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Otopathogens: characterization and alternative treatment

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DECLARAÇÃO

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É AUTORIZADA A REPRODUÇÃO PARCIAL DESTA DISSERTAÇÃO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

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Assinatura:

“Don’t let the fear of losing be greater than the excitement of winning”.

Albert Einstein

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A minha dissertação assinala o final de uma caminhada de 5 anos repletos de persistência, ambição, determinação e, sobretudo de esforço. Desta forma, acredito que o caminho para o sucesso é feito de pequenas vitórias e deve ser realizado de forma sustentada.

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ABSTRACT

Otitis media is an inflammatory disease of the middle ear, which comprises 3 main types: Acute Otitis media, Otitis media with Effusion and Chronic Otitis media. This disease affects, especially, children younger than 2 years of age worldwide. Otitis media is a polymicrobial disease, which involves microorganisms, such as *Moraxella catarrhalis*, *Streptococcus pneumoniae* and *Haemophilus influenzae*, however a co-infection caused by both bacteria and viruses is common in 28% to 70% of the Otitis media cases.

In the last years, bacteriophage therapy has emerged, constituting an efficient alternative to fight against antimicrobial resistance and treat middle ear infections, being lytic bacteriophages the best choice in case of therapeutic applications.

Multiplex PCR was used to determine the serotypes of different clinical isolates of *M. catarrhalis*, *S. pneumoniae* and *H. influenzae*. *M. catarrhalis* isolates were classified as serotype A (56%) and the remaining as non-identified (44%). Regarding *S. pneumoniae* clinical isolates, six pneumococcal serotypes were identified: serotype 1 (11%), serotype 19F (7%) and serotypes 15B/C, 12F/12A/12B, 6A/B and 9N/L with 4% each. More than a half of pneumococcal isolates tested were classified as non-typeable (66%). In what *H. influenzae* clinical isolates are concerned, 100% of them were non-typeable.

All of *M. catarrhalis* strains were resistant to Ampicillin besides 3.7% and 7.4% of *S. pneumoniae* clinical isolates were resistant to Moxifloxacin and Levofloxacin, respectively. *H. influenzae* showed 29.2%, 12.5% and 8.3% of resistant strains to Ampicillin, Amoxicillin-clavulanic acid and Trimethroprim-sulfamethoxazole, respectively.

Related to pneumococcal bacteriophages, three lytic ones - Cp-1, Dp-1 and MS1 – that where obtained from the Félix d'Hérelle Reference Centre for Bacterial Viruses - were tested against planktonic bacteria and bacteriophage Cp-1 was considered the most effective, contributing to the immediate bacteria cell lysis of the host strain.

Finally, it was possible to verify that none of the clinical isolates of *S. pneumoniae* was infected by any of the bacteriophages, meaning that these viruses have narrow host ranges and the host strain (R6st) is non-typeable.

Keywords: Otitis media, Bacteriophages, Antibiotic resistance, Serotypes

SUMÁRIO

A Otite média é uma doença inflamatória do ouvido médio que engloba três tipos principais: Otite média Aguda, Otite média com Efusão e Otite média Crónica. As Otites médias afetam principalmente crianças com menos de 2 anos de idade. As principais bactérias responsáveis por causar Otites médias são: *Moraxella catarrhalis*, *Streptococcus pneumoniae* e *Haemophilus influenzae*, contudo 28% a 70% dos casos de Otites médias devem-se a uma coinfecção causada por bactérias e vírus.

Nos últimos anos, a terapia fágica tem vindo a emergir, constituindo uma alternativa promissora contra a resistência antimicrobiana e que permite o tratamento de infeções do ouvido médio, sendo que os fagos virulentos constituem a melhor escolha para fins terapêuticos.

Vários isolados clínicos de *Moraxella catarrhalis*, *Streptococcus pneumoniae* e *Haemophilus influenzae* foram serotipados. Os isolados de *Moraxella catarrhalis* apresentaram maioritariamente serótipo A (56%) e os restantes foram classificados como não tipáveis (44%). Quanto aos isolados clínicos de *Streptococcus pneumoniae*, foram identificados 6 serótipos: serótipo 1 (11%), serótipo 19F (7%) e os serótipos 15B/C, 12F/12A/12B, 6A/B e 9N/L, cada um com uma percentagem de 4%. Cerca de 66% dos isolados clínicos de *Streptococcus pneumoniae* foram classificados como não tipáveis. Os isolados de *Haemophilus influenzae* foram, na sua totalidade, classificados como não tipáveis.

Todas as estirpes de *M. catarrhalis* revelaram ser resistentes à Ampicilina e, quanto aos isolados de *S. pneumoniae,* 3.7% e 7.4% deles foram resistentes a Moxifloxacina e a Levofloxacina, respetivamente. *H. influenzae* demonstrou 29.2% de estirpes resistentes a Ampicilina, 12.5% a Amoxicilina-ácido clavulânico e 8.3% a Trimetroprim-sulfametoxazol.

Algum trabalho experimental foi também realizado com bacteriófagos de *S. pneumoniae*: Cp-1, Dp-1 e MS1 que foram obtidos da coleção de Félix d'Hérelle e que foram testados contra bactérias planctónicas, mostrando que o bacteriófago Cp-1 foi o mais eficaz na lise da estirpe hospedeira (R6st), proporcionando uma lise celular bacteriana imediata.

Finalmente, foi possível verificar que nenhum dos isolados clínicos de *S. pneumoniae* foi infetado por nenhum dos bacteriófagos testados, revelando a sua elevada especificidade, bem como o seu reduzido espetro lítico, tendo também em atenção que a estirpe hospedeira é não tipável.

Palavras-chave: Otite média, Bacteriófagos, Resistência antimicrobiana, Serótipos

TABLE OF CONTENTS

Acknownledgments v

Abstract vii

Sumário ix

List of Figures xiii

List of Tables xv

List of Abbreviations and Symbols xvii

Motivation and Aim of the Thesis 1

1. Introduction 1

1.1 Otitis media 1

1.1.1 Anatomy of the middle ear 1

1.1.2 Types of Otitis media 3

1.1.3 Risk factors of OM 6

1.1.4 The microbiome in OM 7

1.1.5 Diagnosis and treatment of OM 9

1.2 Vaccination Plan 11

1.3 Bacteriophages 12

1.3.1 Bacteriophages life cycles 14

1.3.2 Classification of bacteriophages 16

1.3.3 Bacteriophage therapy in Otitis Media – State of the art 17

2. Materials and Methods 19

2.1 Typing of *Moraxella catarrhalis* 19

2.1.1 Bacterial Isolates and culture conditions 19

2.1.2 PCR typing 20

2.2 Typing of *Streptococcus pneumoniae* 21

2.2.1 Bacterial Isolates and culture conditions 21

2.2.2 PCR typing 22

2.3 Typing of *Haemophilus influenzae* 25

2.3.1 Bacterial Isolates and culture conditions 25

2.3.2 PCR typing 27

2.4 *Streptococcus pneumoniae* bacteriophages 29
2.4.1 Bacterial strains and culture conditions 29
2.4.2 Bacteriophage source and Host 29
2.4.3 Optimization of bacteriophages media 29
2.4.4 Production and titration of bacteriophages in liquid medium 30
2.4.5 Lytic spectra 31
3. Results and Discussion 33
3.1 Typing clinical samples 33
3.1.1 Typing of *Moraxella catarrhalis* 33
3.1.2 Typing of *Streptococcus pneumoniae* 34
3.1.3 Typing of *Haemophilus influenzae* 38
3.2 Antimicrobial susceptibility testing 41
3.2.1 *Moraxella catarrhalis* clinical isolates 41
3.2.2 *Streptococcus pneumoniae* clinical isolates 42
3.2.3 *Haemophilus influenzae* clinical isolates 44
3.3 Bacteriophages 46
3.3.1 Assay with planktonic bacteria 46
3.3.2 Lytic spectra 48
4. Conclusion and Future Perspectives 51
References 55

LIST OF FIGURES

Introduction

Figure 1.1 – Anatomy of a healthy human ear. 1

Figure 1.2 – Behavior of the Eustachian tube in Infants versus Adults. 2

Figure 1.3 – Evolution of a healthy middle ear to different degrees of AOM: A. Healthy middle ear with normal tympanic membrane; B. Uncomplicated AOM; C. Nonsevere AOM; D. Severe AOM. 4

Figure 1.4 - A. Healthy middle ear (no fluid); B. Middle ear in case of OME (full of fluid). 5

Figure 1.5 - A. Healthy middle ear; B. Middle ear in case of CSOM (perforation of the TM). 5

Figure 1.6 - Categories of risk factors of OM: host and environmental factors. 6

Figure 1.7 - A. Representation of the Otoscopy procedure (otoscope assessing the eardrum); B. Otoscope with pneumatic bulb to perform Pneumatic Otoscopy. 10

Figure 1.8 - A. Small incision in the tympanic membrane (Myringotomy); B. Grommet insertion. 11

Figure 1.9 - Simple representation of a typical bacteriophage. 13

Figure 1.10 - Schematic representation of: A. Lytic lifecycle associated to virulent phages and B. Lysogenic lifecycle associated to temperate phages. 15

Materials and Methods

Figure 2.1 – Different combinations of solid and liquid media tested to observe a clear action of the bacteriophages. 30

Results and Discussion

Figure 3.1 - Distribution of *Moraxella catarrhalis* serotypes in clinical samples obtained from Hospital de Braga. A refers to serotype A and Non identified belong to non-typeable strains. 33

Figure 3.2 - Distribution of *Streptococcus pneumoniae* serotypes in clinical samples obtained from Hospital de Braga. 35

Figure 3.3 - Distribution of *Haemophilus influenzae* serotypes in clinical samples obtained from Hospital de Braga. 39

Figure 3.4 - Action of the phages on planktonic bacteria throughout the time. A – Action of phage Cp-1 on planktonic bacteria throughout the time (1st assay); B - Action of phage Cp-1 on planktonic bacteria throughout the time (2nd assay); C - Action of phage MS1 on planktonic bacteria throughout the time (1st assay); D - Action of phage MS1 on planktonic bacteria throughout the time (2nd assay); E - Action of phage Dp-1 on planktonic bacteria throughout the time (1st assay); F - Action of phage Dp-1 on planktonic bacteria throughout the time (2nd assay). 47

LIST OF TABLES

Introduction

Table 1.1 - Microbiome in AOM. 7

Table 1.2 - Some details about the most prevalent bacteria present in OM. 8

Table 1.3 - Dosing vaccination schedule according to the NIP. 12

Table 1.4 - Classification and characteristics of phages families. 17

Materials and Methods

Table 2.1 - *Moraxella catarrhalis* strains used in the study, with the respective ages and sex of the patients they were recovered 19

Table 2.2 - Primers used for *Moraxella catarrhalis* clinical isolates serotyping determination, the respective product sizes and target serotypes 20

Table 2.3 - *Streptococcus pneumoniae* strains used in this study, with the respective ages and sex of the patients they were recovered 21

Table 2.4 - Primers used for *Streptococcus pneumoniae* clinical isolates serotyping determination, the respective product sizes and target serotypes 22

Table 2.5 - *Haemophilus influenzae* strains used in the study with the respective ages and sex of the patients they were recovered 26

Table 2.6 - Primers for *Haemophilus influenzae* clinical isolates serotyping determination, the respective product sizes and target serotypes 27

Results and Discussion

Table 3.1 - Incidence of *Moraxella catarrhalis* LOS types and non identified isolates 34

Table 3.2 - Incidence of *Streptococcus pneumoniae* capsule serotypes and non-typeable isolates 36

Table 3.3 - Incidence of *Haemophilus influenzae* non-typeable isolates 40

Table 3.4 - Antibiogram profile of *Moraxella catarrhalis* isolates (n=25) (%) 42

Table 3.5 - Antibiogram profile of *Streptococcus pneumoniae* isolates (n=27) (%) 44

Table 3.6 - Antibiogram profile of *Haemophilus influenzae* isolates (n=48) (%) 45

Table 3.7 - Lytic spectra of Cp-1, Dp-1 and MS1 bacteriophages against 27 *S. pneumoniae* clinical isolates ... 49

LIST OF ABBREVIATIONS AND SYMBOLS

°C – Degrees Celsius

µL – Microliter

µM – Micromolar

AOM – Acute Otitis media

BHI – Brain Heart Infusion

bp – Base pair

$CaCl_2$ – Calcium Chloride

CO_2 – Carbon Dioxide

COPD – Chronic Obstructive Pulmonary Disease

COM – Chronic Otitis media

COME - Chronic Otitis Media with Effusion

CSOM - Chronic Suppurative Otitis Media

DNA – Deoxyribonucleic acid

ds – Double stranded

EUCAST – European Committee on Antimicrobial Susceptibility Testing

FBS – Fetal Bovin Serum

Hib - *Haemophilus influenzae* type b

HCl – Hydrogen Chloride

ICTV - International Committee on Taxonomy of Viruses

IPD – Invasive Pneumococcal Disease

Kb – Kilobase

LOS – Lipooligosaccharides

LPS – Lipopolysaccharide

$MgSO_4$ – Magnesium Sulfate

$MnCl_2$ – Manganese Chloride

mL – Milliliter

mM – Milimolar

NAD – Nicotinamide adenine dinucleotide

NIP - National Immunization Plan

OD – Optical Density

OM – Otitis media

OME – Otitis media with Effusion

PBP – Penicillin-Binding Protein

PNSP – Penicillin-Nonsusceptible *Streptococcus pneumoniae*

PFU – Plaque Forming Units

PCV – Pneumococcal Conjugate Vaccine

PCR – Polymerase Chain Reaction

RNA – Ribonucleic acid

R6st – *Streptococcus pneumoniae* R6 strain

rpm – Rotations per minute

ss – Single stranded

THB – Todd Hewitt Broth

TSA – Tryptic soy agar

TSB – Tryptic soy broth

TM – Tympanic membrane

U – Enzyme unit

URTI – Upper Respiratory Tract Infections

V – Volt

WHO - World Health Organization

MOTIVATION AND AIM OF THE THESIS

The work developed within this dissertation is related with the use of bacteriophage therapy to fight against Otitis media pathogens. Otitis media is highly prevalent worldwide, affecting a significant percentage of children younger than 2 years old diminishing their life quality.

Since the discovery of Penicillin, antibiotic therapy became the most commonly used treatment of middle ear infections and the selective pressure that arise from its use led to the development of antibiotic resistant strains. Indeed, the high possibility of this treatment failure allied to the increasing concern about bacterial resistance renewed interest in searching for novel alternative strategies to treat Otitis media.

Bacteriophage therapy appears to be a promising strategy to treat Otitis media and fight against the exponential augment of bacterial resistance in the last decades, showing capability to control biofilms as well as planktonic bacteria. Moreover, bacteriophage therapy is non-toxic to human cells, making this therapy suitable for using in therapeutic applications.

The aim of this thesis was to determine the serotypes of clinical isolates using a Multiplex PCR approach. Another aim was to access the antimicrobial effect of three lytic bacteriophages specific for *S. pneumoniae* and this involved media optimization, bacteriophage production, infection of planktonic cultures, and further testing these bacteriophages against the clinical isolates.

1. INTRODUCTION

1.1 Otitis media

Otitis media (OM) is one of the most common generic terms for all types of inflammatory diseases of the middle ear. Approximately 90% of the OM cases affect children younger than 2 years of age. In developed countries OM is registered as the most usual reason why children need pediatrician consultation, receive antibiotics or go through a surgery and is a contributing disease to the widespread of antimicrobial resistance [1, 2].

OM consists of an inflammation of the middle ear, where its cavity and ossicles are included, characterized by the presence of fluid in the same structure that prevents the tympanic membrane of vibrating properly, blocking the transmission of the sound in the middle ear and can possibly lead to a permanent or temporary hearing loss [1, 2]. According to World Health Organization (WHO) it is estimated that there might exist nearly 42 million people who suffer from hearing loss, mostly caused by OM. In the US alone, the annual direct costs account for US$ 3-5 billion spent on the treatment of patients with this disease [1, 3, 4].

1.1.1 Anatomy of the middle ear

The middle ear is a structure which includes the cavity that enables communication with the nasopharynx by the Eustachian (auditory) tube and the ossicles – malleus, incus and stapes – that are linked to the tympanic membrane (or eardrum), as shown in Figure 1.1 [2, 5].

