways and their involvement in a range of infections such as otitis media, adenotonsillitis, COPD and cystic fibrosis (Bakaletz, 2012; Galli *et al*., 2007; Murphy *et al*., 2005; Starner *et al*., 2006). Other studies have provided physical evidence via direct microscopic observation of *H. influenzae* biofilm structures in a chinchilla model of otitis media (Fig. 15) (Ehrlich *et al*., 2002; Post, 2001). Despite all these observations, biofilm formation by *H. influenzae* remains controversial because of the lack of a specific polysaccharide associated with the extracellular matrix and the biofilm-specific phenotype (Moxon *et al*., 2008).

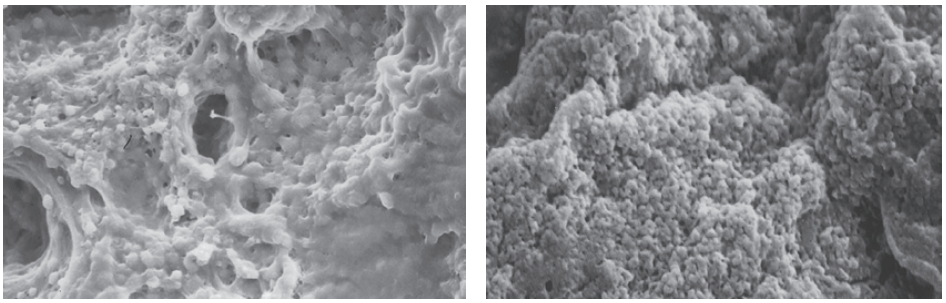

Figure 15: SEM images of NTHi biofilm from chinchilla middle-ear mucosa. Figure from Post *et al*., 2001 (Post, 2001).

Several studies of the factors involved in *H. influenzae* biofilm formation have been performed. As a result of these experiments, a list of factors involved in biofilm formation has been described. These factors can be divided into two groups: surface structures (expression of type IV pili, sialylation and PCho addition to the LOS, and production of extracellular DNA) and quorum sensing (Swords, 2012a).

- Expression of type IV pili: these pili may represent a structural component which links bacterial cells and may stabilize the biofilm. Moreover, type IV pili can bind DNA, and so may provide a substrate in which the bacteria can form the biofilm. These data are supported by experiments in which *pilA* mutants formed less robust biofilms in the middle ear in a chinchilla model and were unable to adhere to the epithelial cells (Jurcisek *et al*., 2007).

- Incorporation of sialic acid in the LOS: sialylated LOS glycoforms contribute to the biofilm initiation and formation both *in vitro* and *in vivo*. Mutants for the *siaB* gene, involved in sialic acid incorporation, formed less biofilm than the wild type strain in experiments performed *in vitro*. Furthermore, this mutant was less able to colonize and persisted in otitis and pulmonary animal models due to its low capacity to form biofilm (Swords *et al*., 2004).

- Incorporation of PCho in the LOS: addition of PCho to the bacterial surface promoted full biofilm maturation *in vitro* as well as *in vivo*. It has been reported that *licD* mutants form a less dense biofilm than wild type strains in continuous-flow systems *in vitro*. Moreover, in an otitis animal model, strains that were able to incorporate PCho in the LOS formed denser biofilms than the *licD* mutants, and promoted persistence in the middle ear (Hong *et al.*, 2007b; Hong *et al.*, 2007a).

- Adhesin expression: the importance of protein components in the biofilm matrix of *H. influenzae* has been shown by experiments using proteinase K, which inhibits biofilm formation and causes detachment from the previously formed biofilm (Izano *et al*., 2009).

- Production of extracellular DNA: the presence of DNA within biofilms formed by NTHi has been described (Jurcisek *et al*., 2007). The importance of this DNA as a major component in the biofilm matrix of *H. influenzae* was tested in experiments using DNase I, which inhibited biofilm formation and caused rapid detachment from pre-formed biofilm (Izano *et al*., 2009).

- Quorum sensing: although the *luxS* mutants retained the capacity to form biofilm, their biofilms lost density in comparison with the biofilms formed by wild type strains. In view of these experiments, it was suggested that a luxS-dependent mediator modulated the LOS composition, affecting on biofilm maturation and bacterial persistence *in vivo* (Armbruster *et al*., 2009; Swords, 2012b).

OBJECTIVES

II. JUSTIFICATION OF THE STUDY AND OBJECTIVES

H. influenzae is an opportunistic pathogen that colonizes the human nasopharynx and forms part of the normal microbiota. However, this microorganism is able to cause several acute invasive and non-invasive infections, and it can persistently colonize the lower respiratory tract in patients with chronic respiratory diseases. The introduction of the vaccine against Hib was a major breakthrough in the epidemiology of *H. influenzae*. Since then, the frequency of this serotype has decreased dramatically, allowing the increase in NTHi as an etiological agent. Due to this shift in strain, continuous monitoring of NTHi in all the infections in which *H. influenzae* is the causal agent is important, placing the emphasis on the genetic relationship among these strains and on the molecular characterization of the antimicrobial resistance. For these reasons, we designed several epidemiological studies to identify the importance of this pathogen in healthy carriers and in the main infections caused by *H. influenzae* in adults. In addition, due to the involvement of biofilm formation in chronic infections and because NTHi is an important pathogen in some chronic respiratory infections such as COPD, we included the study of biofilm formation in NTHi in this research.

As a result, the present thesis focuses on three major objectives in the epidemiology of *H. influenzae*:

1. To study the colonization rate, antimicrobial resistance and clonal composition of encapsulated and nontypeable *H. influenzae* in healthy children.

2. To determine the clinical and molecular epidemiology of *H. influenzae* as a causal agent of invasive and non-invasive diseases in adults in Barcelona.

   2.1 To analyse the role of NTHi as a cause of acute exacerbations in patients with severe or very severe Chronic Obstructive Pulmonary Disease.

   2.2 To analyse the clinical and molecular characteristics of NTHi causing non-bacteraemic community-acquired pneumonia.

   2.3 To determine the clinical and molecular epidemiology of *H. influenzae* causing invasive disease.

2.4 To study the rates of resistance to β-lactams and fluoroquinolones, and to determine the mechanisms of resistance to these antimicrobial groups in *H. influenzae*.

2.5 To investigate the importance of *H. haemolyticus* among *Haemophilus* spp. isolates collected from different infections.

3. To analyze various factors involved in biofilm formation by *H. influenzae* as a cause of persistence and invasion.

3.1 To analyze the role of biofilm formation by NTHi in carriage and diseases caused by this microorganism.

3.2 To determine the role of PCho in biofilm formation in colonizing and clinical NTHi isolates.

RESULTS

III. RESULTS

A) COLONIZATION

Objective 1: To study the colonization rate, antimicrobial resistance and clonal composition of encapsulated and nontypeable *H. influenzae* in healthy children.

Paper 1: Oropharyngeal colonization by nontypeable *Haemophilus influenzae* among healthy children attending day care centers.

Puig C*, Marti S*, Fleites A, Trabazo R, Calatayud L, Liñares J, Ardanuy. Microbial Drug Resistance. 2014, 20(5):450-5.

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MICROBIAL DRUG RESISTANCE
Volume 20, Number 5, 2014

DOI: 10.1089/mdr.2013.0186

Oropharyngeal Colonization by Nontypeable *Haemophilus influenzae* Among Healthy Children Attending Day Care Centers

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Haemophilus influenzae colonizes the upper respiratory tract and can spread causing otitis and sinusitis. This work aimed to study the oropharyngeal carriage rate in healthy < 5-year-old children attending day care centers in Oviedo, Spain in two consecutive years (January to March 2004–2005). The carriage rate was 42% (400/960) and highly variable among centers (range, 12% to 83%). Isolates were mainly identified as nontypeable *H. influenzae* (NTHi, 99%). Epidemiologically, 127 different genotypes were identified by PFGE with a minimum of two genotypes per center. One hundred fourteen children (12%) were included in both studies and none of them harbored the same strain over a period of time. The isolates only showed resistance to cotrimoxazol and ampicillin, presenting a shift in the level of ampicillin reduced susceptibility, showing a predominance of PBP3 mutations in 2004 and a predominance of β-lactamase production in 2005. This study proved the great genetic variability of NTHi isolates that present similar genotypic patterns in both years with no long-term carriage of the same strain.

Introduction

THE FASTIDIOUS GRAM-NEGATIVE coccobacilli *Haemophilus influenzae* form part of the indigenous nasopharyngeal microbiota and can also cause acute respiratory infections.[1,8,15,18] The presence or absence of a polysaccharide capsule segregates this bacterial species in two well-defined groups; a group of encapsulated strains and a second group of noncapsulated strains, commonly referred as nontypeable *H. influenzae* (NTHi).[1,15,18]

NTHi isolates were initially associated with asymptomatic colonization; nevertheless, these unencapsulated bacteria are also pathogenic and frequently identified as the etiologic agent of otitis media, sinusitis, conjunctivitis, chronic bronchitis, and community acquired pneumonia.[1,8,15]

The pathogenesis of NTHi starts with an initial colonization of the upper respiratory tract, followed by the bacterial migration to other neighboring parts where the bacterial pathogen initiates an inflammatory response.[15,18,22] Strain transmission occurs frequently within households and it has also been observed at very high rates among children sharing the same Day Care Center (DCC).[1,15]

Many day care colonization studies investigate *Streptococcus pneumoniae* carriage, but information on *H. influenzae* colonization of young children is scarce and has not been reported from DCCs in Spain. On this ground, the objectives of this study were to investigate the colonization rate and level of antimicrobial resistance in the major DCCs and schools from Oviedo, Spain.

Materials and Methods

Study design and children selection

Two prospective point-prevalence studies were conducted in DCCs and schools from Oviedo (Spain) between January and March of 2004 and 2005. Informed written consent for participation in the study was obtained from the children's parents. The study was approved by the Ethics Committee from the Hospital Universitario Central in Asturias (Spain).

Children between 1 and 5 years were recruited from 16 DCCs (age 1–3) and three public schools (age 3–5). The exclusion criteria were falling out of the age range, respiratory infection, absence in the center on the sampling day, and inability to obtain the sample. Every child presented a

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This work was presented as a poster at the 52nd annual ICAAC in San Francisco, California.

parental filled in questionnaire on the children's illnesses and antimicrobial consumption in the 6 months previous to the study.

Sample collection

Oropharyngeal swabs were collected by a trained nurse and preserved in the STGG medium (3% Tryptone Soya Broth, 0.5% glucose, 2% skim milk, and 10% glycerol). Sterile cotton-tipped wooden swabs were wiped across the respiratory tract mucosa lining at the rear of the oropharynx with care not to touch the teeth, gums, or tongue. Bacterial identification was performed by standard microbiological methods.[3] Serotyping was achieved by latex agglutination with the Phadebact® Haemophilus Test (Bactus AB, Huddinge, Sweden) and by polymerase chain reaction (PCR) as previously described by Falla *et al.*[9] Species differentiation between *H. influenzae* and *H. haemolyticus* was performed by PCR detection of *iga*, *fucK*, and *lgtC* genes as described by Binks *et al.*[4]

Antimicrobial susceptibility testing

Bacterial susceptibility was determined by standard disc diffusion with the following antibiotics (Oxoid, Madrid, Spain): ampicillin, amoxicillin–clavulanic acid, cefotaxime, ciprofloxacin, chloramphenicol, sulfamethoxazole–trimethoprim, and tetracycline. Susceptibility was defined according to the CLSI guidelines.[5] The β-lactamase activity was determined by the chromogenic cephalosporin test using nitrocefin as a substrate and following the manufacturer's directions (BD, Madrid, Spain). Ampicillin and amoxicillin–clavulanic acid minimum inhibitory concentrations (MICs) were performed by e-test on all the isolates with a disk inhibition zone ≤ 28 mm for both antibiotics; these strains were selected for a molecular characterization of the mutations in the PBP3 by sequencing an inner region of the *fstI* gene, as described by Dabernat *et al.*[6]

Pulsed-field gel electrophoresis

Molecular typing of *H. influenzae* was performed by pulsed-field gel electrophoresis (PFGE). Genomic DNA embedded in agarose plugs was restricted with *Sma*I, and fragments were separated by PFGE in a CHEF-DRIII apparatus (Bio-Rad, Madrid, Spain) as previously described.[17]

Statistical analysis

All the statistical analyses were performed using the SPSS v.16.0 (SPSS, Inc., Chicago, IL) software package. Differences were evaluated using the Fisher's exact test or chi-squared test with Yate's correction. A p-value of <0.05 was considered significant. Logistic regression analysis was used to identify the independent risk factors for *H. influenzae* carriage. Variables with $p<0.05$ in the univariate analysis and those found in previous studies were included in the multivariate analysis.

Results

Sample collection

Sixteen DCCs and 3 schools participated in the study. A total of 960 oropharyngeal swabs were examined, with 482 healthy children included in 2004 and 478 children in 2005. An average of 25 samples was obtained per center (range, 6 to 59), which remained constant in both studies.

Questionnaire analysis

Data from the questionnaires were only used as an indicator of global health. Sixty-seven percent of the children had been healthy for the 3 months before the sampling period. On the other hand, 24% of the children had otitis media, 5% asthma, 2% sinusitis, and 2% respiratory tract infections or allergies.

Table 1. Baseline Characteristics of the Study Children Population According to the *Haemophilus influenzae* Oropharyngeal Carriage

	2004 (n = *481*)				*2005* (n = *476*)			
Characteristics	*Total* (n = *481*)	*HINF-carriage* (n = *206*)	*HINF-non carriage* (n = *275*)	p*	*Total* (n = *476*)	*HINF-carriage* (n = *194*)	*HINF-non carriage* (n = *282*)	p*
Age ≤ 36 months	400 (83.2%)	161 (78.2%)	239 (86.9%)	**0.01**	356 (74.8%)	149 (76.85)	207 (73.4%)	0.40
Sex								
Male/female center	258/223	112/94	146/129	0.78	256/220	99/95	157/125	0.32
DCC/school center size	338/143	138/68	200/75	0.17	344/132	142/52	202/80	0.71
>35 children	408 (84.8%)	181 (87.9%)	227 (82.5%)	0.11	382 (80.3%)	145 (74.7%)	237 (84.0%)	**0.01**
>40 children	323 (67.2%)	151 (73.3%)	172 (62.5%)	**0.01**	267(56.1%)	106 (54.6%)	161 (57.1%)	0.59
Antibiotic consumption								
Previous 6 months	310 (64.4%)	136 (66.3%)	174 (63.5%)	0.52	188 (39.5%)	108 (55.7%)	173 (61.3%)	0.35
Previous month	131 (27.2%)	53 (25.7%)	78 (28.4%)	0.53	193 (40.5%)	45 (23.2%)	77 (39.7%)	0.33
Sampling day	37 (7.7%)	9 (4.4%)	28 (10.2%)	**0.02**	187 (39.3%)	5 (2.6%)	21 (7.4%)	**0.03**
Previous otitis media	116 (24.1%)	43 (20.9%)	73 (26.5%)	0.16	110 (23.1%)	38 (19.6%)	72 (25.5%)	0.15
History of asthma	23 (4.8%)	8 (3.9%)	15 (5.5%)	0.16	23 (4.8%)	8 (4.1%)	15 (5.3%)	0.55
Brothers	249 (51.8%)	104 (50.5%)	145 (52.7%)	0.63	251 (52.7%)	106 (54.6%)	145 (51.4%)	0.49

*Bold: statistically significant.
DCC, Day Care Center; HINF, *Haemophilus influenzae.*

On average, 27% of the children were treated with at least one antibiotic in the month previous to the sampling, and 62% received antimicrobial therapy in the previous 6 months. A low percentage of children also received a course of antimicrobial therapy in the morning before sample collection (7%). The most common treatment was an antimicrobial agent from the β-lactam group (67%), mainly amoxicillin–clavulanic acid.

Oropharyngeal colonization

Colonization by *H. influenzae* was observed in all the centers included in this study with an overall carriage rate of 42% (stable over the two tested periods). The isolates were identified mainly as NTHi; only three strains (1.5%) in 2004 and three (1.5%) in 2005 were capsulated *H. influenzae* serotype f.

Table 1 shows the main baseline and demographic characteristics of all children compared by groups according to the *H. influenzae* oropharyngeal carriage. Univariate analysis identified the center size and antibiotic consumption in the sampling day as factors associated with *H. influenzae* oropharyngeal carriage in 2004 and 2005, whereas age over 36 months was a risk factor associated only in the 2004 group. A logistic regression model with *H. influenzae* carriage as a dependent variable, adjusted for age, sex, and center size, and previous antibiotic consumption identified age (odds ratio [OR] −0.18; 95% confidence interval [CI] −0.35 to −0.02, $p=0.029$) and antibiotic consumption in the sampling day (OR −0.19; 95% CI −0.36 to −0.02, $p=0.03$) as independent risk factors for *H. influenzae* colonization in the 2004 children's group, whereas the center size (OR 0.19; 95% CI 0.06 to 0.33, $p=0.005$) and antibiotic consumption in the sampling day (OR −0.23, 95% CI −0.43 to −0.04, $p=0.019$) were identified in the 2005 children's group.

Strain genotyping

Overall, 127 different genotypes were identified (at least two genotypes per center). Half of the isolates were classified in small clusters (≥80% genotypic similarity) with less than five strains; from them, 40 isolates (32%) were genotypically unique, while 56 genotypes (44%) were distributed among different centers and 31 genotypes (24%) were present in a single center. Only 20% of the isolates were present in large clusters with more than 10 genetically related isolates. The genotypic analysis for each point-prevalence study grouped the isolates from 2004 into 79 different genotypes and in 2005, in 76 genotypes. Twelve percent (114/960) of the children were included in both studies; only 17 of them were colonized by *H. influenzae* in both occasions and none of them harbored the same strain in the second year.

Antimicrobial susceptibility

The isolated strains only showed resistance to cotrimoxazol and ampicillin. The frequency of ampicillin-resistant strains in 2005 was almost double than in 2004 (24% vs. 13%), while cotrimoxazol resistance remained constant (33% vs. 36%). There was no association between antimicrobial treatment in the previous month (as indicated in the questionnaires) and ampicillin resistance.

TABLE 2. DISTRIBUTION OF AMINO ACID SUBSTITUTIONS IN THE TRANSPEPTIDASE DOMAIN OF PBP3 IN 46 ISOLATES OF NONTYPEABLE *HAEMOPHILUS INFLUENZAE*

Centers	*Year*	*No isolates*	*PFGE genotype*	*MIC (μg/ml)*		*BL*	*PBP3 group*	*Amino acid substitutions*						
				AMC	*AMP*			*Asp350*	*Ala368*	*Met377*	*Ser385*	*Gly490*	*Ala502*	*Arg517*	*Asn526*
19	2004	7	22; 28	1–1.5	1	−	I	.	.	Ile	Thr	.	.	His	.
19	2004	1	19	2	2	−	IIa	.	.	.	.	.	.	.	Lys
24; 31	2004	2	107; 108	2–4	>256	+	IIb	.	.	.	.	.	Val	.	Lys
1; 3; 5; 18; 26; 35	2004	9	5; 7; 33; 100	0.75–3	1–3	−	IIc	.	.	.	.	.	Thr	.	Lys
29	2004	2	12	0.25–0.75	0.5–1	−	IIc	.	Thr	.	.	.	Thr	.	Lys
3; 5; 6; 11; 18; 31; 35	2004	10	30; 37; 57; 64; 74; 91; 92; 97	0.75–2	1–3	−	IIc	Asn	.	.	.	.	Thr	.	Lys
24	2004	1	9	4	>256	+	IIc	Asn	.	.	.	.	Thr	.	Lys
3; 6	2005	5	94	2	>256	+	IIb	Asn	.	Ile	.	Glu	Val	.	Lys
16	2005	1	10	2	1.5	−	IIb	.	.	.	.	.	Val	.	Lys
10	2005	1	33	2	2	−	IIc	.	.	.	.	.	Thr	.	Lys
19	2005	2	82	2–3	>256	+	IIc	Asn	.	.	.	.	Thr	.	Lys
18; 29; 35; 36	2005	5	36; 46; 51; 69; 74	0.25–2	0.25–2	−	IIc	Asn	.	.	.	.	Thr	.	Lys

AMP resistant ≥4 μg/ml; AMP susceptible ≤1 μg/ml. AMC resistant ≥8 μg/ml; AMC susceptible ≤4 μg/ml.
(.) indicates no amino acid substitutions.
AMC, amoxicillin–clavulanic acid; AMP, ampicillin; BL, β-lactamase production (+) positive (−) negative; MIC, minimum inhibitory concentration; PFGE, pulsed-field gel electrophoresis.

Molecular characterization of ampicillin-resistant isolates

Among the 400 strains isolated in both point-prevalence studies, 76 isolates showed reduced susceptibility to ampicillin; 96% were resistant due to β-lactamase production, and 4% (negative for β-lactamase) presented reduced ampicillin susceptibility.

A molecular characterization of mutations in the PBP3 was performed on 67 strains that showed an inhibition zone ≤ 28 mm for ampicillin and amoxicillin–clavulanic acid. Eighteen of those isolates were already resistant to ampicillin due to β-lactamase production and were mainly strains isolated during 2005 (5 and 13, in 2004 and 2005, respectively).

Sixty-nine percent of the tested isolates (46 out of 67) showed mutations at the *fts*I gene; among them, 10 β-lactamase-producing isolates also showed mutations in the *fts*I gene (Table 2), and 36 isolates with a reduced susceptibility to ampicillin only presented mutations in the *fts*I gene. The remaining 21 isolates with reduced susceptibility did not have any mechanism of ampicillin resistance.

Discussion

Clinical and epidemiological factors associated to *H. influenzae* infections have changed in the last 20 years.[1] Before vaccine development, *H. influenzae* serotype b (Hib) was the most common cause of meningitis in young children,[11] but the introduction of the Hib conjugate vaccine reduced the nasopharyngeal carriage of this serotype.[1] As expected, we did not isolate any Hib, possibly because in Spain, the vaccine was incorporated to the national immunization schedule in 1998.[16]

Although NTHi is considered less virulent than Hib, it can be responsible for severe diseases, especially among children with previous comorbidities.[11] Several studies have been performed to establish the *H. influenzae* carriage rate after vaccine introduction,[2,7,10,12–14,19–21] although to our knowledge, no studies have been reported from Spain. Longitudinal studies performed on one or two selected DCCs give information about the dynamics of colonization, but fail to give an overall view of the situation in similar centers of the same geographical area. Our work presents two consecutive point-prevalence studies in 19 centers, and we observed that children sampled in both occasions did not harbor the same strain. With this approach, we could examine different colonization behaviors while targeting a big and heterogeneous group, although still vulnerable to the seasonality and environmental factors.

In addition, we have incorporated into the study, 5-year-old children from three public schools and found that, despite the typical differences between both centers (*i.e.,* age range, hours in the classroom, number of children enrolled), there was no association between the type of center and the level of colonization.

Other risk factors such as gender and respiratory problems showed no direct association with *H. influenzae* colonization, in agreement with previous works.[7,14,19,21] However, risk factors such as age, size of the center, and antibiotic consumption on the sampling day were linked to *H. influenzae* colonization. The age factor (> 36 months) has been associated to higher colonization levels only in the first year of study, probably because the study was unintentionally biased toward this age range as a result of the randomness of the sample. In the same way, in 2004, centers with a high number of children enrolled had an increased colonization level. Those results suggest that children's colonization is variable and cross-sectional studies have to be interpreted with caution, as their results cannot be contrasted.

By contrast, taking antibiotics on the sampling day showed a reduced colonization in both years, suggesting that antibiotic uptake produces a rapid effect on *H. influenzae* colonization. A study by Barbosa-Cesnik *et al.*[2] also suggested that children taking antibiotics on the day of culture were less likely to carry NTHi strains, and Raymond *et al.*[19] isolated less *H. influenzae* strains in children who received an antibiotic treatment over the 15 days previous to sample collection.

In our study, the carriage rate associated to *H. influenzae* was kept stable during the two point-prevalence studies, ranging from 43% to 41% in both consecutive years. The prevalence of *H. influenzae* was similar or lower than other colonization studies conducted on worldwide centers.[2,7,10,13,14,19,20] Recently, Carvalho *et al.*[7] reported NTHi colonization in 32% of the children from DCCs in a large Brazilian city with a frequent antibiotic use (> 80%) within the studied population,[7] while studies in the United States showed a carriage rate of 64%.[2,10]

Antimicrobial susceptibility was high probably due to the low range of antimicrobial agents given to treat infections in children. Raymond *et al.*[19] reported resistance to ampicillin (56%), cotrimoxazol (25%), and tetracycline (24%) in isolates from a French orphanage, while in northern Taiwan, a high-level resistance to several antimicrobial agents was found.[21] However, data on antimicrobial susceptibility among healthy young children are scarce, as most epidemiological studies only identify the presence or absence of β-lactamase-producing isolates. In our current work, the rate of ampicillin resistance due to β-lactamase production was very low (24% in 2005 and 13% in 2004) compared to other worldwide studies that ranged between 35% and 45% of the *H. influenzae* isolates.[2,6,7,12,19,21] Despite this low prevalence, ampicillin resistance increased significantly in the second year where we observed a twofold increment in the β-lactamase-producing strains. The isolate characterization was completed with a determination of the PBP3 mutations associated to ampicillin resistance. So far, the studies on amino acid modifications in the transpeptidase domain of the PBP3 were hardly ever performed on samples from young healthy children. In our study, 69% of the tested isolates showed mutations at the *fts*I gene, which represented 12% of the *H. influenzae* isolates (46/400) and were detected twice as often in strains isolated in 2004 (Table 2); those mutations alone were not enough to confer ampicillin resistance, but they were associated to a reduced susceptibility phenotype (MIC 1 to 3 μg/ml).

Overall, this study provides an overview of the colonization diversity within a whole community from a country where there is scarce information on this subject. The study has determined the carriage rate of *H. influenzae* in children attending DCCs and schools in a large geographic area, with emphasis to the level of ampicillin resistance detected in the

isolates. We have shown that more than a tenth of the children were colonized with isolates presenting mutations in the PBP3, together with a shift in the level of ampicillin-reduced susceptibility with a predominance of PBP3 mutations in 2004 and a predominance of β-lactamase production in 2005.

Acknowledgments

The authors wish to thank nurse M. Garrán from the Hospital Universitario Central de Oviedo who contributed to this project by collecting the oropharyngeal samples from the young children, and to the center's directors for their collaboration.

Funding

This study was supported by a grant from the Fondo de Investigaciones Sanitarias de la Seguridad Social (PI0901904), by the RETIC G03/103, and by CIBER de Enfermedades Respiratorias (CIBERES—CB06/06/0037), run by the ISCIII (Instituto de Salud Carlos III, Madrid, Spain).

C.P. was supported by grants from AGAUR-FI (Generalitat de Catalunya, Spain) and from FPU (Formación de Profesorado Universitario, Ministerio de Educación, Spain). S.M. was supported by the ''Sara Borrell Postdoctoral contract CD10/00298'' from the Instituto de Salud Carlos III (ISCIII), Madrid, Spain.

Disclosure Statement

No competing financial interests exist.

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B) INFECTION AND ANTIMICROBIAL RESISTANCE

Objective 2: To determine the clinical and molecular epidemiology of *H. influenzae* as a causal agent of invasive and non-invasive diseases in adults in Barcelona.

Paper 2: Infectious etiology of acute exacerbations in severe COPD patients.

Paper 3: Molecular epidemiology of nontypeable *Haemophilus influenzae* causing community acquired pneumonia in adults.

Paper 4: Clinical and molecular epidemiology of *Haemophilus influenzae* causing invasive disease in adult patients.

Paper 5: Molecular characterization of fluoroquinolone resistance in nontypeable *Haemophilus influenzae* clinical isolates.

Paper 6: Identification of *Haemophilus haemolyticus* in clinical samples and characterization of their mechanisms of antimicrobial resistance.

Objective 2.1: To analyse the role of NTHi as a cause of acute exacerbations in patients with severe or very severe Chronic Obstructive Pulmonary Disease.

Paper 2: Infectious etiology of acute exacerbations in severe COPD patients.

Domenech A*, **Puig C***, Marti S, Santos S, Fernández A, Calatayud L, Ardanuy C, Liñares J. Journal of Infection. 2013, 67:516-23.

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Journal of Infection (2013) **67**, 516—523

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Infectious etiology of acute exacerbations in severe COPD patients

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Accepted 3 September 2013
Available online 20 September 2013

KEYWORDS
Chronic obstructive pulmonary disease;
Acute exacerbation;
Pseudomonas aeruginosa;
Streptococcus pneumoniae;
Haemophilus influenzae

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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0163-4453/$36
http://dx.doi.org/10.1016/j.jinf.2013.09.003

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 ($n = 71$)

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).

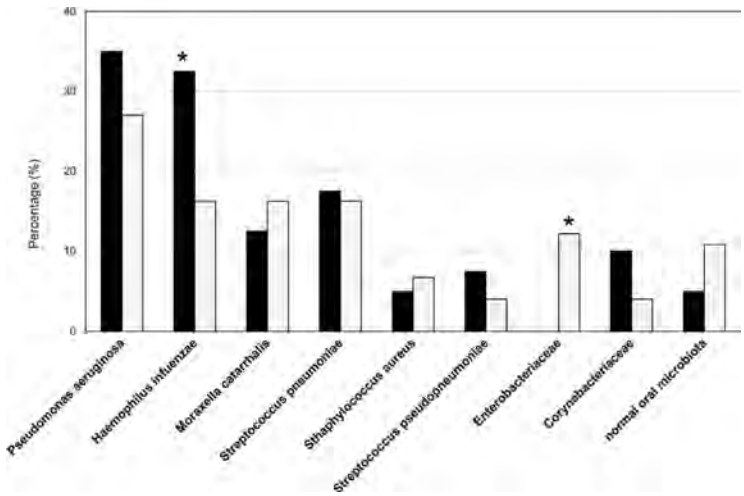

Figure 1 Distribution of the main bacteria isolated from patients with a low frequency (black bars) and high frequency (white bars) of acute exacerbations. *Significant differences between groups ($P < 0.05$).

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 antipseudomonal 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 $\leq$2 mg/L), ampicillin (susceptible $\leq$2 mg/L) and cefotaxime/ceftriaxone (susceptible $\leq$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.

Funding

This work was supported by grants from the Fondo de Investigaciones Sanitarias de la Seguridad Social [PI 0901904] and by CIBER de Enfermedades Respiratorias, CIBERES; [CB06/06/0037], run by the Instituto de Salud Carlos III (ISCIII), Madrid, Spain.

AD and CP were supported by two grants from Formación de Profesorado Universitario (FPU; Ministerio de Educación, Spain). SM was supported by a "Sara Borrell postdoctoral contract CD10/00298" from the Instituto de Salud Carlos III (ISCIII), Madrid, Spain.

Transparency declarations

None to declare.

Acknowledgments

We wish to thank Jordi Niubó and Dolors García-Somoza of the Microbiology Laboratory and Maria Jose Manuel of the Respiratory Medicine Department of the Hospital Universitari de Bellvitge for their contributions to this project on a daily basis.

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

Objective 2.2: To analyse the clinical and molecular characteristics of NTHi causing non-bacteraemic community-acquired pneumonia.

Objective 2.4: To study the rates of resistance to β-lactams and fluoroquinolones, and to determine the mechanisms of resistance to these antimicrobial groups in *H. influenzae*.

Paper 3: Molecular epidemiology of nontypeable *Haemophilus influenzae* causing community acquired pneumonia in adults.

Puig C, Calatayud L, Marti S, Tubau F, García-Vidal C, Carratalà-Fernández J, Liñares J, Ardanuy C. PLoS One 2013 8(12):e82515.

OPEN ACCESS Freely available online

PLOS ONE

Molecular Epidemiology of Nontypeable *Haemophilus influenzae* Causing Community-Acquired Pneumonia in Adults

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Abstract

Nontypeable *Haemophilus influenzae* (NTHi) is an opportunistic pathogen which causes a variety of respiratory infections. The objectives of the study were to determine its antimicrobial susceptibility, to characterize the β-lactam resistance, and to establish a genetic characterization of NTHi isolates. Ninety-five NTHi isolates were analyzed by pulsed field gel electrophoresis (PFGE) and multi locus sequence typing (MLST). Antimicrobial susceptibility was determined by microdilution, and the *fts*I gene (encoding penicillin-binding protein 3, PBP3) was PCR amplified and sequenced. Thirty (31.6%) isolates were non-susceptible to ampicillin (MIC≥2 mg/L), with 10 of them producing β-lactamase type TEM-1 as a resistance mechanism. After *fts*I sequencing, 39 (41.1%) isolates showed amino acid substitutions in PBP3, with Asn526→Lys being the most common (69.2%). Eighty-four patients were successfully treated with amoxicillin/clavulanic acid, ceftriaxone and levofloxacin. Eight patients died due either to aspiration or complication of their comorbidities. In conclusion, NTHi causing CAP in adults shows high genetic diversity and is associated with a high rate of reduced susceptibility to ampicillin due to alterations in PBP3. The analysis of treatment and outcomes demonstrated that NTHi strains with mutations in the *fts*I gene could be successfully treated with ceftriaxone or fluoroquinolones.

Citation: Puig C, Calatayud L, Martí S, Tubau F, Garcia-Vidal C, et al. (2013) Molecular Epidemiology of Nontypeable *Haemophilus influenzae* Causing Community-Acquired Pneumonia in Adults. PLoS ONE 8(12): e82515. doi:10.1371/journal.pone.0082515

Editor: Oliver Schildgen, Kliniken der Stadt Köln gGmbH, Germany

Received September 4, 2013; **Accepted** November 4, 2013; **Published** December 13, 2013

Funding: This study was supported by a grant from the Fondo de Investigaciones Sanitarias de la Seguridad Social (PI0901904), by CIBER de Enfermedades Respiratorias (CIBERES - CB06/06/0037), run by the ISCIII (Instituto de Salud Carlos III), Madrid, Spain, and by Spanish Network for Research on Infectious Diseases (REIPI, RD12/0015), run by ISCIII, Madrid, Spain. CP was supported by grants from AGAUR-FI (Generalitat de Catalunya, Spain) and from FPU (Formación de Profesorado Universitario, Ministerio de Educación, Spain). SM was supported by a "Sara Borrell Postdoctoral Contract, CD10/00298" from the Instituto de Salud Carlos III (ISCIII), Madrid, Spain. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Haemophilus influenzae is a human-restricted pathogen which forms part of the normal nasopharyngeal microbiota [1–4]. This bacterial species is commonly divided into two different groups depending on the presence or absence of the polysaccharide capsule, with six serotypes (a–f) currently described in the encapsulated group. In children, serotype b (Hib) is responsible for most invasive diseases, although incidence has dramatically decreased since vaccine introduction [4]. Non-capsulated *H. influenzae*, also known as nontypeable *H. influenzae* (NTHi), colonizes asymptomatically the nasopharynx in healthy people, and is also a frequent cause of otitis media, sinusitis, conjunctivitis, community-acquired pneumonia (CAP) and exacerbations in chronic obstructive pulmonary disease (COPD) [1–4].

CAP is a common respiratory infection which frequently requires patient hospitalization. Current studies identify *H. influenzae* as either the second most common pathogen causing CAP, after *Streptococcus pneumoniae* [5,6], or the third most common pathogen after *S. pneumoniae* and *Mycoplasma pneumoniae* [7]. In our geographical area, *H. influenzae* has been identified as the aetiological agent in 6–10% of CAP [8].

