Beatriz da Cunha Batista

Licenciatura em Bioquímica

Characterization and Biofilm Assessment of *Haemophilus influenzae* Isolates from Patients with Chronic Obstructive Pulmonary Disease and Otitis Media

Dissertação para obtenção do Grau de Mestre em Bioquímica

Orientador: Maria Paula Bajanca Lavado, Investigadora auxiliar, Instituto Nacional de Saúde Doutor Ricardo Jorge

Co-orientador: Maria Luísa Forte Marques Jordão, Investigadora auxiliar, Instituto Nacional de Saúde Doutor Ricardo Jorge

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FACULDADE DE CIÊNCIAS E TECNOLOGIA
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Resumo

Haemophilus influenzae é um microrganismo comensal da nasofaringe humana, responsável por doenças invasivas e não-invasivas. Este trabalho focou-se no estudo de 93 estirpes de *H. influenzae*, isolados entre 2013 e 2018, de indivíduos com duas doenças não-invasivas epidemiologicamente relevantes: Doença Pulmonar Obstrutiva Crónica (DPOC) e Otite Média (OM). As estirpes foram caraterizadas, fenotípica e molecularmente, relativamente à presença de cápsula, produção de β-lactamase, suscetibilidade a antibióticos, diversidade genética por MLST, presença/ausência de fatores de virulência (*pilA*, *hifA*, *hmw1A*, *hmw2A*, *hia* e *ompP5*) e a capacidade para formar biofilmes.

A DPOC incidiu numa população adulta (100%), enquanto a OM foi predominante em crianças (98,2%). Verificou-se que o *H. influenzae* não capsulado (HiNC) foi o agente etiológico responsável pela DPOC (97,4%) e OM (100%). Relativamente à susceptibilidade aos antibióticos, há a destacar a produção de β-lactamase em 15,1% das estirpes. O MLST revelou uma elevada diversidade genética, tanto na DPOC como na OM, com a caraterização de 31 STs em 41 estirpes analisadas. Os genes *pilA* e *ompP5* foram identificados em mais de 50% dos isolados de DPOC e OM. Os genes *hifA* e *hia* foram encontrados em menos de metade dos isolados, tendo-se verificado uma maior prevalência na OM. Os genes *hmw1A* e *hmw2A* foram identificados, respetivamente, em 25,5% e 32,7% das estirpes de OM, estando ambos presentes em 76,3% das estirpes de DPOC. A formação de biofilmes foi observada em 14,0% e 29,0% das estirpes após 24h e 48h, respetivamente. Não foi possível estabelecer uma relação entre formação de biofilmes e origem clínica, nem com a presença de fatores de virulência (*pilA*, *hmw1A* e *hmw2A*) envolvidos na produção de biofilmes.

DPOC e OM são doenças frequentemente associadas a HiNC. Não existindo uma vacina disponível, é importante a sua monitorização, pelo impacto social e económico que representam em Saúde Pública.

Palavras-chave: *Haemophilus influenzae*, DPOC, OM, fatores de virulência, biofilmes, HiNC

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Abstract

Haemophilus influenzae is a commensal microorganism of the human nasopharynx, responsible for both invasive and non-invasive diseases. This work focused on the study of 93 *H. influenzae* isolates, collected between 2013 and 2018, from patients with two epidemiologically relevant non-invasive diseases: Chronic Obstructive Pulmonary Disease (COPD) and Otitis Media (OM). Phenotypical and molecular characterizations were performed for the isolates, regarding capsular typing, β-lactamase production, antibiotic susceptibility, genetic diversity by MLST, presence/absence of virulence factors (*pilA*, *hifA*, *hmw1A*, *hmw2A*, *hia* and *ompP5*) and ability to produce biofilms.

COPD isolates were collected from adults (100%), while 98.2% of OM isolates were collected from children. Non-typeable *H. influenzae* (NTHi) was the aetiological agent in COPD (97.4%) and OM (100%). Regarding antibiotic susceptibility, it should be noticed that 15.1% of the isolates were β-lactamase producers. MLST revealed a high genetic diversity among COPD and OM isolates, with 31 STs in 41 analysed isolates. *pilA* and *ompP5* genes were present in more than 50% of COPD and OM isolates. *hifA* and *hia* genes were identified in less than half of the isolates, with a higher prevalence of these among OM isolates. *hmw1A* and *hmw2A* genes were respectively identified in 25.5% and 32.7% of OM isolates, while both *hmw* genes were present in 76.3% of COPD isolates. Biofilm production was observed for 14.0% and 29.0% of all isolates after 24h and 48h, respectively. No relationship between biofilm production and clinical source could be established, as well as with the presence of virulence factors (*pilA*, *hmw1A* e *hmw2A*) involved in biofilm production.

COPD and OM are frequently associated with NTHi. Since no vaccines are available, monitoring of these diseases is highly recommended, as these constitute a Public Health threat associated with a high economic and social burden.

Keywords: *Haemophilus influenzae*, COPD, OM, virulence factors, biofilms, NTHi

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Index

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Index of Figures

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Index of Tables

List of Abbreviations and Acronyms

***adK* –** Adenylate Kinase gene

Am – Ampicillin

AOM – Acute Otitis Media

***atpG* –** ATP Synthase F1 Subunit Gamma gene

Aug – Amoxicillin-K clavulanate

Azi – Azithromycin

BHI – Brain Heart Infusion

BLNAR – β-Lactamase-Negative Ampicillin-Resistant

BLNAS – β-Lactamase-Negative Ampicillin-Susceptible

BLPACR – β-Lactamase-Positive Amoxicillin-Clavulanate-Resistant

BLPAR – β-Lactamase-Positive Ampicillin-Resistant

bp – Base-Pairs

C – Chloramphenicol

CAP – Community Acquired Pneumonia

CEACAM1 – Carcinoembryonic Antigen-Related Cell Adhesion Molecule-1

Cft – Cefotaxime

Cl^- – Chloride Ion

COPD – Chronic Obstructive Pulmonary Disease

Cp – Ciprofloxacin

Cpe – Cefepime

Crm – Cefuroxime

CSOM – Chronic Suppurative Otitis Media

DGS – Direcção-Geral da Saúde

DLV – Double Locus Variant

DNA – Deoxyribonucleic Acid

dsDNA – Double Stranded DNA

ECDC – European Centre for Disease Prevention and Control

EPS – Extracellular Polymeric Substance

EU/EAA – European Union and European Economic Area

EUCAST – European Committee on Antimicrobial Susceptibility Testing

FASTA format – Text format for representation of nucleotide, or peptide, sequences

Fe^{3+} – Ferric Ion

***frdB* –** Fumarate Reductase Iron-Sulfur Protein gene

***fucK* –** Fuculokinase gene

Hap – *Haemophilus* Adhesion Protein

Hgp – Hemoglobulin:Haptoglobolin Binding Proteins

Hia – *Haemophilus influenzae* Adhesin

Hia – Encapsulated *Haemophilus influenzae*, serotype a

Hib – Encapsulated *Haemophilus influenzae*, serotype b

Hic – Encapsulated *Haemophilus influenzae*, serotype c

Hid – Encapsulated *Haemophilus influenzae*, serotype d

Hie – Encapsulated *Haemophilus influenzae*, serotype e

Hif – Encapsulated *Haemophilus influenzae*, serotype f

HifA – Major structural subunit of the fimbria gene cluster *hifABCDE*

HMW – High Molecular Weight

Hsf – *Haemophilus* Surface Fibril

HxuC – Heme-Hemopexin

IgA – Immunoglobulin A

INSA – Instituto Nacional de Saúde Doutor Ricardo Jorge

LOS – Lipooligosaccharide

***mdh* –** Malate Dehydrogenase gene

Mer – Meropenem

MIC – Minimum Inhibitory Concentration

MIC_{50} – Minimum Inhibitory Concentration for 50% of isolates

MIC_{90} – Minimum Inhibitory Concentration for 90% of isolates

MLEE – Multilocus Enzyme Electrophoresis

MLST – Multilocus Sequence Typing

MTS – Minimum Spanning Tree

NAD – Nicotidamide Adenine Dinucleotide

NTHi – Nontypeable *Haemophilus influenzae*

OD – Optical Density

$OD_{cut\text{-}off}$ – Optical Density defined as the Cut-off

$OD_{isolate}$ – Optical Density defined as mean $OD_{570\ nm}$ for each isolate

OD_{nc} – Optical Density of the Negative Control

OM – Otitis Media

OME – Otitis Media with Effusion

OMP – Outer Membrane Protein

PBP – Penicillin-Binding Protein

PCR – Polymerase Chain Reaction

***pgi* –** Glucose-6-Phosphate Isomerise gene

PHiD-CV – 10-valent Pneumococcal Nontypeable *Haemophilus influenzae* Protein-D Conjugate Vaccine

PilA – Major structural subunit of the type IV pilus *pilABCD*

PRP – Polyribosylribitol Phosphate

***recA* –** RecA protein gene

Rif – Rifampicin

SD – Standard Deviation

SD_{nc} – Standard Deviation of the Negative Control

SLV – Single Locus Variant

ST – Sequence Type

T/S – Trimethoprim/Sulfamethoxazole

TBE – Tris-Borate-EDTA

Tbp – Transferrin Binding Protein

TBS – Tris-Buffered Saline

Te – Tetracycline

Tfp – Type IV Pilin

TLV – Triple Locus Variant

TSB – Trypic Soy Broth

USA – United States of America

UK – United Kingdom

WGS – Whole Genome Sequencing

WHO – World Health Organization

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1. Introduction

1.1. *Haemophilus influenzae*

1.1.1. *Pasteurellaceae* family, *Haemophilus* genus and *Haemophilus influenzae*

Pasteurellaceae family belongs to the *Pasteurellales* order and *Gammaproteobacteria* class.[1] It is composed of a wide group of gram-negative opportunistic pathogens and commensal organisms, which have an important impact on human and animal health.[2]

Currently, the family is composed of 26 genera, including *Actinobacillus*, *Aggregatibacter*, *Avibacterium*, *Avibacteriumendocarditidis, Basfia, Bibersteinia, Bisgaardia, Chelonobacter, Cricetibacter, Frederiksenia, Gallibacterium*, *Haemophilus*, *Histophilus*, *Lonepinella*, *Mannheimia*, *Mesocricetibacter, Muribacter, Necropsobacter, Nicoletella*, *Otariodibacter, Pasteurella*, *Phoconobacter, Testudinibacter, Ursidibacter, Vespertiliibacterpulmonis, and Volucribacter*.[3] *Haemophilus*, *Actinobacillus* and *Pasteurella* are considered the three main classic genera.[2]

Of the *Haemophilus* genus, four species possess host specificity for animals (*H. parasuis*, *H. felis*, *H. paracuniculus* and *H. haemoglobinophilus*) and nine for humans (*H. influenzae*, *H. aegyptius*, *H. haemolyticus*, *H. parainfluenzae*, *H. ducreyi*, *H. pittmaniae*, *H. sputorum*, *H. paraphrohaemolyticus* and *H. parahaemolyticus*).[2]

H. influenzae was first described by physician Richard Pfeiffer in 1892. The bacilli were detected during the epidemic of the *influenza* virus, in sputum samples of patients suffering from this infection and was then named as "The *influenza* Bacillus".[4] In 1931, Margaret Pittman detected two different types of colonies grown on agar plates.[5] These two types differentiated in size and opaqueness. One type of colonies was described as large, smooth, mucoid, slightly opaque and iridescent when observed in obliquely transmitted light and were denominated as S (Smooth) isolates; the second type of colonies were smaller, translucent, non-iridescent with transmitted light, with a rough surface, being denominated as R (Rough) isolates. S isolates appeared to be more virulent for laboratory animals and, in all of them, it was detected the presence of a capsule. This finding led to a differentiation of *H. influenzae* into isolates with a capsule, or typeable, and isolates lacking a capsule, also referred to as Nontypeable isolates (NTHi) (Fig. 1.1).

H. influenzae are small gram-negative, coccobacilli, pleomorphic and fastidious bacteria. This bacterium grows optimally with temperature between 35 and 37 ºC, in an atmosphere supplemented with 5–10 % of CO_2. Regarding *in vitro* growth, it is dependent on X-factor and V-factor – also known

as haemin and Nicotinamide Adenine Dinucleotide (NAD), respectively.[6] The X-factor consists of a Ferric Ion (Fe^{3+}) inserted in the centre of a porphyrin ring (protoporphyrin IX), with Chloride Ion (Cl^-) as a ligand.[7] It can be used, optionally, for anaerobic growth. The V-factor is a pyridine nucleotide that possesses important roles in metabolic conversions, such as signal transducers, plays a major role as electron carrier in oxidoreductase reactions and is essential for growth.[8,9]

H. influenzae is an important human-restricted commensal of the nasopharynx. A longitudinal study conducted in Portugal by Sá-Leão and colleagues[10] collected 414 nasopharyngeal samples of children from a day care center, during one year. The authors stated that *H. influenzae* was present in 87% of the nasopharyngeal samples. In addition, all children were colonized with this bacterium, at some point of the study and 34% were persistently colonised, highlighting the importance of this bacterium as a colonizer of the respiratory tract.

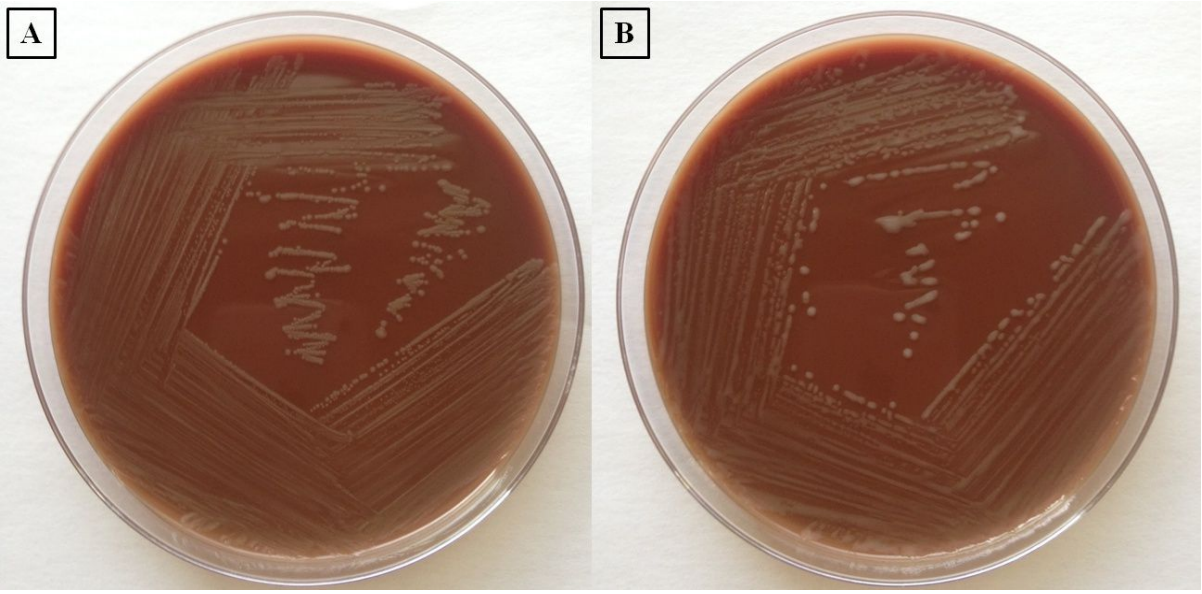

Figure 1.1 | (A) NTHi colonies and (B) encapsulated *H. influenzae* colonies in chocolate agar plates supplemented with polivitex. Both photographs were taken during this Master's Thesis, with an Otitis Media isolate included in the study (A) and an invasive serotype e isolate belonging to the collection of the reference laboratory (B).

Similar to all gram-negative microorganisms, the cell wall of *H. influenzae* is composed of a layer of peptidoglycan – cross-linked by Penicillin-Binding Proteins (PBPs) – that confers strength, resistance against antibiotics and protects bacteria against lysis due to variations in osmotic pressure. Outer and inner membranes also protect bacteria against antibiotics, by hampering them from reaching the cytoplasm, where these could interfere with protein synthesis. These membranes serve as barriers of outer, as well as inner permeability.[11] Lipooligosaccharides (LOS) are endotoxins present on the bacterial cell wall and are fundamental for bacterial adhesion.[12] Although *H. influenzae* lacks flagella,

NTHi isolates commonly possess pili structures, which have been associated with twitching motility (non-flagellar based type IV pilin-dependent motility).[13]

1.1.2. *Haemophilus influenzae* serotypes

H. influenzae can be classified as encapsulated, or non-encapsulated based on the production of a capsular polysaccharide.

Encapsulated *H. influenzae* isolates are divided into six distinct serotypes, a, b, c, d, e, f – also referred to as Hia, Hib, Hic, Hid, Hie and Hif – based on the production of different capsular polysaccharides. Capsular polysaccharides confer virulence by avoiding complement system lysis.[14] *H. influenzae* capsular serotypes may be organised into three groups, each composed of two serotypes, based on both the structures of the polysaccharides and an association with their resistance to antibody-free complement lysis effect.[14] Types b and a were considered the most virulent, being composed of a phosphodiester, a neutral sugar and an alcohol (ribitol). Types c, d, e and f were much less virulent, with types c and f being considered of intermediate complement resistance and composed of a phosphodiester, a N-acetylated amino sugar and a monosaccharide. Finally, types d and e possessed the least resistance to complement action and have a repeat unit of a N-acetylmannosamine uronic acid and N-acetylglucosamine.[14] Encapsulated isolates are thus more virulent and tend to be associated with invasive disease, such as septicaemia and meningitis.[6]

Although NTHi isolates lack a polysaccharide capsule, they can be differentiated by Outer Membrane Proteins (OMPs), LOS and High Molecular Weight (HMW) protein profiles, among other virulence factors, such as Hif and Hap adhesins, for instance.[15] NTHi isolates are usually associated with non-invasive diseases, of the upper (e.g.: Otitis Media [OM] and sinusitis) and lower respiratory tract (e.g.: chronic obstructive pulmonary disease [COPD] and cystic fibrosis).[16]

1.1.3. Molecular typing

1.1.3.1. Capsular typing

Capsular typing by molecular methods is considered the most accurate technique to differentiate NTHi from encapsulated isolates and for identification of *H. influenzae* serotypes.[17] The region of the chromosome of *H. influenzae* responsible for capsule expression is named the "*cap* locus", which is described in all six capsular serotypes. It is composed of three different regions: 1, 2 and 3.[18] Regions 1 and 3 are common to all serotypes and are composed of a highly conserved cluster

of genes, essential for processing and exporting capsular material to the cell surface. These two regions flank a serotype-specific region, region 2, which appears to be unique to each capsular serotype and contains genes involved in the synthesis and polymerization of the capsular polysaccharide (Fig. 1.2).[18,19]

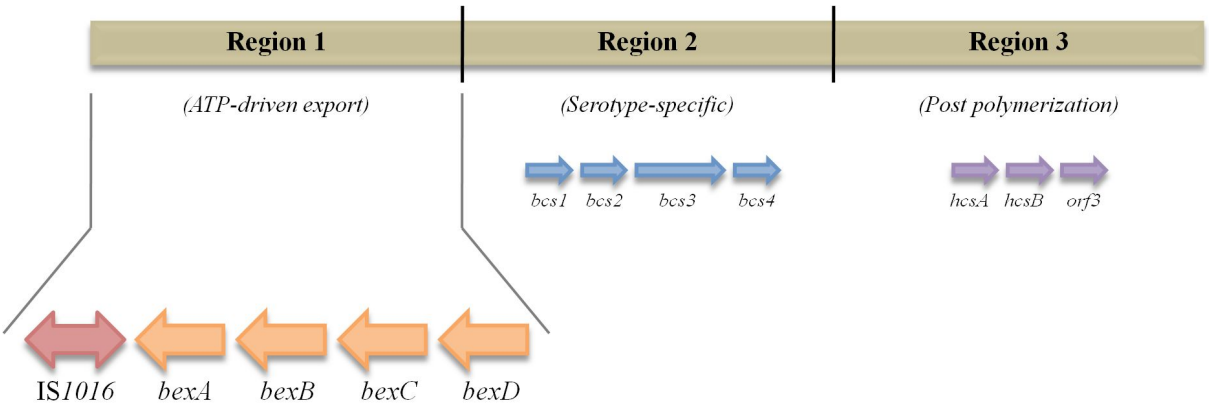

Figure 1.2 | Schematic presentation of the *cap* b locus. *bex* genes are located in region 1 of the *cap* locus, downstream from insertion element IS1016. Region 2 possesses capsule-specific genes and region 3 is responsible for post polymerization. (adapted from[19])

Most Hib isolates usually possess a partial duplication of a DNA fragment. This duplication includes two copies of regions 2 and 3, one copy of region 1 and a truncated copy of region 1 with a deletion between the insertion element IS*1016* and *bexA* sequences.[19] However, these isolates may undergo a recombination event that leads to the loss of the complete copy. The truncated copy would remain, with a loss of part of the coding sequence located downstream of the start of functional *bexA* gene, which is essential for the exportation of the capsular polysaccharide to the cell surface. This results in a capsule-deficient mutant, referred to as b^- mutant, or Hib-minus.[19] Falla and colleagues have developed a Polymerase Chain Reaction (PCR) capsular serotyping method that has been used as a standard protocol for typing *H. influenzae*.[20] This method distinguishes NTHi isolates from encapsulated ones, by amplification of *bexA* gene, which is exclusively present in encapsulated isolates. The authors were able to obtain an amplification product of 343 base-pairs (bp), confirming the presence of this gene in encapsulated isolates. In addition, the authors further designed type-specific primers to all six capsular serotypes enabling a differentiation of each capsular serotype.