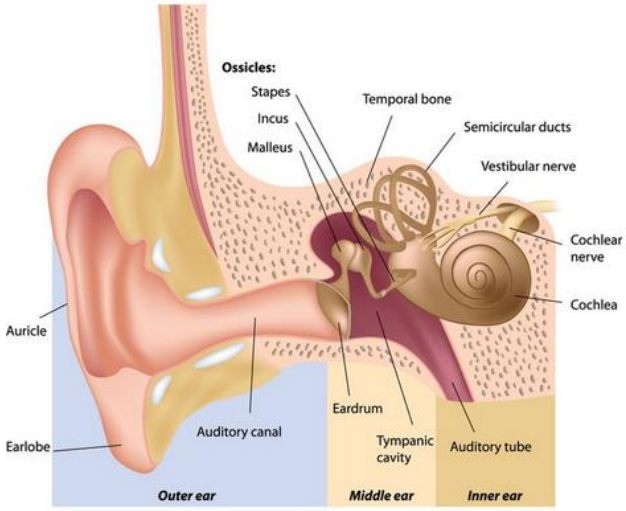


Figure 1.1 – Anatomy of a healthy human ear [6].

The Eustachian tube is an important structure of the middle ear, which plays a vital role on the protection against the entrance of bacterial pathogens and viruses from the nasopharynx, in the middle ear, as well as on the clearance of its secretions. Moreover, it also contributes to keep an equal pressure between the middle and the outer ear, in order to allow the tympanic membrane (TM) movement, when it receives the sound waves. A ventilatory function is also assured by the Eustachian tube [2, 3].

The fast and spontaneous recovery of children with OM is thought to be a sign of the natural and gradual maturation of their immune system. Firstly, their Eustachian tubes are still short, horizontal and function poorly, therefore they are more prone to contract middle ear infections (Figure 1.2). Those characteristics prevent clearance of secretions and lead to a negative pressure in the middle ear space, contributing to the suction of contaminated bacterial secretions from the nasopharynx. In this way, the maturation of the Eustachian tube, that occurs by 7 years old, might contribute to the reduction of the OM risk after this age, because the Eustachian tube angle increases with the age, limiting the pathogens transmission. Colonization of the nasopharynx has a peak in young children, then declines towards adulthood and finally increases in the elderly [3, 5].

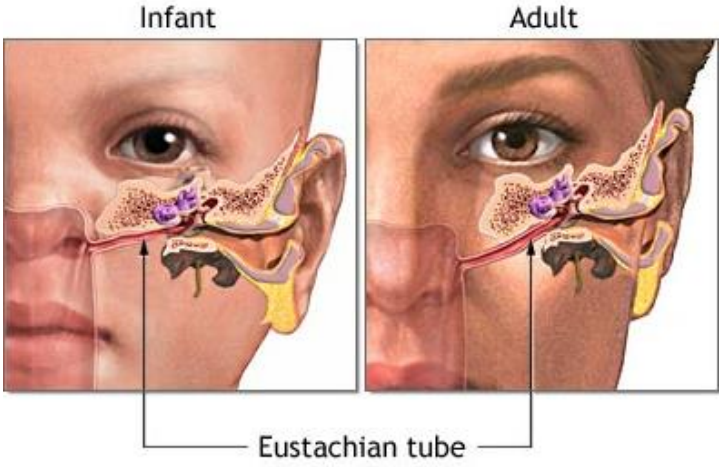


Figure 1.2 – Behavior of the Eustachian tube in Infants versus Adults [7].

The Eustachian tube capability of protecting the middle ear is due to the presence of ciliated respiratory epithelial cells in its epithelium, which are responsible for the production of antimicrobial proteins, such as lysozymes (that contribute to the bacterial cell wall lysis), pro-inflammatory cytokines and of goblet cells that produce serous mucus that retain bacteria. Moreover, the direction of the flow from the Eustachian tube to the nasopharynx constitutes another factor in favor of the middle ear protection against bacterial colonization [3].

1.1.2 Types of Otitis media

OM comprises a group of diseases that include Acute Otitis media (AOM), Otitis media with Effusion (OME) and Chronic Otitis media (COM), each subtype with its own characteristics.

A. Acute Otitis media (AOM)

AOM is characterized by the presence of fluid in the middle ear accompanied by the fast onset of signs and symptoms of acute infection, such as: otalgia, fever, otorrhea and bulging tympanic membrane. Respiratory viruses and bacteria may be both (or separately) responsible for the development of AOM. A synergism between viral upper respiratory tract infections (URTI) and OM pathogens in 70% of the cases, mostly prevalent among children has been clearly identified [8].

Generally, nasopharyngeal bacterial colonization, which is initially asymptomatic, is triggered by the viral infection that appears when the person contracts a common cold. The URTI leads to the inflammation of the nasopharynx and dysfunction of the Eustachian tube which cause inflammatory responses, including the generation of cytokines and inflammatory mediators and an increase of nasopharyngeal bacterial colonization and adherence to epithelial cells. The properties of these substances secreted alter the properties of the mucous, which become thicker and reduces the mucociliary clearance. Hence, this leads to the dysfunction of the Eustachian tube as well as to a negative middle ear pressure facilitating the entry of bacteria and respiratory viruses coming from the nasopharynx to be sucked into the middle ear cavity, causing middle ear inflammation and finally contributing to the development of AOM [8, 9].

Generally, AOM is most prevalent in children aged less than 2 years old and the peak incidence is registered between 6 and 18 months with 22% eventually developing the condition. It is also noticed that 80% of the patients have had at least one occurrence of AOM, by their third year of life [3, 8, 10].

Some children can develop various episodes of AOM and if there are registered three or more events in a period of 6 months, this will then be classified as Recurrent AOM commonly affecting 10%-20% of 1-year-old children. The otitis patients are considered prone to this infection when 6 events of AOM have occurred in their first 6 years of life [3, 8, 11].

There are distinct stages of AOM (Figure 1.3) that vary from uncomplicated to severe. Uncomplicated AOM is characterized by the absence of otorrhea and nonsevere AOM by mild pain and intense erythema or mild bulging of the tympanic membrane in which the body temperature remains lower than 39°C. Lastly, severe AOM is characterized by moderate to severe pain which extends for more than 48 hours

and moderate to severe bulging of the tympanic membrane, nevertheless and in contrast to nonsevere AOM, the body temperature is equal or greater than 39°C [8, 10].

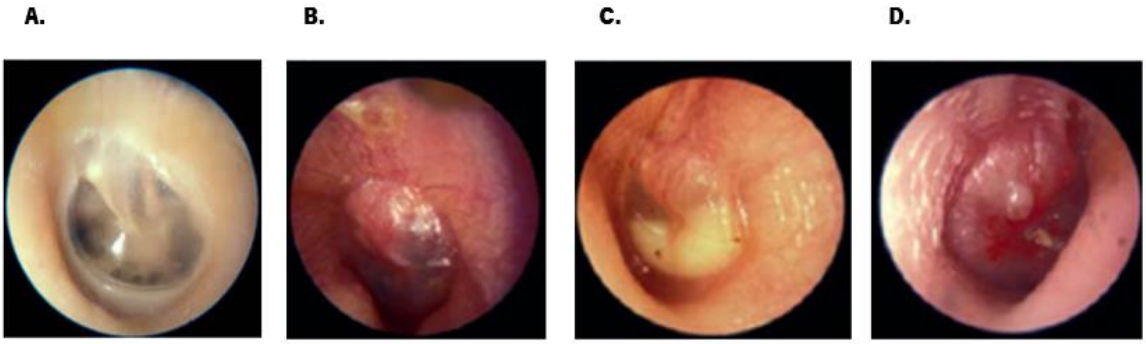


Figure 1.3 – Evolution of a healthy middle ear to different degrees of AOM: **A.** Healthy middle ear with normal tympanic membrane; **B.** Uncomplicated AOM; **C.** Nonsevere AOM; **D.** Severe AOM [10].

B. Otitis media with Effusion (OME)

OME ("glue ear" or serous OM) is another stage of OM, but in contrast to AOM, this one is not associated with signs or symptoms of acute infection, despite existing fluid collection in the middle ear, more precisely, behind the tympanic membrane (Figure 1.4). Mucin is the most prevalent component of the middle ear effusion which confers thick viscous properties to the glue-like fluid [12].

However, there is a link between these two types of OM, because OME may precede the onset of AOM or it may follow AOM, as a result of the inflammatory response that subsides, but the middle ear effusion persists. In the majority of cases, OME is asymptomatic and can only be detected by screening tests, such as pneumatic otoscopy, which become difficult in infants, because of the size of the ear canal, the lack of infants' cooperation, as well as the presence of cerumen and the difficulty to remove it. In those cases where symptoms are present, the most reported is the transient hearing loss, because middle ear effusion frequently resolves spontaneously, which may cause speech delay, educational problems, sleep disorders, loss of appetite and ear pain [12, 13].

OME has its peak incidence on children aged 1 year and by 3 years old most of them have experienced one or more episodes of OME. An examination of the eardrum found OME in 15% to 40% of children between the ages of 1 to 5 years old. As OME persists, viscosity of middle-ear fluid increases and it is almost certain that children who had OME for more than 12 months had fluid with high viscosity [14].

According to previous studies, 1 month after the diagnosis of AOM, 50% of children have OME, 2 months after 30% and 3 months after 10% of them had the condition [15–17].

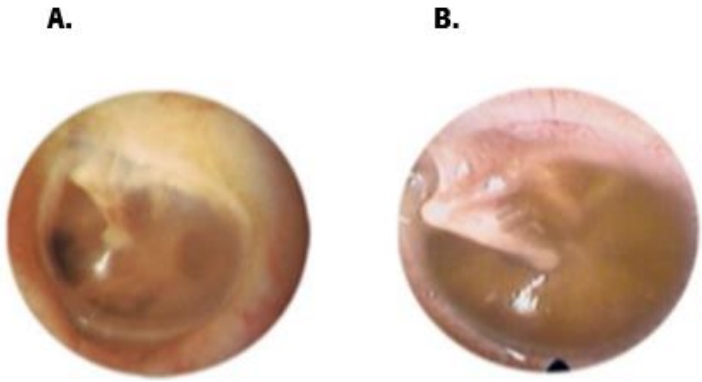


Figure 1.4 - A. Healthy middle ear (no fluid); **B.** Middle ear in case of OME (full of fluid) [15].

C. Chronic Otitis media (COM)

COM comprises two inflammatory conditions of the middle ear: Chronic Otitis Media with Effusion (COME) and Chronic Suppurative Otitis Media (CSOM). Chronic OME occurs when OME persists for a period of more than 3 months and CSOM (Figure 1.5) consists of a chronic inflammation of the middle ear and mastoid cavity also lasting more than 3 months which causes ear discharge, hearing loss and TM perforation [18].

CSOM usually appears in childhood as a sequelae of AOM or OME. This type of infection may occur in the first 6 years of life revealing a peak around 2 years old. CSOM constitutes a serious problem due to the perforated tympanic membrane that enables bacteria to enter into the middle ear by the external ear canal. The infection of the middle ear mucosa will lead to a persistent ear discharge. A recent worldwide review revealed that there are around 31 million new cases of CSOM and 22.6% of them are from children under 5 years old [19, 20].

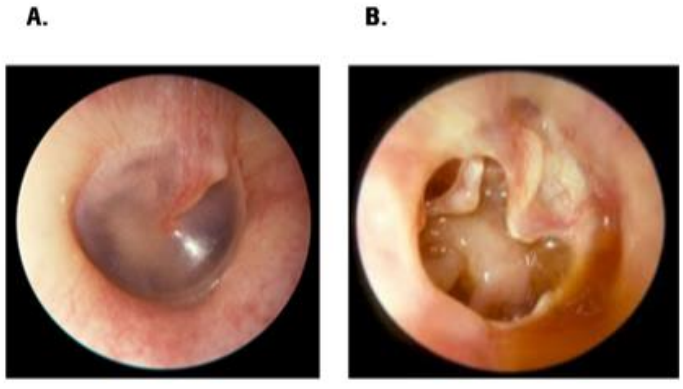


Figure 1.5 - A. Healthy middle ear; **B.** Middle ear in case of CSOM (perforation of the TM) [21].

1.1.3 Risk factors of OM

OM is a multifactorial disease and some of these factors will be here enunciated and briefly described.

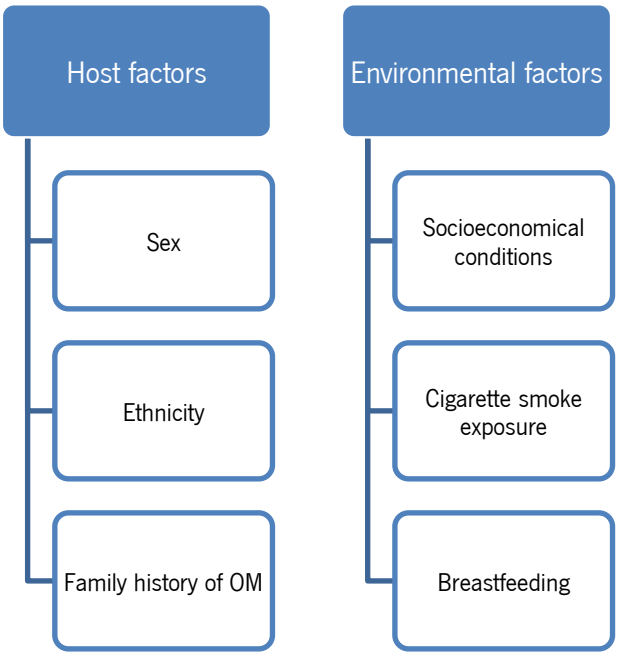


Figure 1.6 - Categories of risk factors of OM: host and environmental factors [22].

Sex predominance is one of the host factors that influence propensity of AOM. It is known that AOM occurs more often in males than in females, with 66% and 86% of males versus 53% and 77% of females having an episode of OM in their first and third years of life, respectively. This tendency can be explained by the insufficient antibody production of IgG2 or its delayed maturation. A deficiency of IgG2 is related to an augment of the host susceptibility to bacterial infections [22].

Regarding ethnic differences, it is not clear whether the ethnicity itself offers protection or not against Otitis or if it is solely related to different accesses to medical care. For example, the Afro-American black children are less prone to OM because they have anatomic differences in the Eustachian tube structure. In contrast, Australian aboriginal children have the highest incidence of OM in the world that can be explained by an early and dense nasopharyngeal colonization triggered by specific antibody deficiencies which increase children' susceptibility [23].

Family history of ear infections has also impact on contracting OM. It is known that those patients who had episodes of AOM or Recurrent OM have higher probability of having siblings with OM rather than siblings of children who had never experienced it. This fact is, therefore, related to a genetic basis which will determine the predisposition to middle ear infections [22].

There are some environmental factors that also contribute to the appearance of OM. Smoking is causative of acute respiratory infections, especially until 2 years of age and mothers who smoke double the risk of their children to develop OM. According to some studies, an increase on the rate of cigarette consumption is associated with an increase on the duration of middle ear effusion of OM. Passive smoking can lead to physiological and structural changes in the respiratory tract, pulmonary dysfunction and upper/lower respiratory illnesses, contributing to the weakness of the host and facilitating the appearance of OM. Breastfeeding is a protective factor against OM, mainly due to the fact that human milk contains specific antibodies which confer protection against respiratory tract viruses. It was previously demonstrated that infants breastfed for 4 or more months had 50% less episodes of AOM when compared to those who were not breastfed or even were supplemented with other kinds of food in the same period. Hence, the duration of breastfeeding is correlated with the decreased risk of AOM [22, 24].

Socioeconomic status of the families can be a risk of OM, although it is considered by some authors not very significant on its increased incidence. Normally, in lower social classes chronic infection of the upper respiratory tract are more frequent and can become complicated by middle ear infections, because these families do not tend to go to pediatrician consultation for non-serious diseases. On the other hand, higher social classes will go to the pediatrician consultation to solve every infection episode, reducing the risk of URTI complications [22, 24].

1.1.4 The microbiome in OM

OM is a polymicrobial disease, involving bacteria and viruses, that interact with each other where one or both can be responsible for AOM cases.