Aminopenicillin antibiotics have been used in the treatment of *H. influenzae* infections, and as a result, mechanisms of resistance against this group of antimicrobials have developed [9–11]. The most common mechanism of β-lactam resistance involves the production of a β-lactamase enzyme, usually TEM-1 type or, more rarely, ROB-1 type [12]. Alterations in penicillin-binding proteins (PBP3) have also been reported in different *H. influenzae* strains [13,14]. This phenotype, also known as β-lactamase negative ampicillin resistance (BLNAR), is related to mutations in the *ftsI* gene (encoding the transpeptidase domain of PBP3) [15]. The frequency of resistance to other antimicrobials such as quinolones or azithromycin is, however, low [11,16].

Epidemiological studies of individual patient groups are important for determining the level and mechanisms of antimicrobial resistance. In line with this goal, the present study had three main objectives: to determine the antimicrobial susceptibility of nontypeable *H. influenzae* strains isolated from patients with non-bacteremic CAP, to characterize the β-lactam resistance and to establish the clonal relatedness among these strains.

Materials and Methods

Ethics Statement

This work was 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.

Hospital Setting and Bacterial Strains

This study was carried out at the Hospital de Bellvitge in Barcelona, a hospital for adults serving a population of ca. 600,000 people. A retrospective review of computerized medical charts was performed in all patients seen at the hospital during the study period in order to record those with CAP criteria. Pneumonia was considered when a new infiltrate on a chest radiograph plus one or more of the following symptoms were detected: fever or hypothermia, new cough, pleuritic chest pain, dyspnea or altered breath sounds on auscultation [8]. Overall mortality was defined as death within 30 days of pneumonia diagnosis. Patients were considered cured when clinical findings of pneumonia had disappeared and there was radiological improvement.

A total of 95 NTHi isolates were collected from sputum samples of 92 patients diagnosed with non-bacteremic CAP between 2000 and 2009.

Only *H.* influenzae isolates from good quality sputum samples ($<$10 squamous cells and $>$25 leukocytes per low-power field) and with a predominance of Gram negative coccobacilli forms were considered [17].

Isolates were identified by conventional methodology and preserved by cryopreservation. Additionally, all isolates were identified by mass spectrometry using a MALDI-Biotyper version 3.0 (Bruker), following the manufacturer's recommendations. Differentiation between *H. influenzae* and *H. haemolyticus* was performed by the detection of *fuc*K, *iga* and *lgt*C genes using a previously described methodology [18]. Isolates were identified as *H. influenzae* if they were positive for the three tested genes.

Biotyping, Serotyping and Antimicrobial Susceptibility

Biotypes were determined using three biochemical reactions: urease, indol and ornithine decarboxylase [19]. Serotyping was achieved with the latex agglutination Phadebact® Haemophilus Test (Bactus AB, Huddinge, Sweden) and by PCR as stipulated by Falla et al. [20]. Antimicrobial susceptibility was determined by microdilution according to the criteria of the Clinical Laboratory Standards Institute (CLSI) [21,22]. β-lactamase production was screened using the chromogenic cephalosporin method (nitrocefin disks, BD, Madrid, Spain).

PCR and DNA Sequencing

Identification of β-lactamase type was performed by PCR on all the β-lactamase positive isolates using primers and conditions described previously [23]. For molecular characterization of PBP3, an internal region of the *ftsI* gene (796–1741 pb) was amplified by PCR and sequenced using previously described methodology [24].

Genotype Definition for Ampicillin Resistance

According to previous descriptions [25,26] and on the basis of β-lactamase production and changes in the *fts*I gene, *H. influenzae* isolates were classified into four genotypes: β-lactamase negative ampicillin susceptible (gBLNAS), strains without a detectable resistance mechanism; β-lactamase negative ampicillin resistant (gBLNAR), strains that did not produce a β-lactamase enzyme but which presented mutations in the transpeptidase domain of the *fts*I gene; β-lactamase positive ampicillin resistant (gBLPAR), strains producing β-lactamase but which did not present mutations in *fts*I; and β-lactamase positive amoxicillin/clavulanic acid resistant (gBLPACR), strains which presented both resistance mechanisms (β-lactamase production and mutations in the *ftsI* gene).

Molecular Typing

Pulsed field gel electrophoresis (PFGE). Strain relatedness was determined by PFGE with the restriction enzyme *SmaI* (New England BioLabs, Ipswich, MA, USA), as instructed by the manufacturer. Molecular typing was performed on bacterial suspensions of *H. influenzae* grown on chocolate agar plates, as described by Dabernat et al. [24] but with some modifications. Briefly, bacterial suspensions were prepared in PIV (10 mM Tris-HCl [pH 8], 1 M NaCl) and adjusted to the same final concentration. The bacterial suspension was mixed with an equal volume of melted 1.5% low-melting point agarose (Life Technologies, Madrid, Spain) in order to prepare DNA-agarose plugs with a volume of 20 µl each. These were incubated for 5 h at 37°C in 1 ml of ST buffer (6 mM Tris-HCl [pH 8]; 1 M NaCl; 0.1 M EDTA [pH 8]) containing 0.5% Brij-58, 100 µg/mL lysozyme and 50 µg/ml RNAse. The agarose plugs were transferred into ES buffer (1 M EDTA, 1% sarcosyl) with 1 mg/mL proteinase K (Sigma Aldrich, Madrid, Spain) and incubated over night at 50°C. Finally, the plugs were rinsed three times at room temperature with TE buffer (10 mM Tris-HCl [pH 8]; 1 mM EDTA [pH 8]).

The DNA-embedded plugs were digested with 5 U of *SmaI* for 18 h at 25°C. DNA fragments were then separated in a 1% agarose gel (Megabase, BioRad) with 0.5% TBE buffer (45 mM Tris-base, 45 mM boric acid, 1.0 mM EDTA pH 8.0) in a contour-clamped homogenous electric field system (CHEF DR III; BioRad). The gels were run for 19 h at 14°C, using a constant voltage of 6 V/cm with an angle of 120° and an increasing pulse time from 1 s to 30 s. A bacteriophage λ, low-range PFG marker (New England BioLabs, Ipswich, MA, USA) was used as a size standard.

PFGE band patterns were analyzed using the Fingerprinting II Software 3.0 (BioRad). The similarity of the PFGE banding patterns was estimated with the Dice coefficient, setting the optimization and tolerance at 1%. Isolates with ≥80% relatedness were considered highly genetically related [27].

Multilocus sequence type (MLST). Clinical isolates were analyzed by MLST in order to identify strain relatedness [28]. Allele number and sequence types (ST) were assigned using the *H. influenzae* MLST website (http://haemophilus.mlst.net). The overall database was analyzed using e-BURST v3 in order to define groups available on the *H. influenzae* MLST website.

Results

Patient Characteristics and Antimicrobial Susceptibility

NTHi isolates were recovered from 95 episodes of CAP in 92 patients. Sixty-four patients (69.6%) were men and the mean age was 68.15 years (SD±14.39). Comorbid conditions were present in 97% of patients, with COPD being the most frequent underlying disease (28.3%), followed by chronic heart disease (18.5%), malignancy (15.2%), diabetes mellitus (13%) and chronic renal failure (4.3%). Finally, 59.7% of patients were either current (13%) or past (46.7%) smokers.

Table 1 summarizes the antibiotic susceptibility of the NTHi isolates. All of them were susceptible to ceftriaxone, cefotaxime and levofloxacin. By contrast, 10.5% of the isolates were resistant to ampicillin due to the expression of a TEM-1 β-lactamase, and 23.2% presented intermediate resistance. The rate of resistance to

Table 1. Minimal inhibitory concentrations (MIC) of 10 antimicrobials. MIC against 95 NTHi isolates using the microdilution method according to CLSI breakpoints.

Antimicrobials	MIC_{50}	MIC_{90}	Range	CLSI[a]	
	(mg/L)	(mg/L)		%I	%R
Ampicillin	0.5	2	≤0.25–≥16	23.2	10.5
Amoxicillin/clavulanic acid[b]	1	4	≤0.5–8	0	2.1
Ceftriaxone	<0.06	<0.06	≤0.06–0.12	0	0
Cefotaxime	<0.06	<0.06	≤0,06–0.12	0	0
Cefuroxime	2	4	≤0.5–≥8	3.1	1.1
Tetracycline	≤2	≤2	≤2–≥4	0	2.1
Chloramphenicol	≤2	≤2	≤2–8	0	1.1
Azithromycin	2	2	≤0.5–≥4	0	1.1
Levofloxacin	≤0.5	≤0.5	≤0.5–1	0	0
Cotrimoxazole[c]	≤0.5	>2	≤0.5–≥2	0	32.6

[a]CLSI: Clinical and Laboratory Standards Institute. I: intermediate; R: resistant.
[b]The ratio of amoxicillin/clavulanic acid was 2:1.
[c]The ratio of cotrimoxazole was 1:19.
doi:10.1371/journal.pone.0082515.t001

cotrimoxazole was high (32.6%), whereas the frequency of resistance to amoxicillin/clavulanic acid, cefuroxime, tetracycline, chloramphenicol and azithromycin was low (<4%).

Mutation Patterns in the *fts*I Gene

The sequence of *fts*I encoding the transpeptidase region of PBP3 was determined in all the isolates. Table 2 summarizes the amino acid changes observed, corresponding to 41.1% of the isolates. The most common amino acid substitution was Asn526→ Lys (27/39, 69.2%), followed by Arg517→ His (2/39, 5.1%). The patterns observed were classified into groups I and II according to the criteria of Dabernat et al. [24].

Two isolates were classified as Group I and presented the Arg517→ His substitution alone. Group II included 27 isolates subdivided into three subgroups: i) 5 isolates belonged to the subgroup IIa (1 isolate with Asn526→ Lys, and the remaining 4 isolates with other mutations); ii) 7 isolates were classified as subgroup IIb, defined by Asn526→ Lys and Ala502→ Val substitutions (those isolates also presented the substitutions Asp350→ Asn and Met377→ Ile, and one of them also had a Gly490→ Glu); iii) the subgroup IIc, characterized by Asn526→ Lys and Ala502→ Thr substitutions, was the most common, with 15 isolates. No isolates were observed in subgroup IId or in groups III and III-like (previously described by García-Cobos et al. [10]).

Six patterns (10 isolates) were characterized and classified into the miscellaneous group: four of them (6 isolates) have already been described by García-Cobos et al. [10], while the remaining two were determined in this study and presented the Ala454→ Val and Asp350→ Asn/Thr532→ Asn substitutions.

Fourteen of 36 gBLNAR isolates (38.9%) presented ampicillin MIC within the susceptibility range (≤0.25–1 mg/L). All the isolates with MIC ≤0.25 or 0.5 of ampicillin belonged to the miscellaneous group, suggesting that these mutations were not involved in decreased β-lactam susceptibility.

Phenotypic and Genotypic Characterization

Phenotypically, the most common biotype found was biotype II (39.0%) followed by biotypes III (35.7%), I (16.8%), V (3.2%), VI (3.2%) and IV (2.1%). As a result of positive detection of *lgt*C, *fuc*K and *iga* genes, all the isolates were identified as *H. influenzae*.

Molecular typing by PFGE revealed 47 different PFGE patterns. Twenty-six patterns were genotypically unique and 21 clusters contained between 2 and 15 related isolates (>80% similarity). Furthermore, molecular typing by MLST showed 67 different sequence types, with 28 of them (ST974, ST989 to ST1000, ST1143, ST1162, ST1163, ST1171, ST1172, ST1174, and ST1176 to ST1184) being described for the first time in the present study. The most frequent ST was ST159 (7 isolates). Analysis with e-BURST (including single and double locus variants) revealed 11 groups (≥2 isolates) and 29 singletons (only 1 isolate). Groups 1, 2 and 10 were the largest, with 9 isolates each (Table S1, Supplementary data).

The 39 isolates with mutations in the *fts*I gene were grouped into 25 independent PFGE clusters. Despite the fact that most patterns were unique, five clusters were identified with between two and nine genetically-related isolates (>80% similarity) (Figure 1). Cluster D grouped the majority of isolates with alterations in PBP3 (n = 9), with five different ST: ST159 (n = 4), ST819 (n = 2), ST201 (n = 1), ST414 (n = 1) and ST1177 (n = 1). These nine clonally-related isolates were collected from different patients throughout the study period. Six of these isolates were grouped in the same e-BURST group (ST159/ST819). The isolates in this cluster belonged to different amino acid substitution groups: IIc (n = 4), IIb (n = 2), I (n = 2) and IIa (n = I). Cluster E contained four isolates with three different ST: ST556 (n = 2), ST388 (n = 1) and ST997 (n = 1). Two of these isolates belonged to the miscellaneous group of amino acid substitutions, while the remaining two isolates belonged to subgroups IIb and IIc, respectively. The other three clusters (F, I and K) contained two isolates each. The isolates in cluster F had the same ST (ST142) and were classified into subgroups IIa and IIb. Cluster I comprised isolates with ST1000 and ST1048, which belonged to the same subgroup (IIc). Finally, cluster K was composed of isolates with ST425 and ST998, which were grouped into subgroup IIa and the miscellaneous amino acid substitution groups, respectively.

Treatment and Patient Outcomes

Antibiotic therapy and clinical outcomes were analyzed for all patients included in this study. All patients were treated following the recommendations of the Infectious Disease Society of America and the guidelines of the American Thoracic Society [29].

Forty-one of 46 patients infected by gBLNAR, gBLPAR or gBLPACR isolates were successfully treated, mainly with amoxicillin/clavulanic acid, ceftriaxone and levofloxacin, or by using a combination of two of these antibiotics. The remaining five patients, infected by gBLNAR isolates, were treated with amoxicillin/clavulanic acid and ceftriaxone but died, due to aspiration, during the first 72 h of hospital admission (Table 3).

Forty-three of 46 patients infected by isolates with a genotype susceptible to aminopenicillins (gBLNAS) were successfully treated with ceftriaxone, amoxicillin/clavulanic acid and levofloxacin. The remaining three patients died by aspiration or due to complication of their severe underlying diseases (Table 3).

Discussion

H. influenzae is a common cause of CAP in adults (6–10%) [8] and it is frequently associated with recurrent pneumonia in both children and adults [8,30]. In this study, we analyzed the

Table 2. Amino acid substitutions in the transpeptidase domain of PBP3 identified in 95 NTHi isolates.

Group[a]	Amino acid substitutions											MIC (mg/L)[b]		BL[c]	No isolates	Sequence Type (ST)
	Asp 350	Ala 368	Met 377	Met 391	Ala 545	Gly 490	Ala 502	Arg 517	Asn 526	Ala 530	Thr 532	AMP	AMC			
I								His				0.5–2	1–2	-	2	159 (n = 2)
IIa						Glu			Lys	Ser		2	4	-	1	14
									Lys	Ser		2	4	-	2	142, 414
									Lys			2	4	-	1	998
	Asn					Glu			Lys	Ser		1	1	-	1	201
IIb	Asn		Ile				Val		Lys			≥16	8	+	1	165
	Asn		Ile				Val		Lys			1–2	4	-	3	14, 142, 367
	Asn		Ile			Glu	Val		Lys			1–2	1–2	-	3	204, 556, 1177
IIc	Asn						Thr		Lys			≥16	8	+	1	1171
							Thr		Lys			2	2–4	-	8	1048, 993, 819 (n = 2), 1162, 996, 1000, 409
	Asn						Thr		Lys			1–2	1–4	-	6	556, 648, 1171, 999, 159 (n = 2)
Miscellaneous	Asn											≥16	4	+	1	997
		Thr										≤0.5	1	-	2	267, 1163
	Asn											≤0.5	≤0.5–1	-	2	388, 1143
				Ile								0.5	1	-	1	994
							Val					0.5	1	-	1	85
	Asn										Asn	0.5	1	-	1	425
					Val							≤0.25	≤0.5	-	2	991 (n = 2)
No changes												8–≥16	1–4	+	7	57, 142, 160, 270, 272, 836, 1172
												≤0.25–1	≤0.5–2	-	49	[d]

[a]The isolates were classified into groups I, IIa, IIb and IIc, according to the criteria of Dabernat et al. [24]; the miscellaneous group was classified according to the criteria of García-Cobos et al. [10] and the data from this study.
[b]AMP Resistant: >4 mg/L; AMP Intermediate: 2 mg/L; AMP Susceptible ≤1 mg/L; AMC Resistant: ≥8/4 mg/L; AMC susceptible: ≤4/2 mg/L;
[c]BL: Beta-lactamase production (+: positive; -: negative);
[d]ST11 (n = 3), ST36, ST98, ST103, ST139 (n = 2), ST145 (n = 3), ST159 (n = 3), ST183, ST203 (n = 3), ST241 (n = 2), ST245, ST266, ST270, ST272, ST385, ST408, ST414 (n = 2), ST519 (n = 4), ST582, ST679, ST714, ST974, ST989, ST990, ST992, ST995, ST1174, ST1176, ST1178, ST1179, ST1180, ST1181, ST1182, ST1183 and ST1184.
doi:10.1371/journal.pone.0082515.t002

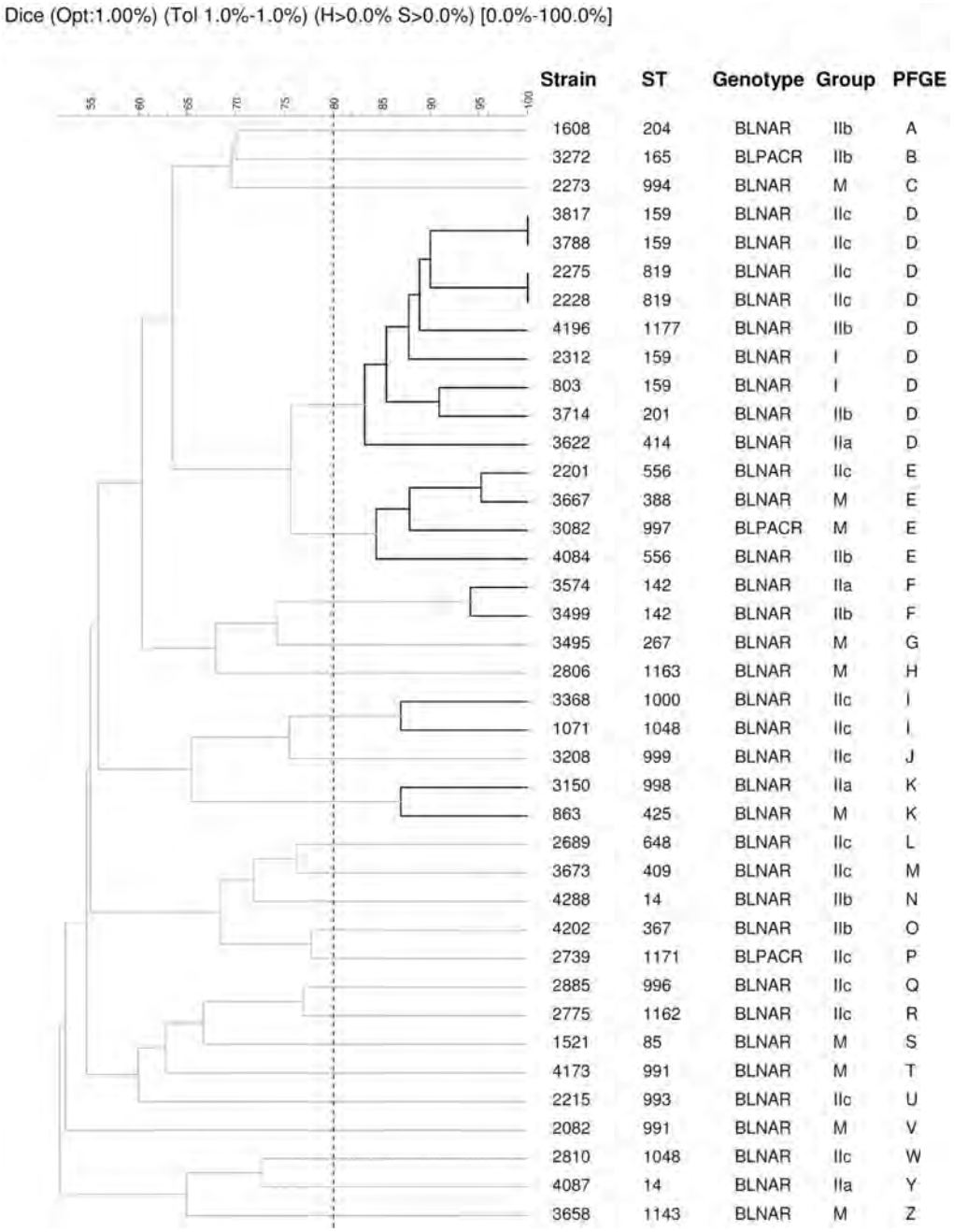

Figure 1. Tree diagram showing the genetic relatedness of 39 nontypeable *H. influenzae* isolates with mutations in the *fts*I gene (gBLNAR n = 36 and gBLPACR n = 3) obtained by PFGE according to Dice's similarity index. Dice coefficients are shown above the tree diagram. Isolates with ≥80% relatedness are considered highly genetically related.
doi:10.1371/journal.pone.0082515.g001

Table 3. Treatment and clinical outcomes for episodes of community-acquired pneumonia caused by NTHi.

Genotype[c]	Outcome	Treatment[a]				
		AMC	CRO	LEV	SXT	Combined therapy[b]
gBLNAS						
Cured	46	12	19	5	1	9
Died	3	2	1			
gBLNAR						
Cured	31	11	15	4		6
Died	5	3	2			
gBLPAR						
Cured	7	3	1			3
gBLPACR						
Cured	3	1	1	1		

[a]AMC: amoxicillin/clavulanic acid; CRO: ceftriaxone; LEV: levofloxacin; SXT: cotrimoxazole.
[b]Combined therapy is β-lactam with fluoroquinolone or fluoroquinolone with another antibiotic.
[c]Genotypes are defined in the Materials and Methods section.
doi:10.1371/journal.pone.0082515.t003

molecular epidemiology of NTHi causing non-bacteremic CAP in adult patients in the Barcelona area of Spain.

β-lactam antimicrobials are the first therapeutic option for treating CAP due to *H. influenzae* [29]. Resistance to ampicillin varies among European countries [31,32]. The rate of reduced susceptibility to ampicillin found in this study was 33.7% (10.5% of isolates were resistant and 23.2% presented intermediate resistance), which is higher than the rate reported (16.2%) in a recent Spanish study by Perez-Trallero et al. [16]. A possible explanation for this high percentage of ampicillin non-susceptibility is that the majority of NTHi isolates were obtained from elderly patients who had received multiple antibiotic courses for their underlying diseases.

β-lactamase are the most common mechanism through which resistance to β-lactam antibiotics is acquired, although the frequency of their involvement fluctuates depending on the geographical area in question [33–35]. In our study, 10.5% of isolates presented TEM-1 β-lactamase production. This result is consistent with an overall downward trend that has been observed in Spain (from 25.7% in 1997 to 15.7% in 2007 [16]), as well as in other European countries and the USA [36]. However, different rates of β-lactam resistance due to alterations in PBP3 have been reported in several countries [15,24,36,37]. In the present study, 41.1% of isolates had amino acid substitutions in the transpeptidase domain of PBP3. The percentage of BLNAR isolates detected in other European countries such as Germany (11.8%), France (0%), Portugal (9.6%) and the UK (1.5%) is lower than that found here [37]. The observed rate of gBLNAR could be due to the fact that most of our patients with CAP received multiple β-lactam antibiotic courses as treatment for their underlying diseases. Furthermore, the consumption of aminopenicillins in Catalonia increased from 46.1% in 1992 to 59.6% in 2007 [38], and this could also explain the frequency of gBLNAR observed in this study. In line with a previous report on Spanish isolates [10], the most frequent mutation found in the *fts*I gene was Asn526→ Lys, followed by Arg517→ His, and this allowed us to use the Dabernat et al. classification to group our isolates [24]. The presence of these mutations conferred a reduced susceptibility on ampicillin and amoxicillin/clavulanic acid (MIC between 1–4 mg/L) although those mutations alone were not enough to confer full resistance. In this set of NTHi, no isolates were found to belong to groups III or III-like (Met377→ Ile and Ser385→ Thr substitutions), which have been related to decreased cefotaxime and cefixime susceptibility [10].

Most of our patients infected with strains that were non-susceptible to ampicillin were successfully treated with amoxicillin/clavulanic acid, ceftriaxone or levofloxacin. In accordance with other studies [16,31], amoxicillin/clavulanic acid, third-generation cephalosporins and quinolones showed excellent in vitro activity and are good therapeutic options for treating non-bacteremic CAP due to NTHi. However, since no gBLNAR isolates with ampicillin MIC ≥4 mg/L were found in our study, the clinical outcomes of patients infected by strains with high ampicillin MIC is unknown.

NTHi strains isolated from CAP episodes were found to be genetically diverse, this being consistent with other surveillance studies performed on respiratory or invasive NTHi isolates [39,40]. Some studies carried out on BLNAR strains have demonstrated the high genotypic heterogeneity and lack of clonal spread in these strains [41,42]. However, recent studies suggest a clonal dissemination of some BLNAR or BLPACR strains [10,43,44]. In our study, some small clusters of gBLNAR strains were found (Figure 1), but only one cluster, comprising two strains, presented the same *fts*I pattern, thereby suggesting a lack of clonal distribution in NTHi from CAP patients.

In conclusion, this study has established the genotypic characterization and antimicrobial resistance of NTHi causing non-bacteremic CAP in adult patients. The results illustrate the high genetic diversity among these strains, as well as the high rate of reduced susceptibility to ampicillin due to alterations in PBP3. Finally, the analysis of treatment and outcomes in this group of patients demonstrated that NTHi strains with mutations in the *fts*I gene (gBLNAR and gBLPACR) could be successfully treated with ceftriaxone or fluoroquinolones.

Supporting Information

Table S1 Groups based on e-BURST analysis with MLST data of 95 NTHi causing non-bacteremic CAP.
(DOC)

Acknowledgments

We acknowledge use of the *Haemophilus influenzae* MLST website. We wish to thank all the staff of the Microbiology Laboratory of Hospital Universitari de Bellvitge who contributed to this project on a daily basis.

Author Contributions

Conceived and designed the experiments: CP CA JL. Performed the experiments: CP. Analyzed the data: CP CA SM JL LC FT CG JC. Contributed reagents/materials/analysis tools: JL. Wrote the paper: CP SM CA JL.

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Table S1. Groups based on e-BURST analysis with MLST data of 95 NTHi causing non-bacteremic CAP.

Group[a]	Sequence type	Allelic profiles							No. isolates
		adk	*atpG*	*frdB*	*fuck*	*mdh*	*pgi*	*recA*	
1	ST14	5	1	1	1	1	2	5	2
	ST183	14	44	1	1	22	1	5	1
	ST267	1	5	1	1	1	83	5	1
	ST367	1	1	1	1	67	1	5	1
	ST408	1	1	1	1	1	21	5	1
	ST582	1	80	1	1	1	13	5	1
	ST974	1	1	1	1	73	42	5	1
	ST1171	1	107	1	1	22	1	5	1
2	ST11	1	8	1	14	9	14	13	3
	ST103	1	1	1	14	9	14	13	1
	ST139	1	1	1	14	45	14	21	2
	ST145	1	8	1	14	22	14	13	3
3	ST266	3	18	53	15	86	14	23	1
	ST993	3	18	53	15	86	14	3	1
	ST1182	3	18	53	15	231	14	23	1
4	ST57	14	7	13	7	17	13	17	1
	ST98	14	7	13	15	17	13	1	1
5	ST648	1	1	35	14	115	1	5	1
	ST679	1	1	10	14	186	1	5	1
6	ST999	6	20	16	15	77	8	43	1
	ST1048	6	20	107	15	77	196	43	2
7	ST388	60	51	16	48	15	1	31	1
	ST997	60	51	107	48	15	2	31	1
8	ST1162	50	12	32	50	147	49	125	1
	ST1174	68	12	32	50	147	183	125	1
9	ST245	1	24	18	18	27	1	5	1
	ST836	1	11	18	18	62	1	5	1
10	ST159	33	8	16	16	17	2	29	7
	ST819	14	8	16	16	17	2	3	2
11	ST409	1	1	1	14	15	111	5	1
	ST1163	50	11	1	14	15	1	5	1
	ST1176	160	1	1	14	15	1	5	1

[a] The remaining 49 isolates were singleton: ST36, ST85, ST142 (n=3), ST160, ST165, ST201, ST303 (n=3), ST204, ST241 (n=2), ST270 (n=2), ST272 (n=2), ST385, ST414 (n=3), ST425, ST519 (n=4), ST556 (n=2), ST714, ST989, ST990, ST991 (n=2), ST992, ST994, ST995, ST996, ST998, ST1000, ST1143, ST1172, ST1177, ST1178, ST1179, ST1180, ST1181, ST1183, ST1184.

Objective 2.3: To determine the clinical and molecular epidemiology of *H. influenzae* causing invasive disease.

Objective 2.4: To study the rates of resistance to β-lactams and fluoroquinolones, and to determine the mechanisms of resistance to these antimicrobial groups in *H. influenzae*.

Paper 4: Clinical and molecular epidemiology of *Haemophilus influenzae* causing invasive disease in adult patients.

Puig C, Grau I, Marti S, Tubau F, Calatayud L, Pallares R, Liñares J, Ardanuy C. PLoS One. 2014 9(11):e112711.

OPEN ACCESS Freely available online

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Clinical and Molecular Epidemiology of *Haemophilus influenzae* Causing Invasive Disease in Adult Patients

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Abstract

Objectives: The epidemiology of invasive *Haemophilus influenzae* (Hi) has changed since the introduction of the Hi type b (Hib) vaccine. The aim of this study was to analyze the clinical and molecular epidemiology of Hi invasive disease in adults.

Methods: Clinical data of the 82 patients with Hi invasive infections were analyzed. Antimicrobial susceptibility, serotyping, and genotyping were studied (2008–2013).

Results: Men accounted for 63.4% of patients (whose mean age was 64.3 years). The most frequent comorbidities were immunosuppressive therapy (34.1%), malignancy (31.7%), diabetes, and COPD (both 22%). The 30-day mortality rate was 20.7%. The majority of the strains (84.3%) were nontypeable (NTHi) and serotype f was the most prevalent serotype in the capsulated strains. The highest antimicrobial resistance was for cotrimoxazole (27.1%) and ampicillin (14.3%). Twenty-three isolates (32.9%) had amino acid changes in the PBP3 involved in resistance. Capsulated strains were clonal and belonged to clonal complexes 6 (serotype b), 124 (serotype f), and 18 (serotype e), whereas NTHi were genetically diverse.

Conclusions: Invasive Hi disease occurred mainly in elderly and those with underlying conditions, and it was associated with a high mortality rate. NTHi were the most common cause of invasive disease and showed high genetic diversity.

Citation: Puig C, Grau I, Marti S, Tubau F, Calatayud L, et al. (2014) Clinical and Molecular Epidemiology of *Haemophilus influenzae* Causing Invasive Disease in Adult Patients. PLoS ONE 9(11): e112711. doi:10.1371/journal.pone.0112711

Editor: Caroline L. Trotter, University of Cambridge, United Kingdom

Received August 7, 2014; **Accepted** October 14, 2014; **Published** November 7, 2014

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper.

Funding: Funding was provided by Fondo de Investigaciones Sanitarias de la Seguridad Social (PI0901904), and by CIBER de Enfermedades Respiratorias (CIBERES - CB06/06/0037), run by the ISCIII (Instituto de Salud Carlos III, Madrid, Spain). CP was supported by an FPU Grant AP2010-3202 (Formación de Profesorado Universitario, Ministerio de Educación, Spain). SM was supported by "Sara Borrell postdoctoral contract CD10/00298" from the Instituto de Salud Carlos III (ISCIII), Madrid, Spain. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Haemophilus influenzae is a human-restricted pathogen that forms part of the normal nasopharyngeal microbiota. The presence or absence of a polysaccharide capsule divides this bacterium into two different groups [1,2]. The variability of capsular polysaccharide means that encapsulated *H. influenzae* strains are classified into six serotypes, labeled a–f. Before the introduction of the conjugate vaccine against *H. influenzae* type b (Hib), Hib was the major serotype responsible for invasive infections in infants and young children, with meningoencephalitis as the most common clinical manifestation [1,3,4]. With the prevention provided by vaccination, colonization rates and invasive infections in children have been considerably reduced [1,3–6]. Non-capsulated strains, also known as nontypeable *H. influenzae* (NTHi), frequently cause respiratory infections such as otitis media in children and exacerbations of chronic respiratory diseases and community-acquired pneumonia in fragile adult populations [1,2,7–9]. Notably, since introduction of the Hib vaccine a strain replacement has been observed in invasive infections and nontypeable strains have become predominant among cases of invasive *H. influenzae* disease in adults [10,11].

The main aims of this study were to analyze the demographic and clinical characteristics of adult patients with invasive *H. influenzae* infections and to determine the antimicrobial resistance and molecular epidemiology of these invasive strains.

Materials and Methods

Ethical Statement

This study has been revised and approved for its publication by the Clinical Research Ethics Committee of Bellvitge University Hospital (PR223/14). Written informed consent was considered not necessary for the study, as it was a retrospective analysis of our usual everyday work. The data of the patients were anonymized for the purposes of this analysis. The confidential information of the patients was protected according to national normative.

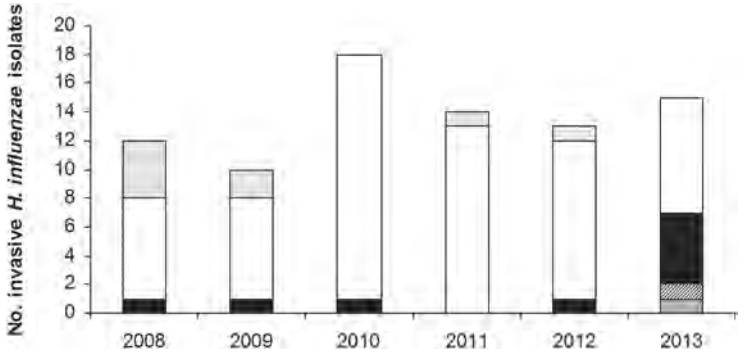

Figure 1. Distribution of 82 invasive *H. influenzae* isolated from adult patients (2008–2013). White bar: nontypeable *H. influenzae*. Black bar: *H. influenzae* serotype f. Dotted bar: no available isolated for serotyping. Grey bar: *H. influenzae* serotype b. Lined bar: *H. influenzae* serotype e. doi:10.1371/journal.pone.0112711.g001

Study design and clinical data

A six-year laboratory-based study (2008–2013) was conducted at the Bellvitge University Hospital, a tertiary care center for adult patients located in the south of Barcelona (Spain), the aim being to analyze the epidemiology of invasive *H. influenzae*. Invasive *H. influenzae* were defined as the isolation of Hi from blood, cerebrospinal fluid (CSF), or pleural fluid with clinical symptoms in the patient. The denominator used to estimate incidence was the number of persons by age group per year recorded in the public database hosted on the website of the Official Statistics Office of Catalonia (http://www.idescat.cat). Clinical and demographical data were retrieved from a prospective protocol of bacteremia cases recorded at our institution; in those patients without bacteremia their clinical records were reviewed.

Bacterial Strains

Invasive *H. influenzae* strains collected from sterile sites in our laboratory were stored at −80°C. Isolates were identified by mass spectrometry using a MALDI-TOF Biotyper version 3.0 (Bruker). Differentiation between *H. influenzae* and *H. haemolyticus* was performed by the detection of *fuc*K, *iga*, and *lgt*C genes, using a previously described methodology [12]. Isolates with a positive detection for all three genes were considered *H. influenzae*.

Capsule Typing and Antimicrobial Susceptibility Testing

Capsular serotype was determined by PCR using primers and conditions described elsewhere [13]. Antimicrobial susceptibility was tested in all 82 isolates by disk diffusion as a part of the normal laboratory routine, following CLSI recommendations. In the 70 available isolates, minimal inhibitory concentration (MIC) was tested by the microdilution method using commercial panels (STRHAE2; Sensititre, West Sussex, England) and following Clinical Laboratory Standards Institute (CLSI) recommendations [14,15]. β-lactamase activity was screened using the chromogenic cephalosporin method (nitrocefin disks, BD, Madrid, Spain). Identification of β-lactamase type was performed by PCR on all the positive β-lactamase isolates using previously described primers and conditions [16].

