Phenotypic characterisation of pneumococcal serotype-1 variants presenting low haemolytic activity

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

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Declaration

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where states otherwise by reference or acknowledgment, the work presented is entirely my own.

Abstract

Background: *Streptococcus pneumoniae* is a significant human pathogen responsible for life-threating diseases such as pneumonia, septicaemia and meningitis. Of the nearly 100 distinct pneumococcal serotypes, pneumococcal serotype 1 is described as one of main causes of invasive disease worldwide. One highly enigmatic feature of serotype 1 resides in its being rarely detected in human nasopharyngeal specimens.

Aim: The aim of this project was to compare serotype 1 lineage A (ST306) and C (ST615) with respect to their immunological and virulence properties using both in vitro and in vivo tools, as well as their differential gene expression profile.

Methods: A series of *in vitro* experiments were performed to assess the ability of serotype 1 to adhere and invade epithelial cells, and to determine its ability to inhibit phagocytosis and gain insight into the mechanisms involved. In parallel, three *in vivo* standardized mouse models of pneumococcal infection were exploited to examine the virulence properties of ST306 and ST615 and their ability to colonise the nasopharyngeal tissue. Finally, RNA-seq analysis of *in vitro* cultures of ST306 and ST615 was carried out in an attempt to identify differential expression patterns.

Results: ST615 serotype 1 was determined to be highly virulent, causing the death of 80-100% of infected mice by around 48h post-infection, while all mice infected with ST306 survived when using pneumococcal doses inductive of invasive pneumonia model. In a nasopharyngeal carriage mouse model, ST306 serotype 1 was shown to be able to establish colonisation persisting up to 28 day post-administration, although at a much lower density compared to ST615. While ST615 was capable of establishing nasopharyngeal colonization, clearance occurred earlier at day 14 compared to ST306. RNA gene expression analysis focused on virulence factors and critical biological functions determined that ST615 serotype 1 presented a profile consistent with its weak colonisation and its invasive properties. The genes associated with capsule synthesis were not differentially expressed between ST615 and ST306 but that other virulence factors such as *psp*, *pav*A, *ply* and *cpbD* were differentially expressed.

Conclusions: Although ST306 and ST615 possesse a unique and virulent capsule, ST306 was not able to cause pneumonia to mice model.

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Abbrreviation

APC = Allophycocyanin

ATP = adenosine triphosphate

BAB = Blood agar base

BCA = Bicinchoninic acid assay

BEAS-2B = Human bronchial-epithelial cell line

BHI = Brain heart infusion

Bp = Base pairs

CAP = Community acquired pneumonia

CBP = Choline-binding protein

CD = Cluster of differentiation

CDC = Cholesterol-dependent cytolysin

CFU = Colony forming units

CLN = Cervical lymph nodes

CP = Capsular polysaccharides

CPS = Capsule locus

CSF = Cerebrospinal fluid

DMEM = Dulbecco’s Modified Eagle’s Medium

DMF = Dimethylformamide

DMSO = Dimethyl sulfoxide

DNA = Deoxyribonucleic acid

DPBS = Dulbecco's phosphate-buffered saline

ECM = Extracellular matrix

EDTA = Ethylenediaminetetraacetic acid

EEP = Early exponential phase

ELISA = Enzyme-linked immunosorbent assay

EMBL-EBI = European Bioinformatics Institute

ESP = Early stationary phase

Fc = Fragment cristallizable

fcR = Fc receptor

FCS = Foetal Calf Serum

FI = Fluorescent intensity 2

FITC = Fluorescein isothiocyanate

HBSS = Hanks' Balanced Salt Solution

HIV = Human immunodeficiency virus

Hyl = Hyaluronidase lyate

Ig = Immunoglobulin

IL = Interleukin

INF = Interferon

IPD = Invasive pneumococcal disease

IVIG = Intravenous immunoglobulin

KC = Keratinocyte chemoattractant

KEGG = Kyoto Encyclopedia of Genes and Genomes

LEP = Late exponential phase

LSP = Late stationary phase

Lyt = Autolysin

MEM = Minimum Essential Medium

MEP = Mid-exponential phase

MIP = Macrophage Inflammatory Protein

MLST = Multi locus sequence type

MHC = Major histocompatibility complex

MRC = Medical research council

NADH = Nicotinamide adenine dinucleotide

NALT = Nasal associated lymphoid tissue

Nan = Neuraminidase

NCTC = National collection of type cultures

NLRP3 = NOD-like receptor family pyrin domain containing 3 inflammasome

OD = Optical density

OPKA = Opsonophagocytic killing assay

OR = Odds ratio

PAGe1 = Pneumococcal African Genomic Consortium 1

PavA = Pneumococcal adherence and virulence factor A

PBS = Phosphate-buffered saline

PCho = Phosphorylcholine

PCR = polymerase chain reaction

PCV = Pneumococcal conjugate vaccine 3

PE = Phycoerythrin

PE/Cy7 = Phycoerythrin/Cyanine dye7

pIgR = Polymeric immunoglobulin receptor

PLNA = Pneumolysin deficient pneumococcus

Ply = Pneumolysin

PPV = Pneumococcal polysaccharide vaccine

PsaA = Pneumococcal surface adhesion

PspA = Pneumococcal surface protein A

PTS = Phosphotransferase system

RNA = Ribonucleic acid

RPMI = Roswell Park Memorial Institute medium

Sec = Secretion systems

SNP = Single nucleotide polymorphism

SP = *Streptococcus pneumonia*

SpxB = Pyruvate oxidase

Srt = Sortase

SSA = Sub-Saharan Africa

ST = Sequence type

TGF = Transforming growth factor

THP-1= Human monocytes

THY= Todd-Hewitt Broth plus 0.5% Yeast Extract

TLR = Toll-like receptor

TNF = Tumour necrosis factor

TOD = Time of death

TPM = Transcripts per million

UK = United Kingdom

USA = United States of America

WHO = World Health Organisation

Xpt = xanthine phosphoribosyltransferase

ZPS = Zwitterionic polysaccharides

Introduction

Introduction

1.1 General characteristics

1.1.1 History of *Streptococcus pneumoniae*

S. pneumoniae was first isolated over a hundred years ago by George Miller Sternberg [11] in the United States and by Louis Pasteur [12] in France. Pasteur isolated *S. pneumoniae* in the saliva of a patient with rabies, while Sternberg isolated the bacteria from his own saliva. A subsequent six years elapsed before Weichselbaum, in Vienna, resolved the disagreement between Carl Friedlander and Albert Fraenkel as to the importance of *S. pneumoniae* as a cause of bacterial pneumonia in humans [13]. In the 1880s, pneumococcus was confirmed as a cause of meningitis, endocarditis, arthritis, and otitis media, and experimental pneumococcal endocarditis was successfully modelled in the rabbit [14]. Between 1880 and 1910, advances were made towards the control of illness caused by pneumococcus; including the recognition by the Klemperers [15] that antiserum could offer a protective value against infection. Following this in 1900, the lytic effect of bile on pneumococcus was discovered and reported by Neufeld [16], which aided their identification. Neufeld and Haendel [17] succeeded in classifying pneumococcal type I and II by 1910. The quelling reaction, in which antibodies can bind to the bacterial capsule of *S. pneumoniae*, became an essential platform in serotherapy three decades later [19], allowing for different pneumococcal types to be visualised under a microscope, previously described by Neufeld in 1902 [18].

Serum therapy of type I and II pneumococcal pneumonia, developed at the Rockefeller Institute [20], led to treatment of those infected with a number of pneumococcal serotypes, with a case-fatality rate reduction of approximately 20%. However, severe haemolytic reactions were observed by Paul Beeson and Walter Goebel [21] attributed to the cross-reactivity of type 14

pneumococcal capsular polysaccharide and type A blood group following treatment with horse antiserum. This led to rabbit antiserum replacing horse antiserum towards the end of the 1930s.

Quinine, which had previously been used to treat experimental pneumococcal infection with little effect, led to the exploration of related compounds, including ethylhydrocupreine hydrochloride (optochin). Optochin showed significant efficacy in laboratory animals, however drug resistance soon began to arise [23, 24] in the form of optochin-resistant pneumococci from treated infected mice in 1912 [22]. Optochin was subsequently abandoned as a treatment for human pneumococcal infection for this reason, as well as in relation to apparent eye toxicity.

Sulfonamides, while initially showing promise due to their ability to treat pneumococcus indiscriminately according to its capsular serotype, was over-shadowed in 1944 [25, 26] due to the drug penicillin showing extraordinary efficacy in the treatment of patients with both non-bacteremic and bacteremic pneumococcal pneumonia, bringing about recovery and reducing case-fatality rate to 5 – 8%. The introduction of tetracyclines, chloramphenicol, and macrolides in the following decade gave further offers of protection, shifting a disease which has been previously feared to one which gave little cause for concern.

In 1943, several laboratory findings reported the appearance of pneumococcal variants with increasing resistance to penicillin. Tillet [26], and Schmidt and Sessler [27] demonstrated resistance in isolations from mice, while Eriksen [28] demonstrated resistance *in vitro*, both following exposure of pneumococci to penicillin. However, these observations appeared to have little effect on clinical practice.

In the early 1960s, reports of resistance to penicillin, as well as tetracyclines, chloramphenicol, macrolides, and cotrimoxazole, began to make more of an impact. Toward the end of the 1970s, an outbreak in a hospital in South Africa [29] of the 19A pneumococcal infection exhibiting

multiple drug resistance occurred, and since then, the complications of infections caused by multiple drug-resistance pneumococci has become a progressively important issue.

To date, vancomycin is currently the only antimicrobial agent that has yet to select drug resistant mutants in pneumococcal infection [40].

In South Africa in 1886, due to the high rate of recruit disease in the mining industry, in the form of lobar pneumonia, and related morbidity and mortality, Sir Almroth Wright, renowned for his work on typhoid vaccine, was recruited to conduct trials with a pneumococcal vaccine of an unknown serotype. The vaccine trial included 50,000 miners [30] and was initially concluded as efficacious, however, subsequent analysis of the data failed to support this. Following this, F. Spencer Lister, a colleague of Wrights', continued with the trial and aimed to characterise the serotypes causing disease. He hypothesised that if one were to immunise 50% of the individuals in a closed population, it would reduce spread of infection among the unimmunised [33]. However, issues with the structure of these trials with the whole killed pneumococci vaccine were disputed and therefore efficacy of immunisation could not be properly demonstrated [31, 32].

In 1927, mice were successfully immunised with homotypic capsular polysaccharide (Schiemann and Casper) [34], followed by reports of immunogenicity in humans in 1930 (Francis and Tillett) [35]. In 1945, purified pneumococcal capsular polysaccharides proved to be effective in preventing infection with homotypic pneumococcal strains (MacLeod, Hodges, Heidelberger, and Bernhard) [36]. It also confirmed Lister's hypothesis that immunised individuals reduced the spread of pneumococcal disease in a closed population. The carriage of pneumococci also proved to be important. Immunisation did not appear to impact on the carrier state, but reduced the likelihood of an individual becoming a carrier after vaccination

by around 50%. Recent studies of several polysaccharide vaccines have also confirmed this finding.

Following this, two hexavalent pneumococcal polysaccharide vaccines were commercially produced, however with the introduction of highly effective penicillin and other agents, they were soon withdrawn from the market. Based on trials in South Africa showing dodecavalent vaccines had the ability to prevent both non-bacteremic and bacteremic pneumococcal pneumonia by approximately 80% [38], a tetradecavalent pneumococcal vaccine was licensed in South Africa in 1977. Other findings regarding the outcome of bacteremic pneumococcal infection treated with penicillin and identification of individuals at particular risk of mortality also had an impact on the re-emergence of vaccine trials for pneumococcus [37]. In 1983, the 23-valent vaccine was introduced. Since only 18 serotypes out of the then known 90 pneumococcal types have been shown to be responsible for the majority of bacteremic illnesses, the opinion was that the introduction of additional serotypes into the 23-valent vaccine showed little necessity.

1.1.2 *Streptococcus pneumoniae* general characteristics

Streptococcus pneumoniae (or pneumococcus) is a well-known commensal bacterium of the upper respiratory tract and can colonise the human nasopharynx asymptomatically. However, it is an opportunistic pathogen and is a major cause of disease in the susceptible host, including pneumonia, meningitis, bacteremia, otitis media and sinusitis [1, 2, 3]. *S. pneumoniae* is a Gram-positive bacterium with a thick cell wall, containing teichoic acid (C-polysaccharide), capable of producing toxin (pneumolysin). The organism is an encapsulated bacterium, an aero-tolerant, anaerobic pathogen (capable of growing in the absence or presence of oxygen), oval

to spherical in shape, with a diameter ranging from 0.5-1.25 µm. Its usual appearance is in pairs (diplococcus) or forming short chains, shown in figure 1 [8, 472]. *S. pneumoniae* has a variety of surface antigens, including pneumococcal surface adhesin A (PsaA), pneumococcal choline-binding protein A (PcpA), pneumococcal surface protein A (PspA), pneumococcal surface protein C (PspC), and pneumococcal autolysin A (LytA). It is a non-spore forming, non-motile, catalase negative, α-haemolytic bacterium. The ideal growth conditions include complex media with a source of catalase (i.e. from blood or serum), incubated at 37°C/5-10% CO_2 [9]. *S. pneumoniae* is sensitive to ethyl hydrocupreine hydrochloride (optochin), an antibiotic used to differentiate pneumococcal from other streptococci. Observation of a growth inhibition halo around the optochin disk indicates detection and identification of *S. pneumoniae* [8]. The haemolytic properties on blood agar can also help classify *S. pneumoniae* and differentiate from others in the Streptococci family; the presence of α-haemolytic bacteria causes a chemical change in the haemoglobin of red blood cells, observed by a green pigment that forms a ring around the colonies [10].

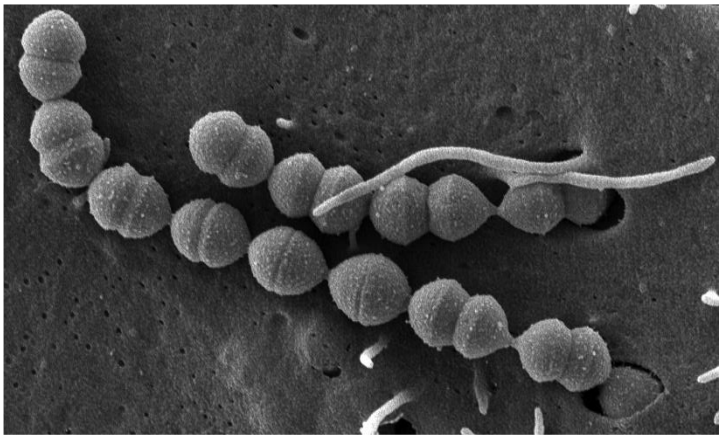


Figure 1. *S. pneumoniae* is a gram-positive bacterium. Its usual appearance is in pairs (diplococcus) or forming short chains. (SEM picture credit: Dr Manfred Rohde, University of Greifswald).

1.1.3 Carriage of *S. pneumoniae*

Carriage of *S. pneumoniae* is found at a higher rate in children compared to adults; the rate of colonisation increases from birth, peaking at the age of around 1 – 2 years old. Following this there is a decline which is age-related [150, 179].

S. pneumoniae carriage typically lasts two weeks, with a duration of more than 30 weeks in some cases [180]. Carriage is also seasonal, peaking between January to March [181]. Bronchial pneumonia and lobar pneumonia are both caused by *S. pneumoniae*. During infection, the organism induces an immune response from the host, causing recruitment of white blood cells which migrate to the lungs. Pneumonia presents as the presence of white blood cells, proliferating bacteria and excessive fluid in this area [184]. A chest X-ray can detect pneumonia, and bacteraemia and septicaemia may occur in around 20 – 30% of cases [185]. The highest fatality rates among those infected with the organism are those presenting with *S. pneumoniae* meningitis (~40%), with symptoms of severe headache, photophobia, neck stiffness and fever, often with long-term consequences in survivors including mental retardation and motor deficiency [186, 187]. Otitis media is a common disease occurring with *S. pneumoniae* infection, presenting as inflammation of the middle ear, with limited mobility and enlarged tympanic membrane, often with recurrent episodes of infection, even despite successful antibiotic treatment. *S. pneumoniae* was found responsible for around 50% of otitis media cases [185]. Other relatively uncommon clinical illnesses known to be caused by *S. pneumoniae* include conjunctivitis, acute tracheobronchitis, endometritis, peritonitis, endocarditis, arthritis, and osteomyelitis [188].

In children below the age of 2 years old, pneumococcal carriage was reported at 45% before the seven-valent polysaccharide conjugate vaccine was introduced in the UK. This was compared to 8% in adults [223].

1.1.4 Transmission of *S. pneumoniae*

Humans are the main host of *S. pneumoniae* due to its successful transmission, occurring through respiratory droplets from carriers of healthy individuals or those with pneumococcal disease [189, 190]. Risk factors for transmission of the organism include number of siblings and numerous visits to the doctor for other mild upper respiratory tract infections [190]. Day centres, military camps, and prisons where there are high numbers of individuals in a closed environment also have high rates of cases [192, 189]. Pneumococcus may be transmitted from children attending day centres, to their older siblings, demonstrated by similarities among isolates of genetic similarity [192]. Pneumococcus is first transmitted to the new host, with subsequent asymptomatic colonisation [180]. Following this, in some cases, the organism may move from the nasopharynx to other areas of the body, including the lungs where pneumococcus can successfully evade the host immune system defences and cause disease [189]. Since acquired pneumococcus is often asymptomatic, it proves difficult to identify the chain of transmission between individuals [197]. Other microflora of the nasopharynx may contribute to the development of disease by symbiosis, or hinder it by competing with the organism [198, 199]. Different serotypes of pneumococcus, such as serotypes 1, 4, 5 and 9A have high rates of related invasive disease, while serotypes 9N, 16F, 20 and 38 have relatively lower rates and have a shorter carriage duration [190]. Opaque and transparent colonies related to phase variation of pneumococcal variants demonstrate as an important factor in the progression of carriage to invasive disease [200, 201]. For example, the opaque form has been commonly isolated from patient samples, while the transparent form has adapted to colonisation of the nasopharynx due to phase variation to increase pneumococcal invasion into human brain microvascular endothelial cells [202].

1.1.5 Invasive pneumococcal disease (IPD)

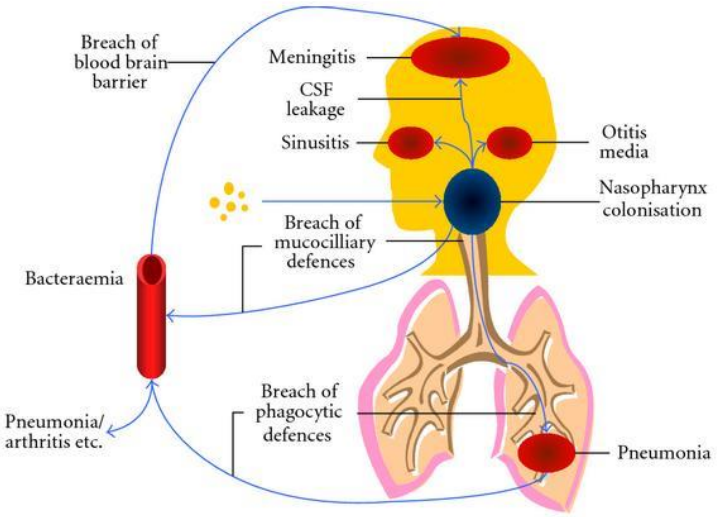


Figure 2. Pathogenic route of *S.pneumoniae* infection [421]

S. pneumoniae initially colonises the nasopharynx, spreading via the airways to the lower respiratory tract, leading to pneumonia, or to the sinuses or middle ear, resulting in medical morbidity [91, 408]. Invasive infections caused by *S. pneumoniae* amounts to approximately 15-30 cases per 100,000 of the population per year in developed countries, with those above 65 years of age with an estimated rate of more than 50 per 100,000, and those below the age of 2 years estimated at more than 150 cases per 100,000 people [407, 410]. These figures are likely to be an underestimation, since they are based only on blood culture results so do not take into account cases diagnosed without a diagnostic blood culture [413]. Pneumococcal disease presents a worldwide public health problem, underlined by the progressively spreading antimicrobial drug resistance to widely used, essential therapies. Vaccination remains the only method to prevent disease caused by *S. pneumoniae* [412]. Individuals particularly at risk of IPD and other pneumococcal disease include children less than 2 years of age, the elderly, children between 2 to 5 years old, and the immunocompromised [377, 378]. *S. pneumoniae* is a major cause of morbidity and mortality, particularly in the developing world, with an

estimated 1.2 million children below the age of 5 years dying each year from pneumonia, approximately 10-30% of all deaths among this age group [405]. Of adults with pneumococcal bacteriaemia, 60-85% of cases were associated with pneumonia, 5-10% with meningitis, and 5-10% with no focal signs of infection [407, 410, 411].

1.1.6 Diagnosis of *S. pneumoniae* and laboratory typing schemes

Since *S. pneumoniae* is present in humans as commensal flora, this has to be taken into consideration in any diagnostic approaches when identifying the organism [93]. Other streptococcus bacteria are also present in the nasopharynx, as well as other organisms such as *Staphylococcus aureus*, so distinguishing between species and other types of bacteria remains important [191]. Gram-negative rods and staphylococci are generally gentamicin-sensitive, whereas pneumococcal and other streptococcus species tend to be gentamicin resistant, therefore the isolation of pneumococcus via the use of gentamicin containing blood agar culture plates can be useful [193-196]. Other streptococcal species, such as *S.mitis* and *S. oralis* tend to be resistant to optochin and bile-insoluble, while *S. pneumoniae* is generally sensitive [191]. Whilst *S. pseudopneumoniae* is generally found to be bile insoluble, it is only optochin resistant in 5% CO_2, but this is not the case if left in room air rather than left within an incubator [209, 210]. *S. pneumoniae* can be identified by its morphology under a microscope, observing its Gram-positive stain, and its diplococcic shape and presentation. Colony morphology and phenotypic identification using α-haemolysis on blood agar can also be used, as well as testing for catalase negativity, optochin susceptibility, and bile solubility [191]. Identification can prove to be difficult for *S. pneumoniae* since some species of Streptococcus may be bile-insoluble [219], and some may have lytA and pneumolysin genes, making genetic testing

limited [220]. There are also some species that produce teichoic acid similar to that produced by *S. pneumoniae.* All of these techniques aid somewhat in the process of elimination however does not exclude all Streptococcus species, with some cases of pneumococcus still being misidentified.

Figure 4. *S. pneumoniae* isolates expressing most capsule types make small round colonies similar to doughnuts on blood agar plate (A) but serotype 3 and 37 pneumococci develop characteristically large mucoid colonies (B) [406].

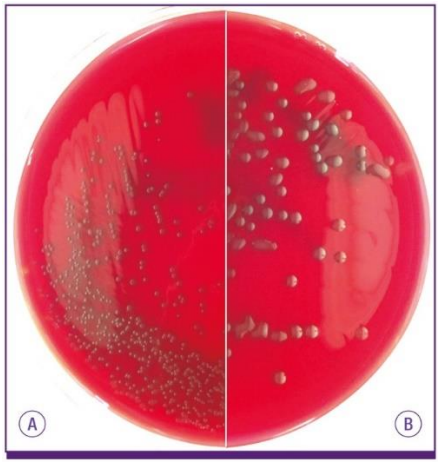
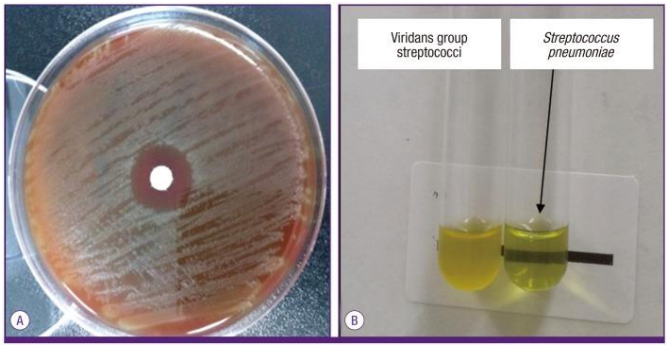


Figure 3. *S.pneumoniae* growth is inhibited around the paper disk containing optochin (A). The test tube containing *S. pneumoniae* shows a loss of turbidity in the presence of sodium deoxycholate (bile salts) due to bacterial lysis while the test tube containing viridans species is turbid (B) [406]



1.1.7 Conventional methods for bacterial identification

Early and accurate identification of pneumonia is of high importance due to a decreased mortality rate, particularly among the elderly when treated early with antibiotics, as well as the reduction of cost associated with excessive clinical investigations and inappropriate treatment, which may lead to complications [224, 225]. Pneumonia is generally diagnosed based on clinical symptoms and a chest X-ray. Symptoms related to pneumonia include cough or difficulty breathing, and tachypnea (rapid breathing) [226]. Diagnostic procedures for Community Acquired Pneumonia (CAP) typically include isolation of bacteria from sterile body fluid, followed by identification and confirmation that the bacteria present is *S. pneumoniae* – this is the gold standard. For identification of pneumococcus from body tissue, a lung biopsy or bronchoscopy to obtain lung tissue would be ideal [227]. An alternative would be to isolate the bacteria via transtracheal aspiration, which has been reported to obtain high yields of *S. pneumoniae* [228, 229]. However, these techniques are not always practical in a clinical setting, therefore the majority of identification for this bacteria is performed commonly with the more readily available bodily fluids, including peripheral sputum, blood samples, and pleural fluids.

1.1.7.1 Sputum Gram stains and culture

Typically, sputum Gram stains and culture are the first technique used for the identification of pneumonia if there is a high quality sample (less than 10 squamous epithelial cells, with more than 25 polymorphonuclear cells at a magnification of x100 under a light microscope). In this case, the diplococcic Gram-positive bacteria may be visualised [230, 231]. While this method

has demonstrated to be highly sensitive and specific in some cases [232], there are some limitations, including inadequate sample collection, particularly from children, and the treatment of antimicrobials before the collection of sputum, which may contribute to a low yield of bacteria [230, 232, 233]. Another issue is the false positives occurring from natural nasopharyngeal carriage of the bacteria.

1.1.7.2 Blood and pleural fluid culture

Since blood and pleural fluid cultures are generally collected from sterile tissues, this can provide a definitive, unambiguous platform for the diagnosis of pneumococcual disease. However, the rate of positive samples for the bacteria within the blood tends to be low, at less than 10% in total in patients with pneumococcus, which may present as false negatives [234-237]. Possible causes of this may be patients presenting with non-bacteraemic pneumonia, autolysis of the bacteria during the stationary growth phase resulting in cell death, treatment with antimicrobials before samples are obtained, or inadequate sample collection, specifically insufficient blood volume [238]. Another issue with culturing bacteria includes the time it takes for bacterial growth and to a level required for identification, usually several days. Similar limitations occur with the collection of pleural fluid [238]. However, using real-time PCR for the detection of bacterial DNA in pleural fluids was a promised technique.

1.1.7.3 Pneumococcal antigen detection methods

Pneumococcal antigen detection method may be used to detect pneumococcus in a variety of tissue samples, including sputum, urine, breath, pleural fluids, and peripheral blood, by rapid detection of pneumococcus molecules [239-242]. An advantage of this technique is that the molecules may not affect after antimicrobial treatment, but still remain detectable. PcpA may

be used as a particularly important target since it can help in distinguishing pneumonia from carriage of the bacteria due to the observation that nasopharyngeal carriage tends to exhibit high levels of manganese, whereas these levels present at a comparatively lower level in the blood [243, 244].

1.1.7.4 Techoic acid (C-polysaccharide) detection

Enzyme-linked immunosorbent assay (ELISA) and latex agglutination can be used to detect techoic acid and capsular polysaccharide antigens in sputum specimens, showing high sensitivity and specificity [239]. A recent addition to diagnostics is the Binax NOW® *S. pneumoniae* assay which is a rapid immuno-chromatography test, introduced in 2003, proving useful as a point-of-care test to detect C-polysaccharide in urine samples. However, in children, specificity with this kit only amounted to around 50–60% due to the presence of high rates of nasopharyngeal carriage in children, subsequently producing high concentrations of techoic aid in the urine [245-165]. A high concentration of urine from children reduced the specificity of this kit further [246], and so is not recommended for the detection of pneumococcal pneumonia in this age group [250]. With the use of pleural fluid, on the other hand, sensitivity is much higher (71-96%) and specificity (71-100%) exceeds that of urine, therefore may prove to be useful in the diagnosis of pneumococcal empyema with the use of pleural fluid [251-256].

In adults, the Binax NOW® *S. pneumoniae* assay showed more promise, with sensitivity and specificity around 74-75% and 94-97%, respectively [93, 191, 193], increasing the rate of diagnosis of CAP by 11-23% above conventional techniques [93, 193]. The detection of bacteraemic *S. pneumoniae* was demonstrated to be more sensitive compared with non-bacteraemic *S. pneumoniae*, with more than 20% difference [194-196, 209, 210]. This assay

may be useful for optimising antimicrobial treatment in terms of distinguishing between bacteraemic and non-bacteraemic pneumococcal pneumoniae. The 2007 IDSA/ATS guidelines have recommended the use of the Binax NOW' *S. pneumoniae* assay during intensive care admissions, failure of out-patient antimicrobial therapy, leukopenia, active alcohol abuse, asplenia, chronic severe liver disease, and pleural effusion [273]. An advantage of this kit is that previous antibiotic use before sampling has less of an impact than with conventional culture methods, however, false positives may arise in cases where there are low levels of C-polysaccharide antigen present in the sample [193], or cross-reactivity with other bacterial species, asymptomatic nasopharyngeal colonisation of pneumococcus, and a history of previous pneumococcal infection showing the presence of antigens. Techoic acid may still be excreted in the urine for more than a month following pneumococcal infection in some cases [274, 193]. There are also concerns about recent vaccination presenting false positive with this kit [275]. In the UK, guidelines for the Management of CAP in Adults in 2009 stated that pneumococcal urine antigen tests should be performed for all patients with moderate or high severity CAP, [472].

1.1.7.5 Capsular polysaccharide detection

ELISA for the detection of the pneumococcus capsule in the urine tends to be variable and dependent serotype and severity of infection. Other limitations include difficulty of testing procedure as well as being unable to detect some capsular serotypes [276]. The multiplexed immunoassay system based on the Luminex' system using monoclonal assays has recently showed promise, using multiplexed serotype-specific urinary antigen detection (UAD) assays [278, 279]. This technique has shown high sensitivity (79-97%) and specificity (99-100%) in the diagnosis of pneumococcal pneumonia [279, 280]. Additional information can also be

detection using this kit, including capsular serotype responsible for CAP, however it can only currently detect a limited number of serotypes (13-14 serotypes in total), and have not yet been introduced into a clinical setting [278, 281].

1.1.7.6 Antibody responses to pneumococcal infection

Serological studies may be used to detect genetically diverse pneumococcal antigens, including pneumolysin, C-polysaccharide, capsular polysaccharides, and PsaA. PsaA is a highly immunogenic lipoprotein, which can be detected in the blood using IgG antibody, with a very high sensitivity and specificity of 85-89% and 83-98%, respectively, in pneumococcal pneumonia diagnosis in Kenyan adults [282, 283]. However, the sensitivity dropped to 42% when applied to detection in children [284], as well as having specificity concerns due to the fact that PsaA may also be found in the cell walls of other streptococcus species [285]. An advantage of using serological testing for pneumococcal pneumonia is that they are not affected by the use of antimicrobials before sampling and do not require the duration of culturing bacteria. However they can potentially be impacted by cross-reactivity to antibodies specific to colonised pneumococci, therefore are not used as a diagnostic tool, but more as an epidemiological surveillance tool [319, 320].

1.1.7.7 Biomarkers

The concentration of biomarkers related to the response of infection, inflammation, and tissue injury can be used to distinguish between bacteraemic and non-bacteraemic infection [286]. Biomarker detection can also play a role in the optimisation of treatment or as a prediction of prognosis. As rapid detection tools, such biomarkers as C-reactive protein (CRP), procalcitonin and TREM-1, a receptor expressed on myeloid cells, may be detected as indications of pneumococcal pneumonia. TREM-1 is an immunoglobulin, upregulated by microbial

components [287]. TREM-1 is capable of stimulating the secretion of numerous cytokines and chemokines of the immune system [288]. The serum soluble form of TREM-1 has been linked to the presence of bacteraemic CAP [288], whereas soluble TREM-1 present in bronchoalveolar lavage fluid has been associated with bacterial pneumonia [287]. During the pro-inflammatory response, particularly in response to bacterial pneumonia, procalcitonin is made by parafollicular cells of the thyroid and neuroendocrine cells of the lungs and intestines, whereas procalcitonin tends to be downregulated during viral pneumonia infection [289], therefore may be a useful biomarker to distinguish between the two. CRP and procalcitonin levels, together, have been demonstrated to be useful in identifying pneumococcal pneumonia in children with non-specific chest X-ray presentation [290, 291]. Procalcitonin showed a good correlation with positive PCR, serological testing, and chest X-ray's in these patients [292], and also appear to correlate well with severity of infection. Elevated procalcitonin and CRP levels showed a strong association in patients presenting with pneumococcal CAP, with a sensitivity of 94.4% and 91.9% for procalcitonin and CRP, respectively [293]. These biomarkers may also be useful in distinguishing between bacterial and viral pneumonia in adults [294, 295, 296]. Biomarker detection has proved promising, however, they will require other detection methods used in conjunction to confirm pneumococcal pneumonia infection, specifically in distinguishing between bacterial and viral pneumonia.

1.1.7.8 Nucleic acid amplification tests (NAAT)

NAAT uses the polymerase chain reaction (PCR) to target and distinguish genetically diverse strains of pneumococcus pneumonia from the blood and respiratory tract. Detectable targets for *S. pneumoniae* include the pneumolysin gene (ply), autolysin gene (lytA), pneumococcal surface adhesin A gene (psaA), wzg/cpsA, and the Spn9802 gene fragment [298].

Using whole blood, *S. pneumoniae* DNA can be detected using PCR directed at the ply gene. Sensitivities have proved to be variable (35-100%) [210, 299-302] and is unable to distinguish *S. pneumoniae* from other streptococcus species due to the fact that some of these also contain the ply gene [303, 304]. In comparison to the low ply specificity, the lytA gene had higher specificity [303, 305, 306], but still presented an issue, in terms of not being able to distinguish between *S. pneumoniae* and *S. mitus* since lytA genes are not as variable between streptococcus strains compared with those between streptococcus species [305]. The Spn9802 PCR is highly specific for *S. pneumoniae*, but is unable to distinguish between *S. pneumoniae and S. pseudopneumoniae* [307]. In terms of quantitative viral loads by real-time PCR from whole blood, a high bacteraemic load has been linked with increased risk of mortality [308, 309], therefore may be used as a tool to predict severity of pneumococcal illness.

Using respiratory tract specimens, ply PCR had a sensitivity of 68-100% [310-313], but specificity presented an issue, particularly in throat swaps between patients with pneumonia and those of the control group [311], suggesting the lack of specificity between pneumococcal pneumonia and nasopharyngeal colonisation. More notably, real-time PCR produced better specificity with Spn9802 PCR, showing sensitivity and specificity of 71% and 100%, respectively, for the detection of pneumococcal pneumonia in patients with more than 10^4 copies/mL bacteria [307]. LytA PCR had sensitivity and specificity level of 82.2% and 92%, respectively, at bacteria levels exceeding $8x10^3$ copies/ml in detecting pneumococcal pneumonia, and distinguishing from nasopharyngeal colonisation. Successful detection rates of CAP using this method increased from 27.1% to 52.5% using this cut-off [314].

1.1.7.9 Multi-locus sequence typing (MLST)

While pneumococcal serotype is important in determining invasiveness, it has become increasingly evident that genotype also plays an important role. Since multilocus sequence typing (MLST) has become available, allowing genotyping of bacterial species using house-keeping genes, a database recognising over 3000 pneumococcal sequence types (ST's) or clones has been established (http://pubmlst.org/spneumoniae/).

MLST has recently been demonstrated as a useful tool to distinguish between different types of Streptococcus species [221-223]. The adapted form of this tool is named Multi-locus sequence analysis (MLSA) and is currently used as a research tool only, but has been demonstrated to be more reliable in distinguishing among Streptococcus species. MLST produces digital nucleotide sequences of approximately 500 base pair segments from seven pneumococcal house-keeping genes selected on the basis of their location within the genome and relative neutrality with regard to selective pressures. These results are portable between laboratories, and can be used in the molecular characterisation of Streptococcus species and *S. pneumoniae*, with the ability to compare isolates on a global scale. Serotype and sequence of pneumococcal can be analysed in conjunction, along with the monitoring of capsular switch and may provide useful information for the development of pneumococcal conjugate vaccines with regard to long-term efficacy [42].

