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Pneumococcal gene expression during its interaction with human basal epithelial cells

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Pneumococcal gene expression during its interaction with human basal epithelial cells

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Preface

The following report is the result of my final research thesis in the master graduation of the Biology Engineering course from University of Algarve in Portugal. This research project took place in the University of Leicester in England during 7 months, in the Department of Infection, Immunity and Inflammation of The School of Medicine.

It was a big challenge for me, not only because I was going to be in other country but also because the genetics and molecular work were always subjects that I had some difficulties to understand, thus the need to improve and learn more. Accepting this challenge gave me knowledge to improve, and the practical work helped me a lot to understand and answer all the questions that I had before. In this research project the *S. pneumoniae* gene expression was studied when these bacteria adhere to nasal epithelial cells. It was an important research, hoping to contribute in some way to the medical field, in specific subjects related to the adherence of this pathogen to human nasal cells.

First of all, I would like to express my gratitude to my parents that always supported me and without them this wouldn't be possible.

I'm very grateful to Professor Peter Andrew that accepted me in his laboratory and gave me the challenge of this research, participating in every step, being always helpful and demonstrating a large interest in this project.

I would like to thank my teacher, Leonor Faleiro, my Portuguese supervisor that helped me a lot in this big adventure, and was always there when I needed.

My supervisor, Claire Smith, was also very important. I would like to thank all the patience, support and knowledge that she gave me during the 7 months that I was in Leicester.

To all the people from Peter's laboratory, a big thanks. They helped me a lot during the first months and showed me all I needed to know about the laboratory, including Sarah Glenn, the lab technician that since the beginning she gave me an enormous support.

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Abstract

The aim of this research was to analyze pneumococcal gene expression during interactions with human nasal epithelial cells and comparison with other different infection models. As the number one cause of bacterial pneumonia and one of the major causes of mortality and morbidity, *S. pneumoniae* has been a target of several studies in past years. Oggioni and colleagues (2006) studied pneumococcal gene expression during infection in two different scenarios, bacteria in the blood and bacteria in the tissue (as brain and lung). In this current study the target genes analyzed were *nanA*, *ply*, *comX*, *nox*, *sodA,* since these were reported as up regulated in the lung (Oggioni *et al.,* 2006), except for *ply*, that was chosen because of its importance in pneumococcal infection.

It has been reported that induction of competence system by the quorum sensing peptide stimulates the bacteria to grow in biofilm and also increases *S. pneumoniae* virulence. Based on these results, we attempted to induce biofilm formation prior to infection with the aim to increase pneumococci virulence before adherence to nasal epithelial cells. However, unlike Oggioni *et al*., (2006), our method was unsuccessful in producing biofilm and therefore it was impossible to proceed. A further idea was to analyze pneumococcal gene expression in apical fluid that is released and present on the surface of human epithelial cells. It was thought that this could elucidate mechanisms of how pneumococci overcome host defense. However, antibiotics that were used for cell culture were detected in the apical fluid and therefore, it was not possible to continue with this experiment since all the bacteria died within 30 minutes of exposure. For these reasons the main aim of this study was to explore the mechanisms of pneumococcal adherence to basal cells. Gene expression analysis was performed using three reference conditions, *S. pneumoniae* in BEBM, in TSB and the non adhered pneumococci. BEBM proved to be the best reference condition and using this we have shown that all the genes we targeted were up regulated within 2h of exposure to basal cells, for adhered and non adhered bacteria, except *comX* and *sodA*. *NanA* (neuraminidase gene) showed the highest increase in expression levels compared to the other genes, 25,47±2,21 for the adhered pneumococcus to patient sp282 basal cells and 23,10±0,47 for the non adhered bacteria to patient 455 basal cells. After 6h, all genes were up regulated except *ply*.

Key words: *S. pneumoniae*; virulence genes; human nasal epithelial cells; adherence

Resumo

Este estudo teve como principal objectivo, avaliar os genes expressos por *Streptococcus pneumoniae* durante a aderência a células epiteliais nasais, analisando e comparando também a expressão do pneumococcus em diferentes modelos de infecção. Para tal, grande parte desta investigação foi baseada numa publicação de Oggioni *et al.,* (2006), onde se investigou a expressão de um grupo de genes (26) por *S. pneumoniae* durante a infecção em ratinhos, em dois padrões diferentes, sangue e tecido (cérebro e pulmão).

Os genes analisados ao longo deste trabalho foram *gyrb* (housekeeping gene), *comX*, *nanA* (neuraminidases), *sodA*, *nox*, *ply.* A escolha destes genes foi baseada no estudo de Oggioni *et al*., (2006), onde a expressão destes genes foi muito elevada no pulmão, com excepção de *ply*, a sua inclusão, neste estudo, foi baseada no conhecimento do seu papel na aderência de *S. pneumoniae* (Rubins *et al*., 1998; Hirst *et* al., 2004).

No estudo de Oggioni *et al*., (2006) foi ainda referido que a indução de competência por parte do péptido quorum-sensing (CSP) não só estimula a bactéria a crescer em biofilme como também aumenta a sua virulência. Com base nestes resultados, optou-se então, por crescer a bactéria *S. pneumoniae*, estirpe D39, em biofilme, no entanto sem sucesso. Apesar de se seguir rigorosamente o protocolo como na publicação de Oggioni *et al*., (2006), não se obtiveram quaisquer resultados no final da experiência. De seguida optou-se por analisar os genes expressos por esta bactéria no fluido apical existente à superfície das células epiteliais. Este procedimento foi realizado de modo a ser possível a comparação da expressão dos genes no fluido apical com a expressão dos genes no processo de aderência da bactéria às células epiteliais. No entanto, devido a presença de antibióticos neste fluido, teve de se interromper esta actividade experimental, visto os antibióticos influenciarem a expressão da bactéria.

Por fim, procedeu-se à aderência de *S. pneumoniae* a células epiteliais nasais. As células utilizadas para este ensaio foram células basais. Os genes expressos foram analisados em três condições de referência diferentes: i) a bactéria em meio TSB, ii) a bactéria em meio BEBM (meio próprio para cultura de células basais) e por último na situação de ausência de aderência da bactéria às células basais. O meio TSB foi utilizado no estudo de Oggioni *et al*., (2006), como condição de referência, pelo que foi igualmente adoptado no presente estudo. Todos os genes apresentaram uma expressão elevada, excepto *sodA* que apresentou um nível de expressão baixo. Nas células basais do

paciente sp430, após 6h de exposição, a bactéria apresentou todos os genes com uma expressão elevada.

O meio que melhor representou uma boa condição de referência foi o BEBM. Visto ser o meio utilizado no ensaio da aderência às células basais, este meio apresenta todos os factores necessários para um bom controlo. Na aderência às células do paciente sp282 e no ensaio da bactéria que não aderiu às células do paciente 455 todos os genes apresentaram uma expressão elevada, excepto *comX* e *sodA*, destacando-se, no entanto o gene responsável pelas neuraminidases, *nanA*, com o nível mais alto de expressão, 25,47±2,21 na aderência às células basais do paciente sp282 e de 23,10±0,47 na bactéria que não aderiu as células basais do paciente 455.

Palavras-chave: *Streptococcus pneumoniae*; genes de virulência; células epiteliais nasais humanas; aderência

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Abbreviations

µg – microgram

µl- microliter

µm – micrometer

ALI – air liquid interface

ATP- Adenosine triphosphate

BAB – Blood Agar Base

BAB – blood agar base

BEBM – bronchial epithelial basal medium

BEGM – bronchial epithelial growth medium

BHI - Brain Heart Infusion

cDNA – complementary DNA

CFU – Colonies formed unities

CO_2 – carbon dioxide

CSP- competence stimulating peptide

DNA – Deoxyribonucleic acid

dNTPs – deoxyribonucleotides

EDTA - ethylenediaminetetraacetic acid

ELISA - Enzyme Linked Immuno Sorbent Assay

FCS – Fetal Calf Serum

g – centrifugal force

h – hours

H_2O_2 –hydrogen peroxide

IU – international units

KDa – kiloDalton

min – minutes

mRNA – messenger RNA

NADH - Nicotinamide adenine dinucleotide

ng – nanogram

nH_2O – nanopure water

nm-nanometer

OD – optical density

PCR – polymerase chain reaction

qPCR – quantitative real time PCR

RNA – Ribonucleic acid

rpm – rotations per minute

RT-PCR – reverse transcriptase PCR

sec - seconds

SOD-Super oxide dismutase

TAE buffer - Tris-acetate-EDTA buffer

TEER - transepithelial electrical resistance

TSB – Tryptone Soya Broth

V – volts

v/v – volume/volume

w/v–Weight/volume

1. Introduction

Streptococcus pneumoniae (the pneumococcus) is a major human pathogen and currently the leading cause of community-acquired pneumonia, meningitis, and bloodstream infections in the elderly, the young, and patients with immunosuppressive illness and chronic diseases; it is also the main causative agent of middle-ear infections in children (Moscoso *et al*., 2006).

The pneumococcus is considered one of the principal causes of otitis media, and responsible for several other diseases including arthritis, osteomyelitis, endocarditis, endophthalmitis, abscesses, necrotizing fasciitis, and sinusitis (Oggioni *et al*., 2006). It is a facultative anaerobic oval or spherical coccus that usually grows in short chains or in pairs (as shown in figure 1).

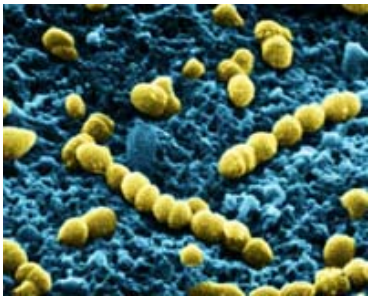

Fig. 1 *Streptococcus pneumoniae (http://www.terradaily.com/reports/Research_Could_Put_Penicillin_Back_In_Battle_Against_Antibiotic_Resistant_Bugs_That_Kill_Millions_999.html).*

The pneumococcus has a polysaccharide capsule that is known to protect against phagocytosis. This enables the bacteria to survive and helps proliferation within tissues (Tonnaer *et al*., 2006). The bacterial cell wall is composed by several proteins and polysaccharides and contains large amounts of teichoic acid, and a lipid bilayer with a large number of proteins. The composition and volume of the pneumococcal capsular polysaccharide varies significantly. Based on the variation of polysaccharides composition, more than 90 pneumococcal serotypes have been identified. Through transformation, it is also possible to convert an unencapsulated phenotype into a capsulated strain.

1.1. The genus *Streptococcus*

The Gram-positive genus *Streptococcus* is responsible for a larger diversity of diseases than any other group. Such diseases include pharyngitis, scarlet fever and pnemococcal pneumonia.

Streptococci do not utilize oxygen but many members of this group are considered aerotolerant and few are obligatory anaerobic. (Prescott, 1996; Tortora, 1995).
Some species of *Streptococcus* have the capacity to produce lactic acid and because of this have practical importance to humans. They are of extreme importance in areas such as the production of buttermilk, silage and other kind of fermented products.

The *Streptococcus* genus is divided in two subgroups, Pyogenes and Viridians, according to the type of haemolysis they cause on blood agar. There are two types of haemolysis, α-haemolysis and β-haemolysis. The organisms responsible for the β-haemolysis produce streptolysin and are surrounded by a large clear region or a complete red blood cell haemolysis, with no color change. The α-haemolysis is characteristic from bacteria that appear on the blood agar with a green or brown zone around the colonies. This appearance is caused by discoloration and loss of potassium from the red cells, not due to a true haemolysis. (Prescott, 1996., Madigan, 1997).

Oral streptococci are present in the oral cavity and upper respiratory tracts of humans and some animals. *Streptococcus pneumoniae* is an example of these streptococci. (Prescott, 1996., Tortora, 1995).

1.2. *Streptococcus pneumoniae*

Streptococcus pneumoniae is 1.0μm in diameter, it is an oval or spherical shape and usually grows in pairs or in chain (Tonnaer *et al*., 2006; Cruickshank, 1988).
This pathogen is a facultative anaerobic, aerotolerant, but most strains prefer an atmosphere enriched with 5-10% CO_2. The optimal growth temperature of these bacteria is 37ºC. The addition of 10% v/v defibrinated horse blood to the medium further enhances streptococcal proliferation. Pneumococcal colonies are opaque and transparent. The opaque variants have less teichoic acid and more capsular polysaccharides, while the transparent strains express more teichoic acid and have less

capsular polysaccharides. These morphologic differences are related with the type of interaction in the host. The opaque variants demonstrated a larger capacity of inducing an inflammation, while the transparent variants showed a bigger capability of adherence. *S. pneumoniae* can survive in several environments as blood and surfaces of the respiratory mucosal because *in vivo,* this pathogen exists in heterogeneous populations, with the two variants, opaque and transparent present at the same time (Tonnaer *et al*., 2006).

On blood agar, pneumococci colonies are small and flat, surrounded by a clearly greenish color (α-hemolytic) like *Streptococcus* viridians. After incubation the colonies appear slightly elevated on the agar. A difference between *S. pneumoniae* and *S. viridian* is that pneumococcus is sensitive to optochin.