PPB3 Sequencing and Genotype Definition for Ampicillin Resistance

An internal region of the *fts*I gene (796-1741 pb) was amplified by PCR and sequenced as previously described [17]. In accordance with previous descriptions [18,19], *H. influenzae* were classified into four ampicillin-resistant genotypes: β-lactamase negative ampicillin susceptible (gBLNAS), strains without a detectable resistance mechanism; β-lactamase negative ampicillin resistant (gBLNAR), strains which presented mutations in the *fts*I gene; β-lactamase positive ampicillin resistant (gBLPAR), strains producing β-lactamase; and β-lactamase positive amoxicillin/clavulanic acid resistant (gBLPACR), strains which presented both resistance mechanisms (β-lactamase production and mutations in the *fts*I gene).

Molecular Typing

Genomic DNA was digested with *Sma*I and the fragments were separated by pulsed-field gel electrophoresis (PFGE), as reported previously [9]. PFGE band patterns were analyzed using the Fingerprinting II Software 3.0 (BioRad). The similarity of the PFGE banding patterns was estimated with the Dice coefficient, setting the optimization and tolerance at 1%. Isolates with ≥80% relatedness were considered highly genetically related [20]. Multilocus sequence typing (MLST) was performed by DNA sequencing of internal fragments of seven housekeeping genes (*adk*, *atp*G, *frd*B, *fuc*k, *mdh*, *pgi*, and *rec*A), as previously described [21]. Allele number and sequence types (ST) were assigned using the *H. influenzae* MLST website (http://haemophilus.mlst.net).

Statistical Analyses

Statistical analyses were performed using GraphPad Prism version 4, using Chi-square or Fisher's exact tests, when appropriate, with $P<.05$ being considered significant.

Results

Clinical characteristics

During the period 2008 to 2013 a total of 3433 *H. influenzae* were isolated from adult patients in our hospital. Of these, 82 isolates (2.4%) caused invasive *H. influenzae* infection in 82 patients. The overall incidence for Hi invasive disease among adults in our area during the study period was 2.12 episodes per 100,000 population. By age group the incidence of invasive disease was higher among those aged 65 or older than among people ≤64 years (6.8/100,000 *vs*. 1.1/100,000; $p<0.01$). We observed no significant change in the incidence of invasive disease over the study period, neither overall nor by age group or serotype.

Table 1 shows the demographics, clinical characteristics, and underlying conditions for the 82 patients with invasive *H.*

influenzae infection. Fifty-two (63.1%) cases occurred in men and the mean age of patients was 64.3 years. Most cases were community-acquired, and pneumonia was the most frequent type of infection (59.8%) The most common comorbidities were immunosuppressive conditions, malignancies, diabetes, chronic obstructive pulmonary disease (COPD), and heart disease (Table 1). In general, older patients had higher rates of underlying conditions than did those ≤64 years, especially for COPD (34.0% vs. 5.7%, p = 0.002) and heart disease (27.6% vs. 5.7%, p = 0.018). The 30-day mortality was 20.7% (n = 17), with no differences between younger and older adults (p = 0.58).

Invasive *H. influenzae*: sample origin and serotypes

The source of strains in the 82 invasive Hi cases was: blood (n = 70), CSF (n = 3), pleural fluid (n = 2), blood plus CSF (n = 5), and blood plus pleural fluid (n = 2). Serotypes of isolates causing meningitis were NTHi (n = 6) and Hif (n = 1), with one isolate being unavailable (n = 1).

Unfortunately, only isolates from 70 cases (70/82, 85.37%) were viable and available for microbiological studies. The majority of these isolates were NTHi (n = 59, 84.3%), and the frequency of capsulated isolates was low (n = 11, 15.7%). Among 11 capsulated strains, 9 were serotype f (Hif), 1 serotype b (Hib), and 1 serotype e (Hie) (Figure 1). Encapsulated *H. influenzae* were mainly isolated from blood (n = 9, 81.8%). Both Hib and Hie strains were isolated from patients with pneumonia. The foci of infection of Hif cases were pneumonia (n = 3), epiglottitis (n = 1), meningitis (n = 1), peritonitis (n = 1), facial cellulites (n = 1), and biliary tract infection (n = 1).

Antimicrobial susceptibility

All 82 isolates tested by disk diffusion presented fully susceptibility to amoxicillin/clavulanic acid, cefotaxime, ceftriaxone, chloramphenicol, tetracycline, and ciprofloxacin. On the other hand, 23% of the isolates (n = 19) were resistant to cotrimoxazole and 8.5% (n = 7) were resistant to ampicillin due to β-lactamase

Table 1. Demographic data, clinical characteristics, and underlying conditions of 82 patients with an invasive *H. influenzae* episode during the period 2008–2013.

	Patients (n = 82)
Characteristics [no. (%)]	
Age (mean ± SD); range	64.3±16.1; 21–96
Male sex	52 (63.4)
Acquisition	
Community-acquired	71 (86.6)
Hospital-acquired	11 (13.4)
Source of infection	
Pneumonia/empyema	49 (59.8)
Meningitis	9 (11.0)
Biliary tract infection	9 (11.0)
Primary bacteremia	7 (8.5)
Epiglottitis	2 (2.4)
Others[a]	6 (7.3)
Underlying conditions [no. (%)]	
Immunosuppressive therapy	28 (34.1)
Solid organ malignancy	26 (31.7)
Diabetes	18 (22.0)
COPD	18 (22.0)
Heart disease	15 (18.3)
Chronic liver disease	11 (13.4)
Hematologic malignancy[b]	9 (11.0)
Cerebrovascular disease	5 (6.1)
Organ transplant[c]	4 (4.9)
HIV	2 (2.4)
Others[d]	10 (12.2)
Shock	18 (22.0)
Neutropenia	8 (9.8)
Mortality	
<30 days	17 (20.7)

[a]Facial cellulites, endometritis, liver abscess, and urinary-tract infection (n = 1, 1.2% each), and peritonitis (n = 2, 2.4%).
[b]Leukemia (n = 3, 3.6%), lymphoma (n = 1, 1.2%), and myeloma (n = 5, 6.1%).
[c]Bone marrow transplant (n = 1, 1.2%), kidney transplant (n = 1, 1.2%), and liver transplantation (n = 2, 2.4%).
[d]Cerebrospinal fluid fistula (n = 2, 2.4%), renal failure, autoimmune disease, and head trauma (n = 1, 1.2% each).
doi:10.1371/journal.pone.0112711.t001

production. Antimicrobial susceptibility of the 70 available isolates was tested by microdilution. All tested isolates were fully susceptible to amoxicillin/clavulanic acid, cefepime, cefotaxime, ceftriaxone, imipenem, chloramphenicol, tetracycline, and ciprofloxacin (Table 2). Capsulated isolates were fully susceptible to all tested antibiotics, with the exception of one strain that was resistant to rifampin. By contrast, NTHi isolates presented higher resistance rates to cotrimoxazole (27.1%), azithromycin (1.4%), and cefuroxime (1.4%) (Table 2). Seven NTHi isolates (10%) were ampicillin resistant: six (6/70, 8.6%) due to β-lactamase production (MIC>4 mg/L) and one due to a modified PBP3 (MIC = 4 mg/L). In addition, three strains (3/70, 4.3%) presented reduced ampicillin susceptibility (MIC = 2 mg/L). All six β-lactamase producers presented the $bla_{\text{TEM-1}}$ gene.

In order to determine the mutations in PBP3 the transpeptidase domain of the *fts*I gene was sequenced in all the isolates. Thirty-four isolates (48.6%) presented mutations in the *fts*I gene (Table 3). The observed mutations allow us to classify the strains into groups I and II, in accordance with Dabernat *et al*. [17]. The most frequent substitutions were those which were classified as group II (22/34, 64.7%). No isolates were observed in subgroup IId or in groups III and III-like. Additionally, eight isolates (23.5%) presented mutations in the *fts*I gene, none of which were at the positions which defined the groups. For this reason, they were considered gBLNAS and classified into the miscellaneous group. All these eight isolates presented similar ampicillin MIC to susceptible strains (≤0.5 mg/L), suggesting that these mutations were not involved in decreased β-lactam susceptibility (Table 3).

Molecular epidemiology

Molecular typing by PFGE revealed 50 different patterns. Fourteen clusters grouped between two and seven related isolates, and 36 patterns were genotypically unique. Hif isolates were grouped into two related clusters, one with seven isolates and the other with two. The other 12 clusters grouped two NTHi isolates each.

Molecular typing by MLST showed high genetic variability among 59 NTHi isolates, which had 51 different sequence types (STs). After eBURST analysis, NTHi STs were distributed into three clonal groups, along with 45 singletons. Clonal group 1 grouped ST103 and ST134, with one isolate each. Group 2 was formed by three isolates, with ST3 (n = 1) and ST367 (n = 2). Finally, clonal group 3 comprised ST14 (n = 1) and a single locus variant (SLV, n = 1). Among the 45 singletons the most frequent ST was ST57, with three isolates. eBURST analysis of all the STs from NTHi isolates published in the MLST database revealed that 75.7% (n = 53) of our invasive isolates belonged to different clonal complexes (CC) defined by the analysis. Twenty-three isolates belonged to the five most prevalent CC among NTHi: CC1, CC3 (n = 5 each), CC41, CC57, and CC472 (n = 3 each).

Capsulated strains, by contrast, were genetically related. All type f isolates belonged to CC124, formed by ST124 (n = 7) and two single locus variants (*rec*A5 and *rec*A43). The type b strain belonged to CC6 (SLV ST6; *rec*A15), while the Hie strain belonged to ST18.

Discussion

The epidemiology of invasive *H. influenzae* has changed since the introduction of the Hib conjugate vaccine for children, with nontypeable strains being the most frequent etiological agent in most cases of invasive Hi disease in adults. The incidence rate observed among our adults (2.12/100,000) is similar to that reported in the USA and in Europe [6,22,23]. As documented in other studies the incidence of invasive Hi disease increased with age (6.8/100,000 in patients ≥65 years old) [6,23].

In the pre-vaccine era, Hib was the most important cause of invasive disease (mainly meningitis) in healthy children under 5 years of age [1,4,22]. In the United States the pre-vaccine incidence of invasive disease in adults was 1.7 cases per 100,000 persons, with Hib being responsible for 50% of invasive diseases due to *H. influenzae* [11]. In the United Kingdom the incidence of Hib infection in adults was low (9%) and it was assumed to be a consequence of transmission from children [24]. Since the widespread childhood immunization program in the 1990s, Hib infection has decreased considerably worldwide [4]. The percent-

Table 2. Antimicrobial susceptibility of 70 invasive *H. influenzae*.

	MIC_{50}	MIC_{90}	Range	% S	% I	% R
Antimicrobial	**(mg/L)**	**(mg/L)**	**(mg/L)**			
Ampicillin[a]	0.25	2	≤0.12–>4	85.7	4.3	10.0
Amoxicillin/clavulanic acid[b]	≤0.5	2	≤0.5–4	100		
Cefuroxime	1	2	≤0.5–8	98.6	1.4	
Cefepime	≤0.25	≤0.25	≤0.25–0.25	100		
Cefotaxime	≤0.06	≤0.06	≤0.06–0.06	100		
Ceftriaxone	≤0.12	≤0.12	≤0.12	100		
Imipenem	0.5	1	≤0.12–2	100		
Chloramphenicol	≤1	≤1	≤1	100		
Tetracycline	≤1	2	≤1–2	100		
Ciprofloxacin	≤0.03	≤0.03	≤0.03	100		
Cotrimoxazole[c]	≤0.5	>2	≤0.5–>2	72.9	1.4	25.7
Azithromycin	1	2	≤0.12–>4	98.6		1.4

[a]β-lactamase production: 8.6% (n = 6).
[b]The ratio of amoxicillin/clavulanic acid was 2:1.
[c]The ratio of cotrimoxazole was 1:19.
doi:10.1371/journal.pone.0112711.t002

Table 3. Amino acid substitutions in PBP3 among 70 invasive *H. influenzae* strains.

BLNAR/BLPACR Genotype	No isolates	Amino acid substitutions													MIC (mg/L)		BL[a]
		Ile 348	Asp 350	Ala 368	Met 377	Met 391	Ala 437	Gly 490	Ala 502	Val 509	Arg 517	Asn 526	Ala 530	Phe 531	AMP	AMC	
No changes[b]	33														≤0.12	≤0.5	–
	3														>4	≤0.5	+
I	3										His				0.5	1	–
	1								Thr		His						–
IIa	1											Lys			1	2	–
	2		Asn					Glu				Lys	Ser		1	1–2	–
IIb	1								Val			Lys			0.5	1	–
	6		Asn		Ile				Val			Lys			0.5–4	1–4	–
	1		Asn		Ile				Val			Lys			>4	4	+
	2		Asn		Ile			Glu	Val			Lys			0.5–1	1–2	–
	1		Asn		Ile			Glu	Val			Lys			>4	4	+
IIc	2								Thr			Lys			1–2	2–4	–
	1								Thr			Lys			>4	4	+
	5		Asn						Thr			Lys			1–2	2–4	–
Miscellaneous[c]	1	Val													0.5	1	–
	1			Thr											≤0.12	≤0.5	–
	1		Asn												0.12	≤0.5	–
	2									Ile					0.25	≤0.5	–
	1													Leu	0.25	≤0.5	–
	2						Ser								0.25	≤0.5	–

[a]BL: Beta-lactamase production: + (positive); – (negative).
[b]Isolates without amino acid changes in PBP3 (gBLNAS).
[c]Isolates grouped in the miscellaneous group were not gBLNAR.
doi:10.1371/journal.pone.0112711.t003

age of Hib infection cases in adults varies depending on the region. In Illinois, for example, 17.7% of cases were Hib, whereas in Utah the figure was 9% [6,11]. Only one of our isolates was identified as Hib (1.4%), a lower percentage than previously reported (4.92%) in another Spanish study [25]. Concerning non-b capsulated strains, Hif is currently the most frequent serotype causing invasive disease [1,5,11,25–27]. However, despite the fact that in our hospital the number of invasive *H. influenzae* isolated from 2008–2013 remained stable, an increment in non-b capsulated strains was observed in 2013, when the number of capsulated isolates doubled due to an increase in serotype f.

During the pre-vaccination era, NTHi were not a frequent cause of invasive disease, even though they had been considered an important respiratory pathogen in adults [8]. However, a strain shift has been observed since vaccination, with NTHi being the strains most frequently responsible for invasive infections in adults [1]. NTHi were the most common cause of invasive Hi infection in adults in Illinois (34%), Utah (43%), Manitoba (57%), and Sweden [6,11,26,27]. A recent publication in Spain reported 62% of NTHi among invasive isolates from adults (2004–2009) [25]. In our study, which analyzed invasive isolates from 2008 to 2013, 85.7% of strains were identified as NTHi, following the trend observed in other studies and adding to previously published data on more recently isolated strains.

As reported in other studies [3,6,11], most invasive *H. influenzae* disease infections occurred in older adults (n = 47 patients were ≥65 years old) and in those with underlying conditions. Increased life expectancy and the growing number of patients with underlying conditions may account for the high proportion of invasive Hi disease found in the present study. Pneumonia was the most common type of infection caused by invasive *H. influenzae* in adults, as reported elsewhere [10,22,24,28]. In line with other studies [3,6,11,28,29], our patients with invasive *H. influenzae* infection showed a high mortality rate, although again this could be associated with age and underlying conditions. Nevertheless, we have to acknowledge the small number of cases identified as a limitation of our study.

Following the clinical and epidemiological evaluation of samples the study aimed to investigate the antimicrobial resistance of invasive *H. influenzae*. Traditionally, the most common mechanism of β-lactam resistance in *H. influenzae* has been β-lactamase production, although this production has decreased over time [30–32]. The percentage of β-lactamase in invasive *H. influenzae* varies depending on the study, ranging from 10–24% [3,25,26,32–38]. In our study the percentage of β-lactamase production was 8.6%, in line with published data although lower than the figures (16.9% and 24.2%) reported in two previous studies about invasive *H. influenzae* performed in 1999–2000 and 2004–2009 in Spain [3,25]. β-lactam resistance due to alterations in PBP3 has also been reported worldwide [18,26,30,39,40]. In the present study, 32.9% of isolates were considered gBLNAR, with ampicillin MIC of 0.5–4 mg/L, presenting relevant mutations in the transpeptidase domain of *fts*I. Although these mutations conferred reduced susceptibility to ampicillin, the isolates in question were not considered resistant according to current CLSI and EUCAST breakpoints, the exception being one strain which presented an ampicillin MIC of 4 mg/L. In our experience, the patients infected by BLNAR strains were successfully treated with amoxicillin/clavulanic acid, cefepime, ceftriaxone, piperacillin/tazobacatam, and quinolones (data not shown). Currently, these strains with altered MICs to β-lactams can be successfully treated with these antibiotics; however, the detection of BLNAR strains in the laboratory could improve the knowledge about the epidemiology of *H. influenzae*. The most common mutations found in invasive isolates were those that classified the strains into group IIb (47.8%), this being consistent with previous data reported by Resman *et al*., Shuel *et al*., and Bajanca *et al*. [26,33,37]. By contrast, however, with Spanish data published by García-Cobos *et al*., who found that group IIc was the most common BLNAR genotype in invasive *H. influenzae* (42.4%) [25].

Genotyping by PFGE and MLST showed a high diversity among NTHi strains, which were distributed into three clonal groups and 45 singletons. Despite the high genetic variability observed in NTHi, the majority of isolates were grouped according to the most prevalent clonal complexes defined by eBURST, using all the NTHi published in the MLST database. Capsulated strains, by contrast, were clonally related. These results are consistent with other studies that also described this difference in diversity between NTHi and capsular *H. influenzae* [26,33,37]. Moreover, all capsulated strains found in our study belonged to international disseminated global clones. For instance, all nine Hif isolates were grouped in CC124 (ST124 and two single locus variants), which has been identified in the USA and other European countries [25,33,37,38]. Hib and Hie isolates belonged, respectively, to the CC6 and CC18 clones, which have been detected worldwide (http://haemophilus.mlst.net/).

In conclusion, NTHi were the most frequent cause of invasive Hi disease in adults, who frequently presented underlying conditions, and they were associated with a high mortality rate. In our hospital, however, there was an increase in capsular strains, generally serotype f, during 2013. It should also be noted that reduced ampicillin susceptibility was observed in a high percentage of invasive *H. influenzae* due to mutations in PBP3. Despite the reduction in Hib, continuous monitoring of invasive *H. influenzae* infections should be performed, not only because of the recent increase in capsulated non-b strains but also in order to detect changes in the epidemiology of invasive *H. influenzae*.

Author Contributions

Conceived and designed the experiments: CP SM CA JL RP. Performed the experiments: CP SM IG. Analyzed the data: CP SM CA JL IG RP. Contributed reagents/materials/analysis tools: FT LC IG RP. Contributed to the writing of the manuscript: CP SM CA JL RP.

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Objective 2.4: To study the rates of resistance to β-lactams and fluoroquinolones, and to determine the mechanisms of resistance to these antimicrobial groups in *H. influenzae*.

Paper 5: Molecular characterization of fluoroquinolone resistance in nontypeable *Haemophilus influenzae* clinical isolates.

Puig C, Tirado-Vélez JM, Calatayud L, Tubau F, Garmendia J, Ardanuy C, Marti S, de la Campa AG, Liñares J. Antimicrobial Agents and Chemotherapy. 2015 59(1):461-6.

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Molecular Characterization of Fluoroquinolone Resistance in Nontypeable *Haemophilus influenzae* Clinical Isolates

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Nontypeable *Haemophilus influenzae* (NTHi) is a common cause of respiratory infections in adults, who are frequently treated with fluoroquinolones. The aims of this study were to characterize the genotypes of fluoroquinolone-resistant NTHi isolates and their mechanisms of resistance. Among 7,267 *H. influenzae* isolates collected from adult patients from 2000 to 2013, 28 (0.39%) were ciprofloxacin resistant according to Clinical and Laboratory Standards Institute (CLSI) criteria. In addition, a nalidixic acid screening during 2010 to 2013 detected five (0.23%) isolates that were ciprofloxacin susceptible but nalidixic acid resistant. Sequencing of their quinolone resistance-determining regions and genotyping by pulse-field gel electrophoresis and multilocus sequence typing of the 25 ciprofloxacin-resistant isolates available and all 5 nalidixic acid-resistant isolates were performed. In the NTHi isolates studied, two mutations producing changes in two GyrA residues (Ser84, Asp88) and/or two ParC residues (Ser84, Glu88) were associated with increased fluoroquinolone MICs. Strains with one or two mutations ($n = 15$) had ciprofloxacin and levofloxacin MICs of 0.12 to 2 μg/ml, while those with three or more mutations ($n = 15$) had MICs of 4 to 16 μg/ml. Long persistence of fluoroquinolone-resistant strains was observed in three chronic obstructive pulmonary disease patients. High genetic diversity was observed among fluoroquinolone-resistant NTHi isolates. Although fluoroquinolones are commonly used to treat respiratory infections, the proportion of resistant NTHi isolates remains low. The nalidixic acid disk test is useful for detecting the first changes in GyrA or in GyrA plus ParC among fluoroquinolone-susceptible strains that are at a potential risk for the development of resistance under selective pressure by fluoroquinolone treatment.

Haemophilus influenzae is a human-restricted pathogen that forms part of the normal nasopharyngeal microbiota. It is classified either as encapsulated or as nontypeable *H. influenzae* (NTHi), depending on the presence of a polysaccharide capsule. Before the introduction of the conjugate vaccine against *H. influenzae* type b (Hib), this serotype was the most common cause of meningoencephalitis in young children. Since the introduction of the Hib vaccine, strain replacement has been observed and NTHi has become the predominant species among both invasive and noninvasive diseases such as otitis media, sinusitis, conjunctivitis, chronic bronchitis, and pneumonia (1–4). Fluoroquinolones (FQs) are frequently used as antimicrobial therapy in respiratory tract infections in adults and have shown good activity against respiratory pathogens such as *H. influenzae*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, and *Moraxella catarrhalis* (5, 6). Since their first description in 1993, FQ-resistant *H. influenzae* isolates have been detected all over the world (7–16). Although resistance in this bacterial pathogen remains low (8, 17), treatment failure with ofloxacin or levofloxacin (LVX) has already been described (15, 18). FQ resistance in *H. influenzae* is due mainly to chromosomal point mutations in the quinolone resistance-determining regions (QRDRs) of the genes encoding DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) (19). As in other Gram-negative bacteria, DNA gyrase is a primary target and topoisomerase IV is a secondary target for FQs. Mutations in the *H. influenzae* QRDRs have been shown to occur in a stepwise manner: a first mutation in *gyrA* produces reduced susceptibility to quinolones, but MICs remain in the susceptible range according to currently established breakpoints (20, 21). Strains susceptible to ciprofloxacin (CIP) or LVX could harbor first alterations in the QRDRs (22). Corkill et al. found that nalidixic acid (NAL) was a good indicator of reduced CIP susceptibility (23), and it has been proposed as a useful indicator for testing of low- and high-level quinolone resistance (24). Double mutations in both FQ targets generate a resistant phenotype that is detectable by using the current Clinical and Laboratory Standards Institute (CLSI) and European Society of Clinical Microbiology and Infectious Diseases (EUCAST) breakpoint interpretations (20, 21).

The aims of this study were (i) to analyze the genotypes of FQ-resistant NTHi clinical isolates collected in our hospital over a 14-year period (2000 to 2013), (ii) to detect CIP-susceptible isolates with NAL resistance that could harbor a first mutation in their QRDRs, and (iii) to characterize the mechanisms of resistance to FQs in clinical isolates.

Received 31 July 2014 **Returned for modification** 31 August 2014
Accepted 29 October 2014

Accepted manuscript posted online 10 November 2014

Citation Puig C, Tirado-Vélez JM, Calatayud L, Tubau F, Garmendia J, Ardanuy C, Marti S, de la Campa AG, Liñares J. 2015. Molecular characterization of fluoroquinolone resistance in nontypeable *Haemophilus influenzae* clinical isolates. Antimicrob Agents Chemother 59:461–466. doi:10.1128/AAC.04005-14.

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doi:10.1128/AAC.04005-14

MATERIALS AND METHODS

Hospital settings and bacterial strains. This laboratory-based study was carried out at the Hospital Universitari de Bellvitge (HUB), a tertiary-care center for adult patients located in Barcelona, Spain. CIP susceptibility data on all of the *H. influenzae* isolates collected from patients who attended the HUB during a 14-year period (2000 to 2013) were recorded as part of the normal laboratory routine activity. These data were analyzed to determine the proportion of CIP-resistant isolates over the period studied. CIP susceptibility was determined by disk diffusion on *Haemophilus* test medium (HTM) plates and was interpreted by following CLSI criteria (20, 25). CIP-resistant isolates were stored at −80°C. Isolates were grown on chocolate agar plates and incubated at 37°C with 5% CO_2. Informed consent was not required, as this process was part of the normal microbiological routine; patient confidentiality was protected in all cases. To detect isolates harboring first mutations in the QRDRs, isolates collected from 2010 to 2013 that showed susceptibility to CIP and had an inhibitory zone diameter of 21 to 28 mm were screened with NAL disks (30 μg). NAL screening was done by disk diffusion on HTM plates and interpreted according to EUCAST criteria, considering any isolate with an inhibitory zone diameter of <23 mm resistant, since CLSI has not defined breakpoints for NAL (21).

Identification. *H. influenzae* was identified by conventional methodology (26). Isolate identification was confirmed by mass spectrometry with a matrix-assisted laser desorption ionization–time of flight Biotyper, version 3.0 (Bruker). Differentiation between *H. influenzae* and *H. haemolyticus* was performed by amplification of the *fucK*, *iga*, and *lgtC* genes (27); an isolate was considered *H. influenzae* when positive amplification of the genes tested was detected.

Serotyping, susceptibility testing, and sequencing of QRDRs. Capsular serotypes were determined by PCR with the primers and under the conditions previously described (28). MICs were determined by the microdilution method with HTM and commercial panels (STRHAE2; Sensititre, West Sussex, England) and interpreted by following CLSI guidelines (20, 25). *H. influenzae* ATCC 49247 was used as a susceptible control strain. β-Lactamase activity was screened for by the chromogenic cephalosporin method (nitrocefin disks; BD, Madrid, Spain). QRDRs were amplified with specific oligonucleotide pairs as described previously (19, 29). PCR fragments that included *gyrA* nucleotides 137 to 546, *parC* nucleotides 129 to 547, *gyrB* nucleotides 1094 to 1539, and *parE* nucleotides 1002 to 1473 were purified with a GeneJET PCR purification kit (Thermo Scientific) and sequenced on both strands with the same oligonucleotides used for the PCRs and an Applied Biosystems 3730 XL DNA analyzer.

Molecular typing. For molecular typing, genomic DNA was digested with SmaI and the fragments were separated by pulsed-field gel electrophoresis (PFGE) as reported previously (30). PFGE band patterns were analyzed with the Fingerprinting II Software 3.0 (Bio-Rad). The similarity of the PFGE banding patterns was estimated with the Dice coefficient. Isolates with ≥80% relatedness were considered highly genetically related. For multilocus sequence typing (MLST), DNA sequencing of internal fragments of seven housekeeping genes (*adk*, *atpG*, *frdB*, *fucK*, *mdh*, *pgi*, and *recA*) was performed as previously described (31). Allele numbers and sequence types (STs) were assigned by using the *H. influenzae* MLST website (http://haemophilus.mlst.net). The STs were analyzed with e-BURST v3 in order to define the clonal relationship between the isolates.

RESULTS

Patients and CIP-resistant NTHi isolates. Twenty-eight (0.39%) CIP-nonsusceptible isolates (CLSI criteria) were detected among 7,267 *H. influenzae* isolates collected in our Laboratory from 2000 to 2013. The proportion of CIP resistance over the period studied was low and remained stable over time at 0.58% from 2000 to 2004, 0.26% from 2005 to 2009, and 0.36% from 2010 to 2013.

Unfortunately, only 25 out of 28 isolates were available for molecular analysis. All of these 25 were NTHi isolates collected from 19 patients (16 [84%] males) with different episodes of respiratory disease. The mean age of these patients was 72.7 (range, 52 to 88) years. The main underlying diseases were chronic obstructive pulmonary disease (COPD) (n = 10, 52.7%) and bronchiectasis (n = 2, 10.5%), whereas no underlying disease was reported for 7 patients (36.8%).

NAL disk screening of isolates with CIP susceptibility. Among 2,201 isolates collected from 2010 to 2013, 7 (0.32%) had a CIP inhibitory zone dimater of 21 to 28 mm. Five of these were resistant to NAL (inhibitory zone diameter of <23 mm). All of them were NTHi isolates from sputum samples of five patients (three females and two males) with a mean age of 69.4 years suffering from lung cancer, ischemic heart disease, bronchial asthma, COPD, and bronchiectasis.

Genotyping. Thirty NTHi isolates (25 CIP resistant and 5 CIP susceptible but NAL resistant) were grouped into 15 different PFGE patterns (Table 1). Nine genotypes were unique, and the remaining 21 isolates were grouped into six small clusters. MLST results showed 16 different STs, 8 previously described in the MLST database and 8 described as new. Eleven STs were unique, and six clusters were detected (Table 1). The clusters were grouped as follows: cluster 1, isolates 16.1, 18.1, and 24.1, PFGE pattern B and ST 1281; cluster 2, isolates 3.1 and 7.1, PFGE pattern D and ST New4; cluster 3, isolates 15.1 and 23.1, PFGE pattern F and STs 159 and 485, a double-locus variant; cluster 4, isolates 20.1, 20.2, and 22.1, PFGE pattern A and ST New3; cluster 5, isolates 2.1 to 2.5 and 11.1, PFGE pattern C and ST 519; cluster 6, isolates 4.1, 5.1, 10.1, 10.2, and 14.1, PFGE pattern E and ST New5. No relationship between the patients in each cluster could be demonstrated.

Three COPD patients were persistently infected with a particular NTHi strain (ST 519, New 3, or New 5) with a median persistence time of 17.6 (range, 1.33 to 48) months (Table 1).

Antimicrobial susceptibility. NTHi isolates resistant to CIP (n = 25) were also resistant to other antimicrobial agents. The highest proportion of resistance was to cotrimoxazole (56%), followed by resistance to azithromycin (20%). Regarding β-lactams, 4% were ampicillin resistant because of β-lactamase production and 4% were β-lactamase-negative ampicillin-resistant (BLNAR) isolates with intermediate susceptibility to ampicillin and cefuroxime. All isolates were fully susceptible to cefotaxime, imipenem, tetracycline, and chloramphenicol. As for FQs, 22 isolates had CIP resistance (MIC, >1 μg/ml). The remaining three isolates had a CIP MIC of 1 μg/ml, which is considered susceptible according to the CLSI breakpoints, although all of them had a CIP disk diffusion inhibitory zone diameter inside the resistance range and had mutations in their QRDRs. Fifteen (60%) isolates were resistant to LVX (MIC, >2 μg/ml).

Seven CIP-resistant isolates with a CIP MIC of 2 μg/ml were considered low-level CIP resistant whereas 15 isolates were defined as high-level CIP resistant (MICs, 8 to 16 μg/ml). Although low-level CIP-resistant isolates were susceptible to LVX according to CLSI criteria, these isolates had *gyrA* and *parC* mutations that would favor the development of high-level resistance. All of the high-level CIP-resistant isolates showed cross-resistance to LVX (Table 2).

Of the five isolates susceptible to CIP but resistant to NAL, four were resistant to cotrimoxazole and one was considered intermediately resistant to ampicillin.

FQ resistance and amino acid substitutions in the QRDRs. Determination of susceptibility to FQ antimicrobials (by microdi-

TABLE 1 Characteristics of 24 patients and molecular characterization of NTHi clinical isolates[a]

Patient no.	Age (yr), gender	Isolate	Isolation date (day-mo-yr)	PFGE	ST	MLST						
						adk	*atpG*	*frdB*	*fucK*	*mdh*	*pgi*	*recA*
1	73, male	1.1	01-02-00	G	18	18	6	3	7	10	28	12
2	88, male	2.1	01-02-00	C	519	26	1	46	1	79	64	23
2	88, male	2.2	13-12-00	C	519	26	1	46	1	79	64	23
2	88, male	2.3	29-12-02	C	519	26	1	46	1	79	64	23
2	88, male	2.4	23-03-03	C	519	26	1	46	1	79	64	23
2	88, male	2.5	11-04-04	C	519	26	1	46	1	79	64	23
3	81, male	3.1	18-11-00	D	New4	3	18	53	2	7	40	10
4	78, male	4.1	05-01-02	E	New5	33	33	7	7	11	40	48
5	69, male	5.1	30-01-02	E	New5	33	33	7	7	11	40	48
6	77, male	6.1	19-06-02	H	New1	28	33	7	1	236	125	48
7	71, male	7.1	29-01-03	D	New4	3	18	53	2	7	40	10
8	76, male	8.1	02-07-03	I	New6	68	12	32	55	45	120	123
9	74, female	9.1	11-01-05	J	New7	150	60	48	18	23	38	92
10	62, male	10.1	02-03-05	E	New5	33	33	7	7	11	40	48
10	62, male	10.2	12-06-05	E	New5	33	33	7	7	11	40	48
11	84, male	11.1	04-02-06	C	519	26	1	46	1	79	64	23
12	74, female	12.1	07-02-07	K	139	1	1	1	14	45	14	21
13	65, female	13.1	09-03-10	L	New2	14	7	13	7	17	13	4
14	78, male	14.1	15-10-10	E	New5	33	33	7	7	11	40	48
15	77, male	15.1	23-02-11	F	485[b]	33	8	16	16	78	2	3
16	67, male	16.1	14-04-11	B	1281	16	8	16	14	70	1	3
17	67, female	17.1	12-07-11	M	New8	11	100	121	5	59	130	19
18	76, male	18.1	29-03-12	B	1281	16	8	16	14	70	1	3
19	52, female	19.1	05-09-12	O	270	69	54	8	47	80	1	61
20	69, male	20.1	22-04-13	A	New3[c]	14	8	18	11	161	138	3
20	69, male	20.2	31-05-13	A	New3	14	8	18	11	161	138	3
21	76, male	21.1	02-05-13	N	196	14	8	18	11	17	2	3
22	67, male	22.1	15-05-13	A	New3	14	8	18	11	161	138	3
23	62, male	23.1	04-07-13	F	159	33	8	16	16	17	2	29
24	65, male	24.1	14-07-13	B	1281	16	8	16	14	70	1	3

[a] Gray-shaded lines indicate patients with persistent isolates.
[b] eBURST analysis showed that ST 485 is a double-locus variant of ST 159.
[c] ST New3 is a double-locus variant of ST 196 (eBURST analysis).

lution) and characterization (by DNA sequencing) of the QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* were performed. All of the isolates studied had nonsynonymous polymorphisms leading to amino acid substitutions in their QRDRs (Table 2), including the isolates resistant to CIP ($n = 25$) and those that were NAL resistant but CIP susceptible ($n = 5$). Two isolates presenting the single GyrA Ser84Leu change with CIP and LVX MICs of 0.12 to 0.5 μg/ml were considered susceptible. Thirteen isolates had two changes in equivalent positions of GyrA (Ser84 or Asp88) and ParC (Ser84 or Glu88) and had CIP MICs of 0.5 to 2 μg/ml and LVX MICs of 0.5 to 1 μg/ml. We also assessed five isolates from the same patient (isolates 2.1 to 2.5) with identical QRDR mutations which, despite having two GyrA (Ser84Tyr, Asp88Tyr) and two ParC (Asp83Gly, Ser84Ala) changes, had a CIP MIC of 2 μg/ml. As far as we know, the ParC Asp83Gly and Ser84Ala changes have not been implicated in resistance in *H. influenzae* or other bacteria and are probably polymorphisms. The mutations in GyrA (Ser84Tyr and Asp88Tyr) may have been the sole causes of the CIP and LVX MICs of the five isolates. Finally, 15 isolates harbored three ($n = 13$) or four ($n = 2$) amino acid substitutions and were fully resistant to CIP (Table 2). Four isolates with high-level resistance showed the Asp420Asn change in ParE. However, the involvement of this change in FQ resistance has not been demonstrated by genetic transformation.