1.1.7. 10 The advances of using whole genome sequencing to study the population biology of *S. pneumoniae*

Whole-genome sequencing (WGS) provides us with a 'top-down' approach to associate genotype with phenotype and has recently sped up learning about the basis of virulence and

antibiotic-resistance acquisition in pathogens [610]. An advantage of the increasing volume of sequence data available for bacteria is that it allows the testing of evolutionary hypotheses across the entire domain. Recent work by Hershberg and Petrov used bacterial WGS data to investigate the spectrum of mutation types. Surprisingly, this revealed that the spectrum varies very little with most biased towards C/G→T/A transitions. This was found universally, even for clades with particularly high GC contents, which suggests that in the absence of selection, bacterial genomes would approach an equilibrium GC content of 20−30%. Given the large variety of GC contents observed for bacteria (~20–80%), this observation suggests that mutational pressures alone are not responsible for the nucleotide composition of the majority of genomes and that selection probably plays an important role [611]. A sample of whole genome sequences makes it possible to adequately reconstruct the natural history of a lineage. The base substitutions used to construct the phylogeny have accumulated over about 40 years and occur, on average, once every 15 weeks. Recombinations happen at a rate approximately 10 fold slower, but introduce a mean of 72 SNPs each. The responses to the different anthropogenic selection pressures acting on this variation are quite distinct. The apparently weak selection by aminoglycosides and chloramphenicol has led to the occasional deletion of loci encoding resistance to these antibiotics. By contrast, resistance to macrolide antibiotics has been acquired frequently throughout the phylogeny, with selection strong enough to drive supplementation or replacement of the resistance afforded by the *mef* efflux pump with the broader-range resistance provided by *ermB*-mediated target modification. The response to vaccine selection is different, involving the depletion of the resident population before it can respond to the selection pressure and thereby opening the niche to isolates that already expressed non-vaccine serotypes [612].

1.1.8 Future diagnostic options for pneumococcal infection

Future diagnostic options for the diagnosis of pneumococcus may include the detection of metabolites, including the analysis of urinary metabolites using nuclear magnetic resonance (NMR) [315], or a breath test to isolate bacteria and detect using mass spectrometry. Sensitivity and specificity to these testing methods are currently being investigated [318]. Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) is currently being used in numerous clinical laboratories for the detection of bacteria, with advantages including ease-of-use, cost-effectiveness and rapid and high-throughput. However, specificity, again, presents an ongoing problem in distinguishing between *S. pneumoniae* and other species and strains of bacteria [316, 317]. Whole-genome shotgun (WGS) assembly of large and complex genomes was once a much-debated question [317]. The advent of next-generation sequencing and the comparative ease and speed with which WGS assemblies can be constructed for mammalian and many other genomes allowed sequencing projects to move beyond these concerns, accepting high quality draft genomes with nearly complete gene spaces [317].

1.2 Pneumococcal serotype 1

1.2.1 Geographic distribution of serotype 1 pneumococcus disease

Serotype 1 pneumococcus has a very low carriage rate and is uncommon with regard to nasal colonisation even in populations with a high prevalence of invasive disease (Figure 5), with the exceptions of outbreaks of this serotype. In 1937, it was found to cause approximately 22% of

pneumococcal pneumonia in children, and 33% in adults, however serotype 1 was estimated to be responsible for only 2% of asymptomatic, colonised adults and children [321].

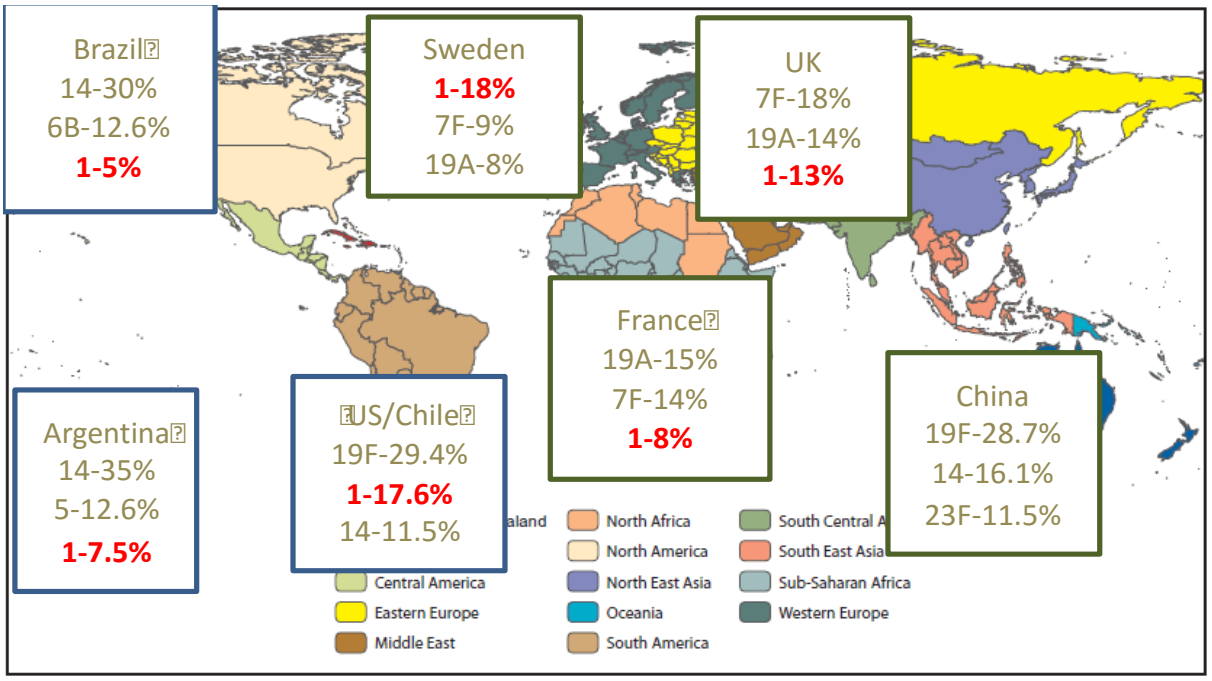


Figure 4. Distribution of serotype-1 IPD worldwide

Serotype 1 pneumococcus is generally associated with bacteraemia, empyema and peritonitis, with epidemics of meningitis arising in Africa. It more frequently affects young adults compared to other known serotypes and mortality and co-morbidity occurs at a low rate. Prisons, homeless shelters and closed environments of young adults in common with regard to outbreaks [322 – 325]. It has been demonstrated to be efficiently passed between close contacts to those infected, for example, healthcare workers working on pneumonia wards, and household contact, particularly with carriage of serotype 1 in homeless shelters [325, 360]. Colonisation tends to be short-lived, relative to other serotypes, while the probability of invasive resulting from serotype 1 infection appears to be very high, an explanation for this has yet to be uncovered [326]. Antibiotic resistance has rarely been reported for first-line antibiotics, such as penicillin, macrolides and quinolones, which is likely due to the shorter

duration of carriage of serotype 1, resulting in less time for recombination leading to genetic diversity, of which antibiotic resistance may be included, as well as reduced chance of selective pressure from antimicrobials [327-330]. It has been noted that there is limited geographical distribution of serotype 1 compared to other serotypes, again, likely due to the short duration of carriage, preventing the organism from spreading farther via international travel [331]. Serotype 1 pneumococcus is one of the most common serotypes found linked to invasive pneumococcal disease (IPD). In the last decade, an increase in serotype 1 pneumococcus causing IPD has been reported in Europe, in countries such as Portugal, Spain, France, Belgium, and the UK [442, 443, 444, 445, 446). Serotype 1 pneumococcus is one of the few serotypes that has been associated with IPD in small or closed communities [447, 448, 449, 450, 451]. In some areas of the world, there has been a marked reduction of serotype 1 bacteraemia reported, particularly between 1940 to 1955. For example, Boston City Hospital, USA [336], Danish national statistics [332], and to a lesser extent due to limited data; Glasgow, UK [337], have all reported a reduced incidence of serotype 1 pneumococcal disease over a period of decades. Other reports from South America [338], Africa [339], and the Indian sub-continent [340] have demonstrated a high prevalence of serotype 1 disease responsible for widespread epidemics, unfortunately historical data for these areas is not available. On the other hand, serotype 1 appears to be less prevalent in areas of South-East Asia [341]. Distribution of serotype 1 and arising epidemics appear to be seasonal; associated particularly with periods nearing the end of the dry season [339]. Serotype 1 IPD generally affects younger adults, which differs from what is usually seen in the general population affected by IPD [347]. Serotype 1 IPD has also been shown to be common among neonates in Europe and North America [348, 349]. A study across England and Wales demonstrated that from 256 cases of IPD in infants identified, 84/256 (33%) developed IPD in the first 48 hours of life. This was following the introduction of PCV7, [476]. Another study of both the pre- and post-vaccine introduction

period demonstrated that of 513 children diagnosed with IPD, 36/513 (7%) of these were in infants younger than 90 days old, [476]. In a study of IPD isolates from Danish infants that were 0-90 days old from 1943-2013, serotypes 7F and 1 were the predominant serotypes found, accounting for 20% and 15%, respectively, of all the IPD cases, [476]. The high incidence of serotype 1 found in young adults may be attributed to a lack of other serotypes able to infect this age group, or the possibility that acquired immunity to serotype 1 IPD is age-independent [350].

Serotype 1 has often been found responsible for the manifestation of empyema in patients. Over the past 15 years in the UK, the incidence of patients presenting with empyema has risen, with serotype 1 typically found to be the cause [351-355].

1.2.2 Contrast in the burden of pneumococcal disease in the industrialised vs. developing world

Carriage rates of pneumococcus in children are notably higher in the developing world. For example, in the Gambia [179], carriage rates reach 80% rate in children less than 5 years of age, and 20% in adults, as reported in 1992 [369], with similarly high rates found throughout the developing world including Papua New Guinea [369], Zambia [372], Pakistan [373], The Philippines [375], and Bangladesh [370]. In contrast, the industrialisedworld appears to have lower rates of carriage overall. In Sweden, in 1992 [371], it was reported that the rate of carriage was 12% in infants aged 3 months, 30% aged 7 months, and 32% aged 12 – 18 months. In 2005, the prevalence of carriage overall in the UK was estimated at 25%, with a carriage rate of 52% in children under 2 years of age, and 8% in adults [368]. In the Netherlands [374], carriage rates were reported in 2008 as 8.3% at 1.5 months of age, 31.3% at 6 months of age,

and 44.5% at 14 months of age. There were an estimated 15 pneumococcal meningitis cases per 100,000 infants under 2 years of age in a UK wide study [376], and an estimated 45 cases of IPD per 100,000 reported in the elderly over 65 years of age in Scotland, prior to the introduction of the PCV-7 vaccine [377].

In urban slums, a population estimated at around 1 billion people, and mostly situated in developing countries, are at particular risk of pneumococcal disease [43], as they do not benefit from current advances in treatment and prevention [44], particularly with regard to immunisation strategies. Conditions of poverty are associated with high rates of pneumococcal carriage and invasive disease, however with regard to transmission of *S. pneumoniae*, and the burden of IPD little is known from these communities. Epidemiological surveillance in Salvador - the third largest city in Brazil with a population of 2.8 million people, 60% of which live in slum settlements - reported that the annual incidence for pneumococcal meningitis was 32 cases per 100,000 in children less than one year of age, and 8 cases per 100,000 in children less than 5 years of age [45]. More than 80% of meningitis cases reported in Salvador were found in individuals residing in slum settlements. Children less than 2 years of age were particularly susceptible to acquiring penicillin resistant strains, compared with those above 2 years of age. Serotype 14 was found to be largely attributed as the cause to around 50% of all penicillin resistance meningitis cases [45, 46].

The surveillance network coordinated by the Pan American Health Organisation, together with the National Reference Laboratories of Argentina, Brazil, Chile, Colombia, Mexico and Uruguay have been monitoring pneumococcal serotype and antimicrobial resistance of *S. pneumoniae* causing IPD in children less than 6 years of age. Between 1993 and 1999, IPD serotypes had been isolated from patients, 44% of which were pneumonia cases, and 41% meningitis cases. From these, thirteen serotypes accounting for 86% of the isolates were found

to be the common causes of IPD. These included serotypes 14, 6A/6B, 5, 1, 23F, 19F, 18C, 19A, 9V, 7F, 3, 9N and 4. Penicillin resistance was detected in 29% of the isolates, 17.3% with intermediate resistance, and 11.3% with high level resistance. Resistance levels increased during this period of surveillance in Argentina, Colombia and Uruguay. Serotypes 14 and 23F accounted for 66.6% of resistance [47].

1.2.3 Serotype distribution of *S. pneumoniae* carriage isolates

Currently, 46 serogroups and 94 known serotypes of *S. pneumoniae*. The most recent serotypes identified were 6C [219], 6D [220] and 11E [221]. Pneumococcal vaccines generally target the capsular polysaccharides based on their high antigenicity. The diversity of virulence, including invasive disease, antibiotic resistance and outbreak potential differ between the different capsular serotypes. *S. pneumoniae* also has the ability to undergo transformation, and recombination of its genetic material can be exchanged among and between species. Minor changes to the capsule can have an effect on the pathogenicity and virulenceof these strains. There is also potential for the occurrence of capsular switch [222], producing clonal isolates, which express different polysaccharide capsule serotypes caused by recombination of heterologous DNA at the capsular locus. Changes in serotype prevalence among pneumococcal populations result from both serotype replacement and serotype (capsular) switching. There have been 36 independent capsular switching events identified to-date, which were found to be either the result of nucleotide substitution and/or deletion, or appeared to be due to recombination. Capsular switching has been found to occurr regularly prior to both PCV7 introduction and widespread antibiotic use [476].

1.2.4 Geographic distribution of the serotype-1 ST306 clone

The clone ST306 was initially identified in Sweden in the 1990's [452], and has subsequently been found in Canada, Denmark, France, Germany, Netherlands, Norway, Poland, Spain and in the USA. The clone has been consistently been associated with IPD [453]. Furthermore, an increase in this clonal group has been reported among healthy carriers following the introduction of the heptavalent conjugate vaccine in Portugal and Spain [454, 433]. The ST306 clone is typically associated with non-lethal pneumonia with or without empyema. ST306 is the most prevalent serotype clone in Europe that is not associated with antibiotic resistance [470].

The ST306 serotype 1 clone has dramatically expanded worldwide during the last two decades and is now the source of >80% of cases of serotype 1 pneumococcus disease [342].

In recent years, serotype 1 has emerged as a much more prominent serotype linked to invasive disease in Europe, first demonstrated in Sweden, where there had been >4-fold increase in the rate of pneumococcal bacteremia over a 10-year period, 1987-1997. A 10-fold increase of type 1 had occurred from 1992 through 1997 and of 20 type 1 isolates examined by MLST, 11 represented the ST306 clone [342]. Other European countries, including England and Wales [343], Scotland [335], and Portugal [344], have reported the presence of the same clone to be linked to a high proportion of IPD cases.

In late 2000 towards early 2001, an epidemic of serotype 1 in New Caledonia was reported, with considerable rise of incidence in the population of serotype 1, lasting a number of months exceeding 50 cases of IPD [333]. A smaller epidemic was noted in 2007 and both were subsequently found to be caused by the same clone of serotype 1 (ST306).

Following the introduction of the PCV-7 vaccine into the childhood vaccination schedule in the UK 2006, considerable and significant changes in serotype and clonal distribution of pneumococcus was observed over time. In Scotland, an increase in IPD caused by non-vaccine serotypes 1, 4 and 6 and a decrease in serotype 14, 19 and 23, which are included in PCV-7, was shown by comparing data of clonal and serotype changes in pneumococcal isolates causing IPD immediately prior to the introduction of PCV-7 between the period from April 2001 to April 2006. When sequence type data was analysed, a significant increase of 135.4% in the prevalence of the ST306 clone associated with serotype 1 was evident over the 5 year period, attributed to clonal expansion of this previously rare clone. The data suggested that PCV-10 and PCV13 vaccines would offer significantly more protection from IPD compared to PCV7 [432]. In Scotland where serotype 1 had previously been dominant, it was shown over time to be replaced by a single clone, ST306 [334, 335]. This type of clonal replacement is rare among the other serotypes, which usually consist of different clonal populations existing simultaneously.

It has also been suggested that the emergence of ST306 clone in Scotland [356] was linked with the increased incidence of paediatric empyema in Aberdeen [351], occurring around the same time period. Paediatric empyema, with serotype 1 pneumococcus as the cause, continues to be reported repeatedly [357-359]. Among 3073 pneumococci isolates identified between 2003 and 2007 in Scotland, ST306 was the second most frequently PMEN clone identified by a combination of serotype and MLST, amounting to 225 isolates. These were all found to be susceptible to penicillin and erythromycin, however continued surveillance for the emergence of antimicrobial and vaccine resistance is advised despite the current low levels of resistance found in Scotland, since the prevalence remains much higher in other European countries with comparable rates of antibiotic use, [430].

After the introduction of the PCV7 vaccine in Spain, the incidence of IPD rates linked to PCV7 serotypes have decreased in both children and adults, however there has been an increase of IPD due to serotypes not included in the PCV7 vaccine, counterbalancing the progress [425, 428). When investigating the epidemiology of IPD in older people in Spain between 2007 and 2009 before the PCV-13 vaccine had been introduced, data showed that from 335 samples collected from patients with IPD, 5.4% of these were serotype 1. 22 clonal groups were identified, including ST306 being one of the most common genotypes (n=17, 3.7%). The increase in serotype 1 between 2007 and 2009 has been attributed to the clonal expansion of the ST306 clone in most European countries [425].

When investigating the clonal structure and 21-year evolution of pneumococcus serotype 1 isolates in northern Spain, an increase in the incidence of IPD from 1.8 per million inhabitants between 1987 to 1993 to 4.0 during the period of 1994 to 2000 was been reported. Between 1987 and 1998 the predominant clone was ST228, with 61.5% (8/13 isolates) of those causing IPD. This decreased to 13.3% (10/75 isolates) after 1999 for this clone. ST306 appeared in 1998. Following the introduction of the PCV7 vaccine in 2001, ST306 became the predominant sequence type (>80%). In northern Spain, between 1998 and 2007, ST306 was found to be the dominant strain for IPD, comprising 74.4% (58/78 isolates) [433].

A high prevalence of serotype 1 pneumococcus in healthy adults with IPD was demonstrated in Barcelona, particularly with the ST306 clone, along with a high association of empyema occurring in serotype 1 patients [426]. In a prospective study during a 20 year period between 1989 and 2008 in Barcelona, 56/347 isolates were identified as serotype 1 in patients <18 years of age, with 39 isolates (71%) of these being the ST306 clone [427]. The incidence of serotype 1 was found to be significantly higher in older children than in children >2 years of age, as well as an increase of serotype 1 prevalence with IPD among children in Barcelona [428].

In a study carried out on the outbreaks of serotype 1 pneumococcus in the South Pacific between the year 2000 and 2007, surveillance of the invasive pneumococcal disease outbreaks demonstrated that the most prevalent serotype was serotype 1 [431]. All 141 serotype 1 strains tested were susceptible to antimicrobials. The majority of serotype 1 isolates belonged to the ST306 clone and was the exclusive clone found across New Caledonia and responsible for the outbreaks in New Caledonia and French Polynesia based on serotype 1 data prior to the outbreaks carried out between 1999 and 2001 and the increased prevalence of ST306 serotype 1 following the outbreaks. The ST306 clone remains an important serotype 1-associated sequence type in the South Pacific, able to cause pneumonia and septicaemia in healthy indigenous people [455].

Surveillance of pneumococcal serotype 1 carriage was carried out during an outbreak of serotype 1 IPD in Central Australia and serotype 1 ST306 was shown to be responsible for the 65% increase in IPD during 2010 compared to the previous year [422]. This outbreak between 2010 and 2012 consisted of 69 confirmed cases of ST306. The majority of cases (84%) were Indigenous, ranging from 3-61 years (mean age 18 years, median age 10 years). The serotype 1 carriage reservoir in this region was investigated further and nasopharyngeal swabbing during the outbreak identified asymptomatic carriers of serotype 1 were highest in children below 10 years of age living in remote communities. Serotype 1 is included in the 10-valent pneumococcal vaccine, PHiD-CV (Synflorix®), and participants that had been vaccinated with PHiD-CV showed no detection of serotype 1 carriage, while participants positive for serotype 1 had either missing immunisation records or had received the PCV7 vaccine with a booster of PPV23.

In Columbia, serotype 1 pneumococcus is one of the most prevalent invasive serotypes, with 11.6% identified among adults, 21.6% among children between 6-14 years of age, and 8.2%

among children below 6 years old. A study of clonal distribution of 135 serotype 1 isolates recovered from patients with IPD in Columbia between 1994 and 2011 showed only two genetic lineages (types E and F). More than half (51%) of the isolates were recovered from children less than 6 years of age, with 58/69 (84%) found to be genetically related to ST306. The other 49% of all isolates were obtained from patients more than 6 years of age, in which 95.4% were genetically related to the ST306 clone. Clinical diagnosis of these patients were typically pneumonia and meningitis. 14/135 isolates showed resistance to antimicrobials, with 9 of these genetically related to the ST306 clone, specifically resistant to SXT, tetracycline or erythromycin [424].

It remains unclear whether these clonal changes observed are due to natural fluctuations in transformable pneumococcal species or are directly driven by vaccine pressure. Serotype 1 IPD was present the introduction of the conjugate vaccine, and there have been no correlations demonstrated between conjugate vaccination and changes in the national incidence of serotype 1 IPD [330]. Interestingly, when Scotland introduced the 13-valent conjugate vaccine, expanded to the serotype 1 capsular antigen, this was followed by a reduction in the incidence of serotype 1 disease in this area. This suggests that previous rises in incidence of serotype 1 in some areas was in fact due to its ability to cause epidemic disease, rather than human intervention [345, 346]. Surveillance of serotype replacement of vaccine types with non-vaccine types is of high importance following vaccine introduction, particularly with serotype 1 since it is commonly associated with outbreaks. This will be vital in responding to changes in the population of pneumococcus. Together with the increase in prevalence of serotype 1 associated with the ST306 clone since the introduction of the PCV7 vaccine, and data suggests that outbreaks of this clone is nearing a pandemic. Fortunately, serotype 1 pneumococcus is rarely carried making it susceptible to antimicrobials due to its infrequent exposure resulting in

low selective pressure. Since carriage is needed for horizontal gene transfer, resistance to ST306 likely remains rare [423].

1.2.4.1 Epidemiology

In the last decade, an increase incidence of serotypes 1 and 7F in invasive disease has been reported in some European countries such as Portugal, Spain, France and UK. In addition, while serotype 5 was increasingly detected in Spain. The few studies that investigated the genotype of these isolates found that most isolates were representatives or close relatives of PMEN clones typically associated with these serotypes, namely, Sweden^{1}-28 (ST306), Sweden^{1}-40 (ST304), Colombia^{5}-19 (ST289) and Netherlands^{7F}-39 (ST191). These serotypes and associated clones are frequently isolated from patients during outbreaks [461].

In terms of carriage distribution, serotypes 1, 5 and 7F in disease, less is known about its epidemiology in carriage. This may be due to the fact that these serotypes are rarely carried. However, Almeida and her colleagues were able to detect serotype 1, 5 and 7F in carriage. These serotypes were able to transmit between children attending day-care centers, and are highly clonal [461].

Serotype 1 is highly invasive and causes sepsis and empyema in Europe and North America (lineage A), whereas it is the major meningitis-causing pathogen among the children above 5 years old individuals in the African meningitis belt (a Sub Saharan region extending from Senegal to Ethiopia). Moreover, the region appears as specific features of infection, such as hyperendemicity, seasonal pattern and high lethality affecting all age groups (lineage B) [462].

There is a strong relationship between site of infection and virulence factors. Both the serotype and genotype distribution of meningitis-related strains are different from those of pneumonia-

related strains. For example, carriage of Pilus-2 and *psrp* genes showed high association with pneumonia, whereas *phtB* was more prevalent in meningitis isolates [462].

1.2.4.2 Pneumolysin variant of ST306

ply is an important gene and plays a role for progression a disease and induces inflammatory responses in the host lung. The pneumolysin amino acid sequence is thought to be highly conserved throughout all pneumococcal serotypes with little variance over time and geographic distance. A number of clinical isolates with mutations in their pneumolysin gene (*ply*) were identified by Jefferies *et al* [361,464].

The mutations in *ply* gene were predominantly in serotypes 1, 7, and 8. It has been reported that 2 isolates of serotypes 7 and 8 possess a threonine to isoleucine substitution at position 172. Both alleles also contained a 2-aa deletion of valine 270 and lysine 271. The only T172I substitution affected the hemolytic activity of pneumolysin [465]. Recently, it has shown that serotype 1, sequence type (ST) 306 pneumococci produce a nonhemolytic pneumolysin [464]. Moreover, ST306 of serotype 1 has allele 5 that contains all the mutations reported for the serotype 8 strain as well as a tyrosine to histidine substitution at position 150 [464].

					Amino acid position															
					14	136	142	150	154	167	172	224	260	265	267	270	271	273	380	402	415
Strain no.	Serotype	ST	Allele	Specific activty	N	Q		Y	T	S	T	K	E	A	I	V	K	A	D	Q	
D39	2	128	1	4.13×10^5																	
TIGR4	4	205	2	4.74×10^5															N		
01-2696	1	227	2	4.20×10^5															N		
2PN00495	8	404	3	6.50×10^3							I	R		S		DEL	DEL				
H040920498	8	944	3	7.43×10^3							I	R		S		DEL	DEL				
00-3645	1	227	4	–															N		INS
01-2884	8	53	5	0				H			I	R		S		DEL	DEL				
01-1204	8	578	5	0				H			I	R		S		DEL	DEL				
01-1956	1	306	5	0				H			I	R		S		DEL	DEL				
01-1199	NT	577	5	0				H			I	R		S		DEL	DEL				
04-2055	1	228	5	0				H			I	R		S		DEL	DEL				
96-5878	2	74	6	3.72×10^3						F	I	R		S		DEL	DEL				
02-3013	NT	448	7	2.95×10^5	D							R									
01-2866	23F	40	8	9.09×10^4														D			
00-1153	9V	156	9	3.14×10^5											M						
01-3862	7F	191	10	1.01×10^5					M					S		DEL	DEL		N		
02-2744	7F	191	10	9.77×10^4					M					S		DEL	DEL		N		
01-4296	27	571	10	1.36×10^5					M					S		DEL	DEL		N		
01-2914	20	591	11	5.95×10^5		K													N		
01-2513	18C	818	12	–															N	E	
00-2328	6A	813	13	3.09×10^5									D						N		
S1-11	1	228	14	–			INS												N		

Figure 5. Pneumolysin alleles dtermined by full sequencing of PLY genes from 22 isolates [464]

Interestingly, despite of the fact that ST306 clone displays a non-cytolytic Ply variant that retains the ability to bind to cholesterol-containing membranes, ST306 cannot form pores. The absence of cytolytic activity in ST306 reduces the virulence compared to a sequencing type has the full cytolytic as D39 [466].

With regard to immunity responses, in previous study it has been shown that strains expressing non-cytolytic Ply may have an advantage more than cytolytic strains as they do not activate the NLRP3 inflammasome and may be more resistance to clearance from the host [467]. The cytolytic activity of Ply enhances increased expression of genes encoding pro-inflammatory cytokines, such as GM-CSF and IL-23α, as well as IFNβ1. *CSF2*, while remained either unchanged or was weakly up-regulated in the strains expressing no or non-cytolytic Ply, but was significantly induced dependant on expression of cytolytic Ply [466].

A previous study was shown that the opportunity of detecting serotype 1 in carriage will increase during IPD associated with outbreaks. The majority of carrying serotype 1 was healthy

young children 5 to 8 years of age from remote communities [468]. That study demonstrated the group of population would carry serotype 1 and they could potentially be driving the outbreak. However, the most outbreaks have occurred by serotype 1 ST306 was after the introduction of PCV7 and the increase in the prevalence of serotype 1 associated with the increased use PCV7 [469].

1.2.4.3 Virulence factors specific to ST306

Virulence genes carried by an extensive collection of invasive *S. pneumoniae* isolates from Europe and Africa were described in terms of their serotype and genotype distribution. The carriage of virulence genes inside the bacterial genome were assessed in African isolates collected between 2002 and 2007 [420]. Most of the studied virulence genes, including the ply allele 5, were found in the invasive Sp1 ST306 clone, circulating in Europe, but were absent on the Sp1 isolates which are genetically distinct from the French strain and are circulating in the African meningitis belt, where a higher level of serious patterns of infection appears to be more prevalent. These virulence genes circulating in different geographic regions, along with their encoded surface proteins which are now considered vaccine candidates, could therefore be important in future vaccine development and global efficacy for *S. pneumoniae*.

Studies using animal models have suggested a link between the pneumococcal serine-rich repeat protein (PsrP), a pneumococcal virulence determinant able to mediate attachment to lung cells and thought to increase the ability of pneumococci to cause pneumonia, and the high prevalence in children with pneumonia with the ST306 clone, since PsrP was detected in all ST306 isolates studied (n=88), [428].

In a study in healthy children carriers and patients with invasive disease, the PspA clade identified in the ST306 clone was clade 1, [434]. There has been a high prevalence of PspA

clade 1 reported from adults with IPD from Australia, the UK and Japan [458, 459]. In contrast, there has been a high prevalence of PspA clade 2 isolated from IPD adults reported in Spain, Canada, Sweden, the USA and France [460, 458]. A number of studies have indicated that PspA family and clade distribution are independent of serotype, but PspA clade classification was related to genotype where all strains with the same ST have the same PspA clade, despite its origin or capsular type. This information will be important if a PspA-based vaccine were to be developed to combat IPD. A PspA-based vaccine inclusive of PspA family 1 and 2 molecules would suggest a beneficial outcome.

1.3 Virulence factors

S. pneumoniae can produce a variety of virulence factors (Figure 7) linked to disease progression, although it is widely acknowledged that pneumococcal strains differ in their ability to generate these virulence factors, and that their full role in disease has yet to be revealed [4, 5].

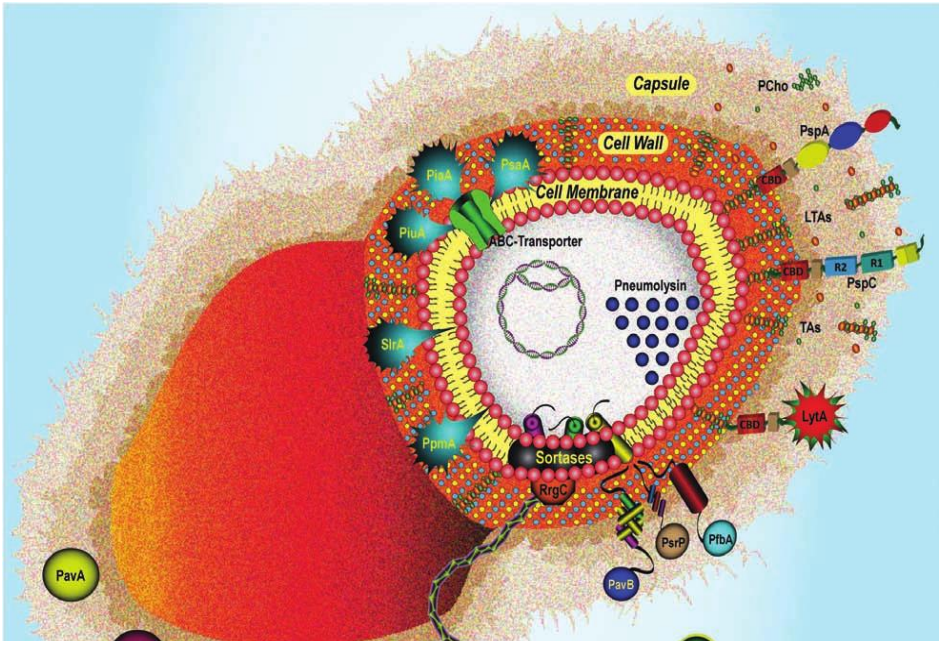


Figure 6. Virulence factors displayed on the surface of pneumococci [100]

1.3.1 The capsule

The polysaccharide capsule is known as one of the most important contributors to virulence of the pneumococcus, due to its anti-phagocytic activity [109]. Seventeen immunogenic proteins have been discovered on the polysaccharide capsule, thirteen of which are age-dependent. Antibodies attach to the pneumococcus cell wall, which then binds to complement. The capsule blocks interaction of the iC3b and the Fc of immunoglobulins on the bacterial cell surface with phagocytic cell surface receptors. A consequence of this is that the bacterium remains extracellular [110]. The capsule also plays an important role in colonisation, preventing the mucus of the host from removing the bacteria [111], as well as limiting autolysis and exposure to antimicrobial drugs [112]. In terms of pathogenicity, the polysaccharide envelope is currently the main area of focus since it is known as a major virulence factor.

1.3.2 Pneumococcal surface protein (PspA)

Pneumococcal surface protein A (PspA) is a choline binding protein that is present in the cell wall, exposed extracellularly on the cell surface [224, 225]. It is conserved across all *Streptococcus pneumoniae* strains [226], though its molecular weight varies between strains, with sizes ranging between 67-99 kDa. PspA has three structural domains; the N-terminal which is composed of repeated α-helices, which extend the cell wall and appear on the outside of the capsule, therefore highly immunogenic [227]. The outer domain of PspA is highly electronegative with anti-complement properties to avoid attachment of *S. pneumoniae* by the host complement system [228-230]. The role of PspA in complement inhibition is uncertain, and studies show conflicting data. While some studies demonstrate that PspA suppresses complement by interfering with complement C4 deposition on the surface of *S. pneumoniae*

[231, 232], other studies demonstrate that PspA is able to interfere with complement C3 activation on the cell surface of pneumococcus, thus protecting the bacteria from complement mediated phagocytosis [233, 234, 230].

PspA has been studied as a potential target in pneumococcal vaccines, since it is abundant across all serotypes and elicits a serotype independent antibody response [235, 227]. PspA can also confer cross-protection against strains regardless of capsular type [236, 237, 238]. Administrating PspA intranasally as a vaccine has showed a promising protective effect against secondary pneumonia as a consequence of influenza, with PspA-specific IgGs showing a fundamental role in protection against *S. pneumoniae* [239]. Immunisation with recombinant fusion proteins consisting of PspA fused with flagellin, administered intranasally was demonstrated to elicit a more effective protective mucosal immune response against *S. pneumoniae* infection in mice, compared to immunisation with PspA alone [240]. PspA has been suggested as a virulence factor, since the PspA-deficient mutants show reduced virulence in a sepsis mouse model [167].

1.3.3 Pneumolysin (PLY)

Pneumolysin (PLY) is a key virulence factor of pneumococcus. It is a pore-forming toxin, which is a member of the family of cholesterol-dependent cytolysins. PLY is produced by the majority of clinical isolates of pneumococcus, hence its importance as a potential target for future vaccine formulations. CDCs also produced by over 20 species of Gram-positive bacteria, and therefore the most widely studied virulence factor for pneumococcus to date. PLY is 53-kDa in size and is expressed during the late log phase of growth [113]. It can be released both during cell autolysis or independently of the major autolysin [114, 115]. PLY first binds to the cholesterol membrane, then undergoes oligomerisation of up to 50 toxin monomers [116],

which subsequently produces large pores of up to 30nm in diameter, which in turn causes potent induction of inflammation and tissue damage.

Using knockdown experiments of the gene for PLY in animal models, a reduction in virulence of the bacterium was observed in both intranasal and systemic routes of pneumococcus infection [118]. When these mutants of pneumococcus were introduced into the mouse lung, much less inflammation was observed as a consequence of infection [119, 120]. Immune cell recruitment in response to infection was delayed and in comparison to wild-type pneumococcus infection there was a reduced number of immune cells [120]. Most notably was the delay and reduction in the neutrophil response, and the redistribution of T-lymphocytes and B-lymphocytes found inside and surrounding the inflamed bronchioles. In the absence of PLY in these mutants, a reduction in the number of pneumococcus bacteria were found in the nasopharynx as a result of intranasal infection [122], however another study indicated PLY did not play a role in colonisation [123].