Table 1.1 - Microbiome in AOM. Adapted from [9]

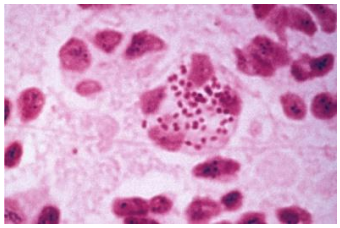
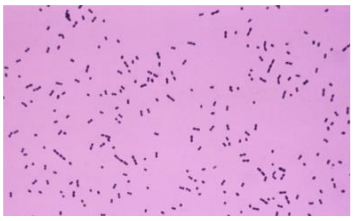
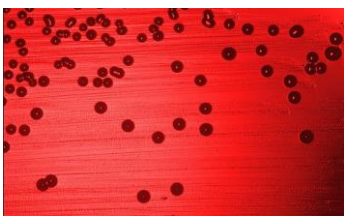
	Distribution
Bacteria	
Streptococcus pneumoniae	25% - 50%
Haemophilus influenzae	15% - 30%
Moraxella catarrhalis	3% - 20%

Virus	
Respiratory syncytial virus	41% - 56%
Coronavirus	50%
Rhinovirus	30% - 44%

The most common bacteria isolated from the middle ear aspirates are: *S. pneumoniae, H. influenzae* and/or *M. catarrhalis* (at least one of the three) and the most common virus coincident with OM are: Respiratory syncytial virus, Coronavirus and Rhinovirus (upper respiratory tract viruses) (Table 1.1). *H. influenzae* is the bacterial pathogen most commonly identified in bilateral disease (coinfection – virus and bacteria) and *S. pneumoniae* equally found either in bilateral or unilateral disease [9, 25].

The next table summarizes the main characteristics of each predominant bacteria responsible for OM.

Table 1.2 - Some details about the most prevalent bacteria present in OM. Adapted from [9].

Microorganisms	
Moraxella catarrhalis	
	Gram-negative bacteria Aerobic diplococcus
Streptococcus pneumoniae	
	Gram-positive bacteria Facultative anaerobic diplococcus
Haemophilus influenzae	
	Gram-negative coccobacillus Facultative anaerobic bacteria

Invasions by *S. pneumoniae* are the leading cause of morbidity and mortality worldwide responsible for causing life-threatening infection diseases, such as meningitis, bacteremia, pneumonia, as well as, less severe diseases: OM, sinusitis and bronchitis. It is important to refer that more than 50% of OM cases are provoked by *S. pneumoniae*, which is considered natural colonizer of the upper respiratory tract of healthy people but is also a serious pathogen responsible for lethal diseases [20, 21].

H. influenzae commonly colonize the upper respiratory tract of humans, which is considered the only known natural reservoir, however it is also a pathogen that can cause not only non-invasive diseases (OM), but also invasive infections – sepsis, pneumonia, purulent meningitis. It is considered the second most common pathogen responsible for AOM in children [22].

M. catarrhalis is the third most prevalent pathogen responsible for up to 20% of OM cases (worldwide) in children and the second most commonly isolated pathogen in case of exacerbations of COPD, in adults. In the past, it was considered a commensal of the upper respiratory tract, but now is emerging as a true pathogen of lower and upper respiratory human tract infections. In patients with immunocompromised immune system, *M. catarrhalis* can cause severe infections, such as: pneumonia, septicemia and meningitis [23].

1.1.5 Diagnosis and treatment of OM

The guidelines for the treatment of OM vary from country to country, however, the idea of minimizing antibiotic treatment in mild OM cases is consensual, in order to control the antibiotic-resistant bacterial strains [5].

Generally, AOM has a high rate of self-resolving episodes within 2 to 14 days, with around 80% of children having a favorable course without antibiotic treatment. Nevertheless, analgesics are important to reduce acute pain, fever and irritability giving priority to a “wait and watch” approach. Ibuprofen is the best analgesic choice regarding its long action duration and low toxicity [5].

When it is necessary to resort to antibiotic therapy, amoxicillin is recommended as first-line therapy, in a high-dosage of 80-90 mg/kg/day divided in 2 to 3 times a day. The choice of this antibiotic is due to its efficacy against the most common pathogens, its pleasant taste, reduced cost and low incidence of side effects. If patients are allergic to amoxicillin, second or third-generation cephalosporins (cefuroxime, ceftriaxone) constitute the first-line therapy. Macrolides may also be considered in cases of sever allergy (azithromycin, clarithromycin), although its efficacy against *S. pneumoniae* and *H. influenzae*

is limited. Antibiotics should be prescribed in case of children older than six months with severe signs or symptoms and for children younger than 2 years old with bilateral AOM [30].

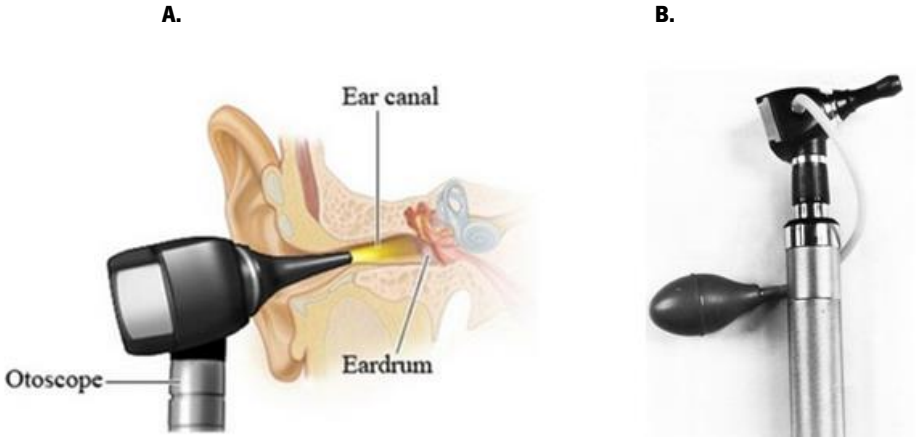


Figure 1.7 - **A.** Representation of the Otoscopy procedure (otoscope assessing the eardrum); **B.** Otoscope with pneumatic bulb to perform Pneumatic Otoscopy [94].

Otoscopy is the main modality used for AOM diagnostic, but it can also be used for CSOM (Figure 1.7A.). This procedure implies the assessment of the color, opacity, position and integrity of the tympanic membrane. A bulging tympanic membrane is the principal sign of AOM. A discharging TM perforation is an indicator of CSOM [1].

Regarding OME, Pneumatic Otoscopy is its primary diagnostic procedure providing a higher-quality diagnostic in comparison with Otoscopy alone (Figure 1.7B.). The restricted mobility of the TM is a clear sign of OME. The examination is based on pressure change in the ear canal by using an otoscope with a bulb and notice the reaction of the TM. A normal TM moves when pressure is applied, in contrast with no motion that means a non-intact TM [31].

OME is known to have spontaneous resolution in 50% of the children after 3 months. A period of 3 months is required for watchful waiting prior to surgical intervention [2].

Grommet insertion is one of the most common surgeries performed in the UK to treat OME. A grommet or ventilation tube is inserted through the TM, allowing the ventilation of the middle ear. Nevertheless, some side effects, such as: infection (2-26%) and permanent perforation of the TM (3%) led to the reduction of the number of operations from 43300 (1994) to 25300 (2008) [25].

In children with Recurrent AOM it is frequent to involve surgeries of Myringotomy alone or combined with the insertion of a grommet. Myringotomy is a procedure that consists of making a small incision in the TM to allow the fluid (blood, pus and/or water) trapped in the middle ear to drain out

(Figure 1.8A.). A grommet can be inserted to maintain drainage (Figure 1.8B.). There are some reasons to undergo Myringotomy: to restore hearing loss, to place grommets/ventilation tubes, to help treating ear infections unresponsive to medical treatment and even to take sample fluid from the middle ear to examine [25, 32].

Taking all this information into account, it is important to focus on local delivery of antibiotics directly to the ear, in opposition to systemic administration [19].

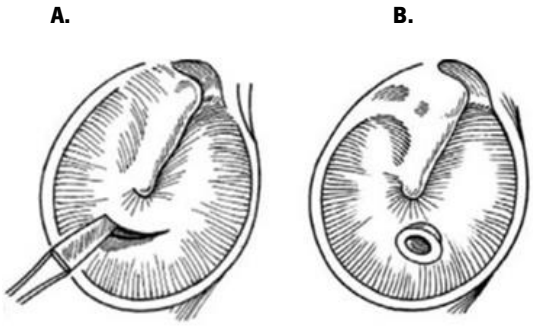


Figure 1.8 - **A.** Small incision in the tympanic membrane (Myringotomy); **B.** Grommet insertion [94].

1.2 Vaccination Plan

The National Immunization Plan (NIP) in Portugal was created in 1965 and although it is in constant revision and development, its aim is to vaccinate the larger number of people with the most suitable vaccines, to promote, not only their own individual protection but also the public health [33].

The NIP comprises specific vaccines which are selected according to the epidemiology of diseases, their expected impact, their relation cost-effectiveness and their availability in the market (Table 1.3). This program is free, universal, subsidized by the state and has a specific calendar [33].

Vaccination against infections caused by *S. pneumoniae* and *H. influenzae* type b, are included in the NIP.

Table 1.3 - Dosing vaccination schedule according to the NIP. Adapted from [34].

	Age				
	2 months	**4 months**	**6 months**	**12 months**	**18 months**
Infections caused by *Haemophilus influenzae* type b (Hib)	Hib (1)	Hib (2)	Hib (3)		Hib (4)
Infections caused by *Streptococcus pneumoniae*	PCV13 (1)	PCV13 (2)		PCV13 (3)	

*Pneumococcal Conjugate Vaccine (PCV)

The Hib conjugated vaccine comprises capsular oligosaccharides or polysaccharides of Hib conjugated with a bacterial protein. *H. influenzae* is transmitted by saliva droplets that spread in the air, when the patients cough or sneeze [33, 34].

The Hib vaccine is administered intramuscularly, and its administration is not recommended before 6 weeks of life, because it can lead to immunological tolerance, that is to a disability to react to additional doses of the vaccine. The clinical effectiveness of this vaccine is around 95%. This vaccine is administered in 4 doses, but the latter is considered as a booster dose [34].

The PCV13 vaccine comprises capsular polysaccharides of 13 *S. pneumoniae* serotypes conjugated with a protein called CRM197 (non-toxic mutant of diphtheria toxin functioning as a carrier protein). *S. pneumoniae* is spread by saliva droplets or mucus, when the patients cough or sneeze.

This vaccine is administered in 2 doses in the first year of life and 1 booster dose in the second year of life. The clinical effectiveness of PCV13 is considerably high, however it depends on the serotypes that it comprises [33, 34].

1.3 Bacteriophages

The existence of bacteriophages was firstly reported by Frederick Twort, in 1915 and two years later, by Felix d'Herelle [35]. Bacteriophages, also known as phages, are viruses that specifically infect bacteria and they are considered as natural predators of bacteria. These organisms are the most abundant entities on the planet and exist wherever their bacterial host are present. However, some bacteriophages are extremely specific and have narrow host ranges while others have broad host ranges within a bacterial species. They can be divided into monophages that solely recognize one type of

receptors and polyphages that possess a broader host range and, therefore, recognize more than one type of receptor [30, 32].

From 90 years ago on, bacteriophages have been used in therapeutic applications in Eastern Europe and in the former Soviet Union, for both humans and animals. Bacteriophages were successfully used to treat bacterial infections 10 years before the discovery of penicillin, however this therapy was abandoned to enable the use of the new emerging antibiotics [36].

Bacteriophages can exhibit different morphologies, although the majority of them present the constitution shown in Figure 1.9. They have a protein head or capsid often with the shape of an icosahedron, which contains the viral genome. The base plate is responsible for coordinating the bacterial host recognition and attachment with tail sheath contraction (when it exists). The movement starts at the base-plate and then it is propagated through the sheath. Bacteriophages that have tail fibers, can have contractile structures. Tail fibers are connected to the base plate and contain receptors at their tips that will be responsible for the recognition of the attachment sites on the bacterial cell surface [37].

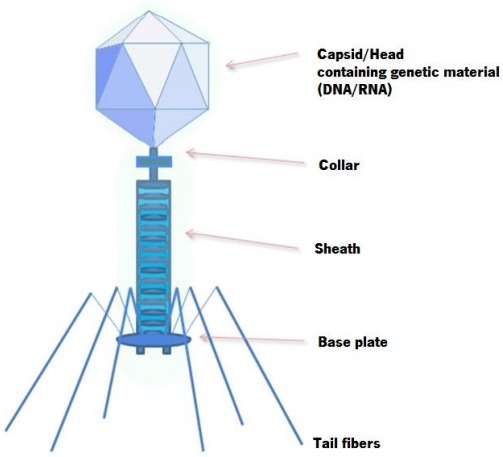


Figure 1.9 - Simple representation of a typical bacteriophage. Adapted from [37].

Bacteriophages are considered generally safe, based on their abundance in nature and our consequent exposure to them without any harmful events registered. Moreover, their composition just comprises nucleic acids and proteins, which are non-toxic. They also possess a self-reproducing capability on the host, which means that few quantities of phage are necessary to control an infection. As the viruses multiply within the bacterial hosts the initial dose increases exponentially and is released. It is also said that bacteriophages are able to penetrate poorly vascularized tissues, as well as cross the blood-brain barrier. Bacteriophages are also easier and cheaper to produce in comparison with antibiotics. In spite

of having these characteristics, it is hard to develop lytic and broad-spectrum bacteriophages effective for therapy mostly due to regulatory issues [26, 30, 31]. However, the major drawback of bacteriophage therapy resides on the possibility of bacteria developing resistance to bacteriophages, but this is circumvented using cocktails of bacteriophages [32].

As antimicrobial resistance is a global and increasing health issue, bacteriophage therapy has potential to become one of the successful and efficient alternatives to fight against this problem. Furthermore, bacteriophages kill biofilm cells, that are known to be more resistant to antibiotic therapy than planktonic cells [29, 30].

1.3.1 Bacteriophages life cycles

There are two types of life cycles that bacteriophages can exhibit: either virulent or temperate. Virulent bacteriophages are the most suitable to therapeutic use and they have lytic life cycles.

Their action starts with the attachment of the virus to the bacterial host via specific receptor sites on the cell surface – proteins, oligosaccharide, teichoic acid, peptidoglycan and lipopolysaccharide – or on the cell capsule, pili or flagella. After the attachment, occurs the injection of bacteriophage genetic material into the host cell that can occur in different ways according to the morphology of the virus but, generally, it involves contraction of bacteriophage tail and formation of a hole in the bacteria cell wall. Many bacteriophages circularize their DNA to protect them from the action of exonucleases and restriction enzymes. Then, the RNA polymerase of the host cell transcribes the viral genome into early mRNA using the machinery of the host bacteria and new virus components start to be assembled. After the formation of new bacteriophage particles, they are released to the environment. For that, lytic enzymes – endolysins - that attack the bacterial peptidoglycan are developed by the majority of dsDNA bacteriophages within the cytoplasm. Nevertheless, another enzyme called holin is needed to help on the disruption of the bacteria membrane and then, the endolysin cleaves peptidoglycan, cause the lysis and the bacteriophages are released and will infect other bacteria (Figure 1.10A.) [26, 33].

In what temperate bacteriophages are concerned, they integrate their DNA into the host cell DNA. When the bacterial DNA replication occurs, the bacteriophage DNA is also replicated, therefore each resultant cell contains the viral DNA – prophage - assuming a quiescent state. Lysogenic cells may undergo various divisions and can, occasionally and spontaneously, start a lytic cycle and liberate the bacteriophage (Figure 1.10B.) [26,33].