DISCUSSION

Since its first description in 1993, FQ-resistant *H. influenzae* has been isolated mainly from elderly patients with chronic lung diseases who received frequent antimicrobial treatments, including quinolones (7, 15, 16, 32). Resistance to FQs in *H. influenzae* remains very low worldwide. A global study (SENTRY) performed by American and European institutions found that 0.15% of *H. influenzae* isolates were resistant to FQs (8). In Hong Kong, Japan, and South Korea, *H. influenzae*-resistant isolates were first described in 2009, when Hirakata et al. reported 0.1% CIP resistance in Japan (33, 34); by 2014, the level had increased to 1.3% (29). A recent surveillance study published in Taiwan showed a major increase in LVX resistance from 2% in 2004 to 24.3% in 2010 (35). In the United States in 2006, the percentage of FQ resistance was 0.1% (36), similar to the percentage found in Spain in 2011 (0.2%) (17). In our study, we found a low percentage of FQ-resistant *H. influenzae* isolates (0.39%), in accordance with the data published in other parts of the world but higher than previous reports in Spain (17). However, the proportion of *H. influenzae* isolates resistant to CIP may depend on the criteria used (CLSI or EUCAST) because of the difference in the breakpoints. In the disk diffusion method, the current EUCAST breakpoint for CIP susceptibility is ≥26 mm, whereas the CLSI breakpoint is ≥21 mm. This differ-

TABLE 2 Mutations in the QRDR of and CIP and LVX MICs for 30 NTHi clinical isolates resistant to NAL

Isolate(s)[a]	Disk diffusion inhibitory zone diam (mm)		MIC (μg/ml)		Mutation(s) in QRDR[b]		
	NAL	CIP	CIP	LVX	GyrA	ParC	ParE
13.1	12	28	0.12	0.12	**Ser84Leu**	—	—
17.1	10	26	0.5	0.5	**Ser84Leu**	—	—
15.1, 23.1	6	24	0.5	0.5	**Ser84Leu**	**Ser84Ile**	—
1.1	9	20	0.5	0.5	**Ser84Tyr**	**Glu88Lys**	—
21.1	6	22	1	0.5	**Ser84Leu**	**Ser84Ile**	—
6.1	10	20	1	0.5	**Ser84Leu**	**Ser84Ile**	—
3.1	6	20	1	1	**Ser84Leu**	**Glu88Lys**	—
12.1	6	20	2	1	**Ser84Leu**	**Glu88Lys**	—
5.1	10	16	2	1	**Asp88Tyr**	**Glu88Lys**	—
2.1–2.5	6	20	2	1	**Ser84Tyr, Asp88Tyr**	Asp83Gly, Ser84Ala	—
11.1	6	15	8	4	**Ser84Tyr, Asp88Tyr**	**Ser84Asn**	—
7.1	6	6	8	4	**Ser84Leu, Asp88Tyr**	**Glu88Lys**	—
10.1, 10.2, 14.1	6	6	16	16	**Ser84Leu, Asp88Tyr**	**Glu88Lys**	—
8.1	6	6	16	16	**Ser84Leu, Asp88Asn**	**Gly82Asp**	—
19.1	6	6	16	16	**Ser84Leu, Asp88Asn**	**Ser84Arg**	—
16.1	6	6	16	8	**Ser84Tyr, Asp88Gly**	**Ser84Ile**	Asp420Asn
18.1	6	6	16	16	**Ser84Tyr, Asp88Gly**	**Ser84Ile**	Asp420Asn
24.1	6	6	16	16	**Ser84Leu, Asp88Gly**	**Ser84Ile**	Asp420Asn
20.1, 20.2, 22.1	6	6	16	16	**Ser84Leu, Asp88Asn**	**Ser84Ile**	Asp420Asn
4.1	6	6	16	16	**Ser84Leu, Asp88Tyr**	**Gly82Cys, Glu88Lys**	—
9.1	6	6	16	16	**Ser84Tyr, Asp88Tyr**	**Ser84Ile, Glu88Lys**	—

[a] Isolates are numbered accord to their origins of isolation as defined in Table 1.
[b] Changes at positions classically involved in resistance are shown in bold. Additional amino acid changes, found also in susceptible isolates, were ParC S133A (six isolates), ParC N138S (eight isolates), ParE R368H and S458L (one isolate), and GyrB A400V (three isolates). No amino acid changes have been detected in GyrB. —, no changes.

ence is also observed when using susceptibility microdilution breakpoints (CIP MICs of ≤1 μg/ml for CLSI and ≤0.5 μg/ml for EUCAST (20, 21). The use of current CLSI criteria underrecognizes a proportion of the low-level CIP-resistant strains with first-step mutations in *gyrA* and *parC* (22, 37, 38). In the present study, we identified five isolates with CIP MICs of 0.12 to 0.5 μg/ml and three strains with a MIC of 1 μg/ml that had at least one amino acid alteration in a position of the FQ targets involved in resistance. According to the current EUCAST resistance breakpoints for disk diffusion, 28 of the 30 isolates studied could be considered resistant. In order to detect nonsusceptible CIP strains with first-step mutations, the EUCAST guidelines have proposed screening with NAL as an indicator of resistance (22–24). In our study, five (0.23%) isolates collected from 2010 to 2013 with CIP inhibitory zone diameters of 21 to 28 mm were resistant to NAL and had changes in GyrA and/or ParC. Other authors have recommended general screening with NAL in order to identify these strains and to avoid therapeutic failures (22–24). Although FQs present good activity against *H. influenzae*, their use in respiratory tract infections merits special attention. To the best of our knowledge, two reports of FQ treatment failure have been published to date (15, 18). In 1999, Vila et al. reported a case of FQ resistance after treatment with ofloxacin in a patient with recurrent respiratory infections (15), and in 2003, Bastida et al. reported a case of LVX treatment failure in a patient with community-acquired pneumonia who had previously been treated with LVX and moxifloxacin (18).

Some specific mutations involved in FQ resistance in *H. influenzae* were originally described in 1996 (19). Subsequent studies have confirmed the mutations described by Georgiou et al. and described new mutations involved in resistance (15, 16, 18, 19, 22, 24, 29, 33, 37, 39). FQ resistance is acquired gradually with increasing numbers of mutations. Strains harboring one or two mutations in *gyrA* and *parC* have low-level resistance to FQs, while those with three or more mutations in *gyrA*, *parC*, and *parE* show high-level resistance (19, 40). In our study, strains with a single change in GyrA or one change in GyrA plus one in ParC had CIP MICs of 0.12 to 2 μg/ml, while those with three or four mutations (in GyrA, ParC, and ParE) had higher MICs (8 to 16 μg/ml). In the present study, the most common changes in GyrA were Ser84 to Leu or Tyr and Asp88 to Tyr, Asn, or Gly, which have been reported to contribute to resistance in *H. influenzae* (15, 16, 18, 19, 22, 29, 33, 37, 39). In ParC, the most common changes were Ser84Ile and Glu88Lys, which have been widely described in the literature (16, 18, 19, 22, 29, 33, 39), and Ser84Arg, a change also reported by other authors as an alteration involved in resistance (15, 16, 22, 24, 29, 33, 39). In addition, our study presents two strains harboring two new previously unidentified ParC changes, Ser84Asn and Ser84Ala. The strain carrying Ser84Asn had two additional changes in GyrA and had a CIP MIC of 8 μg/ml, suggesting its involvement in FQ resistance. In contrast, the strain carrying the ParC Ser84Ala change had a CIP MIC of 2 μg/ml, suggesting that this change would not be involved in resistance. Besides these changes at residues 84 and 88 of ParC, two strains had previously described changes in Gly82 (to Asp or Cys) (16, 22, 29, 39) and an Asp83Gly change that was not linked to any increase in the CIP MIC. This change (Asp83Gly) was already described by Pérez-Vázquez et al., but its involvement in quinolone resistance has not yet been established (22). Only one change was detected in ParE, Asp420Asn, which has been previously reported

(11, 29, 33), although its role in resistance has not been proved by genetic transformation.

In spite of a clonal spread previously described in long-term care facilities (11, 12, 41), our study reports a high genetic diversity among FQ-resistant NTHi isolates. Although we found six small clusters, the majority of the strains were isolated from patients who had no relationship to each other. In a recent publication from Taiwan, regional clonal emergence was found in different areas of the country (35). None of the STs published in Taiwan were found in our study, suggesting that the evolution of FQ-resistant strains is regional (35).

A relevant finding in our study was the persistence of genotypically identical quinolone-resistant isolates in COPD patients. During the period studied, these three patients had more than one isolate that had the same PFGE pattern and ST. It is well known that COPD patients have several impairments in innate lung defenses, facilitating microorganism persistence (42). Groeneveld et al. found COPD patients persistently infected with the same *H. influenzae* strain for up to 23 months, and their antibiotic treatment was not effective in eradicating the strains (43). In addition, Sethi et al. reported that a quarter of the acute exacerbations of COPD were caused by a persistent strain when bacterial pathogens were present in sputum (44). Long persistence of NTHi was also described in patients with cystic fibrosis (32).

In conclusion, although FQs are commonly used to treat respiratory infections, the proportion of FQ-resistant NTHi isolates during the period studied remained low. Long persistence of FQ-resistant isolates was identified in three COPD patients. The NAL test is recommended to detect FQ-susceptible strains with first mutations in the QRDRs that may acquire full resistance under selective pressure with FQ therapy and cause treatment failure.

ACKNOWLEDGMENTS

This work was supported by grants from the Fondo de Investigaciones Sanitarias de la Seguridad Social (PI 0901904) and the Plan Nacional de I+D+I of Ministerio de Ciencia e Innovación (BIO2011-25343) and by CIBER de Enfermedades Respiratorias, CIBERES; (CB06/06/0037), run by the Instituto de Salud Carlos III, Madrid, Spain. C.P. was supported by FPU grant AP2010-3202 (Formación de Profesorado Universitario, Ministerio de Educación, Spain). S.M. was supported by Sara Borrell Postdoctoral contract CD10/00298 from the Instituto de Salud Carlos III, Madrid, Spain.

We have no conflict of interest to declare.

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Objective 2.5: To investigate the importance of *H. haemolyticus* among *Haemophilus* spp. isolates collected from different infections.

Paper 6: Identification of *Haemophilus haemolyticus* in clinical samples and characterization of their mechanisms of antimicrobial resistance.

Marti S, **Puig C**, de la Campa AG, Tubau F, Domenech A, Calatayud L, Garcia-Somoza D, Ayats J, Liñares J, Ardanuy C. Submitted to Journal Antimicrobial Chemotherapy.

Identification of *Haemophilus haemolyticus* in clinical samples and characterization of their mechanisms of antimicrobial resistance

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Running title: *Haemophilus haemolyticus* frequency and resistance.

Keywords: *H. haemolyticus*, resistance, QRDR, PBP3, epidemiology

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Synopsis

Objectives: The aims of the study were to establish the frequency of *Haemophilus haemolyticus* in clinical samples, to determine the antimicrobial resistance rate and to identify the mechanisms of resistance to β-lactams and quinolones.

Methods: Species differentiation was performed by matrix-assisted laser desorption and ionization with time-of-flight mass spectrometry and compared before and after the introduction of a database update. *H. haemolyticus* isolates were confirmed by polymerase chain reaction (PCR). Antimicrobial susceptibility was studied by microdilution. Ampicillin- and ciprofloxacin-resistant isolates were studied by PCR to identify β-lactamase type and mutations in the penicillin-binding protein 3 and quinolone resistance-determining region (QRDR).

Results: We obtained 1,633 clinical isolates of *Haemophilus* spp. between October 2012 and September 2014. Of these, 63 (4%) *H. influenzae* isolates had been misclassified and were reclassified as *H. haemolyticus*. The frequency of *H. haemolyticus* was low in respiratory samples and higher in genitourinary samples, with no differences to *H. influenzae*. We found among *H. haemolyticus* isolates low antimicrobial resistance rates, with 8.7% for ampicillin, 8.7% for cotrimoxazole, 7.2% for tetracycline and 4.3% for ciprofloxacin. Mutations in the *ftsI* gene were identified in isolates where ampicillin had minimum inhibitory concentrations (MICs) of 0.25-1 mg/L; mutations in the QRDR were identified in isolates with ciprofloxacin MICs ≥2 mg/L.

Conclusions: The frequency of *H. haemolyticus* was low, especially in respiratory samples where *H. influenzae* remained the main pathogen of this genus. Although

antimicrobial resistance rates were low, the emergence of *H. haemolyticus*-resistant isolates supports the need for epidemiological surveillance of these microorganisms.

Introduction

The genus *Haemophilus* can be phenotypically divided into two main groups according to the need for haem in the medium (growth factor X). The *H. influenzae* group contains the X-factor-dependent species *H. influenzae, H. aegyptius* and *H. haemolyticus*; in contrast, the *H. parainfluenzae* group contains the X-factor independent species *H. parainfluenzae, H. parahaemolyticus, H. paraphrohaemolyticus, H. pittmaniae* and *H. sputorum.*[1] Within the haem-dependent group, *H. influenzae* is the main pathogen associated with human infection, and the presence or absence of a polysaccharide capsule divides it into either capsulated or non-capsulated non-typeable *H. influenzae* (NTHi) respectively.[2]

The prevalence of *H. influenzae* serotype b (Hib) has been significantly reduced by the introduction of the Hib conjugate vaccine, making NTHi the predominant cause of invasive *H. influenzae* disease in adults.[3,4] In response to this increased prevalence of NTHi in recent years, many epidemiological studies have been carried out which have uncovered further difficulties in differentiating between NTHi and *H. haemolyticus*, two non-capsulated species from the haem-dependent group.[1,2,5] However, because standard microbiological methods have failed to separate these two species, differentiation has been by 16S ribosomal RNA (rRNA) and additional polymerase chain reaction (PCR)-based techniques to detect the lipooligosaccharide gene (*lgtC*), the conserved IgA protease gene (*iga*), the fuculose kinase gene (*fucK*), and the protein D gene (*hpd*).[5,6] Recently, modifications in the matrix-assisted laser desorption and ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) database have improved the reliability of MS for the identification of *H. haemolyticus* by rapid discrimination between both microorganisms.[7]

Accurate identification of these species is important because *H. haemolyticus* is considered a respiratory tract commensal that is rarely associated to infection, a fact that can prevent unnecessary antibiotic therapy.[5,7,8] However, recent studies show no agreement on the proportion of *H. haemolyticus* misclassified in respiratory samples, with data from the Centres for Disease Control and Prevention (CDC) even suggesting that *H. haemolyticus* should not be considered a strict non-pathogenic commensal.[1] For this reason, we designed a study to determine the frequency of misclassified *H. haemolyticus* isolates and their association with clinical infection. In addition, we evaluated their resistance patterns and mechanisms of resistance to the antimicrobial agents.

Materials and Methods

Study design and clinical data

This study was carried out at the Hospital Universitari de Bellvitge in Barcelona, a tertiary care centre for adults serving a population of approximately 600,000 people. A two-year laboratory-based study was conducted between October 2012 and September 2014 to determine the frequency of *H. haemolyticus* in clinical samples previously identified as *H. influenzae* by conventional microbiological techniques and MS. We included 1,633 *Haemophilus* isolates obtained from all clinical samples including those from respiratory disease, invasive infections, genitourinary infections and abscesses. In addition, we retrospectively searched for *H. haemolyticus* isolates in two collections: a) *Haemophilus* spp. from vaginal swabs isolated between 2010 and 2012 and b) *Haemophilus* spp. from invasive disease isolated between 2008 and 2012. The search periods were determined for each clinical group according to the initial date for routine

cryopreservation of *Haemophilus* spp. by the Microbiology laboratory. Informed consent was not required as this was part of the normal microbiological routine. However, patient confidentiality was protected throughout.

Differentiation between *Haemophilus* spp

Conventional methodology

All *Haemophilus* spp. isolated in our laboratory were initially identified by conventional methodology as described in the Manual of Clinical Microbiology.[9] Differentiation between the *H. influenzae* group and the *H. parainfluenzae* groups was by their growth requirement for haemin (factor X) and nicotinamide adenine dinucleotide (NAD, factor V). No differentiation between *H. influenzae* and *H. haemolyticus* was performed on isolates identified during the normal microbiological routine.

Mass spectrometry

All *Haemophilus* spp. isolated from October 2012 to September 2014 were further identified by MS using a MALDI Biotyper (Bruker Daltonics GmbH, Bremen, Germany) as previously described.[10] Briefly, a single colony was placed on a polished steel MSP 96-target plate (Bruker Daltonics), overlaid with 1 µl of matrix solution (α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile-2.5% trifluoroacetic acid) and dried at room temperature. Identification was performed using software version 3.1 of the MALDI Biotyper (Bruker Daltonics), with species-level identification accepted as correct for scores ≥2.

In May 2014, the spectrum database was updated by Bruker to include spectrum data to differentiate between *H. haemolyticus* and *H. influenzae*. Consequently, all the spectra from previously identified *Haemophilus* spp. were retrieved and re-analysed using the

updated database. Bacteria classified as *H. haemolyticus* by MALDI-TOF MS were corroborated by PCR detection of the *fucK*, *iga* and *lgtC* genes, as previously described.[6] Isolates with a positive detection for all three genes were considered to be *H. influenzae*.

Molecular genotyping

Genomic DNA embedded in agarose plugs was digested with *Sma*I and the fragments separated by pulse field gel electrophoresis (PFGE) as previously described.[11] Band pattern analysis was with the Fingerprinting II software (version 3.0, BioRad) with 1% optimization and tolerance in the Dice coefficient setting; isolates assessed as having a ≥85% match were considered genetically related.

Biotyping, serotyping and antimicrobial susceptibility

All of the identified *H. haemolyticus* biotypes were determined using three biochemical reactions (urease, indol and ornithine decarboxylase) following the methodology already described for *H. influenzae*.[12] The capsular serotype was determined by PCR using primers and conditions previously described.[13] The minimal inhibitory concentration (MIC) was tested by microdilution using commercial panels (STRHAE2; Sensititre, West Sussex, England) and interpreted following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines.[14]

β-lactamase detection and penicillin-binding protein 3 sequencing

We screened β-lactamase activity using chromogenic cephalosporin discs (nitrocefin discs, BD, Madrid, Spain). The identification of the β-lactamase type was performed by PCR on all the β-lactamase-positive *H. haemolyticus* isolates using the primers and conditions previously described for *H. influenzae*.[15]

For molecular characterization of penicillin-binding protein 3 (PBP3), an internal region of the *ftsI* gene was amplified by PCR and sequenced using the primers previously described for *H. influenzae* and specific primers for *H. haemolyticus*. The primers and amplification conditions are described in Table 1. Sequences were compared to the internal region of the *ftsI* gene of *H. influenzae* Rd KW20 and *H. haemolyticus* ATCC33390.

Characterization of quinolone resistance

The quinolone resistance-determining regions (QRDRs) were amplified and sequenced with specific oligonucleotide pairs, as previously described for *H. influenzae* (Table 1). PCR fragments were then purified with a GeneJET PCR purification kit (Thermo Scientific) and sequenced on both strands with an Applied Biosystems 3730XL DNA analyser.

Results

Differentiation among *Haemophilus* spp

Between 2012 and 2014, we isolated 1,633 *Haemophilus* spp. from adult patients by conventional microbiology techniques and MALDI-TOF MS. Of these, *H. influenzae*, *H. parainfluenzae* and *H. parahaemolyticus* accounted for 1,323, 248 and 62 isolates respectively. No *H. haemolyticus* were initially identified with the MALDI-Biotyper software. After applying the new database, 63 of the *H. influenzae* isolates (4%) were reassigned as *H. haemolyticus*, and two additional isolates could not be identified at the species level, showing 50% identity with both *H. influenzae* and *H. haemolyticus*. Species identification was then validated in 60 isolates by the absence of PCR

amplification of the *fucK*, *iga* and *lgtC* genes, which confirmed the diagnosis of *H. haemolyticus* obtained by MALDI-TOF MS. The other three isolates were not identified as *H. haemolyticus* by PCR-based techniques. The distribution of the 1,633 *Haemophilus* spp. after analysis with the updated software was as follows: 77% were *H. influenzae*, 15% were *H. parainfluenzae*, 4% were *H. haemolyticus* and 4% were *H. parahaemolyticus*.

All 65 isolates identified by MALDI-TOF MS were used for the studies of antimicrobial resistance (i.e., 63 *H. haemolyticus*; 2 showing 50% identity with both *H. influenzae* and *H. haemolyticus*).

Distribution of the *Haemophilus* spp. in clinical samples

Most of the *Haemophilus* spp. were collected from respiratory samples (91%), with lesser amounts from genitourinary (5%), invasive (2%) and other (2%) infection sources (Fig. 1A). Within the respiratory isolates, the frequency of *H. haemolyticus* was low (4%) compared with *H. influenzae* (81%); in genitourinary isolates, the frequency was similar to that observed for *H. influenzae* (10.5% and 11.5%, respectively), with *H. parainfluenzae* (78.0%) being the main pathogen in those samples. Thus, all *Haemophilus* spp. isolated from vaginal swabs since 2010 were retrospectively analysed and we identified four additional *H. haemolyticus* isolates, corroborating the slightly higher frequency of this microorganism in vaginal swabs (Fig. 1B). We also retrospectively analysed invasive *Haemophilus* isolates but only one invasive *H. haemolyticus* strain (1.2%) was found in a pleural effusion sample from 2008.

Antimicrobial resistance

Antimicrobial resistance was only assessed in 62 of the 63 *H. haemolyticus* isolates identified by MALDI-TOF MS in the two-year study; unfortunately, one isolate could

not be recovered. In addition, we included the two isolates not identified to the species level (*H. influenzae/H. haemolyticus*) and the additional *H. haemolyticus* isolates identified in the retrospective studies: one invasive isolate and four isolates from vaginal swabs. Table 2 summarises the antimicrobial susceptibility of the 69 *H. haemolyticus* isolates that were included.

All isolates were susceptible to amoxicillin/clavulanic acid, cefuroxime, cefepime, cefotaxime, ceftriaxone, imipenem and meropenem, using the breakpoints established by EUCAST for *H. influenzae*. The antimicrobial resistance rates for *H. haemolyticus* were low, with 8.7% of the isolates resistant to ampicillin (due to the expression of a TEM-1 β-lactamase), 8.7% to cotrimoxazole, 7.2% to tetracycline and 4.3% to chloramphenicol. Although resistance to azithromycin was low (1.4%), 97.1% of the isolates were classified with intermediate resistance to macrolides according to the EUCAST criteria.[14]

Interestingly, we also identified three *H. haemolyticus* isolates with high-level resistance to ciprofloxacin (MIC = 1 mg/L) and one isolate with low-level resistance (MIC = 0.5 mg/L).

Amino acid substitutions in the QRDRs

The four *H. haemolyticus* isolates with MICs ≥0.5 mg/L had nonsynonymous polymorphisms leading to amino acid substitutions in the QRDRs (Table 3). The isolate with an MIC of 0.5 mg/L, although considered susceptible by the EUCAST criteria, had a nalidixic acid MIC ≥8 mg/L and presented a change associated with resistance: GyrA, (Ser-84→Leu). The three isolates resistant to ciprofloxacin (MIC = 1 mg/L) also had a nalidixic acid MIC ≥8 mg/L and presented two changes associated with resistance:

GyrA (Ser-84→Leu) and ParC (Ser-84→Ile). In addition, we found two polymorphisms not involved in resistance: ParC (Asn-138→Ser) and ParE (Asp-420→Asn).

Mutations in the *ftsI* gene

The sequence of the region encoding the transpeptidase domain of the PBP3 gene, *ftsI*, was determined from 35 isolates with an ampicillin MIC >0.125 mg/L. Because amplification with previously described primers failed in 12 isolates, we used ClustalW analysis of 53 *H. haemolyticus* strains to design new primers in the most conserved region of the *ftsI* gene (Table 1). Sequences were compared with those of the *ftsI* gene of *H. influenzae* strain Rd Kw20 and grouped according to a previous classification of β-lactam-resistant *H. influenzae* (by Dabernat).[16] The amino acid changes identified are summarised in Table 4.

Only nine of the 35 isolates (26%) fitted the Dabernat classification. Of these, all carried the Asn-526→Lys substitution: one was considered Group I (characterised by the Arg-517→His substitution), and eight were considered Group II. The Group II isolates were then further subdivided into three subgroups: i) three isolates were from subgroup IIa, with no additional substitution; ii) three isolates were from subgroup IIb, with the additional Ala-502→Val change; and iii) two isolates were from subgroup IIc, with the additional Ala-502→Thr substitution. The other isolates were classified into Group III-Hhae (14 isolates) and Group IV-Hhae (12 isolates) with mutations considered unrelated to resistance in *H. haemolyticus* because all the non-β-lactamase producing isolates remained susceptible to ampicillin. However, most of the isolates (74%) were classified into those two groups, which consequently contained more mutations. When these sequences were compared against *H. haemolyticus* ATCC 33390, the fragments of the *ftsI* gene were identical for the Group III-Hhae isolates (Table 5). In contrast, the *ftsI*

gene from the Group IV-Hhae isolates, which did not amplify with the primers for *H. influenzae*, was different from the *ftsI* genes in *H. influenzae* Rd Kw20 and *H. haemolyticus* ATCC 33390. These differences introduced the possibility that these isolates could represent *H. intermedius*, a proposed subspecies that can only be differentiated from *H. haemolyticus* by their capacity to ferment mannose.[1] However, none of these 12 isolates from Group IV-Hhae could ferment mannose and we considered them true *H. haemolyticus*.

The five isolates with non-reliable identification to the species level were classified into different groups: one in Group I, two in Group IIa, one in Group IIc and one in Group III-Hhae.

Phenotypic and genotypic characterisation

Phenotypically, the most common biotype was III (63%), followed by biotypes II (19%), VII (13%), VIII (3%), and I (2%). The biotypes were determined with the established methodology for *H. influenzae*, because biotypes have not been defined for *H. haemolyticus* to date. Molecular typing by PFGE showed high variability among the isolates, with 57 different patterns identified (> 85% similarity). Only four small clusters were identified, three of them contained two isolates each, and one contained three isolates.

Discussion

The introduction of the Hib conjugate vaccine dramatically decreased the prevalence of clinical infection with *H. influenzae* type b, irrevocably changing the disease's epidemiology[1] and leaving a niche that has been filled by NTHi and, occasionally, the

capsulated *H. influenzae* serotype f.[1,3,4,17] With the increased prevalence of NTHi, the ability to differentiate it from *H. haemolyticus* has become particularly relevant for clinical microbiology, especially given that the latter tends to be considered rare and of little clinical relevance.[1]

Initial studies on species differentiation suggested a high prevalence of *H. haemolyticus* isolates in respiratory samples. In 2007, Murphy *et al* reported a high percentage of misclassified *H. haemolyticus* isolates in nasopharyngeal (27.3%) and sputum (39.5%) samples.[18] Conversely, other studies differentiating between colonization and clinical samples have suggested a moderate prevalence of 15%-20% in healthy colonized subjects and a low prevalence of 0.5%-2% in invasive clinical samples.[1] Discrepancies among studies could be due to the difficulties in differentiating *H. haemolyticus* from NTHi isolates, which should be considered when interpreting the literature on respiratory tract colonization and infection.[1] However, since the incorporation of new algorithms and improved databases, differentiation between both species may now be routinely achievable in the clinical laboratories by MALDI-TOF MS.[1,7]

Our clinical laboratory received updated databases in May 2014. Following their incorporation, we designed a two-year study to determine the frequency of *H. haemolyticus* in clinical samples. The previous *Haemophilus* spp. MS data were retrieved and compared against the new database to identify all cases of *H. haemolyticus* that had previously been misclassified. Although most isolates were identified to the species level by MALDI-TOF MS and PCR, two isolates (3%) could not be differentiated, and three additional isolates (4%) were identified as *H. haemolyticus* despite being positive for the *fucK*, *iga* and *lgtC* genes. These non-classified rates were consistent with data obtained using PCR-based techniques for discrimination.[19]

The clinical samples we studied were obtained from respiratory (91%), genitourinary (5%), invasive (2%) and other (2%) disease sources. To date, studies have focussed on respiratory tract samples for identifying the prevalence of *H. haemolyticus* as either a colonizer or infective agent.[8,18,20-23] In this study, we wanted to establish the frequency of *H. haemolyticus* in all clinically relevant samples sent to a clinical microbiology laboratory. Our results corroborate the previous reports of a low frequency of *H. haemolyticus* in respiratory samples (4%). However, we found that its frequency in genitourinary samples (16.5%) for the last five years was similar to that for *H. influenzae* (18%). The incidence of *H. influenzae* in genitourinary infections tends to have been associated with biotype IV (cryptic genospecies biotype IV), which although rare, has been recognized as a neonatal, maternal and urogenital pathogen.[1,24] For this reason, we determined whether the genitourinary-associated *H. haemolyticus* isolates belonged to biotype IV. However, the *H. haemolyticus* isolates from vaginal swabs only belonged to biotypes II (4/11, 36%), III (5/11, 46%) and VII (2/11, 18%). Despite the low number of samples and the inability to link *H. haemolyticus* with genitourinary infection, it is important to acknowledge the frequency of this microorganism in samples other than from the respiratory tract.

Despite being considered a colonizing agent rarely associated to infection, *H. haemolyticus* could act as a potential reservoir for determinants of antimicrobial resistance.[18,20] Therefore, we assessed both the level of antimicrobial resistance and the mechanisms associated with β-lactam and quinolone resistance. In our study, six of the 69 *H. haemolyticus* isolates (8.7%) were resistant to β-lactam antibiotics due to β-lactamase production, which is slightly lower than the 13.2% previously reported for this microorganism,[19] but similar to the resistance observed for respiratory and invasive *H. influenzae* disease in our institution.[4,11] The remaining 63 isolates were susceptible to

ampicillin despite mutations in the transpeptidase domain of *ftsI*, as reported by Witherden *et al*.[19] Following the Dabernat classification for *ftsI* mutations in *H. influenzae*,[16] only nine *H. haemolyticus* isolates (13%) could be classified into groups I and II. Consistent with this data, Witherden *et al* classified 31% of their *H. haemolyticus* isolates as Group II, with the remaining isolates consistent with *H. haemolyticus* ATCC 33390.[19] We also identified 14 isolates with *ftsI* sequences identical to that strain; but, in addition, we identified 12 isolates with sequences that were different from both *H. haemolyticus* ATCC 33390 and *H. influenzae* Rd Kw20 strains. This group corresponded to the isolates that could not be amplified with previously described primers for the *ftsI* gene and suggested that Group IV-Hhae could include isolates from the *H. intermedius* subspecies. However, these isolates were confirmed as *H. haemolyticus* because they could not ferment mannose. Although most of the mutations within this group did not confer resistance to β-lactams for *H. haemolyticus*, further studies are necessary to determine their effect in *H. influenzae*.

Fluoroquinolones are the second-line antimicrobials for treating NTHi infections in adults. To date, no cases of ciprofloxacin-resistant *H. haemolyticus* have been described. In an epidemiological study to identify the prevalence of *H. haemolyticus* in patients with acute pharyngotonsillitis, Hotomi *et al* reported levofloxacin MIC values ranging from <0.06 to 2 mg/L, but provided no additional information about the isolates.[20] We identified three isolates that were resistant to ciprofloxacin and levofloxacin, with alterations in GyrA (Ser-84→Leu) and ParC (Ser-84→Ile) that have already been described in *H. influenzae* as responsible for the increase in quinolone resistance.[25-28] In addition, we identified one isolate with low-level resistance to ciprofloxacin but with a nalidixic acid MIC ≥8 mg/L. This isolate presented the Ser-

84→Leu mutation in GyrA and could become resistant with the addition of a second mutation in ParC.

The frequency of clinical reporting of *H. haemolyticus* could increase with improved differentiation by techniques such as MALDI-TOF MS. Previously, microbiological laboratories have not sought this microorganism because the differentiation between *H. haemolyticus* and *H. influenzae* was time consuming and unsuitable for routine use. Therefore, differentiation was restricted to clinical research, where authors highlighted the importance of correct identification to avoid unnecessary treatment,[7,8,18] and of the need to monitor resistance patterns given the potential for other *Haemophilus* spp. to act as reservoirs for resistance determinants in *H. influenzae*.[18-20,29]

Conclusion

We identified a low number of *H. haemolyticus* in respiratory samples and invasive disease, but found that its frequency in genitourinary samples was similar to that of NTHi. We also identified isolates that were resistant to β-lactams and quinolones caused by β-lactamase production and modifications in PBP3 and the QRDR.

Acknowledgements

We wish to thank all the staff of the Microbiology Laboratory of Hospital Universitari de Bellvitge who contributed to this project on a daily basis.

Funding

This work was supported by grant from Plan Nacional de I+D+I of Ministerio de Ciencia e Innovación (BIO2011-25343) and CIBER de Enfermedades Respiratorias (CIBERES - CB06/06/0037), run by the Instituto de Salud Carlos III (ISCIII, Madrid, Spain).

SM was supported by “Sara Borrell Postdoctoral contract CD10/00298” from the ISCIII. CP was supported by a grant from FPU (Formación de Profesorado Universitario, Ministerio de Educación, Spain).

Transparency declarations

None to declare

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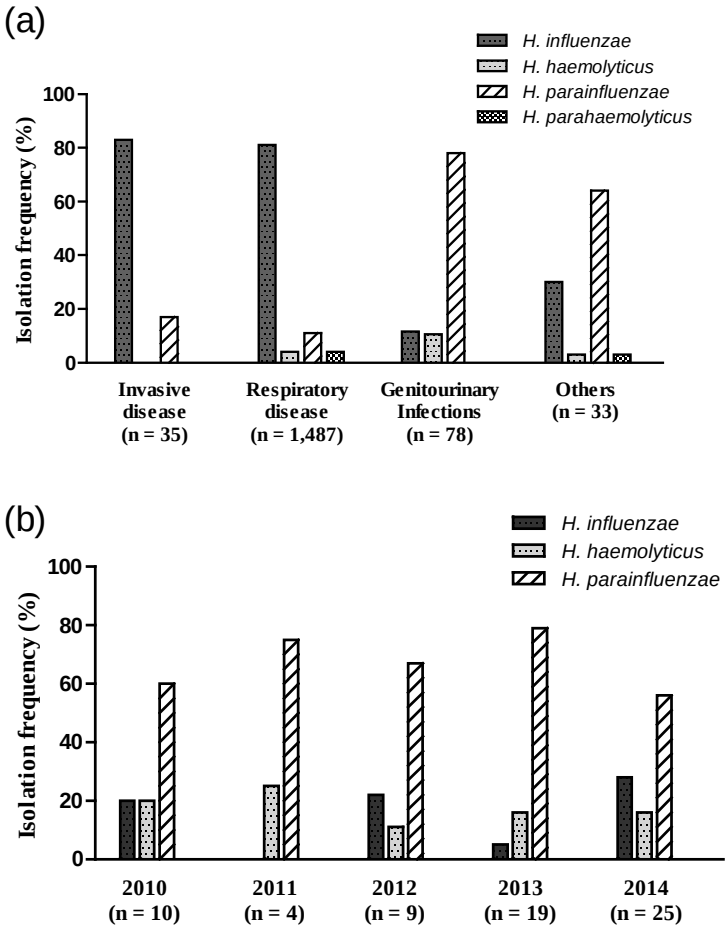
461 **Figure**



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464 **Figure 1: Clinical origin of all the *Haemophilus* isolates identified in this study by**
465 **MALDI-TOF MS.** (a) Distribution of *Haemophilus* spp. in clinical samples from
466 October 2012 to September 2014; (b) Distribution of *Haemophilus* spp. identified in
467 vaginal swabs since 2010.