1.1.3.2 Multilocus Sequence Typing

H. influenzae genetic diversity studies have been conducted since the 1980's. Musser and colleagues[21] showed that encapsulated isolates tended to be phylogenetically related in a limited

number of clusters according to each serotype, which suggested that isolates belonging to a certain serotype show limited genetic diversity. The authors hypothesised that since *H. influenzae* is a naturally competent microorganism, the capsule may serve as a barrier for the uptake of extracellular DNA, which probably contributes to the clonal structure of encapsulated isolates. In fact, genetic diversity in NTHi isolates is mostly due to horizontal genetic exchange.[12]

NTHi isolates have been shown to be electrophoretically distant from serotype b and even Hib-minus isolates, presenting a very heterogeneous population, unlike encapsulated population. Considering NTHi heterogeneous population structure, Chiara and colleagues[15] were able to organize NTHi population into six different clades (I-VI) according to the presence/absence of six virulence factors, supporting a capacity of clonal evolution in the NTHi population.

Initial studies were mostly conducted by Multilocus Enzyme Electrophoresis (MLEE), with a main focus on the electrophoretic mobility patterns of major OMPs. However, despite of the elucidations provided by MLEE regarding the clonal structure of the encapsulated population and great diversity of NTHi isolates, an alternative method – Multilocus Sequence Typing (MLST) – was developed as a standard for studies of genetic diversity in *H. influenzae* species.

MLST aimed to address two major issues related to epidemiological surveillance: first, to understand if the isolates collected from a certain outbreak were equal, or different from the one that started the outbreak. Second, to know if a possible correlation between isolates causing a disease in a specific geographic area and worldwide collected isolates could be established.[22] Therefore, a focus was turned into identifying alleles in the nucleotide sequences of housekeeping genes. Considering that MLST provided analysis of nucleotide sequences instead of enzymatic electrophoretic patterns, it enabled the identification of more genetic variations, than MLEE. Furthermore, this technique permitted comparisons of sequences data between laboratories, due to the existence of worldwide databases.[23] MLST analyses internal fragments of 450–500 bp from seven selected housekeeping genes. Each gene has its sequence assigned to an allele number and the alleles at the seven loci provide an allelic profile, which in turn defines the Sequence Type (ST) for each isolate. Since the accumulation of nucleotidic changes in housekeeping genes is considered a slow process, the allelic profile of a certain bacterial isolate is stable enough in time, allowing this method to be regarded as ideal for epidemiologic studies.[22] The STs are then displayed in a dendrogram that enables to establish a correlation between identical, or highly similar, allelic profiles of isolates. Thus, a phylogenetic relation may be inferred.[24]

In the specific case of *H. influenzae*, Meats and colleagues[23] selected Adenylate Kinase (*adK*), ATP Synthase F1 Subunit Gamma (*atpG*), Fumarate Reductase Iron-Sulfur protein (*frdB*), Fuculokinase (*fucK*), Malate Dehydrogenase (*mdh*), Glucose-6-Phosphate Isomerise (*pgi*) and RecA protein (*recA*) genes that were separated in the genome by a minimum of 120 kb, with the exception of *recA* and *fucK* (only separated by 22 kb). The sequences for each allele are uploaded to

Haemophilus influenzae MLST Database (*https://pubmlst.org/hinfluenzae/*), where a number is assigned to each allele and an ST to each isolate profile.[23]

1.2. *Haemophilus influenzae* infections

H. influenzae is a human-restricted commensal microorganism found in the nasopharynx of the respiratory tract, which makes it an ongoing source of potential infections in the upper and lower respiratory tract, as it is the case of OM, COPD, pneumonia, cystic fibrosis and bronchitis, in addition to invasive diseases.[25]

1.2.1. Invasive disease

In 2018, the European Commission[26] updated the definition of invasive disease caused by *H. influenzae* as the isolation of this bacterium, or its nucleic acids from a clinical sample of a biological fluid considered sterile, such as blood, or cerebrospinal fluid, for instance. In Europe, the Surveillance Report of the European Centre for Disease Prevention and Control (ECDC) showed that the distribution of invasive *H. influenzae* disease tends to follow a seasonal pattern and the highest number of reported cases seems to occur during winter months, with a great decrease in August. From this month on, the number of cases increases until the end of the year.[25,27]

1.2.2. Non-invasive disease

1.2.2.1. Chronic Obstructive Pulmonary Disease

World Health Organization (WHO) has defined COPD as a disease that can be characterized by an airflow limitation, which is usually progressive and associated with alveolar or airway abnormalities of the lungs to certain noxious gases and particles. Patients with this disease usually present a chronic inflammation of the airways and parenchymal destruction.[28] Inflammation, pathological changes in the peripheral airways and lesions of the lungs are known to be induced by cigarette smoke, in addition to inhaled toxicants and possible occupational exposures to dust and smoke.[28,29] Chronic inflammation of bronchi and bronchioles leads to the development of emphysema

and alteration of the airways and vasculature. Airway obstruction and tissue destruction ultimately reduce the capacity of lungs for gas exchange.[30]

COPD is currently the fourth leading cause of worldwide death and it is estimated to rank as third in 2020.[28] According to WHO, there was a prevalence of 251 million worldwide cases of COPD in 2016 and 3.17 million deaths were caused by this disease in 2015.[31] Additionally, this organization stated that despite of COPD being previously more common in men than women, due to increased tobacco smoking among women (in high-income countries) and a higher risk of exposure to indoor pollution (in low-income countries), this disease now equally affects both genders.[32]

Exacerbations are changes in the baseline dyspnea, sputum and/or cough of the patient that goes beyond the usual variability.[33] Interestingly, it is estimated that approximately 50% of all COPD exacerbations are actually caused by bacterial infections, being NTHi, along with *Streptococcus pneumoniae* and *Moraxella catarrhalis*, the most frequent aetiological agents.[34,35] NTHi may be present in both stable and exacerbated states and it has been shown that patients colonised with this bacterium in the stable state tend to present more symptoms and sputum purulence during an exacerbation than non-colonised patients. Additionally, these patients present more cough, may take a longer period of time to recover from a peak flow at an exacerbation and present a higher exacerbation frequency.[36] Furthermore, a patient can be colonised by more than one isolate of NTHi and acquisition of a new isolate is related to occurrence of an exacerbation. Sethi and colleagues[37] have demonstrated that, in the majority of cases, after an exacerbation related to NTHi, serum antibodies are produced specifically to target that newly acquired isolate. However, this immunological response is only efficient against homologous isolates, and has no effect in newly acquired heterologous isolates. The authors postulate that these results may elucidate a mechanism that explains recurrent exacerbations, in COPD patients, in the presence of *H. influenzae*.

Given the significant role of bacteria in exacerbations, most of the patients are treated with antibiotics. However, since clinical features do not distinguish between bacterial and non-bacterial exacerbations, the benefits of the treatment would not be as successful in non-bacterial exacerbations.[38] In Portugal, empirical treatment recommended is amoxicillin, with or without clavulanic acid, macrolides, or doxycycline. However, it is recommended that antibiotics against COPD should only be taken by a patient previously confirmed to have bacterial exacerbations and not by a patient in a stable-state.[39]

1.2.2.2. Otitis Media

Otitis Media (OM) are inflammatory and infectious conditions that affect the middle ear. Infection occurs when bacteria migrate, through the Eustachian tube, from the nasopharynx to the middle ear, which is an event usually triggered by a viral infection.[40]

OM may be manifested either by acute, or chronic episodes. Three of its most common conditions are Acute Otitis Media (AOM), Otitis Media with Effusion (OME) and Chronic Suppurative Otitis Media (CSOM). AOM may be caused by either viruses or bacteria and common symptoms include otalgia and fever.[41] OME, on the other hand, is a chronic inflammation.[41] Common symptoms are glue ear fluid (presence of fluid in the middle ear space behind the eardrum) with absence of acute signs, and hearing loss is a possible consequence.[42] CSOM is a chronic inflammation characterized by suppurative middle ear infection established for a long period of time and with perforation in the tympanic membrane, in most of the cases.[41]

In 2012, Monasta and colleagues[43] conducted a review with data from 1980 to 2008 with relevant worldwide information regarding AOM and CSOM. From the information they gathered, the authors observed that the global AOM incidence rate is estimated as 709 million cases per year, with 51% of the cases being registered in children under the age of five. In Central Europe, 40% of the cases occurred in children with ages ranging from less than one to five years old. CSOM, on the other hand, registered 31 million cases each year, with 22.6% of these cases occurring in children under the age of five years old. As for mortality rates, the authors estimated that approximately 21,000 people die, each year, due to complications related with OM. The authors stated that, although the mortality rates associated with OM are relatively low, when considering the overall combination of AOM and CSOM, plus respective sequels, the numbers should be considered relevant, more specifically in the first five years of life.[43]

The most common treatment against AOM, in Portugal, is antibiotic administration. Amoxicillin, or amoxicillin-clavulanate are the elected choices for treatment of OM infections. Second, or third, generation cephalosporins may be an alternative in the case of ineffectiveness of the first-line antibiotic, or for penicillin allergic children.[44]

A 10-valent pneumococcal nontypeable *Haemophilus influenzae* protein-D conjugate vaccine – PHiD-CV (Synflorix™) – was developed against *H. influenzae* and 10 serotypes of *S. pneumoniae*.[45] Using protein D as carrier relates to it being a surface protein mostly conserved in both encapsulated and NTHi isolates, serving as antigen.[45,46] Thus, this vaccine contains 8 serotypes of *S. pneumoniae* individually conjugated to a non-lipidated recombinant form of protein D, one serotype conjugated with tetanus toxoid and the remaining with diphetheria toxoid. A protective effect of protein D as carrier was demonstrated in a predecessor study of an 11-valent vaccine that showed a reduction of 57.6% and 35.3% of AOM episodes caused by *S. pneumoniae* and NTHi, respectively. The effectiveness of the 10-valent vaccine was then expected to be similar to that of the 11-valent vaccine.[45,46]

1.3. Epidemiology

Due to the severity of invasive diseases, most epidemiologic studies available in literature are focused on these. Limited information is available regarding non-invasive diseases caused by *H. influenzae*. Therefore, the surveillance data here presented are related to invasive disease reports.

Conjugated Hib vaccine induces bactericidal antibodies to respond against Polyribosylribitol Phosphate (PRP), a capsular polysaccharide, regarded as a major virulence factor.[47]

In the pre-vaccine era, serotype b was one of the major causes of meningitis in children, particularly under five years old.[48] In the United Kingdom (UK), a survey conducted in this period[49] revealed that from all *H. influenzae* reported cases, 82% were caused by Hib, 88% were registered in children under five years old, and meningitis was the most common infection. In the United States of America (USA), 3–6% of infected children would die and in 20–30% of survivors, permanent mental retardation, or mild hearing loss were common sequels.[47]

Hib vaccines were first available in the European Union and European Economic Area (EU/EEA) in 1989.[48] WHO reported that Hib vaccine is currently used as part of the routine immunization programme in 192 countries,[50] including all EU/EEA member states.[25]

In the post-vaccine era, although a decrease of infections caused by Hib was observed, invasive disease caused by NTHi increased. Among encapsulated isolates, a major incidence has been reported for serotype f, in Europe.[25]

An epidemiologic study comprising the years of 2007 and 2014 in 12 European countries[51] showed that, overall, 78% of invasive cases were caused by NTHi and low notification rates for Hib supported the efficacy of Hib vaccine. The authors highlighted that these trends may be a result of improved surveillance programs, physicians awareness and even better serotyping techniques that were not available in the pre-vaccine era.

In 2015, ECDC reported 3,162 cases of invasive disease, caused by *H. influenzae*, confirmed in 30 European countries (Fig.1.3). Invasive cases were reported, mostly in children under the age of one and in elderly people over 65 years old.[25] It was further reported that 82% of all cases were caused by NTHi and that these were the most common cause of infection in all ages. Regarding encapsulated isolates, Hif, Hib and Hie accounted for 9%, 4% and 3% of all cases, respectively and the remaining cases were caused by Hia, Hic and Hid. Hie and Hif serotypes seemed to mostly affect people over 45 years of age.

Despite of worldwide surveillance data, comparisons of *H. influenzae* disease incidence between countries and overtime trends of infection should be carefully evaluated, since these are dependent on policies and surveillance systems of each country, along with reporting processes and case detection methods.[48]

Since Hib vaccines specifically target the polysaccharide capsule of serotype b, these are ineffective against NTHi isolates and even against the remaining encapsulated serotypes. Several

studies testing protein-D and protein-E have been conducted, but the development of an effective vaccine against NTHi is still an ongoing subject of research.[52,53]

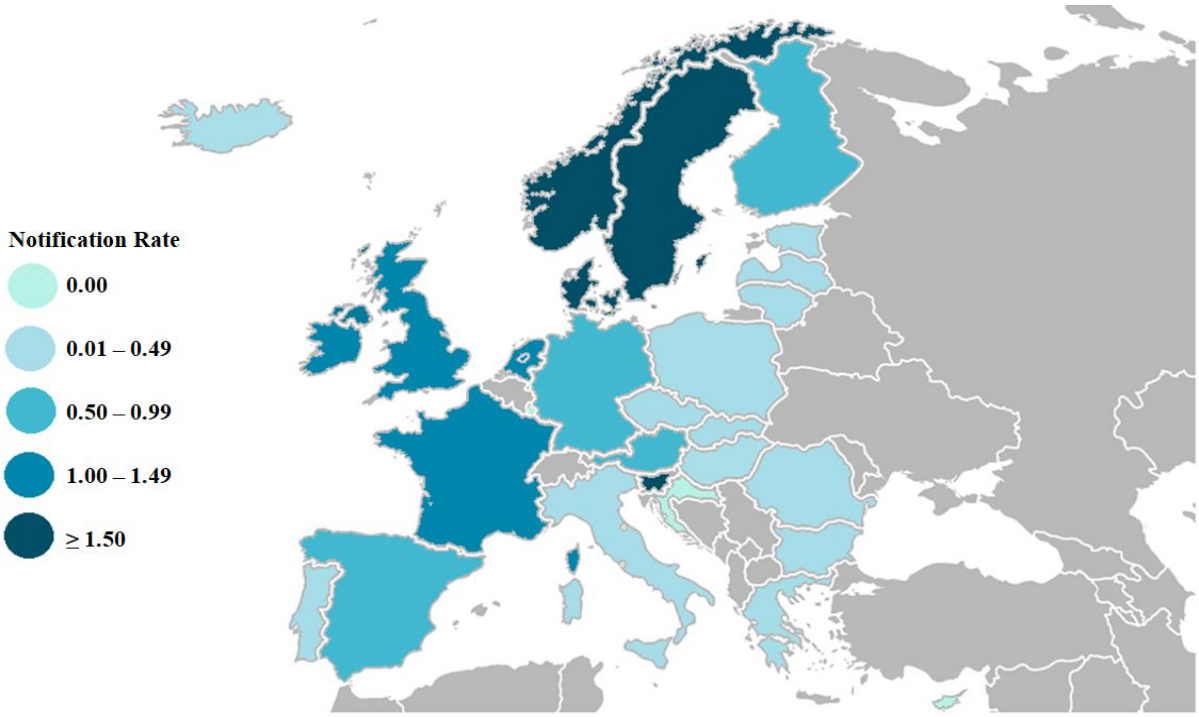

Figure 1.3 | Distribution of invasive *H. influenzae* disease cases per 100,000 population, in each EU/EEA country, in 2015. (adapted from[25])

In Portugal, epidemiological data covers three distinct periods: 1989–2001,[54] 2002–2010[55] and 2010–2014,[56] with the last period being specific to paediatric cases. All these studies were related to *H. influenzae* invasive disease and the results were in agreement with studies described above.[25,51]

Hib vaccine was available in Portugal, in 1994, being then recommended for children under five years old. It was implemented as part of the National Vaccination Programme in the year 2000. The first study period, 1989–2001, includes the pre-vaccine era. During this period, a majority of Hib isolates was characterized (60.5%) followed by NTHi (38.6%) and the first case of a Hif isolate was reported. Along with a decrease in the number of Hib infections, a decrease in multidrug resistance was also reported, which may be explained due to most of the resistant isolates being, then, of serotype b.[54]

Between the years 2002 and 2010[55] prevalence of Hib invasive disease was much lower (13.2%), NTHi isolates accounted for the majority of cases (77.1%) and non-b serotypes appeared to be emerging, with reports of serotypes a (2.1%) and f (6.9%). Serotype d is a rare serotype that was characterized in Portugal for the first time in 2009 and since then, no other serotype d isolate was found among Portuguese disease.[57]

The paediatric study in the period 2010–2014[56] reported NTHi isolates as being responsible for the majority of the cases (65.7%). Hib was characterized in nine children (23.7%), six of which were considered vaccine failures.

1.4. Antibiotics in *Haemophilus influenzae*

Antibiotics are biological, or synthetical, substances that either possess the ability to inhibit bacterial growth, or kill bacteria, which characterizes them as bacteriostatic, or bactericidal, respectively.[58] Nowadays, it is recognized that infectious diseases are still a leading cause of worldwide death and antimicrobial resistance is a topic of great concern. Antibiotics with a similar structure present identical patterns of toxicity, effectiveness and potential side effects.[58]

β-lactams usually possess a highly reactive ring – as a common structure – and include penicillins (e.g.: ampicillin, amoxicillin), cephalosporins (e.g.: cefepime, cefotaxime, cefuroxime) and carbapenems (e.g.: meropenem).[58]

These antibiotics usually target PBPs, which are fundamental for synthesis of the bacterial cell wall, as they cross link peptide units in peptidoglycan production. Covalent bonding with antibiotic disrupts the process by weakening peptidoglycan and the cell eventually bursts, due to osmotic pressure.[58-60]

Macrolides (e.g.: azithromycin) and tetracyclines bind to bacterial 50S and 30S ribosome subunit, respectively, which stops addition of amino-acids to polypeptide chains, ultimately resulting in inhibition of protein synthesis. Quinolones (e.g.: ciprofloxacin) interfere with DNA replication, by inhibiting the action of DNA gyrase, an enzyme that introduces negative supercoils in DNA during replication. Other agents, such as chloramphenicol, rifampicin and trimethoprim-sulfamethoxazole, may act as inhibitors of the 50S ribosome subunit, DNA replication and folic acid metabolism, respectively.[58,59,61]

For treatment of *H. influenzae* infections, β-lactams are commonly used, since the outer bacterial membrane presents little resistance to penetration of this class of antibiotics.[60] However, resistance mechanisms against them have been reported and thoroughly studied over the last decades.