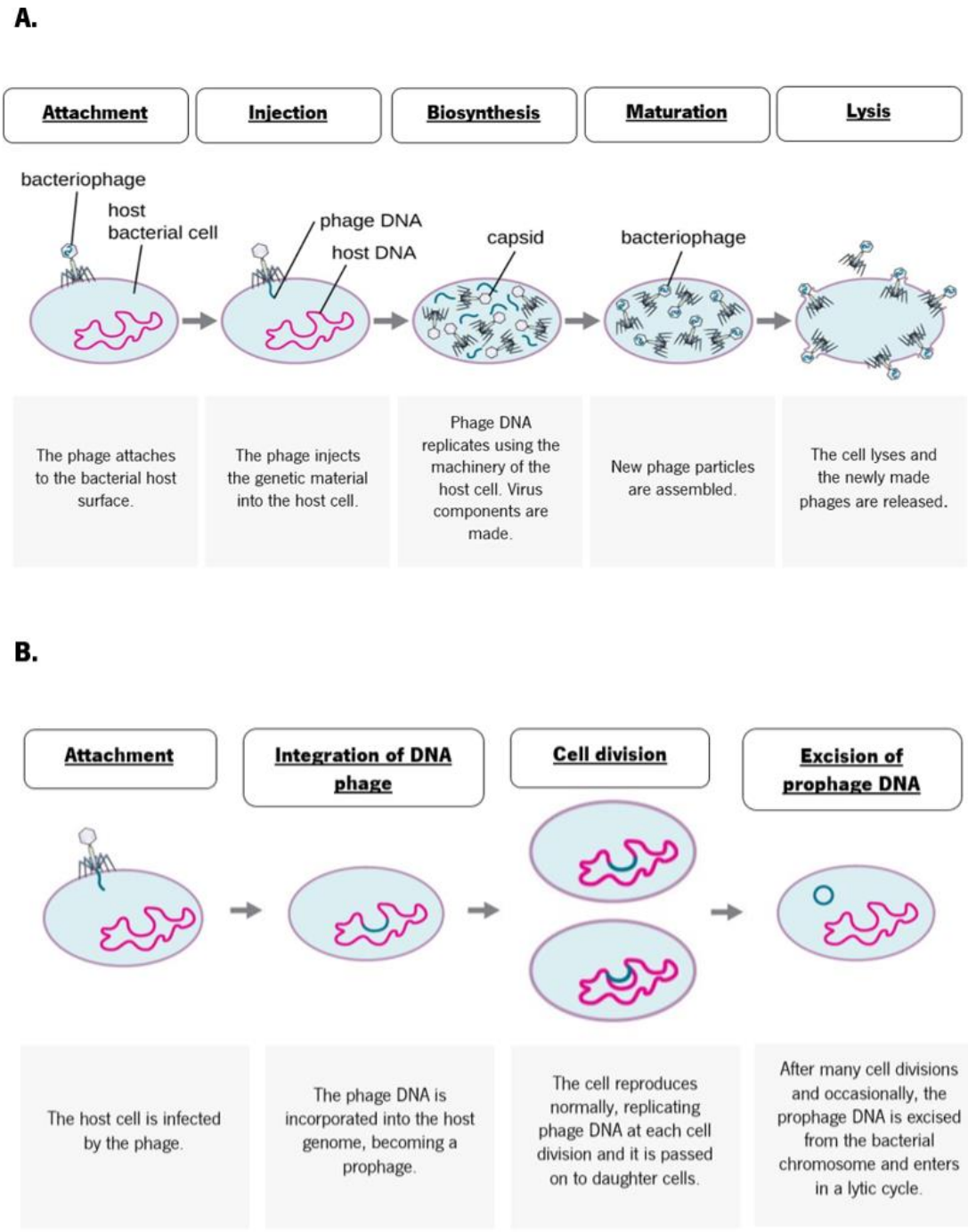


Figure 1.10 - Schematic representation of: **A.** Lytic lifecycle associated to virulent phages and **B.** Lysogenic lifecycle associated to temperate phages [95].

Temperate bacteriophages are not the best choice to be used for therapy and there are three reasons that can explain it. The first one is related to lysogenic conversion in which occurs the insertion of genes of the bacteriophage that can contribute to the change of the host cell phenotype. Briefly, there

are temperate bacteriophages that encode genes which are responsible for increasing bacterial virulence, and, therefore, their potential to cause disease [25].

The second concern has to do with lysogenic cells that are immune to superinfection by the same bacteriophage or related bacteriophages (from the same immunity type) [25].

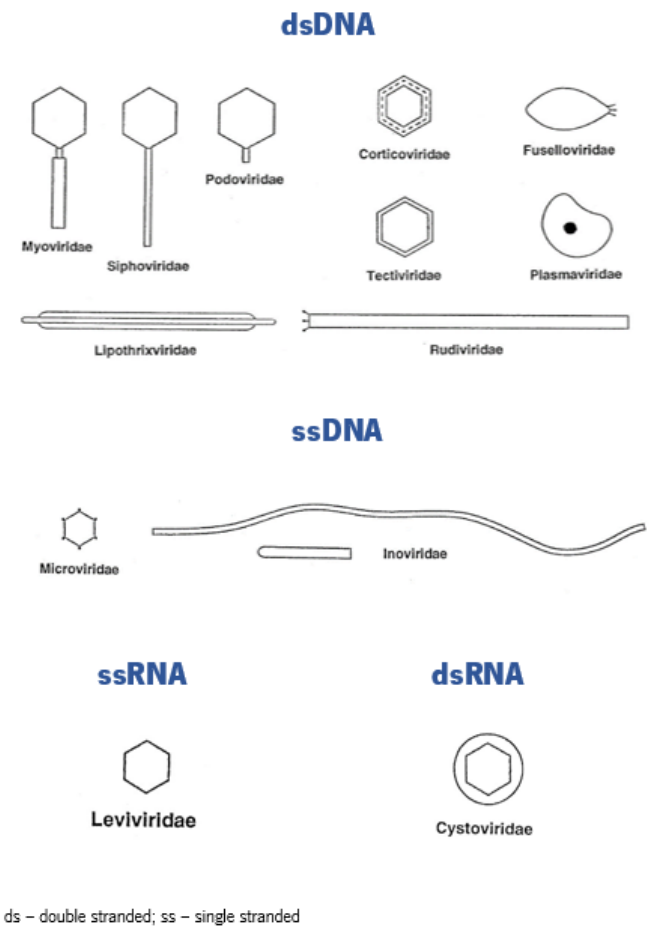
Finally, the third reason is associated with the capability of some temperate bacteriophages to undergo generalized transduction, meaning that the bacterial genes adjacent to the prophage DNA can be incorporated into the bacteriophage capsid and then transmitted from one host to another, increasing the potential of the bacteria to cause disease [25].

1.3.2 Classification of bacteriophages

In 1973, the International Committee on Taxonomy of Viruses (ICTV) was created to organize the taxonomic classification and nomenclatures of bacteriophages according to their morphology, genetic material and major characteristics. In what genetic material is concerned, bacteriophages can be divided in 4 groups: single stranded DNA (ssDNA), double stranded DNA (dsDNA), single stranded RNA (ssRNA) and double stranded RNA (dsRNA) bacteriophages [34].

Since 1968, there have been discovered more than 5100 bacteriophages and more than 90% of them have tails (order *Caudovirales*) and belong to the *Myoviridae, Siphoviridae* and *Podoviridae* families (Table 1.4) [35].

Table 1.4 - Classification and characteristics of phages families. Adapted from [37]

Family	Main characteristics
	***Myoviridae*** – Contractile tail/sheath; ***Siphoviridae*** – Long, flexible and non-contractile tail/sheath; ***Podoviridae*** – Short and non-contractile tail/sheath; ***Corticoviridae*** – Icosahedral capsid with lipid layer without tail/sheath; ***Fuselloviridae*** – Pleomorphic, lipidic, without capsid and tail/sheath; ***Tectiviridae*** – Icosahedral capsid with internal lipoprotein vesicle without tail/sheath; ***Plasmaviridae*** - Pleomorphic, lipidic, without capsid and tail/sheath; ***Lipothrixviridae*** – Enveloped filaments and lipidic; ***Rudiviridae*** – Helical rods; ***Microviridae*** – Icosahedric capsid without tail/sheath; ***Inoviridae*** – Filamentous or rod-shaped without tail/sheath; ***Cystoviridae*** – Icosahedral capsid, lipidic, enveloped without tail/sheath; ***Leviviridae*** – Quasi-icosahedral capsid without tail/sheath.
ds – double stranded; ss – single stranded	

1.3.3 Bacteriophage therapy in Otitis Media – State of the art

Only a few studies based on bacteriophage or bacteriophage derived enzyme therapy have been tested on different types of OM. One of the studies focused on the use of bacteriophages against *Pseudomonas aeruginosa* control in COM. Six bacteriophages were tested in patients suffering from COM. The number of patients in the trial was 24 and only one ear was treated since COM was unilateral. The ear infection was due to the acquisition of antibiotic-resistant *P. aeruginosa* strains, which were found to be sensitive to one or more of the six bacteriophages. By the final trial day, the 12 patients treated with bacteriophages showed reduction of 39.5% from 56.9% to 17.4% of *P. aeruginosa* levels. Regarding the remaining 12 patients without treatment *P. aeruginosa* levels decreased 32.7% however these reductions were from 141.6% to 108.9%. Although a significant reduction in *P. aeruginosa* levels was obtained in the

bacteriophage treated group, it was not possible to completely eradicate *P. aeruginosa* levels, as the patients chose for the study represented complicated cases of COM which had not responded to previous treatments with antibiotics and even surgeries. Bearing this in mind, it was challenging to notice improvements just with a single dose of bacteriophage. Some conclusions were recovered from this study: bacteriophage therapy used one single dose with a bacteriophage concentration of 1×10^5 PFU/mL (containing 2.4 ng of protein and 0.06 ng of DNA); and bacteriophage replication occurred during a mean of 23 days after the single bacteriophage dose given to the patients [38].

Another study was performed in order to prevent OM caused by *S. pneumoniae*, using lysins that are cell wall hydrolases. Cp1-1 lysin specific for *S. pneumoniae,* was tested in a mouse model and showed to prevent AOM. This study revealed 100% of effectiveness of Cp1-1 lysin in preventing AOM, however none of the animals treated with it developed other bacterial infections after virus infection. Finally, Cp1-1 lysin neither showed any topical or systemic toxicity nor triggered any clinical or significant histopathological observations in mice [39].

2. MATERIALS AND METHODS

2.1 Typing of *Moraxella catarrhalis*

2.1.1 Bacterial Isolates and culture conditions

Twenty-five clinical isolates of *M. catarrhalis* were provided by Hospital de Braga. Samples were collected from patients whose ages are ranged between 0 and 91 years old in Braga district. Clinical isolates were plated onto TSA [TSB with 1.2% (w/v) agar] supplemented with 5% (v/v) defibrinated horse blood and incubated overnight at 37°C in 5% CO_2.

Table 2.1 - *Moraxella catarrhalis* strains used in the study, with the respective ages and sex of the patients they were recovered

Strains	Source /Description	Age	Sex
1	Sputum isolate	67 years	M
2	Middle-ear fluid isolate	5 years	M
3	Bronchia aspirate	81 years	M
4	Sputum isolate	60 years	M
5	Sputum isolate	88 years	M
6	Sputum isolate	39 years	F
7	Sputum isolate	76 years	F
8	Sputum isolate	65 years	M
9	Sputum isolate	66 years	M
10	Sputum isolate	58 years	M
11	Sputum isolate	4 months	F
12	Sputum isolate	39 days	M
13	Sputum isolate	73 years	M
14	Sputum isolate	13 months	M
15	Sputum isolate	53 years	F
16	Sputum isolate	90 years	F
17	Sputum isolate	80 years	F
18	Sputum isolate	86 years	F
19	Sputum isolate	92 years	F
20	Sputum isolate	91 years	M

21	Bronchoalveolar lavage isolate	72 years	M
22	Sputum isolate	84 years	M
23	Ocular exudate isolate	21 days	F
24	Sputum isolate	87 years	F
25	Sputum isolate	78 years	F

2.1.2 PCR typing

a) Primers: Three primers (*Invitrogen*), previously designed [40], were mixed in multiplex PCR to target lipooligosaccharides (LOS) serotypes: A, B and C (Table 2.2).

Table 2.2 - Primers used for *Moraxella catarrhalis* clinical isolates serotyping determination, the respective product sizes and target serotypes [40]

Primer pair name	Primer sequence (5'→ 3')	Product size (bp)	Serotype
Pr 406	CAAAAGAAGACAAACAAGCAGC	3300/4300*	B/C
Pr 408	CATCAAAAACCCCCCTACC		
Pr 649	ATCCTGCTCCAACTGACTTTC	1900	A
Pr 408	CATCAAAAACCCCCCTACC		

*The same pair of primers targets two different serotypes.

b) Multiplex PCR scheme: Colony PCR was performed by resuspending a single bacterial colony in 200 µL of NZY bacterial cell lysis buffer (NZYTech), followed by heating at 95°C for 15 minutes. The PCR amplifications were performed in 25 µL-volumes with each reaction mixture containing: KAPA Taq DNA polymerase (1 U), KAPA Taq Buffer (1x), dNTP mix (0,2 mM each), forward and reverse primers (0,4 µM each) (Table 2.2), template DNA (unknown concentration) and nuclease-free water up to 25 µL. Amplification was performed with an initial denaturation at 95°C for 5 minutes, followed by 35 denaturation cycles at 95°C for 30 seconds, annealing at 51°C for 30 seconds, extension and final extension at 72°C for 4 minutes and 30 seconds each and a final hold step at 4°C during forever. The PCR products were separated on 1% agarose gel in 1× TAE buffer stained with *GreenSafe* (NZYTech) by gel electrophoresis at 100 V for 30 minutes. Before loading the samples on gel, they were mixed with DNA loading dye (Thermo Scientific) and finally visualized in ChemiDoc™ XRS + System with Image Lab™ Software (Version 5.2.1 Bio-Rad

Laboratories). The sizes of the PCR products were determined by comparison with the molecular size standard (1 kb DNA ladder, Thermo Scientific).

2.2 Typing of *Streptococcus pneumoniae*

2.2.1 Bacterial Isolates and culture conditions

Twenty-seven clinical isolates of *S. pneumoniae* were provided by Hospital de Braga. Samples were collected from patients whose ages are ranged between 0 and 90 years old in Braga district. Clinical isolates were plated onto TSA [TSB with 1.2% (w/v) agar] supplemented with 5% (v/v) defibrinated horse blood and incubated overnight at 37°C in 5% CO_2.

Table 2.3 - *Streptococcus pneumoniae* strains used in this study, with the respective ages and sex of the patients they were recovered

Strains	Source /Description	Age	Sex
1	Sputum isolate	67 years	M
2	Middle-ear fluid isolate	22 months	F
3	Sputum isolate	90 years	F
4	Sputum isolate	85 years	M
5	Sputum isolate	62 years	M
6	Sputum isolate	66 years	M
7	Bronchia aspirate	77 years	F
8	Sputum isolate	65 years	F
9	Sputum isolate	57 years	M
10	Blood culture	45 years	M
11	Sputum isolate	89 years	M
12	Sputum isolate	55 years	M
13	Sputum isolate	80 years	M
14	Middle-ear fluid isolate	6 months	M
15	Middle-ear fluid isolate	6 months	M
16	Middle-ear fluid isolate	3 years	F
17	Middle-ear fluid isolate	6 years	M
18	Middle-ear fluid isolate	27 days	M
19	Blood culture isolate	83 years	M
20	Sputum isolate	88 years	F

21	Liquor isolate	39 years	F
22	Blood culture isolate	68 years	F
23	Sputum isolate	77 years	M
24	Blood culture isolate	87 years	M
25	Sputum isolate	6 years	F
26	Sputum isolate	39 years	M
27	Blood culture	54 years	M

2.2.2 PCR typing

a) Primers: Fifty-four primers (Invitrogen), previously designed [41], were organized into seven sets and then mixed in multiplex PCR to target different serotypes: 1, 2, 3, 4, 5, 6A/B, 6C/D, 7A/F, 7C, 9V/9A, 9N/L, 11A, 12F/12A/12B/44/46, 14, 15B/C, 16F, 17F, 18A/B/C, 19A, 19F, 20, 22F/22A, 23F, 23B, 23A and 33F/33A/37. It was also included in each set, a primer pair – cpsA-f and cpsA-r – functioning as a positive control to target cps conserved gene sequences common to all pneumococci. These primers were grouped together based on product size and have different concentrations (Table 2.4).