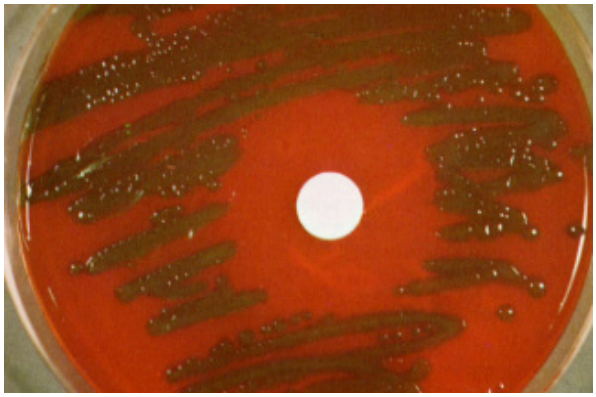

Fig. 2 *Streptococcus pneumoniae* on blood agar illustrating α-haemolysis and sensitivity to optochin (Website: www.bact.wisc.edu/themicrobialworld/Spalpha.jpg).

Pneumococcal replication within host tissues induces disease and consequent inflammatory responses. These host responses are induced by the bacteria cell wall components. After bacterolysis the (lipo)teichoic acids are released, and during bacterial growth and lysis, pneumolysin toxin is released (Tonnaer *et* al., 2006).

S. pneumoniae can be found in two different physiological states, sessile and planktonic. The latter refers to the bacteria that grow in liquid culture, while the sessile state refers to bacteria that grow as biofilm. A biofilm is defined as an organized community of microorganisms attached to an inert or a living surface, which is enclosed

in a self-produced polymeric matrix. In 1995, was reported the importance of a peptide for bacterial adherence in the biofilm model (Havarstein *et al*., 1995); this is known as competence stimulating peptide, CSP. This peptide is responsible for cell to cell signaling in competence development, enabling the quorum-sensing phenomenon (Havarstein *et al*., 1995). Oggioni *et al.,* (2006) demonstrated that only in presence of CSP was it possible to find pneumococci attached on plastic surfaces and that gene expression levels in pneumococci found in tissue (meningitis and pneumonia) displays similar patterns to those found in a biofilm model. However, in blood infection, or sepsis, bacterial gene expression resembles that of bacteria grown in liquid culture medium. Several studies have shown that in nature pneumococci are generally organized in biofilms, but the importance of these biofilms in medical microbiology is still unknown (Oggioni *et al.,* 2006).

1.3. *S. pneumoniae* virulence genes

Many genes have been reported as important factors in *S. pneumoniae* virulence including those related to the pore-forming toxin pneumolysin, the polysaccharide capsule, and surface proteins such as neuraminidases (NanA), pneumococcal surface protein A (PspA), pneumococcal surface protein C (PspC), hyaluronidase and the zinc metalloproteinases (Oggioni *et al*., 2006).

The major virulence factor in invasive infection is usually the polysaccharide capsule, but when it comes to pneumococcal adherence this appears to have no contribution, impeding it in some cases. The cell wall components, on the other hand, appear to be one of the major factors on pneumococcal adherence (Rubins *et al*., 1998; King *et al*., 2005).

Pneumolysin is a 53Kd pore-forming toxin causes a range of effects, even in low sublytic concentrations, including apoptosis induction, the activation of the host complement and in immune cells may induce the proinflammatory response. Furthermore, previous studies have demonstrated that pneumolysin is of extreme importance in nasopharynx colonization, including a pneumolysin-deficient mutant showing that *S. pneumoniae* was less capable of colonization. (Rubins *et al*., 1998; Hirst *et* al., 2004). An essential characteristic of *S. pneumoniae* is the fact that undergoes autolysis, caused by a peptidoglycan hydrolase, LytA (autolysin). This protein causes

the cell to lyse, releasing all intracellular contents including pneumolysin and other pneumococcal virulence factors.

Neuraminidase has also been shown to be important to pneumococcal colonization and is particularly important for middle ear infection (King *et al*., 2005). This enzyme is responsible for the cleavage of the terminal sialic acid residues associated with galactose or N-acetylgalactosamine. Previous studies have suggested that NanA increases the number of available ganglioside receptors that are important for pneumococcal adherence. The removal of the terminal sialic acids by this enzyme could be the major cause of the exposure of these surface receptors for *S. pneumoniae* (King *et al.*, 2005; Cámara *et al*., 1994).

Competence induction by CSP is caused by the production of the comX protein. *ComX* has been implicated as a key regulator gene, fundamental to the CSP dependent expression of many competence specific genes. This regulator plays an important role as a transient alternative sigma factor (Oggioni *et al.,* 2004, Luo and Morrison, 2003).

Many reactive oxygen intermediates cause damage to living organisms by peroxidation of membrane lipids and damage of DNA strands. Hydrogen peroxide (H_2O_2), superoxide anion ($^{\cdot}O2^{-}$) and hydroxyl radical ($^{\cdot}OH^{-}$) are some examples of these intermediates. Several aerobic organisms have developed enzymatic and non enzymatic mechanisms in order to detoxify these active compounds. Enzymatically, four enzymes play an important role in the elimination of these oxygen radicals: super oxide dismutase, catalase, glutathione and glutathione redutase. Classified as metalloenzymes, the super oxide dismutases (SODs) catalyze the conversion of superoxide molecules into molecular oxygen and hydrogen peroxide. For this reason, SODs are considered one of the cell's major defense mechanisms against oxidative stress. Three types of SODs have been described, depending on the metal cofactor: SodA (Mn- cofactor), SodB (Fe- cofactor) and SodC (Cu-Zn- cofactor). Cu-ZnSOD exists in many eukaryotic organisms, in the cytosol, although many studies have reported the presence of this enzyme in some prokaryotes as well. MnSOD and FeSOD are enzymes typically found in prokaryotes but also present in eukaryotes mitochondria's. Not only important in superoxide radical's detoxification, SOD has also been demonstrated to be a significant

virulence factor in many pathogenic bacteria. The absence of catalase in *S. pneumoniae* suggests that SOD may be an important key against oxidative stress, interfering in the virulence and in the survival of these bacteria. Aeration of *S. pneumoniae* influences the amount of SOD, with more MnSOD (sodA) activity detected in aerobic conditions than anaerobic. This capacity suggests that adaptation to the oxidative stress is part of pneumococcus virulence. (Yesilkaya *et al*., 2000).

D39HY1, a *sodA* mutant of *S. pneumoniae* did not survive in presence of oxygen. *SodA* inactivation is lethal to this pathogen in oxygen or superoxide environments. An intranasal infection with D39HY1 in an *in vivo* model of pneumonia showed that the lack of *sod* reduced the virulence of this pathogen. No changes were observed in pneumococcus virulence, between the wild type and D39HY1, after intravenous infection. These observations demonstrated that the route of infection is an important factor in the virulence of *S. pneumoniae*. These variations in pneumococcus virulence may be related with different levels of oxidative stress in lung and blood. (Yesilkaya *et al*., 2000).

Another enzyme with a significant importance in O_2 detoxification is the NADH oxidase. This protein catalysis the reduction of molecular O_2 to H_2O or H_2O_2. It has been reported that this enzyme not only plays an important role in pneumococcus detoxification but also influences its competence. The competence state, as mentioned before, appears during a particular time in the exponential growth phase, and is associated with a metabolic stimulation. A higher ATP amount per glucose derived from a more efficient energetic metabolism, or a higher rate of glucose degradation can be associated with this metabolic stimulation. (Auzat *et al*., 1999).

Most of the energy that *S. pneumoniae* uses is thought to derive from the glycolytic breakdown of glucose into lactate. In the fermentation reaction, one consumed glucose yields at most 2 ATP molecules, releasing lactate as the by-product of NADH reoxidation at the expense of pyruvate. Reported as a soluble flavoprotein, NADH oxidase has an important role in the glycolytic NADH reoxidation, using O_2 instead pyruvate improving the glucose catabolism efficiency and leaving a high level of ATP available for the DNA transformation. (Auzat *et al*., 1999).

Nox is the gene that encodes NADH oxidase. Using *S. pneumoniae* mutant strains, with a disruption in the *nox* gene, no differences were observed in pneumococcus growth behavior under aerobic or anaerobic conditions, although, the competence efficiency for genetic transformation during pneumococcus growth was clearly affected. (Auzat *et al*., 1999).

Despite all the information related to *S. pneumoniae* virulence, a lot is still unknown about pneumococcal metabolism and its pathogenic mechanisms.

1.4. Epithelial cells

In a pneumococcal infection, bacteria colonize the nasopharyngeal epithelium and penetrate the nasopharynx or lung epithelium achieving the vascular compartment (Lagrou *et al*., *2003*). The alveoli can become filled of fluid and inflammatory cells. The infection is immediately spread to adjacent alveoli, infecting a large pulmonary area, indispensable for the gas exchange (McCormick, 2006).

The respiratory, gastrointestinal and urogenital tracts are composed of a mucosal layer, which is covered by epithelial cells. Besides being responsible for the regulation of ion flow and solutes, the epithelial cells are also important in detecting and responding to external stimuli, thus forming a protective barrier separating the apical surface of the mucosa from the tissue below (Rojas and Apodaca, 2002).

Despite this protective barrier, the human respiratory tract is frequently colonized by different pathogens. The fundamental step for a successful colonization appears to be bacterial adherence as implicated in several diseases such as bacterial endocarditis, prosthetic device infections, urinary tract infections, gonorrhea, streptococcal pharyngitis, dental caries and gastroenteritis. *Streptococcus pneumoniae* along with other pathogenic and non pathogenic bacteria are responsible for the oral cavity colonization and have been particularly related with the posterior and nasopharynx (Selinger and Reed, 1979).

The epithelial surface of the respiratory tract is formed primarly by basal layers, composed of small round cells, generally covered by elongated ciliated epithelial cells and goblet cells. The goblet cells are mucin producers and can be found in variable number (McCormick, 2006). The epithelial cells of the trachea and bronchi are also covered by ciliated columnar epithelium.

Ciliated cells are considered of extreme importance in the defense mechanism of the respiratory sytem. As terminal cells, the ciliated cells are generally replaced trough the differentiation of basal and /or secretory cells. It has been demonstrated in rats, that these cells compose 40% of the trachea epithelium and each one contains in its apical surface, approximately 200 cilia (Ostrowski *et al*., 1999).

One of the most important local defenses of the respiratory tract is nasal mucociliary clearance, which captures foreign material and prevents infection. The physiological control of the ciliated cells and the rheological (viscosity) properties of the mucus layer are essential factors of the mucociliary clearance system (Dimova *et al*., 2005). The synchronized beating of the cilia origins the force necessary for an efficient mucociliary clearance (Ostrowski *et al*., 1999). While the cilia beat repeatedly and properly, a thin mucin layer entraps the foreign substances and facilitates its exclusion out of the respiratory airway. It has been proved before that an irregular or deficient beat frequency of the cilia leads to numerous infections (McCormick, 2006).

In patients with the inherited disease primary ciliary dyskinesia (PCD) the importance of the clearance defense mechanism is visible. The cilia from these patients do not beat properly, having an abnormal beating or, in some cases, the cilia are completely immotile. Bronchitis, sinusitis and otitis are some examples of diseases that a patient with PCD can suffer, and is believed that an impaired mucociliary clearance can be the cause of these (Ostrowski *et al*., 1999).

Uninfected epithelial cells have confluent cell layers while infected cells demonstrate ruptures on the cellular tight junctions. TEER (transepithelial electrical resistance) is defined as a measure of the integrity of the cell layers. Pnemococci densities close to 1 x 10^7 CFU/ml decreases the TEER of the cell (Lagrou *et al*., 2003). The fluid lining the airways surface epithelium is defined as apical fluid and has been object of several researches. Changes in the ionic composition of this fluid are important and can be a sign of numerous diseases.

1.5. Antibiotics resistance

The medical field is more concerned each day with the excess consumption of antibiotics. The quantity of drugs that people take unnecessarily is a significant problem (Atlas, 1995). This widespread use of drugs has amplified the number of pathogenic microorganisms that demonstrate antibiotic resistance. As consequence, a large variety of antibiotics have lost their efficacy (Madigan, 1997).

Currently, most pneumococcal strains remain sensitive to a large variety of antimicrobial drugs as penicillins, tetracyclines and sulfonamides. However, antibiotic resistance is emerging and therefore understanding pneumococcal infection and finding new targets of drug therapy is becoming increasingly important (Cruickshank, 1988).

The first penicillin-resistant strains were found in the South of Africa, and with these was also discovered for the first time the mechanism of resistance. This mechanism is explained by the reduction of antibiotic binding proteins (PBPs) that causes the decrease in antibiotic affinity by the bacteria.
The study of these resistant-isolates revealed that the cell walls were made from a different composition compared to the penicillin susceptible and nonencapsulated laboratory isolates. This study suggested a possible link between resistance to penicillin and abnormal wall composition (Fischetti *et al*., 2006).