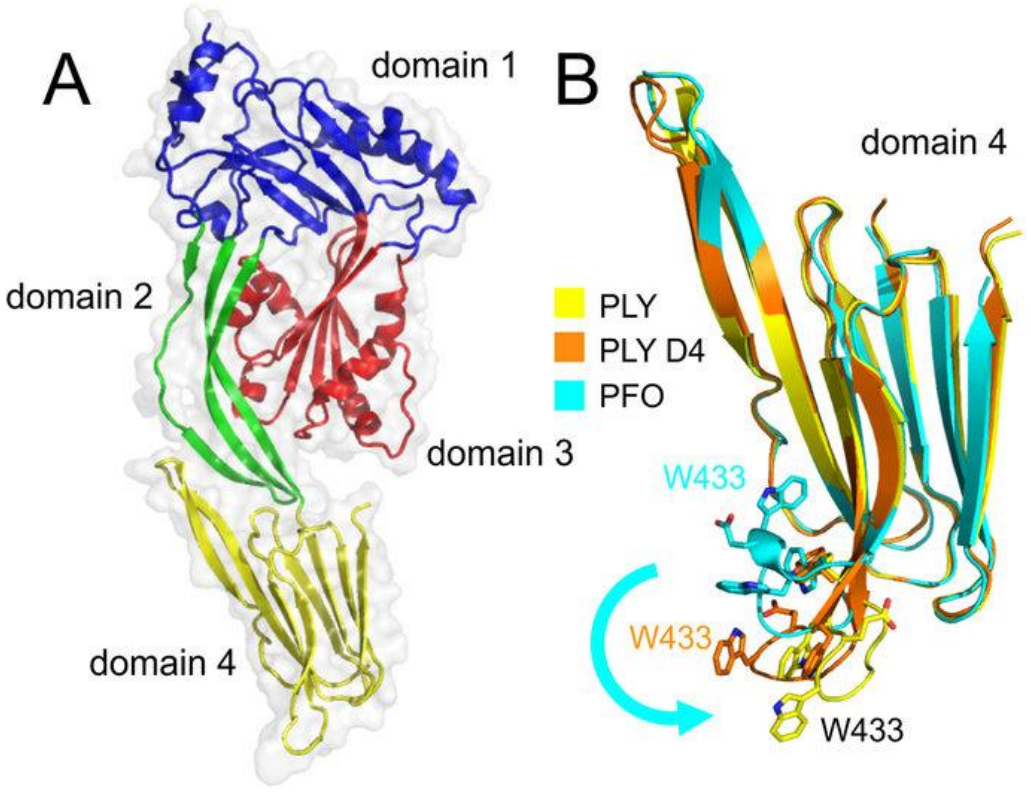


Figure 7. Domain structure of pneumolysin and the likely changes upon membrane binding [435]

At sublytic concentrations, PLY can also activate the classic complement pathway, even in the absence of specific antibody [117]. The pro-inflammatory response is induced and other reactive oxygen intermediates and mediators produced in response to low concentrations of PLY [124]. This is thought to be a result of the interaction between PLY and the lipopolysaccharide receptor, Toll-like receptor 4 (TLR4). A study found PLY was able to synergise with TLR agonists to enhance secretion of proinflammatory cytokines IL-12, IL-23, IL-6, IL-1b, IL-1a and TNF-a by dendritic cells (DC) and enhanced cytokines IL-17A and IFN-c by splenocytes. IL-17A and IFN-Y are known to be required for protective immunity to pneumococcal infection. PLY-induced DC maturation and cytokine secretion by DC and splenocytes was found to be TLR4-independent. When muce were intranasally infected with PLY-deficient pneumococci, significantly less IFN-c and IL-17A in the lungs resulted

compared to infection with wild-type bacteria [125]. In the absence of TLR-4 in mice, macrophages did not prompt an inflammatory response to PLY; colonisation was more widespread and mice were more prone to pneumococcal sepsis [125]. Level of susceptibility was dependent on the presence or absence of PLY. The PLY mutant, with undetectable cytotoxicity and no complement-activating properties, also known as PdT (Figure 8), showed PLY was still active, suggesting the TLR4 interaction was distinct from these actions [125]. Using a solid-phase assay, it was noted that PLY can physically interact with TLR4 [126]. Pore formation induced by PLY can also activate the NLRP3 inflammasome, which leads to the Caspase-1 activation, followed by processing and release of IL1β by the immune system. It has been suggested that pore formation may also be responsible for potassium efflux and lysosomal destabilisation, leading to detection by NLRP3 directly, or through intermediary factors resulting in inflammasome assembly and Caspase-1 activation.

During pneumococcal disease, the role of PLY remains debated. In the chinchilla model, PLY did not stimulate any inflammatory response associated with otitis media [133]. In the rabbit model, a rapid inflammatory response was observed in the brain as a result of intra-cisternal injection; however PLY-deficient and wild-type pneumococcus were not compared to determine the contribution of the PLY toxin in this model [134]. In the mouse [135] and guinea pig model [136], PLY lacked of any inflammatory response in the brain. Specifically, CSF leukocytosis did not appear to be significantly affected by the lack of PLY and the PLN-A inoculum had no detectable hemolytic activity. No difference was observed between the PLY-deficient mutant and wild-type pneumococcus. The PLY-deficient mutant has, however, been shown to reduce protein influx into the cerebrospinal fluid (CSF), accompanied by a reduction in structural damage to the cochlea of infected animals, and a decline in pneumococcus-associated hearing loss [136].

Pneumococcal meningitis has been found to emerge in the rabbit infection model [137], leading to brain damage, with levels of PLY released measuring approximately 20 ng/mL in the CSF [138]. In patients, these levels range from around 1 to 180 ng/mL for those suffering from pneumococcal meningitis. Levels of PLY toxin found in the CSF tends to be dependent on the type of antibiotic used. For instance, more PLY is released into the CSF when ceftriaxone is used to treat infection in rabbits compared to that of a non-bacteriolytic antibody such as rifampicin [138]. This suggests the importance of treatment decisions when treating pneumococcal meningitis and the consequences with regard to bacterial lysis in the release of PLY.

Lytic activity and activation of complement are the major activities linked to PLY. PLY does not appear to play a role in the inflammation linked to meningitis [134-136], but appears to play a role in deafness associated with meningitis [135], along with bacteraemia [140] and pneumonia [118].

1.3.4 Pneumococcal autolysin A (LytA) and other choline-binding proteins

LytA is an important autolysin which promotes lysis of pneumococcus. LytA is located in cell envelope and plays numerous roles in physiological cell function, particularly associated with cell wall growth [241]. The enzyme has been shown to release highly inflammatory cell wall degradation components, as well as PLY from the cytoplasm. The structure of LytA is consists of two domain proteins with an N-terminal N-acetylmuramoyl L-ala-nine amidase domain and a C-terminal choline binding domain. The choline binding domain facilities this enzyme to bind to phosphocholine residues present on the teichoic acids of the cell wall [242]. Cell wall linkage via the choline-binding domain is necessary for LytA activity since choline concentrations suppress cell wall binding and also prevent autolysis [241]. Cell lysis occurs by

cleavage of the lactyl-amide bond that attaches the stem peptides and glycan strands of the peptidoglycan, leading to hydrolysis of the cell wall. LytA orthologs have recently been shown to be conserved through bacteria and in many bacteriophages [243-245].

The role of LytA *in vivo* is still under debate. However, there are three suggestions, firstly, LytA may perform lysis in order to release other virulence factors of pneumococcus, including pneumolysin; secondly, LytA may be released to degrade neighbouring, non-competent pneumococcal cells in a fratricidal manner; and third, LytA may mediate lysis to release either proteins that may contribute to immune evasion, or cell wall components that might interact with the host immune response [246-248].

Some studies have shown that mutations in the LytA gene of the *S. pneumoniae* chromosome result in greatly reduced virulence compared to the parent strain, demonstrated in a mouse intraperitoneal challenge [165]. Moreover, other studies involving LytA have demonstrated that this enzyme prompts a protective response in mice against pneumococcal infection when administrated directly into the lungs [250-252]. The protective property of LytA resulted in a greatly extended survival in mice challenged intranasally with autolysin, making LytA a potential component of novel anti-pneumococcal vaccines [253, 254]. Immunisation, with both autolysin and pneumolysin presented the same degree of protection compared with singular administration, corresponding with the idea that, at least *in vivo*, anti-autolysin antibodies exert their effects first by preventing the secretion of pneumolysin.

LytB, LytC and CbpE aid in colonisation of the nasopharyngeal [161]. The pneumococcal cell wall consists of repeat domains with choline present, which interact with CBPs that attach to the cell surface. Pneumococcal strains have a variable number of CBPs, for example, there are

ten CBPs in the R6 strain and 15 in the TIGR4 strain [162, 163]. LytA, LytB, LytC and CbpE are hydrolytic enzymes present in the cell wall, contributing to virulence [164, 165].

1.3.5 Neuraminidase

Several enzymes encoded by genes of pneumococci have neuraminidase activity. Neuraminidase has the ability to cleave N-acetylneuraminic acid from glycolipids, lipoproteins and oligosaccharides on the cell surface and in body fluids, causing damage to the host cells. This may also uncover binding sites that the bacteria can potentially attach to. Neuraminidase may therefore function in terms of strong adhesion and other processes [255]. Neuraminidase activity degrades sialic acid which subsequently aids spread of the organism up the Eustachian tube to the middle ear. Although neuraminidase possibly promotes colonisation as a result of its action on glycan, its main role in pathogenicity has yet to be identified [256].

There are two forms of pneumococcal neuraminidase enzymes, NanA and NanB. NanA is 108 kDa and NanB 75 kDa in size [155, 258]. The sequence of NanA and NanB display slight homology. Both enzymes show propensity for degradation to smaller fragments during *in vitro* growth and purification, with some of these fragments maintain neuraminidase activity. There are differences between NanA and NanB [155, 258, 259]. Firstly, NanA activity is approximately 100 times greater than that of NanB; secondly, NanA contains a C-terminal sequence that has an LPXTGX motif, which is not present in NanB. This is thought elucidate potent covalent binding of NanA to peptidoglycan structures of pneumococci [258, 260].

It is still not clear why *S. pneumoniae* has two different neuraminidases. However, they are likely to specialise in different environments during exposure during infection and invasion of the host. Notably, the two enzymes elicit different activities at a different pH. NanA is found to have optimal activity at pH 5, while NanB is most active at pH7 [155]. Neuraminidase A

(NanA) has been demonstrated as a virulence factor, playing a role in biofilm production. It has also been found to contribute to colonisation and the development of otitis media when observing pneumococcus infection in the chinchilla model. However, NanA has not been found to play a role in deafness associated with meningitis. NanA activity has been shown to induce the release of sialic acid, which may prove to be of importance to signalling to control pneumococcal virulence.

1.3.6 Pneumococcal surface antigen (PsaA) and other lipoproteins

Pneumococcal surface antigen (PsaA), is part of an ABC transporter that transports manganese and zinc ions into the cytoplasm of the bacteria [261]. Manganese is necessary for normal bacterial growth, thus mutation in PsaA results in impairment of manganese transport, which affects the expression of other pneumococcal genes, including adhesins. Manganese is also important in bacterial ability to resist oxidative stress [154-156], resulting from the production of hydrogen peroxide during pneumococcal metabolism, as well as the output of the reactive oxygen species during the interaction between the host innate immune response and bacteria. When a mutation arises for PsaA, there is decreased adhesion to cells, followed by a decrease in virulence and an increase in sensitivity to oxidative stress, illustrating the disability of PsaA mutant strains of pneumococcal to invade and colonise [262].

PsaA has been identified as the substrate-binding lipoprotein, PsaB as the ATP-binding protein, and PsaC is the permease [153]. The lipoprotein peptidyl prolyl isomerases, SlrA and PpmA have demonstrated roles in virulence and colonisation [157, 158]. The lipoproteins Pia and Piu, known to be involved in iron uptake, are also shown to be virulence factors [159, 160].

1.3.7 Pneumococcal surface protein C (PspC)

Pneumococcal surface protein C (PspC) is an adhesion protein, which binds to the polymeric immunoglobulin receptor, as well as the complement regulatory protein factor H, to provide protection from complement. PspC is the only molecule homologous to PspA, with a similarity in its structure and function [263]. It is a surface exposed protein linked to the bacterial cell wall through specific choline binding motifs [227] and has been characterised as one of the essential virulence factors of *S. pneumoniae*, with an important role in adherence, colonisation and immunogenicity of pneumococcus. *In vitro* binding to sialic acid and epithelial cell residues becomes impaired when infecting with PspC deficient mutants of serotype 2 and 3 and demonstrated decreased nasopharyngeal colonisation and virulence compared to the wild-type strain, in sepsis models [264, 265]. A high degree of genetic variability has been observed between strains for PspC and its contribution to pneumococcal virulence is strain-dependent, not only in lung infection but also during systemic infection [266, 267].

Moreover, PspC plays an important role in evading complement due to its ability to bind factor H (FH), a negative regulator of the alternative pathway, thus preventing immune activity against *S. pneumoniae* [268-270]. The separation of factor B from the alternative pathway C3 convertase (C3bBb) decreases the C3b deposition on the surface of bacteria. Furthermore, factor H can perform as a co-factor in the degradation of C3b via factor I-dependent cleavage of C3b, bound to bacterial surface, to iC3b. Finally, the formation of C3 convertase on the surface of bacteria may be suppressed due to factor H binding to C3b, which can dislocate C3b from factor B and decay the C3 convertase C3bBb [271]. PspC also has ability to bind to the fluid phase complement inhibitor C4b-binding protein, which blocks the activation of the classical complement pathway [272].

1.3.8 IgA1 protease

IgA1 proteases are enzymes able to cleave human IgA1 [48], which predominates in mucosal tissues and secretions [49]. When cleavage occurs, pneumococcal bacteria producing IgA1 become coated with antibody fragments lacking their Fc domains. This allows the bacteria to evade the host immune response, specifically avoiding the recognition by Fc receptors and complement which usually elicit an inflammatory response [50]. Cleavage also results in the organism retaining the Fab fragments on its surface, which aids pneumococcal bacteria in masking its capsular polysaccharide surface antigen. Cleavage of IgA1 protease to human IgA1 also promotes adherence to epithelial cells, enhancing infection, by electrostatic interactions between positively charged Fab fragments coating the bacteria and the negatively charged host cell plasma membrane [51].

1.3.9 LPXRG-anchored surface proteins

17 LPXTG-anchored proteins have been found, although the number may be variable between strains [141]. The LPXTG motif is typically found near the C-terminus of the protein, however it has also been observed near the N-terminus end in some pneumococcal proteins, thought to be involved in the surface location of proteases [142]. The C-terminus sequence is recognised by sortase enzymes, which recognise LPXTG and these attach to the cell surface via covalent linkage of peptidoglycan found in the cell wall. Hyaluronidase, neuraminidase, and serene protease PrtA are LPXTG proteins, and all present as virulence factors. Hyaluronidase is secreted by almost all clinical isolates (99%) [143], and is responsible for the breakdown of hyaluronic acid found in mammalian connective tissue and the extracellular matrix. It has been observed in other micro-organisms, that the degradation of this component can assist bacterial spread and colonisation [144]. Hyaluronidase may increase pulmonary inflammation in pneumococcal pneumonia by interacting with pro-inflammatory cytokines and chemokines [145]. An example of this is the promotion of cytokine secretion induced by hyaluronic acid

binding to CD44 on host cells. The serine protease PrtA gene was found in all clinical isolates [146], and encodes a member of the subtilase family, with hyper-variable region across the central domain of the protein. The catalytic triad of this protein remained conserved. The exact role of PrtA has yet to be uncovered, however it has been demonstrated that PrtA gene expression is co-regulated with the virulence genes for pilus locus and PLY by the transcription factor pneumococcal surface antigen (Psa)R [147]. On the other hand, manganese or zinc has been shown to have opposing effects on the regulation of PrtA by PsaR [148]. When a Prt-A-deficient mutant of D39 was attenuated and introduced into the murine infection model, vaccination provided protection from intra-peritoneal challenge close to that of antibody levels found in healthy control individuals and in patients with invasive disease [146].

1.3.10 Pneumococcal virulence and adhesion

Other virulence factors identified for pneumococcus include the pneumococcal adherence and virulence factor A (PavA) family, glyceraldehyde-3-phosphate dehydrogenase and enolase, which are two glycolytic enzymes, and the pneumococcal histidine triad protein (Pht) family. PavA binds to fibronectin and mediates attachment to endothelial cells, and mutants lacking this protein have been shown to be attenuated in sepsis and meningitis models [168, 169]. Glyceraldehyde-3-phosphate dehydrogenase and enolase enzymes bind to plasminogen [170, 171], which aids in transmigration of the bacteria through the basement membrane [172]. The PhT family has been shown to offer protection against pneumococcus in vaccination models [173, 174], however, mutagenesis of all four proteins are required to attenuate pneumococcus [175]. PhtA, PhtB, PhtD, and PhtE are all members of this family of proteins, which all contain a conserved motif, HxxHHxH, believed to be of importance to the binding of zinc ions. Regulation of the expression of this gene family is controlled by zinc. The Pht family can

reduce binding of complement to the bacterium by recruiting the complement regulator factor H [175].

1.3.11 Pneumococcal pilus

Pili are found on the surface of some strains of pneumococci [149]. The *rlr* locus or pilus islet (PI-I) are the genes responsible for pilus production. They code for three structural proteins and three sortase enzymes, which play a role in the linkage of structural subunits. The pilus is a virulence factor in that it mediates binding of pneumococci to the cell surface (Figure 9), as well as stimulating the pro-inflammatory response in terms of inducing cytokine production [149]. Another pilus type aids adherence of pneumococci to epithelial cells [151]. Certain strains of pneumococci are able to express both types of pili [151].

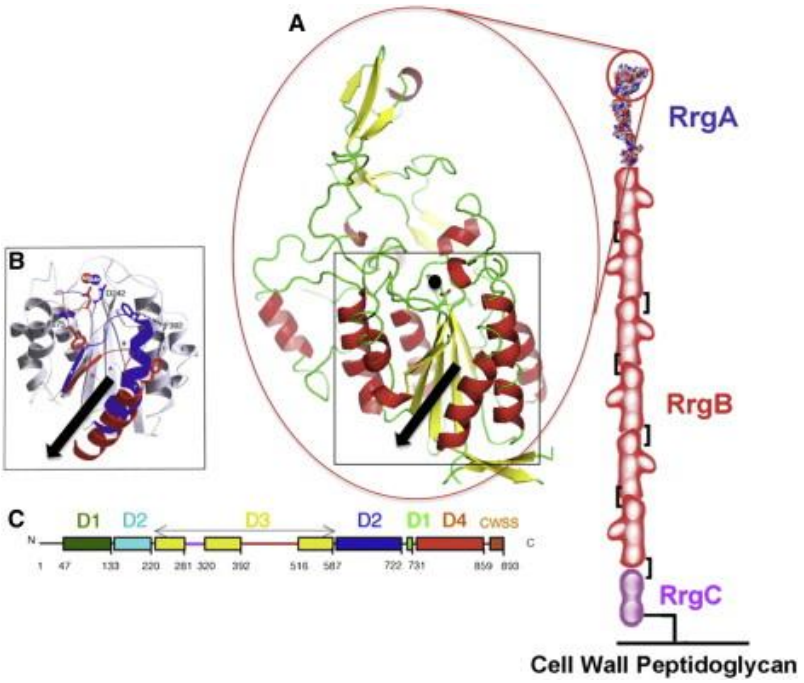


Figure 8. The Pilus of *S. pneumonaie* May Mediate Cell Binding in the Bloodstream [436]

1.3.12 Immune responses to the pneumococcus

The induction of a pro-inflammatory response to pneumolysin and the stimulation of TNF, IL-1, IL8 and IL6 production has been well documented in a number of different host cells [43-47]. Pneumolysin has demonstrated its ability to induce nitric oxide which is an important component of antimicrobial immunity and contributes to tissue pathology, as well as induce the production of CXCL8 and IL-6 in cells of the upper airway. Macrophages are also produced by the upregulation of cox-2 gene expression via an IFNγ- dependent pathway. Other pathways that are thought to play a role against pneumolysin are the MAPK, NFκB and TLR-4 signaling pathways. Several pattern recognition receptors including Toll-like receptors 2 and 4 are also thought to play a role in mediating the inflammatory response to pneumococci [487].

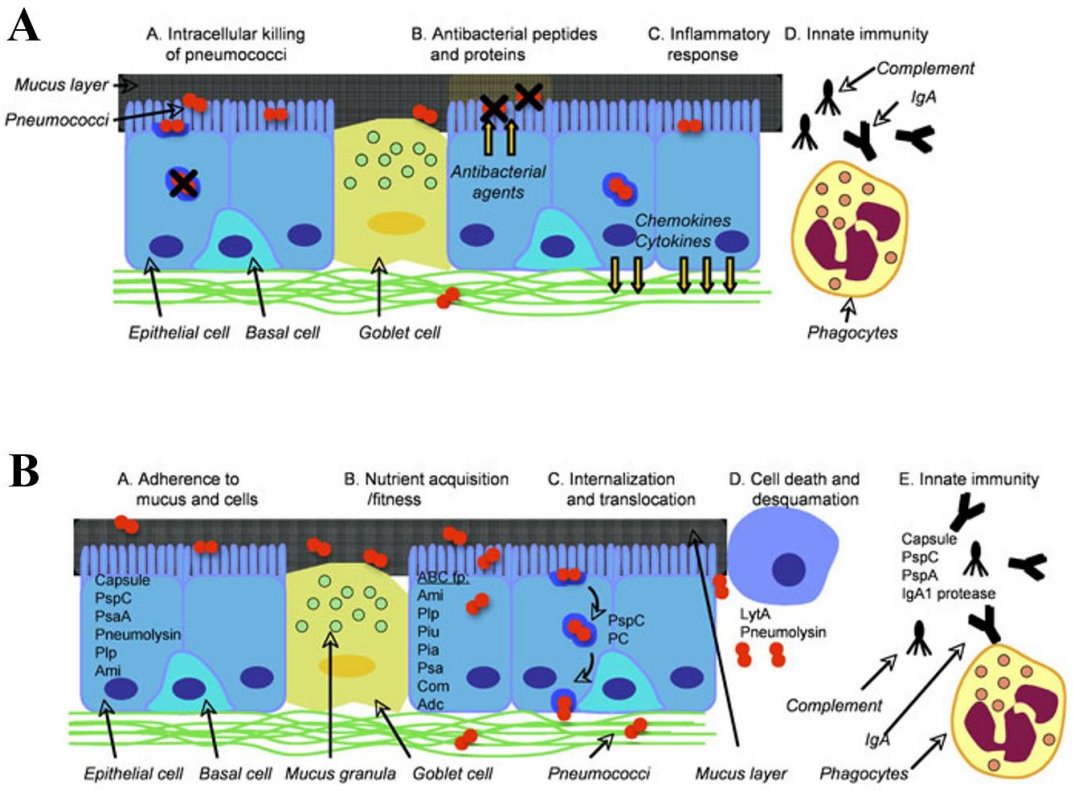


Figure 9. (A) The host mechanisms use to battle pneumococcal colonising and infecting, (B) the main virulence factors of *S. pneumoniae* that have been implicated in colonization and infection [1].

Pro-inflammatory cytokines IL-6, IL1β and the transforming growth factor-β are responsible for the development of the distinct set of T cells, Th17 [129-131]. Th17 cells produce IL-17 cytokine which boosts neutrophil production and migration to areas of infection [128]. Mice that have been immunised by a variety of pneumococcal proteins have been shown to stimulate IL-17 production, however it is uncertain whether this result is primarily IL-17 production from the spleen or whether there were contributory pro-inflammatory cytokines involved [132]. IL-17 has been shown to contribute to the immune response against pneumococcus colonisation, also dependent on the presence of PLY [127]. Inflammasome signaling is important for the promotion of IL-17A responses, which are thought to play an important role in resistance to both pneumococcal carriage and pneumonia, [487].

Pneumolysin has been shown to have complement activating activity and may play a role in induction of memory T cell responses, as well as its role in T cell recruitment and T cell cytokine production [487].

1.4 Pneumococcal disease interventions

1.4.1 Antimicrobial treatment of pneumococcal disease

Increasing emergence of antimicrobial resistance is progressively hindering attempts at treating pneumococcal infections [84-86]. Treatment using β-lactam agents, such as ampicillin, with individuals presenting with CAP is generally not associated with poorer outcomes, but this is not the case with high-level macrolides resistance or fluoroquinolone resistance, in which treatment failure is likely to occur [84-86]. In the treatment of severe CAP, recommended therapy is a combination of β-lactam and macrolides agents, such as azithromycin, or clindamycin (a macrolide-like agent), or fluoroquinolone alone [52, 53]. Combination therapy

has been demonstrated to show a more beneficial effect in bacteraemic pneumococcal CAP and in severe pneumococcal bacteraemia compared to monotherapy [53, 54].

1.4.1.1 Adjuvant anti-inflammatory strategies in severe pneumococcal disease

The invasion of the host immune cells, particularly epithelial and endothelial cells [56], as well as erythrocytes [57], can protect the bacteria against β-lactam agents, though with more lipophilic agents such as macrolides, protection is to a lesser extent due to these agents being poorly taken up by eukaryotic cells [58]. Furthermore, β-lactams have been shown to cause disintegration of pneumococcal bacteria, followed by release of pro-inflammatory cell wall components including lipoteichoic acid and peptidyoglycan, as well as the cytotoxin pneumolysin, resulting in detrimental inflammatory responses [59]. Adjunctive anti-inflammatory therapy, such as the use of macrolides in combination therapy, aims to optimise treatment by suppressing the production of pneumococcal adhesins, invasins, cytotoxins, and immunosubversins, while simultaneously limiting the potentially harmful host inflammatory response by acting as an anti-inflammatory [60]. Macrolides have the ability to inhibit the potentially harmful effect of neutrophil-mediated inflammation, which mobilise chemokines and cytokines interleukin-8 (IL8) and tumour necrosis factor (TNF) by epithelial cells, fibroblasts and smooth muscle cells in the airways, and lung macrophages [61, 62]. Macrolides are thought to interfere with the transcription process of genes encoding these pro-inflammatory proteins 61-64]. Treatment with macrolides have been associated with reduced mortality in patients presenting with pneumonia and severe sepsis caused by macrolide-resistant bacteria [65], pneumonia caused by pneumococcus resistant strains [66], and in acute lung injury as a cause of pneumonia [67].

1.4.1.2 Corticosteroids

Corticosteroids are broad spectrum anti-inflammatory agents, currently suggested as adjuvant treatment for individuals with penicillin-susceptible meningitis [68], due to higher survival rate, reduced duration of hospital stay, and less neurological and auditory complications in both adults and children [69-72]. However, it is known that neutrophils are relatively insensitive to corticosteroids as an anti-inflammatory [73], and recent reports show that high levels of the endogenous corticosteroid, cortisol, is predictive of severe disease and mortality in individuals with severe CAP [74]. Furthermore, recent studies have failed to confirm the benefit and efficacy of systematic administration with corticosteroids [75-76].

1.4.1.3 Statins

Statins inhibit the enzyme 3-hydroxy-3-methylglutaryl co-enzyme A reductase and can act as therapeutic agents capable of controlling hypercholesterolemia and reduce the risk of cardiovascular disease and stroke. In addition to these functional properties, statins also possess anti-inflammatory properties and can be used to control harmful neutrophilic inflammation. Firstly, they appear able to interfere with G-protein receptor-mediated signalling mechanisms in immune and inflammatory cells [77], and secondly, they are able to induce the enzyme hemeoxygenase-1 which mediates the induction of anti-inflammatory proteins [78]. Statins have been shown to provide a significant survival advantage among individuals with bacterial CAP [79-80], as well as with individuals with pneumococcal disease [81]. Statins may also provide protection against acute cardiac complications triggered by pneumonia [79], along with potential interference with cytotoxic and pro-inflammatory processes of the cholesterol-binding pneumococcal toxin, Pneumolysin [83].

1.4.2 Pneumococcal vaccines

Current pneumococcal vaccines consist of capsular polysaccharide antigens, or vaccines conjugated with proteins to boost the immunological response in order to match the response seen during natural infection with pneumococcus, termed protein-polysaccharide conjugate vaccines [152]. The most often used pneumococcal vaccines to date are the 7-, 10-, and 23-valent vaccines [367]. It is thought that this is due to the emergence of infections caused by pneumococcal serotypes absent from the current vaccine spectrum [404]. Current research is focussed on evaluating potential adjuvants to polysaccharide or conjugate vaccines, or extending the range of coverage to more protein antigens common to all pneumococcal serotypes, forming novel vaccine candidates [364-366].

	Serotypes
Pneumovax	1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A,11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, 33F
Prevnar	4, 6B, 9V, 14, 18C, 19F, 23F
Prevnar-13	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F

Figure 10. Coverage of serotypes for pneumococcall vaccines [407]

1.4.2.1 Capsular polysaccharide vaccines

In 1911, a crude, whole-cell vaccine was developed aimed at preventing pneumococcus [89]. Following this, pneumococcus became known to consist of different capsular serotypes, and so numerous studies initially focussed on single type-specific vaccines, and then vaccines

including two or four serotypes. Towards the end of the 1940s, two hexavalent vaccines were marketed, until highly effective antibiotics against pneumococcus became available a few years later. In 1964, it became obvious that vaccines were still a necessity, due to reports that the incidence of mortality due to pneumococcal bacteraemia remained very high despite the use of antibiotics [90]. In a large hospital in New York, it had been estimated during a longitudinal study over 10 years that approximately 25% of all patients with bacteraemia died, with around 50% of these consisting of patients over 60 years of age. During 1977, after the revival of vaccine interest, a polysaccharide 14-valent vaccine, including 14 capsular serotypes became available for use in the USA. This vaccine was replaced in 1983 with the 23-valent vaccine, which includes purified capsular polysaccharide antigens for the following 23 serotypes (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F), representing more than 90% of serotypes known to cause invasive pneumococcal disease [91].

1.4.2.2 Immunogenicity

Antigenic differences of the capsular polysaccharide remain the basis of pneumococcal vaccines [92]. These antigens are capable of eliciting specific antibody responses that can provoke opsonisation, phagocytosis and killing of the bacteria by phagocytic cells [91]. The T-cell independent mechanism is primarily responsible for the induction of antibodies against pneumococcal polysaccharide antigens, thus, children below 2 years of age may respond poorly due to an under-developed immune system. This makes immunisation particularly important in this age group since they have little protection from numerous opportunistic bacterial infections in the first few years of life. In the elderly, antibody response is usually adequate [95-96], with only a subset who may only respond to some of the 23 serotype antigens [97-98].

They may also have lower functional activity, in terms of avidity, opsonisation and phagocytosis, induced by antibodies against the vaccine serotypes [99]. Despite these potential low responders, in general vaccination can induce a two-fold or greater increase in specific antibodies against capsular polysaccharide antigens within 2-3 weeks [94].

1.4.2.3 Pneumococcal 7-valent conjugated vaccine

In 2000, the protein conjugate vaccine (PCV-7 Prevenar, Pfizer, previously Wyeth), was recommended for use in the USA by the Committee on Infectious Diseases. By 2008, 22 countries had introduced PCV7 to their vaccination schedule. It included protection from 7 different serotypes of the bacteria based on their polysaccharide capsular antigens – 4, 6B, 9V, 14, 18C, 19F and 23F, which covers 80-95% of serotypes known to be responsible for invasive pneumococcal infection in the USA [161]. Conjugated to this is CRM_{197}, which is a non-toxic diphtheria variant carrier protein which is highly immunogenic, able to elicit a strong T-cell dependent response, and therefore improve vaccine efficacy. Since this vaccine was introduced in the United States, data has reported that there has been a reduction by 94% in pneumococcal disease in children under the age of 5 years, confirming the effectiveness of this particular vaccine [162]. However, PCV7 does not include optimal protection for all serotypes included in the vaccine [176]. The most notable effectiveness in terms of serotypes protection was against serotypes 14, 9V and 23F, which showed the most significant reduction (more than three-fold) in occurrence after the 7-valent vaccine was introduced. Serotypes 18C and 6B showed a modest reduction of around 2-fold of cases, while serotype 19F showed little reduction overall. Serotypes not included in the vaccine coverage continued to show a rise in

the frequency of infections, as did the strains resistant to antibiotics. There was promise in terms of resistance levels among the serotypes included in vaccine coverage, with a 1.5-fold decrease, however, those not included within the vaccine continued to double in frequency of resistance. Non-invasive serotypes also showed a rise in resistance to multiple (two or more) antimicrobial factors. All 7 serotypes have been associated with high-antibiotic resistance 162, 176].

1.4.2.4 PCV10

In 2010, the WHO announced the pre-qualification of the 10-valent pneumococcus vaccine (PCV10) [177] and some countries, such as Poland, saw an increase in the number of people being vaccinated, though unfortunately there was not a matched decrease in incidence of pneumococcal disease [178]. While PCV-7 is gradually being removed from the market, PCV10 and PCV13 are licensed for immunisation of infants and children from 6 weeks to 5 years of age against IPD and acute otitis media caused by the vaccine serotypes of *S. pneumoniae*. PCV13 is also licensed for the prevention of pneumococcal disease in adults >50 years of age [478].

1.4.2.5 PHiD-CV and PCV13

PHiD-CV (Synflorix®) and the conjugate 13-valent pneumococcus vaccine (PCV13, Prevenar 13) are both currently available in the UK, with PCV13 replacing PCV7 in the UK immunisation program in April 2010, [478]. These aimed to widen the spectrum of coverage in terms of serotypes responsible for the increasing numbers of infection, but not previously included in the vaccine [177]. PHiD-CV was developed by GlaxoSmithKline and consists of

two capsular polysaccharide types conjugated to either diphtheria (serotype 19F) or tetanus (serotype 18C) toxoid, and 8 other serotypes (1, 4, 5, 6B, 7F, 9V, 14 and 23F) conjugated to non-typable *Haemophilus influenzae* (NTHi) protein D [362]. NTHi protein D is thought to offer additional protection against acute otitis media (AOM) caused by *H. influenza* [362]. PCV13 consists of 6 additional polysaccharide antigens alongside those 7 previously included in PCV7. Additional serotypes included in PCV13 were 1, 3, 5, 6A, 7F and 19A, combined with the diphtheria toxoid, CRM_{197} [363]. These additional serotypes were most often seen in Africa, Asia and Latin America and known to be responsible for 70% of invasive pneumococcal disease worldwide [177, 415]. This vaccine was also documented to be useful in vaccinating children infected with the human immunodeficiency virus (HIV), and offers some protection against pneumococcal infection [414]. PCV13 has now been introduced in over 40 countries; however there is lack of data to report the impact of PCV13 on the incidence of pneumococcal infection caused by the spectrum of serotypes included in this vaccine [177]. Serotype 22F is not included in either the PHiD-CV or PCV13 vaccine, which is of concern since the prevalence of 22F IPD has recently shown a dramatic increase in children less than two years of age in England and Wales [340], as well as ranking sixth in Scotland for causes of IPD in children less than 5 years of age. Currently, PCV13 appears to be the most effective vaccine against pneumococcus and has been introduced into numerous childhood immunisation programs worldwide.

1.4.2.6 23-valent polysaccharide vaccine (PS)

Polysaccharide 23-valent vaccine (PPV23) offers the highest coverage across the different pneumoccoccal serotypes*;* however, PPV23 presents weak immunogenicity in children, [480-484]. Despite this, the application of this vaccine among the risk groups and older people has

gained some ground [178]. The effectiveness of the 23-valent polysaccharide vaccine is uncertain in its capability to protect against pneumococcal pneumonia. However, in the protection against invasive pneumococcal disease, severe pneumonia and serotype-specific otitis media in children below 2 years of age [386], the vaccine has shown promising efficacy, is cost-effective and has demonstrated a protective effect in the immunocompetent population, including the elderly [417, 418], and in groups of immunocompromised individuals, such as those with HIV infection or infants with sickle cell disease, [485]. It may also be useful in limiting the spread of antibiotic-resistant pneumococci. The 23-valent polysaccharide vaccine is currently recommended to individuals aged 2 years or over who are at high risk of pneumococcal disease, and to all elderly individuals over 55-65 years of age. Following primary vaccination, a booster vaccination is recommended when 5 years have elapsed [416]. The 23-valent polysaccharide vaccine (PPV, Pneumovax®, Merck) has been marketed for almost 30 years, and is primarily used to protect adults at risk of pneumococcal disease, and the elderly. While this vaccine is not recommended in children below 2 years of age due to under-developed T-cell-independent immune responses in these individuals [380], conjugate vaccines have been introduced to improve the overall efficacy. The Joint Committee on Vaccination and Immunisation have reported the protective ability of the vaccine as largely unsuccessful in the elderly population in the UK, which is disappointing [336].

1.4.2.7 Vaccine coverage and serotype replacement

When PCV7 was introduced into the UK routine childhood immunisation program by the Department of Health in 2006, it was predicted to reduce the incidence of pneumococcal disease and reduce pneumococcal antibiotic no-susceptibility [381-384]. However, surveillance data for England and Wales showed only a 41% reduction in the number of IPD

cases among those aged 5 years and under (794 cases in 2005–2006, to 470 cases in 2007-2008) [385]. This can be attributed to invasive pneumococcal disease caused by serotypes included in the PCV7 vaccine, which previously accounted for 70% of cases in those less than 5 years of age during 2005 and 2006 and reduced to 24% in 2007-2008. This overall trend has also been demonstrated in Scotland [387]. Indirectly, herd immunity has also been a co-factor in the protection against serotypes-specific IPD since PCV7 immunisation. In the unvaccinated population, a reduction in IPD has also been noted in children over 5 years of age and in adults [385]. Herd immunity due to reduced exposure appears to correlate with number of doses given to the population [388]. Decreased carriage and transmission from vaccinated individuals improves cost-effectiveness and impact of vaccine efficacy [382, 389]. For PhiD-CV and PCV13, serotype coverage in England and Wales was 53% and 74% respectively for 2007-2008. This showed a reduction in coverage compared to the previous years' 2005-2006 which were 81% and 92% for PhiD-CV and PCV13, respectively, likely due to serotype replacement and disease replacement [382, 385]. Serotype replacement describes the shift in prevalent circulating serotypes present in the populating with the ability to cause disease due to vaccination [390]. Following the introduction of PCV7, this shift was observed in the USA and predicted to occur in the UK as well [391]. Serotype replacement has now documented across all age groups in the UK, with a reported increase in IPD cases caused by serotypes 7F, 19A and 22F [385]. In the post PCV7 period, an increased incidence of IPC caused by serotype 7F has also been reported in Portugal [392], and the same phenomenon was observed for 19A and 22F IPD in the US [393]. The ST320 clone known to incur high antibiotic resistance was noted as particularly important in the rise of serotype 19A IPD in the USA [394, 395]. In contrast, the ST199 clone appeared predominantly responsible for the rise of serotype 19A IPD in the UK [396]. This suggests that intrinsic factors other than that of antibiotic resistance were responsible for serotype replacement. Interestingly, the fact that 19F was included in

conjuguate vaccines, has not offered cross-protection to serotype 19A [397]. This may be due to the fact that 19F presents the lowest immunogenicity out of all serotypes covered in the PCV7 vaccine [398]. Cross-reactive antibodies of 19F measured against 19A have also been shown to elicit a weak response, *in vitro* [399]. Following PCV7 introduction in the UK, other serotypes not comprised in the coverage of the vaccine have shown an increase, including serotype 6C [400, 401, 403], despite the presence of 6B antigen in the vaccine. Similarly, the inclusion of 6B polysaccharide in the vaccine formulation did not protect against serotype 6A either [402]. Serotype replacement has contributed to the shift in the pneumococcus niche more than originally predicted, likely due to vaccine pressure among the serotypes able to cause IPD, causing a reduction in the protective efficacy of PCV7 [404]. Serotype replacement was also demonstrated in Scotland, with the example of serotype 7F reported to cause little disease incidence between 2005-2006, and now becoming the most common cause of IPD in children less than 5 years of age, responsible for 12% of cases [385], post PCV-7 introduction. In the UK, a highly significant increase in serotype 1 was reported prior to the introduction of routine PCV7 immunisation [360, 361]; demonstrating serotype dynamics are also prone to fluctuations [404]. Therefore, long-term epidemiological surveillance remains important in treatment responses to these changes.