Table 2.4 - Primers used for *Streptococcus pneumoniae* clinical isolates serotyping determination, the respective product sizes and target serotypes [41]

Primer pair name/Primer concentration*	Primer sequence (5'→ 3')	Product size (bp)	Serotype
Set 1			
19A-f	GTTAAGATTGCTGATATTAATTGATATCC	566	19A
19A-r (1 µM)	GTAATATGTCTTTAGGGCGTTTATGGCGATAG		
3-f	CCACTAAAGCTTTGGCAAAAGAAA	371	3
3-r (1,5 µM)	CCCGAACGTAAAGCTTCTTCA		
22F/22A-f	TCTATTAAATAACCCATTGGAATTGAAACG	643	22F/22A
22F/22A-r (1,5 µM)	TCGCAATTGAAGACCACATAAACTG		
6A/B-f	AATTTGTATTTTATTCATGCC TATATCTGG	250	6A/B
6A/B-r (0,5 µM)	TTAGCGGAGATAATTTAAAATGATGACTA		

Set 2			
4-f	CTGTTACTTGTTCTGGACTCT CGATAATTGG	430	4
4-r	GCCCACTCCTGTTAAAATCCTACCCGCATTG		
(1,5 µM)			
14-f	GAAATGTTACTTGGCGCAGGTGTCAGAATT	189	14
14-r	GCCAATACTTCTTAGTCTCTC AGATGAAT		
(1 µM)			
12F/12A/12B/44/46-f	GCACCCACGGGTAAATATTCTAC	376	12F/12A/12B/44/46
12F/12A/12B/44/46-r	CAACTAAGAACCAAGGATCCACAG		
(1,5 µM)			
9V/9A-f	GGGTTCAAAGTCAGACAGTGAATCTTAA	816	9V/9A
9V/9A-r	CCATGAATGAAATCAACATTGTCAGTAGC		
(2 µM)			
Set 3			
23F-f	GTAACAGTTGCTGTAGAGGGAATT GGCTTTTC	384	23F
23F-r	CACAACACCTAACACTCGATGGCTATATGATTC		
(1,5 µM)			
7A/F-f	TCCAAACTATTACAGTGGGAATTACGG	599	7A/F
7A/F-r	ATAGGAATTGAGATTGCCAAAGCGAC		
(1,5 µM)			
11A-f	GGACATGTTCAGGTGATTTCCCAATATAGTG	463	11A
11A-r	GATTATGAGTGTAATTTATTCCAACTTCTCCC		
(1 µM)			
33F/33A/37-f	GGAACTGGTTCAGCAACTATACG	338	33F/33A/37
33F/33A/37-r	GGTTCTAAGACCGTCTGAAATACC		
(1 µM)			
Set 4			
19F-f	GTTAAGATTGCTGATCGATTAATTGATATCC	304	19F
19F-r	GTAATATGTCTTTAGGGCGTTTATGGCGATAG		
(1,5 µM)			
16F-f	TAATGTTATGACCTTGGTAATCTTCCC	988	16F
16F-r	TCCCAAAGGATAATCAATAACTTTTAGAAG		
(2 µM)			
23B-f	CCACAATTAGCGCTATATTCATTCAATCG	199	23B
23B-r	GTCCACGCTGAATAAAATGAAGCTCCG		
(0,5 µM)			
18A/B/C-f	CTTAATAGCTCTCATTATTCTTTTTTTAAGCC	573	18A/B/C
18A/B/C-r	TTATCTGTAAACCATATCAGCATCTGAAAC		

(1,5 µM)			
Set 5			
5-f	CATGATTTATGCCCTCTTGCAA	362	5
5-r (1,5 µM)	GACAGTATAAGAAAAAGCAAGGGCTAA		
15B/C-f	TTGGAATTTTTTAATTAGTGGCTTACCTA	496	15B/C
15B/C-r (1,5 µM)	CATCCGCTTATTAATTGAAGTAATCTGAACC		
2-f	TATCCCAGTTCAATATTTCTCCACTACACC	290	2
2-r (1 µM)	ACACAAAATATAGGCAGAGAGAGACTACT		
23A-f	TATTCTAGCAAGTGACGAAGATGCG	722	23A
23A-r (1,5 µM)	CCAACATGCTTAAAAACGCTGCTTTAC		
Set 6			
1-f	CTCTATAGAATGGAGTATATAAACTATGGTTA	280	1
1-r (1,5 µM)	CCAAAGAAAATACTAACATTATCACAATATTGGC		
20-f	GAGCAAGAGTTTTTCACCTGACAGCGAGAAG	514	20
20-r (1,5 µM)	CTAAATTCCTGTAATTTAGCTAAAACTCTTATC		
23A-f	TATTCTAGCAAGTGACGAAGATGCG	722	23A
23A-r (1,5 µM)	CCAACATGCTTAAAAACGCTGCTTTAC		
4-f	CTGTTACTTGTTCTGGACTCTCGATAATTGG	430	4
4-r (1,5 µM)	GCCCACTCCTGTTAAAATCCTACCCGCATTG		
Set 7			
6C/D-f	CATTTTAGTGAAGTTGGCGGTGGAGTT	627	6C/D
6C/D-r (2 µM)	AGCTTCGAAGCCCATACTCTTCAATTA		
7C-f	CTATCTCAGTCATCTATTGTTAAAGTTTACGACGGGA	260	7C
7C-r (1,5 µM)	GAACATAGATGTTGAGACATCTTTTGTAATTTC		
17F-f	TTCGTGATGATAATTCCAATGATCAAACAAGAG	693	17F
17F-r (1,5 µM)	GATGTAACAAATTTGTAGCGACTAAGGTCTGC		
9N/L-f	GAACTGAATAAGTCAGATTTAATCAGC	516	9N/L

9N/L-r (1,5 µM)	ACCAAGATCTGACGGGCTAATCAAT		
cpsA-f	GCAGTACAGCAGTTTGTTGGACTGACC	160	-
cpsA-r (0,5 µM)	GAATATTTTCATTATCAGTCCCAGTC		

*The different primer concentrations used in the Multiplex PCR were previously optimized [35].

b) Multiplex PCR scheme: Colony PCR was performed by resuspending a single bacterial colony in 200 µL of NZY bacterial cell lysis buffer (NZYTech), followed by heating at 95°C for 15 minutes. The PCR amplifications were performed in a reaction volume of 25 µL containing: KAPA Taq DNA polymerase (1 U), KAPA Taq Buffer (1×), dNTP mix (0,2 mM each), forward and reverse primers (at concentrations described in Table 2.4), template DNA (unknown concentration), $MgCl_2$ (2,5 mM) and nuclease-free water up to 25 µL. Initial denaturation step was performed at 95°C for 5 minutes, followed by 35 denaturation cycles at 95°C for 30 seconds, annealing temperature at 54°C for 30 seconds, extension and final extension at 72 °C for 1 minute each step and finally completed with a hold step at 4°C during forever. The PCR products were electrophoresed on a 3% agarose gel in 1x TAE buffer stained with GreenSafe (NZYTech) for 30 minutes at 100V. Before loading the samples on gel, they were mixed with DNA loading dye (Thermo Scientific) and finally visualized in ChemiDoc™ XRS + System with Image Lab™ Software (Version 5.2.1 Bio-Rad Laboratories). The 100 bp DNA ladder marker (BioLabs) and 1 kb DNA ladder (Thermo Scientific) were used for molecular weight reference.

2.3 Typing of *Haemophilus influenzae*

2.3.1 Bacterial Isolates and culture conditions

Forty-eight clinical isolates of *H. influenzae* were provided by Hospital de Braga. Samples were collected from patients whose ages are ranged between 0 and 91 years old in Braga district. Clinical isolates were plated onto BHI with 1.2% (w/v) agar suitably supplemented with hemin and NAD (nicotinamide adenine dinucleotide) and incubated overnight at 37°C in 5% CO_2.

Table 2.5 - *Haemophilus influenzae* strains used in the study with the respective ages and sex of the patients they were recovered

Strains	Source / Description	Age	Sex
1	Sputum isolate	33 years	F
2	Sputum isolate	5 years	M
3	Sputum isolate	63 years	M
4	Sputum isolate	39 years	F
5	Sputum isolate	22 months	M
6	Sputum isolate	60 years	M
7	Sputum isolate	73 years	M
8	Bronchia aspirate	64 years	M
9	Sputum isolate	72 years	M
10	Bronchia aspirate	55 years	M
11	Bronchia aspirate	49 years	M
12	Sputum isolate	90 years	F
13	Bronchia aspirate	49 years	M
14	Sputum isolate	85 years	M
15	Sputum isolate	78 years	M
16	Bronchia aspirate	66 years	M
17	Sputum isolate	88 years	F
18	Sputum isolate	90 years	F
19	Bronchia aspirate	46 years	M
20	Sputum isolate	4 months	M
21	Bronchia aspirate	42 years	M
22	Sputum isolate	81 years	M
23	Bronchia aspirate	42 years	M
24	Sputum isolate	86 years	M
25	Sputum isolate	81 years	M
26	Bronchia aspirate	75 years	F
27	Sputum isolate	79 years	F
28	Bronchia aspirate	82 years	M
29	Sputum isolate	39 years	F
30	Sputum isolate	84 years	F
31	Sputum isolate	73 years	M
32	Sputum isolate	91 years	F
33	Sputum isolate	74 years	M
34	Sputum isolate	47 years	M
35	Sputum isolate	89 years	M

36	Sputum isolate	81 years	M
37	Sputum isolate	71 years	F
38	Sputum isolate	91 years	M
39	Sputum isolate	86 years	M
40	Sputum isolate	81 years	M
41	Sputum isolate	80 years	M
42	Sputum isolate	74 years	M
43	Sputum isolate	60 years	M
44	Sputum isolate	86 years	F
45	Sputum isolate	89 years	F
46	Sputum isolate	91 years	F
47	Sputum isolate	64 years	F
48	Sputum isolate	12 years	M

2.3.2 PCR typing

a) Primers: Seventeen primers (Invitrogen), previously designed, were prepared to use in multiplex PCR [42, 43] to target different serotypes: a, b, c, d, e and f. Nevertheless, the primer pair – HI-1 and HI-2 – that functioned as a positive control to distinguish typeable from non-typeable *H. influenzae* strains was used at first and if the result was positive, the other seventeen primers were then mixed in the reaction.

Table 2.6 - Primers for *Haemophilus influenzae* clinical isolates serotyping determination, the respective product sizes and target serotypes [42, 43]

Primer pair name	Primer sequence (5'→3')	Product size (bp)	Serotype
a2	GAATATGACCTGATCTTCTG	180	a
a3	AGTGGACTATTCCTGTTACAC		
b1	GCGAAAGTGAACTCTTATCTCTC	480	b
b2	GCTTACGCTTCTATCTGGTGAA		
c1	TCTGTGTAGATGATGGTTCA	250	c
c2	CAGAGGGCAAGCTATTAGTGA		
d1	TGATGACCGATACAACCTGT	150	d
d2	TCCACTCTTCAAACCATTCT		

d2	TCCACTCTTCAAACCATTCT	100	d
d3	CTCTTCTTAGTGCTGAATTA		
e1	GGTAACGAATGTAGTGGTAG	1350	e
e2	GCTTTACTGTATAAGTCTAG		
e2	GCTTTACTGTATAAGTCTAG	1160	e
e3	CAGCTATGAACAAGATAACG		
f1	GCTACTATCAAGTCCAAATC	450	f
f2	CGCAATTAGGAAGAAAGCT		
f1	GCTACTATCAAGTCCAAATC	400	f
f3	AATGCTGGAGTATCTGGTTC		
HI-1	CGTTTGTATGATGTTGATCCAGAC	343	-
HI-2	TGTCCATGTCTTCAAAATGATG		

b) Multiplex PCR scheme: Colony PCR was performed by resuspending a single bacterial colony in 200 µL of NZY bacterial cell lysis buffer (NZYTech), followed by heating at 95°C for 15 minutes. The PCR amplifications were performed in a reaction volume of 25 µL containing: KAPA Taq DNA polymerase (1 U), KAPA Taq Buffer (1×), dNTP mix (0,2 mM each), forward and reverse primers (0,8 mM), template DNA (unknown concentration), $MgCl_2$ (1,6 mM) and nuclease-free water up to 25 µL. Initial denaturation step was performed at 95°C for 5 minutes, followed by 35 denaturation cycles at 95°C for 30 seconds, annealing temperature at 55°C for 30 seconds, extension and final extension at 72°C for 1 minute and 15 seconds each step and finally completed with a hold step at 4°C during forever. The PCR products were electrophoresed on a 3% agarose gel in 1× TAE buffer stained with *GreenSafe* (NZYTech) for 45 minutes at 100 V. Before loading the samples on gel, they were mixed with DNA loading dye (Thermo Scientific) and finally visualized in ChemiDoc™ XRS + System with Image Lab™ Software (Version 5.2.1 Bio-Rad Laboratories). The 100 bp DNA ladder marker (BioLabs) and 1 kb DNA ladder (Thermo Scientific) were used for molecular weight reference.

2.4 *Streptococcus pneumoniae* bacteriophages

2.4.1 Bacterial strains and culture conditions

The three virulent pneumococcus bacteriophages: Cp-1, Dp-1 and MS1 were propagated on the unencapsulated strain *S. pneumoniae* R6 on TSA [TSB with 1.2% (w/v) agar] supplemented with 5% (v/v) defibrinated horse blood at 37°C in the presence of 5% CO_2. These bacteriophages were previously characterized and isolated and were obtained from the Félix d'Hérelle Reference Center for Bacterial Viruses.

2.4.2 Bacteriophage source and Host

Bacteriophage Dp-1 was the first virulent pneumococcus infecting virus being isolated in 1975, followed by bacteriophage Cp-1 firstly isolated in 1981 and finally, the most recent one isolated in 2017 called MS1. Bacteriophages Cp-1 and MS1 belong to the *Siphoviridae* family and phage Dp-1 to the *Podoviridae* family, with a linear double stranded DNA genome [44–46]. All of these 3 bacteriophages were isolated from throat swab of patients with upper respiratory tract infections (URTI). These bacteriophages were propagated in the same host strain: *S. pneumoniae* R6st (streptomycin-resistant strain).

2.4.3 Optimization of bacteriophages media

Five different solid media and two different liquid media were tested with the aim of finding the best combination for each bacteriophage, that is, the best media where it was possible to observe the clearest action of the bacteriophage.

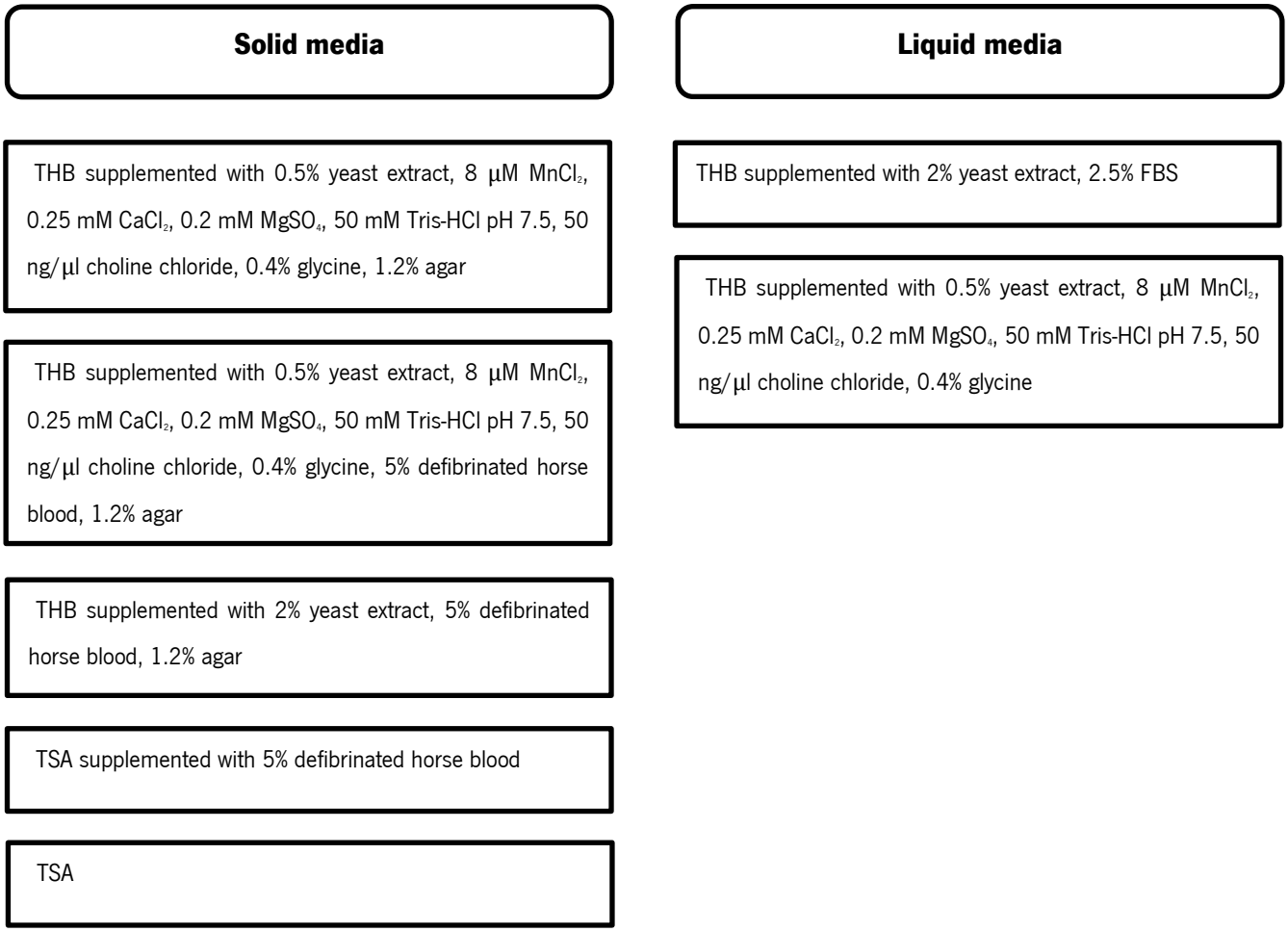


Figure 2.1 – Different combinations of solid and liquid media tested to observe a clear action of the bacteriophages.