The most common bacterial resistance mechanisms include efflux pumps, modification of the target molecule and inactivation of the antibiotic through enzymatic action.[59]

1.4.1. β-lactams resistance mechanisms in *H. influenzae*

In *H. influenzae*, both enzymatic and non-enzymatic mechanisms have been reported and production of β-lactamase constitutes the most common resistance mechanism against β-lactams.[62]

1.4.1.1. β-Lactamase-Positive Ampicillin-Resistant (BLPAR) isolates

In 1972, the first report of ampicillin resistance in *H. influenzae* was registered and was related to the production of TEM-1 β-lactamase, which is encoded by $bla_{\text{TEM-1}}$ gene. *H. influenzae* rarely produces a second β-lactamase, ROB-1, encoded by the bla_{ROB} gene.[62] β-lactamases hydrolyse the ester-amide bond in β-lactams, which interferes with the structure of the antibiotic and, ultimately, its effectiveness (Fig. 1.4).[59,63]

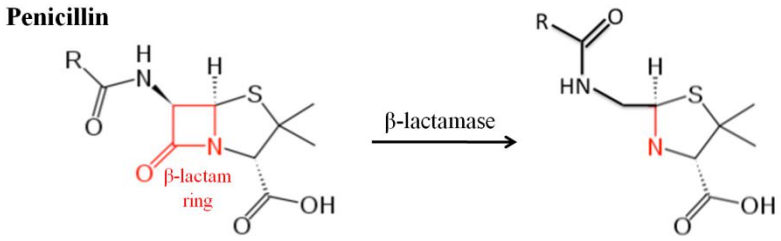

Figure 1.4 | Penicillin inactivation through β-lactamase production. (adapted from[63])

β-lactamases may be divided into four classes, from A to D, according to similarities in their structure. Classes A, C and D possess a serine residue in the active site, whereas class B are metalloproteins that require zinc as a cofactor.[64] TEM-1 and ROB-1 are class A β-lactamases.[62]

As a solution for β-lactamase producing *H. influenzae* isolates, alternative treatments that combine amoxicillin and clavulanic acid have been administered to patients.[65,66] In the presence of clavulanic acid, β-lactamase irreversibly binds to its β-lactam ring, which inactivates the enzyme and prevents it from binding to β-lactams.[67]

1.4.1.2. β-Lactamase-Negative Ampicillin-Resistant (BLNAR) isolates

Ampicillin-resistant isolates that do not produce β-lactamase have been reported and designated as BLNAR. Usually, these isolates possess altered PBPs with reduced affinity for β-lactams, due to amino-acid substitutions in this protein.[68] In addition to ampicillin, BLNAR isolates also present reduced susceptibility for other β-lactams, such as cephalosporins.[62] In opposite to β-

lactamase producing isolates, clavulanic acid has no effect in BLNAR isolates, due to lack of the target enzyme.[69]

H. influenzae possesses eight PBPs, PBP1 – PBP8.[62] Mutations in *ftsI* gene, which encodes PBP3, have been reported and are useful to confirm phenotypically identified BLNAR isolates. More specifically, amino-acid substitutions close to conserved KTG (K512TG) and SSN (S379SN) motifs in the transpeptidase domain of PBP3 are believed to be mostly responsible for antibiotic resistance.[68] However, there is still not a clear definition for BLNAR isolates, since some authors define them based on ampicillin-resistance breakpoint, while others consider ampicillin-intermediate isolates.[62] These isolates can only be accurately identified by sequencing the *ftsI* gene.[68]

1.4.1.3. β-Lactamase-Positive Amoxicillin-Clavulanate-Resistant (BLPACR) isolates

BLPACR isolates possess both the production of β-lactamase and modified PBPs, which means that these are resistant to β-lactams, such as ampicillin and amoxicillin-clavulanate as well. Most authors define these isolates considering the amoxicillin-clavulanate breakpoint, but mutations identified in the *ftsI* gene ultimately serve as confirmation.[62,69,68] BLPACR isolates have higher amoxicillin resistance levels than BLNAR isolates, due to the presence of β-lactamases.[69]

1.5. Virulence factors

Identifying and understanding how virulence factors influence pathogenic, or commensal behaviour is very important and this is, nowadays, a main focus in NTHi research.[12] *H. influenzae* has developed several mechanisms to resist immune responses and to adhere, persist and, consequently, invade host cells.[70,71]

1.5.1. Adhesion

The very first step in the NTHi pathogenesis is adherence of bacteria to the mucosa, after initial interaction.[70,71]

To avoid complement system immune responses, encapsulated isolates usually rely on the presence of a capsule for protection against deposition and/or binding of host complement factors, or immunoglobulins. NTHi isolates, however, have to develop alternative defence mechanisms against host immune responses.[70] One of the most important is inactivation of Immunoglobulin A (IgA) which

is the most common immunoglobulin in nasal secretions. Both Hib and NTHi isolates may produce IgA protease, which cleaves IgA1 antibodies into Fc and Fab fragments.[72]

LOS are lipopolysaccharides, with shorter saccharide chains, considered to be essential for interaction of bacteria with host cells.[12] These structures vary among bacterial cells of the same isolate and also between different isolates, which naturally affects interaction and invasion of host cells. LOS are present in both Hib and NTHi isolates.[71]

For adherence to host mucosa, *H. influenzae* also counts on the expression of several adhesins, which are proteins that facilitate this action.[73] Five important adhesins, HifA, HMW1, HMW2, Hia/Hsf and Hap, have been identified in encapsulated (Hib) and NTHi isolates.[71] Piliated adhesins include PilA and HifA proteins. PilA was shown to be essential for NTHi adherence to epithelium and twitching motility, since experiments with *pilA*-mutant isolates demonstrate a significant reduced ability of isolates to properly accomplish these mechanisms.[13] Kubiet and colleagues[74] have also shown that for both Hib and NTHi isolates, absence of HifA pili significantly decreases the ability of isolates to adhere to mucin and that pre-treatment with antibodies that specifically targeted HifA has a similar effect.

However, isolates that lack pili structures still have the ability to adhere to human epithelium, which suggests expression of non-piliated adhesins. For instance, non-piliated high molecular weight proteins, HMW1 and HMW2, are present in encapsulated non-type b and most NTHi isolates, in addition to Hia protein, which seems to be a substitute for HMW-deficient isolates.[75-77]

1.5.1.1. *pilA*

pilA is a highly conserved gene that is present in, approximately, 100% of NTHi isolates,[13,78] (Table 1.1). This gene is one of the four-member gene cluster *pilABCD*, expressing Type IV Pilin (Tfp). Bakaletz and colleagues[13] have showed that expression of *pilA* is essential for pilus structures on the bacterial surface. *pilA* expression has been shown to be necessary for proper adherence, biofilm formation and epithelium colonization. Jurcisek and colleagues[79] demonstrated that *pilA* mutants showed diminished capacity for *in vitro* adherence, which translated into loss of ability to colonize *in vivo* and decreased stability of biofilms in chinchilla middle ear. Tfp structures additionally play a significant role in competence, as these help the uptake of extracellular DNA through bacterial membranes.[80] Pili structures have also been suggested to mediate interbacterial interaction, since *pilA*-mutants present thinner and biomass decreased biofilms.[80]

1.5.1.2. *hifA*

HifA, encoded by *hifA* gene, is a structural subunit of the pilus structure encoded by *hifABCDE* cluster. This protein mediates adherence to eukaryotic cells, by specifically binding to sialyl-lactosylceramide ganglioside receptor on epithelial cells. Expression of *hifA* is essential for adherence, since inactivation of this gene highly hampers the ability of *H. influenzae* to adhere to epithelial cells.[81] Analysis of amino-acid sequences revealed conserved and variable regions, in which variable regions possibly result in different antigenic sites.[82] Geluk and colleagues[82] found that *hifABCDE* cluster is present in both Hib and NTHi isolates with 18% of NTHi isolates possessing *hifABCDE* cluster (Table 1.1). The authors additionally suggested that *H. influenzae* probably loses the ability to express fimbriae structures inside tissues, as a defence mechanism to avoid clearance. Although HifA plays a role in adherence to mucosa, other factors such as HMW proteins are also important for establishment of binding to epithelium.[74,82]

1.5.1.3. *hmw1A* and *hmw2A*

hmw1A and *hmw2A* genes encode HMW1 and HMW2 proteins, respectively. These genes present 80% similarity and are 71% identical.[83] Despite of highly similar, HMW1 and HMW2 proteins possess different binding specificities: while HMW1 is specific for sialylated glycoprotein receptor containing sialic acid, a recent study has shown a high affinity of HMW2 to 2-6 linked N-acetylneuraminic acid, suggesting that this may be the receptor.[84] Both proteins mediate attachment to Chang epithelial cells.[76,85] van Schilfgaarde and colleagues[86] conducted a study for comparison of adherence, *hmw* presence/absence and expression of HMW proteins in NTHi isolates. The authors found that isolates presenting both *hmw* genes and HMW proteins represented 72% of all isolates capable of adherence. These results highlighted a correlation between HMW and capacity for adherence. Other studies also corroborate the importance of expression of *hmw* genes for adherence.[76,78] A study conducted by Ecevit and colleagues[87] found, in NTHi isolates, percentages of 51% and 23% for *hmw1A* and *hmw2A*, respectively, while others have found prevalence for both genes from 55 to 100% of isolates (Table 1.1).[78,88] NTHi isolates that do not express HMW proteins are still able to adhere, since other adhesins, such as Hia and OMP P5, may serve as alternative adherence mechanisms.[78]

1.5.1.4. *hia*

Hia adhesin is encoded by *hia* gene and has been described for 32% to 55.6% of NTHi isolates (Table 1.1).[87,89] Hia seems to act as a substitute for HMW1/HMW2 with adherence function

when these are absent, since *hmw* and *hia* genes are mutually excluded in both NTHi and non-type b encapsulated isolates.[75,77] In fact, *hia* has been described to be present in over 80% of NTHi isolates lacking both HMW1 and HMW2 proteins.[90] However, a receptor for Hia still remains unidentified.[91] In the chromosome, *hia* and *hsf* are located in the same region. Although these genes encode proteins with similar functions, *hia* is present in NTHi isolates, while *hsf* is the major non-pilus adhesin of Hib isolates. Non-type b encapsulated isolates also possess a gene homologous to *hsf*.[75]

1.5.1.5. *ompP5*

OMP P5 is a β-barrel outer membrane protein. It is encoded by *ompP5* gene and is a member of the OmpA protein family,[92,93] which has been identified from 52% to 100% of NTHi isolates (Table 1.1).[78,92] OMP P5 binds to mucin and surface-expressed Carcinoembryonic Antigen-Related Cell Adhesion Molecule-1 (CEACAM1) receptor.[93] Duim and colleagues analysed NTHi isolates from patients with chronic bronchitis and verified that, regarding *ompP5*, all isolates had conserved sequences, in addition to diverse regions located on the cell surface. These diverse regions resulted in OMPs with different molecular weights. The authors explain that this diversity may have resulted from selective pressure, which becomes an advantage of these isolates in persistence during a chronic infection.[94] Similar results have been found by other authors when analysing isolates from patients with non-respiratory and respiratory diseases, as well as with exacerbations of chronic diseases (COPD isolates included).[92] Vuong and colleagues described that 13% of isolates lacking *hmw1A* and *hmw2A* genes, had *ompP5* gene and these were still capable of adherence. The authors stated that although these results were not statistically significant, these may elucidate the importance of OMP P5 in adherence.[78]

1.5.2. Persistence and invasion

Ability of *H. influenzae* to adhere and persist on the mucosa is especially favoured in patients with underlying diseases of the upper and lower respiratory tract, in which mucociliary clearance is hampered.[74] After adherence has been established, persistence on the mucosa is the following step. In order to persist, *H. influenzae* depends upon a constant supply of iron and heme, among additional nutrients from the surrounding environment. Uptake of nutrients not only promotes survival, but also bacterial replication. Furthermore, bacteria must develop further mechanisms to resist host immune system responses.[70,71]

Most of iron in mammalian hosts is kept as heme components, inside ferritins, or bound to transferrins, meaning that its availability is much limited for invading microorganisms. Hib and NTHi

isolates count on Transferrin-Binding Proteins (Tbp), Tbp1 and Tbp2, for direct iron uptake from transferrins.[95] For heme uptake, *H. influenzae* uses hemoglobulin and Hemoglobulin:Haptoglobolin binding Proteins (Hgp), encoded by *hgp* genes and Heme-Hemopexin (HxuC), the most important complexes for heme binding and transfer to cytoplasm.[96]

Table 1.1 | Distribution of virulence genes among NTHi isolates.

Gene	Prevalence		Reference	
pilA	~ 100 %		Bakaletz *et al.*, 2005[13] and Vuong *et al.*, 2013[78]	
hifA	~ 18 %		Geluk *et al.*, 1998[82]	
hmw 1A	51%	55–100 %	Ecevit *et al.*, 2004[87]	Vuong *et al.*, 2013, [78] and Busher *et al.*, 2004[88]
hmw 2A	23%		Ecevit *et al.*, 2004[87]	
hia	32% ; 55.6%		Ecevit *et al.*, 2004[87] and Cardines *et al.*, 2007[89]	
ompP5	52% ; 100 %		Vuong *et al.*, 2013[78] and Martí-Lliteras *et al.*, 2011[92]	

In addition to genetic diversity, *H. influenzae* has the ability to express phase variable virulence factors. Phase variation is an adaptation mechanism developed when bacteria are faced with changes in the surrounding environment, or must resist certain immune responses. It involves a variation of a structure and is usually associated with the number of nucleotides in a gene sequence.[71] This means that each isolate may independently express some genes and switch-off different ones, considering the surrounding environment. The result is hundreds of different *H. influenzae* virulence phenotypes in a single culture, which can be translated to different structures of LOS, or expression of fimbriae surface antigens. Ultimately, the phenotype that better fits the environment requirements is the one that persists. Therefore, there is a very high probability that phenotypes of the same isolate observed *in vivo* and *in vitro* may quite differ.[12,91] Key targets of phase variation are structures important for virulence behaviour, such as HMW1 and HMW2 proteins, or OMP P5. Loss of fimbria structures, once inside tissues, may also be helpful for avoidance of clearance.[73,82,94]

Hap protein was shown to be a major virulence factor for establishment of interaction between Hib, or NTHi isolates and host cells and as an adhesin, by binding to laminin, fibronectin and collagen IV, which are components present on the respiratory epithelium. Additionally, considering the opportunistic behaviour of *H. influenzae*, Hap is also important for interaction and consequent invasion of damaged epithelium.[97] Naturally, it is expected that other factors besides Hap, such as proteins D and E, may also contribute to invasion of the epithelium.[70,71]

1.6. Biofilms

Biofilms are assemblies of microorganisms enclosed in a self-produced Extracellular Polymeric Substance (EPS) matrix, adhered to a surface.[98] Changing from a planktonic to a biofilm state enables a multicellular behaviour that favours bacterial survival in unfavourable conditions.[99] Cell signalling (e.g.: quorum sensing) is a fundamental mechanism for bacterial communication and, ultimately, biofilm production.[100]

Biofilm production is a dynamic process divided in three main stages. The first is adhesion and involves attachment to a surface, plus formation of microcolonies. The second phase is maturation of biofilm, which involves an adaptation to conditions of the surrounding environment and the production of an EPS matrix. The final stage is biofilm dispersion that can be triggered by different factors, such as nutrient availability, or antibiotic action, among others (Fig.1.5).[99]

Attachment may occur on any surface – whether biotic, or abiotic – such as host tissues, or medical devices. Biofilms that may be a source of infection for humans pose a major concern of public health, since the presence of biofilms in humans constitutes a major virulence behaviour, being mostly associated with chronic infections (e.g.: recurrent OM, CSOM, pneumonia, cystic fibrosis, endocarditis and osteomyelitis). Biofilms serve as a protection mechanism for bacteria and can be repeatedly produced.[100,101] Bacteria commonly associated with biofilms include *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus epidermidis*, *Enterobacter cloacae* and *Klebsiella pneumoniae*. Biofilms formed by these bacteria are related to several human infections of the urinary tract, respiratory tract and soft tissue, for instance. Most of these bacteria are associated with hospital-acquired infections.[101]

Challenges related to biofilm production include spreading of bacteria to bloodstream, causing secondary infections, resistance to antibiotics in the presence of a mature biofilm and, ultimately, recurrence of infection.[101,102] Furthermore, routine bacterial cultures are designed to detect planktonic bacteria, meaning that these methods are less sensitive in detecting bacteria inside a biofilm. This may lead to false negative cultures.[103]

As referred, bacteria in biofilms usually present increased resistance to drug treatment. Resistance may come from hampering the penetration of biofilm, or from inhibition mechanisms through the action of enzymes (e.g.: β-lactamases), for instance, that inactivate antibiotics and protect bacteria.[101,102] Therefore, the conventional use of antibiotics may not be efficient in these cases and surgical interventions, or alternative therapies that directly target the EPS matrix, or inhibition of quorum sensing signalling molecules, for example, have to be considered.[100]

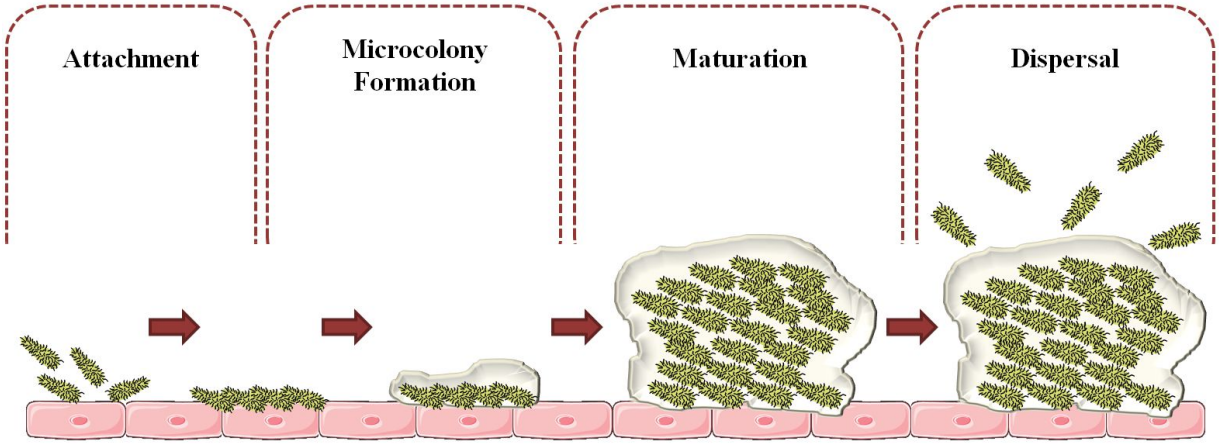

Figure 1.5 | Schematic presentation of the three main stages of biofilm production: adhesion, maturation and dispersal (adapted from[99]).