1.4.2.8 PCV-15

PCV-15 is currently in pre-clinical trials. PCV-15 was produced in order to create a vaccine which covered a broader range of serotypes to allow for the changing epidemiology of pneumococcus, particularly regarding serotype 19A, which after the introduction of Prevnar into the infant vaccination schedule in the US and Europe, showed an increase in prevalence of disease in infants due to serotype replacement. Serotypes 22F and 33F are the next two most prevalent emerging serotypes causing IPD, based on studies in US infants <5 years of age,

children and older adults [309, 437, 438, 439]. PCV-15 consists of the 13 original pnueumococcal polysaccharide serotypes included in the licensed PCV-13 vaccine, plus two additional serotypes (22F and 33F). These additional serotypes have been shown to account for approximately 10% of IPD cases in adults in the US in 2007, [309].

A study describing the development and pre-clinical evaluation of PCV-15, demonstrated that the antibody response to this vaccine was comparable to the antibody response in infant-rhesus monkeys to the PCV-7 vaccine of the 7 serotypes it has in common. PCV-15 was shown to induce strong immunological responses to all 15 polysaccharide components within the vaccine, including the 7 serotypes included in PCV-7 and the 8 new serotypes, however correlate of protection and sero-conversion in human infants has yet to be established. Immunisation with PCV-15 in infant rhesus monkeys did not show serotype interference of antibody responses between the 7 original serotypes and the additional oligosaccharide conjugates. In terms of functional antibody responses based on opsonophagocytosis activity to *S. pneumoniae*, PCV-15 demonstrated excellent responses to all 15 polysaccharide serotypes [440].

In 60 healthy adults immunised with the PCV-15 vaccination, PCV-15 was shown to have an acceptable saftey profile, although had a higher incidence of injection-site and systemic adverse effects, as well as some incidences of elevated body temperature and fever, when compared to recipients of PCV-7. PCV-15 was able to induce both IgG and opsonic activity to all 15 serotypes included within the vaccine [441].

1.4.2.9 Future prospects of novel pneumococcal vaccine formulations

The immunogenicity of current polysaccharide antigens may be improved with the use of adjuvants capable of boosting the immune response [415]. Another potential technique is to convert polysaccharide antigens to T-cell dependent forms of protein-conjugation, further enhancing the immune response. In mice studies, interleukin-12 (IL-12) was found to give an enhanced response of IgG2a, which plays a role in mediating complement fixation and opsonisation, and IgG3, in treatment of pneumococcus when given in conjunction with the pneumococcal polysaccharide vaccine. IL-12 is was also demonstrated to have an important role on the basis of its ability to enhance cell-mediated immunity through the activation of T-cells and natural killer (NK) cells, which suggested this cytokine could potentially be used as an adjuvant to boost the protective efficacy when treating humans with the pneumococcal vaccine. However, when tested in phase I/II randomised, double-blind vaccine studies, results demonstrated a disappointing IgG and antibody isotype response, and antibody avidity, in comparison to the placebo group given the 23-valent pneumococcal vaccine only, without recombinant human IL-12 (rh-IL-12). Furthermore, the co-administration of rhIL-12 and 23-valent pneumococcal vaccine was associated with a high incidence of local and systemic side-effects [420].

Surface protein A (PspA) is the most widely studied antigen common to all serotypes [100]. Being highly immunogenic, it has been demonstrated to induce antibodies protective against pneumococcal infections in mice [101-103], as well as being produced in human sera, both adult and children, in response to previous infection [104]. It is thought that PspA antibodies may play an important role in the protection against pneumococcal invasive infection due to the significantly reduced antibody level of PspA antibody in children aged 7-36 months with invasive pneumococcal infection, compared to other infections. Other targets for protein vaccine candidates currently being explored include the pneumococcal surface adhesin 1

(psaA), choline-binding protein A (cbpA), and *S. pneumoniae* secretory immunoglobulin-A (IgA) binding protein (SpsA). These proteins have common functional characteristics, likely revolving around the process of bacterial adherence [105-107]. Pneumolysin [108] and neuraminidase [100] are two proteins playing an important role in the virulence of pneumococcus, so are also important targets for vaccination. Current vaccine efforts are largely focussed on the above mentioned pneumococcal proteins, as well as whole-cell vaccines, which may provide extended, serotype-independent protection.

1.5 Pneumococcal genetic plasticity

1.5.1 Recombination events

Recombination is an important factor related to the evolution of *S. pneumoniae* compared to other factors responsible for genetic variation of the organism. For instance, the rate of recombination has been documented as 10 times higher than the rate of mutation [203], showing hundreds of recombination events in a single lineage of pneumococcus, with 74% of the genome being subjected to recombination in at least one isolate [204]. However, it has yet to be uncovered whether this rate is similar in all pneumococcal strains, or whether there are other driving factors such as geographical location. High rates of pneumococcal recombination may be a result of the presence of the high density of repeat elements in the genome that may contribute to the integration of foreign DNA into the genome and consequently lead to rearrangement of its structure [205]. *S. pneumoniae* has been reported to have the greatest density of repeats (1 every 500 bp) in comparison to 51 other prokaryotic genomes [206]. BOX, RUPS and Rho-independent Terminator-like Element (SPITE) have all been identified as important repeat elements for recombination. SPITE has also been found to play a role in the

termination of transcription [207]. *S. pneumoniae* consist of an open pan-genome, of an infinite size which means the organism can adapt rapidly to changes in its environment in the form of genetic evolution [208]. High rates of recombination in this organism mean distinct pneumococcal strains or clones may form, but these may be less stable in comparison to other bacteria [203, 208].

Genetic exchange of material between *S. pneumoniae* species in the form of recombination is commonly observed in streptococcus of the mitis group [211], including *S. pneumoniae, S. mitis, S. oralis, S. infantis, S. sanguis, S. gordonii, S. pseudopneumoniae, S. cristatus, S. oligofermentans, S. parasanguinis, and S. peroris.* Some of these organisms from the mitis group also colonise in the nasopharynx, making genetic exchange easier [212]. *S. mitis* and *S. oralis* have been documented to be donors of genetic information passed to *S. pneumoniae* resulting in evolution of mosaic penicillin-binding protein genes [213]. Pneumococcal isolates which had elements of hyper-recombination showed selective advantage, with a significantly higher rate of resistance for various antibiotics, including penicillin, erythromycin, tetracycline, chloramphenicol, and cefotaxime, compared the pneumococcus isolates that had no evidence of recombination. Defects in the mechanism of the DNA mismatch repair system are thought to play a role in hyper-recombination [214, 215, 216].

1.5.2 Genes expressed during infection and colonization

In the last decades, Pasteur and Sternberg first described the pneumococcus, a number of virulence factors involved in invasive disease have been identified. Major virulence determinants such as capsular polysaccharide, pneumolysin, and choline-binding proteins have obviously established roles in pathogenesis [227]. Recently, massive identification of *S. pneumoniae* virulence determinants has been attempted. Studies, such as signature-tagged

mutagenesis (STM), have used transposons and suicide vectors to pepper the chromosome of the bacteria with mutations and identify genes required for virulence [55].

Characterization of pneumococcal virulence determinants should ideally include analysis of gene expression during invasive disease. Confirmation of their expression in vivo would not only verify the contribution of these genes to pathogenesis but would also clarify their contribution to discrete forms of disease (e.g., genes expressed during pneumonia versus those in the blood during bacteremia). However, analysis of in vivo bacterial gene expression, much less at discrete sites, has been limited by the difficulties of isolating sufficient quantities of pure and intact bacterial RNA from infected host tissues [87]. To challenge the requirement for RNA, investigators have used differential fluorescence induction (DFI) to identify *S. pneumoniae* promoters that are induced during disease [87]. Pneumococcal genes play critical roles either with pneumonia and sepsis, or pneumonia and carriage, or sepsis and carriage. For example, *comDE* up-regulated during pneumonia and sepsis, while dwon-regulated during colonisation in nasopharynx [496,497, 498, 499]. A recent study was found a mutation in *sodA* gene resulted in significant attenuation of virulence in a mouse intranasal challenge model [496].

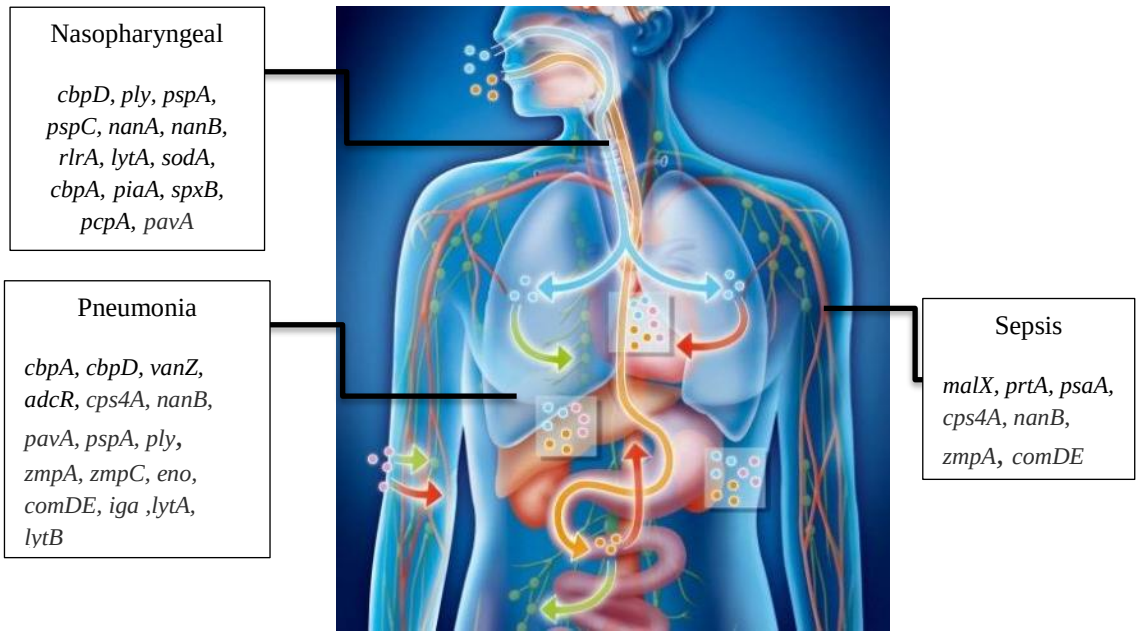


Figure 11. Important genes expression during inftection by S.pneumoniae [607]

Infection	Involved genes
Nasopharyngeal carriage	*cbpD,ply, pspA, pspC, nanA, nanB, rlrA,lytA, sodA, cbpA, piaA, spxB, pcpA, pavA*
Pneumonia	*cbpA, cbpD, vanZ, adcR, cps4A, nanB, pavA, pspA, ply, zmpA, zmpC, eno, comDE, iga ,lytA, lytB*
Sepsis (blood circulation)	*malX, prtA, psaA, cps4A, nanB, zmpA, comDE*

Table 1. Important genes expression of pneumococcal during infection

1.6 Aims and objectives

1.6.1 Aims

This study aimed to characterize the carriage, invasive and immunological properties of *S. pneumoniae* serotype 1 ST306 and ST615 clinical isolates using in vitro and in vivo models. Meanwhile, The ST306 was compared directly with ST615 by using RNA-seq.

1.6.2 Objectives

Host-pathogen interactions of *S.pneumoniae* were observed with a focus on cellular immune responses; colonisation and invasive properties of naturally occurring ST-306 which exhibit low haemolytic activity were investigated; and novel key virulence genes were identified by comparing the expression profiles of *S. pneumoniae in vitro*. In this project, we merely focus on ST306 and ST615.

Chapter 2 describes the materials and methods used in this study, including the characterisation of pneumococcal strains originating from Uruguay and Switzerland, ST615 and ST306, respectively. It also highlights the spontaneous mutations in pneumolysin found to be present in ST306 isolates, which resulted in attenuated bacteria. All of the procedures undertaken in this section have enabled us to illustrate the differences between sequence types and will assist in explaining the interaction between bacteria and their host cells *in vitro* and *in vivo*, including those involved in genetic expression of the bacterial genome identified in chapter three.

Chapter 3 is focused on the *in vitro* results describing the differences in pneumolysin between predominate sequence types in Europe and South America. A haemolytic assay and PLY ELISA were used to measure the concentration of pneumolysin produced, followed by the use of Detroit and BEAS-2B cell lines to characterise adherence and invasion abilities of both sequences. To measure the host response, a complement deposition and opsonizing phagocytosis killing assay were performed. With regard to molecular and genetic techniques, RNA-Seq was used to compare gene expression during the growth phase of bacteria in normal media without any additional nutrients. This chapter is also focused on the in vivo methodology. It includes the study of the behaviour of both *S. pneumoniae* serotype 1 (ST306 and ST615) in mouse models of carriage, pneumonia and sepsis. . First, the ability of

pneumococcal to cause pneumonia and bacteremia after intranasal infection for both the spontaneous mutation strain and non-mutant strain was observed, with mouse survival indicated in this particular experiment. A competition experiment was then carried out using ST306 and ST615 isolates by co-infecting mice with both strains to determine their interaction within the host. Finally, the most significant experiment included intranasally infecting mice with a mixture of the spontaneous strain (ST306) and pure pneumolysin.

Chapter 4 is focused on transcriptome analysis of ST306 comparing to ST615. Analysis of certain genes differential RNA expression provides us with greater insights into biological pathways and molecular mechanisms.

Chapter 5 provides a conclusion to the overall thesis and presents prospects for future work.

Materials and Methods

2.1 Pneumococcal bacterial strains

The pneumococcal isolates used in this project are listed in **Table 2**. All serotypes are clinical isolates, with the exception of laboratory strain D39 and its pneumolysin-deficient variant, PLN-A, which are laboratory-adapted strains [499]. Pneumococci ST306 and ST304 were kindly provided by our collaborator, Dr Lucy Hathaway, Institute for Infectious Diseases, University of Bern, Switzerland, while the strain belonging to ST615 was provided by Prof Francois Trottein, at the Pasteur Institute in Lille, France.

Table 1. List of pneumococcal strains used in this project

Strain ID	Sequence type	Serotype	Carriage/ Invasive	Reference paper/Country of origin
D39	**ST128**	**2**	**Lab strain**	
PLN-A			**[499]**	
B915	**ST306**	**1**	**Invasive**	**Switzerland**
BIL777			**Invasive**	**Switzerland**
BIL450			**Invasive**	**Switzerland**
202			**Carriage**	**Switzerland**
308			**Carriage**	**Switzerland**
204			**Carriage**	**Switzerland**
879			**Carriage**	**Switzerland**
628			**Carriage**	**Switzerland**
455			**Carriage**	**Switzerland**
201			**Carriage**	**Switzerland**
106	**ST304**	**1**	**Carriage**	**Switzerland**
NCTC7465	**ST615**	**1**	**Invasive**	**Uruguay**

Strains were confirmed optichin-sensitive (Oxoid limited, Hampshire, UK), alpha-hemolytic on 5% sheep blood agar, and soluble in 2% sodium desoxycholate.

2.2 Culture media

2.2.1 Blood agar

For the preparation of blood agar base (BAB, Oxoid) plates, 39g of blood agar base was added to 1L of distilled water. The mixture was sterilised by autoclaving at 121ºC for 15 minutes, then cooled down to 50ºC. Horse blood (HB, Oxoid), warmed to room temperature, was added to the media at a final concentration of 5% and flask was swirled to mix thoroughly. The blood agar was then poured into 100x15 mm sterile petri dishes under aseptic conditions, left until solidification, then plates were inverted and stored at 4ºC until use.

2.2.2 Brain heart infusion broth (BHIB)

To prepare BHIB (BHIB, Oxoid), 37g of BHI powder were added to 1L of distilled water. The pH of the broth was adjusted to 7.4 and autoclaved at 121ºC for 15 minutes. Media was poured into sterile plates, left until solidification, then plates were inverted and stored at 4ºC until use.

2.3 Solutions

2.3.1 Phosphate buffer saline (PBS)

To prepare PBS, 10 tablets of phosphate buffer saline (PBS, Oxoid, Thermo Scientific) were dissolved in 1L of distilled water and autoclaved at 121°C for 15 minutes.

2.3.2 DNA extraction lysis buffer

The following chemicals were added to make up the lysis buffer: distilled water 1L, 1M Tris-HCl 20ml (Tris-HCl, Sigma-Aldrich); 10% SDS 20ml (SDS, Sigma-Aldrich); 0.5M EDTA 40ml (Sigma-Aldrich).

2.3.3 Freezing media for immune cell storage at -80°C

The following ingredients were added to make up the freezing media: 90ml of fetal bovine serum (FBS, Sigma-Aldrich); and 10ml dimethyl sulfoxide, DMSO (Sigma-Aldrich).

2.4 Bacterial growth and cultures

2.4.1 Preparation of bacteria liquid stocks

One streak of the respective *Streptococcus pneumoniae* isolates incubated overnight on a 5% blood agar plate was inoculated into 10mL of 20% FCS in BHI broth and further incubated for 18 hours at 37°C under anaerobic conditions, and aliquots of 1ml were stored at -80°C until use.

2.4.2 Bacterial growth curve

Growth curves were established to compare the growth kinetics of different pneumococcal strains. For each strain, a single colony of *S. pneumonia*e was inoculated into 50ml of Brain Heart Infusion broth (BHIB), and grown at 37°C under anaerobic conditions to an absorbance value of 0.5-0.6 (OD_{500nm}). Growth was measured using a spectrophotometer (Thermo Helios, Fisher Scientific) to confirm attainment of the mid-logarithmic growth phase. A 500µl derived from these cultures was further inoculated in 50ml of BHI overnight under the same growth conditions. Aliquots were chosen at each hour to perform viable counts. Results were recorded and analysed to plot the growth curve respective of each strain of interest.

2.5 Determination of *S. pneumoniae* viable counts on blood agar plates

The Miles-Misra technique [501] was used to enumerate the number of colony-forming units (CFU) per ml in stored aliquots of pneumococcal serotypes or mouse tissues. Pneumococcal liquid aliquots or freshly homogenized mouse tissues were serially diluted ten-fold in duplicate on a 96-well microplate, to a final dilution of 10^6. A 5% blood agar plate was divided into six sectors and 60µl of every dilution were spotted over each section. This process was repeated on a second plate to confirm results. Plates were incubated overnight in a candle jar at 37ºC. The following day, the dilution sector containing the optimal numbers of colonies (between 20-200 colonies) was identified and viability calculated using the following formula:

CFU per ml = mean number of colonies in section × Dilution × 1,000/60

2.6 DNA extraction for MLST analysis

Pneumococcal strains were grown on two blood-agar plates to high density. Cells were collected using a plastic loop and resuspended in 1ml of 50mM Tris-10mM EDTA pH 8 in an Eppendorf® tube. 20µl of 50mg/ml lysozyme was added to each tube and incubated at 37°C for 15mins on a shaker. 5µl of 20mg/ml proteinase K was added to each tube and incubated at 37°C for 40mins on a shaker. 10µl of RNAse at a working concentration of 10µg/ml was added to each tube and incubated at room temperature for 2 minutes. 100µl of 10% SDS (100mg/ml) was added to each tube and incubated at 37°C for a maximum of 2 hours on a shaker until lysis was complete. Tubes were centrifuged for 5 minutes at 13,000 rpm and 900µl supernatant transferred to a new tube. 900µl of phenol/chloroform/isoamyl alcohol was added and mixed carefully, then centrifuged for 5 minutes at 13,000rpm. 800µl of supernatant was transferred to a fresh tube and 800µl of phenol/chloroform/isoamyl alcohol was added and mixed carefully.

Tubes were centrifuged at 13,000rpm and 700µl supernatant was transferred to a fresh tube. 700µl of chloroform was added and mixed carefully, followed by centrifugation for 5 minutes at 13,000rpm. 600µl of supernatant was transferred to a fresh tube and 1 ml of ice cold 100% ethanol added and mixed. Tubes were incubated at -20°C for 15 minutes, centrifuged for 5 minutes at 13,500rpm at room temperature, followed by removal of supernatant. Pellets were allowed to try at room temperature, and then DNA was dissolved in 50µl of DEPC water. DNA was stored at -20°C until further use.

2.7 PCR amplification for MLST analysis

Seven house-keeping genes were used [501] to identify and confirm the sequence type of the pneumococcal isolates.

- aroE (shikimate dehydrogenase)
- gdh (glucose-6-phosphate dehydrogenase)
- gki (glucose kinase)
- recP (transketolase)
- spi (signal peptidase I)
- xpt (xanthine phosphoribosyltransferase)
- ddl (D-alanine-D-alanine ligase)

The primer pairs used for the PCR amplification of internal fragments of these genes are:

aroE-up, 5'-GCC TTT GAG GCG ACA GC

aroE-dn, 5'-TGC AGT TCA (G/A) AA ACA T(A/T)T TCT AA

gdh-up, 5'-ATG GAC AAA CCA GC(G/A/T/C) AG(C/T) TT

gdh-dn, 5'-GCT TGA GGT CCC AT(G/A) CT(G/A/T/C) CC

gki-up, 5'-GGC ATT GGA ATG GGA TCA CC

gki-dn, 5'-TCT CCC GCA GCT GAC AC

recP-up, 5'-GCC AAC TCA GGT CAT CCA GG

recP-dn, 5'- TGC AAC CGT AGC ATT GTA AC

spi-up, 5'-TTA TTC CTC CTG ATT CTG TC

spi-dn, 5'-GTG ATT GGC CAG AAG CGG AA

xpt-up, 5'-TTA TTA GAA GAG CGC ATC CT

xpt-dn, 5'-AGA TCT GCC TCC TTA AAT AC

ddl-up, 5'-TGC (C/T) CA AGT TCC TTA TGT GG

ddl-dn, 5'-CAC TGG GT(G/A) AAA CC(A/T) GGC AT

Master mix was prepared and contained 6µl 5X buffer, 0.5µl 40mM dNTPs, 0.5µl of each 20µM primer, 0.25µl 5000U/ml NEB Phusion taq, 1µl DNA from bacterial strain, and dispensed at a volume of 21.25µl H_2O, per tube. PCR conditions used were: 98°C for 30secs, 98°C for 10 secs, 54°C for 30 secs, 72°C for 45 secs (35 cycles), and 72°C for 10 mins. PCRs were performed using a Techne Flexigene thermal cycler (Thermo Fisher, Loughborough, UK).

2.8 Haemolytic assay

The haemolytic activity of the different isolates was determined using a modified version of a method published by James C. Paton in 1983 [503]. The bacterial isolates were grown in BHI to a late exponential phase (OD500 < 1) and then lysed by treatment with 0.1% sodium deoxycholate. The total protein of the lysates was determined using the BCA Protein Assay Kit (Thermo Scientific, Loughborough, UK). Approximately 85µg of total protein was used for the determination of the haemolytic activity of each isolate by making serial dilutions of the lysates and incubating them with a 4% solution of sheep red blood cells (Oxoid). After a 30-minute incubation period at 37°C, the solution was centrifuged at 3000g for 5 minutes and the OD540

of the supernatants was measured to determine the haemolytic activity. The results presented are representative of three independent experiments.

2.9 Pneumolysin detection using ELISA

A 96-well ELISA microplate (eBioscience, Cheshire, UK) was coated with 1µg/well of PLY domain 4 mouse antibody (IgG1) (Abcam) mixed with coating buffer (eBioscience, Cheshire, UK) and left overnight at 4ºC. Wells were washed with 200µl 1X PBS, 0.05% Tween/PBS wash buffer (eBioscience, Cheshire, UK), then blocked with 200µl/well 20% FBS in PBS, incubated at 37ºC for 4 hours. Wells were washed and 100µl per well of sample and standards were added. The plate was incubated for 1 hour at 37°C (or overnight at 4°C). Wells were washed (as above) and 1µg PLY polyclonal antibody (Abcam, Cambridge) in 100µl blocking buffer was added to each well, and then incubated at 37°C for 30 minutes. Wells were washed, then anti-rabbit IgG alkaline phosphatase was added at a 1:5,000 dilution in blocking buffer, and left to incubate at 37ºC for 30 minutes. Wells were washed and then colour reagent pPNPP (50µl/well) was added and left to incubate for 30 min in the dark. 100µl/well of 1M NaOH was added to stop the reaction, then absorbance read on an automated plate reader using a BMG Labtech FLUOstar OMEGA microplate reader (BMG Labtech, Aylesbury, UK) at 405nm.

2.10 Tissue culture

The cell lines used for this study were the human bronchial-epithelial cells BEAS-2B (ATCC® CRL-9609™), the human nasopharyngeal epithelial cells Detroit-562 (ATCC® CCL-138™) and the promyelocytic leukaemia human cell line HL-60 (ATCC® CCL-240™).

The BEAS-2B is an adherent cell line that was maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% inactivated Foetal Calf Serum (FCS) and 1% Penicillin-Streptomycin solution. The flasks were kept in horizontal 75cm^2 flasks at 37°C and 5% CO_2 until confluent. Once confluent, approximately every 2-3 days, the cells were split into a new flask after being detached using 5ml of 1xTripsin/EDTA. The Detroit-562 is also an adherent cell line and was maintained in Minimum Essential Medium Eagle (MEM) supplemented with 10% FCS and 1% Penicillin-Streptomycin solution. This cell line was kept under the same conditions as the BEAS-2B and the cells were split after incubation with 5ml 1xTrispin to induce the detachment of the cells from the flask.

The HL-60 cell line was maintained in suspension in RPMI-1640 Phenol Red free media (Invitrogen, Thermo Scientific, Loughborough, UK) supplemented with 20% FCS. The cells were kept in vertical 75cm^2 flasks at 37°C and 5% CO_2, and were fed every other day. This cell line was used for opsonophagocytic killing assays, for which the cells had to be differentiated into polymorphonuclear lymphocytes (neutrophils) by incubating 2x10^{5}cells/ml in 100ml with 750µl of dimethylformamide (DMF) for 4 days. For the opsonophagocytic killing assay, the viability of the cells after differentiation needed to be above 90%, which was determined with trypan blue.

2.11 Adhesion and invasion assays

Adhesion and invasion assays were performed using the lung epithelial cells BEAS-2B and the nasopharyngeal epithelial cells Detroit-562 following a modified protocol [155].

A density of 10^5 cells per well were seeded into 24-well plates and grown in their respective media (DMEM for BEAS-2B, and MEM for Detroit-562) in the presence of FCS and antibiotics. The cells were grown at 37°C and 5%CO2 until confluent, approximately 48h after seeding. Frozen stocks of known concentration of bacteria were used. The bacteria were washed and re-suspended in the respective media supplemented with FCS but without antibiotics to a final concentration of a ratio 1:10 (cell: bacteria) . Following 3 washes with PBS (Oxoid), one ml of bacterial suspension was added to the cell monolayer. The 24-well plates were then incubated for 2 hours at 37°C and 5% CO_2. For each independent experiment, two 24-well plates were used at a time, one of them to assess the adherence of the bacteria to the surface of the cells, the other one to assess the invasion of the bacteria into the cells.

Following 2 hours of incubation, both plates were washed 5 times with PBS to remove the excess of bacteria that did not adhere or invade the cells. To determine the invasion ratios bacteria, 1ml of media supplemented with FCS and 5ul of penicillin was added to each well and incubated at 37°C and 5%CO2 for a further 2 hours. To assess adhesion ratios, 100µl of 1xTripsin/EDTA were added to each well and the plate was incubated for 10 minutes at 37°C and 5% CO2. Once the cells were detached they were lysed by incubating them for 10 minutes in 1ml of 0.05% Triton X-100 media with FCS. Finally, the viable bacterial number was determined using the Miles and Misra method [501].

After the 2 hour-period of incubation with penicillin, cells were washed 3 times with PBS to remove penicillin. Cells were then detached and lysed in order to assess invasive bacteria by using viable bacterial numbers were determined using the Miles and Misra method [501]. The number of adhered bacteria was determined by subtracting the number of invading bacteria (invasion plate) to the total number of bacteria (adhesion plate).

These experiments were performed in triplicate on three independent days and appropriate controls were used.

2.12 Capsule thickness

The bacterial capsule thickness was determined using a modified version of the FITC-dextran exclusion method originally published by Gates in 2004 [504]. This method consists in measuring the zone of exclusion of FITC-dextran which correlates with capsule size.

Briefly, a single colony from an overnight culture in 5% BAB plates was incubated for approximately 8-12 hours at 37°C in 10ml of BHI to mid-log phase. The bacteria were then sub-cultured to again, a mid-log phase. Finally, 2µl of 200kDa FITC-dextran (Thermo Scientific) was added to 10µl of the culture and pipetted onto a microscope slide. The slides were observed using a Nikon Eclipse 80i fluorescence microscope (Nikon, Kingston Upon Thames, UK). At least 100 individual bacteria were analysed for each sample using the ImageJ software. These experiments were kindly done by Mansoor, a PhD student in the lab.

2.13 Complement deposition assay

A volume of 5 ml of bacterial stock solution at $1x10^5$CFU/ml in BHI was prepared and incubated at 37°C for 15 minutes. 100µl suspension was removed at 0.5 OD_{500}nm (viable count determined using the Miles and Misra technique). The suspension was centrifuged at 3,359 x g for 10 minutes then supernatant removed and pellet resuspended in 5ml PBS. Suspension was distributed evenly across 5 x 1.5ml Eppendorf tubes. Tubes 1-3 contained 1) Bacteria, 2) Bacteria and Thiazol Orange, and 3) Bacteria, plus human sera from healthy donors, mouse anti-human C3 and anti-mouse conjugated to APC fluorochrome (abcam, Cambridge, UK), tubes 4) and 5) contained bacteria, complement and anti-mouse APC conjugate. Tubes were centrifuged at 17,000 x g for 3 minutes, supernatants removed, and pellets reconstituted in

100µl of 20% human serum in PBS with1% gelatin veronal buffer. Tubes were incubated at 37°C/5% CO_2 for 30 minutes, washed with 900µl PBS per tube and centrifuged at 17,000 x g for 3 minutes. Supernatants were removed, and pellet from tube 3 was reconstituted in 100µl of mouse anti-human C3 (1:300 dilution), while pellets in tubes 1 and 2 were reconstituted in PBS only. Tubes were incubated at 37 °C/5% CO_2 for 30 minutes, washed with 900µl PBS per tube and centrifuged at 17,000 x g for 3 minutes. Supernatants were removed and tube 3 pellet reconstituted in 100µl of anti-mouse APC, while tube 1 and 2 pellets resuspended in 100µl PBS. Tubes were incubated at 4 °C in the absence of light for 30 minutes, washed with 900µl PBS per tube and centrifuged at 17,000 x g for 3 minutes. Supernatants were removed, pellets washed with 100µl PBS and centrifuged at 17,000 x g for 3 minutes. Supernatants were removed and pellets resuspended in 500µl PBS, followed by the addition of 5µl Thiazol Orange at a 1:1,000 (0.5ul/ml) dilution to tubes 2 and 3. The tubes were briefly vortexed and incubated for 5 minutes at room temperature, then stored at 4°C in the absence of light until acquisition on a flow cytometer (BD Accuri™ C6, BD Biosciences). Once the samples acquired, the mean fluorescence intensity (MFI) was calculated to quantify C3 deposition.

2.14 Opsonophagocytosis killing assay (OPKA)

Differentiated HL-60 cells were used in an opsonophagocytic assay at an effector: target cell ratio of 40:1. The volume required for 4 x 10^5 cells/well per 100 wells in a microtitre plate were harvested by centrifugation at 160 x *g* for 10 minutes at room temperature, the supernatant was discarded removing any residual medium. The pellet was resuspended in Hanks' buffer without Calcium and without Magnesium (Life Technologies), using 5ml for every 50ml of centrifuged cell culture. Resuspended cells were kept at 37°C/5% CO_2 for 10 minutes until use in the functional OPK assay. To carry out the OPK assay, intravenous immunoglobulin (IVig) was

serially diluted (2-fold) in 10ml of opsonophagocytosis buffer (20% of FBS in DPBS) for a total of 8 dilutions, from 1:8 to 1:1,024. 40µl of IVig was added in duplicate to the first row of a round-bottomed microtitre plate (Costar, Cambridge). Bacterial suspensions diluted from frozen stocks, were added at $1x10^5$ CFU per well in 10µl opsonophagocytosis buffer consisting of 4ml Hanks' buffer with Ca^{++} and Mg^{++} (Life Technologies) and 0.1% gelatin. The assay plate was incubated at 37°C/5% CO_2 for 15 minutes. Following incubation, sterile baby rabbit serum, (Sigma-Aldrich) was thawed and 5µl/well of this complement source was added to the plate. Harvested differentiated HL-60 cells were washed as described above, and the pellet gently resuspended in opsonophagocytosis buffer. 4 x 10^5 cells in a 40µl volume were added to each well immediately after the addition of complement. The assay plate was incubated at 37°C for 45 minutes with horizontal shaking (220 rpm) to promote the phagocytic process. Using a multichannel pipette, 10µl from each well was plated onto solid medium prepared from 5% horse blood and 4% agar. Aliquots were allowed to air dry, and culture plates were incubated overnight at 37°C/5% CO_2. Viable colony counts were performed after approximately 18 hours incubation period. Complement control wells included all test reagents except IVIg. A viable count of the initial number of bacteria added per well at time zero (*T*0) was included in each run. The difference of complement control counts and number of bacteria counts should not be more than 60% .The purpose of that experiment was to determine the resistant and sensitive strain against phagocytosis.

2.15 Purification of rPLY

Full-length recombinant PLY (rPLY) was prepared as described previously [505]. Briefly, the *ply* gene was cloned into the pQE-31 vector (Qiagen, Manchester, UK), and the recombinant vector was transformed into *Escherichia coli* SG13009 (Qiagen) harboring a pREP4 plasmid,

which contains *lacI* and kanamycin resistance genes. rPLY was produced in *E. coli* cells as a six-His-tagged protein by incubation of the transformants with 2 mM isopropyl-β-D-thiogalactopyranoside at 25°C for 6 h. The *E. coli* cells were then harvested by centrifugation, incubated with lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 1 mg/ml lysozyme, 200 U DNase I, pH 8.0), and disrupted by vortexing with 0.1-mm zirconia-silica beads (Bio-Spec Products, UK). rPLY was then purified from the soluble fraction by use of a nickel-nitrilotriacetic acid column (Qiagen) under native conditions according to the manufacturer's instructions. Contaminating LPS was extensively removed using a Detoxi-Gel endotoxin-removing gel (Thermo fisher, Loughborough, UK). The purity was analyzed by Coomassie brilliant blue staining and immunoblotting using an anti-His-tag monoclonal antibody (penta-His antibody; Qiagen) after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

To inhibit its cytolytic activity, rPLY was treated with 20 μg/ml of cholesterol for 30 min on ice. Heat-treated PLY and LPS were prepared by heating the aqueous stock suspensions, in 50% glycerol-water (vol/vol) for PLY and in PBS for LPS, in a boiling water bath for 60 min. This experiment was kindly done by Hesham, a PhD student in the lab.

2.16 Mouse models

2.16.1 Models of Intranasal infection

2.16.1.1 Invasive pneumonia model

Bacteria were grown until desirable optical density and used to perform these experiments. Female MF1 mice (Charles River Laboratories) at 6-8 weeks of age were challenged with 50μl, containing 1×10^6 pneumococci, via the intranasal route. Mice were divided into two groups: ST306 (n=45 mice) and ST615 (n=45 mice). All mice involved in the experiment were

assayed using the following pain scoring scale: 1) normal, 2) hunched, 3) stary and piloerected, 4) lethargic, 5) moribund and 6) dead, as described by Morton [506]. The scoring sheet used can be found in Appendix 1.

Bacterial CFU counts were determined in the inoculum. each group (ST306 and ST615) 5 mice from were humanely euthanised at time zero (i.e, approximately 5 minutes post-infection), and their lungs and nasopharynx were collected, homogenised and processed for viable counts (Miles and Misra) and flow cytometry analysis.