2.4.4 Production and titration of bacteriophages in liquid medium

In 10 mL of the respective media stated in the section above, 100 μL of overnight culture of *S. pneumoniae* were added, as well as, 100 μL of bacteriophage (Cp-1, Dp-1 and MS1). A control tube (just with bacteria) was also prepared. Then, all the tubes were incubated at 37°C with 5% of CO_2, overnight. After the incubation, a filtration was performed on a 0.45 μm filter.

Drops of 20 μL of each bacteriophage dilution were spotted over THB plates [Cp-1 - THB supplemented with 0.5% yeast extract, 8 μM $MnCl_2$, 0.25 mM $CaCl_2$, 0.2 mM $MgSO_4$, 50 mM Tris-HCl pH 7.5, 50 ng/μl choline chloride, 0.4% glycine and 1.2% agar; Dp-1 - THB medium supplemented with 2% yeast extract, 5% defibrinated horse blood and 1.2% agar; MS1 - THB supplemented with 0.5% yeast extract, 8 μM $MnCl_2$, 0.25 mM $CaCl_2$, 0.2 mM $MgSO_4$, 50 mM Tris-HCl pH 7.5, 50 ng/μl choline chloride, 0.4% glycine, 1.2% agar and 5% defibrinated horse blood]. The plates were left to dry for 10 minutes and

then were incubated overnight at 37°C with 5% CO_2. Plaques formation was checked and calculated according to the following equation:

$$Plaque\ forming\ units\ (PFU)/mL = \frac{(Number\ of\ plaques)\ \times\ (Dilution\ factor)}{(Bacteriophage\ volume\ plated)\ (mL)}$$

2.4.5 Lytic spectra

The three bacteriophages: Cp-1, Dp-1 and MS1 – were tested against all the *S. pneumoniae* clinical isolates (27 strains) in order to see what strains are infected by these bacteriophages, assessing their lytic spectra.

The 27 strains were grown overnight, and the lawn was composed by 100 µL of inoculums and 3 mL of THB supplemented with 2% yeast extract top agar 0,4% put on THB supplemented with 2% yeast extract and 1.2% agar plates. Then, 20 µL drop of each bacteriophage were spotted over the lawn and were left to dry. The plates were incubated overnight at 37°C with 5% CO_2.

By the next day, the susceptibility of each strain to each bacteriophage was evaluated by the presence or absence of a lysis zone.

3. Results and Discussion

3.1 Typing clinical samples

3.1.1 Typing of *Moraxella catarrhalis*

LOS is a surface-exposed lipooligosaccharide which is a major component of *M. catarrhalis* outer membrane. It constitutes an important virulence factor for these bacteria and is similar to the lipopolysaccharide (LPS) of other gram-negative organisms, consisting of an oligosaccharide part and lipid A, however it lacks O-antigenic side chains (repeating units), which are characteristic of Gram-negative enteric pathogens [47, 48].

Bearing this in mind, there are three different LOS documented serotypes of *M. catarrhalis*: serotypes A, B and C. Throughout the years, serotype A presents as being the most prevalent one (60%), followed by B (30%) and C (5%) respectively and unidentified strains (5%). The distinction between the three LOS serotypes resides in the oligosaccharide part of the molecule [49].

In this study, a similar distribution of LOS serotype A was found with 56% of isolates having it. Neither serotype B, nor serotype C strains were identified, and a substantial increase was observed on the distribution of unidentified strains ranging from 5% to 44% (Figure 3.1).

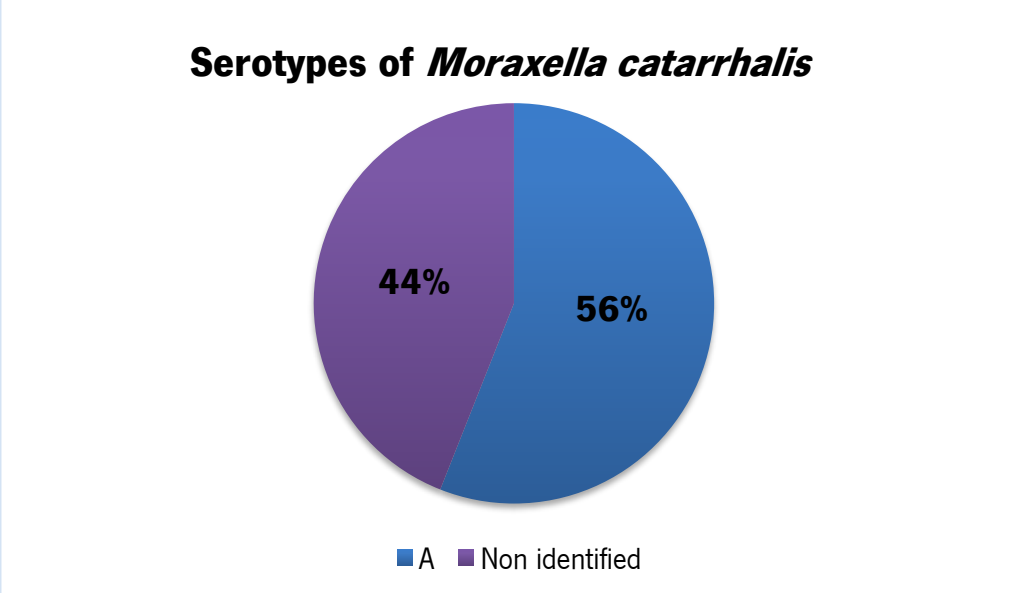


Figure 3.1 - Distribution of *Moraxella catarrhalis* serotypes in clinical samples obtained from Hospital de Braga. A refers to serotype A and Non identified belong to non-typeable strains.

Table 3.1 - Prevalence of *Moraxella catarrhalis* LOS types and Non identified isolates

	LOS type	**Non identified**
	A	
Age range (years)		
0 – 9	4	1
10 – 24	-	-
25 – 64	4	0
≥ 65	6	10

LOS type A showed to be as prevalent in isolates from adults (25-64) as in children (0-9), noticing the prevalence of an untypeable children isolate. Isolates from at least 65-year-old adults assumed the leadership with 6 LOS type A and 10 untypeable strains. Attention must be paid to the arise of untypeable isolates, especially in elderly people with a compromised immune system or with chronic obstructive pulmonary disease (COPD). Moreover, *M. catarrhalis* is a frequent cause of lower respiratory tract infections in older people, which can evolve to severe invasive infections, such as pneumonia, that can be life-threatening [50, 51].

Until now, just three LOS serotypes have been discovered, becoming imperative the discovery of more, as well as the development of vaccines against *M. catarrhalis*, in order to diminish the prevalence of *M. catarrhalis*-mediated diseases. The challenge remains in the antigen identification. *M. catarrhalis* is not known to secrete antigens that are usually good vaccine components, such as exotoxins and polysaccharide capsules. Since the bacteria seem to lack these antigens, the search for vaccine ones has focused primarily on the bacteria outer surface: outer membrane proteins and LOS [52–54].

3.1.2 Typing of *Streptococcus pneumoniae*

More than 90 pneumococcal serotypes have been identified to date based on the structure of *S. pneumoniae* polysaccharide capsule, therefore the potential of each pneumococcal isolate to cause disease has been associated with the capsule expressed. Despite the high number of pneumococcal serotypes known, just about 20 of them are responsible for the majority of invasive diseases [55].

Although this enhances the probability of developing effective vaccines targeting the most frequent serotypes, the challenge remains on the fact that the distribution of them depends on factors, such as: age, geography and time, making difficult the development of vaccines comprising all these aspects [56].

In this study, there were identified 6 different pneumococcal serotypes: 1, 15B/C, 12F/12A/12B, 6A/B and 9N/L. More than a half of pneumococcal isolates tested were classified as non-typeable (66%) and it was verified a significant difference in comparison with the other serotypes found: serotype 1 (11%), serotype 19F (7%) and serotypes 15B/C, 12F/12A/12B, 6A/B and 9N/L with 4% each (Figure 3.2.).

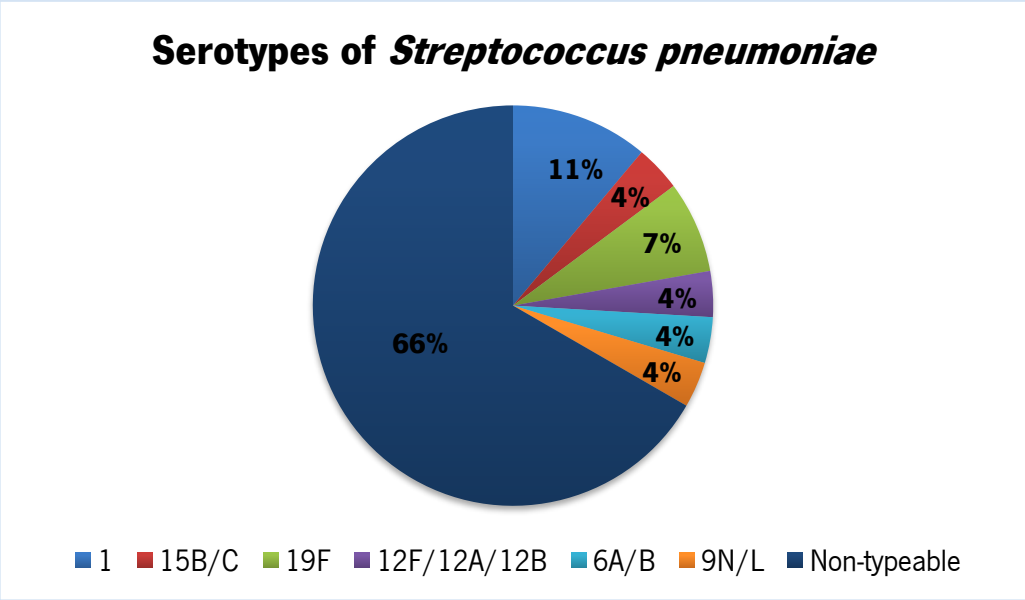


Figure 3.2 - Distribution of *Streptococcus pneumoniae* serotypes in clinical samples obtained from Hospital de Braga.

S. pneumoniae is known, worldwide, as a bacterium able to cause non-invasive diseases and also life-threatening diseases – invasive - particularly among young children and the elderly (especially with immunocompromised immune system) [55].

Indeed, that is what the data presented in Table 3.2 suggest: infections caused by the specific serotypes obtained (1, 15B/C, 19F, 12F/12A/12B, 6A/B, 9N/L) are more usual in people with more than 65 years old and non-typeable strains usually affect children younger than 9 years old. A total of 2 cases of infection were registered by serotypes 1 and 19F, each, in the elderly. Regarding non-typeable strains there were found 7 cases in children between 0 and 9 years old and 6 noticed in people older than 65 years old.

Table 3.2 - Prevalence of *Streptococcus pneumoniae* capsule serotypes and non-typeable isolates

	Capsule serotype						Non-typeable
	1	15B/C	19F	12F/12A/12B	6A/B	9N/L	
Age range (years)							
0 – 9	-	-	-	-	-	-	7
10 – 24	-	-	-	-	-	-	-
25 – 64	1	-	-	1	-	-	5
≥ 65	2	1	2	-	1	1	6

In order to fight infections against pneumococcal serotypes, pneumococcal conjugate vaccines (PCV) were developed comprising a limited serotype valency. The aim of developing the PCVs was not only to have impact on invasive diseases, but also to reduce colonization by vaccine serotypes [57].

The introduction of the 7-valent pneumococcal conjugate vaccine (PCV7) into the vaccination calendars was performed by some countries, such as: Belgium, Italy, France, Ireland, Spain (Madrid region), however Portugal did not do so. PCV7 was just given under medical prescription and despite neither being included in the National Immunization Plan (NIP) nor subsidized by the state, it still had a widespread and high usage, suggesting that Pediatricians frequently prescribed PCV7 and parents were willing to pay for it. PCVs (7, 10, 13 and 23) were not introduced in the NIP until 2015, nevertheless they were commercially available in the private market without any reimbursement by the state. In Portugal, just PCV13 entered in the NIP in August 2015 [58, 59].

In general, it is known that most of the children <6 years old attend day-care centres, making them major reservoirs of pneumococci, contributing to pneumococcal transmission to other people, however a study showed that the availability of PCV7 in Portugal, in 2001 helped counteracting this reality. Since 2002, a decrease in the prevalence of PCV7-serotypes has been observed in Portugal, reducing the number of infections from 56% in 1999-2002 to 17% in 2006-2008, particularly in children. This fact enabled the decline of the prevalence of these 7 serotypes covered by PCV7, not only among vaccinated children, but also among unvaccinated children and adults, because of the reduced nasopharyngeal carriage of pneumococcus in vaccinated children and consequently the reduced transmission to unvaccinated people [60, 61].

Despite the reduction of PCV7 serotypes, a significant serotype replacement has been observed by 2009-2010. Serotypes not included in the vaccine emerged to replace the decline of the ones covered by PCV7, however they vary according to geographic location and age groups [58].

Regarding otitis media and comparing pre-PCV7 era with post-PCV7 era, studies show that there was a reduction of more than 28% in recurrent otitis media and more than 43% in AOM, in children aged <2 years old [62].

Later, PCV7 was substituted by PCV13, a higher-valent PCV, covering 13 serotypes and a decrease was observed in IPD, particularly in young children with a reduction from 52.1 (2008/2009) to 25.1 (2011/2012) in children <1 year and from 31.6 (2008/2009) to 16.1 (2011/2012) in children 1-2 years of age (numbers in a 100 000 population). In the adult population, PCV13 serotypes responsible for IPD decreased from 70% (2008) to 54% (2011) [63].

PCV23 was also a vaccine which became available in 2015, covering 23 serotypes, but just recommended for people that belong to risk groups, more prone to acquire invasive disease by pneumococcus: people more than 65 years old, people with chronic lung disease, diabetes, weakened immune system and smokers. In line with some European reports, this vaccine seems to be restricted to meningitis and bacteremic manifestations [64, 65].

Comparing the results obtained with the data presented above of a study of 2010, in Portugal, the most prevalent PCV7 serotype was 19F (Figure 3.2), carried by 4.6% (2010) and 7.4% (2017) of all participants. It was verified an increase on the percentage of serotype 19F carriage, despite the total number of participants being different (481 people – in 2010 – and 27 people – in 2017). This serotype was carried by adults with more than 65 years old, meaning that, probably they were not vaccinated against pneumococcus infections. Indeed, by 2009-2010, all PCV7 serotypes had become rare, except for serotype 19F, which had decreased, but its carriage was still higher than the other PCV7 serotypes [63].

According to previous studies, serotype 19F is associated with carriage, suggesting it has a low invasive disease potential. This result suggests that, for this particular serotype (19F) higher vaccine coverage is needed to achieve a lower carriage among the population, mainly among non-vaccinated people [66].

Regarding PCV13 serotypes, the most prevalent one was 19A in 2010 and 1 in 2017 (Figure 3.2), with carriage percentages of 8.3% and 11.1%, respectively. According to the study of 2010 in Portugal, serotype 1 was either absent or with a very low prevalence (< 2%) and 7 years after, it emerged with a percentage of 11.1%. Serotype 1 showed to be carried by a person with age between 25 and 64 years old and the others by people >65 years old, considering any of them had taken the vaccine [63].

The emergence of serotype 1 happened after the introduction of PCV7 use, in Portugal, and accompanied its increased use. This emergence may have been associated to the impact of PCV7 use

on colonization, so that the replacement of serotypes from vaccine types by non-vaccine types occurred. Previous studies showed that serotype 1 had a strong association with age, especially carried by older children and adults, however, according to the present study, it was solely found in adults. Contrarily to serotype 19F, serotype 1 was found to have enhanced tendency to cause invasive disease [67].