1.6.1. *H. influenzae* biofilms

Until recently, *H. influenzae* biofilms were a topic of controversy, as to whether isolates were able to produce an EPS matrix and its significance for virulent behaviour.[12,104] However, studies have shown a capacity of *H. influenzae* to aggregate in biofilms, which affect bacterial virulence.[105]

Considering that attachment is the first step towards biofilm production, various studies have focused on inhibiting bacterial adhesion, thus preventing biofilm production.[99]

Several virulence factors expressed by *H. influenzae* were suggested to contribute to adhesion and to be constituents of the EPS matrix, including LOS, PilA, OMPs, HMW1 and HMW2 proteins. Jurcisek and Bakaletz[106] have described the presence of dsDNA, LOS, OMPs and type IV pilin structures in the EPS matrix of NTHi biofilm. They hypothesised that pilin proteins may have a fundamental role in the structural stabilization of the biofilm, as interbacterial bridges. The fact that a PilA-mutant isolate could not form a biofilm as robust as isolates expressing this protein and that it presented a decreased adherence to the middle ear of a chinchilla host, further supported this statement. Similar to what has been reported for other bacterial biofilms,[99] these authors[106] described a compartmentalization of the EPS matrix constituents with dsDNA being mostly located in the outer edges of the biofilm. This possibly served for stabilization of the structure. A more recent study further

supported such results, since antisera treatment targeting PilA protein translated into a significant inhibition of biofilm production by NTHi isolates.[107]

Furthermore, HMW1 and HMW2 are thought to be major constituents of *H. influenzae* biofilms, as well, since antisera specific for these proteins has been shown to cover most of NTHi biofilm surface, suggesting a wide distribution of the adhesins on the EPS matrix.[108]

Biofilms are known to provide protection against antibiotics, as previously referred. In the specific case of *H. influenzae*, *in vitro* studies have demonstrated a diminished effect of antibiotics against bacteria inside a biofilm. In fact, for the same bacterial isolate, the required antibiotic concentration to inhibit bacterial growth in a biofilm may be 100 folds higher than the dose required to inhibit the planktonic form.[109] These results demonstrate that, in a biofilm associated infection, a patient may receive an empirical treatment that may, ultimately, have no effect, leading to bacterial survival and persistence of infection. The fact that routine culture methods may not detect bacteria inside biofilms and that there are no standard methods of antibiotic susceptibility tests for bacteria in biofilms, further hampers a proper treatment of infection.[102,103] Moreover, it has been suggested that sub-inhibitory concentrations of β-lactams – the antibiotics most commonly used to treat *H. influenzae* infections – may actually induce biofilm production of NTHi isolates.[110]

An additional challenge related to biofilm production is the development of polymicrobial biofilms in infected tissues, resulting from interplay of different bacterial species. *H. influenzae*, for example, has been shown to form polymicrobial biofilms with *Moraxella catarrhalis* and *Streptococcus pneumoniae*.[111,112]

When compared to biofilms formed by *H. influenzae* alone, *in vitro* and *in vivo* polymicrobial biofilms with *Moraxella catarrhalis* protected susceptible *H. influenzae* isolates against ampicillin and trimethoprim-sulfamethoxazole action, allowing an increase of the number of viable bacteria inside the biofilm. Furthermore, it was demonstrated that quorum sensing was fundamental for polymicrobial biofilm production, as previously described, since a quorum signalling-deficient *H. influenzae* isolate hampered biofilm production and bacterial persistence within it.[111] Polymicrobial biofilm of *H. influenzae* and *Streptococcus pneumoniae* may lead to several phenotypes that enhance a capacity for both adaptability and persistence. For instance, different combinations of gene expression, that result in variations of surface proteins, are mechanisms that might be affected by the interaction between these two bacterial species and may help in the adhesion/cohesion of biofilms.[112]

1.6.2. *H. influenzae* biofilms in patients with COPD and OM infections

Although several studies have demonstrated the capacity of *H. influenzae* isolates from patients with both COPD and OM to form biofilms, only a limited number of these have established comparisons between biofilm production and underlying diseases and, even so, the results of these studies may be contradictory.[105,113-119]

Puig and colleagues[105,114] have shown that isolates recovered from patients with OM tend to form denser biofilms than isolates collected from patients with lower respiratory tract diseases, such as COPD or Community Acquired Pneumonia (CAP). Additionally, the authors showed that biofilms formed by isolates from healthy carriers presented similar biofilm production to that of patients with lower respiratory tract diseases. One of these studies,[114] further demonstrated that OM and invasive NTHi isolates possessed a higher and faster capacity for adherence than COPD and CAP isolates. Together, these studies could establish a correlation between disease and capacity for biofilm production.

However, a different study conducted by Obaid and colleagues,[119] which included isolates from patients with different clinical backgrounds (OM, conjunctivitis, lower respiratory tract diseases, cystic fibrosis and oropharyngeal normal flora) found no relation between clinical source and biofilm production. These results were in agreement with findings from Murphy and Kirkham,[113] who compared OM and COPD isolates and also found that biofilm production was not related with the clinical group of isolates.

1.7. Aim of the work

H. influenzae is a commensal human-restricted microorganism of the nasopharynx. Due to its opportunistic behaviour, it is frequently associated with infections of the upper and lower respiratory tract.

This work aimed to characterize non-invasive NTHi isolates recovered from patients with COPD and OM. Antibiotic susceptibility, genetic diversity and relatedness and assessment of the presence/absence of six virulence genes were performed with the final goal of establishing a relationship between these factors and the respective disease. The ability to assemble biofilms was assessed in order to explore a possible correlation between the studied diseases and the presence of virulence genes (*pilA* and *hmw1A/2A*) known to influence biofilm production.

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2. Methods

2.1. Clinical isolates

Isolates used in this Master Thesis belong to the collection of the "Laboratório Nacional de Referência de Infeções Respiratórias" in the "Instituto Nacional de Saúde Dr. Ricardo Jorge (INSA)", Lisboa and were collected between the years of 2013 and 2018.

A total of 93 isolates were included in the study. These were collected from patients with two non-invasive diseases: COPD and OM. Bacterial isolates were recovered from 10 Portuguese hospitals. Overall, 64.5% (60/93) isolates were from men and 35.4% (33/93) from women. Ages for COPD patients ranged from 50 to 89 years old and, for OM patients, from four months to 21 years old (Table 2.1).

The thirty-eight *H. influenzae* isolates recovered from patients with COPD and the 55 isolates collected from patients with OM were analysed regarding capsular typing, ability to produce β-lactamase, susceptibility to 12 antibiotics, genetic diversity, presence/absence of six virulence genes and ability for producing biofilms.

Data regarding isolation date, gender of the respective patient, β-lactamase production, presence/absence of a capsule, presence/absence of the six virulence factors and biofilm production after 24h and 48h is detailed for each isolate on Tables 5.1 and 5.2 of the Appendix.

Table 2.1 | Sample characterization.

Epidemiological data \ Disease		COPD (n=38) n (%)	OM (n=55) n (%)	Total (n=93) n (%)
Hospital	North	19 (**50.0%**)	22 (**40.0%**)	41 (**44.1%**)
	Centre	17 (**44.7%**)	3 (**5.5%**)	20 (**21.5%**)
	Lisboa	2 (**5.3%**)	4 (**7.3%**)	6 (**6.5%**)
	Alentejo	0	25 (**45.5%**)	25 (**26.9%**)
	Açores and Madeira islands	0	1 (**1.8%**)	1 (**1.1%**)
Isolation Date	2013	5 (**13.2%**)	14 (**25.5%**)	19 (**20.4%**)
	2014	3 (**7.9%**)	18 (**32.7%**)	21 (**22.6%**)
	2015	11 (**28.9%**)	5 (**9.1%**)	16 (**17.2%**)
	2016	7 (**18.4%**)	0	7 (**7.5%**)
	2017	11 (**28.9%**)	16 (**29.1%**)	27 (**29.0%**)
	2018	1 (**2.6%**)	2 (**3.6%**)	3 (**3.2%**)
Age in Years	<1	0	15 (**27.3%**)	15 (**16.1%**)
	1 - 4	0	36 (**65.5%**)	36 (**38.7%**)
	5 - 9	0	1 (**1.8%**)	1 (**1.1%**)
	10 - 25	0	2 (**3.6%**)	2 (**2.2%**)
	50 - 59	5 (**13.2%**)	0	5 (**5.4%**)
	60 - 69	8 (**21.1%**)	0	8 (**8.6%**)
	70 - 80	13 (**34.2%**)	0	13 (**14.0%**)
	≥80	12 (**31.6%**)	0	12 (**12.9%**)
Gender	Male	32 (**84.2%**)	28 (**50.9%**)	60 (**64.5%**)
	Female	6 (**15.8%**)	27 (**49.1%**)	33 (**35.4%**)

2.2. Bacterial growth and conservation

Bacteria were grown on chocolate agar plates supplemented with polivitex (*bioMérieux*) and then incubated, for 18–24h, at 35ºC in a 5% CO_2 atmosphere.

H. influenzae isolates were stored in Tryptic Soy Broth (TSB) with 20 % glycerol at -80 ºC.

2.3. DNA extraction

DNA extraction was performed by a boiling procedure: six to eight colonies were suspended in 100 µL of nuclease free ultra-pure water. The sample was boiled for 10 minutes (*VWR™ – VMS-C7*), in order to disrupt the membranes of bacterial cells and was then centrifuged for 3 minutes at 13000 rpm (*eppendorf® – Centrifuge 5418*). The supernatant, which contained the DNA, was stored at -20ºC.

2.4. Antimicrobial susceptibility testing

2.4.1. β-lactamase production

β-lactamase production was determined by a chromogenic cephalosporin assay: five to seven colonies of bacteria were suspended in 30 µL of nitrocefin (*OXOID™ – Nitrocefin Solution, 1mg*). If a change in colour, from yellow to red, was observed, it was an indicative of a positive result (production of β-lactamase).[120]

2.4.2. Minimum Inhibitory Concentration

Minimum Inhibitory Concentration (MIC) was determined for 12 antibiotics: Ampicillin (Am), Amoxicillin-clavulanate (Aug), Cefuroxime (Crm), Cefotaxime (Cft), Cefepime (Cpe), Meropenem (Mer), Azithromycin (Azi), Tetracycline (Te), Ciprofloxacin (Cp), Chloramphenicol (C), Rifampicin (Rif), and Trimethoprim/Sulfamethoxazole (T/S).

Commercial microdilution panels (*Beckman Coulter – MICroSTREP Plus*) were used to determine MICs, according to supplier's instructions.

In brief, 4 to 5 colonies of bacteria were suspended in 3 mL of sterile water (*Beckman Coulter*). Turbidity was compared to that of 0.5 of the McFarland barium sulphate standard (*bioMérieux*). Then, 100 µL of the suspension were added to 25 mL of *Haemophilus* Test Medium (*Beckman Coulter*). Suspension (200 µL) was poured into the 96-well microplate with the different antibiotic concentrations (Table 2.2) and was incubated at 35 ºC for 18 to 24 h in a non-CO_2 incubator. MICs for each isolate were registered on a panel worksheet, as shown in Fig. 5.1 of the Appendix.

Table 2.2 | Antibiotic concentrations on the microdilution panels.

Antibiotic	Concentrations of Antibiotic (mg/L)								
Am	0.03	0.06	0.12	0.25	0.5	1	2	4	8
Cft	0.03	0.06	0.12	0.25	0.5	1	2	4	8
Crm	0.25	0.5	1	2	4	8			
Te	1	2	4						
Rif	0.5	1	2						
Mer	0.06	0.12	0.25	0.5	1	2	4		
Aug	0.25/0.12	0.5/0.25	1/0.5	2/1	4/2				
C	1	2	4	8					
T/S	0.25/4.75	0.5/9.5	1/19	2/38					
Cpe	0.12	0.25	0.5	1	2				
Cp	0.06	0.12	0.25	0.5	1	2			
Azi	0.25	0.5	1	2	4				

2.5. Capsular typing

2.5.1. Amplification

Capsular typing, by PCR, was previously described by Falla and colleagues.[20]

The reaction mix, with a final volume of 25 µL, was composed as follow: 15.85 µL of pure bi-distilled water, 2.5 µL of 10x buffer (*Qiagen*), 2.5 µL of 10 mM dNTPs (*dATP, dTTP, dCTP and dGTP, Roche, 2.5 µM* each), 0.4 µL of each of the primers (forward and reverse) (*Thermo Fisher Scientific, 10 µM)*, 1.75 µL of 25 mM $MgCl_2$ (*Qiagen*), 0.1 µL of Taq DNA polymerase (*Qiagen,* 5 U/µL) and 1.5 µL of DNA.

A positive and a negative control were included in the PCR.

Sequences of the primers and the expected length of the amplified product are presented on Table 5.3 of the Appendix. PCR was conducted in a thermocycler (*Applied Biosystems – GeneAmp® PCR System 9700*) and the protocol is schematically presented on Table 2.3.

Table 2.3 | Capsular typing PCR protocol.

	Temperature	Time	Cycles
Denaturation	94^{0}C	5 min	1
Denaturation	94^{0}C	1 min	
Annealing	55^{0}C	1 min	30
Extension	72^{o}C	1 min	
Final extension	72^{o}C	10 min	1
Conservation	4^{o}C	∞	

2.5.2. Analysis of fragments by gel electrophoresis

Analysis of the results was conducted by observing the presence/absence of bands of the amplified gene fragment in a 2% (w/v) agarose (*Lonza - SeaKem®LE Agarose*) gel in Tris-Borate-EDTA 1x (TBE 1x). The gel contained 5% of Sybr Safe (*Roche*), in order for the bands to be visible in a UV-transilluminator (*BioRad – Gel Doc™ XR*). Electrophoresis was conducted for 30 minutes at 100 V.

Molecular weight marker VIII (*Roche Diagnostics GmbH*), which presents standard bands between 19 and 1114 pb, was used for band weight comparison.

Before loading the samples in the gel, 7 µL of each sample were added to 3 µL of loading buffer (0.25 % bromophenol blue, 0.25% cyanol xilene and 50% glycerol, in water).

If an isolate was encapsulated, a band with, approximately, 343 bp should be visible.

In order to characterize the capsule type, a second PCR should be performed. In this case, specific primers (*Eurogentec Gold, 10 µM*) for each capsular type were used. PCR serotyping protocol was the same as described in 2.5.1. section. Sequences of the primers and the expected length for the amplified products are presented on Table 5.3 of the Appendix.

2.6. Multilocus Sequence Typing

2.6.1. Amplification

MLST protocol was previously described by Meats and colleagues.[23]

Seven housekeeping genes were amplified for each *H. influenzae* isolate: *adK*, *atpG*, *frdB*, *fucK*, *mdh*, *pgi* and *recA*.

For each allele, a reaction mix with a final volume of 25 µL was constitute as follow: 12.5 µL of HotStart Taq DNA Polymerase master mix (*Qiagen*), 1 µL of the specific primer, forward and reverse (*Thermo Fisher Scientific, 20 µM*), 5.5 µL of nuclease free water (*Qiagen*) and 5 µL of DNA.

Sequences of the primers and the expected length for the amplified product are presented on Table 5.4 of the Appendix. PCR was conducted in a thermocycler (*Applied Biosystems – GeneAmp® PCR System 9700*) and the protocol is schematically presented on Table 2.4.

Table 2.4 | MLST amplification protocol.

	Temperature	Time	Cycles
Denaturation	95^{0}C	15 min	1
Denaturation	95^{0}C	0:30 min	
Annealing	47^{0}C	0:30 min	34
Extension	72^{o}C	1 min	
Final extension	72^{o}C	10 min	1
Conservation	4^{o}C	∞	

2.6.2. Purification

Purification protocol was performed as described by *Applied Biosystems* by *Thermo Fisher Scientific*.[121]

Each amplification product (5 µL) was added to 2 µL of ExoSAP-IT™ PCR Product Cleanup Reagent (*Thermo Fisher Scientific*).

PCR was conducted in a thermocycler (*Applied Biosystems – GeneAmp® PCR System 9700*) and the protocol is schematically presented on Table 2.5.

Samples were loaded into a 2% (w/v) agarose gel in TBE 1x, to verify amplification and purification of all seven housekeeping genes, as described in 2.5.2. section.

Table 2.5 | Purification PCR protocol.

Temperature	Time	Cycles
37^0C	15 min	1
80^0C	15 min	1
4ºC	∞	

2.6.3. Sequencing

After purification, 1 µL of each purified product was added to a mix, containing 4 µL of nuclease and protease free water, 3.2 µL of 5x Big Dye Buffer (*Applied Biosystems*), 0.8 µL of Big Dye (*Applied Biosystems*) and 1 µL of M13 Phage forward primer (*invitrogen, 20 µM*).

PCR was conducted in a thermocycler (*Applied Biosystems – GeneAmp® PCR System 9700*) and the protocol is schematically presented on Table 2.6.

Table 2.6 | Sequencing PCR protocol.

	Temperature	Time	Cycles
Denaturation	96^0C	0:30 min	1
Denaturation	96^0C	0:10 min	
Annealing	50^0C	0:05 min	25
Extension	60ºC	4 min	
Conservation	4ºC	∞	

Samples were forwarded to the "Unidade de Tecnologia e Inovação" from the "Departamento de Genética Humana", in INSA, where sequencing was performed, by capillary electrophoresis, in a genetic analyser (*Applied Biosystems – ABI 3130XL*).

2.6.4. Multilocus Sequence Typing analysis

After sequences for the seven alleles of each isolate were obtained, these were analysed by the *Finch TV* software. A FASTA document was created for each sequence and uploaded to the *Haemophilus influenzae* database (*http://pubmlst.org/hinfluenzae/*). Each sequence of the seven housekeeping genes were compared with the same locus sequences in the database.

If the sequence matched a previously assigned allele, the same allele number was attributed. If the sequence did not match any allele, it was necessary to submit the sequence to the database for assignment of a new allele number. When the combination of the numbers for the seven alleles matched a combination in the database, a ST number was attributed to the isolate. In the case of a new combination of the STs for the seven alleles, all seven allele numbers had to be submitted to the database for assignment of a new ST number.

To display allelic differences between obtained STs, goeBURST analysis was performed using PHYLOViZ platform. STs that differed in one, two, or three genes were identified as Single Locus Variant (SLV), Double Locus Variant (DLV) and Triple Locus Variant (TLV), respectively. A Minimum Spanning Tree (MTS) presenting STs as nodes was obtained and STs would be joined if these were related up to DLV.

2.7. Virulence factors

Analysis of the presence/absence of six virulence genes was based on the protocols previously described by Vuong and colleagues (for *pilA*, *ompP5*, *hmw1A*, *hmw2A*),[78] Geluk and colleagues (for *hifA*),[82] and Cardines and colleagues (for *hia*).[89] Original protocols were adapted, in order to reduce unspecific amplified products.

The reaction mix, with a final volume of 22.5 µL, was composed as follow: 12.7 µL of pure bi-distilled water, 5 µL of 5x Buffer (*Promega*), 1 µL of 10 mM dNTPs (*Roche*), 1 µL of each primer, forward and reverse (*Invitrogen, 20 µM*) specific for each gene, 1.5 µL of 25 mM $MgCl_2$ (*Promega*), 0.2 µL of GO Taq® DNA polymerase (*Promega*, 5 U/µL) and 2.5 µL of DNA. This master mix was common to all analysed genes, except for *hia* gene, for which 0.15 µL of Ex Taq (*Takara*, 5 U/µL) was used.

A positive and a negative control were included in the PCR.

Sequences of the primers and the expected length of the amplified products are presented on Table 5.5 of the Appendix. PCR was conducted in a thermocycler (*Applied Biosystems – GeneAmp® PCR System 9700*) and the protocol is schematically presented on Table 2.7 for *pilA*, Table 2.8 for *hifA*, Table 2.9 for *hmw1A/2A*, Table 2.10 for *hia* and Table 2.11 for *ompP5*.

Table 2.7 | *pilA* PCR protocol.

	Temperature	Time	Cycles
Denaturation	94^{0}C	5 min	1
Denaturation	94^{0}C	1 min	
Annealing	50^{0}C	0:30 min	35
Extension	72^{o}C	1:30 min	
Final extension	72^{o}C	5 min	1
Conservation	4^{o}C	∞	

Table 2.8 | *hifA* PCR protocol.