The following time points were targeted:

0 hour: 50ul/mouse *S. pneumoniae* intranasally administration

0 hour post-challenge: Schedule 1 n=5 mice per sequence type

6 hour post-challenge: Schedule 1 n=10 mice per sequence type

12 hour post-challenge: Schedule 1 n=10 mice per sequence type

24 hour post-challenge: Schedule 1 n=10 mice per sequence type

48 hour post-challenge: Schedule 1 n=10 mice per sequence type

2.16.1.2 Nasopharyngeal carriage model

Frozen stocks of bacteria grown to exponential phase were freshly thawed to run these experiments. Female MF1 mice (Charles River) at 6-8 weeks of age were intranasally challenged with 10µl pneumococci at a final concentration of 1×10^5 (CFU/10µl). Mice were divided into two groups: ST306 (n=65) and ST615 (n=65).

Bacterial CFUs were determined before and after the infection of mice. 5 mice from each

group; ST306 and ST615, were humanely euthanised at time zero and their nasal mucosa, lymph nodes, lungs and nasopharynx collected, homogenised and processed for either viable counts on BAB plates, or flow cytometry analysis. Whole blood was collected by cardiac puncture. Animals will be anaesthetised with a mixture of O_2 and isofluorane, and infected intranasally with 10^5 CFU *S. pneumoniae*/mouse in a volume of 10µl. Mice were closely monitored for the visual development of clinical signs of disease, as described previously [506]. Mice Nasopharynx and lungs were removed at predefined timepoints (as detailed below). Whole blood samples were collected by cardiac puncture into sterile heparinized tubes.

The following time points were targeted:

Day 0: 10ul/mouse *S. pneumoniae* intranasally administration to all n=130 mice.

Day 0 post-challenge: Schedule 1 n=10 mice, 5 mice per sequence type.

Day 1 post-challenge: Schedule 1 n=20 mice, 10 mice per sequence type.

Day 3 post-challenge: Schedule 1 n=20 mice, 10 mice per sequence type.

Day 7 post-challenge: Schedule 1 n=20 mice, 10 mice per sequence type.

Day 14 post-challenge: Schedule 1 n=20 mice, 10 mice per sequence type.

Day 21 post-challenge: Schedule 1 n=20 mice, 10 mice per sequence type.

Day 28 post-challenge: Schedule 1 n=20 mice, 10 mice per sequence type.

2.16.2 Pneumococcal sepsis model

Frozen stocks of bacteria were freshly thawed to run these experiments. Female MF1 mice (Chiral river) were challenged intravenously with 50µl pneumococci containing $1x10^6$ CFU. Mice were divided into 2 groups (n=6) and received either ST306 or ST615 pneumococci. Bacterial viable counts were determined in the inoculum and in postmortem samples, approximately 20 minutes immediately after infecting the mice. All mice involved in the

experiment were subjected to pain scoring at regular interval and at the most critical points to monitor the severity of the symptoms over time.

Tail bleeding was performed, at 6, 12, 24 and 48 hours after infection. Blood samples were collected in 0.5 ml eppendorf tubes containing 1µl heparin to prevent clotting, and viable counts were determined.

2.15.3 Mouse survival experiment

This experiment was carried out to determine and compare the in vivo virulence of ST306 vs. ST615 isolate. The procedure used in this experiment is similar to that used in the intranasal model of infection in section 2.16.1.1. However, in this procedure mice were carefully monitored throughout the infection period and blood was collected from mouse tails in 0.5ml heparinised eppendorf tubes. Female MF1 mice (Charles River Laboratories) were challenged with 50µl pneumococci via the intranasal route in a final concentration of $1x10^6$ CFU. Mice were divided into two groups: ST306 and ST615 mice. In total, 10 mice received 50µl of ST306 pneumococci intranasally, while another 10 mice received 50µl of bacteria ST615 pneumococci. Mice were monitored and scored using the pain scoring system (1-5) discussed above up to 7 days.

2.16.4 Induction of streptomycin resistant pneumococci

Colonies of ST615 pneumococci were exposed to increasing concentrations of streptomycin in BHI broth until they became growth resistant to a concentration of 40 µg/ml of streptomycin. This experiment was performed in order to differentiate viable counts of ST306 and ST615 on blood agar plates.

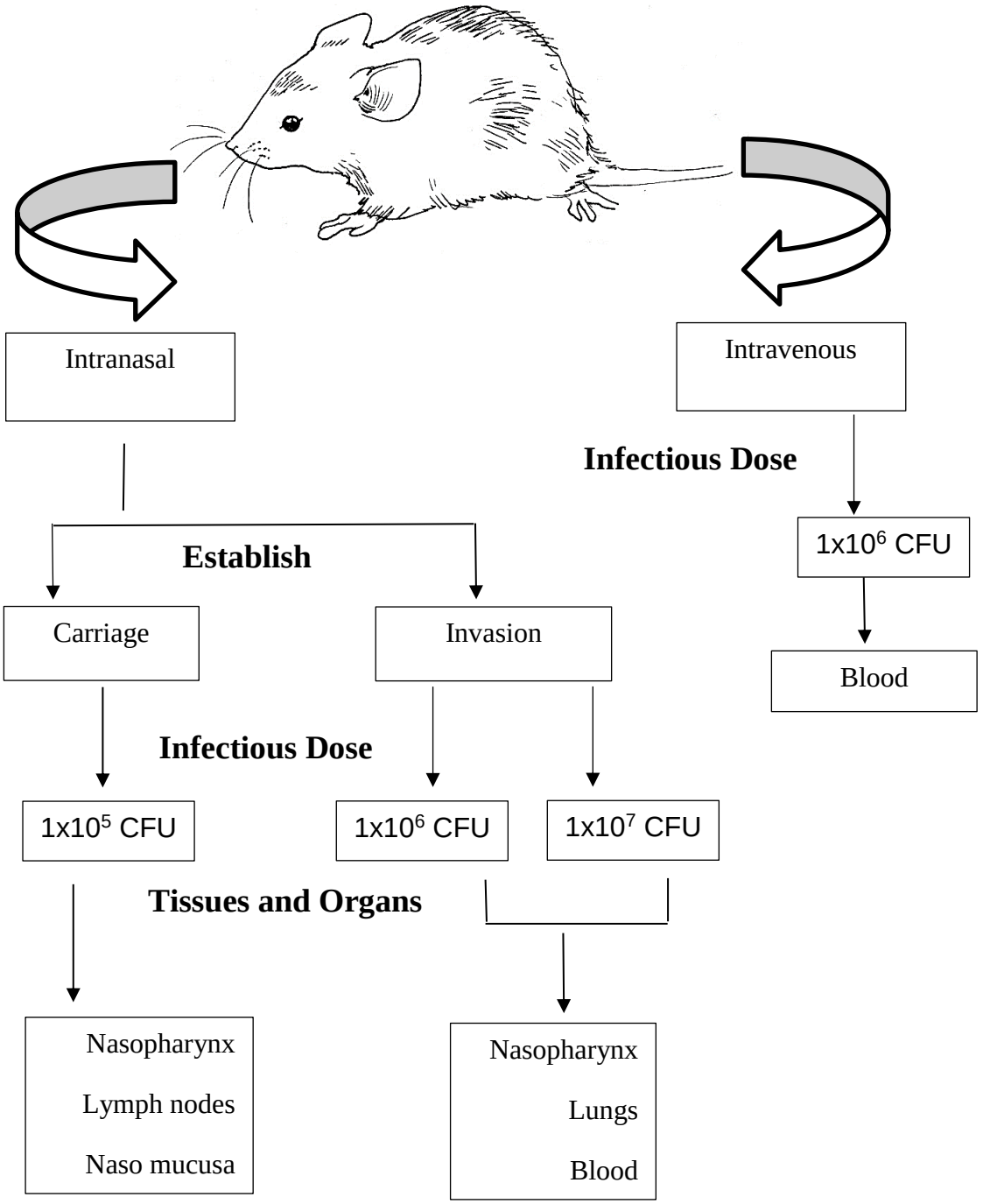


Figure 12. Schematic diagram of experimental animal models. MF1 female mice were infected as described in the Materials and Methods section. Infected mice were sacrificed at mentioned days and nasopharyngeals, lungs and blood were collected to assess the levels of pneumococcal infection and characterise immune cells.

2.16.5. Pneumococcal ST306-ST615 co-infection model

Female MF1 mice (Charles River Laboratories) at 6-8 weeks of age were challenged with 10µl of a mixture 1:1 of ST306:ST615 pneumococci, at a final concentration of $2x10^5$ CFU, via the intranasal route. Five mice per group from ST306- and ST615-challenged mice, were humanely euthanised at time zero and their lungs and nasopharynx collected, homogenised and processed for viable counts. Blood was collected by cardiac puncture.

On days 1. 3 and 7, 10 mice from each group; ST306 and ST615, were humanely euthanised. Mice from each group; ST306 and ST615, were humanely euthanised and their lungs and nasopharynx collected using the same procedure.

2.16.6. Pulse experiment using purified pneumolysin and ST306

Female MF1 mice (Charles River Laboratories) at 6-8 weeks of age were anaesthetised with a mixture of O_2 and isofluorane and challenged with 50µl of ST306 pneumococci (10^6 CFU per mouse) mixed with 100ng of LPS-free recombinant pneumolysin, via the intranasal route. Five mice were humanely euthanised at time zero, and their lungs and nasopharynx collected, homogenised and processed for viable counts (Miles and Misra) and flow cytometry analysis. Mice were then closely monitored for the visual development of clinical signs of disease, as described previously [506]. Any animal judged to have reached 2+ lethargic (minimal movement in the absence of application of finger pressure) was humanely euthanized. Mice were monitored up to 48 hours post-infection, at which point the experiment will end. Nasopharynx, lungs, blood and brain tissues, and whole blood samples were collected for further analysis.

The following time points were targeted:

0 hour: 50ul *S. pneumoniae* + Ply pulse intranasal administration

0 hour post-challenge: Schedule 1 n=5 mice per sequence type

6 hour post-challenge: Schedule 1 n=10 mice per sequence type

12 hour post-challenge: Schedule 1 n=10 mice per sequence type

24 hour post-challenge: Schedule 1 n=10 mice per sequence type

48 hour post-challenge: Schedule 1 n=10 mice per sequence type

2.17 Flow cytometry analysis

FACS staining was performed with multiple antibodies, using the BD FACScalibur machine and data analysed using the BD CellQuest Pro software.

2.17.1 Staining Buffer

FACS staining was performed using PBS supplemented with 3% foetal-calf serum (FCS) or PBS containing 1% bovine serum albumin (BSA). All buffers were filter sterilised before use.

2.17.2 Preparation of single cell suspension

Lung tissues were placed into a clean 10 cm dish/petri dish and cut into fine pieces using disposable scalpel blades. Tissues were transferred into 1.5 ml eppendorf containing 500 µl PBS supplemented with collagenase then incubated at 37°C for 30 minutes, to digest the connective tissue, and releasing the haematopoietic cells.

Tissues were placed into a cell strainer placed onto open top 50 ml Falcon tubes and pushed through the cell strainer using a plunger from 2 ml or 5 ml disposable syringes. Any remaining cells attached to the cell strainers were pushed through by flushing with 10 ml PBS using a 10 ml pipette. Cell strainers were discarded and cap was placed back on the Falcon tube, then centrifuged at 300g for 5 minutes at 4°C. The supernatant was decanted and cell pellet reconstituted in 5 ml of erythrocyte lysis solution (ACK lysing buffer, Invitrogen), diluted as recommended by the manufacturers. The tube was further incubated at room temperature for 5 minutes then quenched with 5ml PBS. Following centrifugation at 300g for 5 minutes at 4°C, supernatant was discarded. Cell pellet was resuspended in 3% FCS for staining at a concentration of no more than 10^7cells/ml, at an approximately equal cell densities between samples.

Lungs were resuspended in 5 ml and 100 μl of the resuspended cells used for each individual stain; lung or cervical lymph nodes were resuspended in 750 μl, using 100 μl per stain; mediastinal lymph nodes or nasal mucosa were resuspended in 500 μl, using 100 μl per stain.

2.17.3 Cell staining procedure

All staining was performed on ice and samples covered with foil during the incubation periods to protect the fluorochromes from light. Appropriate isotype controls were used for each staining. For example, if a rat anti-mouse CD3-PE of IgG2a isotype was used, then a Rat IgG2a-PE isotype control was included. In addition, an unstained sample from every tissue was included in the FACS analysis to aid with the gating strategy.

Staining was performed in 1.5ml eppendorfs or on round-bottomed 96-well plates. Cells were transferred to tubes or plates and centrifuged at 300g for 5 minutes at 4°C. Supernatant was removed by pipette or decanting. Cells were resuspended in 50 μl PBS 1% BSA or PBS 3% FCS supplemented with anti-CD16/32 (Fc-block, BD Biosciences) at 1:200 dilution. Cell

suspension was incubated for 20 minutes on ice then centrifuged at 300g for 5 minutes at 4°C. Pellet was resuspended in 50µl PBS 3% FCS supplemented with antibodies at pre-optimised concentrations (see Table 3), then incubated for 30 mins on ice. 200 µl PBS 1% BSA or PBS 3% FCS was added to the stained cells and centrifuged at 300g for 5 minutes at 4°C. Supernatant was removed and pellet washed. Each sample was resuspended in 300 µl PBS 1% BSA or PBS 3% FCS and transferred to FACS tubes. Samples were acquired using a BD FACS Calibur (BD Biosciences) and Cell Proquest software.

Panel A	**Panel B**
APC - CD45 (Leukocyte surface marker) Clone I3/2.3	FITC – CD45 (Leukocyte surface marker) 30-F11
PE – FoxP3 (T regulatory cells) Clone FJK-16S	APC – F4/80 (Macrophages) Clone BM8
PE/Cy7 - CD4 (T cells) Clone GK1.5	PE/Cy7 – Gr1 (Neutrophils) RB6-8C5
FITC – CD3 (T cells) Clone 17A2	PE – CD19 (B cells) eBio1D3

Table 2. Panel A &B showing the antibodies were used in flow cytometry

2.18 RNA extraction protocol

Growth curves of NCTC7465 strain (ST615) and B915 strain (ST306) of serotype 1 were performed by inoculating 250ml of BHI with $2.5x10^6$ bacteria and incubating them at 37ºC in a water bath. Samples were taken every hour to determine the optical density (OD500). This experiment was done in triplicates and plotted to determine the five phases of growth: early exponential phase (EEP), mid-exponential phase (MEP), late exponential phase (LEP), early stationary phase (ESP) and late stationary phase (LSP).

Ribonucleic acid (RNA) from each growth phase was stabilised by incubating the bacteria with a solution of 5% phenol (Invitrogen) and 95% ethanol for a minimum of 30 minutes in ice. Following stabilisation, the RNA was extracted using a phase separation method using TRIzol® (Invitrogen) [507]. Briefly, the cells were centrifuged and re-suspended in TRIzol® to allow bacterial lysis. The lysed bacteria were mixed with chloroform to separate the RNA from DNA, lipids and proteins. Once the aqueous phase was collected (RNA), the sample was washed and the RNA was precipitated using ethanol. Finally, the RNA was re-suspended in DNase/RNase free distilled water (Invitrogen). The RNA extraction was done in an RNase-free area and using RNase-free materials to avoid RNA degradation.

The quantity of RNA was determined using the Qubit® Fluorometer (Thermo Scientific). A 2100 Bioanalyzer (Agilent Technologies, Stanta Clara, USA) was used to assess the quality of the RNA. Once confirmed that the RNA was not degraded the samples were sent to Vertis Biotechnologie AG (Freising, Germany) for Illumina NextSeq 500 sequencing (Illumina).

2.19 RNAseq transcriptomic analysis

The raw sequencing data were prepared by Vertis Biotechnologie AG in FastQ format. Chrispin Chaguza, PhD student, annotated the genomes. Assembly of the sequence reads was performed using Velvet and SPADes assembly programs. The resulting contigs of the genomes were then ordered by ABACAS and annotated using a combination of the RATT and PROKKA prokaryotic annotation pipelines. Orthologous genes between the NCTC7465 strain (GenBank assembly Accession number: GCA_001457635.1) and the B915 strain (Genebank Accession number: FQ312039) were identified using CD-HIT. The mapping of the RNA-Seq reads against the annotated genomes to infer transcripts was done by Dr. Karsten Hokamp at Trinity College, Dublin, Ireland, as previously described [508]. Finally, the expression analysis of the

transcriptome was done in collaboration with Prof. Jay Hinton (Institute of Infection Biology, University of Liverpool) using the GeneSpring 7.3 software (Agilent Technologies).

2.20 Details of mapping and RNA-seq reads

RNA-seq was performed by Vertis Biotechnologie AG, followed by annotation that done by Chrispin Chaguza, and RNA-seq was mapped against annotated genomes to infer transcriptome by Dr. Karsten Hokamp at Trinit, College Dublin, as previously described. Expression analysis of the transcriptome was performed in collaboration with Professor Jay Hinton, University of Liverpool using the GeneSpring 7.3 software (Agilent Technologies).

2.21 Statistical analysis

All data from this experiment was analysed with Student t-test and ANOVA with a significant p-value set at 0.05, using GraphPad Prism®5 software.

Phenotypic characterization of ST306 *In vitro & in vivo*

3.1 Introduction

Recent epidemiological studies have reported the clonal expansion of pneumococcal isolates expressing Ply with either low or non- hemolytic activity, in particular those associated with serotype 1, 7F, and 8, which, coincidentally, are also described among the most invasive pneumococcal strains [415]. As such, the reduced hemolytic activity of Ply in pneumococcal isolates readily capable of causing invasive pneumococcal disease (IPD) has questioned the importance and contribution of Ply to the pathogenesis of pneumococcal disease [413]. Interestingly, Ply possesses a number of immunomodulatory properties that perform independently of the toxin's cytolytic activity. These properties include antibody-independent activation of the classical complement pathway [414], Toll-like receptor 4 (TLR4) [414],-dependent and -independent activation of the NLRP3 inflammasome [414]. However, it remains unclear whether the noncytolytic properties of Ply are sufficient or essential for IPD or whether a drastic reduction in hemolytic activity diminishes Ply's overall contribution to

IPD. Furthermore, it is not clear whether pneumococcal strains presenting low hemolytic activity will escape from vaccine formulations which include Ply-specific antigens [415].

Epidemiological data related to pneumococcal serotype 1 are still scarce, though, several reports showed an increased prevalence of invasive pneumococcal disease (IPD) caused by serotype 1, in Scotland [416], Sweden and Denmark [417] as well as throughout Europe, South America, Africa and Asia [418]. On the other hand, simultaneously diseases caused by serotype 1 tend to show a decreased prevalence in North America and Egypt [418]. Pneumococcal serotype 1 is classically associated with clinical features such as complicated pneumonia, pulmonary empyema and peritonitis. It has been directly linked to mortality, irrespective of factors such as age, environment, and co-infection. Along with serotypes 5 and 7, serotype 1 is also associated with a higher ratio of hospitalisation versus ambulatory care compared with pneumococcal infections from other serotypes [415].

There are ten mouse strains were used for pneumococcal research which showed their susceptibility for different model of infections such as pneumonia, otitis media and sepsis, these strains are MF1, BALB/C, DBA/2, CBA/Ca, AKR, C57BL/6, NIH, FVB/n, CSH/He and C3H/HeJ [613]. MF1 strain was used for these experiments as this strain is susceptible for all model of pneumococcal infection; survival rate of this strain is around 48 hours and quite cheaper than other strains [613].Multiple infection mouse in vivo models were utilized in our study: an intranasal model of invasive pneumonia, an intravenous model of sepsis, and an intranasal model of nasopharyngeal carriage.

The overall aim of this project was to compare the key microbial, immunological and invasive properties of pneumococci serotype 1 ST306 and ST615 which has fully active pneumolysin using both in vitro and in vivo experimental approaches.

3.2 Multilocus sequence typing (MLST)

MLST for the 12 serotype 1 isolates revealed that 10 of these isolates were ST306 and 1 was ST304, and there was also 1 ST615.

3.3 Bacterial Growth Curve

The different isolates were grown in order to monitor the growth kinetics specific to each sequence type, and compare them to the well characterised laboratory serotype 2 strain D39. Bacteria were grown in 20% BHI-serum for around 19h and growth measured throughout at 500nm every 30 minutes (Thermo Helios, Fisher Scientific). As observed in Figure 8, all the isolates followed the same pattern of growth: at first, a lag phase reflecting the time required to adapt to a new growth environment, followed by a replication and growth phase commonly described as the exponential phase [328]. The final bacterial growth phase, also known as the stationary phase, reflects the exhaustion of available nutrients, or the increased accumulation of waste products and toxic metabolites that are detrimental to bacterial growth. During the stationary phase, the bacteria undergo cell death due to the prolonged lack of nutrients [328].

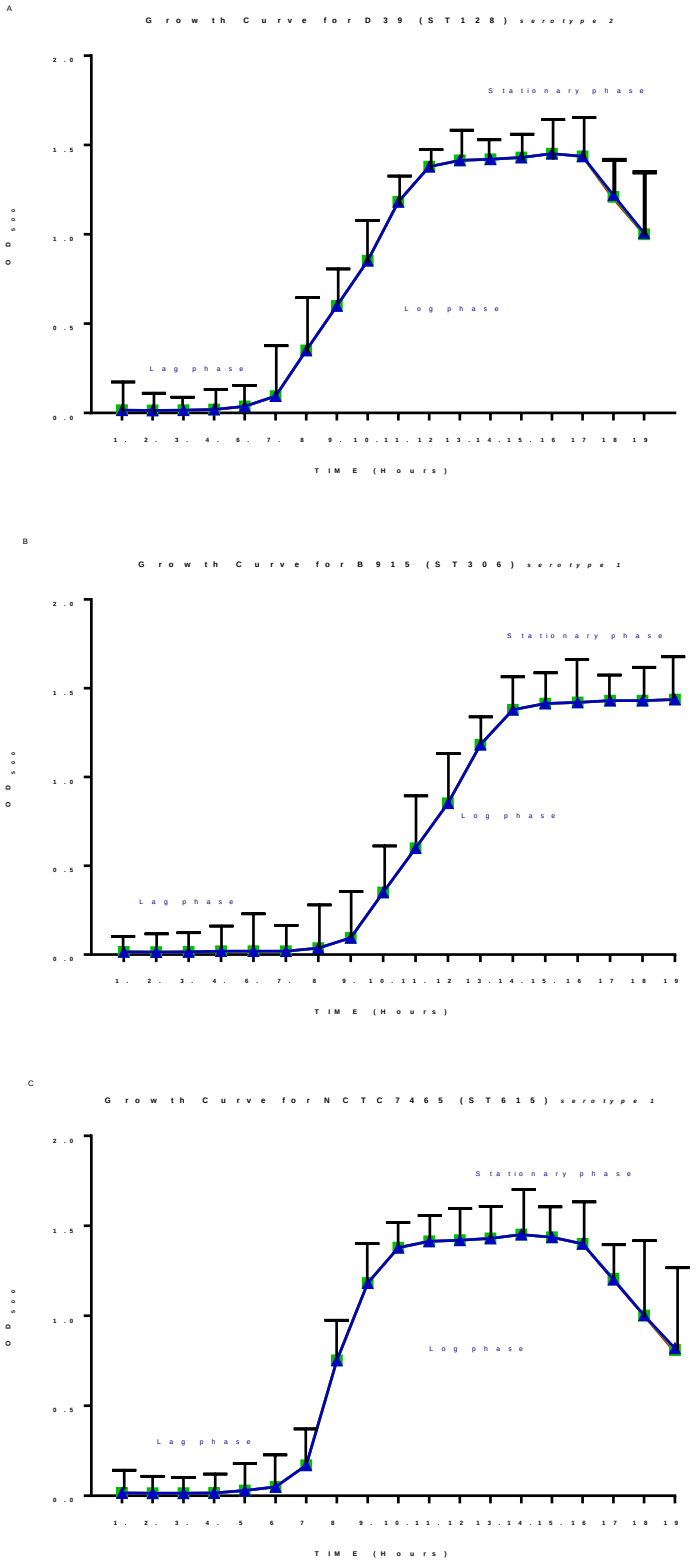


Figure 13. Growth profiles of strains (A) D39, (B) ST306 and (C) ST615 in BHI media without serum by using spectrophotometer at OD_{500}. The experiment was repeated twice with three replicates.

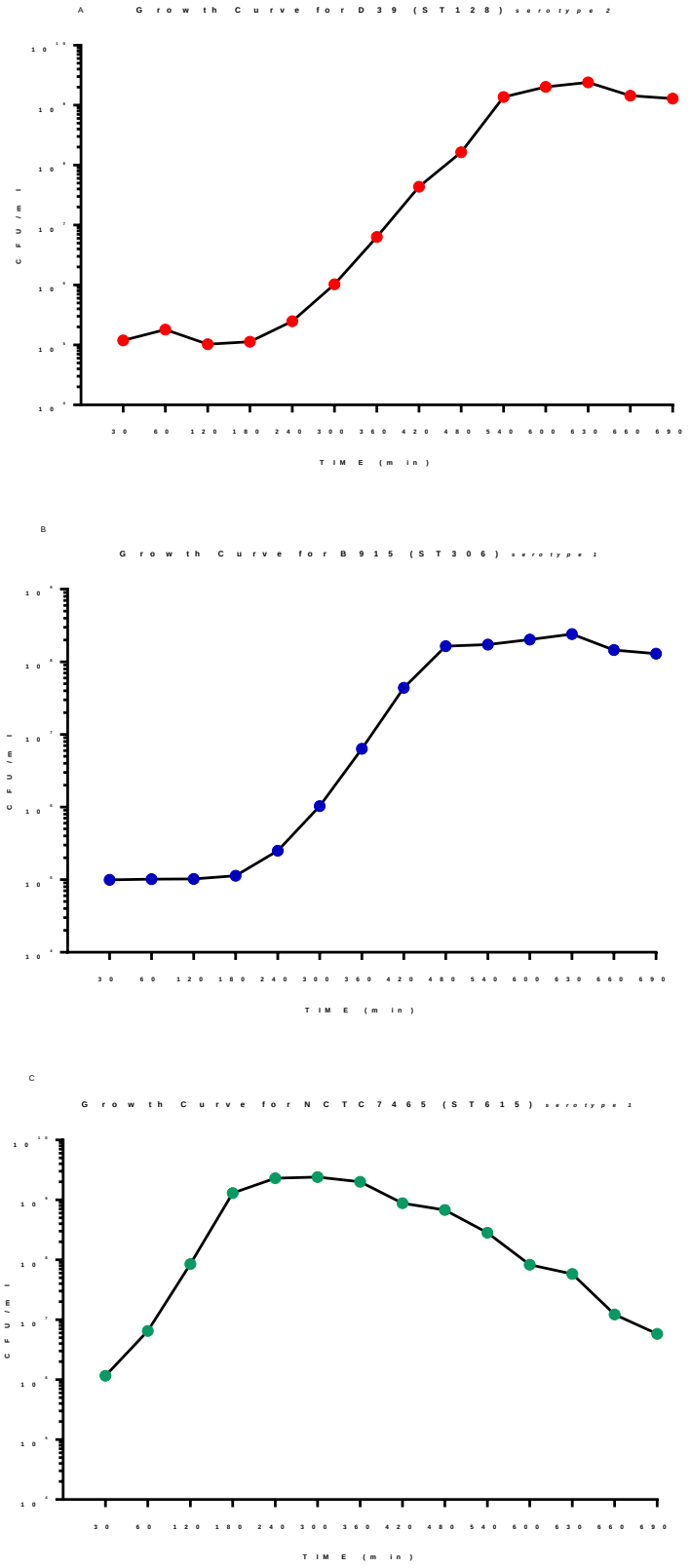


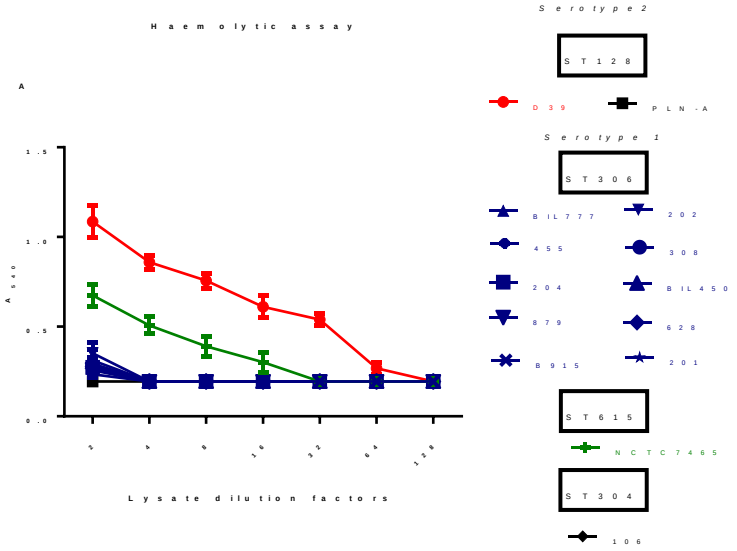
Figure 14. Growth profiles of strains (A) D39, (B) ST306 and (C) ST615 in BHI media without serum by using bacterial counts

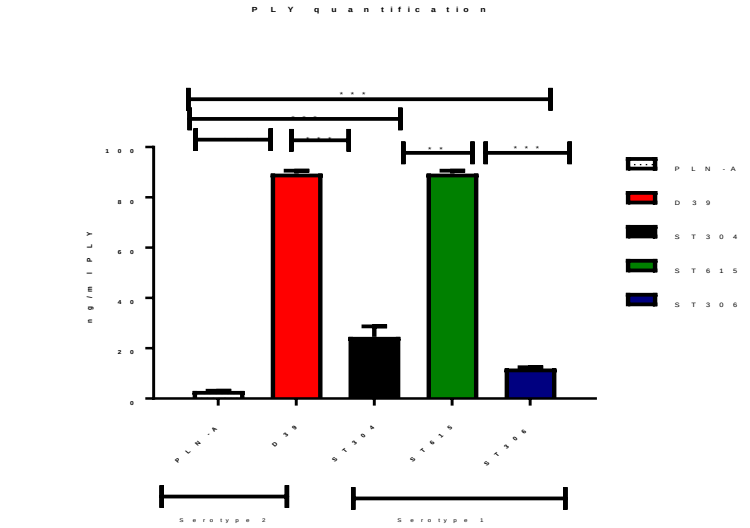
Growth curve analyses showed a pattern typical of bacterial growth for all three strains ST128, ST306 and ST615. Variations were observed between isolates from serotype 1 at the lag phase with ST615 growing faster than ST306, while ST128 (D39) was similar to ST306. Data collected during these growth phases illustrate the generation time which is the average time between two consecutive generations in the lineages of a population. Generation time, defined as time (minutes or hours)/ logB-logA, was equal to 20 min for ST128, 18 min for ST615 and 36 min for ST306. The growth of all isolates steadily increased between 6 and 8 hours onward and reached exponential phase after 7-9 hours post incubation.

The stationary phase started earlier 10 hours after inoculation in ST615 compared to ST128 and ST306, which suggested that cells underwent reductive cell division and became more resistant to osmotic and acid stress. Thus, the count remained stationary due to balance between multiplication and death rate. On other hand, ST128 and ST306 did not reach the stationary phase as rapidly because the generation time was longer.

3.4 Determination of the haemolytic activity of serotype 1 and 2 isolates

The haemolytic activity of pneumococcal lysates was determined using a previously described haemolytic assay [503]. The haemolytic activity of pneumolysin produced by ST306, ST304 and ST615 isolates was compared to that produced by D39 and PLN-A, control isolates [73]. An ELISA assay was also used to quantify the content of pneumolysin (PLY) protein.


A
Haemolytic assay
A620
1.5
1.0
0.5
0.0
Lysate dilution factors
Serotype 2
ST128
D39
PLN-A
Serotype 1
ST306
BIL777
202
455
308
204
BIL450
879
628
B915
201
ST615
NCTC7465
ST304
106


B
PLY quantification
ng/ml PLY
100
80
60
40
20
0
PLN-A
D39
ST304
ST615
ST306
Serotype 2
Serotype 1

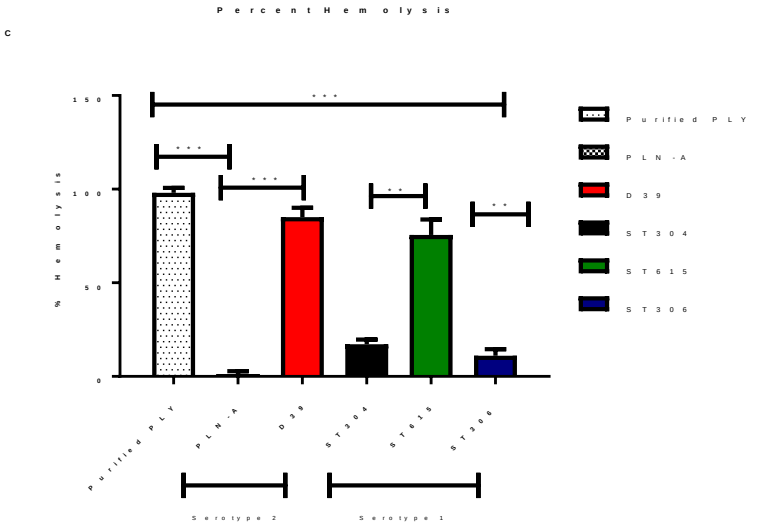


Figure 15. Haemolytic assay and Pneumolysin ELISA quantification. (A) Serial dilution was carried out (n=3 per strain) from bacterial stock solutions, different sequence types of serotype 1 isolates displayed as mean OD540 ± SEM of the bacterial lysates after incubation with a 4% red blood cell solution B) Quantification of PLY content in pneumococcal strains as determined by ELISA. Error bars denote standard deviation (C) Percentage hemolysis (n=3 per strain) for the strains of interest. Percentage was determined by comparison to purified pneumolysin 0.75mg/ml as a positive control and PBS as a negative control. ***, *p<0.005*, **, *p<0.05*. D39, PLY-deficient (PLN-A) of D39, ST304, ST306 and ST615 were used in these experiments.

3.4 Concentration of total PLY content in different strains of *S. pneumoniae* and *in vitro* haemolytic activity

Hemolysis assays were performed to characterise the PLY hemolytic activity in a selected sequencing type (615, 304 and 306). PLY hemolytic activity relies on the toxin's ability to lyse RBCs. In **Figure 16A**, pneumococci D39 reached max OD_{540} = 1.1 when being compared to other strains. Moreover, NCTC7465 which belongs to ST615 remained able to lyse higher than 0.5 unit OD_{540} of red blood cells. However, all strains of ST306 (designated BIL777, 202, 308, 204, BIL450, 879, 628, 455, 201) and one strain of ST304 (designated 106) were able to lyse a few number of red blood cells and the max OD_{540} was lower than 0.5nm.

In Figure 16, panel B, strains D39 and NCTC7465 - a serotype-1 pneumococcal isolate with sequence type 615 – were found to contain a significantly higher PLY content than any other strains tested ($p<0.05$) This coincides with the observation that the total content of pneumolysin (85ng/ml +/-…) in this strain was also the highest. Strains D39 and NCTC7465 were not significantly different in their total PLY content. The serotype 1 pneumococcal strain B915 which belongs to the group of sequence type ST306 had a significantly smaller PLY content (12ng/ml +/- …) compared to the other tested strains ($p<0.05$).

In Figure 16, panel C are presented the percentage hemolysis of a selected panel of pneumococcal cell lysates. PLN-A, a serotype 2 pneumolysin deficient strain [249], was included in the assay as a negative control. The strains tested included D39 strain belonging to serotype 2, and three other strains designated 106, FT and B915, all belonging to pneumococcal serotype 1. Percentage hemolysis was determined relatively to recombinant pneumolysin 75 ng/ml as a positive control (100% hemolysis) and PBS as a negative control (0% hemolysis). Strains were considered to have low PLY activity if hemolysis was < 50% and high PLY activity if hemolysis was >80%, relative to the 100% lysis control. D39 and sequence type 615 were designated high PLY activity strains with 80.5% and 67% hemolysis, respectively (Figure 4A). Sequence types ST304 and ST306 were designated low PLY activity strains with 15.4% and 8.2 % hemolysis, respectively.

3.5 Adhesion and invasion assays

It is commonly accepted that, in order to cause infections, pneumococci must first colonise their host. Bacterial pathogens express various molecules able to enhance attachment to host cells [511]. These adhesins establish stable interactions between the host cell surface receptors or soluble proteins. Adhesion is a fundamental first step prior to invasion and/or secretion of toxins. Furthermore, adhered bacteria frequently induce a broad range of cellular responses,

including T-cells and specific antibody [512]. Bacterial adhesion on host cells and their invasion therefore play important roles to determine pneumococcal pathogenesis [511]. In this study, human nasopharyngeal epithelial cells (Detroit-562) and human broncho-alveolar epithelial cells (BEAS-2B) were used and infected with serotype 1 (ST304, ST306 and ST615) as well as D39, a serotype 2 laboratory strain.