468

469

Table 1: Primers used for the amplification of the *fts*I gene and the QRDRs of *gyrA*, *parC* and *parE* genes

Primer name	Sequence (5' – 3')	Gene	Position in gene	Fragment size (bp)[a]	Source
PBP3S-F	GATACTACGTCCTTTAAATTAAG	*ftsI*	1048-1070	551	[19,30]
PBP3S-4	GCAGTAAATGCCACATACTTA	*ftsI*	1578-1598	551	[19,30]
F1	GTTAATGCGTAACCGTGCAATTACC	*ftsI*	936-960	705	[16]
F2	ACCACTAATGCATAACGAGGATC	*ftsI*	1618-1640	705	[16]
Hae_ftsI_Fw	AATGCTGAGTCTGGTACTGC	*ftsI*	826-845	902	This study
Hae_ftsI_Rv	ATTATGGGCTATGCTTTGCG	*ftsI*	1708-1727	902	This study
GyrAF	CCGCCGCGTACTATTCTC	*gyrA*	138-154	476	[26,31]
GyrAR	GTTAGTTGCCATCCCCACCGC	*gyrA*	525-476	476	[26,31]
ParCF	CCCGTTCAACGTCGTATTG	*parC*	130-148	418	[26,31]
ParCR	CTGTGGCCATCCCCACCGC	*parC*	529-547	418	[26,31]
ParEF	GAACGCTTATCATCACGCCA	*parE*	1003-1022	471	[27]
ParER	AGCATCCGCGAGAATACAGA	*parE*	1454-1473	471	[27]

[a] The size refers to the PCR amplification with the oligonucleotides indicated.

Table 2: Minimal Inhibitory Concentrations (MIC) of 13 antimicrobials against 69 *H. haemolyticus* isolates using the microdilution method according to EUCAST breakpoints

Antimicrobial	MIC_{50} (mg/L)	MIC_{90} (mg/L)	Range (mg/L)	% S	% I	% R
Ampicillin	<0.12	1	<0.12 – >4	91.3		8.7
Amoxicillin/clavulanic acid	<0.5/0,25	1/0.5	<0.5/0.25 – 2/1	100.0		
Cefuroxime	<0.5	1	<0.5 – 2	97.1	2.9	
Cefepime	<0.25	<0.25	<0.25	100.0		
Cefotaxime	<0.06	<0.06	<0.06	100.0		
Ceftriaxone	<0.12	<0.12	<0.12	100.0		
Imipenem	<0.12	0.25	<0.12 – 0.5	100.0		
Meropenem	<0.25	<0.25	<0.25	100.0		
Chloramphenicol	<1	<1	<1 – >8	95.7		4.3
Tetraycline	<1	<1	<1 – >4	92.8		7.2
Ciprofloxacin	<0.03	<0.03	<0.03 – 1	95.7		4.3
Cotrimoxazole	<0.5/9.5	<0.5/9.5	<0.5/9.5 – >2/38	91.3		8.7
Azithromycin	1	2	<0.12 – >4	1.4	97.1	1.4

Table 3: MIC of ciprofloxacin (CIP), levofloxacin (LVX) and nalidixic acid (NAL) with the QRDR mutations for four isolates with reduced susceptibility to ciprofloxacin

	MIC (mg/L)			Mutation(s) in QRDR[a]		
Isolate	NAL	CIP	LVX	GyrA	ParC	ParE
9259	≥8	0,5	<0,5	S84L	N138S	D420N
8306	≥8	1	1	S84L	S84I, N138S	None
8424	≥8	1	1	S84L	S84I, N138S	None
10051	≥8	1	1	S84L	S84I, N138S	None

[a] Changes at positions classically involved in resistance in *H. influenzae* are underlined.

Table 4: Amino acid substitutions in PBP3 among 35 *H. haemolyticus* isolates with an ampicillin MIC >0.125 mg/L against *H. influenzae* strain Rd Kw20

	Amino Acid substitutions[a]																								MIC (mg/L)					
Group	Phe 332	Lys 344	Ile 348	Asp 350	Thr 352	Ser 353	Lys 355	Leu 356	Met 377	Ser 406	Pro 408	Val 418	Ala 437	Val 461	Gly 490	Ala 502	Arg 517	Ile 519	Asn 526	Val 547	Asp 551	Asn 569	No. Isolates	AMP	AMC	BL	Biotype	Origin
I																Val	**His**					Ser	1	0,5	1/0,5	No	II	Resp
IIa		Arg		Asn	Gly		Thr	Val	Ile						Glu				**Lys**	Ile	Asn	Ser	1	1	1/0,5	No	VII	Resp
		Arg		Asn	Gly		Thr	Val	Ile						Glu				**Lys**	Ile		Ser	1	1	2/1	No	II	Resp
																			Lys		Ala		1	1	2/1	No	III	Resp
IIb		Arg		Asn	Gly		Thr	Val	Ile						Glu	**Val**			**Lys**	Ile			1	0,5	1/0,5	No	VII	Resp
		Arg		Asn	Gly		Thr	Val	Ile						Glu	**Val**			**Lys**	Ile		Ser	1	0,5	1/0,5	No	VII	Resp
		Arg		Asn	Gly		Thr	Val	Ile						Glu	**Val**			**Lys**	Ile		Ser	1	1	2/1	No	II	GenUr
IIc				Asn												**Thr**			**Lys**	Ile		Ser	1	0,5	1/0,5	No	III	Resp
				Asn												**Thr**			**Lys**	Ile		Ser	1	0,25	<0,5/0,25	No	VII	Resp
III-Hhae		Arg		Asn	Gly		Thr	Val	Ile											Ile		Ser	11	0,25-0,5	<0,5/0,25	No	I; II; III; VII	Resp; GenUr
		Arg		Asn	Gly		Thr	Val	Ile											Ile		Ser	2	>4	<0,5/0,25	Yes	III	GenUr; Pleural
		Arg		Asn	Gly	Ala	Thr	Val	Ile											Ile		Ser	1	0,25	<0,5/0,25	No	II	Resp
IV-Hhae	Leu	Arg	Val	Asn	Gly		Thr	Val	Ile	Gly	Ser	Ala	Ser	Ile				Leu		Ile		Ser	8	0,25-0,5	<0,5/0,25	No	III	Resp
	Leu	Arg	Val	Asn	Gly		Thr	Val	Ile	Gly	Ser	Ala	Ser	Ile				Leu		Ile		Ser	3	>4	<0,5/0,25	Yes	II; III	Resp
	Leu	Arg	Val	Asn	Gly	Ala	Thr	Val	Ile	Gly	Ser	Ala	Ser	Ile				Leu		Ile		Ser	1	0,25	<0,5/0,25	No	III	Abscess

AMP: ampicillin; AMC: amoxicillin-clavulanic acid; BL: β-lactamase; Resp: respiratory; GenUr: genitourinary; Pleural: pleural fluid.

[a]Amino acids in bold represent the modifications involved in resistance described by Dabernat *et al* [16].

Table 5: Amino acid substitutions in PBP3 among 35 *H. haemolyticus* isolates with and ampicillin MIC >0.125 mg/L against *H. haemolyticus* strain ATCC 33390

Group (No. isolates)	Amino Acid substitutions																					
	Phe 332	**Arg 344**	Ile 348	**Asn 350**	**Gly 352**	Ser 353	**Thr 355**	**Val 356**	**Ile 377**	Ser 406	Pro 408	Val 418	Ala 437	Val 461	Gly 490	Ala 502	Arg 517	Ile 519	Asn 526	**Ile 547**	Asp 551	Ser 569
Group I (1)	·	Lys	·	Asp	Thr	·	Lys	Leu	Met	·	·	·	·	·	·	Val	His	·	·	·	·	·
Group IIa (3)	·	·	·	·	·	·	·	·	·	·	·	·	·	·	Glu	·	·	·	Lys	·	Asn	·
	·	·	·	·	·	·	·	·	·	·	·	·	·	·	Glu	·	·	·	Lys	·	·	·
	·	Lys	·	Asp	Thr	·	Lys	Leu	Met	·	·	·	·	·	·	·	·	·	Lys	Val	Ala	Asn
Group IIb (3)	·	·	·	·	·	·	·	·	·	·	·	·	·	·	Glu	Val	·	·	Lys	·	·	Asn
	·	·	·	·	·	·	·	·	·	·	·	·	·	·	Glu	Val	·	·	Lys	·	·	·
Group IIc (2)	·	Lys	·	·	Thr	·	Lys	Leu	Met	·	·	·	·	·	·	Thr	·	·	Lys	·	·	·
Group III-Hhae (14)	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
	·	·	·	·	·	Ala	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Group IV-Hhae (12)	Leu	·	Val	·	·	·	·	·	·	Gly	Ser	Ala	Ser	Ile	·	·	·	Leu	·	·	·	·
	Leu	·	Val	·	·	Ala	·	·	·	Gly	Ser	Ala	Ser	Ile	·	·	·	Leu	·	·	·	·

[a]Amino acids in bold represent differences between *H. influenzae* and *H. haemolyticus* type strains.

C) ADHESION AND BIOFILM FORMATION

Objective 3: To analyze various factors involved in biofilm formation by *H. influenzae* as a cause of persistence and invasion.

Paper 7: Increased biofilm formation by nontypeable *Haemophilus influenzae* isolates from patients with invasive disease or otitis media *versus* strains recovered from cases of respiratory infections.

Paper 8: Incorporation of phosphorylcholine into the lipooligosaccharide of nontypeable *Haemophilus influenzae* does not correlate with the level of biofilm formation *in vitro*.

Objective 3.1: To analyze the role of biofilm formation by NTHi in carriage and diseases caused by this microorganism.

Paper 7: Increased biofilm formation by nontypeable *Haemophilus influenzae* isolates from patients with invasive disease or otitis media *versus* strains recovered from cases of respiratory infections.

Puig C, Domenech A, Garmendia J, Langereis JD, Mayer P, Calatayud L, Ardanuy C, Liñares J, Marti S. Applied Environmental Microbiology. 2014 80(22):7088-95.

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Increased Biofilm Formation by Nontypeable *Haemophilus influenzae* Isolates from Patients with Invasive Disease or Otitis Media versus Strains Recovered from Cases of Respiratory Infections

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Biofilm formation by nontypeable (NT) *Haemophilus influenzae* remains a controversial topic. Nevertheless, biofilm-like structures have been observed in the middle-ear mucosa of experimental chinchilla models of otitis media (OM). To date, there have been no studies of biofilm formation in large collections of clinical isolates. This study aimed to investigate the initial adhesion to a solid surface and biofilm formation by NT *H. influenzae* by comparing isolates from healthy carriers, those with noninvasive respiratory disease, and those with invasive respiratory disease. We used 352 isolates from patients with nonbacteremic community-acquired pneumonia (NB-CAP), chronic obstructive pulmonary disease (COPD), OM, and invasive disease and a group of healthy colonized children. We then determined the speed of initial adhesion to a solid surface by the BioFilm ring test and quantified biofilm formation by crystal violet staining. Isolates from different clinical sources displayed high levels of biofilm formation on a static solid support after growth for 24 h. We observed clear differences in initial attachment and biofilm formation depending on the pathology associated with NT *H. influenzae* isolation, with significantly increased biofilm formation for NT *H. influenzae* isolates collected from patients with invasive disease and OM compared with NT *H. influenzae* isolates from patients with NB-CAP or COPD and healthy colonized subjects. In all cases, biofilm structures were detached by proteinase K treatment, suggesting an important role for proteins in the initial adhesion and static biofilm formation measured by crystal violet staining.

Nontypeable (NT) *Haemophilus influenzae* is an opportunistic pathogen which is highly adapted to colonize the human upper respiratory tract and which can subsequently progress to cause mucosal infections in children and adults (1–3). This Gram-negative unencapsulated microorganism is responsible for causing upper respiratory tract infections (otitis media, sinusitis, and conjunctivitis), community-acquired pneumonia (CAP), and acute exacerbations of lower respiratory tract infections in adults with chronic obstructive pulmonary disease (COPD) or cystic fibrosis (CF) and is increasingly present in invasive disease (1, 2, 4).

The pathogenesis of many human infections, including chronic and recurrent respiratory infections, has been associated with biofilm communities; these biofilms represent a protective mechanism that enhances bacterial resistance to clearance (5–7). This mechanism has been observed in CF-related pulmonary infections, mainly involving *Pseudomonas aeruginosa* (8–10). However, recent data have also revealed the presence of NT *H. influenzae* in biofilm communities in the lower and upper airways, and physical evidence has been shown in experimental models of otitis media (OM) with the detection of biofilm-like structures in the middle-ear mucosa of chinchillas (11, 12). Moreover, these biofilms could be important in early lung injury and could facilitate colonization and infection by *P. aeruginosa* (1, 7). Despite these observations, biofilm formation by NT *H. influenzae* remains a controversial topic, because NT *H. influenzae* lacks a specific polysaccharide associated with the extracellular matrix (13).

To date, biofilm formation by NT *H. influenzae* has been studied in only a limited number of strains, and a repertoire of genes and bacterial surface structures have been implicated in biofilm formation and maturation. These include type IV pili (PilA) overexpression (14), the presence of fimbriae (13), quorum sensing (15), the presence of outer membrane proteins (OMPs) P2 and P5 (16), and the presence of phosphorylcholine (PCho) and sialic acid in the lipooligosaccharide (LOS) molecule (17, 18). Despite the previously shown role of PCho in biofilm growth (19), a longitudinal study on NT *H. influenzae* isolates from patients with chronic respiratory disease found no clear correlation between biofilm growth and the presence of PCho in the LOS molecule (20). We previously showed the absence of a clear correlation between *in vitro* biofilm formation and the presence of PCho in the LOS of NT *H. influenzae* using a collection of 111 clinical isolates from different clinical sources (21). Despite the limitation imposed by the number of isolates, our previous study suggested that isolates from the middle ear fluid of children with OM formed

Received 31 July 2014 **Accepted** 3 September 2014
Published ahead of print 5 September 2014
Editor: H. L. Drake
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doi:10.1128/AEM.02544-14

denser biofilm structures than isolates from patients with either COPD or nonbacteremic community-acquired pneumonia (NB-CAP). Given that no association had previously been observed between the sample source and biofilm formation for NT *H. influenzae*, our study suggested the need for further investigation. Therefore, to provide a more comprehensive analysis of the differences in biofilm formation among different clinical sources, we significantly expanded our collection to 352 isolates and included NT *H. influenzae* isolates obtained from patients with invasive disease. This study assessed the initial bacterial adhesion to and biofilm formation on a solid surface by these isolates.

MATERIALS AND METHODS

Bacterial strains and culture conditions. We analyzed 352 NT *H. influenzae* strains from five different patient groups. These included the following: (i) 92 isolates from sputum samples from patients with NB-CAP; (ii) 60 isolates from sputum samples from patients with COPD; (iii) 29 isolates from the middle ear fluid of children with OM; (iv) 54 isolates from the blood, cerebrospinal fluid, and pleural fluid of patients with invasive disease; and (v) 117 oropharyngeal isolates from healthy children in day care centers.

Isolates from NB-CAP (22), COPD (23), and invasive disease were obtained from the Hospital de Bellvitge, Barcelona, Spain. Informed consent was not required, as this process formed part of the normal microbiological routine; patient confidentiality was always protected.

OM isolates were obtained from the University Medical Center, St. Radboud, Nijmegen, The Netherlands (24), and approved by the Committee on Research Involving Human Subjects of the Radboud University Medical Centre, Nijmegen (CMO 2007/239, international trial registry number NCT00847756).

Isolates from healthy children were obtained in a point prevalence study conducted in day care centers and schools in Oviedo, Spain, and approved by the Ethics Committee of the Hospital Universitario Central in Asturias, Spain (25).

All NT *H. influenzae* isolates were identified according to standard microbiological procedures (26). Additionally, all isolates were identified by mass spectrometry (matrix-assisted laser desorption ionization [MALDI] Biotyper, version 3.0; Bruker) according to the manufacturer's instructions and preserved in frozen stocks at −80°C. *H. influenzae* and *Haemophilus haemolyticus* were differentiated by detection of the *lgtC*, *fucK*, and *iga* genes, as previously described (22). Capsular serotype was determined by PCR using primers and conditions previously described (27); only nontypeable strains were considered for this study. Isolates were cultivated on brain heart infusion (BHI; BD) supplemented with 10 μg/ml hemin (Sigma-Aldrich) and 10 μg/ml NAD (Merck) (sBHI). Growth was performed at 37°C in a 5% CO_2 atmosphere.

Molecular genotyping. Molecular typing was performed on bacterial suspensions by pulsed-field gel electrophoresis (PFGE) as previously described (22). Genomic DNA embedded in agarose plugs was digested with SmaI, and the fragments were separated using a CHEF-DRIII apparatus (Bio-Rad). The PFGE band patterns were analyzed using Fingerprinting II software 3.0 (Bio-Rad). Similarity of PFGE banding patterns was estimated with the Dice coefficient (1% optimization and tolerance), and isolates that were ≥85% similar were considered genetically related.

Biofilm formation. The static biofilm formation assay was performed on 96-well plates with crystal violet staining, as previously described (21). Before staining, the optical density at 600 nm (OD_{600}) was determined to assess bacterial growth. We obtained biofilm values by calculating the mean absorbance from at least three independent tests and comparing it with the absorbance of negative controls (sBHI). Isolates were defrosted and used without additional passages for each repetition. The cutoff for biofilm formation was three times the value of the negative control. Strong biofilm formation was defined as three times the value of this cutoff, and any value between was considered indicative of moderate biofilm formation.

Bacterial adhesion assay. The speed of initial bacterial adhesion was evaluated by the BioFilm ring test (Biofilm Control, St Beauzire, France). Biofilm and adhesion assays were performed in parallel to reduce differences in strain behavior. The adhesion assay was performed on modified 96-well polystyrene plates obtained from Biofilm Control, as described by Chavant et al. (28). Briefly, bacterial suspensions were mixed with magnetic beads, incubated for 2 or 4 h at 37°C, and placed on a magnetic block. Free beads migrated to the center of the well and formed a spot, while bead migration was blocked in the presence of adherent bacteria. We used the BioFilm Control software to obtain the biofilm index (BFI); values of >7 corresponded to a total lack of bacterial adherence, while values of <5 were associated with different degrees of bacterial adherence.

Classification regarding adhesion and biofilm. The BioFilm ring test method was used to determine the speed of initial bacterial adhesion to a surface, independently of bacterial biofilm formation after 24 h growth. This is because faster and slower adhesion could be associated with different bacterial adhesion mechanisms. Regarding the association between initial adhesion and biofilm formation, four groups can be identified and defined as follows: B^+Ad^+, biofilm formation at 24 h with a fast initial adhesion to the surface; B^+Ad^-, biofilm formation at 24 h with a slow initial adhesion to the surface; B^-Ad^+, no biofilm formation at 24 h with a fast initial adhesion to the surface; B^-Ad^-, No biofilm formation at 24 h with a slow initial adhesion to the surface.

Biofilm detachment assays. Biofilms were developed in 96-well plates for 24 h as described. Biofilms attached to the bottom of the plate were washed with water and separately treated with 10 mM sodium metaperiodate or with 100 μg/ml proteinase K, as previously described (29). After treatment for 2 h at 37°C, biofilms were washed and then stained with crystal violet. The detachment assay was performed on a selection of 150 strains (52% of the biofilm-forming isolates) distributed among clinical sources and with different intensities of biofilm formation.

The effect of proteinase K on bacterial viability was assessed on five randomly selected isolates. Bacterial cultures were treated with 100 μg/ml proteinase K at 37°C for 2 h. After serial dilutions were performed, treated and untreated cultures were plated to determine bacterial viability by calculating the number of CFU/ml.

Statistical analysis. Statistical analysis were performed using the GraphPad Prism 5 software. Differences were evaluated using the Fisher exact test or the chi-squared test with Yates' correction. A P value less than 0.05 was considered statistically significant. Means ± standard errors of the means of at least three independent replicates are depicted. One-way analysis of variance with the Newman-Keuls multiple-comparison *post hoc* test was used for statistical analysis.

RESULTS

Molecular typing associated with adhesion and biofilm formation. Molecular typing was performed on all the studied isolates, and their clonal relationship was compared among the independent clinical sources (Table 1). (i) Ninety-two NB-CAP isolates from 92 adult patients were separated into 48 genotypes. (ii) Sixty COPD isolates from 60 adult patients were separated into 57 genotypes. (iii) One hundred seventeen carrier isolates from 117 children were separated into 85 genotypes. (iv) Twenty-nine OM isolates from 29 children were separated into 27 genotypes. (v) Fifty-four invasive isolates from 54 adult patients were separated into 47 genotypes.

Genotypically identical isolates obtained from unrelated patients displayed phenotypic differences. Thus, the initial adhesion to the surface and biofilm formation patterns were not always maintained; in fact, only half of the genotypes from unrelated patients had the same initial adhesion and biofilm formation profiles.

TABLE 1 Genotype distribution within the five groups of NT *H. influenzae* isolates considered in this study

Source of NT *H. influenzae*[a]	No. of isolates	No. of genotypes		No. of clusters with:						
		Total	Unique	2 isolates	3 isolates	4 isolates	5 isolates	6 isolates	7 isolates	8 isolates
NB-CAP	92	48	27	10	7	1	1	0	1	1
COPD	60	57	54	3	0	0	0	0	0	0
Carriers	117	85	67	13	1	1	1	2	0	0
OM	29	27	25	2	0	0	0	0	0	0
Invasive disease	54	47	40	7	0	0	0	0	0	0

[a] OM, otitis media; NB-CAP, nonbacteremic community-acquired pneumonia; COPD, chronic obstructive pulmonary disease.

Relationship between NT *H. influenzae* adhesion and bacterial origin. The speed of initial adhesion to a solid surface was determined by the BioFilm ring test after 2 and 4 h static growth at 37°C, where NT *H. influenzae* strains that adhere were defined by a BFI less than 5 (Fig. 1), being inversely proportional to the number of bacteria adhered to the surface. The number of NT *H. influenzae* isolates that adhered after 2 h was low for all the bacterial groups tested (range, 3% to 14%). Isolates collected from patients with invasive disease and from pediatric patients with OM showed a significantly higher adhesion than isolates from carriers and those from patients with COPD and NB-CAP. OM isolates also showed a significantly higher adhesion rate than isolates from invasive disease (Fig. 1A). After 4 h growth, the percentage of adherent NT *H. influenzae* isolates was variable among the groups of isolates (NB-CAP, 35%; invasive disease, 52%; healthy carriers, 56%; COPD, 58%; OM, 83%). NB-CAP isolates showed significantly less adhesion than those from patients with invasive disease, COPD, and OM and from healthy children (Fig. 1B). In addition, following the trend observed after 2 h growth, the OM isolates showed a significantly higher adhesion than isolates from the other sources (NB-CAP and COPD strains, invasive strains, and isolates from healthy children).

Relationship between NT *H. influenzae* biofilm formation and bacterial origin. Most NT *H. influenzae* isolates from the five clinical sources showed a clear ability to form biofilms (i.e., the OD_{570} was more than three times that of the negative control) on a static solid support after 24 h growth (range, 67% to 100%); however, the percentage of strongly biofilm-forming isolates (the OD_{570} was more than three times the cutoff) was variable among the groups (range, 18% to 63%). As in the case of initial adhesion, differences in the intensity of the biofilm formed were observed between the five isolate groups, with no significant differences in the stationary-phase culture (Fig. 2A). Isolates collected from patients with invasive disease and OM formed denser biofilms, as measured by crystal violet staining, while isolates from NB-CAP patients exhibited a lower capacity for biofilm formation (Fig. 2B) than all the other groups studied. Although the lower biofilm formation observed for NB-CAP isolates was not statistically significant compared to that of isolates from COPD patients and healthy carriers, the number of isolates that did not form biofilms (i.e., that were biofilm negative [B^-], defined as having an OD_{570} less than three times that of the negative control) was significantly higher (Fig. 3). Conversely, isolates from patients with COPD and from healthy children showed similar levels of biofilm formation.

Correlation between NT *H. influenzae* adhesion and biofilm formation. The relationship between initial adhesion to a solid surface and biofilm formation varied between isolates. Four independent groups were identified based on the amount of biofilm formed and the speed of the initial adhesion to the surface (Fig. 3): B^+Ad^+, B^+Ad^-, B^-Ad^+, and B^-Ad^-.

Most OM isolates (>80%) showed a fast initial adhesion to the surface, which translated into strong biofilm formation after 24 h of growth, while invasive isolates presented a high level of biofilm formation independently of the speed of the initial adhesion to the surface (Fig. 3). NB-CAP isolates were mostly associated with slow adhesion, although some (>40%) were able to form biofilms after 24 h of growth. Isolates from COPD patients and healthy carriers had similar patterns of adhesion and biofilm formation. As shown

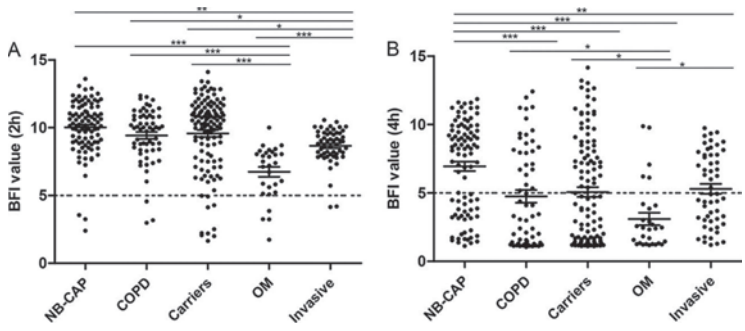

FIG 1 Initial adhesion to a solid surface determined by the BioFilm ring test after 2 h (A) and 4 h (B) of static growth at 37°C. The biofilm formation index (BFI) was adjusted by the test software and is inversely proportional to the number of adherent bacteria. Dotted lines represent the cutoff for adhesion (BFI = 5), with values of <5 representing high levels of adhesion to the surface. Means ± standard errors of the means for at least three independent replicates are presented. One-way analysis of variance with the Newman-Keuls multiple-comparison *post hoc* test was used for statistical analysis (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Abbreviations: OM, otitis media; NB-CAP, nonbacteremic community-acquired pneumonia; COPD, chronic obstructive pulmonary disease.

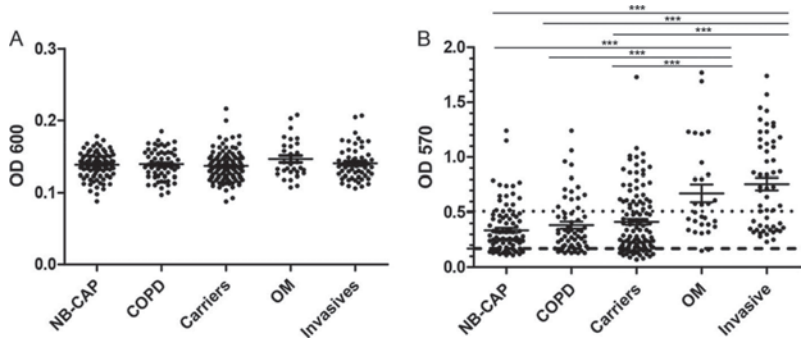

FIG 2 Stationary-phase cultures and biofilm formation determined for 352 NT *H. influenzae* isolates from patients with NB-CAP, COPD, OM, and invasive disease and from healthy carriers. (A) Stationary-phase cultures measured by optical density at 600 nm (OD_{600}); (B) biofilm formation measured by crystal violet light absorbance at 570 nm (OD_{570}). Means ± standard errors of the means of at least three independent replicates are presented. One-way analysis of variance with the Newman-Keuls multiple-comparison *post hoc* test was used for statistical analysis (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Abbreviations: OM, otitis media; NB-CAP, nonbacteremic community-acquired pneumonia; COPD, chronic obstructive pulmonary disease. The dashed line shows the OD_{570} that is more than three times that of the negative control; the dotted line shows the OD_{570} that is more than three times the biofilm breakpoint.

in Fig. 4, no significant relationship was observed between initial adhesion and biofilm formation for individual NT *H. influenzae* isolates in any of the five groups studied.

Biofilm detachment. To compare the nature of the biofilm structures formed by the clinical isolates, a biofilm detachment assay was performed on 150 strains from the five sources. We also considered the four categories established (B^+Ad^+, B^+Ad^-, B^-Ad^+, and B^-Ad^-) to determine differences in biofilm composition. The biofilm structures of all the studied isolates were sensitive to proteinase K and were highly resistant to sodium metaperiodate. These findings were independent of the amount of biofilm formed, the speed of adhesion to the solid surface, and the isolate origin, indicating that initial attachment and biofilm formation depends on the presence of proteins but not on sugar components in the extracellular matrix (Fig. 5). Proteinase K treatment did not affect viability.

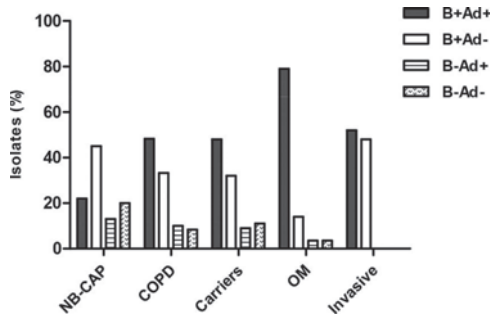

FIG 3 Distribution within the NT *H. influenzae* clinical sources of four independent groups regarding the amount of biofilm formed and the speed of initial adhesion to the surface. OM, otitis media; NB-CAP, nonbacteremic community-acquired pneumonia; COPD, chronic obstructive pulmonary disease. B^+Ad^+, biofilm formation with a fast initial adhesion to the surface; B^+Ad^-, biofilm formation with a slow initial adhesion to the surface; B^-Ad^+, no biofilm formation after 24 h with a fast initial adhesion to the surface; B^-Ad^-, no biofilm formation after 24 h with a slow initial adhesion to the surface.

DISCUSSION

Biofilm formation in chronic and recurrent infections is a persistence mechanism used by a wide range of microorganisms (10, 30, 31). Biofilm structures are common, both in nature and in clinical settings, and protect bacteria from bactericidal agents, bacteriophages, or host clearance mechanisms (30).

Despite the controversy over the inability to identify a specific polysaccharide link to the extracellular matrix, biofilm formation by NT *H. influenzae* has been widely described (11–13, 32). Additionally, there is evidence that bacterial adhesion to human epithelial cells in the respiratory epithelium leads to microcolony and biofilm formation (2). For this reason, a comprehensive analysis of the capacity of NT *H. influenzae* isolates from different clinical sources to form biofilms will contribute further insights into their involvement in bacterial infection.

Bacterial adhesion. Previous studies used crystal violet staining after 2 h growth to determine the initial adhesion to solid surfaces (33, 34). This staining is an established method for quantifying biofilm formation; however, in common with other approaches that involve washing the surface where bacteria adhere, this is a controversial technique for assessing initial adherence (33, 35). In fact, initial adhesion is a reversible process based on physicochemical interactions (36) and, for this reason, repeated washes can remove bacteria from the surface (33, 35, 37). Consequently, we used the BioFilm ring test, a system based on the immobilization of beads by adherent sessile bacteria (28) which allows quantification of initial attachment while avoiding the washing steps. Furthermore, it has been shown to be suitable for the study of adhesion with *Campylobacter* spp. (38).

To date, no studies have shown the initial surface adhesion of NT *H. influenzae*. After 4 h growth, NT *H. influenzae* isolates from OM patients presented higher adherence levels than the other isolates, while NB-CAP isolates showed the lowest level of adhesion. It has been stated that bacterial isolates from different niches can exhibit differences in adhesion patterns (34, 39). However, why NT *H. influenzae* OM isolates should adhere faster than the other isolates remains unresolved.

Biofilm formation. A biofilm starts to develop after bacteria

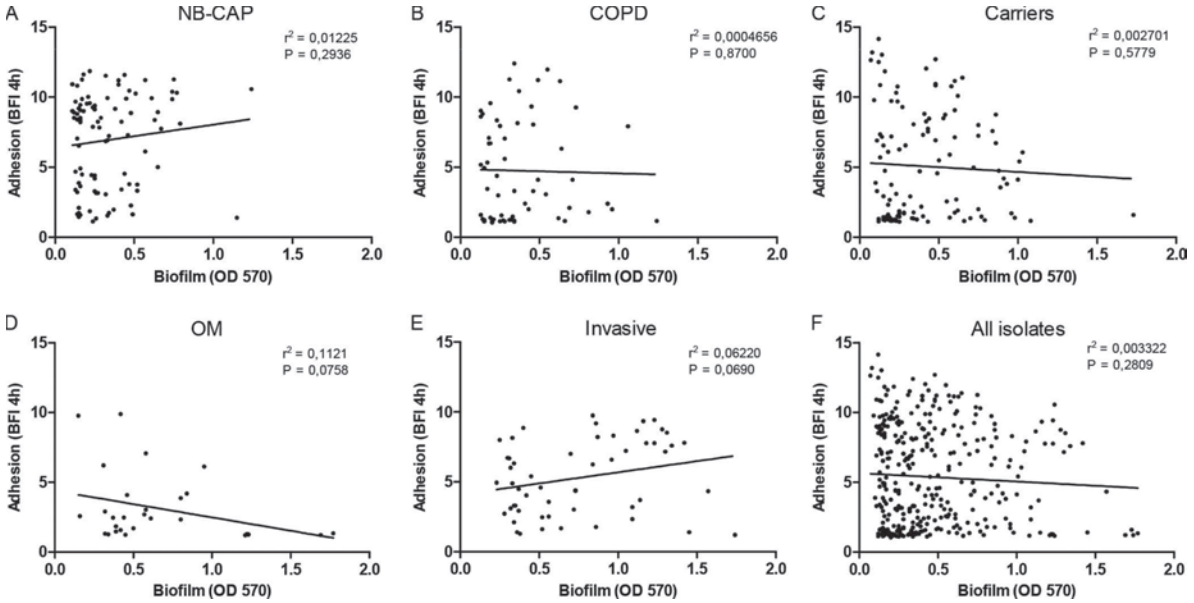

FIG 4 Correlation between initial adhesion to a solid surface by the BioFilm ring test after 4 h growth and levels of biofilm formation measured by crystal violet light absorbance at 570 nm (OD_{570}) in 352 NT *H. influenzae* isolates from different sources. (A) NB-CAP patients; (B) COPD patients; (C) healthy children; (D) children with OM; (E) patients with invasive disease; (F) all groups combined. Abbreviations: OM, otitis media; NB-CAP, nonbacteremic community-acquired pneumonia; COPD, chronic obstructive pulmonary disease.

have irreversibly attached to the surface (30, 36). After 24 h growth, the biofilm was more resistant to washing, and crystal violet staining was selected for quantification. Biofilm formation was significantly stronger for isolates from OM and invasive disease, supporting our previous findings on the increased ability of OM isolates to form biofilm (21). OM isolates were obtained from The Netherlands, and therefore we cannot completely exclude the possibility of differences due to geographic variation. However, studying 15 COPD and 15 OM isolates, Murphy and Kirkham found no relationship between biofilm formation and the clinical source of the sample (5). Their findings might have been related to the limited number of isolates included in the study.