	Temperature	Time	Cycles
Denaturation	94ºC	5 min	1
Denaturation	95ºC	1 min	
Annealing	55ºC	1 min	35
Extension	72ºC	2 min	
Final extension	72ºC	8 min	1
Conservation	4^{o}C	∞	

Table 2.9 | *hmw1A/2A* PCR protocol.

	Temperature	Time	Cycles
Denaturation	94^{0}C	5 min	1
Denaturation	94^{0}C	1 min	
Annealing	52^{0}C	0:30 min	35
Extension	72^{o}C	1 min	
Final extension	72^{o}C	10 min	1
Conservation	4^{o}C	∞	

Table 2.10 | *hia* PCR protocol.

	Temperature	Time	Cycles
Denaturation	95ºC	5 min	1
Denaturation	95ºC	1 min	
Annealing	53ºC	1 min	30
Extension	72ºC	5 min	
Final extension	72ºC	10 min	1
Conservation	4ºC	∞	

Table 2.11 | *ompP5* PCR protocol.

	Temperature	Time	Cycles
Denaturation	94ºC	5 min	1
Denaturation	94ºC	0:30 min	
Annealing	53ºC	0:30 min	35
Extension	72ºC	2 min	
Final extension	72ºC	10 min	1
Conservation	4ºC	∞	

Samples were loaded into a 2% (w/v) agarose gel in TBE 1x, to verify if all samples contained the amplified product, as described in 2.5.2. section. Molecular weight marker VII (*Roche Diagnostics GmbH*) which presents standard bands between 359 and 8576 bp, was used for analysis of *hia* gel bands, while molecular weight marker VIII (bands between 19 and 1114 bp) was used for analysis of the remaining genes.

2.8. Biofilms

Regarding biofilm production, in addition to the 93 NTHi isolates, 14 invasive isolates were included in this study. Invasive isolates had been previously analysed by Whole Genome Sequencing (WGS) and were selected as positive controls for possessing *hmw1A/2A* genes, which have been associated with biofilm production. Invasive isolates comprised the years 2002, 2006, 2008–2012, 2014–2016 and were all NTHi.

Biofilm formation protocol was based on the modified Christensen's method with small modifications.[122]

The assay was conducted using 96-well flat-bottomed cell culture plates (*Thermo Scientific – Nunc™*) with four replicates for each isolate and negative control. Briefly, bacterial suspensions were prepared in saline to a final concentration of, approximately, 10^8 CFU/mL ($OD_{600\ nm}$ = 0.2 [*Thermo Scientific – GENESYS™ 20*]) from *H. influenzae* isolates grown overnight in chocolate agar plates. Bacterial suspensions were diluted in Brain Heart Infusion (BHI) broth (1:10) for a final volume of 200 µL and distributed in each well. BHI broth was used as negative control. Plates were incubated, at 37 ºC, for 24h and 48h (*Thermo Scientific – HERATherm™*). Each well was, then, aspirated and all wells were washed three times with sterile distilled water, for removal of non-adherent bacteria. Attached bacteria were stained with 100 µL of crystal violet for 15 min at room temperature. After crystal violet was removed, each well was washed three times with sterile distilled water. To each well, 200 µL of 96% ethanol were added, to elute crystal violet. Optical density was measured at an $OD_{570\ nm}$ in a microplate reader (*Thermo Labsystems – Multiskan Ascent™*).

Biofilm formation by *H. influenzae* was classified as described by Stepanović and colleagues.[123]

A mean $OD_{570\ nm}$ for each isolate ($OD_{isolate}$) plus the negative control (OD_{nc}) replicates and standard deviation (SD) were calculated. Cut-off for biofilm formation ($OD_{cut\text{-}off}$) was defined as the mean of the negative control adding to 3 times the respective SD (SD_{nc}) ($OD_{cut\text{-}off} = OD_{nc} + 3SD_{nc}$).

Isolates were classified as non-producers, weak, moderate and strong biofilm producers, according to the criteria described in Table 2.12.

Statistical significance of the obtained results was evaluated by the chi-square test, where a *p* value inferior to 0.05 ($p < 0.05$) was regarded as statistically significant.

Table 2.12 | Criteria for classification of *H. influenzae* isolates ability to assemble biofilms.

Classification	OD Range
Non-producer	$OD_{isolate} \leq OD_{cut\text{-}off}$
Weak producer	$OD_{cut\text{-}off} < OD_{isolate} \leq 2OD_{cut\text{-}off}$
Moderate producer	$2OD_{cut\text{-}off} < OD_{isolate} \leq 4OD_{cut\text{-}off}$
Strong producer	$OD_{isolate} > 4OD_{cut\text{-}off}$

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3. Results

3.1. Clinical isolates

This study was focused on two epidemiological relevant non-invasive diseases: Chronic Obstructive Pulmonary Disease and Otitis Media. Ninety-three isolates were included in this study: 38 were collected from patients with COPD and the remaining 55, from patients with OM. Isolates were recovered from ten hospitals, during the period of 2013–2018 (Table 2.1).

COPD patients age ranged between 50 and 89 years old, with most of the cases being collected from patients within the range 75 to 84 years old (14/38, 36.8%) (Fig. 3.1). OM patients age was comprised between less than one and 21 years of age, with the majority of these (30/55, 54.6%) being from children less than one year and between two and three years old (Fig. 3.2). The isolates from patients in the last age group, presented on Fig. 3.2, include two children with the ages of five and ten and an adult of 21 years old.

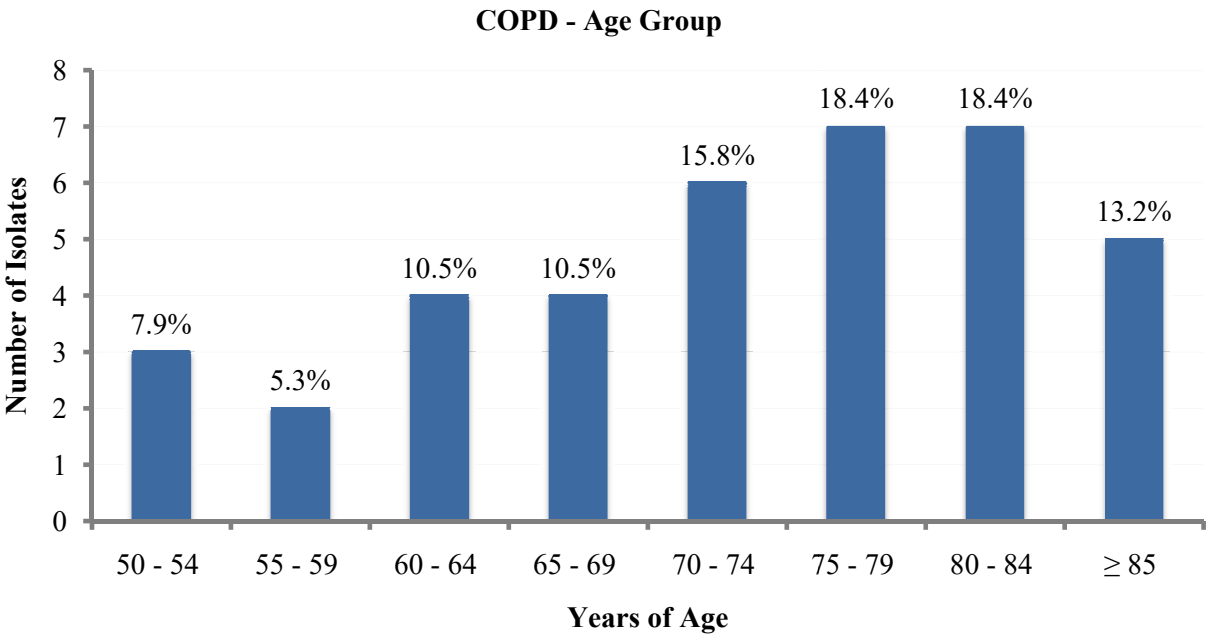

Figure 3.1 | Distribution of COPD isolates according to the age of patients.

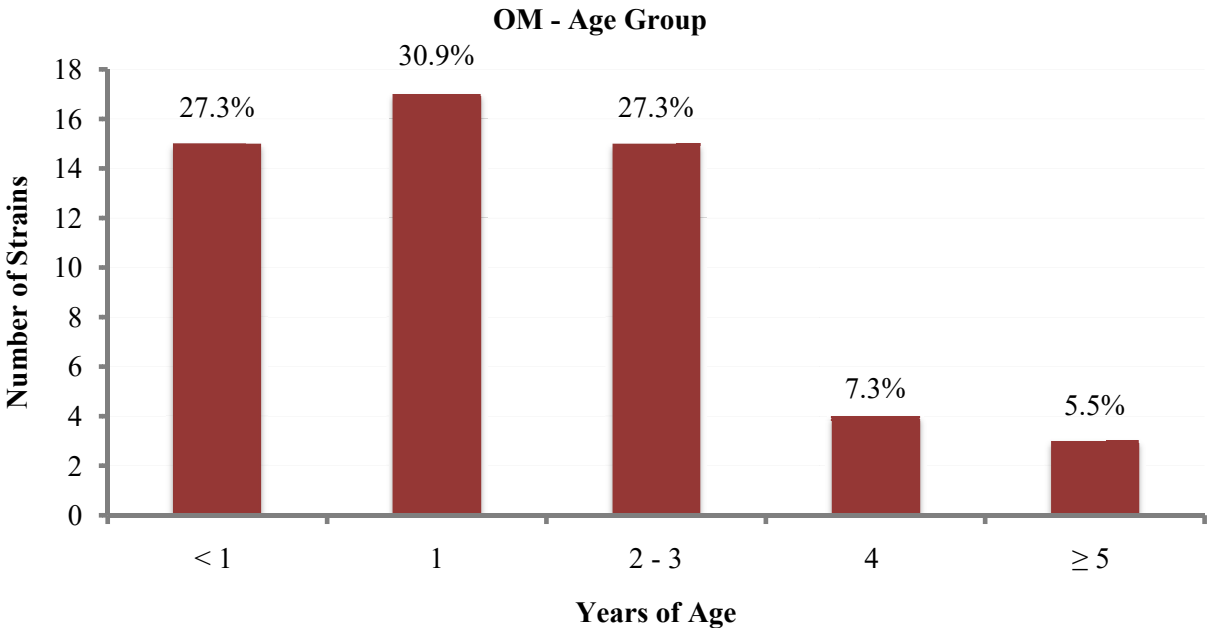

Figure 3.2 | Distribution of OM isolates according to the age of patients.

3.2. Capsular typing

Capsular typing was performed for all 93 isolates. Thirty-seven COPD isolates (37/38, 97.4%) were characterized as NTHi. Capsule was detected in only one of the isolates and was characterised as serotype f.

Regarding OM clinical group, all 55 isolates were NTHi.

3.3. Antibiotic susceptibility

All 93 isolates were analysed regarding β-lactamase production and susceptibility to 12 antibiotics. The antibiotics studied included Ampicillin (Am), Amoxicillin-clavulanate (Aug), Cefuroxime (Crm), Cefotaxime (Cft), Cefepime (Cpe), Meropenem (Mer), Azithromycin (Azi), Tetracycline (Te), Ciprofloxacin (Cp), Chloramphenicol (C), Rifampicin (Rif), and Trimethoprim/Sulfamethoxazole (T/S).

3.3.1. β-lactamase production

The majority of isolates did not produce β-lactamase (79/93, 84.9%). Only 15.8% (6/38) of COPD and 14.5% (8/55) of OM isolates were identified as β-lactamase producers (Fig. 3.3).

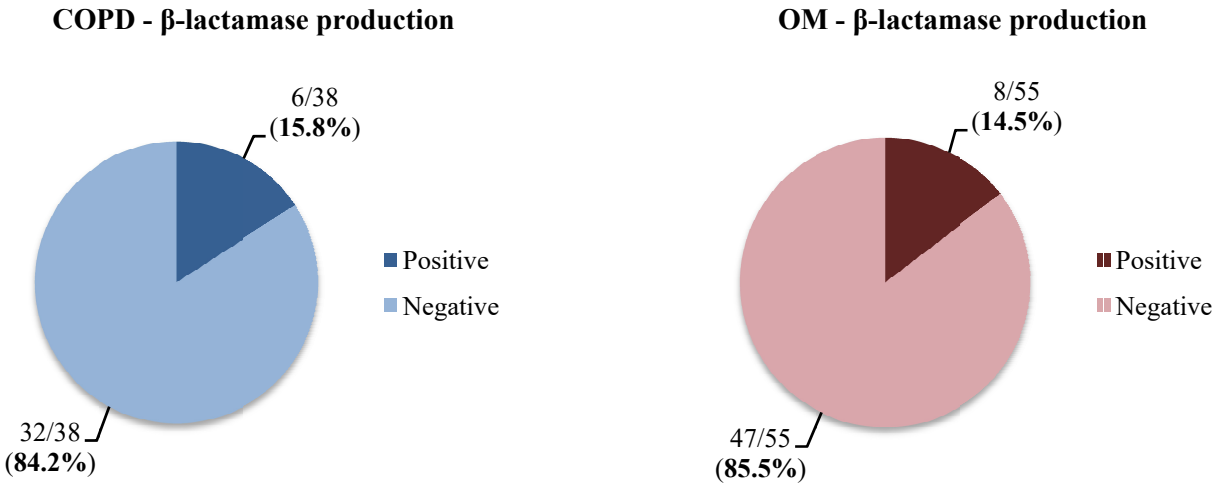

Figure 3.3 | Schematic presentation of β-lactamase producer (Positive) and non-producer (Negative) COPD and OM isolates.

3.3.2. Antibiotic susceptibility – Minimal Inhibitory Concentration

Antibiotic breakpoints for susceptible, intermediate and resistant isolates were based on the 2018 European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines.[61] However, in this latest version, there are no defined breakpoints for azithromycin. Therefore, we analysed our results considering the previously defined breakpoints (Table 3.1).[124]

Table 3.1 | Antibiotic breakpoints for *H. influenzae*.

Antibiotic	Susceptible (mg/L)	Intermediate (mg/L)	Resistant (mg/L)
Am	≤ 1	-	> 1
Cft	≤ 0.125	-	> 0.125
Crm (oral)	≤ 0.125	0.125 – 1	> 1
Te	≤ 1	1 – 2	> 2
Rif (for prophylaxis only)	≤ 1	-	> 1
Mer (indications other than meningititis)	≤ 2	-	> 2
Aug	≤ 2	-	> 2
C	≤ 2	-	> 2
T/S	≤ 0.5	0.5 – 1	> 1
Cpe	≤ 0.25	-	> 0.25
Cp	≤ 0.06	-	> 0.06
Azi*	≤ 0.125	0.125 – 4	> 4

*For azithromycin, 2017 EUCAST breakpoints were used

Tables 3.2 and 3.3 show the MIC_{50}, MIC_{90}, MIC range and percentage of susceptible, intermediate and resistant isolates for COPD and OM isolates, respectively.

The majority of both COPD and OM isolates was susceptible to all 12 tested antibiotics.

Among COPD isolates, resistance to ampicillin was verified for 15.8% (6/38) of isolates, all of which were β-lactamase producers. Resistance was also observed for cefuroxime (8/38, 21.1%), trimethoprim/sulfamethoxazole (11/38, 28.9%), ciprofloxacin (1/38, 2.6%) and azithromycin (1/38, 2.6%) (Table 3.2). It should be noted that *H. influenzae* resistance to ciprofloxacin was characterized for the first time in the laboratory (MIC > 32 mg/L, by E-test).

Intermediate resistances to cefuroxime and azithromycin were identified. Regarding cefuroxime, 71.0% (27/38) isolates were considered intermediate. For azithromycin, the lowest concentration tested was 0.25 mg/mL. The 2017 susceptible breakpoint was ≤ 0.125 mg/L. Considering that MIC_{50} and MIC_{90} for this antibiotic were 0.5 and 2 mg/mL, respectively, all COPD isolates with a breakpoint inferior to 4 mg/mL (37/38, 97.4%) were classified as intermediate (Table 3.2).

Table 3.2 | Antibiotic susceptibility of COPD isolates.

Antibiotic	COPD (n=38)					
	MIC_{50} (mg/L)	**MIC_{90} (mg/L)**	**MIC range (mg/L)**	**Susceptible** n (%)	**Intermediate** n (%)	**Resistant** n (%)
Am	0.25	>8	0.12 – >8	32 (**84.2%**)	0	6 (**15.8%**)
Cft	≤0.03	≤0.03	≤0.03 – 0.12	38 (**100%**)	0	0
Crm	0.5	2	≤0.25 – 4	3 (**7.9%**)	27 (**71.0%**)	8 (**21.1%**)
Te	≤1	≤1	–	38 (**100%**)	0	0
Rif	≤0.5	≤0.5	–	38 (**100%**)	0	0
Mer	≤0.06	0.12	≤0.06 – 0.25	38 (**100%**)	0	0
Aug	0.5	2	≤0.25 – 2	38 (**100%**)	0	0
C	≤1	≤1	–	38 (**100%**)	0	0
T/S	≤0.25	>2	≤0.25 – >2	27 (**71.1%**)	0	11 (**28.9%**)
Cpe	≤0.12	0.25	≤0.12 – 0.25	38 (**100%**)	0	0
Cp	≤0.06	≤0.06	≤0.06 – >2	37 (**97.4%**)	0	1 (**2.6%**)
Azi*	0.5	2	≤0.25 – >4	0	37 (**97.4%**)	1 (**2.6%**)

*For azithromycin, 2017 EUCAST breakpoints were used

Among OM isolates, 18.2% (10/55) of isolates were considered resistant to ampicillin. Eight of these were β-lactamase producers, while the remaining two were non-β-lactamase producers. Of the non-producers, one was considered resistant to amoxicillin-clavulanate (MIC=4 mg/mL), the other intermediate (MIC=2 mg/mL) and both isolates presented reduced susceptibility to other β-lactam antibiotics. Considering these, both isolates were phenotypically identified as BLNAR. Resistance rates were observed for cefuroxime (11/55, 20.0%), trimethoprim/sulfamethoxazole (14/55, 25.5%), cefepime (2/55, 3.6%) and azithromycin (1/55, 1.8%), as well (Table 3.3).

Thirty-five (35/55, 63.6%) isolates were considered intermediate to cefuroxime. Similar to the results for COPD isolates, MIC_{50} and MIC_{90} of azithromycin for OM isolates were 1 and 2 mg/mL, respectively and 54/55 (98.2%) isolates were considered intermediate (Table 3.3).

Table 3.3 | Antibiotic susceptibility of OM isolates.

	OM (n=55)					
Antibiotic	**MIC_{50} (mg/L)**	**MIC_{90} (mg/L)**	**MIC range (mg/L)**	**Susceptible** n (%)	**Intermediate** n (%)	**Resistant** n (%)
Am	0.25	>8	0.06 – >8	45 (**81.8%**)	0	10 (**18.2%**)
Cft	≤0.03	≤0.03	≤0.03 – 0.06	55 (**100%**)	0	0
Crm	0.5	4	≤0.25 – 8	9 (**16.4%**)	35 (**63.6%**)	11 (**20.0%**)
Te	≤1	≤1	–	55 (**100%**)	0	0
Rif	≤0.5	≤0.5	–	55 (**100%**)	0	0
Mer	≤0.06	0.12	≤0.06 – 1	55 (**100%**)	0	0
Aug	0.5	1	≤0.25 – 0.5	54 (**98.2%**)	0	1 (**1.8%**)
C	≤1	≤1	–	55 (**100%**)	0	0
T/S	≤0.25	>2	≤0.25 – >2	41 (**74.5%**)	0	14 (**25.5%**)
Cpe	≤0.12	0.25	≤0.12 – 0.5	53 (**96.4%**)	0	2 (**3.6%**)
Cp	≤0.06	≤0.06	–	55 (**100%**)	0	0
Azi*	1	2	≤0.25 – >4	0	54 (**98.2%**)	1 (**1.8%**)

*For azithromycin, 2017 EUCAST breakpoints were used

3.4. Multilocus Sequence Typing

Forty-one isolates – 16 COPD and 25 OM isolates – were analysed by MLST. Isolates were selected according to date of collection and antibiotic susceptibility pattern: (**i**) all isolates collected in 2017 and 2018 and (**ii**) all β-lactamase producers. Besides these, MLST was also performed for the 2 BLNAR, the ciprofloxacin resistant and the serotype f isolates.