In the present study, non-typeable pneumococci represent the largest percentage of strains and the increasing trend will keep on, while pneumococcal vaccines available were just targeted against the polysaccharide capsule (Figure 3.2). The widespread vaccination will lead to the increased prevalence of unencapsulated pneumococci, as the conjugate vaccines do not have any impact on non-typeable strains [68].

As it was not possible to know if all samples analyzed were recovered from patients that had been vaccinated in the past, there is no way to recognize the real coverage of the vaccines in this study, as well as whether they were infected even being vaccinated. However, it is supposed that children until 9 years old were, necessarily, vaccinated with PCV13, as it was already in de NIP, therefore, 6 children from the study population of 27 should have taken the vaccine against *S. pneumoniae* infections. Precisely, the 6 clinical isolates recovered from the children are non-typeable, thus, they do not express any serotype covered by PCV13.

Bearing in mind the serotypes covered by PCV13 and supposing that all the participants have taken the vaccine, the coverage of PCV13, based in the obtained results, should be 22.2%, because just 3 identified serotypes belong to PCV13 serotypes coverage (1, 19F, and 6A/B).

3.1.3 Typing of *Haemophilus influenzae*

The majority of *H. influenzae* strains are unencapsulated – non-typeable – however there also exist strains with a polysaccharide capsule. Capsulate *H. influenzae* isolates can express one of six capsular serotypes from a to f, although they are less frequent [42].

Non-typeable *H. influenzae* is most frequently connected with mild inflammatory diseases of the mucosa, such as otitis media, nevertheless it can also be responsible for invasive diseases [69].

There is a region of the chromosome, called cap, which is divided in three regions: regions 1 and 3 are common to all serotypes and region 2 specifically expresses the capsule type [70].

In this study, it was possible to identify 100% of non-typeable strains, in a total of 48 clinical isolates tested (Figure 3.3).

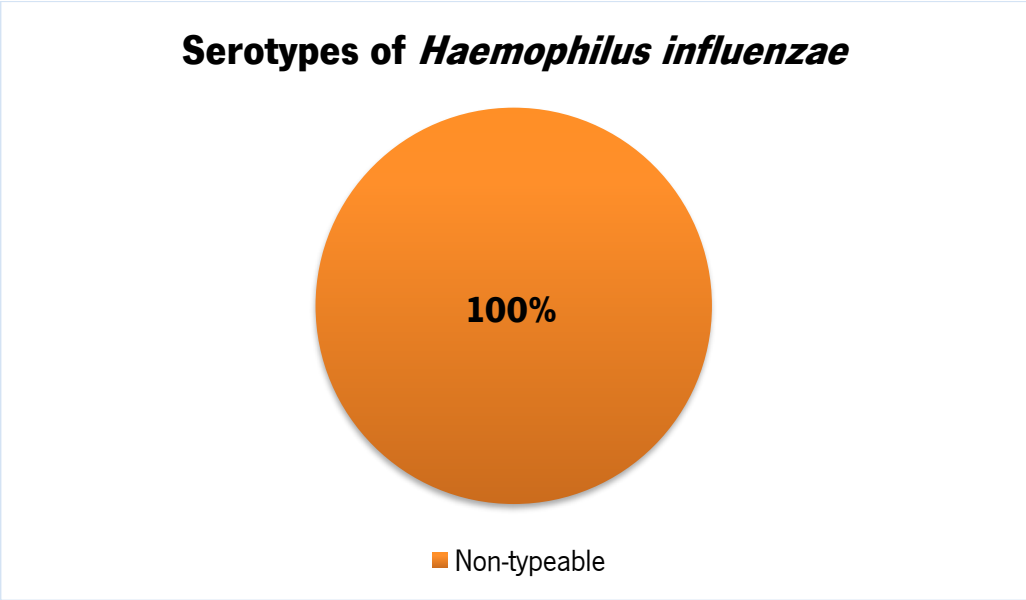


Figure 3.3 - Distribution of *Haemophilus influenzae* serotypes in clinical samples obtained from Hospital de Braga.

In spite of the high frequency of unencapsulated strains noticed nowadays, in the 1990s and before the implementation of *H. influenzae* type b (Hib) vaccination, more than 95% of disease was caused by serotype b and constituted the major cause of invasive disease, especially in children [43].

H. influenzae type b vaccine is the only one available against *H. influenzae* infections and entered in the NIP, in Portugal, in 2000, however its coverage was already high since 1996, although it was just administered under medical prescription [33].

The introduction of this vaccine had an effective impact on the decrease of *H. influenzae* type b infections, therefore the awareness towards the other capsular types (a, c, d, e and f) and non-typeable strains had increased. Hib vaccine has strong immunogenic properties responsible for its success on the decrease of serotype b infections, though this protection is limited to serotype b. It was suggested that the reduction on carriage provoked by type b vaccine could open an ecologic niche, permitting increased colonization by non-type b *H. influenzae* strains in which Hib vaccine do not offer any protection [71].

Generally, different countries around the world have faced a huge increase on the percentage of non-typeable strains, for example, in the Netherlands, the number of invasive non-typeable strains cases increased from 20, in 1992 to 115 in 2013. In Portugal, from 2002 to 2010, nearly 77% of the total serotyped *H. influenzae* isolates (n=144) were identified as non-typeable and just 13% as serotype b [72].

Table 3.3 - Prevalence of *Haemophilus influenzae* non-typeable isolates

	Non-typeable
Age range (years)	
0 – 9	3
10 – 24	1
25 – 64	15
≥ 65	29

According to previous studies, the highest incidence of Non-*H. influenzae* serotype b was observed especially in the most vulnerable people at the age spectrum extremes: in children <1 year and adults ≥ 65 years old. However, it is said that nonencapsulated strain infections are more common in adults and associated with cases of pneumonia, whereas infections caused by encapsulated strains tend to affect young children, mainly causing meningitis and bacteremia [70].

By the observation of the results obtained represented on Table 3.3, adults with more than 65 years old, followed by adults with ages ranged between 25 and 64 years old were the most afflicted by non-typeable strains infections, being in agreement with literature data. The 29 cases of infection noticed on people more than 65 years old can be explained by the higher vulnerability, possibly undergoing on other medical illnesses, weakening their immune system. These conditions reunited make these people an easier target to be colonized and initiate disease.

Besides the vaccine-mediated strains replacement, there are other possible explanations to the emergence of non-typeable *H. influenzae* strains. One of them can be the increase on the non-typeable strains virulence, as a result of the acquisition of different virulence factors. This acquisition is facilitated by the ability of these strains to frequently exchange pieces of DNA between them [69].

Another explanation could be the changing on the *H. influenzae* epidemiology. At first, mostly of the invasive diseases were caused by serotype b, and over the past 20 years, non-type b serotypes and non-typeable ones started contributing to the statistics. People with some risk factors, such as: coronary artery disease, smoking, congestive heart failure are more prone to invasive diseases than the general healthy population - for example, the number of patients with COPD, which is the third leading cause of death worldwide, is increasing and non-typeable strains choose to colonize their lungs, therefore they might contribute to the augmented incidence of non-typeable *H. influenzae* cases [69].

Moreover, it is known that serum IgG levels to *H. influenzae* protein D decline with age, mainly in adults with coexisting conditions as COPD compared with healthy ones. Protein D is a highly conserved

antigen and the absence of natural antibodies against it, may contribute to increased susceptibility to invasive non-typeable disease [69].

Finally, the binding of IgM to the bacteria surface plays an important role in the innate protection against non-typeable strains. It was found that patients with hyper-IgM syndrome were less prone to colonization by non-typeable strains, fact that emphasizes the importance of IgM in the defense against *H. influenzae* [69, 73].

Taking into consideration the changing epidemiology of invasive *H. influenzae*, the development of vaccines against non-type b and non-typeable *H. influenzae* strains is now essential, in order to reduce the prevalence of infections caused by these bacteria, as they are often responsible for causing pneumonia in patients with COPD and OM in children. Monitorization is also needed for *H. influenzae* serotype b strains to guarantee the maintenance of its low prevalence [69].

3.2 Antimicrobial susceptibility testing

Antibiotics are powerful medicines used to kill or inhibit the growth of susceptible bacteria that cause diseases, however they can change or mutate, giving them the ability to resist antibiotics – antibiotic resistance - possibly leading to treatment failures. Antibiotic resistance can be inherent - characteristic of all isolates of specie or acquired [74].

Antibiotic consumption in Portugal remains higher than the European average [75]. Finding strategies to fight against the spread of antibiotic resistance is now, a priority and a major global challenge for public health associated with incorrect prescription and overuse of antibiotics. The increasing tendency of resistant microorganisms is a consequence of selective pressure that arises when antimicrobials are used [76].

3.2.1 *Moraxella catarrhalis* clinical isolates

Antimicrobial susceptibility tests to *M. catarrhalis* clinical isolates were performed in Hospital de Braga under the guidelines of European Committee on Antimicrobial Susceptibility Testing (EUCAST).

Unfortunately, *M. catarrhalis* strains have been developing an increasing resistance to β-lactam antibiotics, as a result of their excessive use, especially in children, contributing to the increase in the number of microorganisms with acquired resistance to this type of antibiotics. The majority of strains are also capable of producing the enzyme β-lactamase, which is considered the main virulence factor of

these microorganisms. This enzyme, through local hydrolysis, will break the β-lactam ring, common to all β-lactam antibiotics, neutralizing their antibacterial properties and facilitating treatment failure [77].

Since 1977, there were found two specific types of β-lactamase, designated BRO-1 and BRO-2, that encode bro-1 and bro-2 genes, respectively and previous studies have reported that more than 95% of global clinical *M. catarrhalis* isolates produce these types of enzymes [78].

Bearing this way, Ampicillin showed nearly 100% of resistant strains, as it was expected, due to the production of β-lactamase. Whereas, clinical isolates also presented high rates of susceptibility to Trimethropim-sulfamethoxazole, Moxifloxacin, Cefotaxime, Amoxicillin-clavulanic acid and Levofloxacin, data that is consistent with international research, because they are non-β-lactam antibiotics and Amoxicillin-clavulanic acid results of a combination of a penicillin (Amoxicillin) and a β-lactamase inhibitor (Clavulanic acid), a strategy used to fight against β-lactamase-producing bacteria (Table 3.4) [77, 79, 80].

In a general view, just the resistance of *M. catarrhalis* to Ampicillin remains a problem presenting a very tiny percentage of susceptibility comparing with the other antibiotics. Regarding the serotypes found, 12 out of 15 clinical isolates are just resistant to Ampicillin and present serotype A.

Table 3.4 - Antibiogram profile of *Moraxella catarrhalis* isolates (n=25) (%)

Antibiotic	Sensitive	Intermediate	Resistant
Ampicillin	4	-	96
Trimethropim-sulfamethoxazole	84	-	16
Moxifloxacin	96	-	4
Cefotaxime	100	-	-
Amoxicillin-clavulanic acid	100	-	-
Levofloxacin	100	-	-

3.2.2 *Streptococcus pneumoniae* clinical isolates

S. pneumoniae is naturally sensitive to the majority of the active antibiotics against Gram-positive bacteria, thus its antibiotic resistance acquisition is easier and constitutes a serious issue [81].

In general, more than 70% of *S. pneumoniae* strains were sensitive to all the antibiotics tested, existing 100% of effectiveness regarding seven antibiotics, as it is shown in the Table 3.5. The lowest

rates of resistance among the studied strains were 3.7% to Moxifloxacin and Levofloxacin and 7.4% to Trimethropim-sulfamethoxazole, on the other hand, resistance to Erythromicin was found in 25.9% of pneumococcal strains. There was also verified intermediate-resistance to Penicillin, Clyndamicin, Erythromicin and Trimethropim-sulfamethoxazole with 18.5%, 11.1%, 3.7% and 3.7% of strains, respectively (Table 3.5).

Beta-lactams such as Penicillin are commonly used for the treatment of *S. pneumoniae* infections, however, the prevalence of Penicillin-Nonsusceptible *S. pneumoniae* (PNSP) varies between countries (temporally and geographically) fact that can be explained by different rates of antibiotics consumption. In Portugal, in 2009 and 2010, PNSP (I+R) rate was 23.5% and 19.6% respectively and eight/nine years later, my study reveals a PNSP rate of 18.5%. These percentages show a positive downward trend of the PNSP rate from 2009 to 2018 in Portugal, on the other hand, this rate presents much higher in other European countries: 53.5% in Spain and 50% in France, data collected in the year of 2013 [63, 82].

Multidrug-resistance was also present in 11.1% of pneumococcal strains and it is defined as being non-susceptible to three or more antibiotic classes has been reported for some authors that a significant proportion of AOM cases are caused by multidrug resistant pneumococcal strains. Nevertheless, it is not known if the patients sample used in this study had received oral antibiotics before contracting OM, therefore it is not possible to determine whether the bacteria became multidrug resistant after successive antibiotic therapies or if the bacteria were already resistant to many classes of antibiotics before infecting the patient [83].

The higher percentage of susceptible strains to some of the antibiotics tested, for example Ceftriaxone may be explained by the fact that it is not widely used as it is just recommended in cases of severe AOM (severe otalgia or fever up to 39°C) [83].

The totality of *S. pneumoniae* isolates were susceptible to Cefotaxime which means that it is a safe choice for treatment of invasive infections in Portugal, in contrast to the Spanish situation, where it has been reported a resistance of 13% to this antibiotic [84, 85].

In this study, serotypes associated with resistant pneumococcal strains are predominantly, non-typeable ones, followed by serotypes 15B/C, 19F, 12F/12A/12B, 6A/B and 1, these ones with low prevalence. Despite serotypes 19F and 1 being included in PCV7 and PCV10/13/23, respectively, the other three serotypes associated with resistance patterns do not belong to any of the current conjugate vaccine formulations (15B/C, 12F/12A/12B and 6A/B). Although serotype 1 is mentioned as being

related to resistant isolates, 2 out of 3 of them remain susceptible to all tested antimicrobials, data that is according to information previously described in Portugal and elsewhere [57].

Table 3.5 - Antibiogram profile of *Streptococcus pneumoniae* isolates (n=27) (%)

Antibiotic	Sensitive	Intermediate	Resistant
Cefotaxime	100	-	-
Chloramphenicol	100	-	-
Imipenem	100	-	-
Linezolid	100	-	-
Moxifloxacin	96.3	-	3.7
Telithromicin	100	-	-
Vancomycin	100	-	-
Ceftriaxone	100	-	-
Levofloxacin	96.3	-	3.7
Penicillin	81.5	18.5	-
Clyndamicin	88.9	11.1	-
Erythromicin	70.4	3.7	25.9
Trimethropim-sulfamethoxazole	88.9	3.7	7.4
Tetracycline	88.9	-	11.1

3.2.3 *Haemophilus influenzae* clinical isolates

In general, Ampicillin showed the highest percentage of resistant *H. influenzae* strains (29.2%), followed by Amoxicillin-clavulanic acid (12.5%) and finally Trimethropim-sulfamethoxazole (8.3%). Erythromycin and Trimethropim-sulfamethoxazole revealed 97.9% and 4.2% of intermediate-resistant *H. influenzae* strains, respectively. Cefotaxime, Chloramphenicol, Levofloxacin, Moxifloxacin, Tetracycline and Rifampicin exhibit 100% of effectiveness against *H. influenzae* isolates (Table 3.6).

Throughout many years, infections caused by *H. influenzae* were effectively treated with Ampicillin and these strains started developing resistance to this antibiotic as well as to other β-lactams. However, frequency of *H. influenzae* infections and their resistance profiles vary from country to country. Indeed, in this study, Ampicillin displayed the highest percentage of resistant strains, probably because of its recurrent use as first line antibiotic of choice [86].

There are two major mechanisms that have been described to understand the development of resistance: production of β-lactamases and changes in the Penicillin-Binding Protein (PBP) [87].

The most common mechanism of resistance in *H. influenzae* is the production of β-lactamases which will hydrolyse the β-lactam ring of the antibiotic, becoming inactive and consequently, neutralizing target antibiotics [87, 88].

Regarding the changes in PBP, there are five known ones in *H. influenzae* – 1A, 1B, 2, 3 and 4 – and alterations in PBP3 were attributed to the increase of resistance against β-lactam antibiotics. Various mutations in PBP3 were discovered, some of which result in a reduction of affinity for Penicillins [87, 88].

The prevalence of strains carrying altered PBPs vary between countries, however it was noticed their raise in many European countries and throughout the world [87]. There are *H. influenzae* strains that can, simultaneously, undergo on alterations in PBP and produce β-lactamases [87, 88].