A research made by the Canadian bacterial surveillance network (CBSN) in 2006 studied streptococcus pneumoniae resistance to different antibiotics. Besides *Streptococcus pneumoniae* resistance to penicillin rose slightly from 14,8% in 2004 to 15,0% in 2005, the high-level resistance appears to have dropped significantly. Over the past 5 years, resistance to penicillin has increased and achieved 15%, suggesting that levels may have stabilized (Marshall *et al*., 2006).
Streptococcus pneumoniae resistance to macrolides, a class of antibiotics used most often to treat infections in the respiratory tract, have demonstrated a steady increase since 1999. An example of one of these macrolides is erythromycin that increased from 18,0% in 2004 to 19, 3% in 2005. According to the principal investigator of this research, Dr. Donald Low, microbiologist-in-chief at Mount Siani Hospital in Toronto "The rate of erythromycin resistance has almost doubled in the last six years, a trend

that has to be watched closely". Besides alarming, the macrolides rates remain lower compared to other countries as Asia, United States and much of Western Europe (Marshall *et al*., 2006).

Pneumococcus resistance to quinolones, demonstrated that levofloxacin, a recent respiratory fluoroquinolone, part of a class of antibiotics used to treat respiratory and urinary tract infections, dropped from 1,5% in 2004 to 1,4% in 2005, remaining almost unchanged.

"Antibiotic resistance is a problem that will remain with us for a very long time, which is why it's important to remember that everyone – healthcare professionals, public health authorities, government, the pharmaceutical industry and the public – has a role to play in fighting this serious public health threat". (Marshall *et al*., 2006).

1.6. q-PCR

q-PCR has proven to be a powerful method for gene expression quantification (Livak and Schmittgen, 2001).

In this project, q-PCR was performed on the LightCycler system from Roche Molecular biochemicals using the Light Cycler DNA Master Sybr Green Kit.

The cycle numbers where all the samples have the same fluorescence levels (the amount of amplified target reaches a fixed threshold) is defined as the Crossing Point (also known as threshold cycle) and its dependent on the initial amount of target in each sample. For example, if the starting amount of target was increased, less cycles would be needed to achieve the specific fluorescence value (Livak and Schmittgen 2001; LightCycler Operator'sManual, version 3.5, Roche Diagnostics).

To calculate gene expression the relative quantification method was applied. This method describes the variation in expression of the target gene compared to some reference group as an untreated control. This relative gene expression was analyzed using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001).

$2^{-\Delta\Delta C}_T$ method (Livak and Schmittgen, 2001)

C_T is related to the threshold cycle,

1. **ΔC_T calculation:**

$$\Delta C_T = (C_{T,X} - C_{T,R}) \quad \text{(eq. 1)}$$

$C_{T,X}$ - threshold cycle of the target amplification (for example the *comX* gene);

$C_{T,R}$ - threshold cycle of reference amplification (in this case *gyrB* is the reference gene)

2. **$-\Delta\Delta C_T$ calculation:**

$$-\Delta\Delta C_T = -(\Delta C_{T,q} - \Delta C_{T,cb}) \quad \text{(eq. 2)}$$

$\Delta C_{T,q} - (C_{T,X} - C_{T,R})$ of any sample

$\Delta C_{T,cb} - (C_{T,X} - C_{T,R})$ of the calibrator (reference condition, in this study three reference conditions were analyzed – D39 in TSB; D39 in BEBM and Non adhered D39 to basal cells).

3. **$2^{-\Delta\Delta C}_T$ calculation:**

$$2^{-\Delta\Delta C}_T = \text{amount of target} \quad \text{(eq. 3)}$$

1.7. Pneumococcal gene expression analysis

In recent years *S. pneumoniae* has been the focus of much research, including pneumococci adherence to cells, gene expression in different culture media, biofilm formation and many others (Oggioni *et al*., 2004; Oggioni *et al*., 2006; Moscoso *et al*., 2006; Lagrou *et al*., 2003).

Oggioni *et al.,* (2006) studied *S. pneumoniae* gene expression during infection of host tissue and blood. Using real time RT-PCR (q-PCR) for quantification and mice as a host, they showed that pneumococci in blood had increased gene expression levels of pneumolysin (*ply*), *pspA* and *hrcA*. The gene expression of pneumococci in tissue was analyzed from infected mouse brain and lung. Using established *in vivo* models, namely meningitis after intracranial infection and pneumonia after intranasal infection, Oggioni *et al*., (2006) detected increased expression of neuraminidases, oxidative stress proteins, metalloproteinases and competence genes (Oggioni *et al.*, 2006). It is known that some virulent genes as *ply* and *nanA*, play an important role in pneumococcal adherence, and when using mutants with a disruption on these genes, *S. pneumoniae* was less able of colonization. (Rubins et al., 1998; King *et al.*, 2005; Cámara *et al*., 1994).

With the information present in Oggioni *et al*., (2006) we thought it would be interesting to analyze pneumococcal gene expression in tissue as well, but instead lung and brain we tested in nasal epithelial cells comparing to Oggioni *et al*., (2006) results and analyzing the role and virulence of more genes (*nanA, ply, comX, sodA* and *nox*) in pneumococcal adherence to these cells, using *gyrb* (gyrase) as the housekeeping gene.

2. Experimental Procedures

All methods were based in Oggioni *et al*., (2006) publication, or discussed and suggested by Claire Smith, Peter Andrew and Chris O'Callaghan.

2.1. Bacterial Growth

Table 1. bacterial strains used in this research

***S. Pneumoniae* strain**	**Serotype**
D39	Serotype 2
TIGR4	Serotype 4

Table 2. Media used for bacterial growth

Media	Application	Preparation
BHI (Brain Heart Infusion) (Supplied by Oxoid, Basingstoke, UK)	Bacterial growth medium	14.8g in 400ml of distilled water. The medium was autoclaved at 121ºC.
TSB (Tryptone Soya Broth) (Supplied by Oxoid, Basingstoke, UK)	Bacterial growth medium	12g in 400ml of distilled water. The medium was autoclaved at 121ºC.
BEBM	Basal cell culture medium	Available from LONZA
BAB (Blood Agar Base) (Supplied by Oxoid, Basingstoke, UK)	Media to make the agar plates with defibrinated horse blood for bacterial growth.	16g in 400ml of distilled water. The medium was autoclaved at 121ºC.

2.1.1. Preparation of BAB culture plates

Once autoclaved at a temperature of 121ºC, the BAB medium left on the bench until it was cool to touch. Defibrinated horse blood was completely mixed and 20ml were added to 400ml of the BAB medium. The Duran bottle containing the medium was then gently agitated by inversion to homogenize the blood. 20ml were added to each plate, using aseptic conditions. The plates were cooled on the bench until the agar had set and were then stored at 4ºC.

2.1.2. Bacteria Growth on TSB and BHI

Pneumococci, stored at -80ºC, was recovered from glycerol (centrifuged at 3000 rpm for 5 min), inoculated into 20 ml of Tryptone Soya Broth and incubated at 37ºC, 5% CO_2. After an overnight incubation the universal tubes were spun for 5 minutes at 3000rpm (ALLEGRA ^{TM} X-22R CENTRIFUGE, BECKMAN COULTER, Inc.), the supernatant was carefully removed and the pellet was resuspended in 1ml of TSB. The suspension was used to inoculate 20 ml of TSB in a new universal or falcon tube. The optical density was measured at 550 nm in regular intervals and stopped at an $OD_{550} \approx 0.8$. The bacteria were aliquoted into microcentrifuge tubes, 1ml into each tube and glycerol was added to make 10% v/v. These bacterial stocks were stored at - 80ºC.

For DNA or RNA extraction, instead of aliquoting the bacterial culture, the universal tubes were spun for 5min at 3000rpm, the pellet was resuspended in 1ml of TSB and stored in microcentrifuge tubes at – 80ºC until a future RNA extraction. (see 2.3.1)

2.1.3. Biofilm model

According to Oggioni 2006, when *S. pneumoniae* is grown in a specific environment that induces biofilm formation, they become more infectious, increasing their virulence. The biofilm model was followed as described by Oggioni 2006, using the CSP, competence stimulating peptide, to induce biofilm formation (Oggioni *et al*., 2006).

1 x 10^8 CFU of a frozen D39 stock was defrosted and diluted 1:100 in 200µl of TSB and 200µl were added to each well of a flat-bottom 96 well polystyrene tissue culture

plate. Then 30ng/ml of CSP1 was added to each well and plates were incubated at 37ºC, 5% CO_2 for 18-24h. Optical density was measured at OD_{590}. The wells were washed 4 times with TSB to remove the most of the planktonic bacteria and at the end 100µl of TSB containing 10% v/v glycerol were added to each well. The optical density was measured and 2sec sonication in a sonicating water bath was performed to detach all the biofilm bacteria. The optical density was measured and detached bacteria were used to CFU counts in blood agar plates. To collect samples for RNA extraction, the bacterial suspension was placed into a falcon tube, harvested by centrifugation, and the pellet frozen at –80ºC.

2.1.4. *Streptococcus pneumoniae* growth curve in BEBM on microtitre plates (12 wells).

In a falcon tube, D39 bacteria stock was added to BEBM in order to have a final concentration of $1x10^7$ CFU. 1ml of this bacterial media was added to the first 6 wells of a microtitre plate and incubated at 37ºC, 5% CO_2. Samples of 20µl were taken of the respective well in the 0h; 0.5h; 1h; 2h; 4h and 6h time points for CFU count. A growth curve was made.

To perform RNA extraction, the sample was taken after 2h incubation.

2.1.5. Viable count

For colonies formed unities (CFUs) counting, dilutions were made adding 20µl of bacteria to 180µl of TSB in a microtitre plate. Six serial dilutions were made taking 20µl from one well to the next, mixing with the pipette. 20µl of each dilution were spotted 3 times onto a blood agar plate.

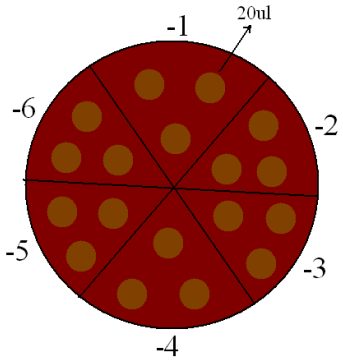

Fig.3. Representation of different D39 dilution drops on a blood agar plate for CFU count

$$CFU/ml = nº\ of\ colonies \times volume\ in\ 1ml \times dilution\ factor \qquad \textbf{(eq. 1)}$$

2.1.6. *Streptococcus pneumoniae* resistance to streptomycin and penicillin.

100µl of a prepared solution of streptomycin (100µg/ml), were spread in one part of the blood agar plate. After the streptomycin had dried, three drops of 20µl of *S. pneumoniae*, at an amount of $1x10^9$ CFUs, were poured in both sides of the plate and after drying, the plate was placed in a 5%CO_2 incubator until next day.

The penicillin test was done in the same way as the streptomycin, but with a concentration of 100IU/ml.

2.2. Cell culture

Table 3. Media for cell growth

Media	Application	Preparation
ALI	Medium to grow cells at air liquid interface	Specifically prepared by the supplier company Lonza
BEBM	Basal cells culture medium	Specifically prepared by the supplier company Lonza
BEGM	Basal cells culture medium with growth factors	Specifically prepared by the supplier company Lonza

2.2.1. Apical fluid

2.2.1.1. *Streptococcus pneumoniae* survival in apical fluid

The apical fluid used in this experiment was recovered with the basal cells from the nose of some patients and then supplied to us by the hospital.

Firstly the sterility of the apical fluid was confirmed making serial dilutions of 10µl of the patient apical fluid and plating these on blood agar for the viable count, proceeding as described in section 2.1.5.

After the sterility of the apical fluid was confirmed, serial dilutions were made in ALI (table 3.) medium (5µl of apical fluid in 45ul of Ali medium), to give the following ratios of apical fluid to Ali medium as shown in Fig.4: 1:1; 1:10; 1:100; 1:1000. To these were added 45µl of D39 bacteria stock, diluted in TSB to 2.2 x 10^8 CFU/ml, changing the final apical fluid ratios to 1:2; 1:20; 1:200; 1:2000, 1:20 ALI + antibiotic and 1:2 ALI. The microcentrifuge tubes were incubated at 37ºC in 5% CO_2. Samples of 10µl were taken for CFU determination at 0h; 0.25h; 0.5h; 1h; 2h; 3h; 4h; 6h; 8h, and respective growth curves were constructed.

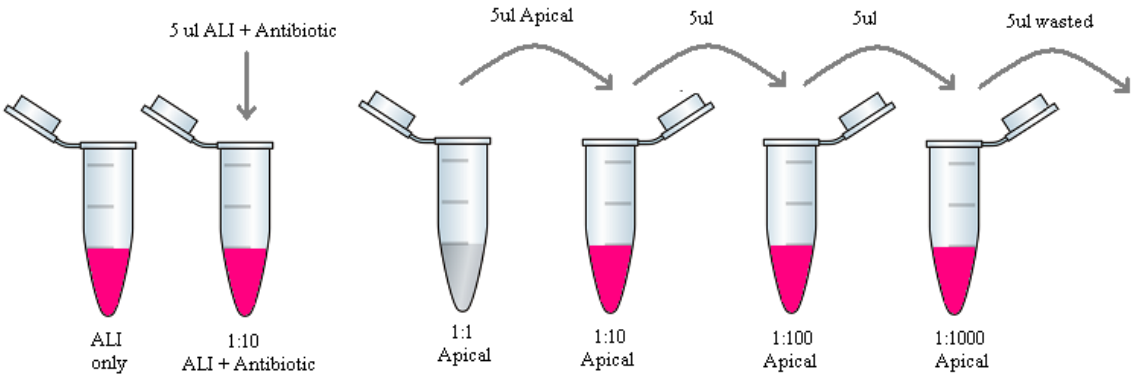

Fig. 4. Representation of the different apical dilutions

2.2.1.2. ELISA

Solutions used:

- Coating buffer: 50mM of Sodium Carbonate pH 9.6
- PBS 10x :
- Washing buffer: PBS-Tween:
  PBS (1x) with 0.5% (v/v) Tween 20.
- Milk blocking buffer (2x)

  100ml of PBS(2x) (from the stock PBS (10x));
  500µl of 100% (v/v) Tween 20;
  50g of Non fat dry milk;
  10ml of a 5% (w/v) NaN_3 (Sodium Azide) solution stock;
  H_2O was added to a final volume of 500ml, and after homogenization the solution was aliquoted in falcon tubes and stored at -20ºC.