Overall, 31 different STs were assigned (31/41, 75.6%). Five of these, were new STs: 1894, 1895, 1896, 1900 and 1901. All new STs belonged to OM isolates, with the exception of ST 1901, which belonged to a COPD isolate (Table 3.4).

Table 3.4 | New STs assigned during the course of this project.

H.influenzae ID	Year	Diagnosis	*adk*	*atpG*	*frdB*	*fucK*	*mdh*	*pgi*	*recA*	ST
13692	2017	OM	69	20	7	1	26	36	29	**1894**
13741	2017	OM	63	32	7	1	46	74	29	**1895**
13744	2017	OM	39	8	53	11	65	48	19	**1896**
12880	2013	OM	3	54	65	1	64	82	48	**1900**
13776	2017	COPD	5	8	58	14	46	13	29	**1901**

A schematic presentation of isolates genetic diversity is shown in Fig. 3.4, where isolates that differed up to two alleles (DLVs) were joined together. STs exclusive to each clinical group – COPD, or OM – were separated according to the respective group. STs shared among both COPD and OM isolates were presented between the two groups.

It was verified a high genetic diversity among NTHi isolates, with 31 different STs assigned to 41 analysed isolates. Serotype f isolate was characterized as ST=124.

There were COPD and OM isolates with the same STs: 155, 389, 103 and 1218. STs 155, 389 and 1218 were shared among two isolates, each: one COPD and one OM. ST 103 was shared among three isolates: one COPD and two OM.

STs 155 and 1281, 1218 and 107 and 1521 and 196 differed in two alleles. STs 474 and 1900 only differed in one allele.

Four STs were assigned to isolates belonging to the same clinical group. Regarding COPD isolates, STs 170 and 388 were both shared among two isolates. In relation to OM isolates, ST 57 was shared among three isolates and ST 241 was shared among two isolates. ST 57 was the most prevalent among OM isolates.

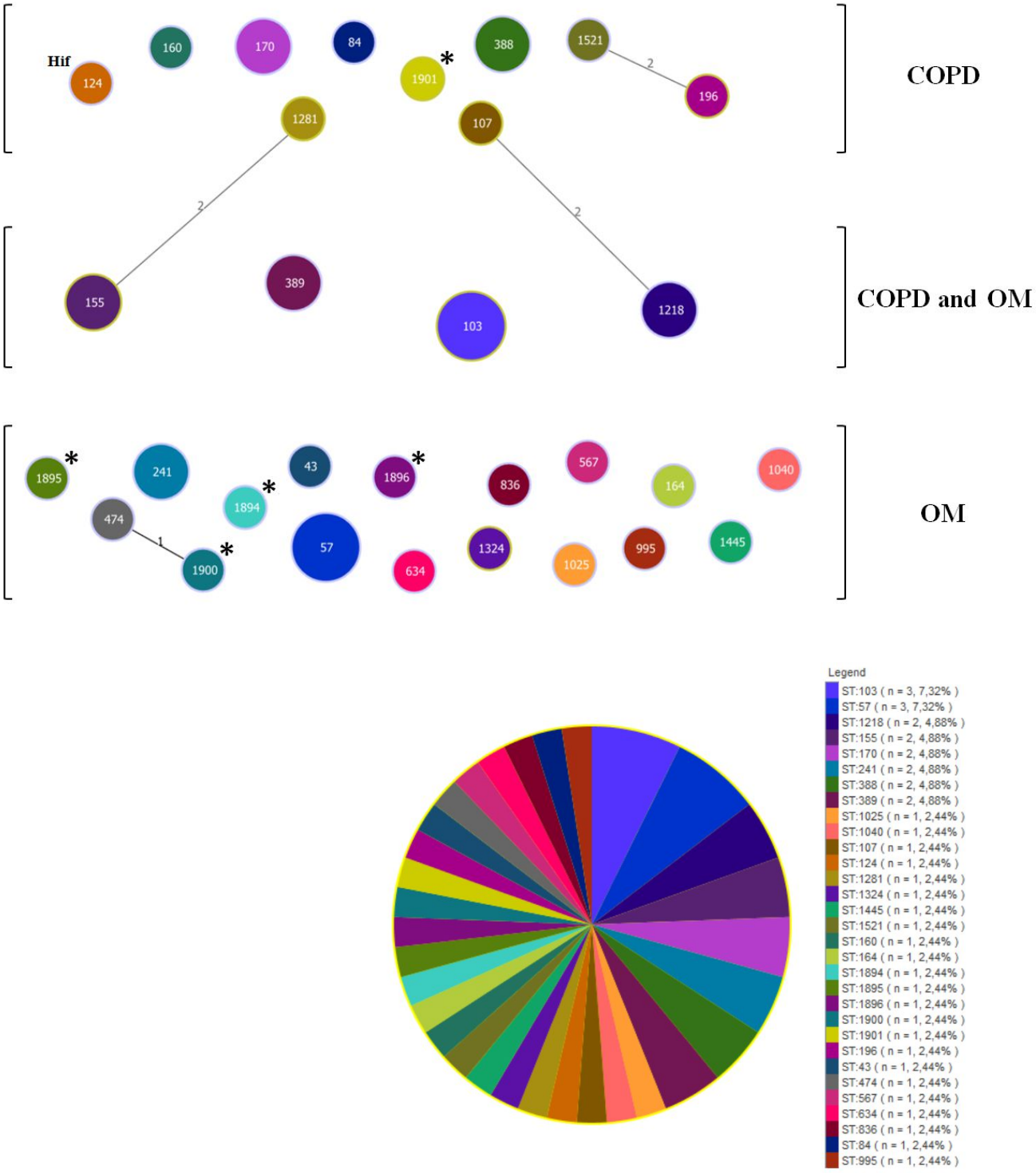

Figure 3.4 | Schematic presentation of genetic diversity among *H. influenzae* isolates (PHYLOViZ 2.0©, with goeBURST algorithm). Thirty-one STs are indicated by numbers within circles. Colours were attributed to each ST number and are described in the Legend. The number (n) of isolates that belong to each ST is also described in the Legend. The size of the circle is proportional to the number of isolates that share that ST. STs were grouped according to the clinical group, COPD or OM. Four STs were shared among COPD and OM clinical groups. STs that presented up to DLVs were joined by dark lines, labelled with the number of allelic differences between STs.

*new STs assigned during the present study

3.5. Virulence genes

All 93 isolates were analysed for the presence of six virulence genes: *pilA*, *hifA, hmw1A*, *hmw2A*, *hia* and *ompP5*.

Presence of *pilA* was observed for the majority of both COPD (38/38, 100%) and OM isolates (51/55, 92.7%). Similarly, the presence of *ompP5* was also verified for most COPD (31/38, 81.6%) and OM isolates (36/55, 65.5%) (Table 3.5).

On the other hand, *hifA* and *hia* were present in less than 50% of either COPD (5.3% and 13.2%, respectively), or OM isolates (25.5% and 41.8%, respectively). However, our results showed a higher prevalence of both *hifA* and *hia* genes in OM isolates, when compared to COPD isolates (Table 3.5).

Regarding *hmw1A* and *hmw2A* genes, these were found together in the majority of COPD isolates (29/38, 76.3%) and in less than 50% of OM isolates (14/55, 25.5%). We highlight four OM isolates where *hmw2A* gene was found, in the absence of *hmw1A* gene, which turned in a higher prevalence of *hmw2A* gene (18/55, 32.7%), when compared with *hmw1A* gene (14/55, 25.5%), as presented on Table 3.5.

Table 3.5 | Distribution of virulence genes in COPD and OM *H. influenzae* isolates.

Gene / Disease	***pilA*** n (%)	***hifA*** n (%)	***hmw1A*** n (%)	***hmw2A*** n (%)	***hia*** n (%)	***ompP5*** n (%)
COPD (n=38)	38 (**100%**)	2 (**5.3%**)	29 (**76.3%**)	29 (**76.3%**)	5 (**13.2%**)	31 (**81.6%**)
OM (n=55)	51 (**92.7%**)	14 (**25.5%**)	14 (**25.5%**)	18 (**32.7%**)	23 (**41.8%**)	36 (**65.5%**)

It was further observed, in both clinical groups, that *hia* gene was present only when both *hmw* genes were absent.

Regarding COPD clinical group, it was verified that the majority of isolates (29/38, 76.3%) possessed both *hmw* genes, with absence of *hia* gene. Only 10.5% (4/38) of COPD isolates lacked all 3 genes (Table 3.6).

In opposite, in the OM clinical group, most isolates lacked both *hmw* genes and possessed *hia* gene (23/55, 41.8%). Curiously, the same number of isolates possessed both *hmw* genes and lacked *hia* (14/55, 25.5%), or lacked all 3 genes (14/55, 25.5%).

It was further observed that in all four isolates that only possessed the *hmw2A* gene, *hia* gene was absent (Table 3.6).

Table 3.6 | Relation between *hmw1A/2A* and *hia* genes in COPD and OM *H. influenzae* isolates.

Genes \ Disease	COPD (n=38) n (%)	OM (n=55) n (%)
***hmw1A/2A* (+) / *hia* (-)**	29 (**76.3%**)	14 (**25.5%**)
***hmw1A/2A* (-) / *hia* (+)**	5 (**13.2%**)	23 (**41.8%**)
***hmw1A/2A* (-) / *hia* (-)**	4 (**10.5%**)	14 (**25.5%**)
***hwm2A* (+) / *hia* (-)**	0	4 (**7.3%**)
***hmw2A* (+) / *hia* (+)**	0	0

3.6. Biofilms

3.6.1. Biofilm production

After 24h incubation, only 14.0% (15/107) of isolates were able to form a biofilm and after 48h, this number had increased to 29.0% (31/107). Therefore, an overall increase of 15.0% in biofilm production was verified from 24h to 48h. Even so, the results suggested a lack of ability for more than 50% of all isolates to form a biofilm after either 24h, or 48h incubation. Measurement of OD_{600nm} at 24h and 48h, enabled to conclude that the overall bacterial growth remained relatively stable after these incubation times.

Results for biofilm production after 24h and 48h incubation and isolate classification regarding biofilm production, according to each clinical group, are summarized on Table 3.7 and schematically presented on Fig. 3.5.

After 24h, the majority of isolates from the three clinical groups was not able to assemble a biofilm (92.1% [35/38], 81.8% [45/55] and 85.7% [12/14] of COPD, OM and invasive isolates, respectively). At 48h, this behaviour persisted for most of these isolates (71.4% [25/35], 86.7% [39/45] and 83.3% [10/12] of COPD, OM and invasive isolates, respectively) and only in a minority biofilm assembly was observed (17.1% [9/35], 13.3% [6/45] and 16.7% [2/12] of COPD, OM and invasive isolates, respectively). Even so, these biofilm assemblers fitted the category of weak producers. Only one COPD isolate (2.9%) became a moderate producer, after 48h incubation.

Few COPD, OM and invasive isolates were weak biofilm producers after 24h (7.9% [3/38], 16.4% [9/55] and 7.1% [1/14] of COPD, OM and invasive isolates, respectively) and while the majority of COPD isolates became moderate producers (66.7% [2/3]) most OM isolates persisted as

weak producers, after 48h (88.9% [8/9]). One OM (11.1% [1/9]) and the invasive isolate (7.1% [1/14]) became non-producers after 48h.

None of COPD isolates were moderate biofilm producers after 24h. Only 1.8% (1/55) of OM and 7.1% (1/14) of invasive isolates were moderate producers after 24h incubation. After 48h, the OM isolate persisted as a moderate biofilm producer, while the invasive isolate became a weak biofilm producer.

No isolates, in the three clinical sets, were strong biofilm producers after either 24h, or 48h.

Although a relation between the production of biofilm and the respective clinical group could be identified, with OM isolates being most capable of biofilm production – mainly after 24h – followed by COPD and invasive isolates (Table 3.7 and Fig. 3.5), this association was not statistically significant ($p = 0.342$).

Similar results were obtained for classification of isolates as weak and moderate biofilm producers. For example, it was possible to conclude that the majority of weak COPD producer isolates became moderate producers from 24h to 48h and that most weak OM isolates maintained as weak producers. However, classifications and correlation with each clinical group after 24h ($p = 0.236$) and 48h ($p = 0.339$) did not meet statistical significance, as well.

3.6.2. Virulence factors and biofilm production

It was verified that all COPD and the majority of OM biofilm producer isolates possessed the *pilA* gene. The majority of COPD biofilm producer isolates also possessed *hmw1A/2A* genes. However, none of OM biofilm producers, after 24h, possessed these genes and few producers after 48h incubation possessed both (n=2). Considering that most COPD and OM non-producer isolates, after either 24h or 48h, also possessed *pilA* and *hmw1A/2A* genes, it appears that no correlation could be established. Furthermore, very few invasive isolates produced a biofilm after 24h (n=2) and 48h (n=3). Since all of these isolates were known to possess *hmw1A/2A* genes, these results further suggest that a correlation could not be established.

Table 3.7 | Biofilm production at 24h and 48h and classification of isolates as weak and moderate producers, according to each clinical group.

			Classification of biofilm producer isolates			
	Biofilm Production n (%)		Weak n (%)		Moderate n (%)	
Pathology	Biofilm (24h)	Biofilm (48h)	24h	48h	24h	48h
COPD (n=38)	3 **(7.9%)**	13 **(34.2%)**	3 **(7.9%)**	10 **(26.3%)**	-	3 **(7.9%)**
OM (n=55)	10 **(18.2%)**	15 **(27.3%)**	9 **(16.4%)**	14 **(25.5%)**	1 **(1.8%)**	1 **(1.8%)**
Invasive (n=14)	2 **(14.3%)**	3 **(21.4%)**	1 **(7.1%)**	3 **(21.4%)**	1 **(7.1%)**	-
Total (n=107)	15 **(14.0%)**	31 **(29.0%)**	13 **(12.1%)**	27 **(25.2%)**	2 **(1.9%)**	4 **(3.7%)**

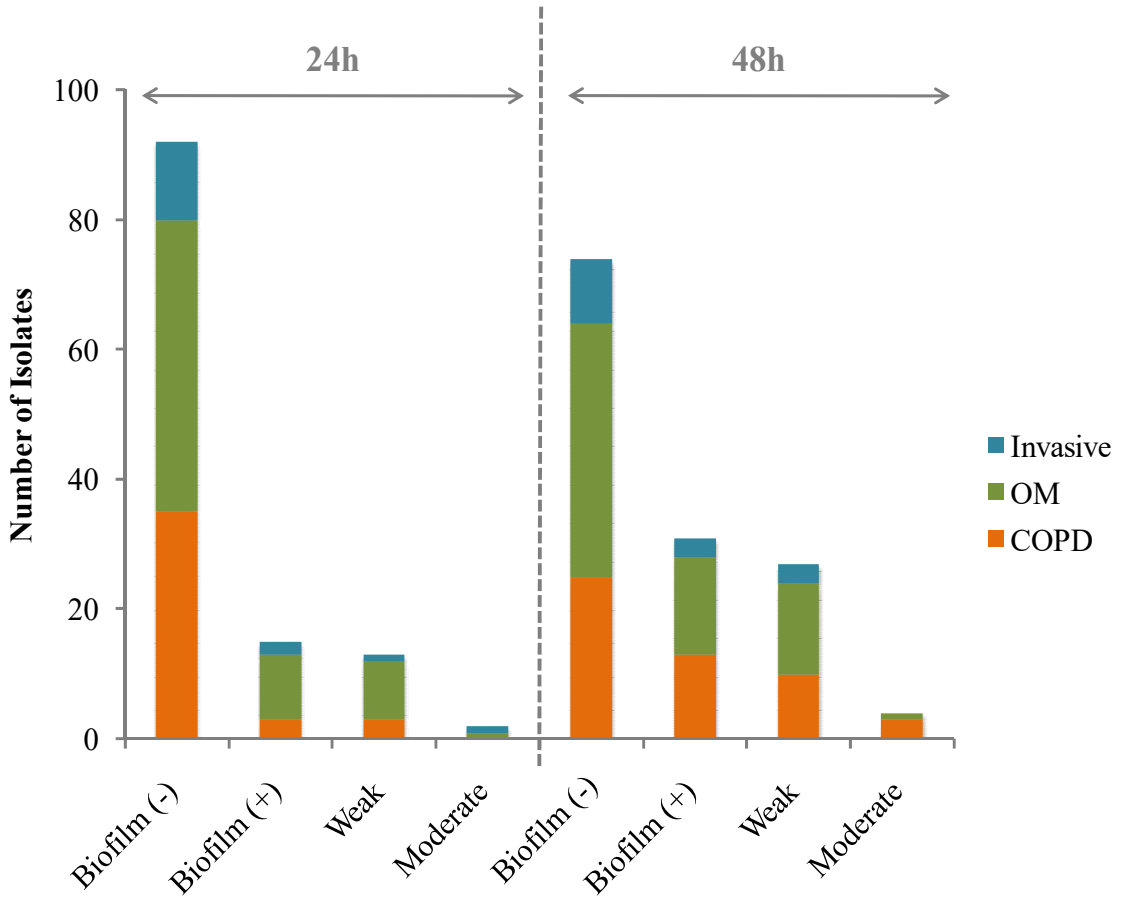

Figure 3.5 | Biofilm production after 24h and 48h.

4. Discussion

4.1. *Haemophilus influenzae* and non-invasive diseases

H. influenzae has been long associated to invasive disease. Therefore, most worldwide epidemiological studies have focused on these infections.[25,27,48,51,125-128] Similarly, in Portugal, epidemiology of invasive disease is published in three studies: one of the pre-vaccine era (1989-2001)[54] and two of the post-vaccine era (2002-2010[55] and 2010-2014[56]). However, since *H. influenzae* is a commensal bacterium in the human nasopharynx, it is also responsible for triggering non-invasive infections that may be associated with diseases of the respiratory tract, such as COPD and OM.

4.1.2. Chronic Obstructive Pulmonary Disease

Due to its chronic nature, COPD is a disease that usually affects adults over 40 years of age. In fact, a review that included data from 19 European countries showed that the mean age for COPD patients was 55.9 years old.[129] Two other studies that analysed COPD data from six European countries and from USA, Mexico, Brazil, Russia, Japan and South Korea[130,131] described a mean age ranging from 57.2 to 66.8 years old for COPD patients. An increase in prevalence of the disease with aging was also reported. Our results were in agreement with these findings, since all clinical isolates were recovered from patients with more than 50 years of age (Fig. 3.1), being the majority of these (36.8%) within the range of 75 to 84 years old. These were also in agreement with data from France and Spain, that also presented a higher rate of disease in the oldest population (70 years old, or older).[130,131] The difference in age range between Portugal and reports for other countries may be related to geographical factors, tobacco smoking, environmental exposure and/or occupational exposure to pollution.