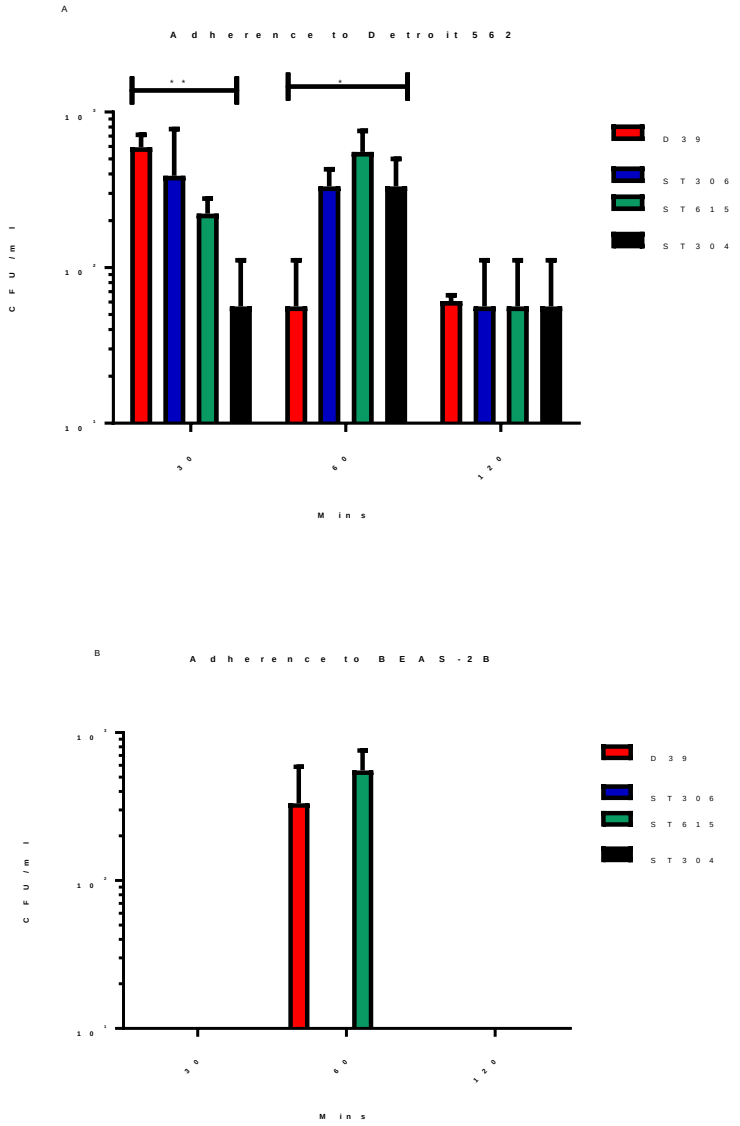


Figure 16. Adherence of different strains of S. pneumoniae to (A) Detroit 562 epithelial cell monolayers (B) BEAS-2B **human bronchial epithelial cells**. To determine pneumococcal adherence, cell monolayer was washed at 30, 60 and 120 min post-infection, and the total number of adherent bacteria

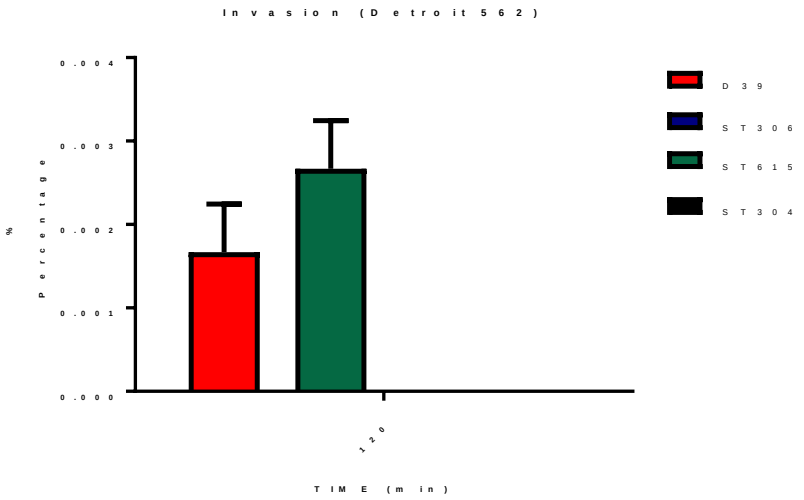
were determined as a percentage of the initial CFU count. The results are represented as mean +/- standard deviation (n=3). ***p<0.001*, **p<0.01*

3.5 Adherence of different strains of *S. pneumoniae* to Detroit 562 epithelial cell monolayers and human bronchial epithelial cell (BEAS-2B)

Previous studies suggested [511, 512, 168] that PLY toxin contributes to nasopharyngeal colonisation, arguing that PLY-deficient strains have reduced adherence to respiratory epithelial cells in vitro compared to PLY-expressing strains. Level of adherence of pneumococcal cells to epithelial cells in vitro may be used to determine whether haemolitically active pneumolysin is a requisite for successful nasopharyngeal colonization, and whether this might be a contributing factor to the invasive potential of *S. pneumoniae* [511]. Moreover, PhtD and PcpA play also important roles in colonisation in a murine model [512]. It was shown that mutation in *pavA* gene reduced level of adhesion to immobilised human fibronectin as PavA mediates adhesion of pneumococci to fibronectin [168].

In Figure 17 are shown the results obtained upon incubation of various pneumococcal isolates with nasopharyngeal Detroit 562 cells at 30, 60 and 120 minutes post-infection with pneumococci. Interestingly, D39 strain (serotype 2) adhered at a higher percentage than serotype 1 strains within half an hour of incubation. This is in line with a previous study showing that D39 displayed low level 0.03% of adherence to human bronchial epithelial cells (BEAS-2B) and the CFU was $6x10^3$ CFU/well after 30 min of incubation [512]. On the other hand, all serotype 1 strains required a longer incubation to adhere to epithelial cells and CFU were only detectable at 60 min post-infection. Strains belonging to ST306 adhered evidently at a higher level than strains belonging to ST615 and ST304. After two hours of incubation, there were no longer any differences in adherence between serotype 1 and serotype 2.

In relation to the adhesion ratios to BEAS, strains D39 and NCTC7465 were able to adhere and the CFU were approximately $5x10^2$ CFU/ml, while other strains of serotype 1 were not detected on blood agar plates. However, the ability of both strains D39 and NCTC7465 to adhere to bronchoepithelial BEAS was lower compared to the adhesion levels observed in Detroit 562. Our findings are in the line with a previous study showing that pneumococci were found to be much lower adherence to pharyngeal cells comparing to other streptococcus species *in vitro* and *vivo* [513].



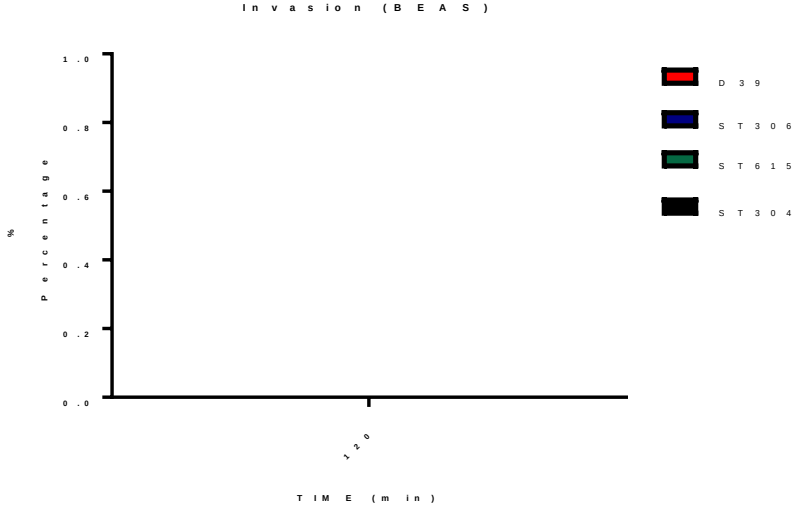


Figure 17. Invasion ratio of different strains of S. pneumoniae in Detroit 562 epithelial cell monolayers (A) and BEAS (B). Extracellular non-adherent bacteria were removed by treatment with penicillin and gentamicin post-infection. After 2 hours of incubation, the cell monolayer was then washed extensively, and the number of intracellular bacteria was determined as a percentage of the initial inoculum. The results are represented as mean +/- standard deviation (n=3).

3.6 Invasion potential of different strains of *S. pneumoniae* in Detroit 562 epithelial cell monolayers

The mechanism of pneumococcal invasion of the respiratory epithelium provides insight into the understanding of pneumococcal pathogenesis. It has previously been reported that invasion of epithelial cells requires specific cell-type and virulent strains [417]. Detroit 562 cells are readily invaded *in vitro* by pneumococci, and therefore provide a useful model to study the invasive potential of pneumococci. Studies have reported that encapsulated strains of pneumococci adhere to and invade cells less efficiently than un-encapsulated strains, while others have shown other virulence factors, such as CbpA are important for efficient colonisation of the nasopharynx [329]. The following experiments investigate the ability of pneumococcal strains to invade to epithelial cells and we discuss how this relates to their invasive potential.

In figure 18 are represented the percentage of pneumococcal cells found upon lysis of Detroit cells at 2 hours post infection. ST615 was the only serotype 1 isolate showing the ability to invade Detroit 562 epithelial cells with approximately 0.003% as a percentage of the initial inoculum, while D39 in the serotype 2 group invaded the Detroit cells with approximately 0.002%. No viable counts of ST306 and ST304 were found suggesting that these two particular sequence types are less invasive. No invasion was observed on BEAS from any strains belonging to either serotype 1 or 2 (Figure 18).

3.7 Complement deposition

Complement is an important part of the innate immunity that acts as a first line of defense against foreign and altered host cells. The activation of the host complement system results in the binding of complement protein C3 on the surface of the pneumococcus resulting in recruitment of phagocytes and engulfment of the pathogen [243]. A small amount of complement deposition would thus suggest that the pathogen in question is more likely to evade immune phagocytosis.

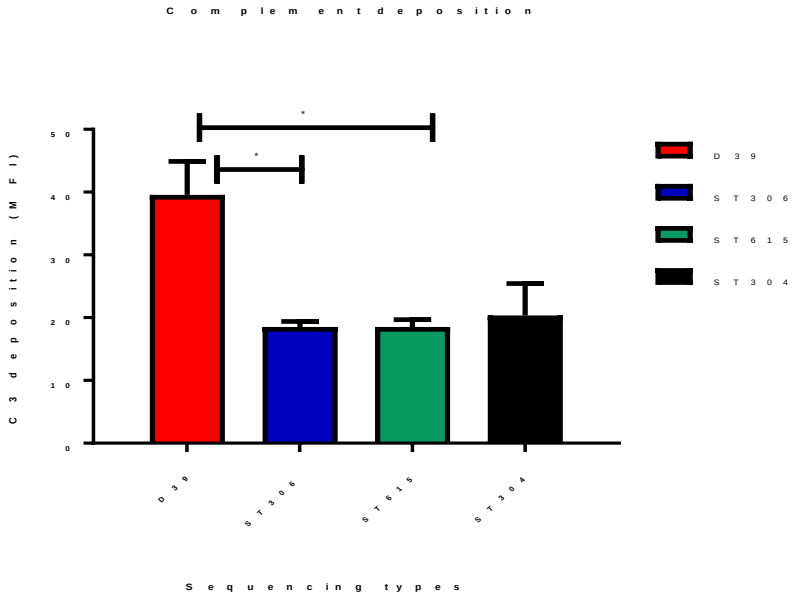


Figure 18. Deposition of rabbit complement C3 on the surface of pneumococcal isolates. C3 deposition is expressed as the median intensity of fluorescence (MIF). The error bars indicate the standard deviation of three independent experiments, *,p< 0.01 (one-way ANOVA) .

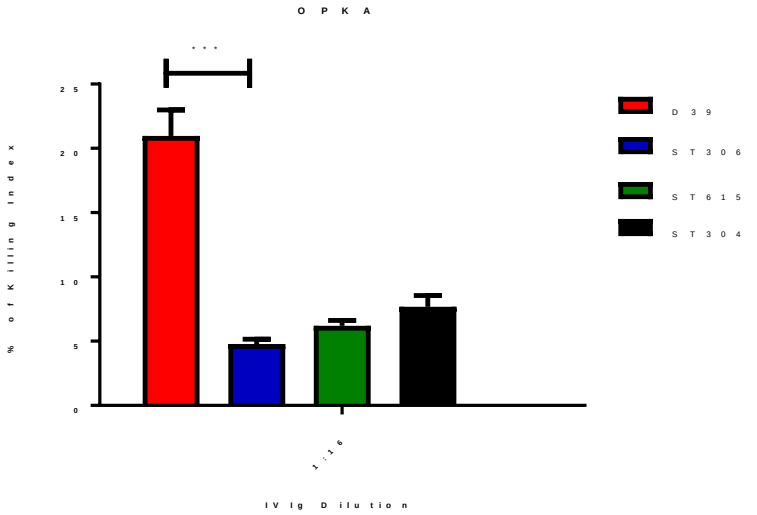
3.7 Deposition of complement C3 on the surface pneumococcal isolates

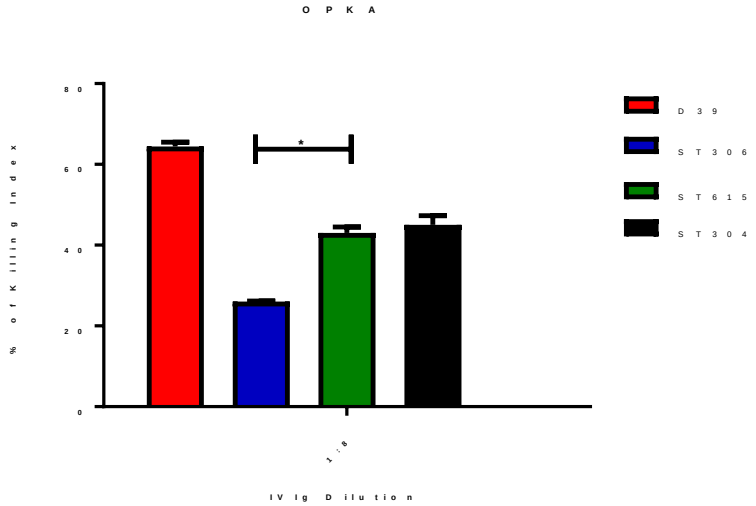
PLY plays an important role in preventing complement activation and contributing to the spread of bacteria to other cells and tissues. PLY, itself, independently of PLY antibody, can activate the classical complement pathway, leading to depletion of complement in the host and reduced opsonic activity, and induce an inflammatory response, aiding the survival and spread of pneumococcal bacteria [517]. This assay was used to assess the propensity of pneumococci (serotype 1 vs. serotype 2) to bind to complement.

Our results indicate that the complement binding percentage was dependent on the strain and not on the serotype. The three sequencing types (ST306, ST304 and ST615) belonging to the serotypes 1 group had almost the same MFI. D39 showed significant binding to C3, whereas ST306, ST304 and ST615 had lower C3 binding activity. Overall, the C3-binding activity on the bacterial surface of all strains of serotype 1 was lower than those in serotype 2.

3.8 Opsonophagocytic killing assay (OPKA)

To understand the ability of the serotype 1 isolates to evade phagocytosis, an opsonophagocytosis killing assay (OPKA) was performed in which the bacteria were opsonised using intravenous Immunoglubulins (IVIG) and then incubated with serum and HL-60-derived neutrophils.


OPKA
***
% of Killing index
25
20
15
10
5
0
1:16
IV Ig Dilution
D39
ST306
ST615
ST304


OPKA
*
% of Killing index
80
60
40
20
0
1:8
IV Ig Dilution
D39
ST306
ST615
ST304

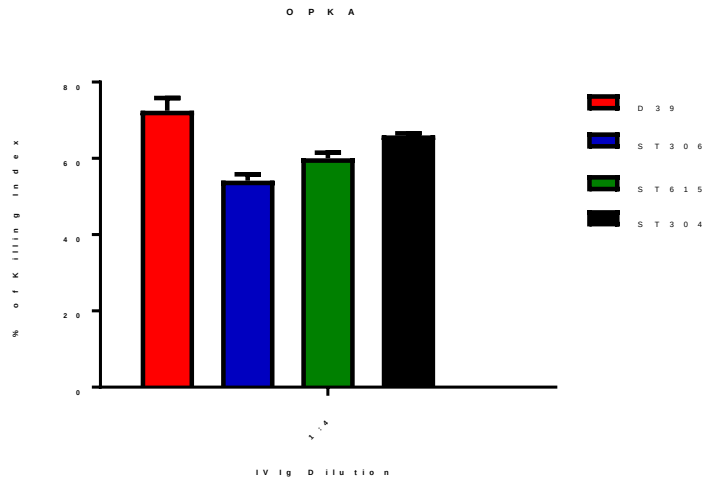


Figure 19. Opsonophagocytic killing of pneumococcal isolates. Each strain was tested with 1:4, 1:8 and 1:16 dilution of IVig antibodies. Results are represented as the percentage of killing different strains in different dilution. The error bars indicate the standard deviation on three independent experiments. *, $p< 0.05$ (one-way ANOVA)

3.8 Opsonophagocytic killing of serotype 1 vs. serotype 2 pneumococci

Host protection against pneumococcal disease is mainly mediated by phagocytosis [519]. An opsonophagocytic assay measures the resistance of pneumococci to phagocytosis, using differentiated HL-60 cells as effector cells. Intravenous immunoglobulin (IVig) was serially diluted (two-fold) in opsonophagocytosis buffer for a total of 8 dilutions, from 1:8 to 1:1,024.

Among the serotype 1 isolates, ST306 showed higher killing percentage at 1:4 dilution of IVIg anticapsular antibodies compared to ST304 and ST615 strains belonging to ST304 and ST615, respectively ($P < 0.05$, one-way ANOVA with post-hoc Tukey's test). However, there were differences between the sequence types of serotype 1, some sequence types more resistant to opsonophagocytic killing than others and was statistically non-significant (one-way ANOVA). Moreover, a previous study showed that there was an increase in killing titer when the strain presented a null mutation on *pspC*. Even the incubation time had an effect on the killing titer [520].

3.9 Colonisation properties of *S. pneumoniae* serotype 1 in a nasopharyngeal carriage mouse model

S. pneumoniae is a common commensal of the nasopharynx of healthy individuals, While particular capsular types, such as serotype 1, are rarely detected during nasopharyngeal carriage 1, serotype 1 is also one of the main causes of pneumococcal invasive disease in Europe and North America and is ranked among the top five most prevalent pneumococcal serotypes in a number of countries [521,331, 522].

ST615 and ST306 serotype 1 strains ST304 and D39 were excluded because the results of ST304 were similar to ST306 and D39 was a control strain) were assessed in a carriage mouse model in which mice were intranasally infected with 10^5 bacteria per mouse. The infection was performed in a total volume of 10µl, mimicking pneumococcal transmission in the nasopharyngeal tissue without translocation to the lungs. For each strain, three mice were culled straight after infection (day 0) and ten mice were culled at each time-point on days 1, 3, 7, 14, 21 and 28 post-infection.

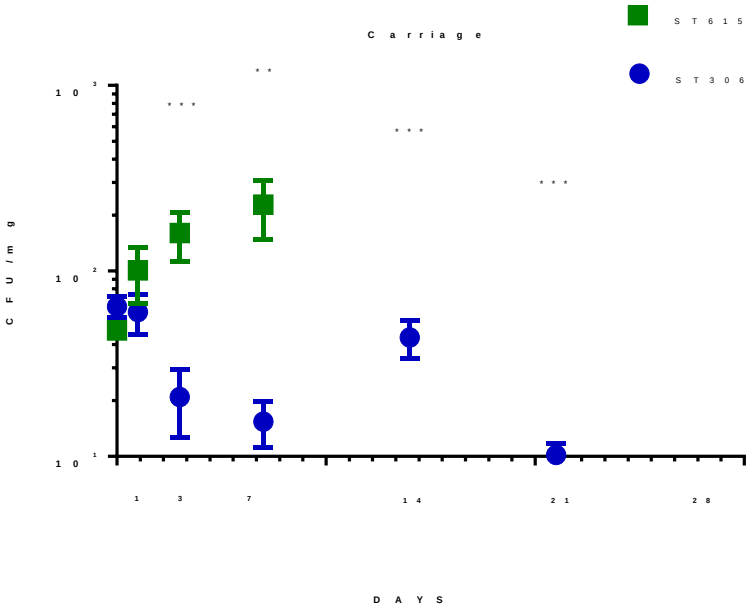


Figure 20. Two groups of mice were infected by either ST306 or ST615. On day 0, mice were challenged intranasally with 10ul of sterile PBS per mouse, containing $1x10^5$ CFU. This figure shows the bacterial density in nasopharyngeal tissue of mice. Data are represented as mean +/- standard deviation, n=10 mice per group.

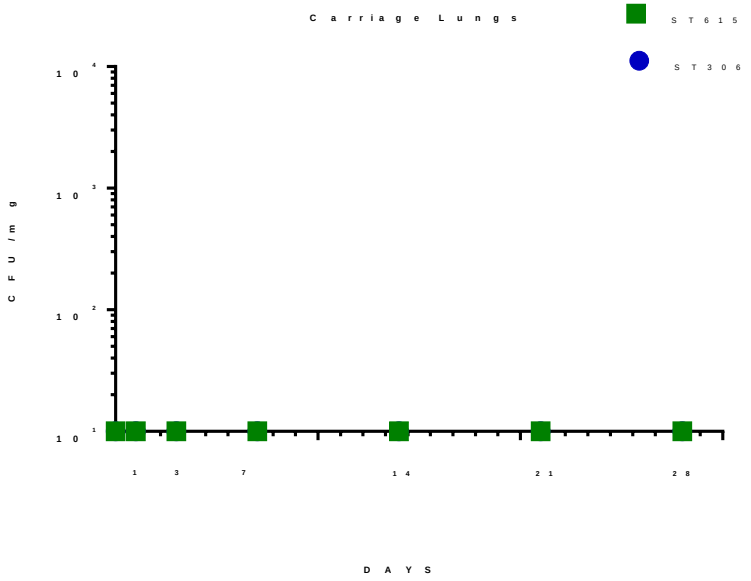


Figure 21. Bacterial density in lung tissues**.** Mice were challenged intranasally with 10ul of sterile PBS containing $1x10^5$ CFU of either ST306 or ST615. This figure shows the absence of detectable amount off pneumococci in the lungs. Data are represented as mean+/- SD, n= 10 mice per group.

3.10 Capsule thickness of pneumococci isolated from nasopharyngeal mouse tissue

The polysaccharide capsule is a major virulence factor of *S. pneumoniae*, which provides protection against opsonophagocytosis by the host immune system. The thickness of the capsule can vary between isolates and at different stages of infection. An increase in the capsule thickness results to an increased protection against the host immune system, while a reduction in the capsule thickness allows pneumococcus to adhere to epithelial and endothelial host cells [111, 515]. Measuring the capsule thickness may assist to determine the relative ability of the isolate to adhere, colonise and invade the host. The capsule thickness of serotype 1 isolates was determined using a FITC-dextran exclusion method [504].

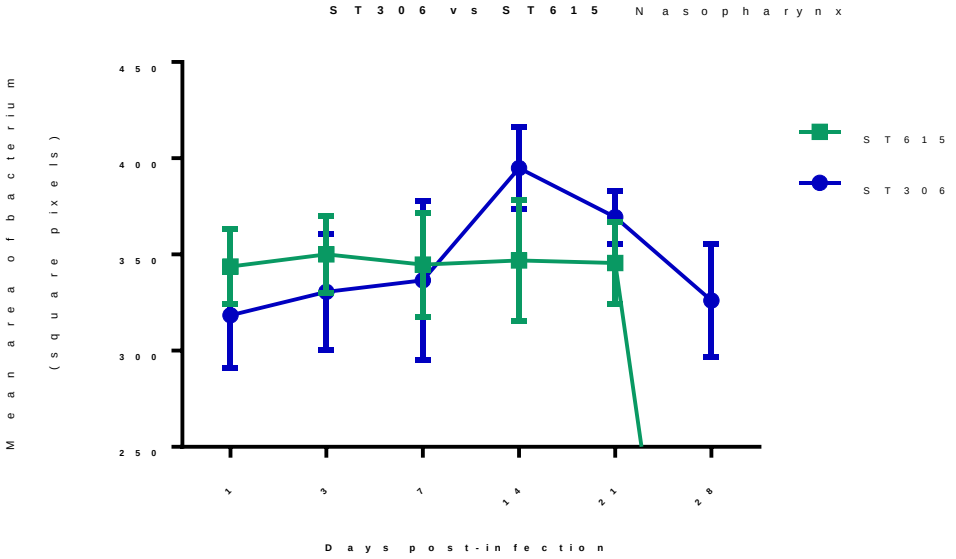


Figure 22. Capsule thickness of ST306 and ST615 of serotype 1 isolates from nasopharynx of infected mice during a carriage experiment. Results are shown as the mean area ± SEM (n=100 bacteria)

The FITC-dextran exclusion method requires the total surface area of the bacterium to be stained with FITC-dextran and then measured and used to compare the thickness of the capsule

of different isolates. The principle of this method is the premise that pneumococci are generally uniform in their size, thus any size differences observed using this method are attributable to differences in capsule thickness. This method is mianly used to measure the size of individual pneumococci not found forming chains.This study suggests that there is maybe a small increase in capsule thickness but there is no difference. These finding are contradictory with a previous study which was showing that a reduction in the capsule thickness is associated with increased adhesion to (specific type of host cells) [522]. This study does not demonstrate any change in capsule thickness between day 0 and day 28, this could be a main reason why serotype 1 is not detected in colonisation, serotype 1 faces difficulty to adapt to the nasopharyngeal niche or does not have the regulatory machinery to change its capsule thickness.

3.11 Competition experiment ST306 vs. ST615 in a nasopharyngeal carriage mouse model

In order to mimic a scenario whereby pneumococcal strains are simultaneously carried in the nasopharyngeal niche, both strains ST306 and ST615 were introduced on day 0, upon infection.

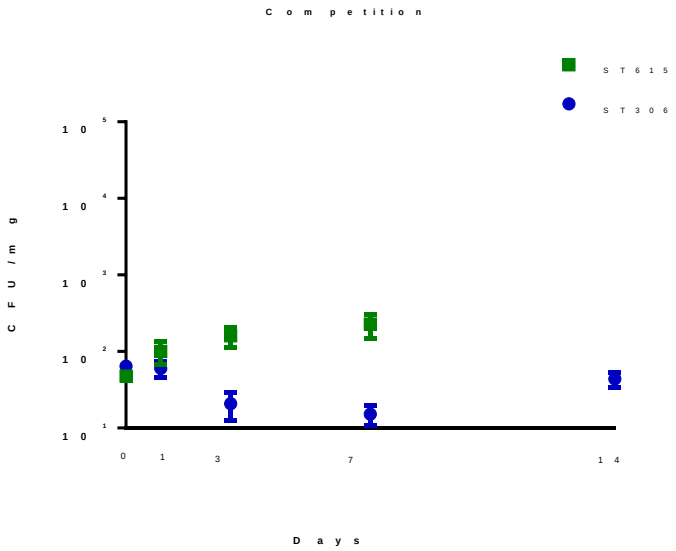


Figure 23. Female MF1 mice were co-infected with ST306 and ST615 at a dose of 2.10^6 CFU/mouse in 10ul of sterile PBS on day 0. Data are represented as mean +/- SD, n= 10 mice per group

Pneumococcal serotype 1 ST306 and ST615 were administered intranasally as described in materials and methods (section 2.16). For each sequence type, ten mice were euthanised at pre-determined time points (days 1, 3, 7, 14, 21, 28 for both isolates) and their lungs and nasopharynx collected, homogenised and processed for viable plate counts and flow cytometric analysis.

This experiment was performed over a 28-day experimental period (Fig. 21). There was no statistically significant difference in the nasopharyngeal bacterial density of mice challenged with ST306- or ST615- at day 0 or day 1 (24 hours) post-infection. However, the bacterial density of ST615 was significantly higher than ST306 on days 3 and 7 (*$p<0.001$, $p<0.01$,* respectively*)*. Interestingly, the scenario was inverted between days 14 and 28. The difference was highly significant on days 14 and 21 (*$p<0.0003$ and $p<0.003$,* respectively*)*, while on day 28, no significant difference was detected between the two sequence types. Interestingly, the lungs did not present any viable pneumococci, except at 24 hours post-infection when

pneumococcal viable counts reached approximately $1x10^1$ CFU/ml in both cases (Fig. 22). It is highly unlikely that bacteremia contributed to the increased bacterial density since no viable counts were detected in the bloodstream of these mice. Blood was monitored for bacteremia at 0, 1, 3, 7, 14, 21 and 28 days after nasal application and no bacteria were detected in the blood.

In a competition experiment, we sought to determine whether the haemolytic activity of ST615 could complement ST306 in its ability to colonise the nasopharyngeal niche. In brief, MF1 female mice were infected with both sequence types at a total dose of $1x10^5$ CFU per mouse in 10ul.

The results in Figure 24 failed to show any competitive advantage between ST306 and ST615 when the mice were infected simultaneously with both sequence types. On day 1, the numbers of bacteria in nasopharynx for ST306 and ST615 were similar at approximately $1x10^2$ CFU/mg tissue. However, ST615 bacterial density was slightly higher than ST306 and the differences were at 0.7 and 1 log at day 3 and 7, respectively. The reduction in ST615 pneumococcal load was noticeable and reached approximately $3x10^2$ CFU/mg at day 14, while ST306 pneumococcus increased again and reached a density similar to day 1, with viable counts of $8x10^2$ CFU/mg.

The tendency of ST306 to establish a lengthier colonization period compared to ST615 – observed in Figure 23 - was not affected. Overall, the results do not suggest that any competition occurred between the two sequence types towards colonising the nasopharyngeal niche. It has previously been shown [525] the ability of pneumococcus to compete with each other for colonization highly depends on the type of capsular polysaccharide expressed by pneumococci and only to a lesser degree on strain or host genetic backgrounds. Moreover, it has been demonstrated the presence of a potent competitive between pneumococcal serotypes

in vivo it depends on the capsule sturcture [526].This study has demonstrated that competitive relationship between different serotypes during host colonisation could affect the order of serotype distribution of nasopharyngeal carriage [526].

3.12 Mouse survival experiment

This survival experiment with serial sacrifice is designed to investigate the effect of introducing pneumococci, on mice in terms of the time course of pathological states. Two groups, infected with ST306 or ST615, are maintained under similar laboratory conditions and observed daily.

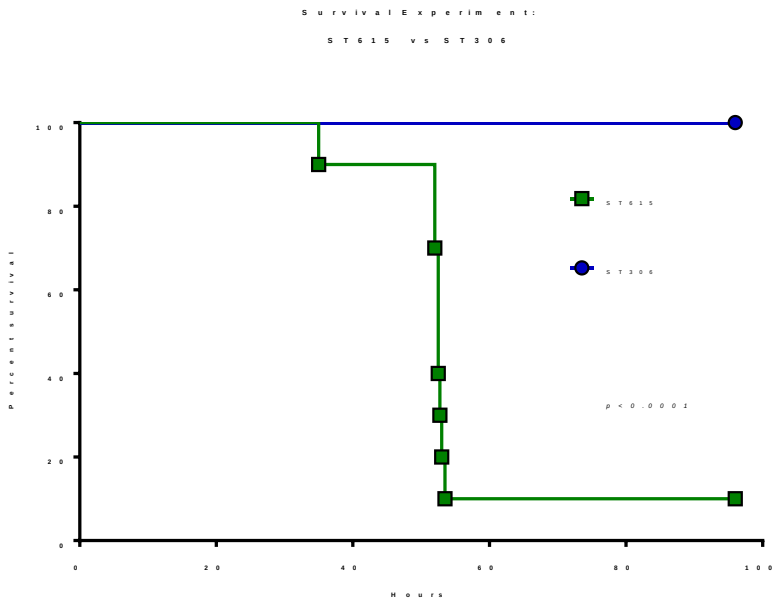


Figure 24. Virulence comparative study using a mouse model of invasive pneumonia.Kaplan-Meier survival chart monitoring the number of mice succumbing to disease at different time points. At the experimental endpoint, all mice (100%) challenged with ST306 mice survived as opposed to 5% survival only in mice challenged with ST615 (n= 10 mice per group). *P<0.0001*

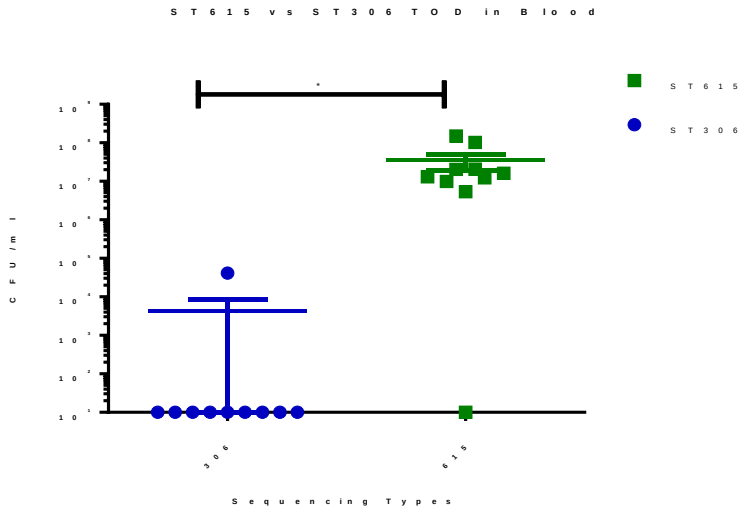


Figure 25. Viable counts (CFU/ml) in peripheral blood after intranasal infection of female MF1 mice with ST306 compared to ST615 pneumococci at time of death (TOD). Data are represented represented as mean+/- SD (n= 10 mice per group). Each mouse is represented by a dot. Statistical significance (student-t-test, * $p<0.05$)

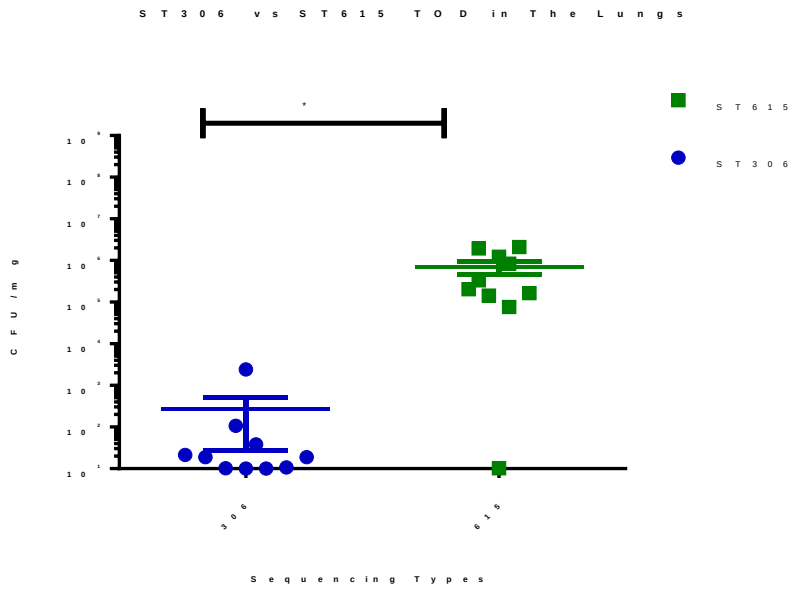


Figure 26. Viable counts (CFU/mg) in lungs after intranasal infection of mice with ST306 compared to ST615 pneumococci at time of death (TOD). Data are represented as mean+/- SD (student-t-test ***p<0.01*)

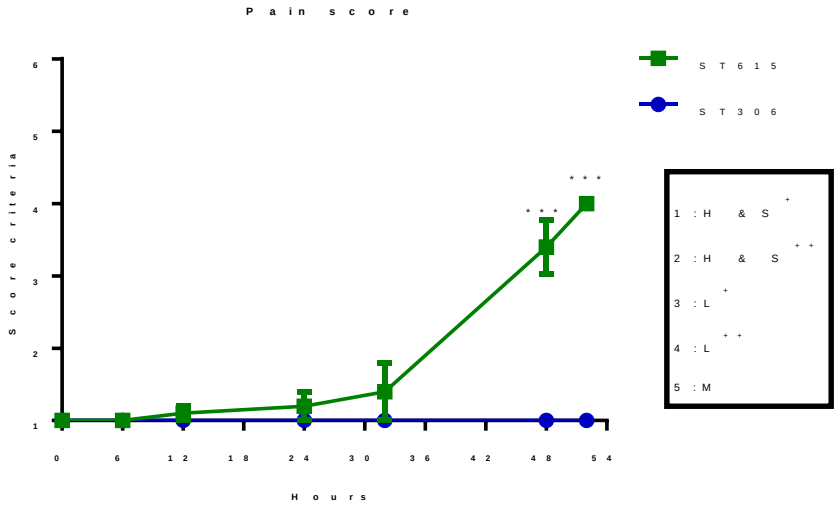


Figure 27. Pain score in ST306 and ST615 mice. Pain scores were assigned as follows: -1 (H &S^{+}): hunched and starry coat +, 2 (H &S^{++}): hunched and starry coat ++, 3 (L^{+}) : lethargic +, 4 (L^{++}): lethargic ++ and 5 (M): moribund (data presented are mean of 10 mice per group). Data are represented as mean+/- SD. Area under curve for ST615=89.7% and ST306=52% at 48 hours and 52 hours was ****p<0.001*

Mice were challenged with serotype 1 pneumococci ST306 or ST615, as described in the methods section (2.16). At the time of death (TOD), bacterial viable counts were determined for ST306 and ST615-challenged mice.