Interestingly, invasive isolates displayed the highest level of biofilm formation. Previous studies on *Streptococcus pneumoniae* showed that biofilm-producing isolates had an enhanced ability to attach to host cells and a reduced ability to cause invasive disease (40). However, other authors have linked *in vitro* biofilm formation to spread through tissue barriers (41, 42) and the adhesion mediated by PilA with meningococcal sepsis (43), and studies on *H. influenzae* type b suggested that fimbrial structures contribute to bacterial spread into the circulation and secondary infection sites (42).

Biofilm detachment. Requirement of a sugar moiety or a protein-based interaction with the surface was determined by treatment with sodium metaperiodate (which cleaves sugar components) and proteinase K (for protein degradation) (29). Biofilm formation by all tested NT *H. influenzae* isolates was sensitive to proteinase K, suggesting that proteins play an important role in adhesion and biofilm formation. Izano et al. demonstrated rapid biofilm detachment in eight NT *H. influenzae* isolates after adding proteinase K and suggested that adhesins existed within the biofilm structure (44). Our study significantly expands this observation based on a large collection of NT *H. influenzae* isolates from different sources. Conversely, treatment with metaperiodate did not affect biofilm, adding to the controversy regarding the role of polysaccharides in NT *H. influenzae* biofilms (32).

Correlation between adhesion and biofilm formation. Bacterial isolates from different areas can exhibit differences in adhesion to solid surfaces (34, 39). We showed that, although adhesion is the first step in biofilm formation, there is no relationship between speed of initial adhesion and biofilm formation. However, given that the adhesion process is due to physicochemical interactions between cellular components and the solid surface (36, 45, 46), differences in the initial surface adhesion could reflect variability in adhesive proteins (since no role was found for extracellular polysaccharide). Further experiments to determine the proteins associated with each type of disease could bring further insights into the mechanisms used to cause infection.

Correlation between adhesion/biofilm and clinical infection. Acute infections are often caused by planktonic bacteria, while biofilm-producing bacteria are mostly associated with chronic infection and colonization (10, 30). Our results partially support this notion, because NB-CAP isolates exhibited slower adhesion and lower biofilm formation than isolates from either patients with chronic infections (COPD and OM) or healthy children. However, isolates from invasive disease showed the highest levels of biofilm formation. A previous study showed that *Acinetobacter baumannii* isolates from blood and from a single meningitis sample formed biofilm, while those isolated from respiratory tract infections were mostly unable to form biofilm (47). This difference could result from the fact that invasive isolates must cross tissue barriers before causing infection.

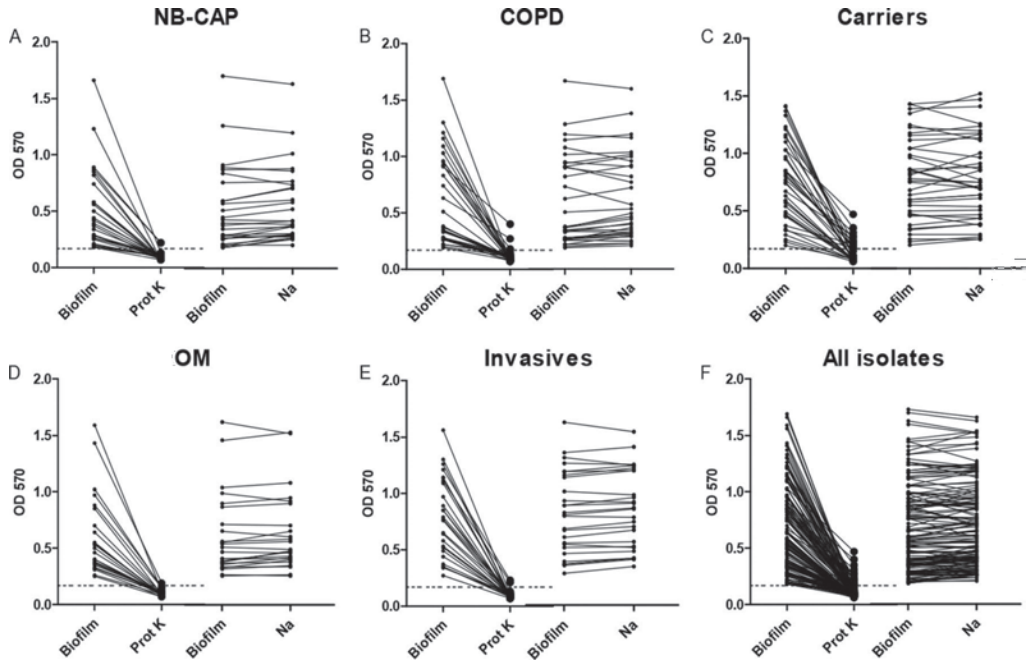

FIG 5 Effect of proteinase K and sodium metaperiodate treatment on 24-h biofilm structures measured by crystal violet light absorbance at 570 nm (OD_{570}) in 150 NT *H. influenzae* isolates from different sources. (A) NB-CAP patients; (B) COPD patients; (C) healthy children; (D) children with OM; (E) patients with invasive disease; (F) all groups combined.

NT *H. influenzae* binds to a variety of receptors in the respiratory tract (16). The OMPs P2 and P5, lipoproteins such as OapA, and proteinaceous adhesins have been attributed early roles in colonization (13, 48). Colonization by *H. influenzae* can result in epithelial damage and eventually reach the circulatory system (49), a process that could depend on the proteins expressed. Previous studies have shown that OMPs are likely to be expressed differently in colonizing bacteria than in invasive isolates (50). Therefore, studies on adhesion to eukaryotic cell lines and the identification of proteins involved in this adhesion would shed light on the differences among groups observed in this study.

Molecular typing associated with adhesion and biofilm formation. PFGE-based genotypically identical isolates from different episodes in the same patient maintained the adherence behavior and biofilm formation (data not shown), but this was not the case for the genotypically identical isolates from unrelated patients whose samples were used in this study. Thus, closely related isolates from different individuals may undergo modifications in the environment or within the host that can alter their ability to adhere and form biofilms. Host-pathogen interactions, including pathology, antimicrobial therapy, and inflammatory responses of different degrees, could be responsible for the variability in adhesion patterns. Bakker et al. suggested that isolates from different sources exhibit modifications in their adhesion patterns, not only because of the environment but because bacterial outer components have adapted through selective pressure over time (39). We acknowledge the limitation imposed by PFGE-based genotyping on bacteria obtained from single colonies and cannot exclude an association between the observed phenotypic differences and nonsynonymous polymorphisms in closely related isolates.

In conclusion, our results suggest differences in biofilm formation depending on the type of disease caused by NT *H. influenzae*. Specifically, there was a clear increase in biofilm-forming ability among isolates from OM and invasive disease. We also found that biofilm stability was dependent on protein interaction; this may represent a novel therapeutic target for disrupting established biofilms *in vivo*.

ACKNOWLEDGMENTS

This study was supported by grants from the Fondo de Investigaciones Sanitarias de la Seguridad Social (PI 0901904) and MINECO (SAF2012-31166) and by CIBER de Enfermedades Respiratorias, CIBERES (CB06/06/0037), run by the Instituto de Salud Carlos III (ISCIII), Madrid, Spain. The work with the BioFilm ring test was performed in the framework of a collaboration with Biofilm Control. C.P. was supported by FPU grant (Formación de Profesorado Universitario, Ministerio de Educación, Spain). S.M. was supported by Sara Borrell postdoctoral contract CD10/00298 from the Instituto de Salud Carlos III (ISCIII), Madrid, Spain.

We thank A. Fleites (Hospital Central, Oviedo, Spain) for providing the samples from healthy children. We also thank M. de Jonge and P. W. M. Hermans (Radboud University Medical Center, Nijmegen, The Netherlands) for providing the otitis media isolates.

We have no conflicts of interest to declare.

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Objective 3.2: To determine the role of PCho in biofilm formation in colonizing and clinical NTHi isolates.

Paper 8: Incorporation of phosphorylcholine into the lipooligosaccharide of nontypeable *Haemophilus influenzae* does not correlate with the level of biofilm formation *in vitro*.

Puig C, Marti S, Hermans PWM, de Jonge MI, Ardanuy C, Liñares J, Langereis JD. Infection & Immunity. 2014 82(4):1591-9.

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Incorporation of Phosphorylcholine into the Lipooligosaccharide of Nontypeable *Haemophilus influenzae* Does Not Correlate with the Level of Biofilm Formation *In Vitro*

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Nontypeable *Haemophilus influenzae* (NTHi) is an opportunistic pathogen that causes otitis media in children and community-acquired pneumonia or exacerbations of chronic obstructive pulmonary disease in adults. A large variety of studies suggest that biofilm formation by NTHi may be an important step in the pathogenesis of this bacterium. The objective of this report was to determine the relationship between the presence of phosphorylcholine in the lipooligosaccharide of NTHi and the level of biofilm formation. The study was performed on 111 NTHi clinical isolates collected from oropharyngeal samples of healthy children, middle ear fluid of children with otitis media, and sputum samples of patients with chronic obstructive pulmonary disease or community-acquired pneumonia. NTHi clinical isolates presented a large variation in the level of biofilm formation in a static assay and phosphorylcholine content. Isolates collected from the oropharynx and middle ear fluid of children tended to have more phosphorylcholine and made denser biofilms than isolates collected from sputum samples of patients with chronic obstructive pulmonary disease or community-acquired pneumonia. No correlation was observed between biofilm formation and the presence of phosphorylcholine in the lipooligosaccharide for either planktonic or biofilm growth. This lack of correlation was confirmed by abrogating phosphorylcholine incorporation into lipooligosaccharide through *licA* gene deletion, which had strain-specific effects on biofilm formation. Altogether, we present strong evidence to conclude that there is no correlation between biofilm formation in a static assay and the presence of phosphorylcholine in lipooligosaccharide in a large collection of clinical NTHi isolates collected from different groups of patients.

Haemophilus influenzae is a Gram-negative human-restricted pathogen that forms part of the normal nasopharyngeal microbiota (1). This species has been classified into two different groups depending on the absence or presence of the polysaccharide capsule (serotypes a to f). Serotype b, as the most invasive serotype, was responsible for invasive diseases in children before the introduction of the successful type b polysaccharide-protein conjugate vaccine in developed countries (2). The second group, commonly known as nontypeable *H. influenzae* (NTHi), is formed by strains lacking the capsular structure. NTHi usually colonizes the nasopharynx asymptomatically in healthy individuals; nevertheless, this opportunistic pathogen is a frequent cause of otitis media (OM), sinusitis, conjunctivitis, community-acquired pneumonia (CAP), and exacerbations of chronic obstructive pulmonary disease (COPD) (3–5).

Chronic infections have been widely associated with the presence of biofilm-forming bacteria (6). Biofilm is defined as a community of microorganisms held together in a polymeric matrix and attached to an inert or living surface (7). This biofilm structure confers protection against the host immune system but also increases antimicrobial resistance (8–10). Despite controversial views with respect to the presence of a specific polymeric matrix (11) or biofilm formation as a controlled survival mechanism (12), NTHi biofilms are suggested to be present during colonization, OM, and exacerbations of COPD (13–15).

Various bacterial factors have been shown to affect NTHi biofilm formation (16), including the presence of sialic acid (NeuAc) (17) and phosphorylcholine (PCho) incorporation into the lipooligosaccharide (LOS) (18). Hong and coworkers presented convincing data where they correlated the presence of PCho in the LOS of three variants of NTHi strain 2019 with biofilm maturation in a continuous flow system *in vitro* as well as in a chinchilla model of OM *in vivo* (19). In that study, a *licD* gene deletion mutant deficient for PCho showed decreased biofilm formation, whereas a phase-locked *licA* gene variant showed increased PCho incorporation and increased biofilm maturation compared to wild-type (WT) strain 2019. These results corroborate recent findings by Morey et al., who showed decreased biofilm formation for a *lic1* mutant of NTHi strain 375 (20). Furthermore, NTHi *licD* mutants of strains 2019 and 86-028NP showed decreased biofilm density and increased clearance in a chinchilla model for OM compared to WT strains (18, 19).

The ability of NTHi to form biofilms *in vitro* is highly strain specific (21–24), but the mechanism that determines whether a particular strain is able to form a biofilm is not known. Based on

Received 11 November 2013 Returned for modification 1 January 2014
Accepted 20 January 2014
Published ahead of print 22 January 2014
Editor: A. Camilli
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doi:10.1128/IAI.01445-13

TABLE 1 Strains and primers used in this study

Strain(s) or primer	Description or sequence[a]	Reference(s)
Strains		
01/1, 02/28, 03/03, 03/05p, 03/09, 03/30, 05/02, 06/1, 06/6, 10/03, 10/12, 11/06, 13/04, 16/11, 16/16, 18/02, 18/14, 19/01, 19/04, 19/11, 21/04, 23/03, 24/01, 26/04, 29/01, 31/4, 35/1, 35/42, 36/1	NTHi isolates from oropharynx of healthy children in day care centers	This study
MEF_C001, MEF_C004, MEF_C006, MEF_C008, MEF_C009, MEF_C013, MEF_C031, MEF_C049, MEF_C052, MEF_C062, MEF_C089-42k, MEF_C089-32j, MEF_C101, MEF_C109, MEF_C115, MEF_R006, MEF_R015, MEF_R020, MEF_R021-1, MEF_R021-2, MEF_R033, MEF_R035, MEF_R038, MEF_R047, MEF_R048	NTHi isolates from middle ear fluid of children with otitis media	29, 45
2-2, 8-3, 11-1, 11-2, 11-3, 12-7, 14-1, 14-5, 19-1, 27-1, 27-2, 33-1, 34-1, 34-2, 35-1, 35-2, 36-1, 36-2, 36-4, 41-2, 42-1, 44-1, 44-2, 44-3, 51-2, 51-3, 71-2	NTHi isolates from sputum samples collected from COPD patients	This study
747, 1298, 1399, 1509, 1532, 1608, 1657, 1755, 2027, 2034, 2082, 2195, 2213, 2215, 2217, 2340, 2377, 2400, 2488, 2580, 2619, 2720, 2739, 2810, 2885, 3272, 3437, 3495, 3499, 3673	NTHi isolates from sputum samples of patients with community-acquired pneumonia	This study
MEF_C008 Δ*licA*	C008 strain with the *licA* gene replaced by a Spec^r cassette	This study
14-1 Δ*licA*	14-1 strain with the *licA* gene replaced by a Spec^r cassette	This study
01/1 Δ*licA*	01/1 strain with the *licA* gene replaced by a Spec^r cassette	This study
16/16 Δ*licA*	16/16 strain with the *licA* gene replaced by a Spec^r cassette	This study
2215 Δ*licA*	2215 strain with the *licA* gene replaced by a Spec^r cassette	This study
Rd	Nonencapsulated type D strain	46
H446	Rd with *lic1D*::Km, constitutively PCho$^-$	38
H457	Rd with *lic1D* Eagan, PCho on HepIII	38
H491	Rd with *lic1A* Δ(CAAT)$_n$, constitutively PCho$^+$, PCho on HepI	38
Primers		
R2866_1107_L1	GCTTGGTTTACCGAACGAAA	This study
R2866_1107_L2	*CCACTAGTTCTAGAGCGGC*TGGAGGAAAAA GGAATGGAA	This study
R2866_1107_R1	AGGCCTGCTGCTAAAATGAT	This study
R2866_1107_R2	GCGTCAATTCGAGGGGTATC*AAAACGCTTA GACGCAGCAT*	This study
R2866_1107_C	TGGAGTTTGATTGATTGATTGA	This study
PBpR412_L	GCCGCTCTAGAACTAGTGG	47
PBpR412_R	GATACCCCTCGAATTGACGC	47

[a] Italic type indicates the overlapping regions of the flanking regions of the R2866 *licA* gene and the spectinomycin cassette.

those previous studies, our work aimed to investigate whether the presence of PCho was associated with the level of biofilm formation by clinical NTHi strains isolated from the oropharynx of healthy children, middle ear fluid of children with OM, and sputum of adult patients with COPD and CAP.

MATERIALS AND METHODS

Bacterial strains and culture conditions. One hundred eleven NTHi strains from different groups of patients were analyzed in this study: (i) 29 isolates from the oropharynx of healthy children in day care centers in Oviedo, Spain; (ii) 25 isolates from middle ear fluid of children with OM at the Radboud University Medical Centre, Nijmegen, The Netherlands; (iii) 27 isolates from sputum samples collected from COPD patients at the Hospital Universitari de Bellvitge, Barcelona, Spain; and (iv) 30 isolates from sputum samples of patients with CAP from the Hospital Universitari de Bellvitge, Barcelona, Spain. All the NTHi isolates were identified according to standard microbiological procedures (25). All strains used in this study are reported in Table 1. The strains were grown in brain heart infusion (BHI) medium (Becton, Dickinson) supplemented with 10 μg/ml hemin (Sigma-Aldrich) and 10 μg/ml NAD (Merck).

Static biofilm formation assay. Bacterial cultures grown overnight were diluted to a final optical density at 620 nm (OD_{620}) of 0.01 in 150 μl of fresh supplemented BHI (sBHI) broth in 96-well plates or 24-well plates with glass slides in triplicate and incubated at 37°C in 5% CO_2 for 24 h. Before biofilm staining, the OD_{620} was determined to assess bacterial growth. Culture broth was removed, the wells were rinsed three times with distilled water, and glass slides were transferred to new 24-well plates. Biofilm was stained with 150 μl of 0.5% crystal violet for 20 min at room temperature and washed three times with distilled water to eliminate traces of unbound dye. Finally, the crystal violet was dissolved with 150 μl of 90% ethanol, and the A_{560} was measured. The experiments were performed on three independent replicates.

Generation of NTHi-directed *licA* mutants. Deletion of the *licA* gene was performed by allelic exchange of the target gene with an antibiotic resistance marker, as described previously (26), with the primers (Biolegio) listed in Table 1. Flanking regions (~1,000 bp) of the R2866 *licA* gene and the spectinomycin cassette with overlapping regions (indicated in

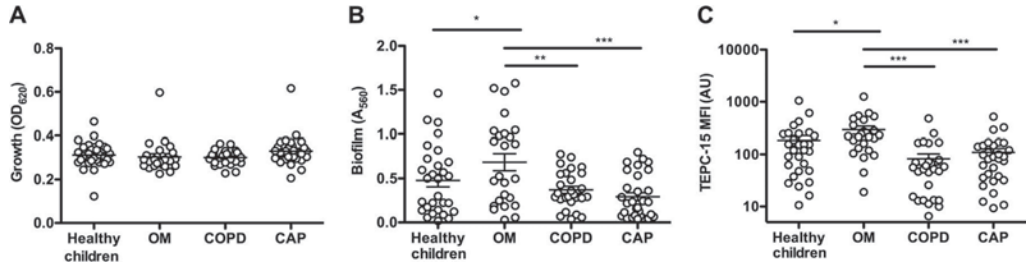

FIG 1 Growth, phosphorylcholine levels, and levels of biofilm formation for 111 clinical NTHi strains. Overnight growth measured by the optical density at 620 nm (OD_{620}) (A), the level of biofilm formation measured by crystal violet light absorbance at 560 nm (A_{560}) (B), and PCho content of planktonically grown bacteria measured by binding of TEPC-15 by flow cytometry shown as mean fluorescence intensity (MFI) in arbitrary units (AU) (C) of NTHi isolates from healthy children, children with otitis media (OM), adults with chronic obstructive pulmonary disease (COPD), and adults with community-acquired pneumonia (CAP) are shown. Means ± standard errors of the means of three independent replicates are depicted. One-way analysis of variance ($P = 0.2458$ [A], $P = 0.0005$ [B], and $P = 0.0002$ [C]) with the Newman-Keuls multiple-comparison *post hoc* test was used for statistical analysis (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). AU, arbitrary units.

italic type in Table 1) were PCR amplified and purified with the Qiagen PCR purification kit (Qiagen). Equimolar concentrations of the flanking regions and spectinomycin cassette were linked together in a second PCR. The megaprimer PCR product amplified from strain R2866 was used to transform M-IV competent NTHi as described previously (27). Strains C008, 14-1, 1/1, 16/16, and 2215 were selected because of their successful transformation.

LOS analysis by Tris-Tricine SDS-PAGE. LOS extraction was performed by the proteinase K-ethanol precipitation method as described previously (28). NTHi was grown to an OD_{620} of ~0.6, and 1 ml of culture was washed twice with phosphate-buffered saline (PBS). The bacterial pellet was lysed in 150 μl lysis buffer (60 mM Tris [pH 6.8], 10 mM EDTA, 2% SDS) and boiled for 5 min. Proteinase K (2.5 μg/ml) was added to the cooled samples and incubated for 16 to 24 h at 37°C. Samples were precipitated by adding 20 μl 3 M sodium acetate (pH 3.0) and 400 μl 100% ethanol, incubated for 1 h at −20°C, and centrifuged for 5 min at 15,000 × *g*. Pellets were washed twice with 500 μl 70% ethanol and suspended in 180 μl H_2O. LOS samples were separated on a Tris-Tricine SDS-PAGE gel with a Protean II xi cell electrophoresis system (Bio-Rad) and visualized by silver staining or transferred onto a polyvinylidene difluoride (PVDF) membrane for Western blotting with monoclonal antibody (MAb) TEPC-15 to detect PCho.

Silver staining. Tris-Tricine gel was fixed for 1 h (45% methanol, 10% acetic acid), washed 3 times for 20 min with 50% methanol, and incubated for 1 min in sensitizing solution (0.02% sodium thiosulfate). The gel was washed 3 times with distilled water and incubated for 20 min in impregnation solution (0.2% silver nitrate, 0.075% formaldehyde). After the gel was washed twice with distilled water, it was treated with develop solution (6% sodium carbonate, 0.02% sodium thiosulfate, 0.05% formaldehyde) until the bands were clearly visible. Development was stopped by incubation in 0.1% acetic acid followed by a 30-min wash with distilled water.

Western blot analysis. PVDF membranes were blocked with 5% bovine serum albumin (BSA) in PBS, incubated for 2 h with TEPC-15 (1:1,000), washed five times for 5 min with PBS plus 0.1% Tween, and subsequently incubated with rabbit anti-mouse Ig (1:5,000) in PBS. Membranes were washed 5 times for 5 min with PBS plus 0.1% Tween and detected with ECL Plus reagent (GE Healthcare).

Complement resistance. Experiments were conducted with pooled normal human serum (NHS) obtained from GTI Diagnostics (catalog number PHS-N100), as described previously (29). NTHi was grown in supplemented BHI medium to an OD_{620} of ~0.5, washed once with PBS, and diluted to an OD_{620} of 0.1 in PBS-Hanks' balanced salt solution (HBSS) without phenol red containing Ca^{2+}/Mg^{2+} (60% PBS–40% HBSS). Samples were finally diluted 10,000-fold in PBS-HBSS to obtain a concentration of ~20,000 CFU/ml. Fifty microliters of the bacterial culture was mixed with 50 μl 10% NHS or 10% heat-inactivated NHS (HI-NHS), diluted in PBS-HBSS, and incubated for 1 h at 37°C. Serial dilutions were plated onto sBHI plates and incubated overnight at 37°C in 5% CO_2. Survival was determined by dividing the CFU counts in 5% NHS with the CFU counts in HI-NHS after 1 h of incubation.

Flow cytometry analysis. NTHi was grown in supplemented BHI medium to an OD_{620} of ~0.5, or NTHi cells present in biofilms after 24 h were scraped from the plate and suspended vigorously by pipetting up and down. TEPC-15 and C-reactive protein (CRP) binding was detected by flow cytometry, as previously described (30). HBSS without phenol red and Ca^{2+}/Mg^{2+}, containing 5% (vol/vol) heat-inactivated fetal calf serum, was used for all dilutions and washes. Surface opsonization was performed by incubating 100 μl bacteria at an OD_{620} of ~0.5 (mid-log growth) with 10 μg/ml TEPC-15 (Sigma) or 5% NHS for 1 h at 37°C with 5% CO_2. Bacteria were fixed for 20 min in 2% paraformaldehyde at room temperature, and surface-bound TEPC-15 or CRP was detected by using 1:200-diluted anti-mouse IgA fluorescein isothiocyanate (FITC)-conjugated antibody (Sigma) or 1:100-diluted goat anti-human CRP (Sigma) and 1:500-diluted donkey anti-goat Alexa 488-conjugated antibody (Life Technology) by flow cytometry using a FACS LSR II instrument (BD Biosciences, San Jose, CA, USA) shown as mean fluorescence intensity (MFI) in arbitrary units (AU). Data were analyzed by using FlowJo version 7.6.3.

Statistical analysis. Statistical analyses were performed with GraphPad Prism version 4, where a *P* value of <0.05 was considered significant. The specific statistical tests that were used for the various experiments are specified in the figure legends.

RESULTS AND DISCUSSION

Phosphorylcholine content and level of biofilm formation of clinical NTHi isolates. This study included a total of 111 NTHi clinical isolates collected from patients with different diseases, including OM, CAP, and COPD, as well as strains isolated from healthy colonized children. Growth of the isolates was consistent and not statistically different between the groups (Fig. 1A). As shown in Fig. 1B, isolates recovered from middle ear fluid of patients with OM showed statistically significant increased levels of biofilm formation compared to isolates from healthy children. In contrast, isolates from sputum samples of CAP and COPD patients presented statistically significant decreased levels of biofilm formation compared to OM isolates. The level of biofilm formation of CAP and COPD isolates was lower than that of isolates

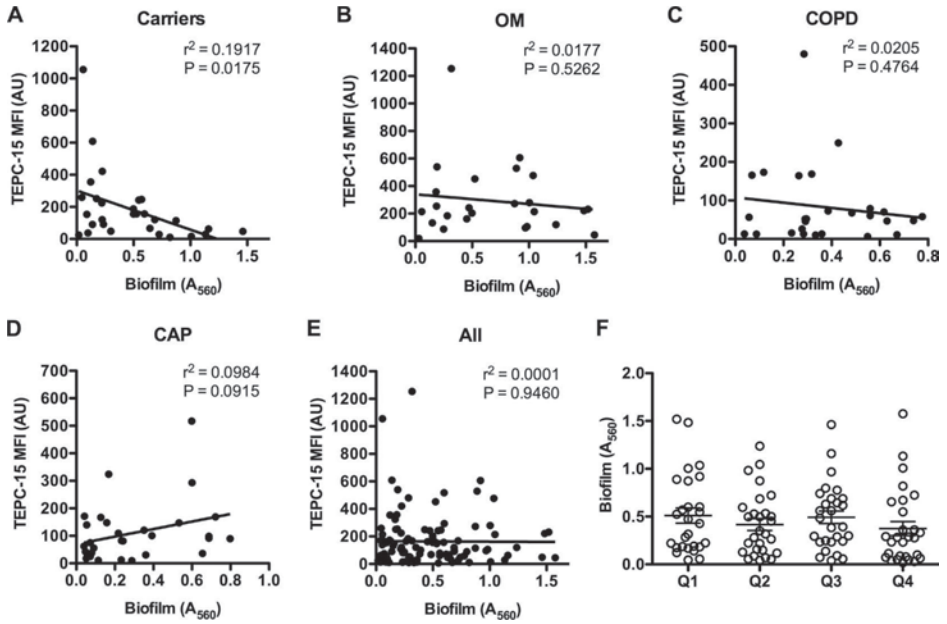

FIG 2 Correlation between phosphorylcholine levels and levels of biofilm formation for 111 clinical NTHi isolates. Shown is the correlation between the level of biofilm formation measured by the A_{560} and the PCho content of planktonically grown bacteria measured by flow cytometry shown as mean fluorescence intensity (MFI) in arbitrary units (AU). (A) NTHi strains from healthy children. (B) NTHi strains from patients with OM. (C) NTHi strains from patients with CAP. (D) NTHi strains from patients with COPD. (E) All NTHi strains combined. (F) Level of biofilm formation measured by crystal violet A_{560} of strains with high (Q1, TEPC-15 MFI of 1,254 to 221 AU), medium high (Q2, TEPC-15 MFI of 214 to 105 AU), medium low (Q3, TEPC-15 MFI of 104 to 46 AU), or low (Q4, TEPC-15 MFI of 45 to 7 AU) PCho levels. Means ± standard errors of the means of three independent replicates are depicted. One-way analysis of variance ($P = 0.4864$ [F]) with the Newman-Keuls multiple-comparison *post hoc* test was used for statistical analysis.

from healthy children, although this did not meet statistical significance.

Isolates collected from the middle ear fluid of patients showed a modest but significant increase in the level of biofilm formation. These results, to some extent, corroborate the results reported by Torretta and coworkers, who showed that biofilm-producing NTHi isolates were present in the nasopharynx of children with recurrent acute OM (22). More striking was the reduced level of biofilm formation by NTHi isolates collected from patients with CAP and COPD. Previously, Murphy and Kirkham showed high variability in the level of biofilm formation but overall showed no association between the source of the sample and biofilm formation (21). Our results imply that isolates from sputum samples from adult patients with COPD or CAP behave differently from isolates collected from the oropharynx and middle ear fluid of children. Whether this is dependent on the age of the patients, location of isolation, or type of inflammatory disease is thus far not known.

The presence of PCho on NTHi grown planktonically was measured by MAb TEPC-15 staining by flow cytometry. PCho is a molecule present on a large number of microorganisms (31). NTHi acquires choline from the environment, which is incorporated into its LOS in the form of PCho, which is regulated by the phase-variable *lic* operon (32). NTHi isolates showed variations in PCho integration into the LOS, being generally higher in strains isolated from healthy children and children with OM than in isolates obtained from COPD and CAP patients (Fig. 1C). This observation could be explained by PCho phase variation, as it has been shown that incorporation of PCho favors colonization and OM in an animal model and recently also in a human colonization model (33–35). A possible factor explaining decreased PCho levels in NTHi isolates collected from CAP and COPD patients is that PCho binds C-reactive protein (CRP), which initiates complement-mediated killing of NTHi (35). A detectable level of CRP was present in sputum samples of COPD patients (36), whereas it was not detected in 30 out of 31 middle ear fluid samples of patients with OM (37). Therefore, NTHi present in the lungs of COPD or CAP patients might decrease PCho incorporation into the LOS in response to increased levels of CRP, thereby preventing complement-mediated killing.

Phosphorylcholine content is not related to the level of biofilm formation of clinical NTHi isolates in a static assay. The evaluation of biofilm formation and PCho incorporation in the LOS of 111 NTHi isolates enabled us to test whether there was a positive relationship between these two conditions. Strains obtained from healthy children showed a very modest ($r^2 = 0.1917$) but significant ($P = 0.0175$) negative correlation (Fig. 2A), whereas no significant correlation between the presence of PCho and biofilm formation was observed for strains isolated from children with OM and patients with CAP and COPD (Fig. 2B to D). The combination of all strains ($n = 111$) also showed no significant correlation between the presence of PCho and the level of biofilm formation (Fig. 2E). In addition, when strains were grouped into four quarters based on PCho expression (quarter 1 [Q1], TEPC-15 mean fluorescence intensity [MFI] in arbitrary units [AU] of 1,254 to 221 AU; Q2, TEPC-15 MFI of 214 to 105

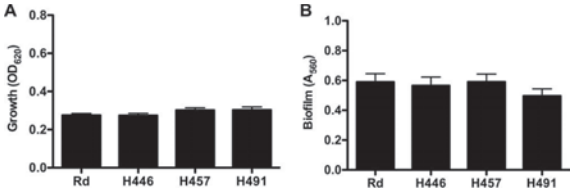

FIG 3 Incorporation of phosphorylcholine on HepI or HepIII extension in LOS does not alter the level of biofilm formation. Overnight growth measured by the OD_{620} (A) and the level of biofilm formation by strains Rd (phase-variable PCho on HepI), H446 (constitutively $PCho^-$), H457 (PCho on HepIII), and H491 (constitutively $PCho^+$ on HepI) measured by the A_{560} (B) are shown. Means ± standard errors of the means of four independent replicates are depicted. One-way analysis of variance ($P = 0.2461$ [A] and $P = 0.5727$ [B]) with Tukey's multiple-comparison *post hoc* test was used for statistical analysis.

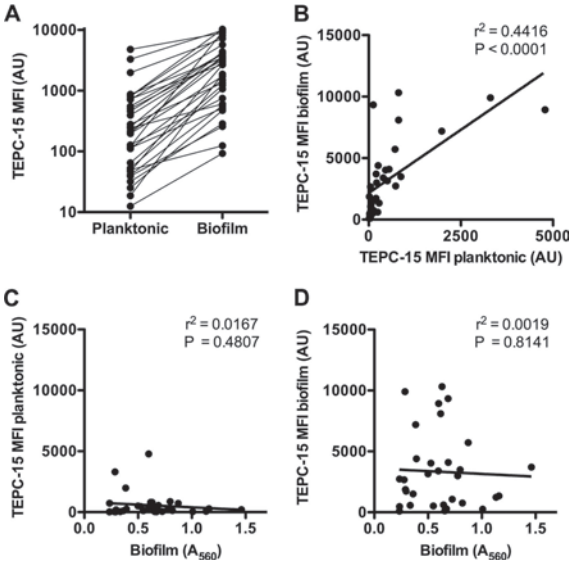

FIG 4 Phosphorylcholine levels on NTHi grown planktonically or in a biofilm shown as mean fluorescence intensity (MFI) in arbitrary units (AU) and the level of biofilm formation. (A) PCho contents of 32 NTHi strains grown planktonically or in a biofilm. (B) Correlation between PCho content of NTHi grown planktonically and in a biofilm. (C) Correlation between the level of biofilm formation and PCho content of NTHi grown planktonically. (D) Correlation between the level of biofilm formation and PCho content of NTHi grown in a biofilm.

AU, Q3, TEPC-15 MFI of 104 to 46 AU; Q4, TEPC-15 MFI of 45 to 7 AU), no differences in the level of biofilm formation were observed.

Different effects of the presence of PCho on NTHi biofilm initiation, formation, and maturation have been observed in static and continuous flow systems previously. It is likely that differences in NTHi strains and biofilm techniques used influence the outcome of the effect of PCho in biofilm assays. For instance, Hong et al. showed a positive correlation between the presence of PCho in the LOS of three variants of NTHi strain 2019 and biofilm maturation in a continuous flow system (19). More representative for our experiments, static biofilm experiments performed with strains 86-028NP and 2019 showed no effects of PCho on biofilm initiation after 10 hours. Therefore, additional experiments were performed to evaluate the relationship between PCho incorporation and the level of biofilm formation in a static assay in more detail by modulating PCho incorporation into NTHi LOS.

The position of phosphorylcholine in LOS does not affect the level of biofilm formation. Incorporation of PCho can occur at multiple positions in NTHi LOS. For example, PCho can be incorporated as a terminal moiety on hexoses extending heptose I (HepI) or heptose III (HepIII), which differentially affects CRP (38) and IgG (39) binding to NTHi. To our knowledge, whether the HepI or HepIII position of PCho affects biofilm formation is not known. Therefore, we tested the level of biofilm formation for strains Rd (phase-variable incorporation of PCho on HepI), H446 (constitutively $PCho^-$), H457 (*lic1D* of the Eagan strain; PCho on HepIII), and H491 (constitutively $PCho^+$ on HepI). We found no differences in growth (Fig. 3A) or the level of biofilm formation (Fig. 3B) between Rd, H491 with PCho on HepI, H457 with PCho incorporated into HepIII, and the constitutively PCho-negative strain H446. These results demonstrate that the level of biofilm formation is not affected by the presence or position of PCho into the LOS of strain Rd.

Phosphorylcholine content in bacteria within a biofilm is not related to the level of biofilm formation of clinical NTHi isolates. Since other studies demonstrated that the PCho content was increased in bacteria growing within a biofilm structure (40), we determined the amount of PCho present in 32 NTHi strains in the planktonic or biofilm state of growth by flow cytometry and analyzed the relationship with the level of biofilm formation.