Considering gender distribution, our data showed that males were the most affected gender (84.2%), with only a small percentage of isolates recovered from women (15.8%) (Table 2.1). These results are in agreement with previous reports for different countries,[130] but recent data suggests that this disease may equally affect both genders, nowadays.[32]

4.1.3. Otitis Media

In opposite to COPD, OM is a disease that mostly affects children in the first years of life.[41] An epidemiological study,[43] that collected worldwide data from all continents, indicated that 51% of annual AOM cases affected children under the age of five, with a higher global incidence in children with less than one and between one and four years old. Our results are in agreement with such data, since the majority of the isolates were recovered from children between the ages of less than one and three years old (85.5%), with a high percentage of cases in the first year of life (30.9%) (Fig. 3.2). Differences in gender were not observed, since 50.9% of the patients were male and 49.1% were female (Table 2.1). These results were in agreement with other studies that reported no risk factors and no significant differences associated with gender.[132,133]

4.2. Capsular typing

NTHi has been associated with infection in patients suffering from COPD, with or without exacerbations.[34,134] Similarly, in patients suffering from OM, NTHi is one of the most common pathogens. It has been further demonstrated that a high prevalence of NTHi in children up to two years old, suffering from AOM, highlights the important infectious role of this bacteria.[135]

In our study, all 55 OM isolates were characterized as NTHi, as well as all but one COPD isolate (37/38, 97.4%). This was expected, since COPD and OM have been extensively described as NTHi diseases.[34,36,134,135,136] Since NTHi is an opportunistic commensal of the human nasopharynx, it can easily spread to the lower respiratory tract, causing infections in COPD patients, or it may spread to the middle ear to cause OM.[136]

4.3. Antibiotic susceptibility

COPD and OM infections are often treated with antibiotics. In Portugal, guidelines from the "Direcção-Geral da Saúde" (DGS) recommend the use of a β-lactam (amoxicillin with, or without clavulanic acid), macrolides or doxycyclines for treatment of COPD exacerbations with sputum.[39] For children with AOM, the first antibiotics to be recommended by DGS are amoxicillin/amoxicillin-clavulanate, or cefuroxime in the case of ineffectiveness of the first antibiotics.[44] However, an increase

of bacterial resistance against antibiotics has been observed over the last decades, which is a great concern.

In the present study, the antibiotic that presented the highest percentage of resistance was trimethoprim/sulfamethoxazole. Our results showed that 28.9% and 25.5% of COPD and OM isolates, respectively, were resistant to trimethoprim/sulfamethoxazole (Table 3.2 and 3.3). These results were in accordance with the percentages described among 144 invasive *H. influenzae* isolates in the previous Portuguese study[55] (20.4%) and among 482 NTHi invasive isolates in a Canadian study[137] (20.7%). Decreased susceptibility to trimethoprim/sulfamethoxazole was also reported for 54.4% of *H. influenzae* isolates, recovered from patients with respiratory tract infections, in China.[138] Although trimethoprim/sulfamethoxazole is not an antibiotic commonly used in Portugal for treatment of either COPD, or OM infections,[39,44] the resistance rate here identified may be related to its use for treatment of other infections, which may ultimately affect susceptibility of *H. influenzae*, in general.

It is worth noting that, although it is not common, one COPD isolate was identified as resistant to ciprofloxacin. This was the first time that a *H. influenzae* isolate resistant to ciprofloxacin was characterized, in our laboratory, in approximately 4,500 isolates. A case study in USA[139] reported a patient, suffering from a chronic lung disease, infected with a *H. influenzae* resistant to ciprofloxacin and presenting a MIC of 8 mg/L. The authors attributed resistance to a long exposure to the antibiotic, which was used for treatment of respiratory tract infections. Curiously, a 2015 Spanish study[140] reported 15 NTHi isolates, recovered from patients with respiratory infections, with ciprofloxacin MICs between 8 and 16 mg/L. The authors related this result with a common use of this antibiotic to treat respiratory infections. However, the authors reported that the percentage of ciprofloxacin resistant isolates remained low. Even so, this antibiotic is not recommended for treatment of COPD, or OM infections in Portugal, which could explain the presence of only one resistant isolate among the 93 analysed isolates.[39,44]

4.3.1. β-lactamase production

In the present study, most isolates (84.9%) did not produce β-lactamase. Ampicillin resistance by β-lactamase production was identified in 15.8% and 14.5% of COPD and OM isolates, respectively, which accounted for 15.1% among a total of 93 characterized isolates (Fig. 3.3). The percentages for ampicillin-resistant β-lactamase producer COPD and OM isolates were slightly higher than that described for NTHi invasive isolates in the 2002-2010 study (12.6%),[55] but similar to those obtained in a Spanish study with 349 NTHi isolates, recovered from patients with respiratory tract infections (15.8%).[141] Due to the common use of β-lactams for treatment of *H. influenzae* infections

(e.g.: COPD and OM) resistance to ampicillin has increased over the years, although resistance rates differ from different countries.[62]

In fact, higher percentages were found in a Canadian study[137] that reported 21.4% β-lactamase producers among NTHi invasive isolates. For NTHi isolates recovered from patients with COPD and other respiratory tract infections, South Korea,[142] China[138] and UK[143] reported even higher percentages for ampicillin resistance, due to β-lactamase production, of 47.2%, 31.0% and 37.5%, respectively. Such percentages were related with common prescriptions of β-lactams for treatment of *H. influenzae* infections in these countries. Curiously, the UK had been previously reported to possess a higher prevalence of β-lactamase producers when compared with other European countries, such as Germany, Italy, Poland, Turkey, The Netherlands and Spain, which presented lower prevalences.[143,144]

4.3.2. β-Lactamase-Negative Ampicillin-Resistance (BLNAR) mechanism

In our work, only 2.2% of the isolates were phenotypically identified as BLNAR (Table 3.3). These isolates were collected from patients with OM and also presented reduced susceptibility to other β-lactam antibiotics, which has been described for BLNAR isolates.[140] When compared with the previous Portuguese study, that described 7.7% genetically defined BLNAR invasive isolates,[55] the percentage obtained in the present study was lower, but more similar to percentages reported for other countries.[138,142,143] Globally, several countries have found low percentages of 4.2%, 5.1%, 6.1% for BLNAR isolates in UK,[143] China[138] and South Korea,[142] respectively, among isolates from patients with respiratory tract infections. However, a higher percentage of 56.0% BLNAR *H. influenzae* isolates has been described in Spain.[141] Likewise, in Japan, before introduction of Hib vaccine, 52.0% BLNAR *H. influenzae* isolates were reported (mostly from patients with respiratory diseases).[145] Similarly, a longitudinal study of meningitis caused by *H. influenzae* observed an increase in BLNAR isolates from 2000 to 2011.[146]

A Portuguese study,[147] with a majority of respiratory isolates, showed that a selected sample characterized as ampicillin non-susceptible isolates (MIC ≥ 1 mg/mL) included 66.7% (94/141) BLNAR isolates with *ftsI* mutations.

Although BLNAR prevalence among COPD and OM isolates was low, surveillance of this resistance mechanism is recommended, since β-lactams are the first line antibiotics commonly used for treatment of these diseases in Portugal.[39,44]

4.4. Multilocus Sequence Typing analysis

MLST is a technique that analyses nucleotide sequences of housekeeping genes. Analysis of variations at gene level enables the genetic comparison of different bacterial isolates, which becomes useful when studying global epidemiology of disease.[24] Different studies have shown that while encapsulated *H. influenzae* isolates are highly clonal and are, therefore, organized in a limited number of clusters, NTHi isolates are a genetically diverse population.[15,23,55]

In our work, 31 STs were assigned to 41 analysed isolates (16 COPD and 25 OM isolates) (Fig. 3.4). Since our isolates were almost all NTHi, these results of great genetic diversity were expected, which is in agreement with the 2002–2010 Portuguese study,[55] as well as with reports from other countries, that also described a highly diverse NTHi population.[15,23] Only four STs (155, 389, 103 and 1218) were shared among COPD and OM isolates, being two of these (STs 155 and 1218) closely related to two COPD STs (STs 1281 and 107) (Fig. 3.4). Therefore, we could not establish a relationship between determined STs and clinical origin. The most common ST was ST 57, shared among three OM isolates (Fig. 3.4) which is in accordance with results from a previous study[148] that found ST 57 to be the most common ST among OM isolates. These authors even postulated that this ST might be representative among OM isolates, contributing to virulence associated with the disease.

4.5. Virulence genes

H. influenzae infection starts by adhesion of the bacteria to the mucosa surface of the host. For adherence, *H. influenzae* expresses several surface adhesins, such as PilA, HifA, HMW1A, HMW2A, Hia and OMP P5.

pilA gene is part of a four-gene cluster, *pilABCD*, and PilA protein represents the major subunit of the type IV pilus in *H. influenzae*.[13,80] In our study, *pilA* was found in all 38 COPD isolates (100%) and in 92.7% (51/55) OM isolates (Table 3.5). Similar results were described by Bakaletz and colleagues and Vuong and colleagues, who found a presence of *pilA* between 91 and 100% of NTHi respiratory isolates.[13,78]

hifA belongs to *hifABCDE* gene cluster and expresses fimbrial HifA, which is the major subunit of the fimbriae encoded by the cluster.[81] Prevalence of *hifA* gene was 25.5% in OM isolates and 5.3% in COPD isolates (Table 3.5). A study by Geluk and colleagues[82] showed a prevalence of 18% for *hifA* in NTHi respiratory isolates. Regarding our results, OM isolates presented a similar percentage, while COPD isolates presented a lower percentage.

hmw1A/2A and *hia* genes encode mutually exclusive adhesins HMW1A/2A and Hia, respectively.[77] *hmw* genes have been shown to undergo phase variation mechanisms related to tandem

repeats of 7-bp (ATCTTTC) in the promoters of *hmw1A* and *hmw2A* genes. That is, protein expression decreases with an increase in the number of repeats.[73] In our study, a prevalence of 76.3% was found for *hmw1A/2A* in COPD isolates (Table 3.5). This is in good agreement with different studies that described a prevalence of both *hmw* genes in NTHi respiratory isolates varying from 55 to 100%.[78,88] However, the same was not observed for OM clinical group. In this case, both genes were present in 25.5% (14/55) of the isolates, while in 7.3% (4/55) of the isolates, only *hmw2A* was present (Table 3.5 and 3.6). Ecevit and colleagues,[87] also found a different prevalence for the *hmw1A/2A* genes in NTHi isolates collected from the respiratory tract, which was similar to our results. Nevertheless, in that study, a higher prevalence was observed for *hmw1A* gene (51%), when compared with that of *hmw2A* gene (23%).

Our study found a higher percentage of *hia* in OM isolates (41.8%) than in COPD isolates (13.2%) (Table 3.5). Presence of *hia* gene in OM isolates was more similar to the percentage of 32.0% , described by Ecevit and colleagues,[87] in NTHi respiratory isolates, than to the percentage of 55.6% described by Cardines and colleagues,[89] for invasive NTHi isolates.

Since it has been described that *hia* is present in the absence of the *hmw1A/2A* genes, this relation was assessed (Table 3.6).[77] Indeed, presence of *hia* was only identified in COPD and OM isolates lacking both *hmw* genes. Furthermore, the four OM isolates that only possessed the *hmw2A* gene also lacked *hia* gene (7.3%). It was further verified that while the majority of COPD isolates (76.3%) possessed both *hmw* genes and lacked *hia*, the majority of OM isolates (41.8%) lacked both *hmw* genes and possessed *hia* (Table 3.6). Curiously, when comparing nasopharyngeal and OM isolates, Dawid and colleagues[73] found that isolates recovered from the ear contained less HMW proteins. The authors hypothesized that, since the middle ear is rich in antibodies against HMW1/2, a lesser content of these proteins could serve as a survival mechanism for persistence. Even so, it appears that this is a controversial topic since results of other studies showed the opposite, with more OM isolates possessing *hmw1A/2A* genes and HMW1/2 expression, than COPD/lower respiratory tract isolates.[86,87]

Nevertheless, it is interesting to notice that, in the present study, *hmw1A/2A* genes were more prevalent in COPD isolates than in OM isolates and that the presence of *hifA* and *hia*, on the other hand, was more prevalent in OM isolates than in COPD isolates. Considering the relatively high prevalence of *hmw1A/2A* genes generally described for NTHi isolates, it might be reasonable to hypothesize a relationship between type of disease and presence of these virulence genes. These differences could be related with adaptations of the bacteria to the different surfaces in each disease and with adaptation to the surrounding environment in the respiratory tract.

ompP5 gene encodes protein OMP P5 and, like *hmw1A/2A*, it was also suggested to be variably expressed in NTHi. Variability in expression of this protein is related to four variable regions in the *ompP5* sequences, which result in amino-acid substitutions in loop regions displayed on the bacterial surface. Naturally, this variability becomes an advantage for *H. influenzae* infection, as a

selective adaptation mechanism.[94] In our study, *ompP5* gene was present in 81.6% and 65.5% of COPD and OM isolates, respectively (Table 3.5), which is within the range presented by previous studies that reported a prevalence for *ompP5* from 20%[78] to 100%[92] in NTHi respiratory isolates.

4.6. Biofilms

In the last two decades, the role of bacterial biofilms has been an important topic of research. Biofilms are dynamic assemblies of microorganisms that serve as a bacterial defence mechanism in biofilm associated infections and are usually related with persistence of chronic diseases.[100,101]

H. influenzae virulence factors such as PilA, HMW1 and HMW2 proteins have been suggested to play important roles on different stages of biofilm formation. In an initial stage, these proteins are important for adhesion[79,86] and, at later stages, these would be secreted and integrated in the EPS matrix.[106,108]

4.6.1. Comparison between different classification methods

Biofilms have been recognized as a serious threat to public health over the last two decades. These structures constitute a bacterial defence mechanism usually associated with chronic infections, which led to a search for new approaches to fight biofilms. However, the need for new efficient therapies resulted in the development of different experimental protocols and testing strategies. The lack of standardized protocols for biofilm assessment highly hampers a direct comparison between results obtained by different laboratories.[149] In fact, this same limitation was identified in a study conducted by Murphy and Kirkam.[113] This problem led Stepanović and colleagues[123] to develop a classification system that expressed results as numbers, for an easier comparison between different studies. This was the classification system used in the present study.

We intended to establish a comparison between different classification systems and how these affected the interpretation of results. Thus, our results were adapted to the classification system described in a study conducted by Puig and colleagues.[114] This study was elected for presenting an experimental protocol similar to that used in the present study. The major difference was in the establishment of cut-offs for biofilm production and classification of biofilm producer isolates. With the classification system of the study conducted by Puig and colleagues,[114] the cut-off OD was higher than that obtained with the classification system described by Stepanović and colleagues.[123] In our study, this translated into a higher number of non-producer isolates and very few producers, either after 24h or 48h. Furthermore, after 24h, no COPD isolates were biofilm producers and only two OM

and one invasive isolate were producers. These results suggest that the definition of a cut-off influences the interpretation of results.

There is a need to describe a standardized and reproducible classification system for biofilm production and for experimental procedures that do not require too much supplies, time and highly specialized personnel.[149] Otherwise, comparisons of results between different studies, that used different protocols and classification systems, are not straightforward and may lead to misleading conclusions.

4.6.2. Production of biofilm

Several studies have shown the ability of *H. influenzae* isolates recovered from patients with OM and COPD to form biofilms, both in *in vitro* and *in vivo* systems.[79,105,113-116,118,119] Those studies conducted *in vitro* showed that most *H. influenzae* isolates were able to form biofilms after 24h incubation. These results were not entirely in agreement with ours, since it was verified that only 14.0% of isolates were able to form a biofilm after 24h incubation (Table 3.7). Furthermore, an overall 15.0% increase in biofilm production from 24h to 48h incubation (Table 3.7) suggested that isolates may require more than 24h to form biofilms. It has been described that efficient attachment is influenced by nutrient availability, roughness and hydrophobicity of the surface and surface conditioning, which is associated with the medium to which a surface is exposed. Properties of the medium, such as specific particles, influence bacterial adhesion to the exposed surface, which will vary according to the surrounding environment.[98] Thus, the fact that bacterial growth did not increase from 24h to 48h and that biofilm production increased, may suggest that biofilm production was a slow process and that bacterial cells possibly required this time to adapt to the surrounding environment and eventual stress conditions, such as nutrient consumption. Even so, approximately 71% to 87% of isolates from the three clinical backgrounds that formed a biofilm after 24h were still not capable of forming a biofilm after 48h.

On the other hand, other *in vitro* studies have also found a low number of biofilm producer isolates among both OM and COPD isolates. Mizrahi and colleagues[117] found that after 24h incubation, 51% of 216 NTHi isolates recovered from patients with AOM (including a control group of 43 isolates) were non-biofilm producers, while a study conducted by Martínez-Reséndez and colleagues[150] described that only 10% of 98 NTHi isolates recovered from patients with lower respiratory tract infections (COPD included) were able to produce a biofilm. These studies presented results more similar to those obtained in the present study, with most of OM and COPD isolates not being able to produce biofilms. The disparity in the results obtained by different studies may be related with geographical variations,[114] which cannot be excluded, but may mostly be due to different

experimental and classification protocols that may affect interpretation of the results, as previously explained.

4.6.3. Correlation between production of biofilm and an underlying disease

Few studies have established comparisons between clinical site of isolation and ability of NTHi isolates to form biofilms. Puig and colleagues have demonstrated, in two different *in vitro* studies,[105,114] that OM isolates were better biofilm producers and formed denser biofilms than COPD isolates, which presented similar biofilm production levels to that of healthy carriers. One of these studies[114] further included invasive isolates that presented the highest levels for biofilm production. Our results, obtained after 24h incubation, partially support these findings: OM isolates were, indeed, the most capable of producing biofilms after 24h, when compared with COPD isolates. However, invasive isolates were the worst biofilm producers, with less biofilm production than OM and COPD isolates (Table 3.7 and Fig. 3.5). The results for invasive isolates were the opposite of the expected, since these were used as positive controls for biofilm formation. This suggests that genomic analysis alone may not have been enough for an inference between presence of the *hmw1A/2A genes* and biofilm production.

The results of our work could establish a relation between origin of the isolates and biofilm production, as follow: from 24h to 48h most COPD isolates tended to increase biofilm production, while OM and invasive isolates tended to stabilize, or decrease production. However, statistical analysis indicated that the results were not statistically significant and such conclusions could not be withdrawn. In addition, it should be considered the small number of isolates that were, in fact, able to produce biofilms after 24h incubation (15/107) (Table 3.7) which further limited the establishment of a correlation between clinical groups and the ability of isolates to produce biofilms.

Other authors were also unable to establish a correlation between origin of isolates and production of biofilm. Murphy and Kirkam[113] conducted an *in vitro* study with 15 COPD and 15 OM isolates and did not highlight significant differences between biofilms produced by NTHi isolates from the two different backgrounds. A more recent *in vitro* study conducted by Obaid and colleagues[119] examined a collection of 60 isolates recovered from oropharyngeal normal floras and patients with different diseases – OM, conjunctivitis, lower respiratory tract diseases and cystic fibrosis – and found that there was no relation between site of isolation and ability of NTHi isolates to form biofilms. These results suggest that a lack of correlation between different clinical groups may not be specific of COPD and OM isolates, since the study conducted by Obaid and colleagues,[119] was unable to establish a correlation between isolates belonging to several respiratory clinical groups. Although these studies are more in agreement with the results here presented, it has to be considered the limited number of isolates included in both these studies.

It should not be excluded the possibility that differences between the results here obtained and results from other studies, could be related with different experimental and/or classification systems for establishment of biofilm production, as explained before.

4.6.4. Virulence factors and biofilm production

Several studies have described a relation between the presence of virulence genes *pilA*, *hmw1A* and *hmw2A* with the ability of *H. influenzae* isolates to produce biofilms.[79,86,106,108]

Apparently, a correlation between presence of the selected virulence genes and production of biofilm could not be established, with our results. While most COPD and OM biofilm producer isolates possessed *pilA* gene, the majority of isolates identified as non-producers also possessed this gene. In addition, while the majority of COPD producer isolates possessed both *hmw* genes, most OM isolates did not. Besides, several non-producer isolates from both clinical groups also possessed these genes, which further supported the assumption that no correlation could be established. Curiously, a recent study conducted by Cardines and colleagues[151] described that isolates with the *hmw1A/2A* genes presented low biofilm production. These assumptions were further supported by our results with invasive isolates, all of which possessed *hmw1A/2A* genes, but very few actually produced a biofilm after 24h and 48h.