- Diethanolamine solution:
  9.7ml of diethanolamine;
  5ml of $MgCl_2$;
  H_2O was added till a final volume of 100ml, the bottle was covered with aluminum foil and stored in the fridge.

Two types of ELISA were used in this experiment: Direct ELISA (Fig.5) and a Sandwich ELISA (Fig.6). In the Direct ELISA the wells were coated overnight with the antigen sample to be analyzed, while in the sandwich ELISA the wells were coated with a capture antibody specific to the antigen. Coating the plate with antigen samples:

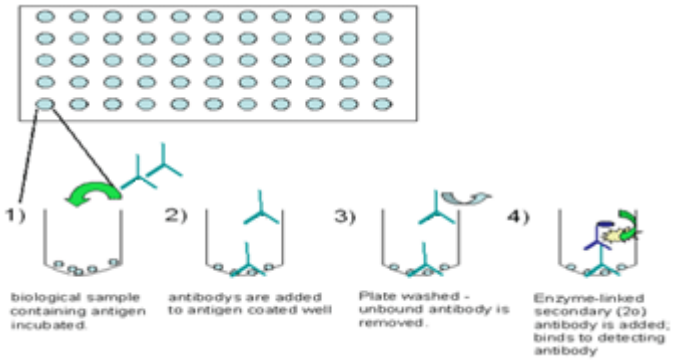

Fig. 5. Direct ELISA, the wells were coated overnight with the antigen solution. (www.elisaassay.com)

Direct ELISA:

Several dilutions of streptomycin and penicillin were made, in order to make a standard curve to calculate antibiotic concentration on the apical fluid. In microcentrifuge tubes, 30µl of the respective antibiotic were diluted several times in 270µl of coating buffer (1x) and 100µl of these dilutions were placed in individual wells of an ELISA microtitre plate. The ELISA plate was sealed with an adhesive lid and left in the fridge overnight.

The following day, each well was washed 5 times with PBS-Tween washing buffer, and after making sure all liquid was removed, 200µl of milk blocking buffer (1x) was added and the plate incubated at 37ºC for 1h. After 1h the wells were washed 5 times with PBS-Tween washing buffer, and 100µl of the antibody solution (1:2000 (v/v) streptomycin and 1:200 (v/v) penicillin) (ab30593 to streptomycin and ab30592 to penicillin, both sheep polyclonal antibodies, ordered from **Abcam Company**) were added. The plate was covered and incubated for 2h. After 2 hours, the ELISA plate was washed 5 times with the PBS-Tween and 100µl of the secondary antibody (ab6748 rabbit polyclonal to sheep IgG, H&L linked to alkaline phosphate, from **Abcam**

Company) solution (1:1500) were added to the wells. After one hour incubation at 37ºC, 100µl of p-nitrophenyl phosphate (PNPP) solution in diethanolamine (p-Nitrophenyl-phosphate was used at 1 mg/ml in 10 % (w/v) diethanolamine buffer (pH 9.8) containing 5 mM MgCl) were added to each well. The lid was removed from the ELISA plate and the A_{405}nm was measured (using a Multiskan Ascent (Thermo) microplate photometer) after 30min and 1h of incubation at 37ºC.

Coating the plate with capture antibody:

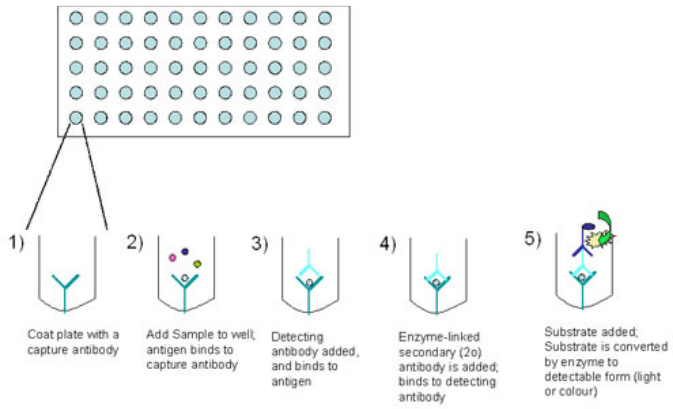

Fig. 6. Sandwich ELISA assay, the wells were coated overnight with a capture antibody. (www.elisaassay.com)

Sandwich ELISA

A sandwich ELISA was also performed. All the procedures were exactly the same as the direct ELISA, but instead coating the plate with the antigen, the plate was coated overnight with a capture antibody, specific to detect the respective antibiotic (ab15071 mouse monoclonal to streptomycin from **Abcam Company**) using the recommended concentration (1/1000). After washed with PBS-Tween washing buffer and applying the milk blocking buffer, the antigen samples were added at the desired concentrations. After this, the experiment was done as previously described in the direct ELISA.

2.2.2. Pneumococcal adherence to basal cells

Coating plates with collagen

The plate wells were coated with 1% (w/v) collagen in filtered sterilized water. To plates with large wells, 1ml was added to each well. Plates with small wells, 150-200µl was added. The plates were left for 5-6h at room temperature and the wells were washed once with filter-sterilized water; the plates could be stored for up to a month at room temperature.

Basal cells culture and pneumococcal adherence

The basal cells were recovered from the nasal cavity of some patients and supplied to us.

For glass slides, 30-60µl of $1x10^5$ CFU/ml basal cells was added in 300µl of BEGM in each well of the coated plate, or 60-80µl of basal cells in 1ml of BEGM to the 12 well plates (larger wells). The cells were fed everyday with new fresh BEGM (300 µl to the glass slides and 1ml to the larger wells) and the plates were inspected using the inverted microscope to check if the cells were confluent (usually it took one week to be confluent). The medium was washed off and to each well was added the same quantity of BEBM (without antibiotic). The plates were left on the 37ºC incubator for 2h. To the glass slides, $1x10^7$ CFU of D39, were added to each well, in 400µl of BEBM. To the 12 well plates, $1x10^7$ CFU were added in 1ml of BEBM. The plates were left on the 37ºC, 5% CO_2 incubator. After 2h, the supernatant was removed to microcentrifuge tubes and centrifuged 5min at 1200rpm (Mikro 22R, HETTICH ZENTRIFUGEN, GMI, Inc.) for future RNA extraction. The wells were washed four times with the same quantity of BEBM, adding at the end 100µl to the glass slide. The wells were scraped with a cell scrapper, in order to release the adhered cells. 400µl of BEBM were added to the 12 well plates to scrape the cells. The medium with the released cells was collected into cleaned microcentrifuge tubes, keeping some sample for CFU count, and for a cell count using a Neubauer counting chamber. The microcentrifuge tubes (adhered cells and non adhered cells) were centrifuged at 1200rpm for 5min, and submitted immediately to RNA extraction or the pellets were ressuspended in RNAlater (a specific solution that preserves the RNA for later extraction) and frozen at -80ºC.

It was done other experiment, but this time with 6h of pneumococcal adherence to compare and analyze the gene expression with the 2h adherence assay.

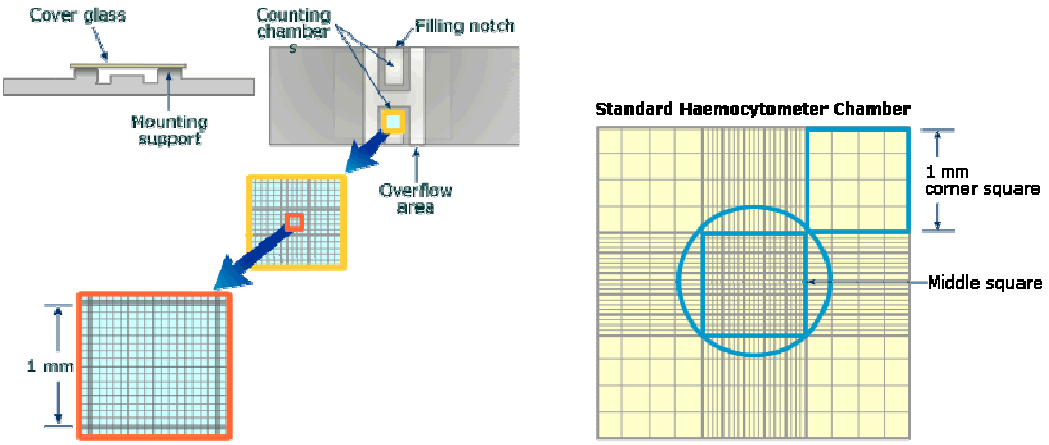

Fig. 7. Neubauer counting chamber (http://toolboxes.flexiblelearning.net.au/demosites/series4/412/laboratory/studynotes/SNHaemo.htm).

To calculate the number of cells per milliliter of sample:

$$\textbf{Nº of cells counted in a square x } 10^4 \qquad \text{(eq. 4)}$$

(10^4 is the chamber conversion factor for neubauer)

Limitations of the neubauer counting chamber method:

1. Small cells are very difficult to identify under the microscope, and some of them are probably missed;
2. Death cells are not distinguished from living cells;
3. For low densities (less than 10^6 cells/ml) this method is not appropriate, being the cells very difficult to count;
4. The precision is difficult to achieve;
5. When samples are not stained, a phase contrast microscope is required.(Madigan, 1997).

2.3. Molecular experiments

2.3.1. RNA extraction

For RNA extraction three different methods were tested. The first one was made manually, step by step, and the other two with specific kits. The third method was the direct application of a specific RNA kit - High Pure RNA Isolation Kit (Roche) being the best method, obtaining higher concentrations of RNA, and more sensitive on extracting RNA from low concentrations of bacteria.

2.3.2. Conversion of RNA into cDNA

Before using the samples in q-PCR, the RNA was converted to cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche).

The transcription reactions to cDNA were performed in PCR tubes as shown in Table 4.

Table 4. Quantities of components added per reaction

Component	Quantity (µl)
Random Primers	2
Rnase free water	6
RNA (Sample)	5
Total	**13**

The RNA was denaturized at a temperature of 65ºC for 10 minutes and after that the PCR tubes were cooled on ice. To each tube was added the solutions described in Table 5, making a total of 20µl per reaction tube.

Table 5. Quantities of each component added per reaction (tube)

Components	Quantity (µl)
Reverse Transcriptase buffer	4
RNase inhibitor	0.5
dNTP mix	2
Reverse Transcriptase	0.5

The reaction tubes were mixed by flicking, and then incubated at the following temperatures:

10 min, 25ºC

30 min, 55ºC

5 min, 85ºC

Storage temperature: 4ºC.

2.3.3. Ethanol precipitation

After converting the RNA into cDNA, 2-2.5x of 100% (v/v) ethanol were added and the sample was left on ice for 30 min. A 20min centrifugation was performed at 12,000x g (Mikro 22R, HETTICH ZENTRIFUGEN, GMI, Inc.), and the supernatant was removed. Freshly made 70% (v/v) ethanol was carefully added to the pellet, and another centrifugation, as described above, took place to remove the supernatant. The pellet was air dried for no longer than 10 min and then resuspended in 10μl of nH_2O.

2.3.4. Electrophoresis

2.3.4.1.Preparation of buffer TAE

A stock solution of TAE buffer was prepared from a concentrated (50x) stock solution of TAE was made by weighing out (242 g Tris base in approximately 750 ml of deionized water, plus 57,1 ml of glacial acid and 100ml of 0,5 M EDTA (pH 8.0) adjusted to a final volume of 1L). This stock solution can be stored at room temperature.
Final solute concentrations were 40 mM Tris acetate and 1 mM EDTA.

2.3.4.2. Preparation of the Agarose gel

A quantity of 100 ml of a TAE buffer (1x) was added to 1g of agarose, in a 100 ml flask and incubated for 2 minutes to a microwave at 50% power with the lid loose. After that, the flask was agitated to homogenize the solution, and placed back for 1 minute more in the microwave. The flask was cooled at room temperature until could be handled with bare hands and 0,5 μg/ml of ethidium bromide were added to the solution. An appropriate electrophoresis cassette was filled with the agaroses solution, and after few minutes the gel was ready to use.

2.3.4.3. Electrophoresis run

The samples for electrophoresis were prepared while the gel was polymerizing. For the RNA or cDNA samples, 5µl of sample to test and 1µl of loading dye were mixed. To prepare the ladder (1 Kb), 4µl of water, 1µl of loading dye and 1µl of Ladder (1 Kb), were mixed in an epperdorf tube.

Each well of the gel was filled with 6µl of the sample to analyze, and the electrophoresis was performed at approximately 96V. A picture of the gel was taken at the end of the run.