Using a pneumonia challenge mouse model, results showed that the survival of ST306-challenged mice was 100% whereas only 5% of ST615-challenged mice survived, as shown in Figure 25. This suggests that ST615 serotype 1 is significantly more virulent than ST306. In terms of inducing disease symptoms, ST306-challenged mice looked healthy and alert. There was a highly significant difference (area under curve value at 48 and 52 hours, was 89.7% for ST615 and 52% for ST306 $p<0.001$) between ST306 and ST615. The group of ST615-challenged mice started showing symptoms after 38 hours and onward (Figure 28).

A significant difference was determined in the viable counts found in the blood of mice challenged with ST306 vs. ST615 (Figure 26). While no bacteria were detected on blood plates in mice challenged with ST306 - except for one mouse presenting a viable count of $1x10^5$ CFU/ml - the number of pneumococci in the group of mice infected with ST615 reached a count of $1x10^8$ CFU/ml at the time of death (student-t-test*$p<0.05$). Interestingly, ST306 pneumococci were found in the lungs at approximately $2x10^4$ CFU/ml which was significantly less than the pneumococcal density found in mice challenged with ST615 (student-t-test**$p<0.01$) (Figure 27). This suggests that ST306 is not able to replicate and survive in the lungs, potentially explained by its longer generation time and low haemolytically active pneumolysin.

3.13 Comparative studies of ST306 vs. ST615 serotype 1 in invasive pneumonia mouse model

Pneumococcal serotype 1 ST306 and ST615 were administrated intranasally, as described in materials and methods (section 2.16). In brief, female MF1 mice (Charles River) were challenged with 50 µl pneumococci via the intranasal route at a final concentration of 1×10^6 CFU/ml. The mice were divided into two groups and infected with ST306 or ST615. The number of tested mice in each time point was 10 mice for each sequence type.

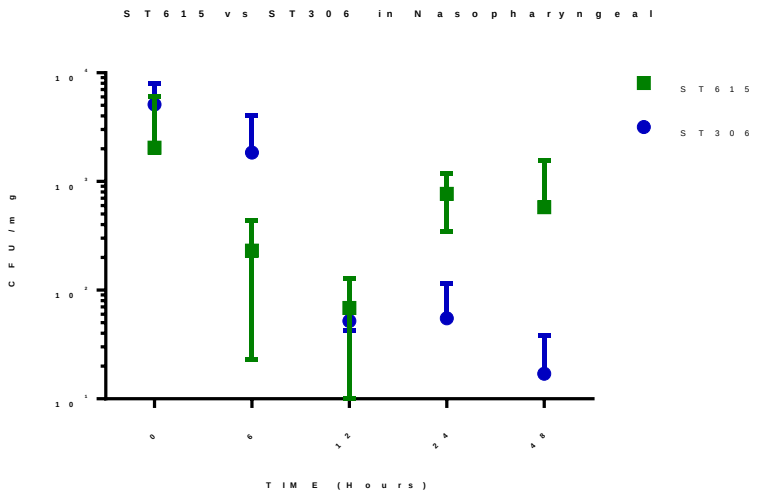


Figure 28. Serotype 1 (ST306 vs ST615) viable counts (CFU/mg) in nasopharyngeal tissue at 0, 6, 12, 24 and 48 hours post-pneumococcal infection. Mice were challenged intranasally with 50µl of sterile PBS containing $1x10^6$ CFU of either ST306 or ST615. Data are represented as mean+/- SD, n= 10 mice per group. The analysis did not show any significant difference between those strains

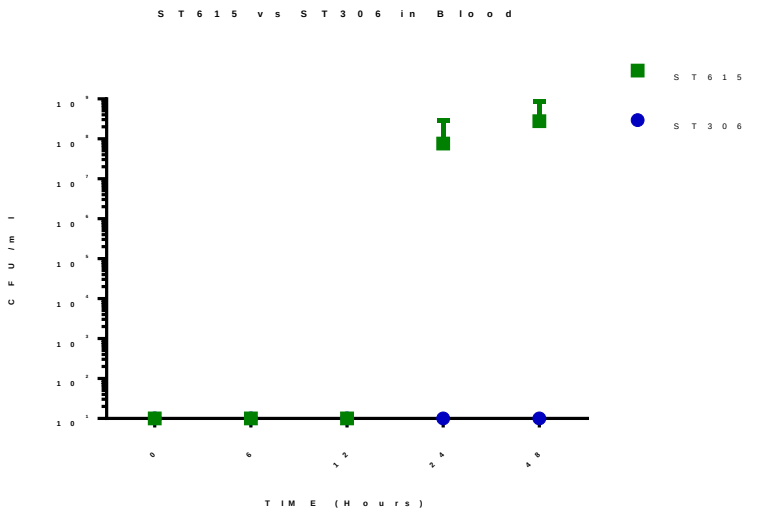


Figure 29. Serotype 1 (ST306 vs ST615) CFU counts in blood at 0, 6, 12, 24 and 48 hours post pneumococcal infection. Mice were challenged intranasally with 50 ul of sterile PBS containing $1x10^6$ CFU of either ST306 or ST615. Data are represented as mean+/- SD, n= 10 mice per group

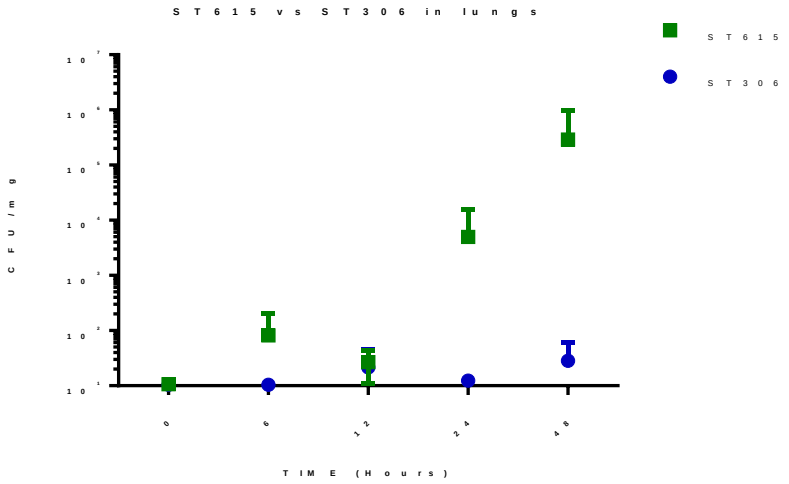


Figure 30. Serotype 1 (ST306 vs ST615) CFU counts in lungs at 0, 6, 12, 24 and 48 hours post pneumococcal infection. Mice were challenged intranasally with 50 ul of sterile PBS containing $1x10^6$ CFU of either ST306 or ST615. Data are represented as mean+/- SD, n= 10 mice per group

On day 0, for each sequence type (ST306 vs ST615), mice (n=10 per group) were humanely euthanised a few minutes following pneumococcal challenge and their lungs and

nasopharynx collected, homogenised and processed for viable counts on blood agar plates and flow cytometry analysis.

Pneumococcal viable count results showed significant differences between all lung and blood samples collected from ST306 vs. ST615 challenged mice at 24 and 48 hours post-infection. The viable counts obtained in the nasopharyngeal tissue were not significantly different although ST615-infected mice presented half to 1 $\log_{10}$ difference after 24 and 48 hours, respectively, compared to the ST306-infected mice.

The major differences in bacterial viable counts were observed at 24 and 48 hours in lungs and nasopharyngeal tissue in ST306-challenged mice. The lungs of ST615 challenged mice presented higher viable counts than those of ST306-challenged mice, a difference of the order of 4 to 5 log10 at 24 and 48 hours, respectively. The higher pneumococcal viable counts observed in ST615 coincide with the more severe clinical symptoms (Figure 31).

3.14 Pneumococcal serotype 1 in intranasal model of invasive pneumonia (high dose)

Briefly, female MF1 mice (Charles River) were challenged with 50µl pneumococci via intranasal administration at a final concentration of 1×10^7 CFU/ml in 50µl.

All mice were observed regularly to monitor kinetics of disease progression over time. At 0, 24 and 48 hours post-challenge, ST306- and ST615-challenged mice were culled, and their lungs and nasopharynx collected. Whole blood was collected by cardiac puncture in all mice. Tissue samples were then homogenised and spotted on blood agar plate for colony-forming unit counts.

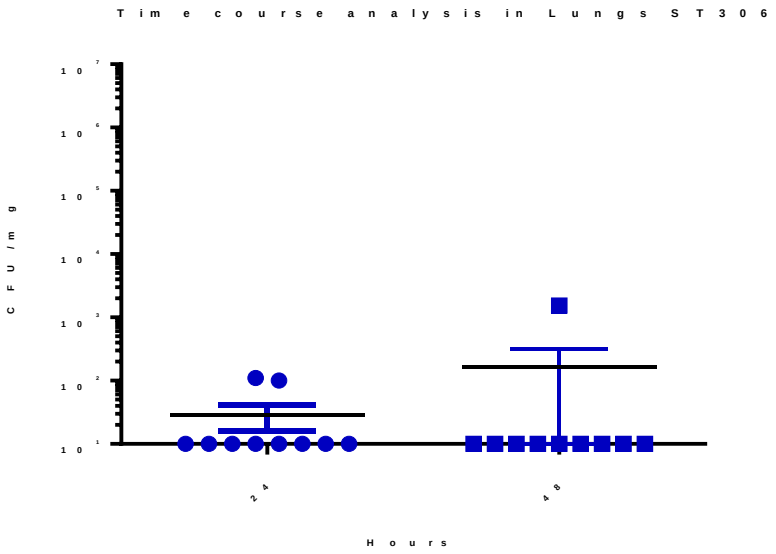


Figure 31. Serotype 1 ST306 CFU counts in lungs at 24 and 48 hours post pneumococcal infection. Mice were challenged intranasally with 50ul of sterile PBS containing $1x10^7$ CFU of ST306. Data are represented as mean+/- SD, n= 10 mice per group

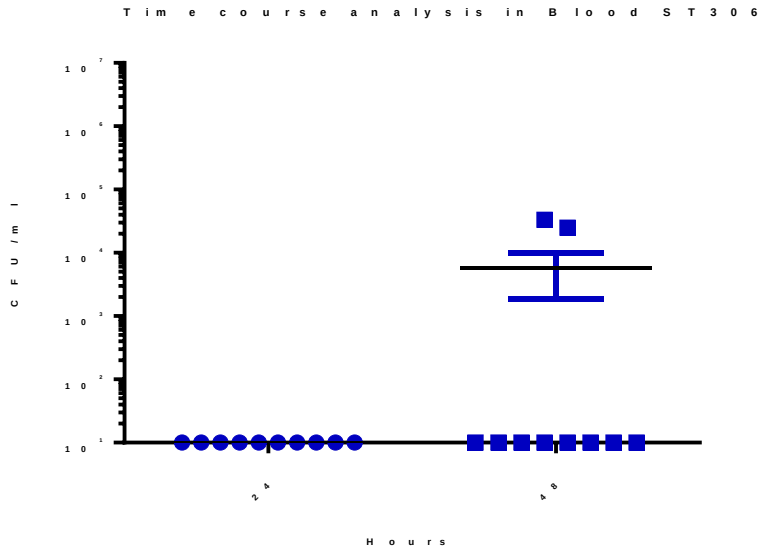


Figure 32. Viable counts in the peripheral blood of ST306-challenged mice at 24 and 48 hours post pneumococcal infection. Mice were challenged intranasally with 50ul of sterile PBS containing $1x10^7$ CFU of ST306. Data are represented as mean+/- SD, n= 10 mice per group

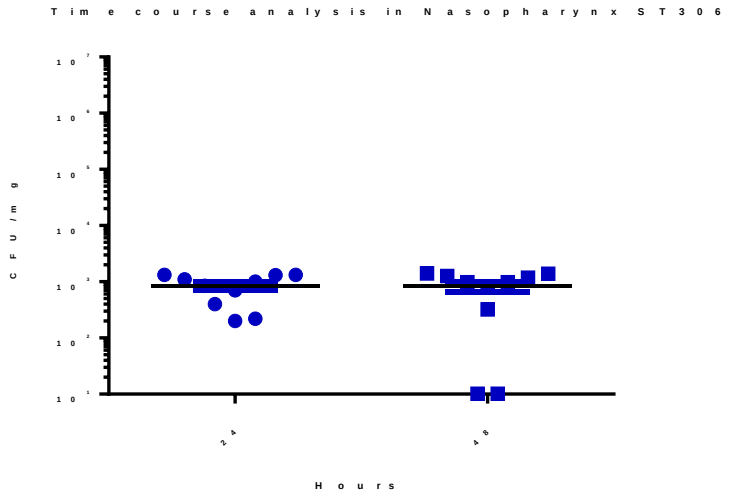


Figure 33. Viable counts in the nasophrynx of ST306-challenged mice at 24 and 48 hours post pneumococcal infection. Mice were challenged intranasally with 50ul of sterile PBS containing $1x10^7$ CFU of ST306. Data are represented as mean+/- SD, n= 10 mice per group

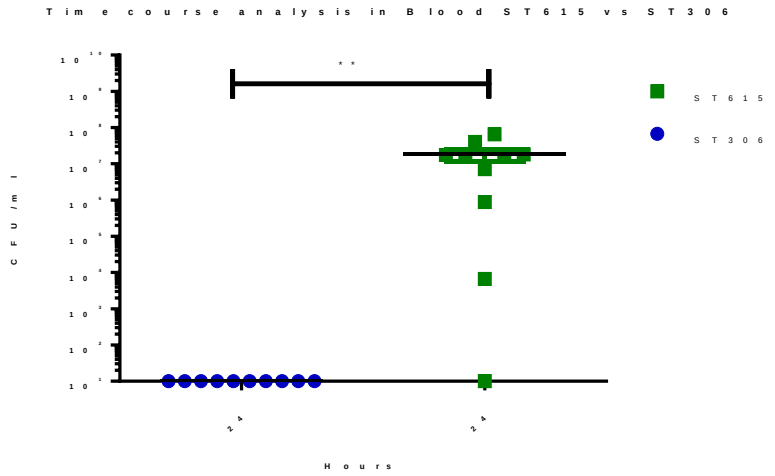


Figure 34. High-dose model -Viable counts in the blood of ST306 vs ST615 -challenged mice at 24 hours post pneumococcal infection. Mice were challenged intranasally with 50ul of sterile PBS containing $1x10^7$ CFU of either ST306 or ST615. Data are represented as mean+/- SD, n= 10 mice per group. (t-test, ** p<0.009)

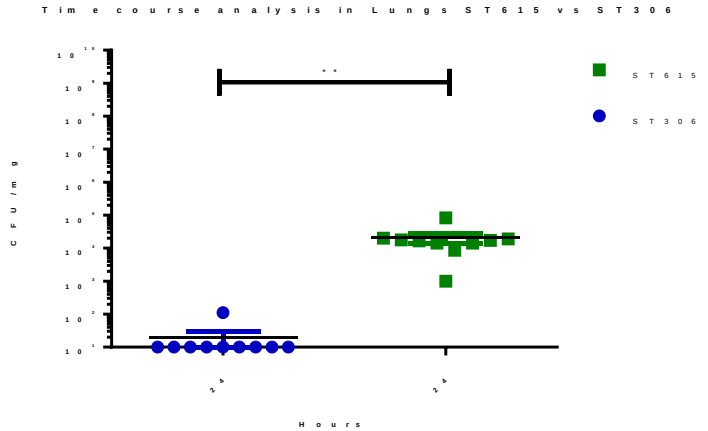


Figure 35. High dose model - Viable counts in the lung of ST306 vs ST615 -challenged mice at 24 hours post pneumococcal infection. Mice were challenged intranasally with 50ul of sterile PBS containing $1x10^7$ CFU of either ST306 or ST615. Data are represented as mean+/- SD, n= 10 mice per group. (t-test,** *p<0.007*)

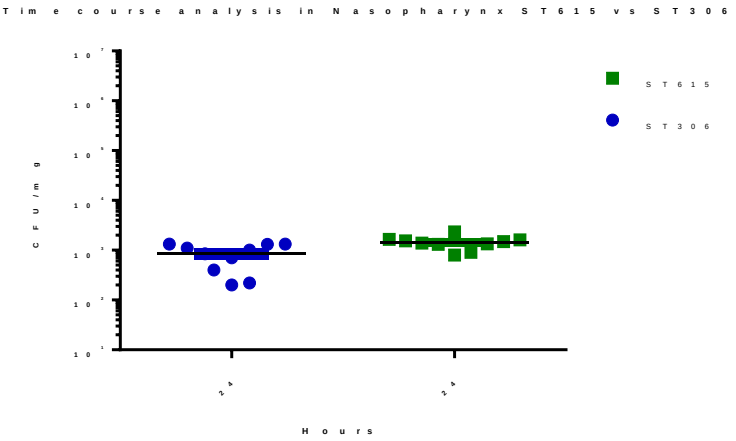


Figure 36. High-dose model -Viable counts in the nasopharynx of ST306 vs ST615 -challenged mice at 24 hours post pneumococcal infection. Mice were challenged intranasally with 50ul of sterile PBS containing $1x10^7$ CFU of either ST306 or ST615. Data are represented as mean+/- SD, n= 10 mice per group

This experiment was performed in order to determine whether ST306 pneumococci could transit from the lung tissue into the blood when administered at a dose one log higher than that used in our standard pneumonia model, i.e. 10^7 CFU rather than 10^6 CFU per mouse. Results show that groups of mice challenged with ST615 caused infiltration of pneumococci into the lung tissues and blood stream at approximately 48 hours. Disease symptoms were obvious and pain score reached a level 4 (i.e., lethargic ++). On the other hand, none of the ST306-challenged mice presented any disease symptoms and viable counts were significant lower (**,*p<0.009)* compared to ST615 challenged mice in peripheral blood as well as in the lung tissues at 24 and 48 hours post-infection (**,*p<0.007).*

In contrast to ST306 pneumococci, the use of a high dose of ST615 altered the survival time of mice to less than 48 hours compared to 53 hours upon administration of 10^6CFU per mouse. Interestingly, pneumococcal viable counts measured in the nasopharynx were similar in both groups of mice i.e., $1x10^3$ CFU/mg. The number of ST615 pneumococci found in the

lungs at 24 hours post-infection was $1x10^5$CFU/mg and coincide with the time the mice started to exhibit disease symptoms (starry and hunched). Nine out of ten mice presented pneumococci in blood at 24 hours, and reached as high as $1x10^7$ CFU/ml in the blood explaining the short survival time in these mice.

Pneumococcal counts in the nasopharynx of ST306-challenged mice showed no significant difference between 24 and 48 post-infection and the viable counts were approximately equal to $1x10^4$ at both time points. Interestingly, no bacteria were detected in the lungs for 9 out of 10 mice and one mouse only presented $1x10^2$ CFU/mg at 24 hours post-infection. Similarly, at 48 post-infection, 9/10 mice challenged with ST306 failed to show any pneumococci in their lungs, except for one mouse with a viable count of 10^2 CFU/mg. No bacteria were found in the blood at 24 hours, while two mice started to develop bacteremia with CFU counts reaching approximately $3x10^4$ cfu/ml. The challenge of mice with a dose of $1x10^7$ CFU per mouse (compared to our standard $1x10^6$ CFU per mouse) failed to promote the ability of ST306 to reach the peripheral circulation. It is likely that using such a high dose may have alerted the immune system and triggered a more efficient elimination of bacteria in the lungs, resulting in rapid clearance

3.15 Virulence of pneumococcal serotype 1 in sepsis and invasive pneumonia models

A sepsis mouse model of infection was set up as described in the section materials and methods (section 2.16). In brief, a fresh liquid stock of ST306 and ST615 pneumococci was used to perform this experiment. Twelve MF1 female mice (Charles river) were intravenously challenged with 50µl pneumococci via the dorsal tail route at a final concentration of 1 ×

10^6 CFU/ml in 50µl. The mice were divided into two groups: six mice in ST306-administered mice and six mice administered with ST615 pneumococci.

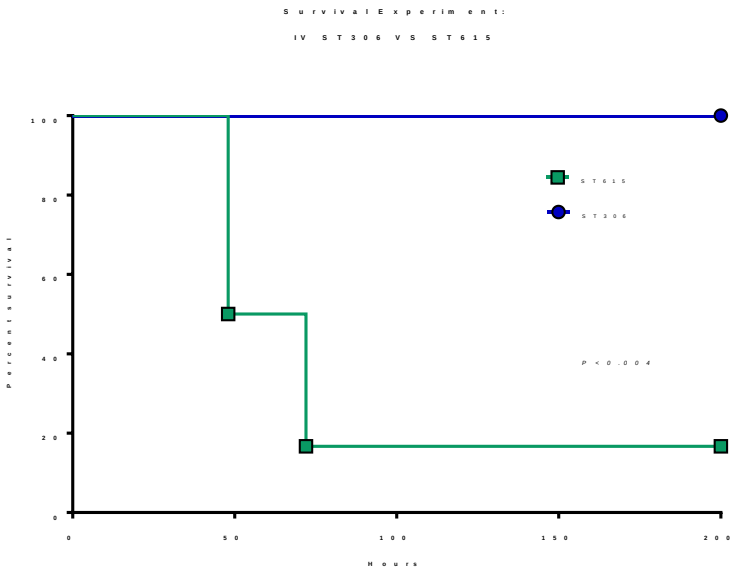


Figure 37. Comparative virulence study in a mouse model of invasive pneumonia. Kaplan-Meier survival chart monitoring the number of mice succumbing to disease at different time points. At the experimental endpoint, data refer to 6 mice challenged with ST306 pneumococci and 6 mice challenged with ST615 pneumococci. At 100 hours, the survival rate of ST615-challenged mice was only ~30%, while the survival rate was 100% in ST306 mice. ST615-challenged mice started to show severe sign of lethargy at 40 hours post-infection

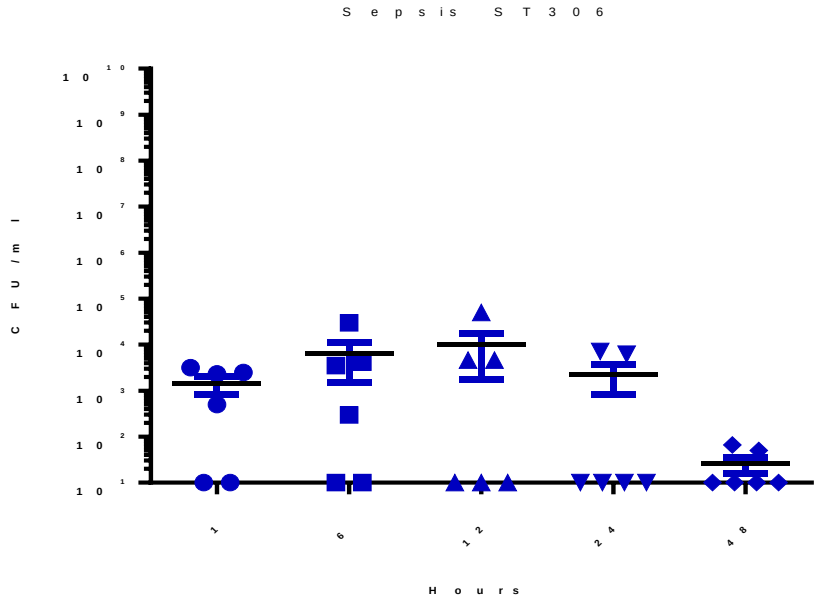


Figure 38. Serotype 1 (ST306) CFU counts in blood at 1, 6, 24 and 48 hours post pneumococcal infection. Mice were challenged intravenously with 50 ul of sterile PBS containing $1x10^6$ CFU of ST306 pneumococci. Data are represented as mean+/- SD, n= 10 mice per group

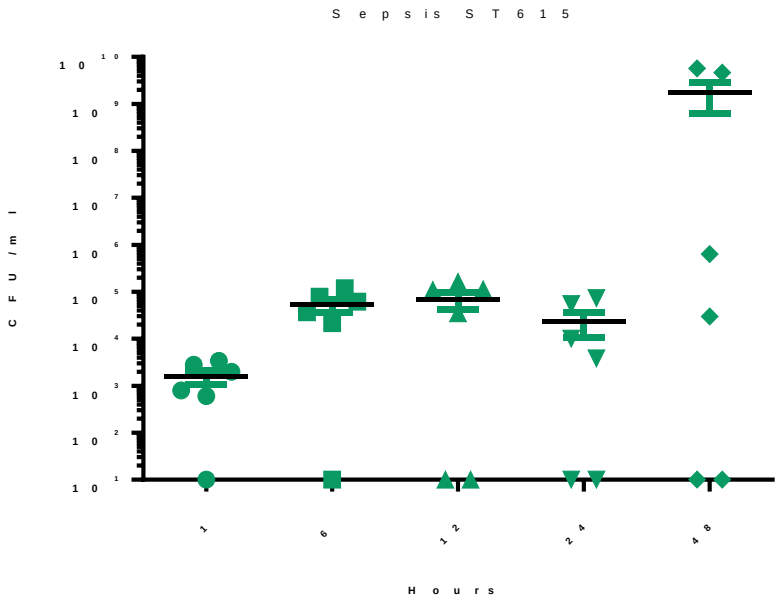


Figure 39. Serotype 1 (ST615) viable counts in blood at 1, 6, 24 and 48 hours post-pneumococcal infection. Mice were challenged intravenously with 50µl of sterile PBS containing $1x10^6$ CFU of ST615 pneumococci. Data are represented as mean+/- SD, n= 10 mice per group

At 1hour post-infection, pneumococcal viable counts in the blood of both ST306 mice and ST615 mice were similar. ST306 pneumococcal viable counts were stable until 24 hours and a reduction in pneumococcal density was observed at 48 hours post-challenge (Figure 39). At 48 hours, ST615 bacterial load was higher than ST306 with more than five log_{10} difference between ST615- and ST306-challenged mice. The major reduction in pneumococcal viability was determined at 48 hours post-challenge, the viability of ST306 pneumococci mouse dropped to 50 CFU/ml. We previously described that mice infected intravenous with ST306 were able to survive until 168 hour post-infection (Figure 38);

however, a previous study showed that 68% was the percentage of survived mice infected with ST306 [526].

3.16 Mouse challenge with ST306 pneumococci supplemented with pure pneumolysin in mouse model of invasive pneumonia

Female MF1 mice were challenged with 10^6 CFU ST306 pneumococci in 50µl supplemented with 100ng LPS-free recombinant pneumolysin via intranasal administration, as described in materials and methods (section 2.16). The mice were divided into two groups; ST306 and ST306 supplemented with LPS-free recombinant pneumolysin mice. The number of tested mice in each time point was 10 mice for each sequencing types.

Pneumococcal viable counts reached a peak at 12 hours in the lungs, while it peaked in the blood at 48 hours. A gradual increase in the number of pneumococci occurred in the lung until 12 hours post-challenge then slightly decreased, while a gradual increase in pneumococcal load was observed in the blood and peaked at 48 hours; however, there was a decline at 72 hours post-infection.

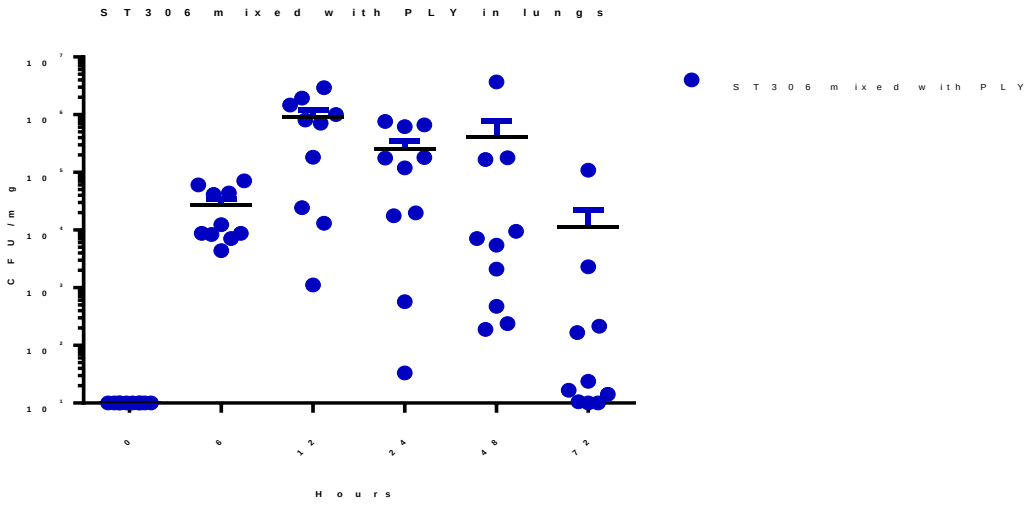


Figure 40. Pulse pneumolysin experiment with ST306-challenged mice: Serotype 1 ST306 was mixed with purified pneumolysin (100ng/mouse) and administered intranasally at 10^6 CFU per mouse in 50µl. Viable counts were determined in lungs at 0, 6, 12, 24, 48 and 72 hours post pneumococcal infection. Data are represented as mean, n=10 mice per group +/- SD

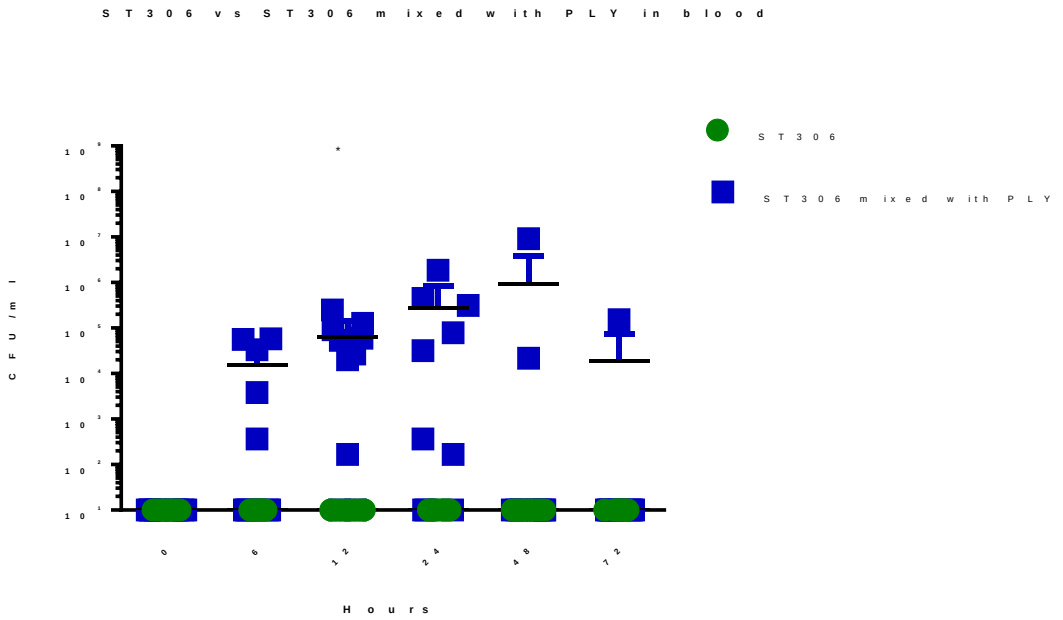


Figure 41. Supplementation experiment: Serotype 1 (ST306) was mixed with purified pneumolysin(100ng/mouse) and administered intranasally at 10^6 CFU per mouse in 50µl comparing to ST306 only. Viable counts in blood at 0, 6, 12, 24, 48 and 72 hours post pneumococcal infection. Data are represented as mean, n=10 mice per group +/- SD. (student-t-test,*p<0.01)

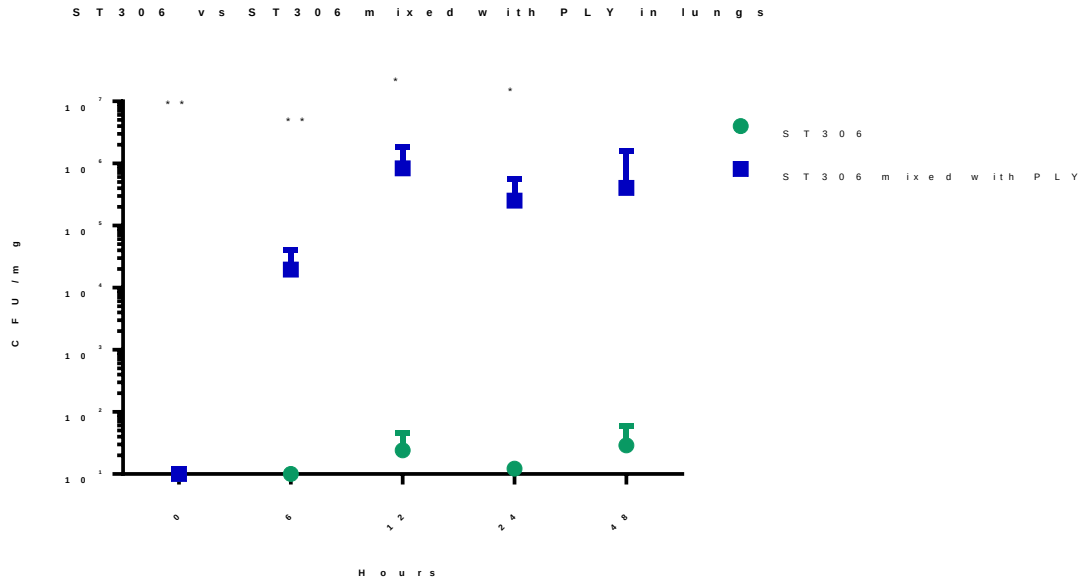


Figure 42. Supplementation experiment: Serotype 1 (ST306) was mixed with purified pneumolysin(100ng/mouse) and administered intranasally at 10^6 CFU per mouse in 50µl comparing to ST306 only. Viable counts in lungs at 0, 6, 12, 24, 48 and 72 hours post pneumococcal infection. Data are represented as mean, n=10 mice per group +/- SD. (student-t-test,*p<0.01, **p<0.001)

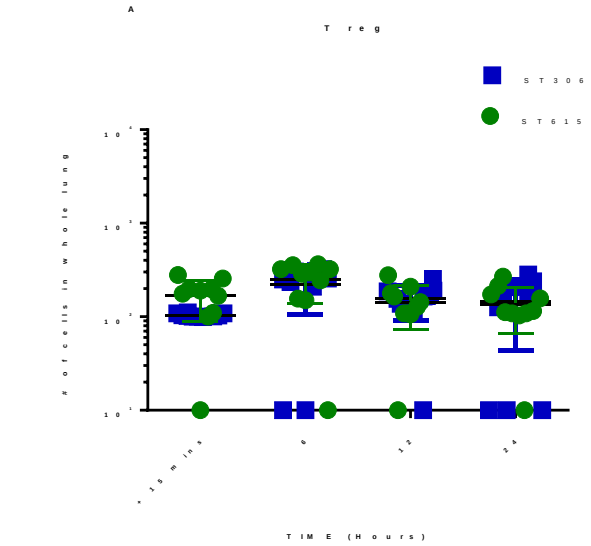
In Figures 42 and 43 are shown the viable counts of mice challenged with ST306 vs. ST306 mixed with recombinant pneumolysin in blood and lungs, respectively, at various time points. In the lungs, a statistically significant difference was found in bacterial viable counts at time zero, 6, 12 and 24 hours between the two groups of mice, while in the blood, a significant difference in bacterial viable counts was only determined at 12 hours.

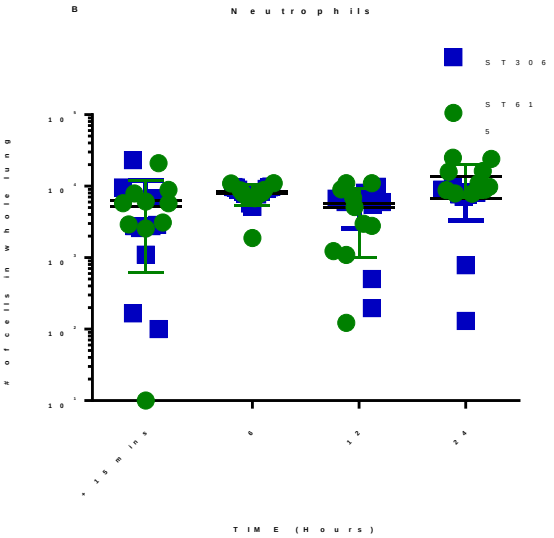
These results suggest that the supplementation of a low haemylotic strain (ST306) with highly haemolitically active pneumolysin enhanced the ability of pneumococci to infiltrate lung and blood cmpartments. These effects were also translated by a more rapid disease progression (Figures 42 and 43).

3.17 Analysis of Immunity

3.17.1 Lung immune cellular profiling during invasive infection with serotype 1

The cellular profile of the lung of 10 MF1 mice infected with the ST306 and ST615 isolates were determined at different stages of infection using flow cytometry. The number of (A) T regulatory cells, (B) neutrophils and (C) macrophages present in the lung of up to ten mice per time point (Figure 44).