It should always be considered that *in vitro* studies do not reflect what occurs *in vivo*. Furthermore, regarding the three virulence factors, it should be noted that it was only assessed the presence of the genes and not the expression levels by transcriptomic analysis. Therefore, the production of PilA and HMW1A/2A proteins may significantly differ from isolates grown *in vitro*, than that observed when bacteria grow in the appropriate environment, which may, eventually, influence biofilm production.

Conclusions and Future Perspectives

Haemophilus influenzae is an important human-restricted microorganism that, in spite of being commonly found in the nasopharynx, it is also able to cause severe invasive diseases, as well as acute and chronic infections. NTHi has long been associated with respiratory tract infections, mainly in patients with underlying diseases, or with a compromised immune system, which is related to the opportunistic behaviour of *H. influenzae*.

A sample of 93 *H. influenzae* isolates collected from patients with COPD and OM, from 2013 to 2018, was studied. As expected, COPD isolates were recovered from adults (100%), whereas OM isolates were mainly recovered from children (98.2%).

Results from this study showed that NTHi infection was associated with both COPD and OM. These results were expected since the presence of NTHi as a commensal of the nasopharynx makes it a potential source of infection in both the upper and lower respiratory tract. Therefore, patients with COPD and OM usually present NTHi associated infections.

The majority of isolates (84.9%) from both clinical groups was susceptible to all antibiotics. Ampicillin resistance, mediated by β-lactamase, was observed for 15.8% of COPD isolates and for 14.5% of OM isolates. It should be noted that one COPD isolate was resistant to ciprofloxacin. This was the first *H. influenzae* ciprofloxacin resistant isolate characterized in the laboratory.

MLST, performed for 41 isolates, revealed a high genetic diversity among isolates from both clinical backgrounds, with 31 assigned STs. This is also consistent with results obtained from other studies that showed a high genetic diversity among NTHi isolates. Although we could not establish a general relation between STs and clinical origin, we observed that ST 57 was the most common among OM isolates. This result, previously described in literature, could suggest an association of a specific genotype with a clinical outcome (in this case, OM).

The presence/absence of six virulence genes, assessed for all 93 isolates, revealed a possible association with clinical origin. Results showed that *pilA* and *ompP5* genes were present in the majority of the isolates. However, *hifA* and *hia* genes were observed for less than 50% of COPD and OM isolates. Even so, the presence of *hifA* and *hia* was higher among OM isolates. In the OM clinical group, *hmw1A* and *hmw2A* were only present in 25.5% and 32.7% of isolates, respectively, while both genes were found together in 76.3% of COPD isolates. We observed a higher prevalence of *hmw1A/2A* genes and a lower prevalence of *hifA* and *hia* genes among COPD isolates, while the opposite was found for OM isolates. It was further observed that *hia* gene was present only when *hmw* genes were absent, which is in accordance with the literature.

Biofilm production was verified for less than 50% of isolates from all three clinical groups (COPD, OM and invasive disease) after 24h and 48h. These results were not in accordance with different reports from several authors that showed NTHi as a strong biofilm producer. However,

comparisons between different studies have to be carefully evaluated, not only due to possible epidemiological factors, but mainly because of a lack in a standardised protocol for biofilm assessment. Furthermore, it was not possible to establish a correlation between clinical origin and ability of isolates to form biofilms. Similarly, no association was found with the presence of three virulence factors: *pilA* and *hmw1A/2A* genes, which express adhesins that were previously described as key factors for adherence and biofilm formation.

It is worth noting that sample size (38 COPD and 55 OM isolates) is a limitation of the present study, which hampered the withdrawn of general conclusions. Furthermore, only genomic studies were conducted for virulence genes. The performance of a transcriptomic and proteomic analysis of these genes could elucidate their role in *H. influenzae* pathogenesis, in general, as well as in biofilm assembly, in particular.

COPD is one of the leading worldwide diseases. It is prevalent in adults over 40 years old and severe patients usually present high rates of morbidity. OM, on the other hand, is the most common paediatric disease and it is highly prevalent in the first five years of life. *H. influenzae* infection contributes for an aggravation of the symptoms and persistence of both these diseases. Given their significant impact in society, monitoring and research studies of NTHi in COPD and OM is fundamental for the development of better, and more appropriate, prevention and combat strategies.

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5. Appendix

Appendix 5.1.

Table 5.1 | COPD isolates data and characterization.

											Biofilm Production*	
***H. influenzae* ID**	**Isolation Date**	**Gender**	**β-Lactamase**	**Capsule**	***pilA***	***ompP5***	***hmw1A***	***hmw2A***	***hifA***	***hia***	**24h**	**48h**
12885	2013	Male	Negative	Non-encapsulated	Present	Present	Absent	Absent	Absent	Absent	-	-
12886		Male	Negative		Present	Present	Absent	Absent	Absent	Present	-	-
12963		Female	Negative		Present	Present	Present	Present	Absent	Absent	-	-
12986		Male	Negative		Present	Present	Present	Present	Absent	Absent	-	1
13007		Male	Negative		Present	Present	Present	Present	Absent	Absent	-	1
13123	2014	Male	Negative	Non-encapsulated	Present	Present	Absent	Absent	Present	Present	-	1
13156		Male	Negative		Present	Present	Present	Present	Absent	Absent	-	1
13303		Male	Negative		Present	Present	Present	Present	Absent	Absent	-	-
13354	2015	Male	Negative	Non-encapsulated	Present	Present	Present	Present	Absent	Absent	1	1
13360		Female	Negative		Present	Present	Present	Present	Absent	Absent	-	1
13390		Male	Negative		Present	Present	Present	Present	Absent	Absent	-	1
13391		Male	Negative		Present	Present	Present	Present	Absent	Absent	-	1
13394		Male	Positive		Present	Present	Present	Present	Absent	Absent	-	1
13395		Male	Positive		Present	Present	Present	Present	Absent	Absent	-	1
13421		Female	Negative		Present	Absent	Present	Present	Absent	Absent	-	1
13424		Male	Negative		Present	Absent	Present	Present	Absent	Absent	-	-
13449		Male	Negative		Present	Absent	Absent	Absent	Absent	Absent	-	-
13463		Male	Negative		Present	Absent	Absent	Absent	Absent	Absent	-	-
13491		Female	Negative		Present	Present	Present	Present	Absent	Absent	1	1

*Regarding biofilm production, numbers indicate the $OD_{isolate}/OD_{cut\text{-}off}$ ratio.

Table 5.1 | COPD isolates data and characterization.

H. influenzae ID	Isolation Date	Gender	β-Lactamase	Capsule	*pilA*	*ompP5*	*hmw1A*	*hmw2A*	*hifA*	*hia*	Biofilm Production*	
											24h	48h
13515	2016	Male	Negative	Non-encapsulated	Present	Absent	Absent	Absent	Absent	Present	-	1
13556		Male	Negative		Present	Present	Present	Present	Absent	Absent	-	1
13558		Male	Negative		Present	Present	Present	Present	Absent	Absent	1	2
13559		Male	Negative		Present	Present	Present	Present	Absent	Absent	-	1
13593		Male	Negative		Present	Absent	Absent	Absent	Absent	Present	2	3
13615		Male	Negative		Present	Absent	Present	Present	Absent	Absent	1	2
13638		Male	Negative	Encapsulated (f)	Present	Present	Absent	Absent	Present	Absent	-	1
13645	2017	Male	Positive	Non-encapsulated	Present	Present	Present	Present	Absent	Absent	-	2
13647		Male	Negative		Present	Present	Present	Present	Absent	Absent	-	2
13655		Male	Negative		Present	Present	Present	Present	Absent	Absent	-	1
13696		Male	Positive		Present	Present	Present	Present	Absent	Absent	1	3
13703		Male	Negative		Present	Present	Present	Present	Absent	Absent	1	2
13716		Female	Negative		Present	Present	Present	Present	Absent	Absent	-	2
13727		Male	Negative		Present	Present	Present	Present	Absent	Absent	1	1
13728		Male	Positive		Present	Present	Present	Present	Absent	Absent	2	3
13758		Male	Negative		Present	Present	Present	Present	Absent	Absent	-	2
13776		Male	Negative		Present	Present	Absent	Absent	Absent	Present	1	2
13777		Male	Positive		Present	Present	Present	Present	Absent	Absent	1	2
13791	2018	Female	Negative	Non-encapsulated	Present	Present	Present	Present	Absent	Absent	2	2

*Regarding biofilm production, numbers indicate the $OD_{isolate}/OD_{cut\text{-}off}$ ratio.

Appendix 5.2.

Table 5.2 | OM isolates data and characterization.

H. influenzae ID	Isolation Date	Gender	β-Lactamase	Capsule	*pilA*	*ompP5*	*hmw1A*	*hmw2A*	*hifA*	*hia*	Biofilm Production* 24h	Biofilm Production* 48h
12579	2013	Male	Negative	Non-Encapsulated	Present	Absent	Absent	Absent	Absent	Absent	1	1
12580		Female	Negative		Present	Absent	Present	Present	Absent	Absent	-	1
12595		Female	Positive		Present	Present	Absent	Present	Absent	Absent	-	1
12608		Female	Negative		Present	Absent	Present	Present	Absent	Absent	-	1
12635		Female	Negative		Present	Present	Absent	Absent	Present	Absent	1	2
12693		Male	Negative		Present	Present	Absent	Absent	Absent	Present	1	2
12717		Male	Negative		Present	Absent	Present	Present	Absent	Absent	1	2
12719		Female	Negative		Present	Present	Absent	Absent	Present	Absent	-	-
12733		Male	Positive		Present	Absent	Present	Present	Absent	Absent	-	1
12734		Female	Negative		Present	Present	Absent	Absent	Present	Absent	2	2
12735		Male	Negative		Present	Absent	Present	Present	Absent	Absent	-	1
12880		Male	Positive		Present	Absent	Absent	Absent	Present	Absent	1	1
12906		Male	Negative		Present	Present	Absent	Absent	Absent	Present	2	2
12931		Male	Negative		Present	Present	Absent	Absent	Absent	Present	2	2
12860	2014	Female	Negative	Non-Encapsulated	Present	Present	Present	Present	Absent	Absent	-	1
12925		Male	Negative		Present	Present	Absent	Absent	Absent	Absent	1	2
12939		Female	Positive		Present	Present	Present	Present	Absent	Absent	-	2
12957		Male	Negative		Present	Present	Absent	Absent	Absent	Present	1	1
12968		Female	Negative		Present	Present	Present	Present	Absent	Absent	-	1
13002		Female	Positive		Present	Present	Absent	Present	Absent	Absent	-	1
13030		Male	Positive		Present	Present	Present	Present	Absent	Absent	-	1
13078		Male	Negative		Present	Present	Absent	Absent	Present	Present	1	2
13083		Female	Negative		Absent	Absent	Absent	Absent	Present	Absent	2	2
13102		Female	Negative		Present	Present	Absent	Absent	Absent	Present	2	2

*Regarding biofilm production, numbers indicate the $OD_{isolate}/OD_{cut\text{-}off}$ ratio.

Table 5.2 | OM isolates data and characterization.

H. influenzae ID	Isolation Date	Gender	β-Lactamase	Capsule	*pilA*	*ompP5*	*hmw1A*	*hmw2A*	*hifA*	*hia*	Biofilm Production* 24h	Biofilm Production* 48h
13164	2014	Male	Negative	Non-Encapsulated	Present	Present	Absent	Absent	Present	Present	-	1
13165		Male	Negative		Present	Absent	Absent	Present	Absent	Absent	-	1
13166		Male	Negative		Present	Present	Present	Present	Absent	Absent	-	1
13167		Male	Negative		Present	Present	Absent	Absent	Absent	Absent	-	1
13223		Male	Negative		Present	Present	Absent	Absent	Absent	Absent	-	1
13224		Female	Negative		Present	Present	Absent	Absent	Present	Present	3	3
13225		Male	Negative		Present	Present	Absent	Absent	Present	Present	-	-
13309		Female	Negative		Present	Present	Absent	Absent	Present	Absent	-	1
13283	2015	Female	Negative	Non-Encapsulated	Present	Absent	Absent	Absent	Absent	Present	-	-
13340		Female	Negative		Absent	Present	Absent	Absent	Present	Absent	-	1
13352		Male	Negative		Present	Absent	Absent	Absent	Absent	Absent	-	-
13425		Male	Negative		Present	Absent	Present	Present	Absent	Absent	-	1
13450		Female	Negative		Present	Present	Absent	Absent	Absent	Present	-	-
13623	2017	Male	Negative	Non-Encapsulated	Present	Present	Present	Present	Absent	Absent	-	1
13628		Male	Negative		Present	Present	Absent	Absent	Absent	Present	1	1
13632		Female	Negative		Present	Present	Absent	Absent	Absent	Present	-	-
13660		Female	Negative		Present	Absent	Present	Present	Absent	Absent	-	-
13673		Female	Negative		Present	Present	Absent	Absent	Absent	Present	-	-
13674		Male	Positive		Present	Present	Absent	Present	Absent	Absent	-	-
13686		Female	Negative		Present	Absent	Present	Present	Absent	Absent	-	-
13692		Female	Negative		Present	Absent	Absent	Absent	Absent	Present	-	-
13693		Female	Negative		Present	Absent	Absent	Absent	Absent	Present	-	1
13739		Male	Negative		Present	Absent	Absent	Absent	Absent	Present	1	1
13740		Male	Negative		Present	Absent	Absent	Absent	Present	Absent	1	1
13741		Male	Positive		Present	Present	Absent	Absent	Present	Present	1	1
13742		Female	Negative		Present	Absent	Absent	Absent	Absent	Present	-	1
13744		Female	Negative		Absent	Present	Absent	Absent	Absent	Present	1	1
13770		Male	Negative		Absent	Present	Absent	Absent	Absent	Present	2	2

*Regarding biofilm production, numbers indicate the $OD_{isolate}/OD_{cut\text{-}off}$ ratio.

Table 5.2 | OM isolates data and characterization.

H. influenzae ID	Isolation Date	Gender	β-Lactamase	Capsule	*pilA*	*ompP5*	*hmw1A*	*hmw2A*	*hifA*	*hia*	Biofilm Production*	
											24h	48h
13787	2018	Female	Negative	Non-Encapsulated	Present	Present	Absent	Absent	Absent	Present	2	2
13795	2017	Male	Negative		Present	Present	Absent	Absent	Absent	Absent	2	1
13801	2018	Female	Negative		Present	Present	Absent	Absent	Present	Present	2	2

*Regarding biofilm production, numbers indicate the $OD_{isolate}/OD_{cut\text{-}off}$ ratio.

Appendix 5.3.

Table 5.3 | Primers used for capsular typing.[20]

Primer name	Sequence (5'- 3')	Use	Length of fragment (bp)
Hi1	CGTTTGTATGATGTTGATCCAGAC	Capsule amplification (*bexA* gene)	343
Hi2	TGTCCATGTCTITCAAAATGATG		
Hia1	CTACTCATTGCAGCATTTGC	Serotype a characterization	250
Hia2	GAATATGACCTGATCTTCTG		
Hib1	GCGAAAGTGAACTCTTATCTCTC	Serotype b characterization	480
Hib2	GCTTACGCTTCTATCTCGGTGAA		
Hic1	TCTGTGTAGATGATGGTTCA	Serotype c characterization	250
Hic2	CAGAGGCAAGCTATTAGTGA		
Hid1	TGATGACCGATACAACCTGT	Serotype d characterization	150
Hid2	TCCACTCTTCAAACCATTCT		
Hie1	GGTAACGAATGTAGTGGTAG	Serotype e characterization	1350
Hie2	GCTTTACTGTATAAGTCTAG		
Hif1	GCTACTATCAAGTCCAAATC	Serotype f characterization	450
Hif2	CGCAATTATGGAAGAAAGCT		

Appendix 5.4.

Table 5.4 | Primers used for MLST.[23]

Primer name	Sequence (5'- 3')	Gene	Expected amplified product (bp)
adK-M13F	TGTAAAACGACGGCCAGTGGTGCACCGGGTGCAGGTAA	*adK*	477
adK-M13R	CAGGAAACAGCTATGACCCCTAAGATTTTATCTAACTC		
atpG-M13F	TGTAAAACGACGGCCAGTATGGCAGGTGCAAAAGAGAT	*atpG*	447
atpG-M13R	CAGGAAACAGCTATGACCTTGTACAACAGGCTTTTGCG		
frdB-M13F	TGTAAAACGACGGCCAGTCTTATCGTTGGTCTTGCCGT	*frdB*	489
frdB-M13R	CAGGAAACAGCTATGACCTTGGCACTTTCCACTTTTCC		
fucK-M13F	TGTAAAACGACGGCCAGTACCACTTTCGGCGTGGATGG	*fucK*	345
fucK-M13R	CAGGAAACAGCTATGACCAAGATTTCCCAGGTGCCAGA		
mdh-M13F	TGTAAAACGACGGCCAGTTCATTGTATGATATTGCCCC	*mdh*	405
mdh-M13R	CAGGAAACAGCTATGACCACTTCTGTACCTGCATTTTG		
pgi-M13F	TGTAAAACGACGGCCAGTGGTGAAAAAATCAATCGTAC	*pgi*	468
pgi-M13R	CAGGAAACAGCTATGACCATTGAAAGACCAATAGCTGA		
recA-M13F	TGTAAAACGACGGCCAGTATGGCAACTCAAGAAGAAAA	*recA*	426
recA-M13R	CAGGAAACAGCTATGACCTTACCAAACATCACGCCTAT		

M13F and M13R sequences are highlighted in grey. M13F primer was used for sequencing

Appendix 5.5.

Table 5.5 | Primers used for each virulence gene.[78,82,89]

Primer name	Sequence (5'- 3')	Gene	Expected amplified product (bp)
*pilA*1	ATGAAACTAACAACACAGCAAACC	*pilA*	416
*pilA*2	AAATAAAGAGGCATCCGTTCC		
*ompP5*1	GCATTAGTAGTTGCTGGC	*ompP5*	1040
*ompP5*2	TGCGATTTCTACACGACG		
*hmw1A*1	GAAACGCRRRTCCTTGAG	*hmw1A*	149
*hmw1A*2	GACTTGATGGTYYYYTGGTTGTAAA		
*hmw2A*1	CGAAACGCGTCCTTGAGAA	*hmw2A*	200
*hmw2A*2	TGCGCCATTACCACTTGAGAA		
*hifA*1	TGCTGTTTATTAAGGCTTTAG	*hifA*	800
*hifA*2	TTGTAGGGTGGGCGTAAGCC		
*hia*1	CAAAATTTTTAACGTTATTTGGAAT	*hia*	3259
*hia*2	AACGCCTGTTTTACCTTGACTAT		

Appendix 5.6.

SIEMENS

MicroScan®

MICroSTREP *plus*® Panel Type 3 Worksheet

S = Susceptible
I = Intermediate
R = Resistant
NS = Not Susceptible

***Haemophilus* spp.**

G										
≤0.03	0.06	0.12	0.25	0.5	1	2	4	8	>8	Am[1,4]
≤0.03	0.06	0.12	0.25	0.5	1	2	4	8	>8	Cft[2,4]
≤1	2	8	16	>16	Cfr					
≤0.25	0.5	1	2	4	8	>8	Crm			
≤1	2	4	>4	Te						

≤1	≤0.25/4.75						
2	0.5/9.5						
4	1/19						
8	2/38						
>8	>2/38						
C[3,4]	T/S						

≤0.12	0.25	0.5	1	2	>2	Cpe[2]	
≤0.06	0.12	0.25	0.5	1	2	>2	Cp[2]
≤0.25	0.5	1	2	4	>4	Az[2,3]	

				≤0.5	1	2	>2	Rif
≤0.06	0.12	0.25	0.5	1	2	4	>4	Mer[2,3,4]
		≤0.25/0.12	0.5/0.25	1/0.5	2/1	4/2	>4/2	Aug

Figure 5.1 | Antibiotic susceptibility panel worksheet.