2.3.5. q-PCR

The sensitivity of the q-PCR was tested. For that purpose, RNA from *S. pneumoniae TIGR4,* 9.5 x 10^8 CFU's was diluted 1 in 10 with TSB and q-PCR was performed with the Light Cycler DNA Master Sybr Green Kit (Roche). In Table 6, the quantities added of each component per reaction tube are shown and the PCR conditions are described in Table 7. The housekeeping gene *gyrb* was also tested in this experiment.

Table 6. Quantities added per tube to perform a q-PCR

Components	Quantity (µl)
Nanopure Water	13.7
$MgCl_2$	1.3
Forward primer	0.5
Reverse primer	0.5
SYBR Green buffer mastermix	2
cDNA template	2
TOTAL	**20µl**

Table 7. PCR conditions

Analysis Mode	Cycles	Segment	Target Temperature	Hold Time	Acquisition Mode
Denaturation					
None	1	1	95ºC	30 s	None
Amplification					
Quantification	40*	Denaturation	95ºC	0 s	None
		Annealing	55ºC	15 s	None
		Extension	72ºC	25 s	Single
Melting Curve					
Melting Curves	1	Denaturation	95ºC	0 s	None
		Annealing	65ºC	15 s	None
		Extension	95ºC	0 s	Continuous
Cooling					
None	1		40ºC	30 s	None

*Depending on the initial target amount, less target more cycles are required; **The annealing temperature of all primers was around 55ºC, so that was the temperature applied on the RT-PCR.

Table 8. Primers sequence used to perform the q-PCR (Oggioni *et al*., 2004; Oggioni *et al*., 2006).

Primers	Primers Sequence
*Gyr**b***	f: CAGATCAAGAAATCAAACTCCAA
	r: CAGCATCATCTACAGAAACTC
comX	f: AGGAAAGTCAGAAGCGTAGATA
	r: GCGTTCTAGTTCTTCTTGTT
nanA	f: AGCAACCTCTGGCAAATGAA
	r: ATAGTAATCTCTTGGAATT
Nox	f: ATTGACGGTGCTGAAGGCTTG
	r: CGCTGTAACTACTTTGTTA
Soda	f: TGAAGACCTTGAAGCCTT
	r: TGTCCGCCACCATTGTTGAT
Ply	f: GCAAATAAAGCAGTAAAT
	r: CCAGGATAGAGGCGAC

3. Results

3.1. Bacterial Growth

3.1.1. Bacterial Growth curve

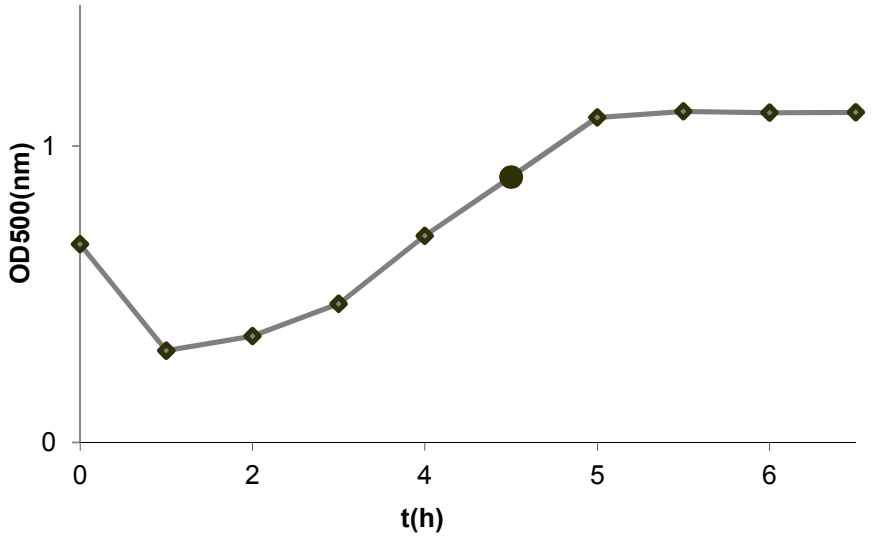

Fig. 8. D39 growth curve in TSB medium.

The bacteria growth was stopped in the mid exponential phase (black dot on the chart of Fig. 8.), and stocks were stored frozen at -80ºC. For RNA extraction, the bacterial culture was centrifuged and the pellet stored at -80ºC.

3.1.2. Biofilm model

The optical density measured after sonication was not significant, showing a low or no turbidity by the detached bacteria on the wells. After centrifuging, no pellet was present making impossible to proceed with a RNA extraction. Serial attempts were made, changing concentrations of CSP and initial bacteria CFU, incubation times, bacteria stocks, plates with larger wells, incubators, but at end the optical density was always insignificant and no pellet was obtained. It was decided not to proceed with these experiments.

3.1.3. *Streptococcus pneumoniae* growth curve in BEBM in microtitre plate

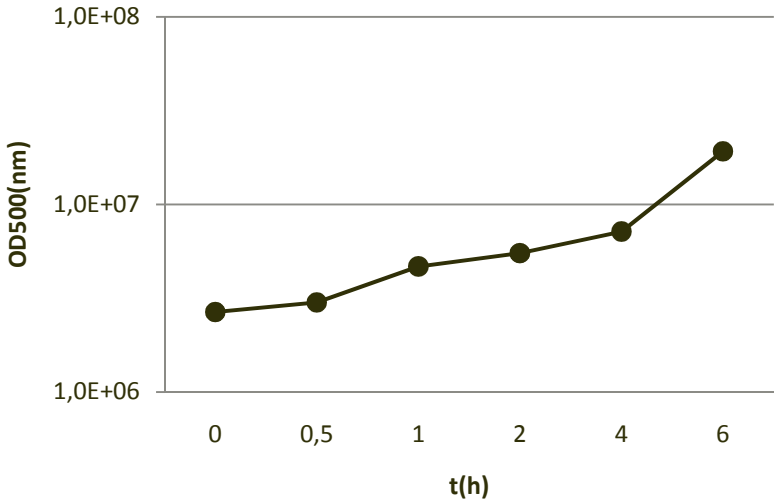

Fig.9. D39 growth in BEBM medium.

The growth in BEBM was done to study *S. pneumoniae* behavior in this medium, since it was going to be used in multiple experiments bacteria adherence to basal cells. Observing the survival curve it was possible to conclude, comparing to other media, that *S. pneumoniae* have a typical exponential growth in BEBM.

3.1.4. *Streptococcus pneumoniae* resistance to streptomycin and penicillin.

After one day incubation, the bacteria only grew in the absence of antibiotic. In the presence of the antibiotics concentrations, streptomycin 100µg/ml and penicillin 100IU/ml, *S .pneumoniae* D39 did not grow, demonstrating that was sensitive to these two antibiotics.

3.2. Cell culture

3.2.1 Apical fluid

. ***Streptococcus pneumoniae*** **growth in apical fluid**

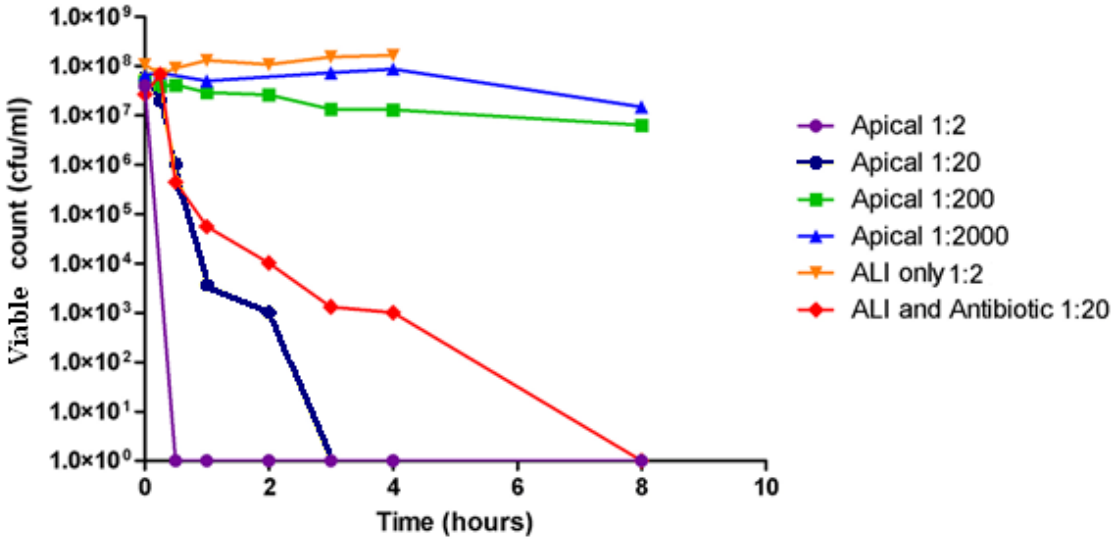

Fig.10. D39 growth in Ali medium and different dilutions of patient SP44 apical fluid.

As can be seen in Fig.10., in 1:2 and 1:20 dilutions of apical fluid, pneumococci were killed with 4h incubation, while in the bigger dilutions the bacteria survived. The cause of bacteria death could be related to several factors, could be a specific component of this of apical fluid (a typical characteristic of this patient apical fluid), could be some rest of antibiotics that remained on the apical fluid even after several washes, could be even caused by some cytokines. To understand better these results, another patient apical fluid was analyzed.

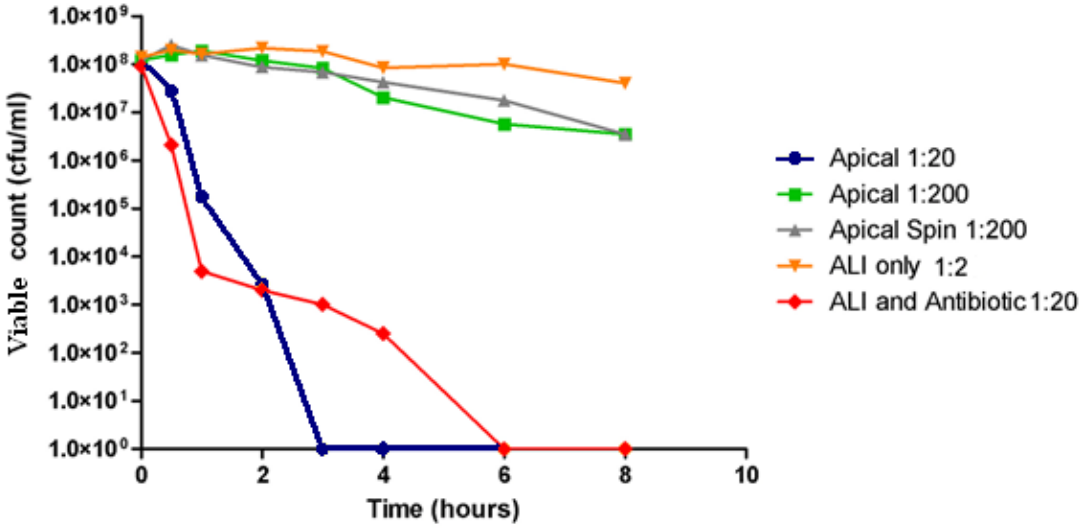

Fig.11. D39 growth in Ali medium and different dilutions of patient SP30 apical fluid.

With the growth curve of *S. pneumoniae* on the apical fluid of other patient, SP30, the same behavior was obtained. In the lowest dilutions the bacteria died after a short period, as shown in Fig.11. Before proceed with RNA extraction for a RT-PCR performance, it was of high priority to understand what was causing this behavior. Only when this problem was solved would be possible to know what control should be used in the RT-PCR gene expression amount.

It was known that contamination of epithelial cell culture was controlled by the addition of two antibiotics, penicillin and streptomycin. Besides the apical fluid being washed before used in this experiment, some rest of antibiotics could remained and have influenced *S. pneumoniae*, D39 growth in the apical fluid, causing its death. The next step was to perform an ELISA test with the specific antibodies to analyze the presence of these antibiotics.

3.2.2. ELISA results

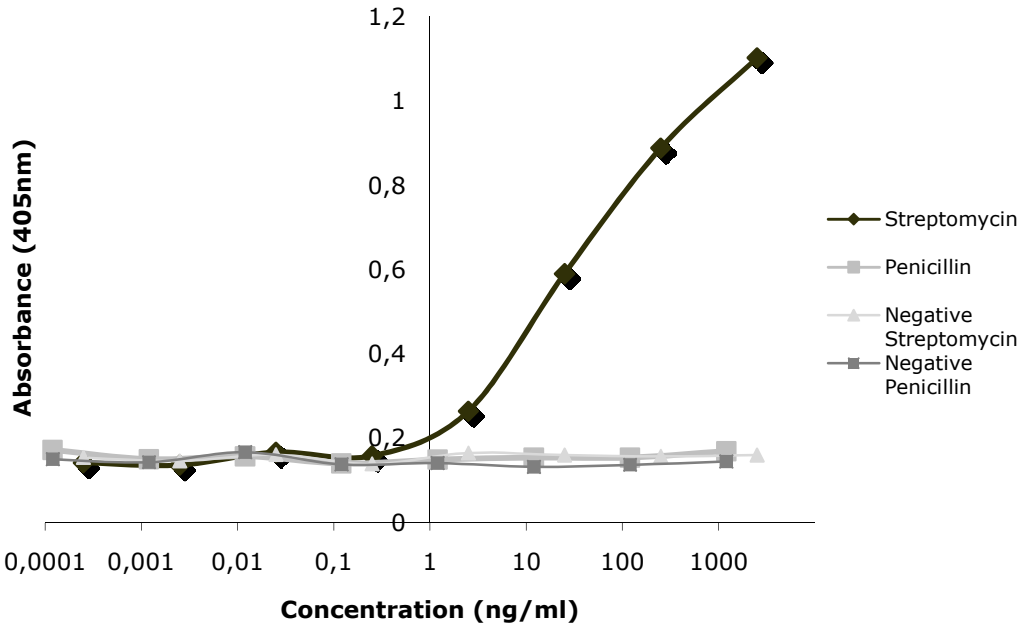

Fig.12. streptomycin and penicillin standard curves from an ELISA assay.