All the tested strains showed increased PCho content in a biofilm compared to planktonic growth (Fig. 4A), and the PCho contents of individual strains in either a planktonic culture or biofilm showed a significant correlation (Fig. 4B). However, we have to mention that it is very likely that we still had sufficient bacterial clumping in the biofilm preparations despite vigorous shearing by pipetting, which increases TEPC-15 binding per particle measured by flow cytometry. Although we attempted to loosen the bacteria in the biofilm samples by vigorous shearing, forward and sideward scatter signals were increased for the biofilm samples (data not shown), which shows the presence of larger particles. Therefore, the PCho content for NTHi samples present in a biofilm might also be higher because of clumping. Nevertheless, as described for planktonic cells (Fig. 4C), no correlation was observed between the PCho content in NTHi strains present in a biofilm and the level of biofilm formation of the particular strain (Fig. 4D). These results show that although the PCho content might be increased within the biofilm, the level of PCho is not related to the level of biofilm formation.

Modulation of phosphorylcholine incorporation affects complement resistance and the level of biofilm formation. In order to address the effects of PCho in the LOS of NTHi on biofilm formation, we constructed five mutant strains by replacement of the *licA* gene with a spectinomycin cassette, which was confirmed by PCR analysis. Replacement of the *licA* gene by a spectinomycin cassette did not affect growth (data not shown). The PCho content of the WT and *licA* mutants was determined by flow cytometry and Western blotting. Flow cytometry analysis confirmed that all *licA* mutants (Fig. 5A, light gray) presented an absence of PCho compared to the WT strains (Fig. 5A, dark gray), which was sig-

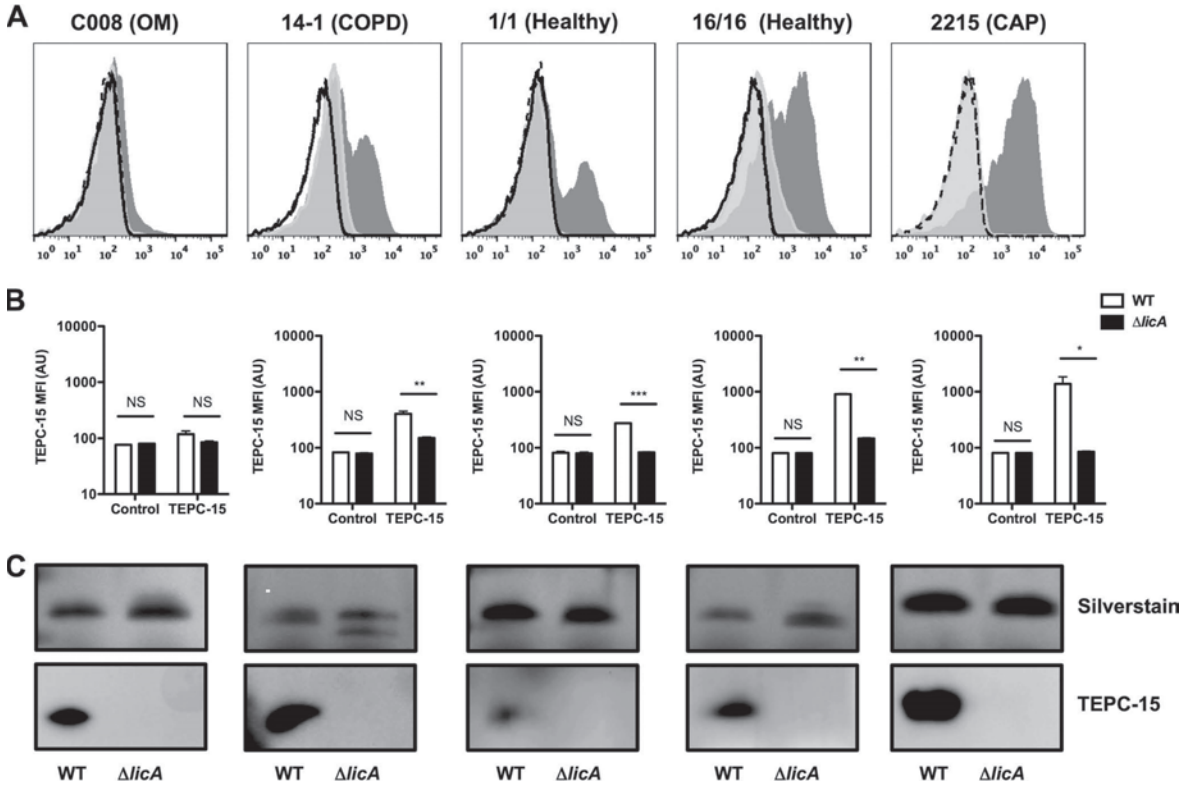

FIG 5 Effect of *licA* deletion on phosphorylcholine incorporation into NTHi LOS. (A) PCho contents in 5 NTHi WT and *licA* mutant strains detected by TEPC-15 binding by flow cytometry in a representative experiment used for analysis in panel B. Solid and dotted black lines are the second antibody controls for the WT and the *licA* mutant, respectively. In dark gray and light gray are TEPC-15 binding with the second antibodies for the WT and *licA* mutant strains, respectively. The *y* axis depicts the relative number of events, and the *x* axis depicts the FITC mean fluorescence intensity (MFI) in arbitrary units (AU). (B) PCho content in 5 NTHi WT and *licA* mutant strains detected by TEPC-15 binding by flow cytometry. Means ± standard errors of the means of two independent replicates are depicted. A two-way analysis of variance with a Bonferroni *post hoc* test was used for statistical analysis (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant). (C) Analysis of LOS purified from *licA* mutants separated by Tris-Tricine SDS-PAGE and visualized by silver staining or by Western blotting using TEPC-15 antibody.

nificant for 4 out of 5 strains (Fig. 5B). Western blot analysis results corroborated the flow cytometry data, with clearly abrogated incorporation of PCho into the LOS of the *licA* gene deletion mutants (Fig. 5C).

PCho has been shown to affect the virulence of NTHi by multiple mechanisms. For instance, PCho was shown to prevent IgG binding, thereby preventing complement-mediated killing (39). However, PCho also binds CRP, which induces classical complement pathway activation (35). Therefore, we determined the effects of *licA* deletion on resistance to complement-mediated killing in pooled normal human serum (NHS) and observed strain-dependent changes. Strains C008, 14-1, 1/1, and 16/16 showed decreased resistance to complement-mediated killing, whereas strain 2215 showed increased resistance (Fig. 6A). The latter strain showed binding to CRP (Fig. 6B) that was significantly reduced upon *licA* deletion, which corresponds to its increased resistance to complement-mediated killing.

Finally, we analyzed the effect of decreased PCho incorporation into LOS on biofilm formation. Biofilm formation showed high variability among the five WT NTHi strains and their respective *licA* mutants (Fig. 7A). The 1/1 *licA* mutant strain formed slightly less biofilm than the WT, although it was not significant. In contrast, the C008 and 16/16 *licA* mutants formed more biofilm than the WT, whereas the 14-1 and 2215 *licA* mutants formed approximately the same amount of biofilm, which is in line with other static biofilm experiments with strains 86-028NP and 2019 (40).

The fact that the *licA* deletion affects the level biofilm formation either positively or negatively might be related to strain-dependent alterations in LOS size or charge. As seen for the 14-1 and 16/16 *licA* mutants, LOS appeared to be slightly altered compared to that of the WT, whereas this was not the case for the other strains. Additionally, alterations in the overall surface charge of NTHi might affect bacterial adhesion and biofilm formation, as was observed for a wide variety of other bacterial species, including *Staphylococcus aureus* (41), *Enterococcus faecalis* (42), and *Campylobacter jejuni* (43). PCho has a positively charged quaternary amine group, and depletion of PCho in the *licA* mutant might affect the overall surface charge depending on the amount of PCho and other charged molecules on the surface of the WT

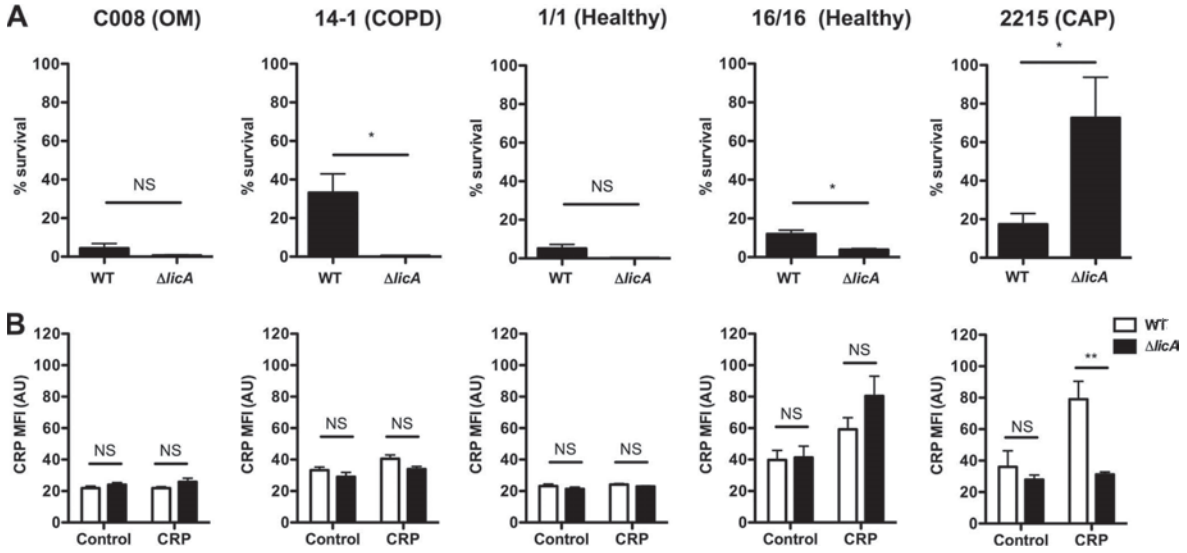

FIG 6 Effect of *licA* deletion on CRP binding and complement resistance. (A) Survival of 5 NTHi WT and *licA* mutant strains in 5% NHS compared to 5% HI-NHS for 1 h. Means ± standard errors of the means of four independent replicates are depicted. A Student *t* test was used for statistical analysis (*, $P < 0.05$; **, $P < 0.01$; NS, not significant). (B) CRP binding to 5 NTHi WT and *licA* mutant strains measured by flow cytometry shown as mean fluorescence intensity (MFI) in arbitrary units (AU). Means ± standard errors of the means of four independent replicates are depicted. A two-way analysis of variance with a Bonferroni *post hoc* test was used for statistical analysis.

strain. Therefore, we tested biofilm formation on glass, since it was shown previously that changes in charge affected adhesion to glass or plastic differently (44). Overall, the ability of the WT and *licA* mutants to form biofilm on glass (Fig. 7B) was similar to the results obtained on plastic (Fig. 7A). Altogether, we show that alterations in PCho affect the level of NTHi biofilm formation in a strain-dependent manner.

Conclusion. An understanding of which general factors are involved in NTHi biofilm formation is an important topic for future research because this knowledge will allow a better compre-

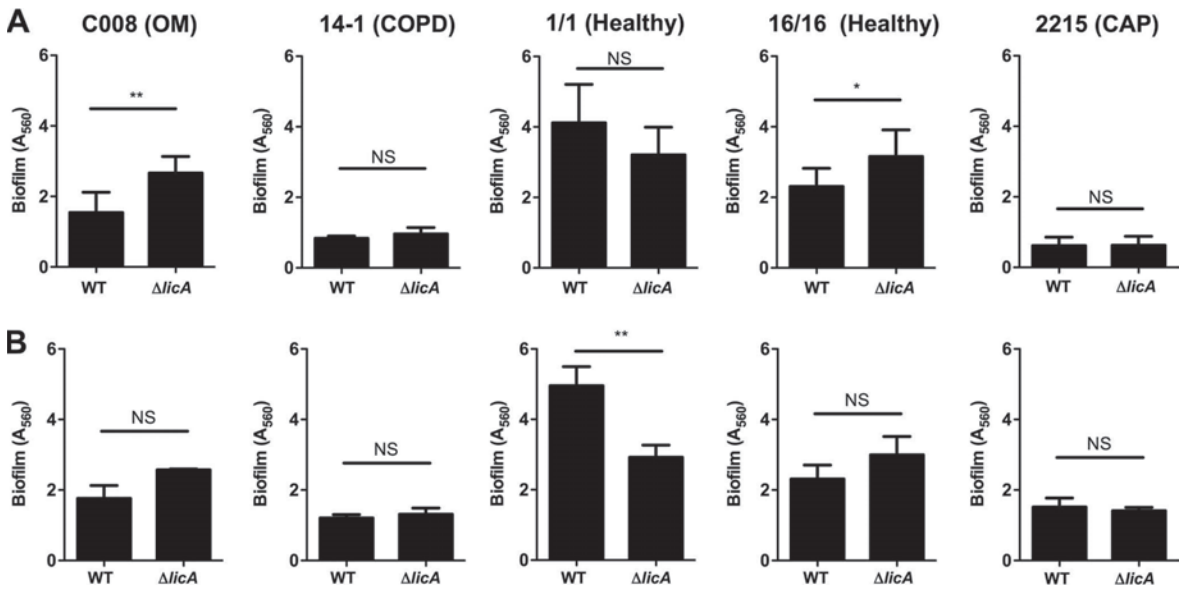

FIG 7 Effect of *licA* deletion on the level of biofilm formation *in vitro*. The level of biofilm formation of the WT and *licA* mutant strains on plastic (A) and glass (B) was measured by crystal violet A_{560}. Means ± standard errors of the means of four (plastic) or three (glass) independent replicates are depicted. A Student *t* test was used for statistical analysis (*, $P < 0.05$; **, $P < 0.01$; NS, not significant).

hension of the infection process, which is important for the evaluation and treatment of diseases caused by NTHi. Our work shows that incorporation of PCho into the LOS of clinical NTHi isolates does not predict the level of biofilm *in vitro*. We observed decreased biofilm formation in a static assay for the *licA* mutant of strain 1/1; however, strains C008 and 16/16 showed increased biofilm formation upon deletion of PCho incorporation. Therefore, we conclude that PCho in NTHi LOS affects the level biofilm formation in a strain-dependent manner and that its presence does not predict the ability to form biofilms in a static assay *in vitro*.

ACKNOWLEDGMENTS

C.P. was supported by an FPU grant (Formación de Profesorado Universitario, Ministerio de Educación, Spain). S.M. was supported by Sara Borrell postdoctoral contract CD10/00298 from the Instituto de Salud Carlos III (ISCIII), Madrid, Spain. J.D.L. was supported by the Nano Cluster of Technology Foundation (STW FES0901, FES HTSM) and a Dutch Lung Foundation long-term fellowship (3.2.12.126FE).

This work was partially possible thanks to the Ayuda de la SEIMC from the Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica. We thank the Hospital Central in Oviedo for providing the samples from healthy children and Jeffrey Weiser for providing the H446, H457, and H491 strains.

We have no conflicts of interest to declare.

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DISCUSSION

IV. DISCUSSION

H. influenzae is an opportunistic pathogen that colonizes the upper respiratory tract and forms part of the human nasopharyngeal microbiota. Despite that, this microorganism is able to cause several infections especially in patients with comorbidities (Gkentzi *et al*., 2012; King, 2012). The turning point in the epidemiology of *H. influenzae* was the introduction of effective childhood vaccination against Hib, the most invasive and virulent serotype. The dramatic decrease in Hib allowed the expansion of NTHi, which has become more relevant in both respiratory and in invasive infections, and is associated with a high case fatality rate (Gkentzi *et al*., 2012). The pathogenesis due to NTHi is not clearly understood but it includes a first step of colonization of the upper respiratory tract and a subsequent spread to the neighbouring lower parts of the respiratory tract where an inflammatory response is initiated. Furthermore, persistence in the host and invasion of eukaryotic cells are also important and poorly understood in the pathogenesis of NTHi (Murphy *et al*., 2009; Rao *et al*., 1999).

In this thesis we have analysed the epidemiology of NTHi, placing the emphasis on molecular genotyping, antimicrobial resistance and biofilm formation. The first step in the epidemiological studies involved the analysis of the population structure of NTHi isolates associated with colonization and infection. Specifically, we characterized NTHi isolated from healthy children genotypically, together with isolates from the main adult infections in which this pathogen is involved, including non-bacteraemic and bacteraemic pneumonia, acute exacerbations in COPD and some other invasive diseases. The second important point in the epidemiology of NTHi concerns the identification of the level of antimicrobial resistance in the clinical isolates. Consequently, the second part of this thesis reports the study of antimicrobial susceptibility of all the NTHi isolated from various clinical origins, with a characterization of the molecular mechanisms involved in β-lactam and fluoroquinolone resistance. The last part focuses on biofilm formation, one of the most important mechanisms involved in persistence and in chronic infections. For this reason we determined the capacity of adhesion and biofilm formation of different NTHi strains, and evaluated other factors that might be involved in the generation of this structure.

Epidemiological relevance of NTHi

The aim of the first part of this thesis was the epidemiological study of NTHi in the colonization of healthy subjects and in the major infections caused by this pathogen in adult patients since the introduction of the conjugate Hib vaccine. The main objective was to genotypically characterise the strains isolated from carriers, patients with CAP, COPD, and with invasive infections. The results of these studies have been reported in **Papers 1, 2, 3, and 4**.

Colonization

The human nasopharynx is the exclusive reservoir of NTHi; therefore, it is essential to know the rates of colonization of this pathogen within the population (Principi *et al.*, 1999). Although several studies have been published in different parts of the world on the carriage rate of *H. influenzae* since vaccine introduction, few data have been reported from Spain (Fontanals *et al.*, 2000). For this reason, we performed a study to establish the colonization rate in healthy children from Oviedo, using oropharyngeal samples collected in two consecutive years (2004-2005), six years after the introduction of the vaccine in Spain. This study was performed in children, as the main reservoir of NTHi, because the colonization rate is higher in childhood and decreases with age (Kuklinska and Kilian, 1984; Mukundan *et al.*, 2007). So far, most of the approaches to identify NTHi colonization have been based on longitudinal studies carried out at one or two centres to determine the dynamics of colonization. We were more interested in the global rate of colonization; therefore, we performed a point-prevalence study at nineteen centres (day-care centres [DCC] and state schools) in two consecutive years. Although co-colonization by more than one strain has been described, we randomly selected only one colony per sample as representative of the whole NTHi population *(*Barbosa-Cesnik *et al.*, 2006; Farjo *et al.*, 2004). The results of this study are described in **Paper 1**.

The *H. influenzae* carriage rate found in the study period remained stable at about 40%, similar to or slightly below the rates in other colonization studies (Barbosa-Cesnik *et al*., 2006; de Carvalho *et al*., 2011; Farjo *et al*., 2004; Fontanals *et al*., 2000; Ito *et al*., 2010; Munsawaengsub *et al*., 2010; Raymond *et al*., 2001; Sa-Leao *et al*., 2008). As expected, no Hib were found in the children's nasopharynx because in Spain

the vaccine against Hib has been part of the recommended vaccination schedule sine 1998. The colonization by other encapsulated strains, in that case serotype f, was very low (1.5%), proving that NTHi has become the most frequent type of *H. influenzae* colonising healthy children. With regard to genotyping, we found a high genetic diversity among NTHi strains within the same DCC or school. DCCs are considered important reservoirs for the transmission of upper respiratory pathogens due to the presence, in crowded spaces, of young children with immature immune systems and with poor hygienic habits (Sa-Leao *et al.*, 2008). However, only small clusters formed by the same strain were detected in DCCs, indicating that although there was transmission among children in those centres, the acquisition of strains also occurred at home or in other locations. In addition, we observed that children sampled in both years did not harbour the same strain, suggesting a short time persistence and a high strain replacement.

Several factors have been associated with colonization such as attendance at DCCs, age, family size, and antibiotic treatment (Raymond *et al*., 2001). Due to the diversity of centres included in the study (DCCs or schools), and the characteristics of the children (age, health status before the sampling, antimicrobial treatment), we were able to examine different colonization behaviours, and observed no association between rate of colonization and types of centre, gender, or respiratory problems. Age and size of the centre had an association in only one of the years studied, suggesting that colonization is highly variable in children. Nevertheless, antibiotic consumption on the sampling day had a clear association with reduced colonization of the oropharyngeal track.

COPD

In the adult population, COPD is the disease in which NTHi is most relevant as an etiological agent (Eldika *et al*., 2006). COPD is an important cause of morbidity and mortality worldwide and is associated with a notable economic and social burden (Lopez *et al*., 2006). Today COPD is considered an important cause of death, and its prevalence is expected to increase mainly due to the continued exposure to risk factors and the changing age structure of the population (Mathers *et al*., 2006). In an acute exacerbation, the patient's symptoms increase due to a variety of factors, among them

viral and bacterial infections (Anzueto *et al*., 2007). Until 2013, the severity of COPD was described according to GOLD classifications, based on the degree of airflow obstruction. In 2013 the COPD guidelines changed, and now take into account the GOLD stage plus the annual number of acute exacerbations (AECOPD) (http://www.goldcopd.org). In order to establish the role of bacteria in AECOPD in accordance with the new guidelines, we designed a one-year prospective study based on the quantitative culture of sputum samples collected from patients with severe COPD. One hundred and eleven patients were included in the study, 40 with only one exacerbation and 71 patients with two exacerbations or more during the year studied. Although the patients were mainly elderly and with underlying diseases, we excluded all those with high comorbidities, immunodeficiency, terminal malignancy or other chronic respiratory diseases, and the episodes related to cardiac failure or other non-infectious causes. The results of this study are presented in **Paper 2**.

We found that *P. aeruginosa* was the most frequent pathogen isolated from AECOPD in patients with advanced airflow obstruction; in fact, this pathogen was more common in patients who suffer ≥ 2 exacerbations per year. This long-term colonization with *P. aeruginosa* causing occasional exacerbations should be taken into consideration in the management of patients with severe COPD, especially with regard to empirical antimicrobial treatment. With respect to the other microorganisms, the frequencies of isolation were similar to those previously reported (Ko *et al*., 2007; Rosell *et al*., 2005; Sethi *et al*., 2008).

Specifically, in our study, *H. influenzae* was the second cause of AECOPD, and it was associated with patients with one exacerbation episode. However, in other studies, *H. influenzae* has been considered as the first bacterial cause of exacerbations (Decramer *et al.*, 2012; Eldika and Sethi, 2006; Sethi and Murphy, 2008). The explanation for this difference may lie in the type of patients included in the studies; all our patients had severe COPD whereas the other studies did not specify the GOLD stage or the number of exacerbations. Probably, the high frequency of this bacterium observed in previous studies is due to the presence of patients in the early stages of COPD who had a lower risk of exacerbations. In addition, *H. influenzae* may also

persist in the respiratory tract without being detected due to the low bacterial concentration, persistance in biofilms, or inside host cells (Murphy *et al*., 2004).

Colonization by multiple NTHi strains has been described as a common event in patients with COPD (Murphy *et al*., 1999). In addition, NTHi strains may differ depending on the site of the respiratory tract they colonize (Bandi *et al*., 2001). These observations have important implications for the isolation of NTHi since sputum cultures may not reliably reflect the diversity in the lower airways. This, in turn, may have significant consequences for antimicrobial treatment (Bandi *et al*., 2001; Murphy *et al*., 1999). However, in our experience, after using PFGE to type eight colonies isolated from each sputum sample from patients with AECOPD and in stable phase, an identical PFGE pattern was found in 22 of the 24 tested samples; only in two samples could we identify two different strains with different PFGE patterns. These two sputum samples were isolated from the same patient, in two stable phases. In both samples, one of the strains was more predominant although they were able to persist for at least 34 days, when the second sputum sample was taken.

Although NTHi is a frequent cause of exacerbation, it is also commonly found in the lower airways of clinically stable patients (Bandi *et al*., 2001; Eldika *et al*., 2006). We isolated NTHi from sputum samples of patients in stable phase with a high bacterial load ($\geq 10^6$ cfu/mL), a trend already observed by Murphy and colleagues in 1999 (Murphy *et al*., 1999). It has been reported that the presence of NTHi in the lower respiratory tract during a stable phase is not innocuous and although there may not be an increase in symptomatology, it may be associated with an increase in inflammation and its consequences. This bacterial colonization may cause an inflammatory response, which together with a damaged lung and impaired innate lung defences, may cause progression of the disease and create a vicious circle (Eldika *et al*., 2006).

Overall, these results suggest that NTHi may present similar behaviour in COPD and cystic fibrosis (CF). In CF, it has been hypothesized that the epithelial damage due to the inflammation by *H. influenzae* facilitates colonization with *P. aeruginosa* (Rosenfeld *et al*., 2001; Smith, 1997; Starner *et al*., 2006). This hypothesis could also explain the role of NTHi in COPD and the higher colonization observed for *P. aeruginosa* in later stages of the disease. In both diseases, NTHi appears in the early

phases: in CF it is predominant in one-year-old children (Rosenfeld *et al*., 2001), and as we have observed, in COPD, NTHi is predominant in patients with moderate airflow obstruction or with severe COPD but with a lower risk of exacerbations. Moreover, in both types of diseases it has been reported that once NTHi enters the lower respiratory tract, it mediates an increase in inflammation and lung damage, conditions that would be beneficial for *P. aeruginosa* colonization and infection (Bandi *et al*., 2001; Rosenfeld *et al*., 2001).

Bacteraemic and non-bacteraemic pneumonia

In addition to AECOPD, pneumonia is a common infection caused by NTHi, especially in the elderly and in patients with COPD (Murphy, 2003; Musher *et al*., 2014). The prevalence of *H. influenzae* as a causal agent of CAP fluctuates between 1.1% and 29.4% depending on the study (Torres *et al.*, 2014), but it is described as a typical etiological agent (Spoorenberg *et al*., 2014; van Gageldonk-Lafeber *et al*., 2013). In fact, *H. influenzae* is the second or third cause of CAP in the elderly population (Simonetti *et al*., 2014) but is less relevant in immunocompetent adults with severe CAP or in patients in Intensive Care Units (ICU) (Cilloniz *et al*., 2011; Khawaja *et al*., 2013). Today, CAP is still one of the most prevalent infections and a major cause of death (Garcia-Vidal *et al*., 2008). Identification of the etiological agent causing CAP is crucial in the management of antimicrobial therapy and patient survival (Cilloniz *et al*., 2011; Garcia-Vidal *et al*., 2008; Kothe *et al*., 2008). Due to the high incidence of this infection in the elderly and the high percentage of patients that need hospital admission, the clinical and economic costs associated with CAP are considerable. Moreover, in developed countries with a continuously ageing population, this health burden is expected to increase (Spoorenberg *et al*., 2014; Torres *et al.*, 2014).

As NTHi is an important cause of pneumonia, we performed two different studies; one analyzing NTHi as a cause of non-bacteraemic CAP, and the other of NTHi from patients with bacteraemic CAP which was included in a larger work on invasive *H. influenzae* disease. The results of these studies are presented in **Papers 3 and 4**, respectively.

In the first study, we analysed the molecular epidemiology of NTHi causing non-bacteraemic CAP in adult patients from 2000 to 2009. A total of 95 isolates collected from 92 patients were included. With regard to invasive disease, we studied the epidemiology of 82 *H. influenzae* isolated from adult patients between 2008 and 2013. In this case, NTHi was directly related to 63 cases of invasive disease, 36 (57.1%) of which presented pneumonia as a source of the invasive infection, either community (83.3%) or hospital-acquired (16.7%).

Our hospital is a tertiary care centre for the adult population. Patients admitted to our institution suffering from CAP were older adults (mean age of 64.04 *vs.* 68.15 years) with underlying diseases. There was a difference in the most common comorbidities between patients with bacteraemic and non-bacteraemic pneumonia. In non-bacteraemic CAP, COPD was the most common underlying disease, whereas in bacteraemic CAP, COPD was the second comorbidity in frequency after malignancies (solid organ or hematological). The 30-day mortality rate was higher in patients with bacteraemic CAP (23.3% *vs.* 8.7%) possibly due to the different underlying conditions of the patients. In fact, it has been reported that patients with bacteraemia presented a greater risk of death (Dworkin *et al.*, 2007).

Regarding the strains causing pneumonia, we found a high genetic variability among the NTHi isolated from bacteraemic and non-bacteraemic CAP, suggesting a lack of clonal distribution in agreement with other reports (Bajanca-Lavado *et al*., 2014; Saito *et al*., 1999; Shuel *et al*., 2011). However, 11.1% of the STs from bacteraemic CAP were identical to STs from non-bacteraemic CAP. In addition, 14.8% of bacteraemic strains were single or double locus variants of STs from the non-bacteraemic strains. Therefore, our results suggest that the medical condition of the patient before infection and a differential expression of bacterial virulence factors could be crucial for determining whether the strain is able to cause an invasive disease.

Prior to the Hib conjugate vaccines, the serotype b was the most prevalent cause of invasive infections, above all meningitis in young children. The widespread use of the Hib conjugate vaccines has caused a remarkable reduction of Hib infections as well as a reduction in the nasopharyngeal carriage, which provides a herd protection effect in the unvaccinated population, and contributes to the high efficacy of the vaccine. The

decreased frequency of Hib strains allowed a replacement of type b strains by NTHi which has become predominant in invasive diseases (Agrawal *et al*., 2011). We observed this reduction in the colonization study (**Paper 1**), where no Hib strains were isolated, and in the invasive study (**Paper 4**), where only one Hib was isolated from a patient with pneumonia. The strain shift has caused a change in the age incidence and in the clinical manifestations of the disease. Currently, invasive infections are more common in older adults and elderly population, usually with underlying diseases, and the most frequent clinical presentation is bacteraemia (Agrawal and Murphy, 2011; Ulanova and Tsang, 2009). Most invasive strains were isolated from blood with pneumonia as a source of infection; NTHi may also cause other types of invasive infections, though their frequency is much lower than bacteraemic pneumonia. The most common focuses were meningitis and biliary tract infection (9.5%), although a high percentage of the cases were primary bacteraemia without a focus (11.1%).

Invasive infections caused by other encapsulated strains are rare and mainly caused by Hif (Adam *et al.*, 2010; Dworkin *et al.*, 2007; Ladhani *et al.*, 2010). In our six-year study, only ten encapsulated-non Hib were found (9 Hif and 1 Hie); these results are consistent with previously reported data (Adam *et al.*, 2010; Ladhani *et al.*, 2010). No differences were observed in the clinical conditions of patients with invasive disease caused by encapsulated and NTHi strains. The majority were older adults with underlying diseases, the most common malignancies being COPD and chronic liver disease. The 30-day mortality rate of encapsulated strains (18.2%) and NTHi (20%) was also similar and agreed with previous studies (Rubach *et al*., 2011). However, unlike NTHi, encapsulated strains were clonally related and belonged to international clones detected worldwide; Hif to clonal complex 124, Hib to CC6 and Hie to CC18 (Bajanca-Lavado *et al*., 2014; Garcia-Cobos *et al*., 2014; Shuel *et al*., 2011).

Despite the efficiency of the Hib vaccine, *H. influenzae* infections remain a significant cause of disease and death. Moreover, changes in the age structure of the population and the increased life expectancy associated with the increasing number of patients with underlying conditions represent a new scenario in *H. influenzae* infections, with NTHi as a main agent. Continued epidemiological studies are needed in order to

establish the transmission and identify potential changes in the strains and the effects in the population.

Epidemiological relevance of *H. haemolyticus*

Among the species classified in the genus *Haemophilus*, the most important human pathogen is *H. influenzae*. Traditionally, species were classified into the *Haemophilus* genus due to their need for the growth factors X (haem) and V (NAD). The implementation of molecular methods for bacterial identification established that the genus *Haemophilus* comprised highly heterogeneous species, and some of them were transferred to other genera such as *Actinobacillus* or *Aggregatibacter*. In spite of this heterogeneity, distinguishing between species may sometimes be challenging, particularly the identification of *H. haemolyticus*, which is often misidentified as NTHi. Moreover, MALDI-TOF and MLSA methodologies showed a high similarity between *H. haemolyticus* and other species such as the cryptic genospecies biotype IV and *Haemophilus intermedius* (Norskov-Lauritsen, 2014). Several markers such as *16SrDNA*, *sodC*, OmpP6, LOS genes, *iga*, *hpd*, and other house-keeping genes have been proposed to differentiate between *H. haemolyticus* and NTHi but no single marker has proved reliable for the identification due to the high genetic similarity and because horizontal gene transfer between bacteria from these species is frequent (Pickering *et al*., 2014). Recently, the mass spectrometry (MALDI-TOF) software has been updated and provides improved bacterial identification, and due to its quick and easy handling it is considered a good tool to use in a clinical microbiology laboratory (Randell, 2014). Some studies have tested the discriminatory power of MALDI-TOF in the differentiation between *H. haemolyticus* and NTHi, generally with good results, but always depending on the databases used in the laboratories (Bruin *et al*., 2014; Frickmann *et al*., 2013; Zhu *et al*., 2013).

With the incorporation in our hospital of the updated MALDI-TOF software with the *H. haemolyticus* spectra, we designed a study to retrospectively analyze all the *Haemophilus* spp. previously identified by mass spectrometry so as to determine the frequency of *H. haemolyticus* in clinical samples. The results of this study are presented in **Paper 6**.

In contrast to previous data which identified 27-40% of the isolates from respiratory samples (sputum samples and nasopharynx swabs) as *H. haemolyticus* (Murphy *et al*., 2007), we found that the frequency in the clinical samples during two consecutive years was low, especially in respiratory samples (4%). Our figures were more similar to other recently reported data (1.5%-6%) (Frickmann *et al*., 2014; Zhang *et al*., 2014), suggesting that the previously published prevalence was overestimated. Furthermore, previous studies also showed that *H. influenzae* was more frequently isolated from the nasopharynx whereas *H. haemolyticus* was normally present in the oropharynx (Frickmann *et al*., 2014). By contrast, in our colonization study (**Paper 1**) the frequency of *H. haemolyticus* in oropharyngeal samples was very low (0.7%), and there was a clear predominance of NTHi. Interestingly, we identified more *H. haemolyticus* in genitourinary samples (10.5%) than in respiratory samples. Although in this ecological niche the predominant *Haemophilus* species is *H. parainfluenzae* (78%), *H. haemolyticus* was as frequent as *H. influenzae* (10.5% and 11.5% respectively). To confirm these observations, we performed a retrospective analysis of all the *Haemophilus* species in vaginal swabs since 2010, observing that *H. haemolyticus* and *H. influenzae* were isolated in approximately 18% of the samples. This is a substantial percentage in this clinical origin where *H. parainfluenzae* is the predominant microorganism from the genus *Haemophilus*.

Regarding the clinical relevance of *H. haemolyticus* species, some authors have considered them as strictly commensal microorganisms because they are not usually isolated from sterile sites or, for instance in COPD, the acquisition of a new *H. haemolyticus* strain has not been associated with an exacerbation, as has already been shown for NTHi (Murphy *et al*., 2007). However, recent studies have reported the first cases of invasive *H. influenzae* isolates from blood which have been re-identified as *H. haemolyticus* (Anderson *et al*., 2012; Morton *et al*., 2012). In the retrospective study of invasive NTHi isolates (**Paper 4**), only one *H. haemolyticus* was found, indicating that although the frequency is low, their prevalence may be underestimated by the misidentification with NTHi. Recently, the Centers for Disease Control and Prevention (CDC) suggested that *H. haemolyticus* should be considered a microorganism with pathogenic capacity, not only a non-pathogenic commensal (Norskov-Lauritsen, 2014). Little is known about the pathogenicity of *H. haemolyticus* but due to the high

frequency of recombination between the two species, it is likely to share some virulence factors with *H. influenzae* which may play a role in its pathogenicity (Anderson *et al*., 2012).