Table 3.6 - Antibiogram profile of *Haemophilus influenzae* isolates (n=48) (%)

Antibiotic	Sensitive	Intermediate	Resistant
Ampicillin	70.8	-	29.2
Amoxicillin-clavulanic acid	87.5	-	12.5
Cefotaxime	100	-	-
Chloramphenicol	100	-	-
Erythromycin	2.1	97.9	-
Levofloxacin	100	-	-
Moxifloxacin	100	-	-
Tetracycline	100	-	-
Trimethropim-sulfamethoxazole	87.5	4.2	8.3
Rifampicin	100	-	-

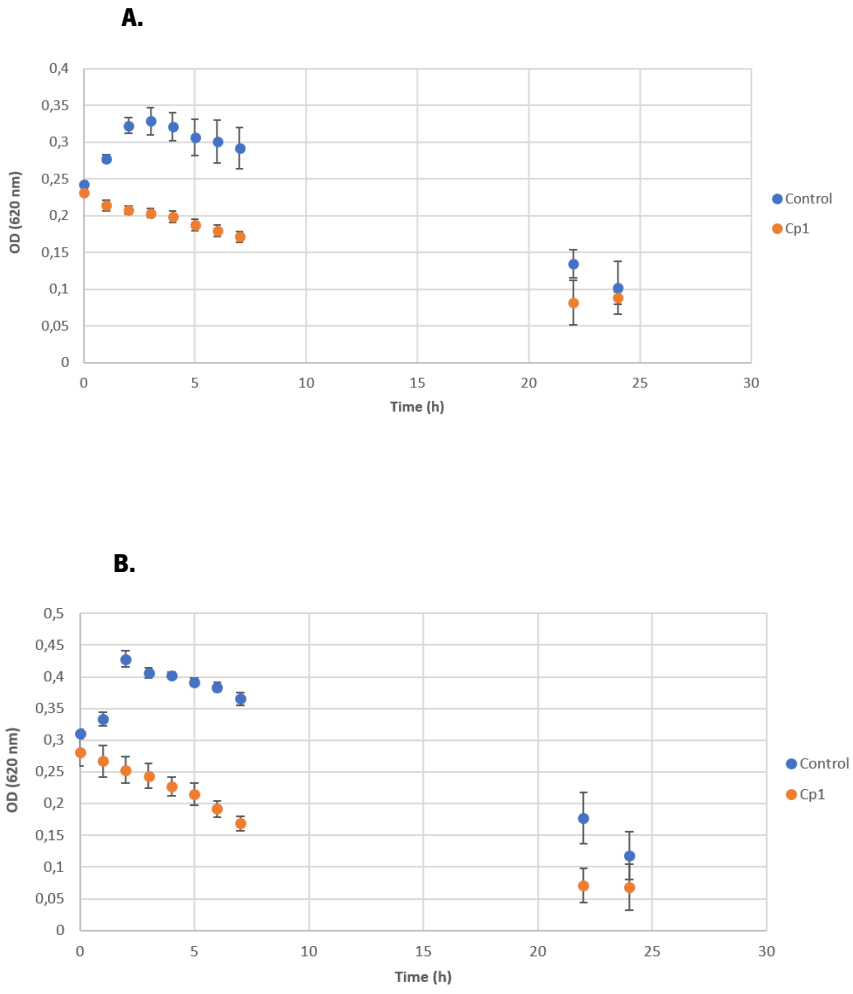
3.3 Bacteriophages

3.3.1 Assay with planktonic bacteria

The majority of antimicrobial and immunological tests are generally developed using planktonic cells [89].

Free-living bacteria (planktonic) have less capability of resisting to adverse environmental conditions in comparison with biofilms. The level of antimicrobials required to produce antibacterial effect on biofilms is nearly 1000 greater than the one needed for planktonic bacteria. According to the information stated before, planktonic bacteria have lower chances of subsistence when compared to biofilms that provide a more secure way for the reproduction and survival of bacteria [90].

Figure 3.4 shows the OD_{620} of planktonic *S. pneumoniae* culture (R6st) and the action of 3 bacteriophages: Cp-1, Dp-1 and MS1 as a function of time.



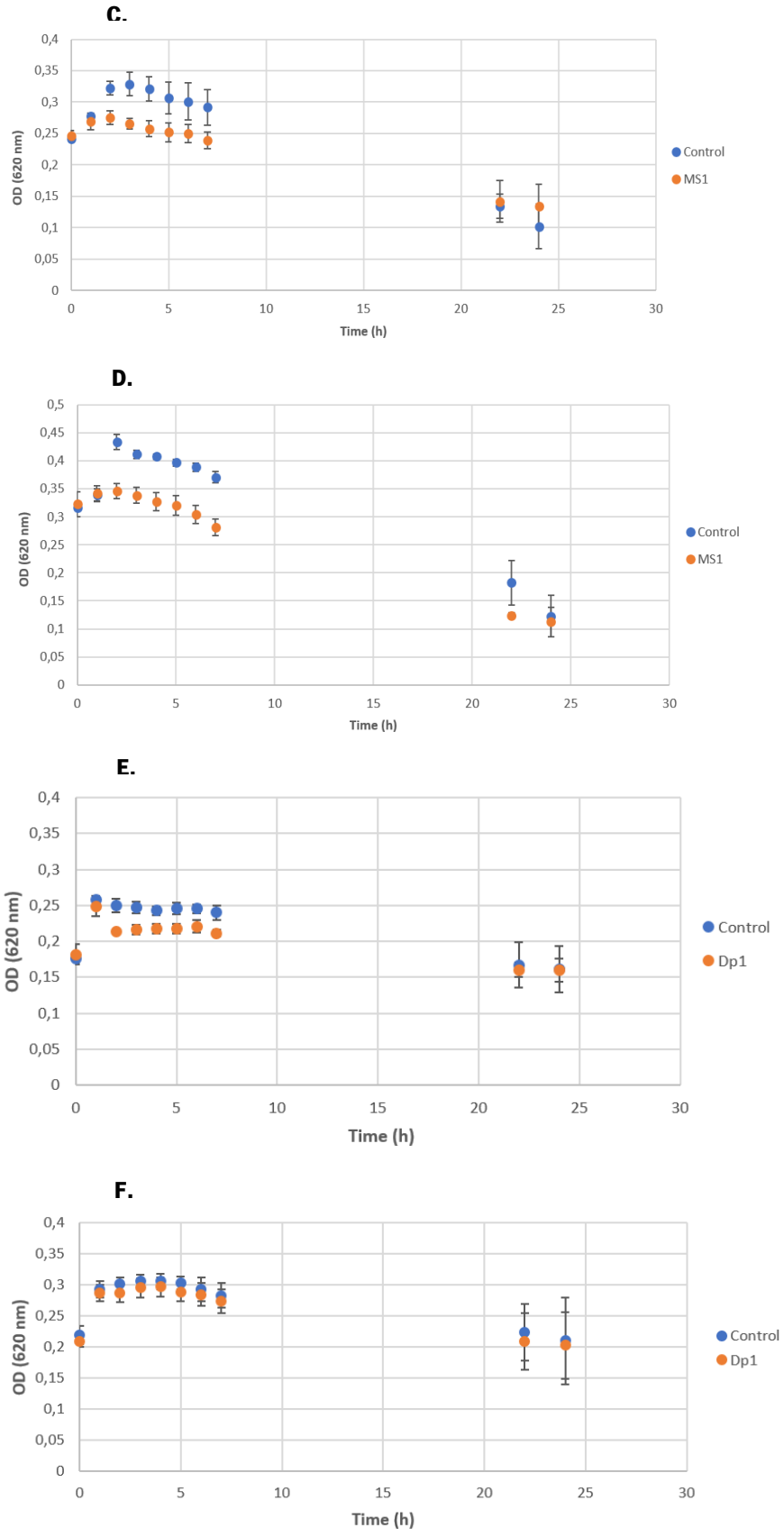


Figure 3.4 - Action of the phages on planktonic bacteria throughout the time. **A** – Action of phage Cp-1 on planktonic bacteria throughout the time (1st assay); **B** - Action of phage Cp-1 on planktonic bacteria throughout the time (2nd assay); **C** - Action of phage MS1 on planktonic bacteria throughout the time (1st assay); **D** - Action of phage MS1 on planktonic bacteria throughout the time (2nd assay); **E** - Action of phage Dp-1 on planktonic bacteria throughout the time (1st assay); **F** - Action of phage Dp-1 on planktonic bacteria throughout the time (2nd assay).

Cp-1, Dp-1 and MS1 are all virulent or lytic bacteriophages. Taking this to account, it was expected a decrease of the optical density throughout the time, which indicates the bacteriophages were causing the bacteria cell lysis and were acting against the bacteria growth.

By the observation of Figures 3.4A. and 3.4B. (1st assay and 2nd assay, respectively), it is possible to notice a gradual reduction of OD from 0.23 to 0.088 (graph A) and from 0.28 to 0.066 (graph B), which is in agreement with what was expected, therefore, bacteriophage Cp-1 acted efficiently towards planktonic bacteria.

Whereas, in Figures 3.4C. and 3.4D. regarding the behavior of bacteriophage MS1, the decrease of the OD_{620} was just observed two hours after through agitation, as in the first two hours a slight increase of the OD_{620} was verified. This means that bacteriophage MS1 action takes more time to be effective and its effect is not as immediate as in the situation of Cp-1.

Finally, analyzing the case of bacteriophage Dp-1, in Figure 3.4E. the reduction of OD starts seven hours later, and it is just verified a reduction of 0.051 on the OD between 7 hours and 24 hours later. In Figure 3.4F., the situation is similar, however the decrease of OD starts 5 hours later with a reduction of 0.086 until 24 hours later. The action of bacteriophage Dp-1 is the most ineffective concerning the insignificant reduction of OD and its behavior throughout time.

Phage Cp-1 shows to be the best choice for therapeutic purposes acting against planktonic bacteria and contributing to the immediate bacteria cell lysis.

3.3.2 Lytic spectra

It is possible to notice by the observation of Table 3.7 that, none of the clinical isolates of *S. pneumoniae* was infected by any of the bacteriophages. Some bacteriophages are very specific, whilst others have a broad host range.

In this specific case, these three bacteriophages are highly specific. This specificity is related to the fact that a bacteriophage can solely infect bacteria which have receptors to which bacteriophages can bind [91] .

Basically, to enter a host cell, bacteriophages attach to specific receptors present on the bacteria surface, including, for example, lipopolysaccharides [92].

The host of bacteriophages Cp-1, Dp-1 and MS1 is *S. pneumoniae* R6 strain (R6st) which is avirulent and unencapsulated, deriving from R36A which also derives itself from D39 and presents serotype 2 [93].

Table 3.7 - Lytic spectra of Cp-1, Dp-1 and MS1 bacteriophages against 27 *S. pneumoniae* clinical isolates and their host

	Bacteriophages		
Clinical isolate	**Cp-1**	**Dp-1**	**MS1**
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	-	-
7	-	-	-
8	-	-	-
9	-	-	-
10	-	-	-
11	-	-	-
12	-	-	-
13	-	-	-
14	-	-	-
15	-	-	-
16	-	-	-
17	-	-	-
18	-	-	-
19	-	-	-
20	-	-	-
21	-	-	-
22	-	-	-
23	-	-	-
24	-	-	-
25	-	-	-
26	-	-	-
27	-	-	-
R6st (Host)	+	+	+

* - Absence of bacteriophage plaques

4. Conclusion and Future Perspectives

Otitis media is an inflammatory disease of the middle ear and is the most common reason why children receive antibiotics in USA, which comprises 3 main types: Acute Otitis media, Otitis media with Effusion and Chronic Otitis media. This disease affects children younger than 2 years of age in 90% of the cases, worldwide. The propensity of young children to acquire Otitis media can be explained by the structure of their Eustachian tubes, that are still short, horizontal and function poorly. The most predominant bacteria responsible for Otitis media are: *Moraxella catarrhalis*, *Streptococcus pneumoniae* and *Haemophilus influenzae*, however a co-infection caused by both bacteria and viruses is common in 28% to 70% of the Otitis media cases.

Normally, Acute Otitis media and Otitis media with Effusion mild cases do not need antibiotic treatment, however analgesics are recommended to reduce acute pain, fever and irritability. When it is necessary, Amoxicillin is the elected antibiotic as first-line therapy. In case of surgical intervention, Grommet insertion is the most performed surgery to treat Otitis media with Effusion and Myringotomy to treat Recurrent Acute Otitis media.

In the last years, bacteriophage therapy has emerged in prejudice of using antimicrobials, constituting an efficient alternative to fight against antimicrobial resistance and treat middle ear infections. Bacteriophages (virus that specifically infect bacteria) can have two different lifecycles: lytic cycles and lysogenic cycles, being the lytic bacteriophages the best choice to therapeutic applications.

Different clinical isolates of *M. catarrhalis*, *S. pneumoniae* and *H. influenzae* undergo Multiplex PCR typing. *M. catarrhalis* isolates were classified as serotype A (56%) and the remaining as non-identified (44%). Serotype A was the most prevalent in people with more than 65 years old, as well as non-identified serotypes.

Regarding *S. pneumoniae* clinical isolates, 6 pneumococcal serotypes were identified: serotype 1 (11%), serotype 19F (7%) and serotypes 15B/C, 12F/12A/12B, 6A/B and 9N/L with 4% each. More than a half of pneumococcal isolates tested were classified as non-typeable (66%). The major number of non-typeable strains was verified in children between 0 and 9 years old and serotypes 1 and 19F were the most incident in people older than 65 years old. The introduction of PCV7 and PCV13 had impact on the reduction of vaccine serotypes and led to serotype replacement.

In what *H. influenzae* clinical isolates are concerned, 100% of them were non-typeable, showing, again, the highest incidence in people with more than 65 years old. The introduction of *H. influenzae* type

b vaccine had impact on the decrease of type b infections, however it does not offer protection against neither other capsular types, nor non-typeable strains.

The level of antibiotic-resistant strains is powerfully associated with the level of antibiotic consumption. Regarding *M. catarrhalis* clinical isolates antimicrobial susceptibility, Ampicillin showed nearly 100% of resistant strains due to the production of β-lactamase. Whereas, clinical isolates also presented high rates of susceptibility to Trimethropim-sulfamethoxazole (84%), Moxifloxacin (96%), Cefotaxime (100%), Amoxicillin-clavulanic acid (100%) and Levofloxacin (100%).

In *S. pneumoniae* clinical isolates, more than 70% of them were sensitive to all the antibiotics tested, existing 100% of effectiveness regarding seven antibiotics. The lowest rates of resistance among the studied strains were 3.7% to Moxifloxacin and Levofloxacin and 7.4% to Trimethropim-sulfamethoxazole. Ampicillin showed the highest percentage of resistant *H. influenzae* strains (29.2%), followed by Amoxicillin-clavulanic acid (12.5%) and finally Trimethropim-sulfamethoxazole (8.3%). Cefotaxime, Chloramphenicol, Levofloxacin, Moxifloxacin, Tetracycline and Rifampicin exhibit 100% of effectiveness against *H. influenzae* isolates.

Pneumococcal bacteriophages: Cp-1, Dp-1 and MS1 were tested against planktonic bacteria and bacteriophage Dp1 was considered the most ineffective. Bacteriophage Cp1 shows to be the best choice acting against planktonic bacteria - R6st (host strain) - and contributing to its immediate cell lysis.

None of the clinical isolates of *S. pneumoniae* was infected by any of the bacteriophages, meaning a high specificity of these bacteriophages and its narrow host range. Therefore, if a bacterial cell does not expose a specific receptor for that specific bacteriophage, it will not be infected.

In conclusion, this work shows that is necessary to discover new ways of fighting against the increase of non-typeable strains to have more impact on the decrease of Otitis media cases. Some of the studied pneumococcal bacteriophages may have potential to treat middle ear infections, however there is still a need to isolate new lytic bacteriophages with broad host ranges, in order to enlarge their lytic spectra and the possibility of a successful treatment.

In future studies and with the aim of optimizing the work that have been developed, there is still some work that can be done and improved:

- New ways of serotyping need to be developed in order to better characterize non-typeable strains;
- Vaccines need to be created against *M. catarrhalis* strains;
- Assays targeting biofilms can be performed to evaluate the action of pneumococcal bacteriophages;

- Combination of bacteriophages in cocktail can be used to assess the result against OM pathogens;
- More attempts should be done to isolate new lytic pneumococcus bacteriophages with broader host ranges.

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