Only the streptomycin standard curve was obtained, showing an increase in absorbance with increasing concentrations of this antibiotic, as shown in Fig.12. In order to confirm the accuracy of these results, negative controls for these antibiotics were also applied. The penicillin standard curve did not show the expected increase in absorbance. Maybe for this antibody (anti-penicillin) higher concentrations of antibiotic were required.

For the streptomycin standard curve, absorbance values from 1-1000ng/ml were used (Fig. 13).

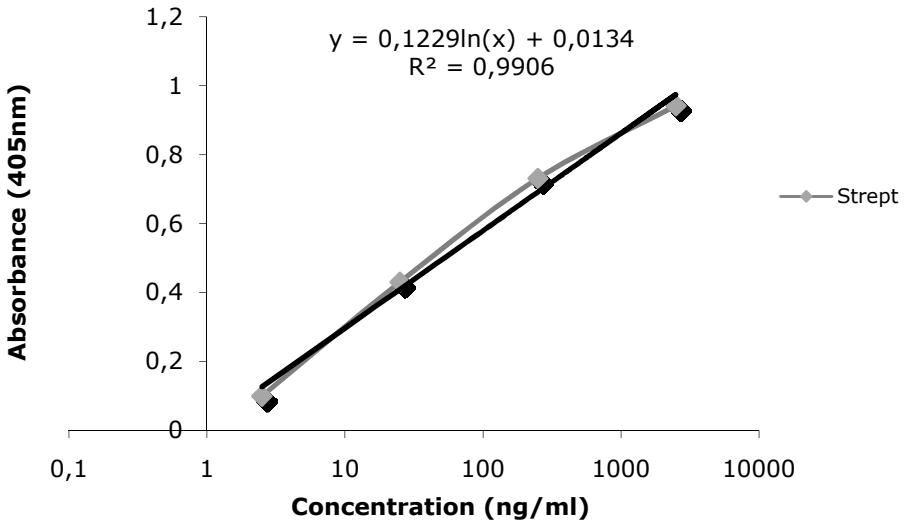

Fig.13. Streptomycin standard curve and its linear regression and equation.

3.2.2.1. ELISA for antibiotic detection in ALI medium

To use the standard curve and see if it has the appropriate values, an ELISA with ALI medium was performed. This Ali medium had a known antibiotic concentration, close to 100ng/ml.Using the formula from the streptomycin standard curve linear regression of the antibiotic concentration present on ALI media was determined (Table 9.).

Table 9. Concentrations and optical densities of streptomycin in different dilutions of ALI medium.

ALI (dilutions)	$A_{(405nm)}$	Strept $A_{(405nm)}$ - Negative $A_{(405nm)}$	Streptomycin concentration (ng/ml)
(1/2)	0,71 0,61	0,45	72
(1/20)	0,54 0,56	0,31	225
(1/200)	0,17 0,21	0,05*	--------
(1/2000)	0,14 0,14	0,02*	-------

*out of the standard curve range

$$y = 0{,}122\ln(x) + 0{,}013$$

y = Absorbance $_{(405nm)}$;

x = concentration (ng/ml).

It was expected to achieve same concentrations in two different dilutions: 1/2 and 1/20, but the final concentration of streptomycin on ALI media 1/20 (225ng/ml) is very different from the concentration on the ALI 1/2 (72ng/ml). Another assay was performed to test these results, this time with the apical fluid.

ELISA for streptomycin detection in patient SP30 Apical fluid

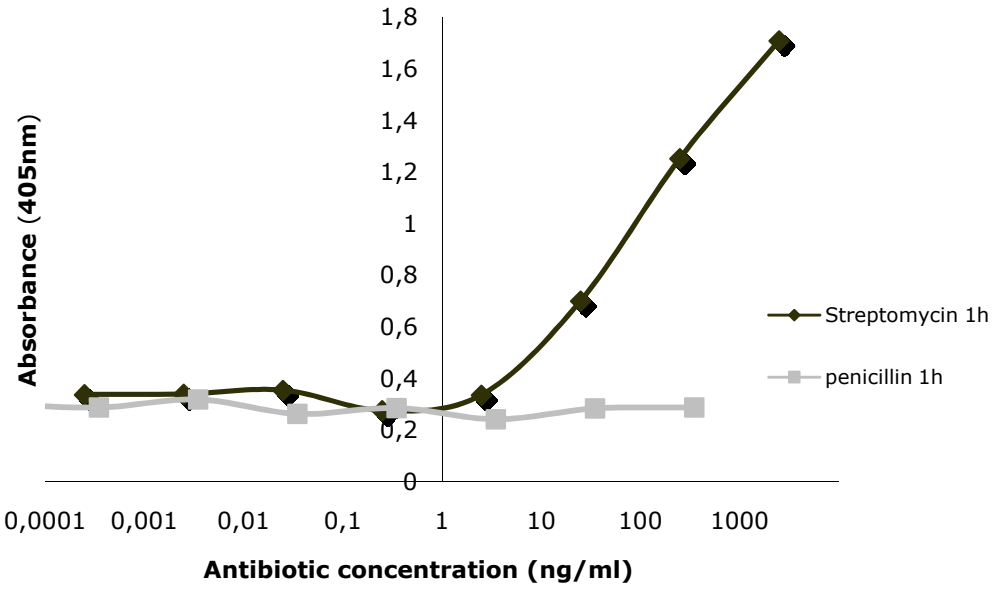

Fig.14. Streptomycin and penicillin standard curves.

Two standard curves were performed on the ELISA plate, and one more time no results were obtained with the penicillin, as shown in Fig.14.

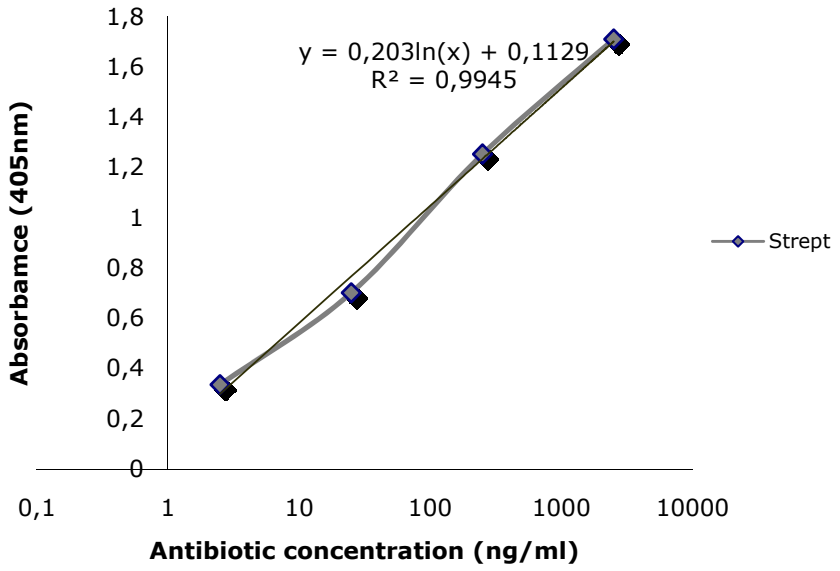

Fig.15. Streptomycin linear regression and its equation to calculate antibiotic concentrations in patient SP30 apical fluid.

Table 10. Concentrations and optical densities of streptomycin in different dilutions of the Apical fluid.

Apical (dilutions)	Absorbance (405nm)	Strept $A_{(405nm)}$ Average - Negative$A_{(405nm)}$	Streptomycin concentration (ng/ml)
(1/20)	0.81 0.82 0.80	0.59	210
(1/200)	0.46 0.47 0.42	0.23*	------

*out of standard curve range.

With the ELISA assay it was possible to detect streptomycin in the apical fluid, however it was not possible to detect the correct concentration of this antibiotic. When the concentration of streptomycin was calculated from different dilutions, each dilution demonstrated a different concentration. And in the last ELISA it was only possible to calculate for one dilution (1/20), since the 1/200 was out of the standard curve. One possible explanation for this was that the streptomycin concentrations chosen to build the standard curve were not the best. It was not possible to detect the presence of penicillin in the apical fluid. This could be due to some problems related to the concentrations used to perform this standard curve, maybe the capture antibody (anti-streptomycin) was not working properly binding not only to the antibiotic.

In order to understand better these results, a capture antibody was ordered from Abcam Company, to coat the plate overnight (sandwich ELISA) and the absorbance was measured using a Multiskan Ascent (Thermo) microplate photometer and analyzed in a different way, using the accompanying software (Ascent v2.6) that calculates immediately the antibiotic concentration.

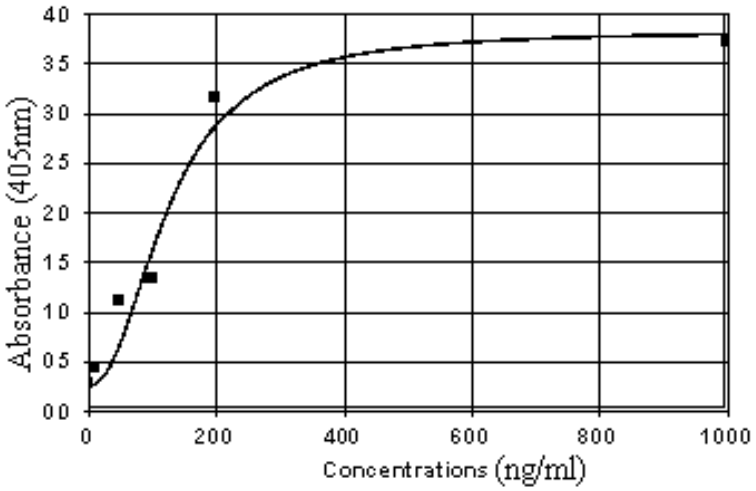

Fig.16. Streptomycin standard curve from a sandwich ELISA assay.

Table 11. Concentrations and optical densities of streptomycin in different dilutions of the Apical fluid.

Apical (dilutions)	Absorbance$_{(405nm)}$	Streptomycin concentration (ng/ml)
(1/10)	0,36	234
(1/100)	0,31	1691

Once again there was no consistency in the antibiotic concentration in the two different dilutions (Table 11).

3.2.3 *S. pneumoniae* adherence to basal cells

Since one assay was not enough to analyze all conditions, it was used basal cells from different patients to perform more assays.

The 2h time for pneumococcal adherence was chosen because it was thought to be the right time for the basal cells not being much damaged, since *in vitro* the area is inferior compared to an *in vivo* assay in lung as in Oggioni. It was also done a 6h adherence assay to compare with the 2h assay and see if there is much difference later in the gene expression.

To initiate D39 adherence to basal cells it was necessary for the cells to achieve confluence. Depending on the wells size it could take 5-7 days to achieve this state and only after, the right concentration of bacteria was added ($1x10^7$ CFU/well). The SP282 basal cells sample with the adhered bacteria was supplied by one of the lab workers, Mina. The sample was ready to proceed to RNA extraction, and it was told that the concentration of the adhered D39 to these basal cells was around 1×10^4 CFU/ml.

After 2h of incubation, the number of adhered pneumococcus to different patients basal cells (SP282; 455; 430) was determined. The basal cells were counted using a Neubauer counting chamber.

Table 12. D39 viable counts and quantity of basal cells per well of each patient

Patient	Cells/well	CFU/well
SP282	-------*	$\approx 1{,}0 \times 10^4$
455	$1{,}8 \times 10^3$	$2{,}3 \times 10^3$
430	$1{,}4 \times 10^3$	$1{,}0 \times 10^3$

*The sample of this patient was gently provided by one of the lab workers, having only the bacteria concentration and no information about the concentration of cells per well.

3.3. Molecular results

3.3.1 RNA extraction using a High Pure RNA Isolation Kit (Roche).

RNA was recovered from $9.5x10^8$ CFUs *TIGR4* and $425x10^8$ CFUs *D39*.

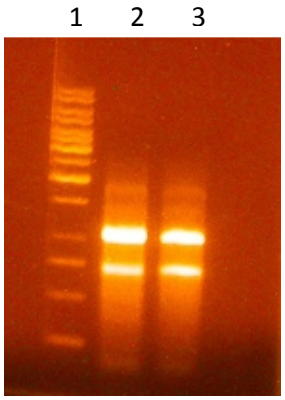

Fig 17. Photo of the electrophoresis gel after RNA extraction. 1 – Ladder 1 Kb; 2 – D39 RNA; 3- TIGR4 RNA.