The genetic diversity of *H. haemolyticus* is as high as the variability in NTHi (Mukundan *et al*., 2007). In our study, we also observed this high genetic heterogeneity in our isolates and we did not detect any cluster of strains according to clinical origin.

Overall, despite the difficulty of differentiating between *H. haemolyticus* and NTHi, a precise identification is important in order to establish the real prevalence and the clinical impact of these bacterial species.

Antimicrobial resistance

Due to the replacement of Hib by NTHi in the most common diseases caused by this pathogen, the continuous monitoring of the antimicrobial resistance among the most important types of infection is essential in order to establish the prevalence and the evolution of this resistance.

The aim of the second part of this thesis was to determine the antimicrobial susceptibility profile of NTHi for all the isolates included in the epidemiological studies, with emphasis on β-lactams and fluoroquinolones as main antimicrobial groups used to treat NTHi infections. Susceptibility data and molecular characterization of β-lactam resistance are described in **Papers 1, 2, 3 and 4**. The prevalence of fluoroquinolone resistance among NTHi was determined in an individual study with isolates collected for a long period of time (2000-2013). In that study, we typified fluoroquinolone-resistant isolates and characterized the molecular mechanisms associated with this resistance. The results are presented in **Paper 5**. Additionally, the antimicrobial susceptibility profile of *H. haemolyticus* was also determined and compared with NTHi. These results are reported in **Paper 6**.

Resistance to β-lactam antibiotics

β-lactams are the antimicrobials most frequently used in the treatment of *H. influenzae* infections. The most common mechanism of resistance to β-lactams in this pathogen involves the production of a β-lactamase enzyme, usually TEM-1 or, more rarely, ROB-1. In our studies, all the β-lactamase producers expressed the TEM-1 enzyme. The prevalence of β-lactamases varies markedly depending on the study and it is also subject to geographical variability: in strains isolated from respiratory infections it ranges from 0% in some European countries such as Germany and the Netherlands to 48 or 52.4% in Korea and Taiwan (Bae *et al*., 2010; Heilmann *et al*., 2005; Jansen *et al*., 2006; Morrissey *et al*., 2008; Niki *et al*., 2011; Qin *et al*., 2012; Wang *et al*., 2011). In addition, despite this regional variation, there is a trend in Europe and the US towards a decrease of β-lactamase producing strains (Heilmann *et al*., 2005; Jansen *et al*., 2006). In Spain, the prevalence is around 16% (Perez-Trallero *et al*., 2010), similar to other European countries such as the UK (13.8%), Portugal (15.4%) and France (17.6%)

(Jansen *et al*., 2006). In our studies, the prevalence of this enzyme ranges from 10.5% in isolates from CAP to 5.4% in isolates from COPD, which is consistent with the overall downward trend observed in Spain (from 25.7% in 1997 to 15.7% in 2007) (Perez-Trallero *et al*., 2010).

The prevalence of β-lactamase producing isolates in invasive disease depends on the study, and ranges from 10% to 24% (Bajanca *et al*., 2004; Campos *et al*., 2004; Garcia-Cobos *et al*., 2014; Giufre *et al*., 2013; Ladhani *et al*., 2008a; Resman *et al*., 2012; Shuel *et al*., 2011). In our study, the proportion of NTHi β-lactamase producers was 10.2%, lower than the rate reported in previous studies in Spain: 24.2% in 1999-2000 and 16.9% in 2004-2009 (Campos *et al*., 2004; Garcia-Cobos *et al*., 2014). Our study covers the period of time since then (2008 to 2013) and the results corroborate the ongoing trend with a decrease in β-lactamase producers.

Among colonizing isolates, the prevalence of β-lactamase producers also varies according to the country, ranging from 0.8% in Japan to 61.5% in Taiwan (Barbosa-Cesnik *et al.*, 2006; de Carvalho *et al.*, 2011; Hashida *et al.*, 2008; Raymond *et al.*, 2001; Wang *et al.*, 2008). In our study, the rate of β-lactam resistance due to β-lactamase was lower (13% in 2004 and 24% in 2005) than in other studies reported worldwide (Barbosa-Cesnik *et al.*, 2006; de Carvalho *et al.*, 2011; Hashida *et al.*, 2008; Raymond *et al.*, 2001; Wang *et al.*, 2008). However, in the second year we found a two-fold increase in the prevalence of β-lactamases which may be due to the spread of a β-lactamase producer clone in 2005 which accounted for 24% of the β-lactamase producer isolates of that year. Without this clone, the prevalence of β-lactamase producers would be 18%, similar to the prevalence in 2004.

Overall, we observed a higher frequency of β-lactamase producing NTHi isolates in children, probably because amoxicillin is the most commonly used antimicrobial in this group. Isolates recovered from children may be subject to higher selection pressure than isolates from adults in whom other antimicrobials, such as cephalosporins or fluoroquinolones are frequently used to treat *H. influenzae* infections.

A second mechanism involved in β-lactam resistance is based on the alteration of PBP3 which reduces its affinity for these antimicrobials. Isolates that harbour this mechanism of resistance are known as BLNAR. The level of resistance caused by this

mechanism is due to an accumulation of mutations (Tristram *et al*., 2007). The first level is acquired with substitutions in positions 517 or 526 (R517H or N526K) and these strains are classified into groups I and II. With the additional S385T substitution, isolates acquire resistance to cefotaxime and are classified into groups III (S385T + N526K) or III-like (S385T + R517H) (Garcia-Cobos *et al*., 2007; Hasegawa *et al*., 2004; Ubukata *et al*., 2001). Finally, the addition of the L389F substitution is associated with higher levels of cephalosporin resistance (Osaki *et al*., 2005). Due to the lack of international consensus on the MIC breakpoints, these isolates are difficult to classify; therefore, we considered BLNAR as non-β-lactamase producing isolates with alterations in PBP3 and ampicillin MIC ≥4 mg/L, and low-BLNAR as the isolates with ampicillin MIC of 0.5-2 mg/L.

The prevalence of non-β-lactamase-mediated β-lactam resistance also depends on geographical location. In respiratory isolates, it ranges from 0% in France and the Netherlands to 52.6% in Japan (Perez-Trallero *et al*., 2010; Qin *et al*., 2012; Heilmann *et al*., 2005; Hasegawa *et al*., 2006; Jansen *et al*., 2006). In Spain, Garcia-Cobos and colleagues compared two collections of isolates obtained mainly from respiratory samples from children, and showed an increase of the BLNAR genotype from 18.2% in 1997 to 27.5% in 2007 (Garcia-Cobos *et al*., 2008). They also analysed a collection of *H. influenzae* isolated between 2001 and 2006 from adults and children with community-acquired respiratory tract infections, and found that the frequency of the BLNAR genotype was 53% (Garcia-Cobos *et al*., 2007). In our study of CAP we found a lower frequency of BLNAR isolates (28.4%), all of them low-BLNAR. It is difficult to compare our data with the data published in 2007 because our isolates were collected from adult patients with CAP whereas the isolates studied in the earlier Spanish report were collected from patients with different ages. Moreover, our isolates were from CAP, whereas the origin of the isolates from their study was not specified.

Among invasive isolates, the prevalence of BLNAR ranges from 5.1% in Italy to 31% in Canada (Bajanca *et al*., 2004; Garcia-Cobos *et al*., 2014; Resman *et al*., 2012; Shuel *et al*., 2011). In our study, among the invasive NTHi isolates, 39% presented relevant mutations in the transpeptidase domain of the *ftsI* gene. Practically all these isolates (22 out of 23) were low-BLNAR, in agreement with the previous Spanish data where all the tested invasive isolates were low-BLNAR, with ampicillin MICs between

≤0.25-2 mg/L (Garcia-Cobos *et al*., 2014). However, the proportion of low-BLNAR strains found in our study (39%) was higher than the rate (19.2%) previously reported (Garcia-Cobos *et al*., 2014). There may be a variety of reasons for this increase; for instance, our isolates were more recent (2008-2013) whereas their previous study assessed isolates from 2004-2009; furthermore, our isolates were from adults whereas they also included isolates from children. In addition, we identified one isolate classified as BLNAR (MIC= 4 mg/L). This isolate had no other alterations in the PBP3 that might explain this MIC, suggesting an overexpression of the AcrAB efflux pump, previously described in *H. influenzae* (Kaczmarek *et al*., 2004).

The high percentage of ampicillin non-susceptible isolates found in respiratory and invasive infection in adults may be explained by the fact that the majority of NTHi were isolated from elderly patients receiving multiple antibiotic courses for their underlying conditions. A second explanation for the high level of resistance may be the increase in the consumption of aminopenicillins in Catalonia, from 46.1% in 1992 to 59.6% in 2007 (Llor *et al*., 2009).

The identification of PBP3 mutations associated with ampicillin resistance has hardly ever been performed in samples from healthy children. However, in a recent study, a low-BLNAR genotype was identified in 29.7% of the NTHi isolated from healthy carriers (Witherden *et al*., 2013). In our study, 12% of the isolates presented alterations in their PBP3 and were considered low-BLNAR because their ampicillin MICs ranged from 1 to 3 mg/L. The percentage of low-BLNAR isolates was lower in carriers than in isolates from respiratory and invasive infection, possibly because we only analysed a selection (67/400 isolates; 16.8%) which presented an inhibition zone for ampicillin and amoxicillin/clavulanic acid ≤ 28 mm. Therefore, we may have missed some isolates with alterations in PBP3 but with MICs of 0.5-1 mg/L.

With regard to other β-lactam antibiotics, our NTHi isolates were susceptible to extended-spectrum cephalosporins and carbapenems. BLNAR isolates with decreased susceptibility to cephalosporins are frequently isolated in Japan (Hasegawa *et al*., 2006; Sanbongi *et al*., 2006; Ubukata *et al*., 2001), but they are very rare in Europe, where the isolation of low-BLNAR strains is more prevalent (Dabernat *et al*., 2012; Garcia-Cobos *et al*., 2014; Resman *et al*., 2012). However, BLNAR isolates classified as III or III-like

have recently been described in Norway, France and Spain (Dabernat *et al*., 2012; Garcia-Cobos *et al*., 2007; Skaare *et al*., 2014). In our studies, only seven NTHi isolates from carriers could be classified as III-like isolates, but they were all susceptible to cefotaxime.

Isolates that present both mechanisms of resistance, β-lactamase and altered PBP3, constitute a second genotype, called BLPACR. Although there is no clear definition for these isolates, some authors use the resistance breakpoint for amoxicillin/clavulanic acid (MIC ≥ 8 mg/L) (Tristram *et al*., 2007). We considered the isolates that expressed both mechanisms of resistance and an amoxicillin/clavulanic acid MIC ≥8 mg/L as BLPACR, whereas the isolates with amoxicillin/clavulanic acid MICs of 2-4 mg/L were considered as low-BLPACR. Although the reported prevalence of BLPACR isolates is low (Bae *et al*., 2010; Hasegawa *et al*., 2003; Heilmann *et al*., 2005) a certain clonal spread of these isolates has been described in Portugal and Japan (Barbosa *et al*., 2011; Ito *et al*., 2010). Despite this, the BLPACR genotype was rare in our studied groups (≤ 5%); most isolates were low-BLPACR, and only two isolates from CAP were identified as BLPACR.

Due to the increase in the prevalence of NTHi isolates with altered PBP3 and the recent description of BLNAR strains with resistance to extended-spectrum cephalosporins in Europe, further epidemiological studies based on molecular characterization of β-lactam resistance in NTHi are needed to monitor the evolution of these resistant strains.

Regarding to β-lactam resistance in *H. haemolyticus*, Witherden and Tristam observed the same mechanisms of resistance already identified in NTHi (Witherden *et al*., 2013). Nonetheless, data on the prevalence of β-lactam resistance in *H. haemolyticus* are scarce. In our study, 8.7% of isolates were β-lactamase producers, below the 13.2% prevalence reported by Witherden *et al*. from healthy carriers, children and adults (Witherden *et al*., 2013). Nevertheless, little is known about the resistance due to altered PBP3 in *H. haemolyticus* (Murphy *et al.*, 2007; Takahata *et al.*, 2007; Witherden and Tristram, 2013). We tested 35 isolates with ampicillin MIC of >0.125 mg/L and found that nine out of 35 (26%) isolates presented the same alterations in the *ftsI* gene described in *H. influenzae*; one of them had the substitution in the 517 position

of the PBP3 (R517H) and the remaining eight the N526K substitution. This frequency was similar to the rate of 24.6% previously reported in carriers (Witherden *et al*., 2013). Furthermore, we also reported two groups of strains that included most of the isolates and could not be classified in any of the groups described. The group called III-Hae included 14 isolates that presented the same sequence as the *H. haemolyticus* ATCC 33390, but they presented several alterations between the positions 344 and 377 with respect to the *H. influenzae* Rd Kw20. The second group, called IV-Hae, included 12 isolates that presented a sequence different from both type strains, *H. haemolyticus* ATCC 33390 and *H. influenzae* Rd Kw20. As all these isolates from groups III-Hae and IV-Hae remained susceptible to β-lactams, it is likely that these alterations are not associated with resistance. However, more studies are needed in order to determine exactly the involvement of these specific alterations in β-lactam resistance in *H. haemolyticus*, and in *H. influenzae* as well, due to the homologous recombination of the *ftsI* gene between the two species.

Takahata *et al*. demonstrated that there is homologous recombination of *ftsI* gene (mosaic structures) between *H. influenzae* and *H. haemolyticus*, two species that share the same ecological niche. With these observations, they have suggested a possible molecular evolution of BLNAR strains based on horizontal gene transfer besides the point mutations caused by selective antimicrobial pressure (Takahata *et al*., 2007). They found homologue sequences between the BLNAR strains, *H. influenzae* and *H. haemolyticus* ATCC 33390 (susceptible strain), around the motif where amino acids located in the resistance-associated positions 377, 385 and 389 were encoded. Based on these results, it has also been suggested that *H. haemolyticus* might be a reservoir of antimicrobial resistance determinants for NTHi because common substitutions detected in BLNAR *H. influenzae* strains might form part of the baseline *fts*I sequence of *H. haemolyticus* (Murphy *et al.*, 2007; Takahata *et al.*, 2007; Witherden and Tristram, 2013). On the other hand, they also showed that primary mutations involved in β-lactam resistance (R517H and N526K) have emerged from point mutations under selective antibiotic pressure because there were no homologue sequences between *H. influenzae* and *H. haemolyticus* downstream of the motif where these amino acids are encoded (Takahata *et al*., 2007). More studies are needed to evaluate the role of *H. haemolyticus*

as a reservoir of antimicrobial resistance determinants and also the effect of R517H, N526K and other substitutions on the PBP3.

Resistance to fluoroquinolones

Fluoroquinolones are the second most commonly used antimicrobial group in the treatment of NTHi infections in adult patients. Although these antimicrobials show good activity against *H. influenzae* and the susceptibility rate is very high, resistant isolates have been detected worldwide since 1993 (Biedenbach *et al*., 2003; Bootsma *et al*., 1997; Elliott *et al*., 2003; Kuo *et al*., 2014; Nazir *et al*., 2004; Shoji *et al*., 2014; Vila *et al*., 1999). As fluoroquinolones are only used in adult therapy, the majority of resistant isolates were collected from patients with chronic lung diseases, usually elderly patients receiving frequent fluoroquinolone treatments (Barriere *et al*., 1993; Campos *et al*., 1996; Vila *et al*., 1999; Yokota *et al*., 2008). Although a Taiwanese study recently published an increase from 2% to 24.3% in the fluoroquinolone resistance rate (Kuo *et al*., 2014), the worldwide prevalence of fluoroquinolone-resistant isolates remains low (<2%) (Biedenbach *et al*., 2003; Critchley *et al*., 2007; Perez-Trallero *et al*., 2010; Shoji *et al*., 2014). In accordance with previous data, in our study we found a low percentage of fluoroquinolone-resistant isolates (0.39%) although it represented twice the prevalence previously published in Spain (0.2%) (Perez-Trallero *et al*., 2010).

Even though the majority of fluoroquinolone-resistant *H. influenzae* have been isolated from adults, in Hong Kong isolates with decreased susceptibility have also been described in children (Ho *et al*., 2004). As fluoroquinolones are not approved for paediatric treatment, the authors proposed three possible explanations for these isolates: i) the approved use of nalidixic acid in paediatric treatment of urinary tract infections; ii) exposure to residues of fluoroquinolones in food from animals treated with these antimicrobials; and iii) cross-transmission from adults to children (Ho *et al*., 2004). In our study of colonization in children we did not find any fluoroquinolone-resistant isolates, and to the best of our knowledge, fluoroquinolone-resistant *H. influenzae* isolates from children have not been described in Spain.

In *H. influenzae*, fluoroquinolone-resistance is due to point mutations in the quinolone resistance-determining regions (QRDRs) of the genes encoding DNA gyrase

and topoisomerase IV, the main targets of these antimicrobials. These mutations are translated into amino acid changes in the positions 84 and 88 of GyrA and ParC which have been associated with resistance (Georgiou *et al*., 1996). Resistance is a consequence of an accumulation of mutations in *gyrA* and *parC* which have been acquired gradually in a stepwise way. Thus, strains with first mutations in *gyrA* and *parC* present a susceptible phenotype or a low resistance level whereas strains with three or more mutations in the QRDRs show high level resistance (Georgiou *et al*., 1996; Li *et al*., 2004). In our study, we found clinical isolates that showed both phenotypes: 12 isolates with one or two mutations had low level resistance to ciprofloxacin and levofloxacin (0.12 mg/L-2 mg/L), and 15 isolates with three or more mutations presented high resistance to ciprofloxacin and levofloxacin (4 mg/L-16 mg/L). With regard to specific substitutions, we observed the amino acid alterations in GyrA (S84Y or L and D88Y, N or G) and ParC (S84I or R and E88K) previously reported elsewhere (Bastida *et al*., 2003; Brenwald *et al*., 2003; Georgiou *et al*., 1996; Hirakata *et al*., 2009; Ho *et al*., 2004; Nakamura *et al*., 2009; Perez-Vazquez *et al*., 2004; Shoji *et al*., 2014; Vila *et al*., 1999; Yokota *et al*., 2008).

Despite the fact that fluoroquinolones showed good antimicrobial activity against *H. influenzae*, two case reports of treatment failure have been described in patients with respiratory infections (Bastida *et al*., 2003; Vila *et al*., 1999). Due to the gradual acquisition of mutations under antimicrobial pressure, it is important to detect isolates that present first mutations because, although they present a susceptible phenotype, they can easily become resistant (Li *et al*., 2004). Unfortunately, these strains are under-recognized in the laboratory routine; for this reason, some authors have suggested the use of nalidixic acid as an indicator of reduced fluoroquinolone susceptibility (Brenwald *et al*., 2003; Corkill *et al*., 1994; Dalhoff, 2012). In order to detect these isolates, we performed a screening with nalidixic acid disk in all isolates collected from 2010 to 2013 with a ciprofloxacin inhibitory zone between 21-28 mm. As a result, we found five isolates susceptible to ciprofloxacin but resistant to nalidixic acid that presented changes in GyrA and/or ParC which we would have missed using ciprofloxacin and CLSI interpretation. Although the reported cases of fluoroquinolone treatment failure are scarce, the detection of these isolates is important because fluoroquinolones may not be the most appropriate therapy in these patients.

Regarding fluoroquinolone resistance in *H. haemolyticus*, few data have been reported. Hotomi *et al*. published antimicrobial susceptibility data in *H. haemolyticus* isolated from patients with acute pharyngotonsillitis where the levofloxacin MIC varied from <0.06 to 2 mg/L (Hotomi *et al*., 2010) but did not present molecular data on these strains. In our study (**Paper 6**), we found four isolates that presented alterations in GyrA and/or ParC associated with an increased ciprofloxacin MIC (0.5-1 mg/L). The alterations found in *H. haemolyticus* were the same as the ones found in *H. influenzae* (S84L in GyrA and S84I in ParC).

Biofilm formation

The aim of the third part of this thesis was to study biofilm formation in NTHi isolates from healthy carriers and from the most common infections caused by this pathogen. Because of the involvement of biofilm structures in chronic infections, we designed several studies to determine the implication of biofilm in NTHi and to find factors that could be involved in this formation. The results of these studies are presented in **Papers 7** and **8**.

Survival in the environment is one of the most challenging tasks for microorganisms. In order to colonize and persist inside the human body, microorganisms have to evade the immune system, and, if they cause infection, they have to be able to resist the antimicrobial therapy. To survive in this hostile environment they have evolved several mechanisms for tolerating these adverse conditions; among them biofilm formation (Costerton *et al*., 1999). Biofilms involved in clinically relevant infections can grow either on natural surfaces such as teeth, heart valves, lungs, middle ear and wounds, or on artificial devices such as catheters and stents (Hoiby *et al*., 2011). Once the microorganism has formed the biofilm, the structure is difficult to eliminate despite antimicrobial treatment and the attempts of the immune system to clear the infection; for this reason, biofilm-associated infections commonly show recurrent symptoms (Costerton *et al*., 1999).

In *H. influenzae*, biofilm communities have been observed directly by microscopy in chinchilla models of otitis media (Ehrlich *et al*., 2002; Post, 2001) and in tympanostomy tubes from children with otorrhea (Post, 2001). Furthermore, there is evidence that biofilm is involved in different respiratory infections such as otitis media, adenotonsillitis, COPD and cystic fibrosis (Bakaletz, 2012; Galli *et al*., 2007; Murphy *et al*., 2005; Starner *et al*., 2006). However, some authors consider that there is insufficient evidence to affirm that NTHi form biofilm, mainly because they lack a specific polysaccharide associated with the extracellular matrix (Moxon *et al*., 2008).

In order to analyze the biofilm formation on clinical NTHi isolates recovered from various infections, we designed a study using 111 NTHi isolates from patients with CAP, COPD, otitis media and healthy children to obtain a preliminary impression

of biofilm formation in clinical isolates. The study was performed in this large collection of isolates because all the previously published biofilm studies used a reduced number of strains. The results of this study, described in **Paper 8**, showed different amounts of biofilm depending on the clinical source, with isolates from otitis media (OM) presenting increased levels of biofilm formation compared with isolates from COPD, carriers and CAP. The results differed from those reported by Murphy *et al*. who found no relationship between biofilm formation and the clinical source of the sample (Murphy *et al*., 2002). Despite the reduced number from COPD and OM patients in Murphy's study, and because these results had not been described before, we decided to corroborate our results by expanding the study with a larger number of strains and including isolates collected from invasive infections. In this new study, described in **Paper 7**, we included 358 isolates collected from CAP, COPD, invasive infections, OM and healthy carriers. Moreover, we decided to include studies of initial adhesion since this is the first step in biofilm formation.

Initial adhesion is considered as the first interaction between bacteria and the surface. This step is caused by physicochemical forces between the two surfaces and it is reversible (Dunne, Jr., 2002). Results from *in vitro* bacterial adhesion studies have generated controversy because of the methodology used in these experiments (Vesterlund *et al*., 2005). With techniques involving washing steps (rinsing and dipping), an unpredictable number of adhering bacteria are detached, leading to non-reproducible results (Gomez-Suarez *et al*., 2001). Consequently, we used the Biofilm Ring Test® technology, a technique based on the adhesion of bacterial cells to the wells wich interferes with the movement of metallic beads when magnetic attraction forces are applied, preventing these beads from migrating to the centre of the well. Depending on the strength of bacterial adhesion, the beads will or will not be attracted to the bottom of the wells. This test allows the measurement of the initial adhesion without any washing steps, avoiding bacterial removal and permitting more reproducible results (Chavant *et al*., 2007). The second step of adhesion is the anchoring process and involves more specific binding between bacterial structures such as adhesins and the surface. Furthermore, during this step bacterial cells start to bind with each other, forming aggregates. At the end of this process, attachment between bacteria and the surface becomes irreversible and the formation of a mature biofilm structure begins

(Dunne, Jr., 2002). After 24h of bacterial growth, a mature biofilm is formed and this structure presents an irreversible attachment to the surface (Donlan, 2002). In this case, we used crystal violet staining to quantify the biofilm; in spite of the washing steps, the biofilm is not removed due to its strong attachment to the surface.

In the second study, we again found a clear difference in biofilm formation between isolates collected from different clinical sources. OM isolates presented the highest levels of adherence and a high level of biofilm formation, whereas isolates from CAP patients presented the lowest levels for both measures, corroborating our previous results. Interestingly, we found that isolates collected from invasive diseases presented the highest level of biofilm formation. It has already been described that acute infections are caused by bacteria in their planktonic state, while bacteria in biofilm structures are more associated with chronic infections and colonization (Donlan, 2002; Hoiby *et al.*, 2011). Accordingly, we observed that CAP isolates formed less biofilm than isolates from chronic infections (COPD and OM) and colonizers. However, isolates from invasive acute diseases such as bacteraemia or meningitis showed the highest level of biofilm formation. To explain these results we suggested that biofilm structures could help these isolates to attach to the host cell surface and facilitate the translocation into the bloodstream. Indeed, a previous report performed with Hib strains described that a fimbrial structure was responsible for the adhesion to heparin-binding extracellular matrix proteins, highly expressed in the meninges, which may play an important role in the pathogenic steps involved in the development of meningitis (Virkola *et al*., 2000). Although this study was performed with Hib strains, NTHi can also express this structure; therefore, its expression by NTHi may also be involved in the translocation into the blood or other locations.

Furthermore, we found different initial adhesion and biofilm formation patterns among the groups of isolates. Generally, isolates from COPD, carriers, OM and invasive infections showed a major pattern of fast adhesion and biofilm formation. Specifically, half of the isolates from COPD and carriers showed fast adhesion to the surface and strong biofilm formation, and approximately 30% of the isolates presented a strong biofilm despite showing a slow initial adhesion. On the other hand, isolates from OM had a predominant fast adhesion and strong biofilm formation pattern whereas

invasive isolates produced strong biofilm independently of the speed of initial adhesion. By contrast, isolates from CAP showed a slow initial adhesion to the surface independently of the amount of biofilm formed after 24 hours. This difference in adhesion could be explained by differential expression of adhesins on the bacterial surface in the different isolates from the studied groups, a hypothesis supported by the report from Bakker *et al*. who described different adhesion patterns in bacteria isolated from different niches (Bakker *et al*., 2004). We show that although adhesion is the first step in biofilm formation, there is no relationship between the speed of initial adhesion and biofilm formation after 24h of bacterial growth. These results have been previously observed by other authors who reported that the capacity to form mature biofilms could not be predicted by initial adhesion (Cerca *et al*., 2005; Simoes *et al*., 2010).

To determine the molecular nature of biofilm formation in NTHi, we designed an experiment to establish involvement of sugar components and proteins in biofilm formation. In this experiment we tested 150 clinical isolates from different clinical sources (carriers, CAP, COPD, OM and invasive). After 24h of growth, the attached biofilms were treated with sodium metaperiodate which degraded sugar components in the matrix, or with proteinase K which degraded proteins. The results are described in **Paper 7**.

Our results suggested that proteins play an important role in biofilm formation in NTHi because all the isolates were sensitive to proteinase K activity. These results supported previously published data based on a small number of NTHi isolates where protein adhesins were important in intracellular adhesion and cohesion of the biofilm structure (Izano *et al*., 2009). As shown by Izano *et al*., the biofilm structure did not depend on polysaccharides because the treatment with sodium metaperiodate did not produce biofilm detachment. Moreover, those authors also reported that the DNA was an important compound of the NTHi biofilm matrix and that cells inside the biofilm were more resistant to various detergents, antiseptics and disinfectants (Izano *et al*., 2009). However, after a revision of the published literature about biofilm in NTHi, Moxon *et al*. concluded that there is not enough evidence to affirm that NTHi is able to form biofilm (Moxon *et al*., 2008).

Several factors have been implicated in biofilm formation by *H. influenzae*, such as expression of type IV pili and other adhesins, sialylation and PCho addition to the LOS, production of extracellular DNA and quorum sensing (Swords, 2012a). In the case of PCho, this is a small molecule commonly found on the surface of a huge variety of organisms which infect humans (Clark *et al*., 2013). However, microorganisms cannot synthesize choline; they take it from the host and use it as a nutrient, as an osmoprotectant and as a mechanism of immune system evasion (Clark *et al*., 2013). *H. influenzae* is one of the pathogens that acquire choline from the host; after phosphorylation, PCho is incorporated into its LOS structure (Fan *et al*., 2003). This incorporation is subject to phase variable mechanisms of expression which results in a high phenotypic variability, allowing the bacteria to adapt fast to the changing environment (Weiser *et al.*, 1998). The $PCho^{+}$ phenotypes are selected in colonization because these variants confer an advantage for evading the immune system (Clark *et al*., 2012); in contrast $PCho^{-}$ phenotypes have an advantage in blood or other niches where the C-reactive protein (CRP) is present because PCho is recognized and bound by CRP, activating the classical complement pathway (Humphries *et al*., 2002).

A positive correlation between addition of PCho into the LOS and biofilm formation has been reported. Hong *et al*. reported a correlation between PCho and the establishment of stable biofilm *in vitro* and *in vivo* in three variants of the NTHi 2019 strain. In this study, a mutant deficient in the incorporation of PCho presented decreased biofilm formation, whereas a mutant with increased PCho incorporation showed increased biofilm formation compared to the wild-type strain (Hong *et al*., 2007b). The same results were reported in other studies with different NTHi strains where the deficient mutants showed decreased biofilm (Hong *et al*., 2007a; Morey *et al*., 2013). As only one strain of NTHi and its respective mutants was studied, we decided to focus on the incorporation of PCho into the LOS and tested the involvement of PCho in biofilm formation in a collection of 111 clinical isolates of NTHi isolated from different clinical sources (CAP, COPD, OM and healthy carriers). The results of this study were reported in **Paper 8**.

Our results of PCho measurement in planktonic cells showed a higher level of PCho incorporation in isolates from carriers and OM than in isolates from CAP and

COPD. These results are consistent with the reported data, which have shown that PCho had a role in the colonization process and promoted OM infection (Poole *et al*., 2013; Tong *et al*., 2000; Weiser *et al*., 1998b). On the other hand, the lower level of PCho found in isolates from CAP and COPD could be attributed to the fact that a decreased incorporation of PCho might help to prevent complement mediated-killing due to CRP (Gao *et al*., 2013). Nevertheless, contrary to previously published data (Hong *et al*., 2007b) we could not show a correlation between PCho incorporation in planktonic cells and biofilm formation using an *in vitro* static assay methodology. In order to verify our results, we performed experiments based on the measurement of PCho within the biofilm because it has been reported to be higher than in planktonic cells (West-Barnette *et al*., 2006). Although we found high levels of PCho in biofilm, no correlation between PCho and biofilm was observed. A role of PCho has been reported in biofilm maturation (Hong *et al*., 2007b) whereas no effects have been showed in biofilm initiation (West-Barnette *et al*., 2006). Furthermore, different methodologies are used to test biofilm based on static and continuous flow assays, so it is likely that the technique might influence the outcome.

We performed additional experiments to evaluate the role of PCho in biofilm formation in clinical isolates. NTHi can incorporate the PCho in different positions of the LOS, in either Heptose I or Heptose III, in order to facilitate the evasion of the immune system (Clark *et al*., 2012; Lysenko *et al*., 2000a). However, its involvement in biofilm formation has not been studied before. For this reason, we tested the biofilm formation in four strains that incorporated the PCho in the different locations in the LOS: strain Rd (phase-variable incorporation of PCho on HepI), H446 (constitutively $PCho^-$), H457 (PCho on HepIII), and H491 (constitutively $PCho^+$ on HepI). We did not find differences in biofilm formation between these strains, thus proving that the position of the PCho in the LOS did not affect the biofilm formation capacity.

In order to address the role of PCho in biofilm formation, we constructed five *licA* mutants that were unable to incorporate PCho. We ensured that the growth of these mutants had not been affected by the deletion of this gene in order to verify that the changes observed between mutants and the wild-type strains were due to the incorporation of PCho. Our results showed a strain-specific variability between the

mutants and the wild-type strains; some mutants formed more biofilm than the wild-type, others less, and others the same amount. Thus, we determined a strain-dependent relationship between PCho and biofilm formation. These differences between strains may be due to the fact that the depletion of PCho might modify the LOS structure in terms of size and charge, affecting bacterial adhesion and biofilm formation.

Overall, the studies reported in this thesis have expanded the understanding of the molecular epidemiology of NTHi in different hosts such as colonized children and adults with different invasive and non-invasive infections. Furthermore, our findings also extend the knowledge of the antimicrobial susceptibility of different clinical NTHi isolates and the molecular mechanisms behind this resistance. Finally, although many questions in the biofilm formation by NTHi remain unresolved, our results have also contributed to clarifying the process of biofilm formation in this pathogen.

CONCLUSIONS

V. CONCLUSIONS

1. Children attending Day-Care Centres (DDC) were heavily colonized by *H. influenzae*, especially NTHi. Due to the high coverage of the conjugate vaccine, serotype b strains were not found in the children's oropharynx. The colonization by other encapsulated strains, in this case serotype f, was very low.

2. The high diversity of genotypes found in isolates from the same centre indicated that the acquisition of strains also occurred outside the DDC. Children sampled in both years did not harbour the same strain, suggesting short time persistence and a high strain replacement.

3. *H. influenzae* was the second cause of acute exacerbations in patients with severe or very severe COPD and it was associated with patients with one exacerbation episode per year. Simultaneous colonization by different *H. influenzae* strains was not frequent in those patients.

4. COPD was the most common underlying disease in non-bacteraemic community-acquired pneumonia (CAP) whereas malignancies were mostly identified in bacteraemic CAP. The 30-day mortality rate was higher in patients with bacteraemic CAP; this result may be due to the different underlying conditions of the patients.

5. High genetic diversity was observed among NTHi isolates from bacteraemic and non-bacteraemic CAP. However, the identification of some small clusters of strains suggested the dissemination of minor clones.

6. Pneumonia is the main focus of NTHi invasive disease. Invasive infections caused by encapsulated strains were rare, and mainly caused by serotype f isolates.

7. Unlike NTHi, encapsulated strains were clonally related and belonged to worldwide detected international clonal complexes: serotype f isolates belonged to CC124, serotype b isolate to CC6, and serotype e isolate to CC18.

8. The most predominant mechanism of β-lactam resistance in NTHi was altered PBP3. The vast majority of these isolates were considered low-BLNAR because they presented low-level β-lactam resistance.

9. The frequency of β-lactamase in clinical NTHi isolates remained below 10%, a finding consistent with the overall downward trend observed in Spain. The high rate found in carriers in 2005 was due to a clonal expansion of a β-lactamase producer strain.

10. The isolation of NTHi isolates presenting both mechanisms of resistance to β-lactams (altered PBP3 and β-lactamase production) was scarce. These isolates were mostly defined as low-BLPACR, because they presented low levels of resistance to amoxicillin/clavulanic acid.

11. The rate of fluoroquinolone resistance in NTHi was low and it was associated with accumulative mutations in the QRDRs of *gyrA* and *parC*. Nalidixic acid was a good indicator for detecting isolates susceptible to fluoroquinolones, but with first mutations in their QRDRs.

12. Clinical isolates of *H. haemolyticus* were rare and genetically diverse, and showed similar antimicrobial resistance mechanisms to NTHi: modified PBP3 in β-lactam resistance and accumulative mutations in the QRDRs of *gyrA* and *parC* in quinolone resistance.

13. There was a difference in biofilm formation depending on the type of disease caused by NTHi, with a clearly increased ability to form biofilm in isolates from otitis media and invasive disease.

14. The incorporation of phosphorylcholine in the LOS of clinical NTHi isolates did not predict the ability to establish biofilm *in vitro*, as it affected biofilm formation in a strain-dependent manner.

15. Biofilm stability was dependent on protein interactions, which might be an attractive target for therapies designed to disrupt established biofilms *in vivo*.

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VI. REFERENCES

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