3.3.2 RT-PCR using Light Cycler DNA Master Sybr Green Kit

Sensitivity test of the Lyght Cycler with different *S. pneumoniae* amounts was done with diluted RNA, using the expression of *gyrb* as the test RNA.

To test the sensitivity of the LightCycler q-PCR machine TIGR4 RNA was used. Beside D39 being the bacteria strain used in this research, TIGR4 was applied first in this experiment and as *S. pneumoniae* strain, also gives the same information about the sensitivity of the LightCycler to low RNA amounts.

Dilutions 4, 5, 6, 7, 8 and 9 demonstrated a similar behavior compared to the negative control, becoming difficult to analyze any expression.

Since D39 was the bacteria strain to be used in the next experiments it was better to perform the test with this kind of bacteria strain. The less amount of bacteria that was expected to have in the next steps of this research (cell adherence by *S. pneumoniae* to basal cells) was $1x10^3$ – $1x10^4$ CFUs, so only dilutions corresponding to these bacteria amounts (d5 and d6) were important to repeat and analyze.

The conditions were the same as the previous test but in order to analyze better the results of this q-PCR machine, the number of cycles was increased to 45.

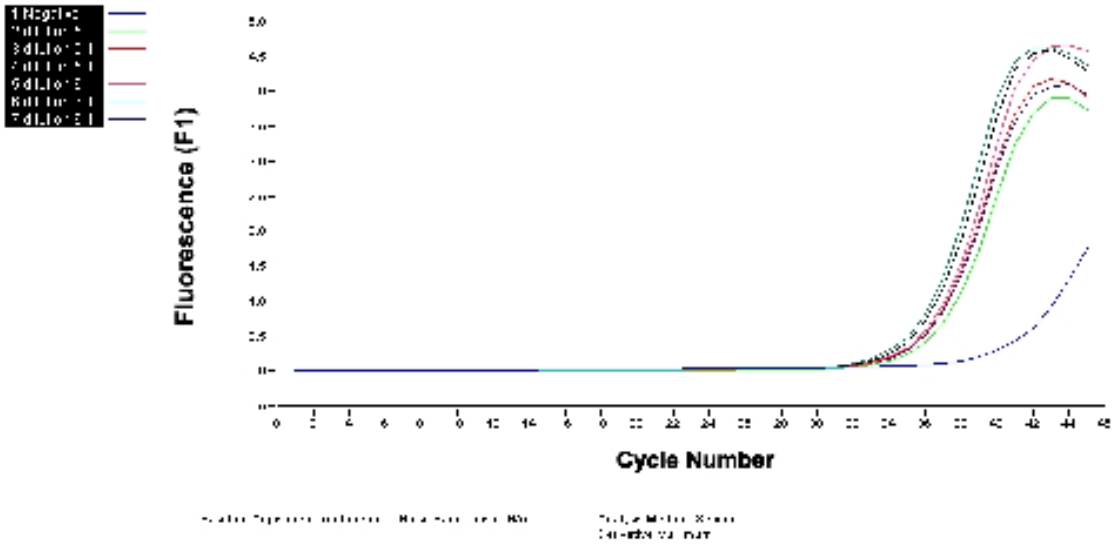

Fig.18. *Gyrb* quantification in different dilutions, using the Light Cycler q-PCR**.**

Increasing the cycle number to 45 cycles made the negative control start to rise up.

The initial amount of D39 frozen stocks was 8.4 x 10^8 CFUs. For each dilution 3 replicates were used.

Table 13. Crossing points of D39 diluted 5 and 6 times and the corresponding concentrations.

Sample	Bacteria amount (CFUs)	Crossing Point	Average
Negative control (Without template)	-------	>41.00	-------
Dilution 5		36.35	
Dilution 5	8,4 x 10^3	35.90	35.94
Dilution 5		35.56	
Dilution 6		35.94	
Dilution 6	8,4 x 10^2	35.28	35.72
Dilution 6		35.93	

The LightCycler software version used was the 3.5 and for quantification 2 analysis methods were available, the Second Derivative Maximum method and the Fit Points method.

Both methods refer to the log-linear phase of PCR for quantification. The reason is because only on this phase is possible to assume that all PCR reactions occur under comparable conditions. Besides using slightly different algorithms for calculation, these two methods are similarly appropriate for quantification. The second derivative maximum gives a quick and automated analysis, while the Fit Points is applied when the user contribution is required to calculation. The table below (Table 14) gives more information about these two methods.

Table 14. Features and steps required for the two different quantification methods.

Method	Features	Steps required
Second derivative maximum	• Automatic data calculation, no user influence. Only standard selection/de-selection to influence the quantification. No user input required. • No influence of background. • Fast calculation. • High reproducibility.	1. Baseline Setting (no influence on calculation, only viewing). 2. Data Analysis: Result Display.
Fit Points	• Data calculation is user-influenced: baseline adjustment, noise band setting, crossing line setting and choice of fit points are required. • Optimization of difficult standard curves is possible.	1. Baseline Adjustment. 2. Noise Band Setting. 3. Data Analysis: Crossing Point Determination and Optimization.

3.3.3. Gene expression

This experiment was of extreme importance since it was going to be finally analyzed the gene expression of D39 adhered to basal cells, demonstrating the most important virulence genes in *S. pneumoniae* adherence.

It was used basal cells from different patients, to analyze better the pneumococcal behavior and because the RT-PCR did not work with some cells, being necessary to repeat the experiment with other cells.

To calculate D39 crossing points, two calculation methods were available on the light cycler q-PCR software, as shown before in Table 14, second derivative maximum and fit points. The first one does the calculation independently from the user, but in some situations was difficult to detect expression by this method. In these cases, the manual method, fit points, was used.

Gene expression of D39 was studied on patient sp282 basal cells; in BEBM; in TSB; on patient 430 after 6h adherence to its basal cells and was studied also in the non adhered bacteria to patient 455 basal cells. D39 gene expression was analyzed using the relative quantification, applying the $2^{-\Delta\Delta Ct}$ method (explained in section 1.6). Three different reference conditions (D39 in three different media) were analyzed to calculate the expression levels: D39 in TSB, D39 in BEBM and non adhered D39.

3.3.4. TSB as reference condition.

Table 15. Gene expression ± standard error of D39 in different conditions, using TSB as the reference condition.

Conditions / Gene	D39 Adhered to sp282	D39 in BEBM	Reference condition: D39 in TSB	non adhered 455 D39	D39 Adhered to 430 6h
comX	6,93 ± 2,23	5,11 ± 0,84	1,00	3,70 ± 0,77	35,73 ± 0,49
nanA	21,22 ± 2,21	0,8332 ± 4,03	1,00	19,25 ± 0,47	2,85
nox	10,61 ± 2,20	2,42 ± 0,99	1,00	13,49 ± 1,47	286,85 ± 2,33
sodA	1,16 ± 0,71	1,32 ± 0,78	1,00	1,01 ± 0,23	9,62 ± 1,24
ply	56,98 ± 0,66	27,03 ± 3,19	1,00	95,89 ± 0,51	34,14 ± 1,36

Expression levels above 2 are considered as significantly up regulated genes and are represented with red, below 0,5 are considered down regulated and are represented with green, and between 0,5 and 2 are not significant.

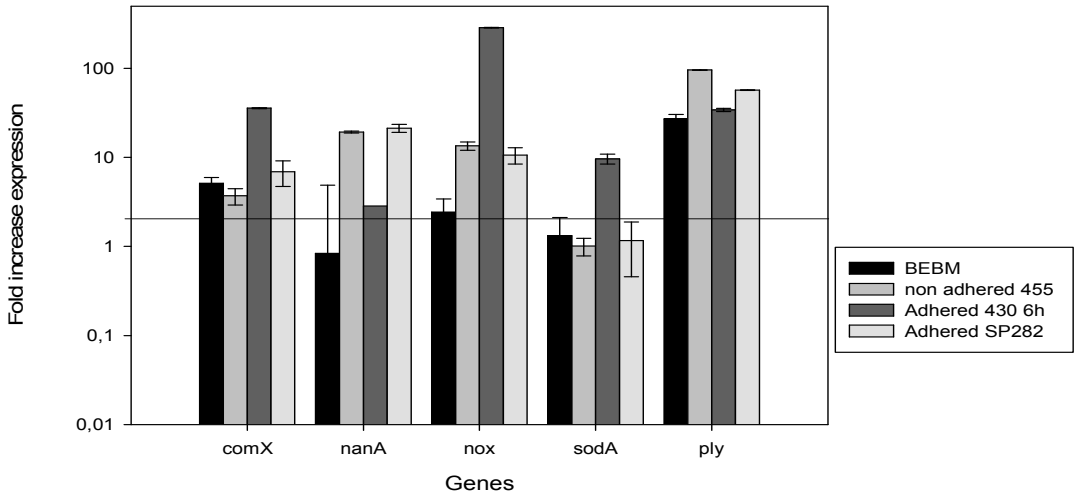

Fig.19. Fold increase in D39 expression using as reference condition D39 in TSB. Any expression level above 2 (black gridline) is related to upregulated genes.

Using as the reference, D39 grown to mid exponential phase in TSB, an increased expression of all genes was obtained, except for *nanA* in BEBM and *sodA* in D39 adhered to SP282 basal cells; D39 not adhered to 455 basal cells and in BEBM. *NanA* and *ply* were the most expressed genes, showing higher expression levels compared to the rest of the genes. In this chart (Fig.19) was applied a logarithmic scale. With a normal scale would be difficult to analyze and compare the results, since some expression levels were really high (*nox* in D39 after 6h adherence).

3.3.5. BEBM as reference condition.

Table 16. Gene expression ± standard error of D39 in different conditions, using BEBM as the reference condition.

Condition / Gene	D39 Adhered to sp282	Reference condition: D39 in BEBM	D39 in TSB	non adhered 455 D39	D39 Adhered to 430 6h
comX	1,36 ± 2,23	1,00	0,20 ± 1,34	0,72 ± 0,77	7,00 ± 0,49
nanA	25,47 ±2,21	1,00	1,20 ± 4,29	23,10 ± 0,47	3,42
nox	4,39 ± 2,20	1,00	0,41 ± 1,91	5,58 ± 1,47	118,60 ± 2,33
sodA	0,88 ± 0,71	1,00	0,76 ± 0,99	0,76 ± 0,23	7,28 ± 1,24
ply	2,11 ± 0,66	1,00	0,04 ± 1,16	3,55 ± 0,51	1,26 ± 1,36

Analyzing D39 in BEBM as the reference condition, it was possible to see some differences, comparing to D39 in TSB as reference condition.

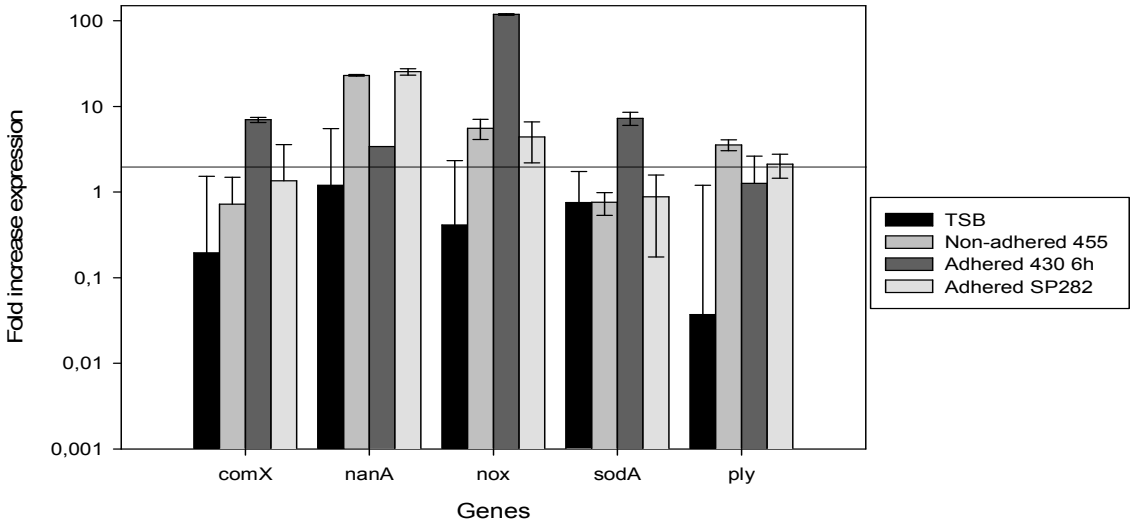

Fig.20. Fold increase in D39 expression using as reference condition D39 in BEBM. Any expression level above 2 (black gridline) is related to upregulated genes.

The D39 adhered to SP282 basal cells and the non adhered D39 to 455 basal cells had a similar expression. *ComX* and *sodA* did not show any significant expression levels. The

genes *nanA*, *nox* and *ply* were as well upregulated, representing *nanA* the most expressed gene.

The adherence by D39 to basal cells of the patient 430 after 6h showed an upregulation of all genes except for *ply*. *Nox* was the gene that demonstrated the highest expression level.

In liquid culture as TSB, *comX*, *nox* and *ply* were downregulated, and the rest were not significant.

3.3.6. Non adhered D39 to 455 basal cells as reference condition

Table 17. Gene expression ± standard error of D39 in different conditions using as reference condition non adhered D39 in presence of basal cells from patient 455.

Condition / Gene	D39 Adhered to sp282	D39 in BEBM	D39 in TSB	Reference condition: non adhered D39 to 455	D39 Adhered 6h to 430
comX	1,87 ± 2,23	1,38 ± 0,84	0,27 ± 1,34	1,00	9,67 ± 0,49
nanA	1,10 ± 2,21	0,04 ± 4,03	0,05 ± 4,29	1,00	0,15
nox	0,79 ± 2,20	0,18 ± 0,99	0,07 ± 1,91	1,00	21,26 ± 2,33
sodA	1,16 ± 0,71	1,31 ± 0,78	0,99 ± 0,99	1,00	9,56 ± 1,24
ply	0,59 ± 0,66	0,28 ± 3,19	0,01 ± 1,16	1,00	0,36 ± 1,36

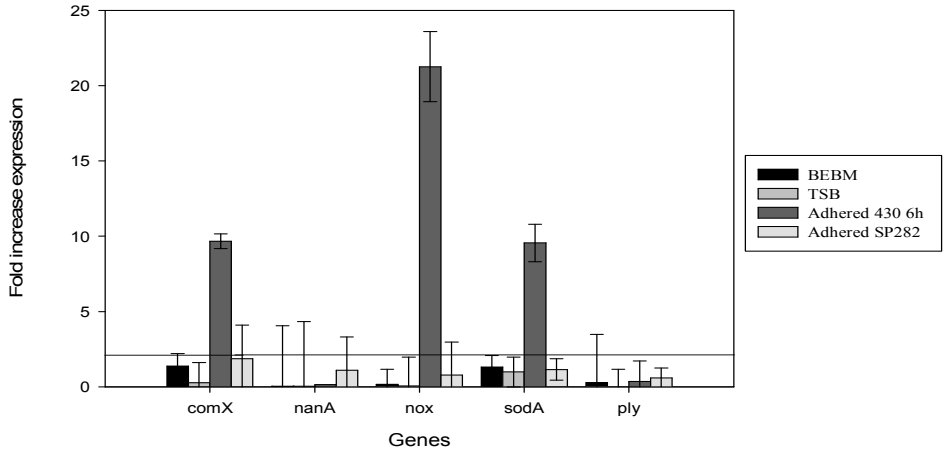

Fig.21. Fold increase in D39 expression using as reference condition the non adhered D39 in presence of 455 basal cells. Any expression level above 2 (black gridline) is related to upregulated genes.

Choosing as reference condition the non adhered D39 to 455 basal cells, it was possible to see that only the adherence after 6h by D39 to 430 basal cells showed upregulation for some genes. *ComX*, *noX* and *sodA* were upregulated, *nanA* and *ply* were downregulated.

There was no significant expression analyzing D39 adherence to patient sp282 basal cells.

In liquid culture as BEBM, all genes were downregulated except for *comX* and *sodA*, these two were considered not significant in what it comes to expression levels. None of the target genes were upregulated in this media culture.

In TSB liquid culture, the genes showed a low expression except for *sodA* demonstrating non significant expression. Once again there was no upregulation of any gene.

4. Discussion

The principal aim of this research project was to study and analyze pneumococcal gene expression when bacteria adhere to human nasal cells and to compare with other infection models. For that purpose the first step was to perform bacterial stocks of *S. pneumoniae*. The bacteria were grown in TSB, liquid culture medium until the mid exponential phase. According to Tomasz and Hotchkiss, 1964, the competence induction in *S. pneumoniae* takes place at a particular cell density in exponential growth cultures. After performed the bacteria stocks, pneumococci concentrations of 1 x 10^8 to 1 x 10^9 CFU were obtained, and the bacteria were frozen with 10% glycerol at -80ºC.

D39 is more virulent than TIGR4, that was one of the reasons why it was used in this research project. The other was to compare to Oggioni's infection with TIGR4 in lung and to see if the gene expression was a typical behavior from that strain or if it was verified in more strains.

4.1. Biofilm model by Oggioni *et al*., 2006

Oggioni 2006 reported that pneumococci detached from microtitre wells (biofilm) become more virulent when infecting human nasal cells than the liquid culture pneumococci. It was demonstrated before that the quorum-sensing peptide, CSP, induces the competence system of *S. pneumoniae* and not only induces biofilm formation *in vitro* but also increases pneumococci virulence *in vivo* (Tomasz, 1965; Oggioni *et al*., 2006). These were some of the reasons why biofilm model was chosen as an important way of inducing pneumococcal virulence on this research. Besides following every step as in Oggioni *et al*., 2006, at the end of this experiment it was not possible to have enough detached bacteria to proceed. There were a lot of possible reasons that could lead to these results, like the CSP solution used was not in the right conditions, the sonication machine was not working properly or the incubator was not with the required CO_2 level. The biofilm model was put aside while other options were analyzed.

4.2. Apical fluid

The Apical fluid was an interesting option to study D39 gene expression and to compare to the adhered D39 to nasal cells. A survival growth curve of D39 in the apical fluid was

done to understand better the bacteria behavior in this new medium. Low dilutions of the apical fluid demonstrated a decrease on the pneumococci survival growth leading to bacteria death after some incubation time, while higher dilutions did not kill *S. pneumoniae*. These results pointed for the presence of something on the apical fluid that influenced the bacteria survival growth. One possible option was antibiotic, considering that streptomycin and penicillin were added before for the cells growth of these patients. To test this option an ELISA was done with anti-streptomycin and anti-penicillin antibodies. The final results showed streptomycin presence, beside not possible the right quantification. It was not possible to confirm penicillin presence, once the anti-penicillin antibody was having some problems to bind to the antigen, even on the standard curve. A resistance test was done before in blood agar plate, showing an inhibition of pneumococci growth in the presence of these two antibiotics, confirming that was the streptomycin presence on the apical fluid that influenced bacteria growth and caused death on the lowest dilutions. It is known the antibiotics influence on bacteria gene expression, becoming not appropriate to continue with this experiment, once the expression would be different and not possible to compare with the adhered bacteria.

4.3. *S. pneumoniae* adherence to basal cells and its gene expression

Finally adherence of D39 on basal cells was studied. Three reference conditions for the gene expression analysis were applied: *S. pneumoniae* gene expression in TSB, BEBM and the non adhered *S. pneumoniae.* The standard errors of each gene were calculated and added to the expression levels. In the adhered bacteria to patient 430h after 6h was not possible to calculate the standard error to *nanA* due to not having enough sample from this patient to analyze more than one replica of this gene.

TSB as control demonstrated that *ply* was the gene with the highest expression levels in the different conditions, except for the 6h adhered pneumococcus, where *nox* was the gene with higher expression.

In conditions like pneumococcus adhered to patient sp282 basal cells, non adhered bacteria to patient 455 basal cells, all virulence genes were upregulated except for s*odA*. The bacteria only in BEBM medium revealed an upregulation for genes like *comX*, *nox*

and *ply*. In pneumococcus adherence after 6h incubation to patient 430 basal cells all genes were upregulated including *sodA*.
Ply was the gene with the highest expression levels in all conditions except for the bacteria adhered for 6h to 430 patient basal cells.
Pneumococcal adherence after 6h incubation demonstrated all genes upregulated, being *sodA* de highest one.

Using BEBM as reference condition, it was possible to observe that adhered bacteria to patient sp282 and the non adhered pneumococcus to patient 455 had similar expression levels. Most of the genes were upregulated in adhered sp282 and non adhered 455 bacteria, except for *comX* and *sodA*. In TSB liquid culture all genes were down regulated except for *sodA* and *nanA* that had a not significant expression. Interesting to analyze was the pneumolysin gene expression in the adhered bacteria to patient 430 after 6h, showing a not significant expression. Excluding in the TSB, in the rest of the different conditions, this gene was always upregulated, but after 6h adherence decreased its expression to a not significant level and *nox* was the gene with highest expression level.

At last, analyzing pneumococcal expression levels with the non adhered bacteria as reference condition, only after 6h adherence to patient 430 it was possible to see upregulated genes. All genes were upregulated except for *nanA* and *ply*. *Nox* showed the highest expression level. In The rest conditions the genes were down regulated or with a non significant expression.

Analyzing these three different reference conditions we conclude that BEBM was the best reference condition to use. As the medium used to grow the basal cell culture, BEBM is a better control to analyze pneumococcal expression. A good reference control should be a medium where all conditions are present except the one that is suppose to study, in this case the bacteria adhered to basal cells. The non adhered bacteria was considered a not good reference condition, beside analyzing expression of not adhered pneumococcus, the basal cells still present in the medium, influencing in the same way pneumococcal expression levels. TSB would be a better control if the medium used to grow the basal cells was this one. With all this information, the best

way to analyze pneumococcal expression in adherence to basal cells is applying the medium used to grow these last ones as reference condition, namely BEBM.

Since BEBM was chosen as the best reference condition to study pneumococcal gene expression, was fundamental to make a deeper analysis to D39 genes in this reference condition:

In TSB all genes were downregulated, except *soda* and *nanA* that had a not significant expression level.

NanA, *nox* and *ply* were upregulated in adhered bacteria and in the non adhered bacteria (adhered to sp282, non adhered to 455). Beside not adhered to the basal cells, the pneumococcus not adhered to 455 had similar expression levels to the adhered sp282. These results suggest that the presence of the basal cells influence pneumococcal expression, even if the bacteria are not adhered. Not only being in presence of basal cells, but also in presence of other adhered bacteria, may influence the non adhered pneumococci to express similar levels as the adhered bacteria.

As shown in Oggioni *et al*., 2006, the neuraminidase protein (*nanA*) and genes related to oxidative stress (*nox*) are important virulence factors in *S. pneumoniae* colonization, agreeing with the fact that these were considered as up regulated in this research.

With an important role in *S. pneumoniae* colonization and with the essential function of increasing the receptors number for pneumococcal adherence (King *et al*., 2005; Cámara *et al*., 1994), it was not surprising that *nanA* demonstrated the highest expression level in this adherence assay.

Other gene of extreme importance in nasopharynx colonization is *ply*, the gene for the pore forming toxin. Several publications have reported the importance of this gene as a virulence key and demonstrated that when using a pneumolysin-deficient mutant, *S. pneumoniae* was less able of colonization (Rubins *et al*., 1998; Hirst *et al*., 2004). This information gives support to the high expression levels achieved by *ply* in the epithelial cells adherence results, specifically in the adhered bacteria to sp282 and non adhered bacteria to 455.

SodA, being a gene related to the oxidative stress, was also important to study in this adherence assay. This gene has an important role in the elimination of many oxygen radicals that can cause several damages to the bacteria. It is known that the aeration conditions influences the amount of *sodA*, detecting more activity from this gene in

aerobic conditions than in anaerobic (Yesilkaya *et al*., 2000). The adherence assay was done in an anaerobic incubator enriched with 5%CO_2. The fact that the oxygen level was low could be the reason why this gene did not show any significant expression level in the adhered bacteria to sp282 and in the non adhered bacteria to 455. In Oggioni's study, the pneumococcal gene expression was analyzed *in vivo*, in lung. The pneumococcus was in presence of a constant gas exchange, demonstrating a high expression level for genes related to oxidative stress. In the present research, the gene expression was analyzed *in vitro* conditions, being this a possible reason for the not significant expression level of *sodA*.

Beside *nox* being related to the oxidative stress too, and responsible for the elimination of some of the oxygen radicals as *sodA*, this gene was upregulated in the adherence assay. Auzat *et al*., (1999), reported that using a *nox*-deficient mutant no differences were observed in pneumococcus behavior under anaerobic or aerobic conditions, although, competence efficiency during pneumococcus growth, for genetic transformation, is clearly affected. The high expression level of this gene may be related with the competence of *S. pneumoniae*. After 6h incubation in the adhered bacteria to 430, this gene is more affected, showing a high expression level of 118,60 ± 2,33.

The 6h adherence of *S. pneumoniae* to 430 may be not the right time to analyze pneumococcus gene expression. The epithelial cells may be damaged after all this time incubation, due to pneumolysin and other components released by the bacteria to the medium. The stress can stimulate a response by the epithelial cells that may influence pneumococcus expression levels.

Beside the standard errors in some genes being significant high, the chosen reference condition revealed clearly the upregulated genes, with no doubt that these three (*nanA*, *sodA* and *ply*) demonstrated a high expression levels. Maybe for *comX* was not that clear, the standard error could mean a possibility of this last being upregulated. Considering that we used 3 samples per gene, in future researches we suggest much more replicas per gene, in order to have more data to minimize the standard error. It would be also interesting to analyze pneumococcal gene expression in adherence to ciliated nasal epithelial cells and compare with the basal cells. We suggest to future researches to analyze more genes, also studied and upregulated in Oggioni *et al*., (2006) publication, as *lytA* (autolysin gene); *comA* (ABC transporter for CSP); *mgrA* (virulence

gene regulator) (Oggioni *et al*., 2006; Oggioni *et al*., 2004). The pneumococcal adherence with the *ply* mutant would be an interesting research as well.

According to all results achieved and to the complementary references, it was shown in this final master thesis that *nanA*, *nox* and *ply* are genes with an important role in *S. pneumoniae* virulence, when this last one is adhered to Human nasal basal cells.

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