A
T reg
ST306
ST615
# of cells in whole lung
15 mins
0
12
24
TIME (Hours)


B
Neutrophils
ST306
ST615
# of cells in whole lung
15 mins
0
12
24
TIME (Hours)

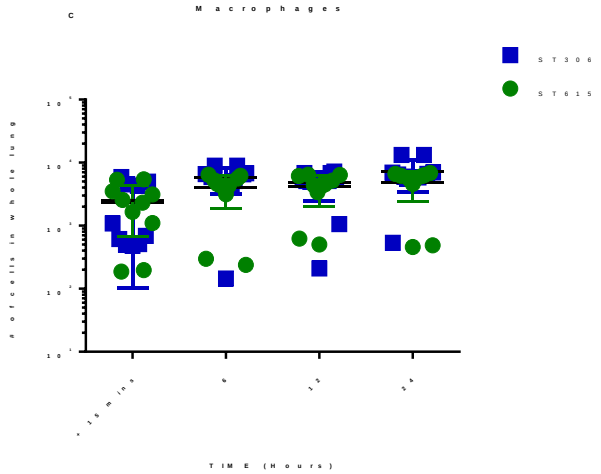


Figure 43. Cellular profiling in the lung of MF1 mice during an invasive infection caused by ST306 and ST615 at times +15mins, 6h, 12h and 24h post-infection shown as mean ± SEM (n=10)

The CD45 marker was used to gate leukocytes, thereby excluding non-immune cells such as endothelial and epithelial cells. The number of T regulatory cells (CD4+/FoxP3+) was found not to vary throughout the duration of the experiment (Figure 43 A) and no difference was observed between ST306 and ST615. The number of T regulatory cells in the whole lung was approximately $5x10^2$ cells at 6h post-infection. The number of macrophages (F4/80+) and neutrophils (Gr1+cells) were stable at a $5x10^3$ macrophages and 10^5 neutrophils per lung throughout the duration of the experiment (Figure 44, panels B and C). There were no significant differences observed in the recruitment of T regulatory cells, neutrophils or macrophages between ST306 and ST615 at all time points.

3.17.2 Cytokine analysis of the lung during invasive infection by ST306 and ST615 serotype 1

Cytokine levels in the lungs of MF1 mice infected with ST306 and ST615 serotype 1 were determined at different stages of infection. This analysis was carried out on the same lungs

used for cellular profiling (Figure 44). A selection of cytokines involved in host immune responses against bacterial infection was analysed: IL-1β, IL-6, INF-γ and TNF-α. The changes in cytokine levels during infection are shown in Figure 45:

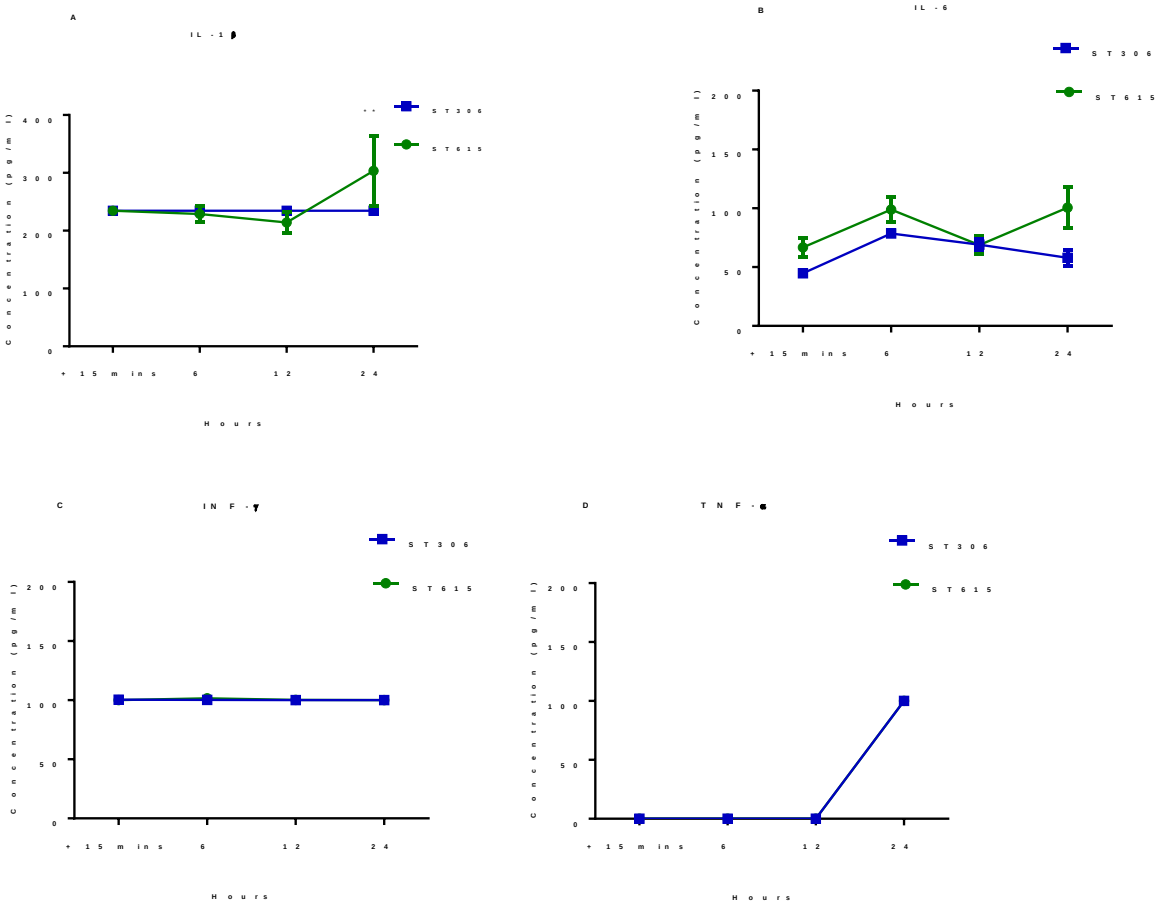


Figure 44. Cytokines analysis of the lung of MF1 mice during an invasive infection caused by ST306 and ST615 at times +15mins, 6h, 12h and 24h post-infection shown as mean ± SEM (n=10). **P-value<0.0001 when analysed using a one-way ANOVA

As observed in Figure 45, panel A the levels of IL-β were 250pg/ml throughout most of the infection, except 24h post-infection for ST615, when there was a significant increase to 300pg/ml comparing to ST306 (*P-value<0.0001). While the levels of IL-6 were found to fluctuate throughout the monitoring period for both sequencing types, the levels of IL-6 were higher in the lung tissue of mice infected with ST615 compared to ST306. IL-6 is classically secreted by macrophages and T lymphocytes, but since our results failed to show any recruitment of these cell types, other cellular sources, such as epithelial cells, might be responsible for the observed increase. Interestingly, however, the increase of IL-6 levels observed in the lungs of mice infected with ST615 at 24h post-infection coincides with no increased number of neutrophils which was also observed in the lung at 24h post-infection.

The levels of TNF-α were virtually null or below detection levels, throughout most of the infection, except at 24h post-infection, when the concentrations were found to increase in both groups of mice (Figure 45, D). TNF-α is a cytokine mainly produced by macrophages, it is involved in cellular apoptosis and immune regulation and is an indication of inflammation.

Hence the increase observed in the concentrations of TNF-α at 24h post infection might reflect the inflammatory response present in the lung when the bacterial numbers are also at their peak titers of 10^4 CFU/mg of lung (Figure 31).

There were no detectable changes in the levels of INF-γ throughout the course of infection (Figures 45, C). Surprinsingly, the levels of INF-γ seemed to remain constant, at approximately 100pg/ml.

3.17.3 Cervical Lymph nodes during carriage infection

The cervical lymph nodes analysed in this study were anterior superficial, which are situated in the cervical area of mice. As with the nasal associated lymphoid tissue, these cervical lymph nodes are the draining lymph nodes for the nasopharyngeal tissue [528, 529].

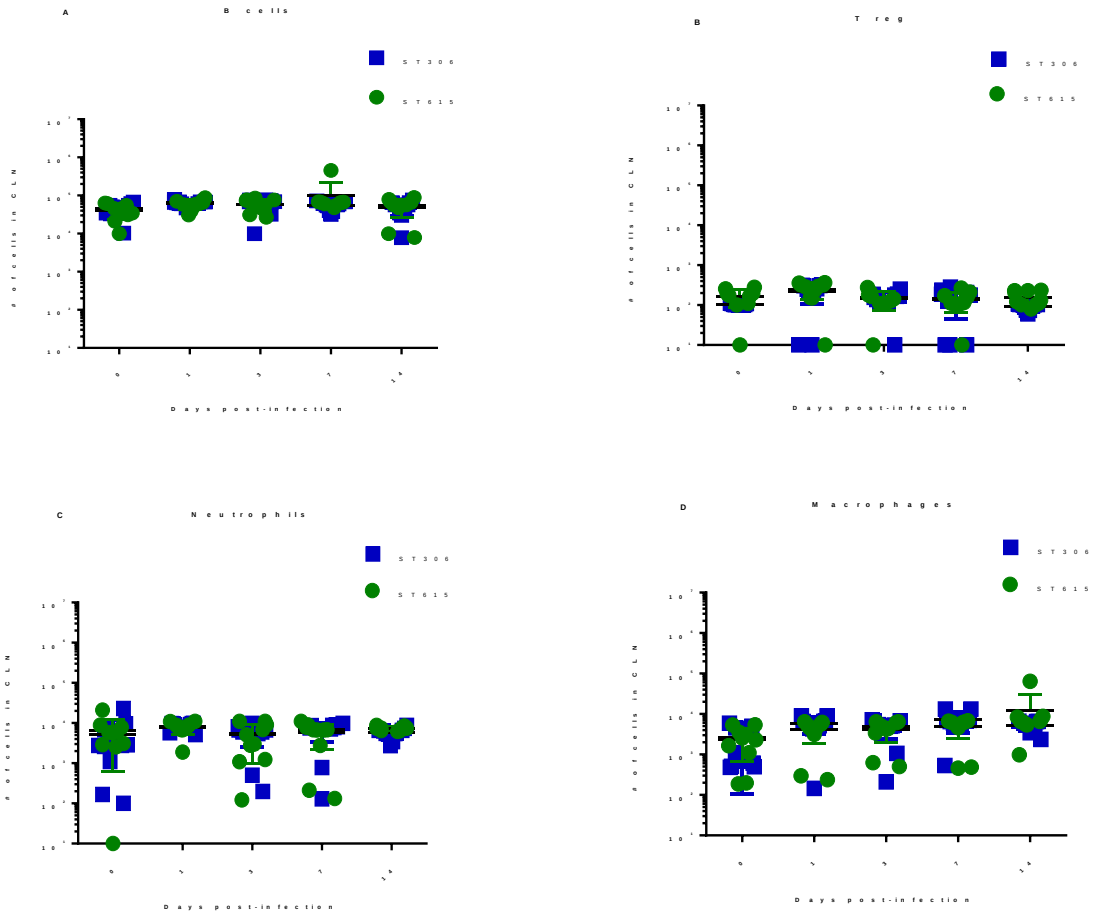


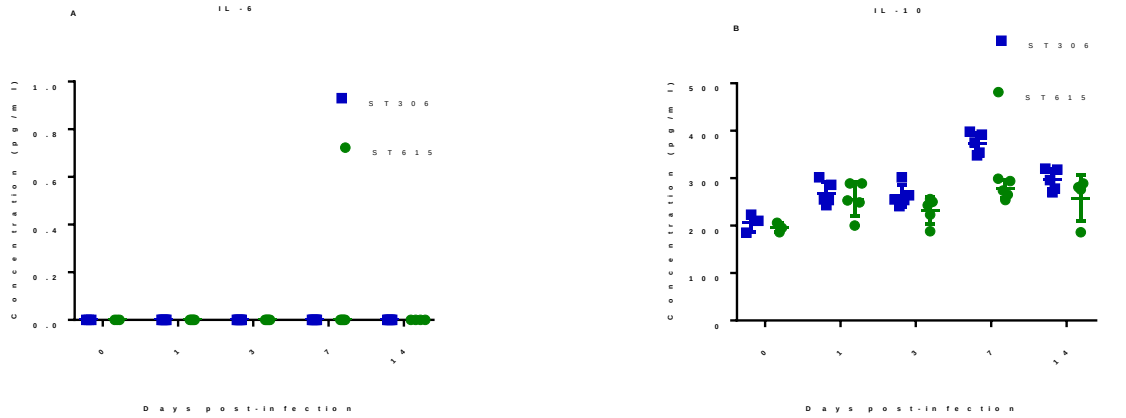
Figure 45. Immune cells phenotypic of (A) B cells, (B) T regulatory cells, (C) neutrophils and (D) macrophages in the cervical lymph nodes during nasopharyngeal carriage with ST306 and ST615 of serotype 1 in MF1 mice at days 0, 1, 3, 7 and 14 post-infection. Three mice were used for the day 0 control, and ten mice were used for the rest of the time-points

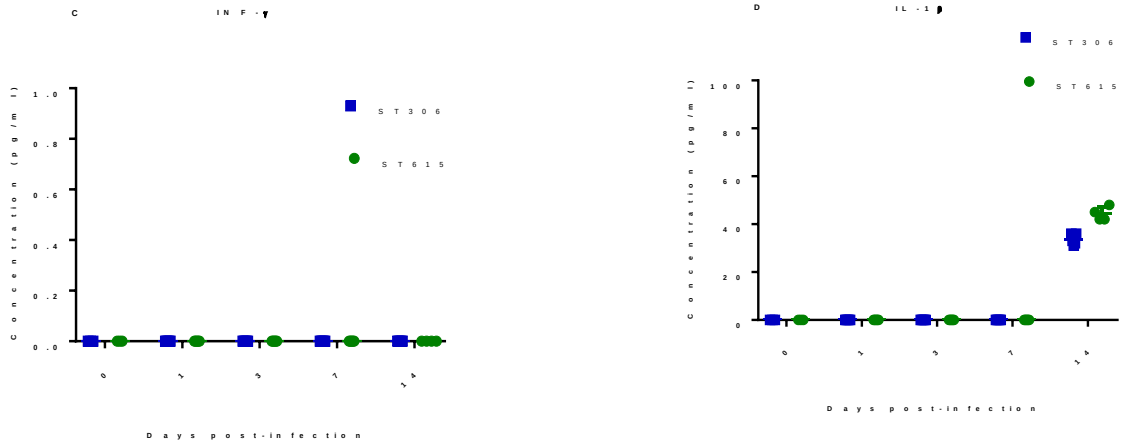
As observed in Figure 46 B, the number of T regulatory cells was approximately $5x10^2$ mg/ CLN tissue and this number remained constant throughout the observation period. The number of neutrophils and macrophages was different in this tissue, with the neutrophils ranging between $8x10^3$ and 10^4 mg/ CLN tissue and the macrophages ranging between 10^3 and 10^4 (Figures 46 C and 46 D). The number of macrophages and neutrophils in the cervical lymph nodes was stable throughout mice and time-points regardless to sequencing types. The clearance of ST615 pneumococci occurred at day 7 post-infection due to the migration of those neutrophils to the site of infection, the nasopharynx.

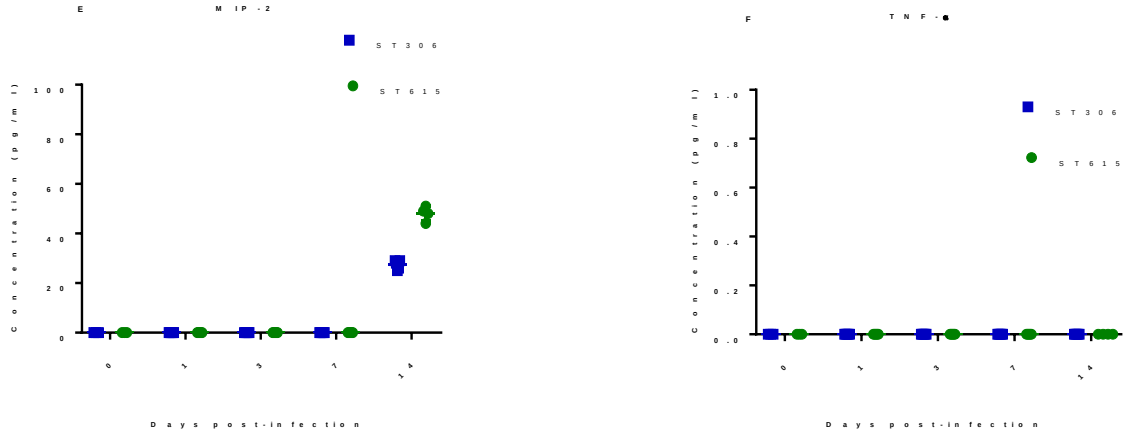
3.17.4 Cytokine production during nasopharyngeal carriage

Cytokines are small secreted proteins released by specific host cells in order to interact and communicate between each other allowing the recruitment and activation of other immune cells. Regulatory cytokines such as IL-10 and TGF-β1, were previously shown to be necessary in maintenance of carriage, while others i.e., IL-17 and IL-1β were documented for their role in pneumococcal clearance during invasive disease [467, 530].

The cytokines studied in the context of nasopharyngeal carriage were IL-6, IL-10, INF-γ, IL1β, MIP-2, TNF-α and active TGF-β1 based on their key roles in inflammation, protective immunity and regulation. In our carriage model, IL-6, INF-γ and TNF-α were found to be below detection level in the nasopharynx of mice at all stages of infection with both ST306 and ST615 (Figure 47 A, 47 C and 47 F). The nasopharyngeal concentration of IL-10 ranged between 250 and 300pg/ml throughout the duration of carriage, with no significant differences observed between time points or sequencing types (Figure 47 B).


A
IL-6
ST306
ST615
Concentration (pg/ml)
Days post-infection
B
IL-10
ST306
ST615
Concentration (pg/ml)
Days post-infection


C
INF-γ
ST306
ST615
Concentration (pg/ml)
Days post-infection
D
IL-1β
ST306
ST615
Concentration (pg/ml)
Days post-infection


E
MIP-2
ST306
ST615
Concentration (pg/ml)
Days post-infection
F
TNF-α
ST306
ST615
Concentration (pg/ml)
Days post-infection

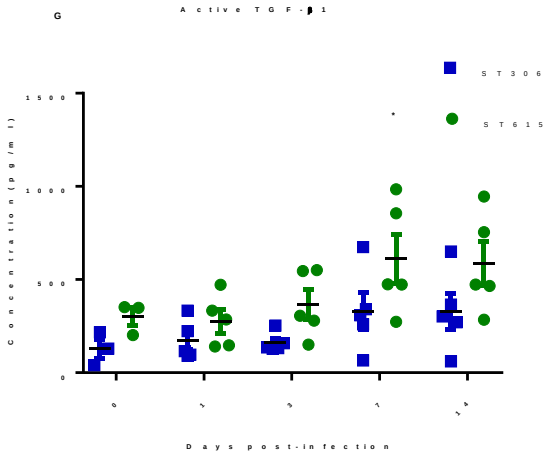


Figure 46. Cytokines analysis of (A) IL-6, (B) IL-10, (C) INF-γ, (D) IL-1β, (E) MIP-2, (F) TNF-α and (G) active TGF-β1 in the nasopharynx of mice infected with ST306 and ST615 during the nasopharyngeal carriage experiment at days 0, 1, 3, 7 and 14 post-infection. Three mice were used for the day 0 control, and five mice were used for the rest of the timepoints. *P-value<0.05 when analysed using a one-way ANOVA

As observed in Figures 47 D, 47 E and 47 G the cytokines analysed (IL-1β , MIP-2 and active TGF-β1) starting at low concentrations at time 0 and either gradually increasing throughout the carriage period to reach its peak on day 14 post-infection (active TGF-β1) or increasing only on day 14 post-infection (IL-1β and MIP-2).

Our group has previously shown, using strain D39 (serotype 2), that, production of TGF-β1 in nasopharyngeal tissue resulted in an elevated concentration of TGF-β1 and recruitment of T regulatory cells which allow carriage to be maintained [467]. Similarly, we found here that the gradual increase, in active TGF-β1 observed in mice carrying ST306 and ST615 occurred concomittantly with an increased pneumococcal density in the nasopharynx.

In the same study that using a D39 strain of serotype 2 it was shown that mice carrying the pneumococcus for a prolonged time had increased levels of IL-10 during the early stages of

carriage [467], while in this study the levels of IL-10 were constant throughout the experimental time. IL-10 is a suppressive cytokine that has anti-inflammatory properties and can be a marker for immunomodulation and tolerance during carriage. Therefore, no variation in the levels of IL-10 in the nasopharynx of mice carrying serotype 1 may maintain the colonisation for ST306.

3.18 Conclusion

Results presented in Figure 16B showed that strains D39 and ST615 release a significantly larger quantity of PLY compared to strains ST304 and ST306. Interestingly, while strain ST304 has almost a two-fold greater of PLY quantity (24.6 ng/ml), compared to B915 (12 ng/ml), percentage hemolysis between the two strains (Figure 16, C) is less than two-fold different. A previous study suggested that the quantity of PLY does not necessarily determine the relative hemolytic activity or lysing ability of a given strain, while presence of mutation in domain 4 residues of PLY resulted in a decrease in hemolytic activity and a reduced affinity for sLeX [530]. The lack of lysing ability of strains ST306 and ST304 observed in (Figure 16, C) suggest that PLY may have critical functions other than that based on its haemolytic activity. Full active pneumolysin is responsible for short duration of colonisation that was obvious in mice infected with ST615. Moreover, PLY is a necessary factor to develop and accelerate diseases.

The study of PLY toxin as a pneumococcal virulence factor has gained importance in the investigation of serotype-independent vaccines to reduce distribution of pneumonia. Previous epidemiological studies [319, 329] have shown various isolates able to express Ply, have low or undetectable hemolytic activity, particularly in serotypes 1, 7F, and 8 strains. While these isolates are capable of causing invasive pneumococcal disease (IPD), it has been suggested that PLY in these isolates may play a minor role in the pathogenesis of these highly invasive isolates

with regard to its hemolytic activity [521, 532]. It is unclear whether reduced hemolytic activity of PLY in strains diminishes its role in IPD. A study has recently shown that non-hemolytic PLY induced a greater pro-inflammatory cytokine response by dendritic cells [532]. Further investigation will be required to determine whether strains with reduced hemolytic ability enable the escape from pneumococcal vaccines with incorporated PLY-specific antigens, or will remain vulnerable to PLY-specific immunization [532].

Previous authors reported [532] that while a reduced hemolytic activity of PLY led to a marginal reduction in virulence, this could also represent a growth advantage in blood during the early stages of infection. This might explain why highly invasive pneumococcal sequence types have expanded despite their apparent lack of PLY haemolytic activity.

Adhesion assays in the presence of Detroit cells showed that D39 (serotype 2) had the highest percentage of adhesion among the tested strains, while ST306 adhered at a higher rate than ST615 or ST304 (Figure 17). All four *S. pneumoniae* strains tested were able to bind to lung epithelial cells *in vitro*. Causing invasive disease depends primarily on the ability of pneumococcus to adhere. It was suggested, that capsule expression is essential for successful colonization of the upper airways [149]. It was also suggested, the present of pili-like on the cell surface of pneumococcus is another important factor to assist the adherence and transmission [149].

Next, we determined that invasion potential was strain specific, with D39 (serotype 2) and ST615 (serotype 1) showing a good ability to invade epithelial cells, while ST306 and ST304 (serotype 1) failed to show any detectable invasive potential (Figure 18). While strain D39 also had quicker adherence kinetic - which may suggest a link with its invasive potential - this was not the case for ST306, which showed higher adherence, but no invasive potential *in vitro*. Pneumococci ST615 showed greater invasive ability compared to all other strains, yet had

relatively low adherence, which may suggest that the adherence ability of strains does not directly influence their invasive potential.

On the basis of these results, the polysaccharide capsule of pneumococcus serotype 1 sequence type inhibit C3b binding, compared to serotype 2 D39, aiding bacterial spread and invasive disease [534]. However, ST615, ST304 and ST306 all had similarly low C3b binding compared to serotype 2 D39, indicating that Ply and its haemolytic activity does not impact on the invasive and adhesion properties of pneumococci. Similarly, the results obtained during the adhesion and invasion assays do not associate with the C3b binding ability of pneumococci.

A mouse model of nasopharyngeal carriage was used in this chapter to illustrate the patterns of nasopharyngeal carriage by serotype 1. The European (ST306) and South America (ST615) strains were used in the *in vivo* nasopharyngeal carriage model to assess the duration and density of carriage of serotype 1, and compare their carriage patterns. However, the two strains followed a different pattern during carriage, the carriage strain ST306 was carried for longer period (Figure 21) suggesting that during colonization, pneumococci serotype 1 may develop a mechanism which allows them to adapt to their new environment and establish colonization. The different behavour in colonisaton may be attributed to evolutionary genetic changes that are influenced by the environment e.g., through single point mutations (SNPs) which may be acquired through random mutations.

These changes can be affected by up-regulation or down-regulation of the expression of critical biological functions or specific virulence genes and is under control tightly regulated networks. Future experiments focused on virulence genes, are required, in order to illustrate whether these mutations exist and whether are permanent or transient.

In the context of a co-infection experiment whereby both ST306 and ST615 were simultaneously administered, it was shown that the pneumococcal density and duration of

carriage of each sequence types was not affected . In other words, the introduction of two sequence types from the same serotype in the nasopharynx of mice did not result to any competition, likely, due to both sequence types sharing the same capsular type.

Overall, we determined that ST306, which express a haemolitically deficient pneumolysin, was able to colonise for a longer period of time than ST615 - which has a fully functional pneumolysin – but these key differences were not reflected in their immunomodulatory properties.

An *in vivo* model of invasive pneumococcal pneumonia was used to study the pathogenicity of ST615 and ST306. Initially, these isolates from Europe and South America, respectively, were used to assess possible differences in virulence. A significant difference in bacterial viable count in blood was observed in the mice infected with ST615 compared to ST306. Mice infected with ST615 developed septicaemia resulting in death of the animals, while the mice infected with ST306 survived, and the mice were healthy and alert.

Flow cytometry analysis was performed to determine the immune cellular profile at different timepoints upon administratin of mice with ST306 or ST615, and cytokines were assayed in order to understand the host immune responses to those sequencing types. The cellular profiling of the lung showed no changes during the timecourse of infection and no differences between ST306 and ST615. Cytokines analysis for IL-1β showed that there was a significant difference between ST306 and ST615.

Additionally, no differences were observed in immune cells between ST306 and ST615 when a model of pneumococcal carriage was studied. However, active TGF-β1 significantly released in mice infected with ST306 on day 7 (* P-value <0.0001).

In a high dose 10^7 administration experiment, ST306 pneumococci were found not able to translocate into the lungs and blood despite the significant dose increase. It remains unclear whether pneumolysin is the only factor involved in these differences observed, and whether others virulence factors have played a role. Interestingly, combining pneumolysin with ST306 followed by intranasal administration led to an enhanced translocation of pneumococci to lungs and blood.

In conclusion, the difference between two sequence types of the same serotype may be due to a differential gene expression patterns rather than the sole expression of haemolitically active pneumolysin. For a future study, researchers should focus on large-scale of virulence factors and investigate if these genes have mutations that will change their functions.

Identification of Orthologous Genes that are Differentially Expressed Between Serotype 1 Strains ST615 and ST306 During In Vitro Growth

4.1 Introduction

A number of studies have investigated the pneumococcal genome and its influence on virulence [536, 537, 538], however while genomic variability between serotypes has been the main focus, there is still the question of how changes in gene expression contribute to pathogenicity particularly when pneumococci translocate from one tissue niche to another [472]. Transcriptomics can be used to compare closely related bacterial species and subtypes. Traditionally, transcriptomics analysis focuses on virulence and metabolic genes and their levels of expression in strains sharing the same virulence and metabolic genes [537, 538, 540]. Currently, little is known about the gene expression of serotype 1, particularly those of the ST306 isolate. The purpose of this study was to compare the transcriptional profiles of two pneumococcal serotype 1 strains B915 (ST306) and NCTC7465 (ST615) to identify differences in the level of expression of known virulence factors.

Most gene expression analyses have largely consisted of real-time PCR assays or DNA microarrays. However, Sirbu *et al*. and Hurd have shown that there are significant limitations associated with microarray technology, particularly since they require the synthesis of specific DNA probes complementary of known genes, with the potential for cross-hybridisation between similar sequences, and background noise interference [541, 542]. RNA-Seq is a recently developed approach to transcriptome profiling that uses deep-sequencing technologies, and allows determination of gene expression levels [541]. A notable advantage of RNA-seq is that it allows for the investigation of non-annotated genes, and is more sensitive than microarray and qRT-PCR in terms of limit of detection, allowing the detection of

transcripts at lower levels with the absence of background noise [541, 542]. In this project, RNA-seq was used in order to determine differences in gene expression levels between serotype 1 strains ST615 and ST306 during five key stages of in vitro growth.

4.2 Gene Expression During Bacterial Growth Phases

Successful infection, survival and persistence in the mammalian host by intracellular pathogens requires rapid adjustment to changing environmental conditions during the passage through the nasopharynx and penetrating into host target cells [463]. Numerous virulence genes, their products, and their coordinated expression, play a key role in allowing the bacteria to invade and colonise host cells by adapting between the extracellular and intracellular phases of infection, to overcome antimicrobial and physical barriers to infections, and to manipulate and modify the actions of host cells [478]. For example, certain bacterial gene products may play a major role in mediating environmental signals involved in the regulation of both the extracellular and intracellular virulence gene products for adaptation to altered growth conditions [543]. Other gene products may play a role in the response to stress and nutrient limitations, such as amino acid starvation, acidic pH, intracellular proliferation in cells such as macrophages during aerobic, late log phase growth conditions, or dependence on the invasive gene expression under oxygen limited conditions [472].

In the past, gene expression profiling has become an important tool for the investigation of bacterial adaptation to infection in host tissue niches. investigation of bacterial adaptation to host tissue niches. The investigation of gene expression in response to certain stressors in vitro, besides animal models, have been useful to gain insight into the nature of the genes involved in the intracellular life of bacteria.

A previous study using microarrays to investigate the gene expression profiles of R6x pneumococcal strain showed that *nanB* was up-regulated during the stationary phase compared

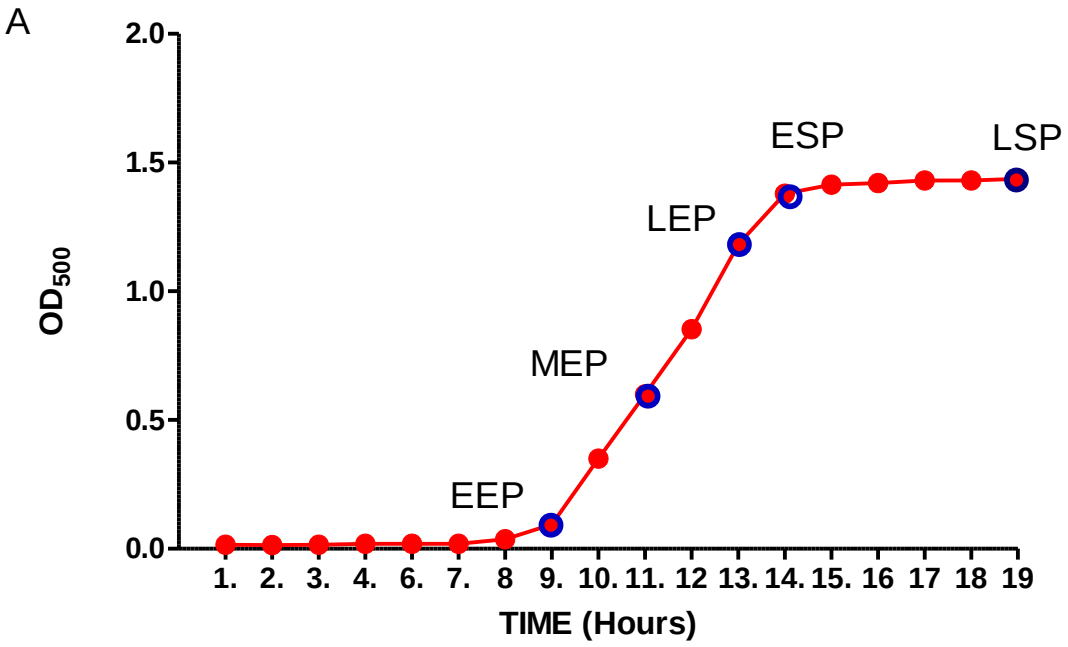
to exponential phase, while *cpsA* was up-regulated during exponential phase compared to stationary phase [576].

4.3 In Vitro Growth Phases

We focused on the five distinct phases of bacterial growth in this study: early exponential phase (EEP), mid-exponential phase (MEP), late exponential phase (LEP), early stationary phase (ESP) and late stationary phase (LSP). The exponential phases describe the period of cell division. The exponential growth of bacteria requires there to be an abundant source of carbon, nitrogen, phosphate, and certain trace elements such as iron, in the growth medium, and involves bacterial growth and replication including multiple rounds of DNA synthesis, along with transcription and translation in order to synthesis the necessary macromolecules. This process is controlled by a number of gene regulatory processes. It is thought that both the accumulation of acetate and carbon starvation in the growth rich medium of LB broth leads to bacteria entering the stationary phase. [471]. The stationary phases begin when environmental conditions become unfavourable for growth of bacteria due to a reduction of nutrient availability and accumulation of toxic metabolites, eventually leading to decreased bacterial metabolic rate. During the late stationary phase, metabolic activity stops, causing the halting of replication and activation of resistance physiology [471].

Previous studies have shown that specific virulence genes were expressed in response to a number of extra- and/or intracellular signals, and that overlapping regulatory networks modulating the expression of these virulence genes had evolved in bacteria to aid its adaptation to different environments and survival [472]. Most interestingly, virulence genes were shown to present growth-phase specific expression profiles in bacterial species such as Salmonella [472]. We thus sought to examine the growth-phase specific gene expression of pneumococcal

serotype 1. The reference strain of serotype 1 *S. pneumoniae* ST615 (NCTC7465), deposited in the National Collection of Type Cultures, London, United Kingdom, in 1948, was used to compare gene expression [257]. The five growth phases for ST306 and ST615 are clearly distinguishable in the growth curve presented in Figure 48. For ST306, the RNA was extracted when bacterial cultures reached the following OD500 values: 0.094 (EEP), 0.57 (MEP), 1.182 (LEP), 1.379(ESP) and the late stationary phase (LEP) extracted 5 hours after ESP extraction. For ST615 the RNA was extracted when bacterial cultures reached the following OD500 values: 0.083 (EEP), 0.067 (MEP), 1.167 (LEP), 1.266 (ESP) and the late stationary phase (LEP) were extracted 5 hours after ESP extraction. The generation time for ST306 and ST615 was already mentioned in the results chapter.



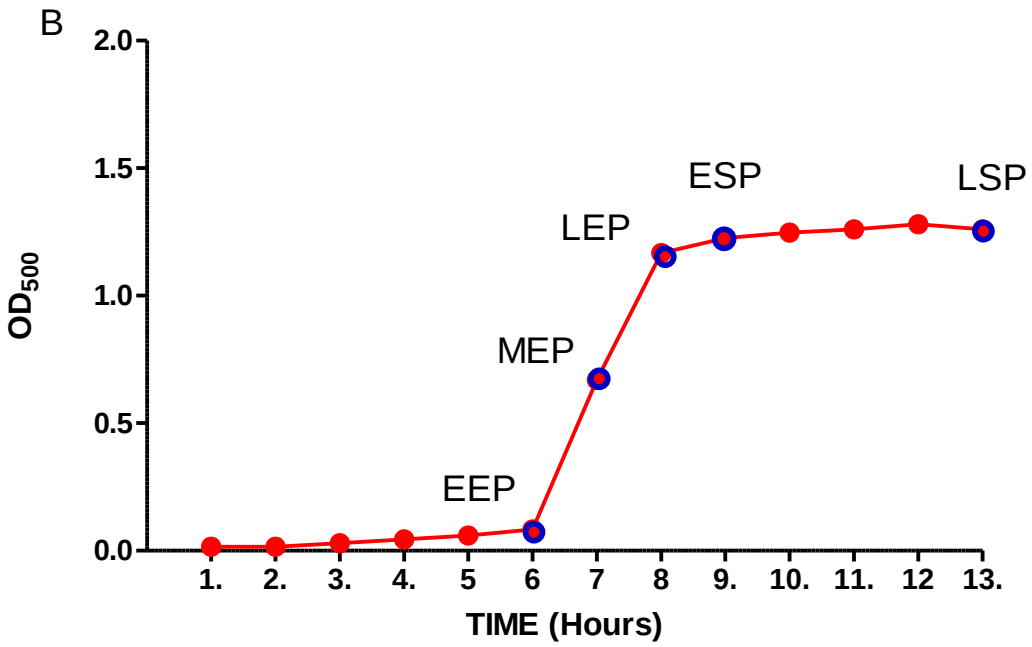


Figure 47. Growth curve of the serotype 1 ST306 (A) and ST615 (B) in BHI broth (OD_{500})

In Figure 49 is presented the pairwise comparison of the expression of orthologous genes between ST615 and ST306. The genes down-regulated in ST615 compared to ST306 are shown in blue, while the up-regulated genes are shown in red. Genes that were not differentially expressed between ST615 and ST306 are shown in white.

It is commonly accepted that a minimum sequencing depth of 5 to 10 million non-rRNA fragments is required to obtain representive bacterial transcriptome profiling [547]. The sequencing depth of the ST306 and ST615 samples was determined sufficient to generate optimal analysis of the transcriptomes, as the total number of read sequenced was higher than 5 million. The percentage of uniquely mapped reads was higher than 78%, these were read

and mapped to only one location in the genome. In this project, the transcriptome analysis was done with uniquely mapped reads to collect high-quality gene expression data.

Sequence type	Growth phase	Total no. of reads sequenced	Number of uniquely mapped reads	Number of multi-mapped reads	Number of un-mapped reads	% of uniquely mapped reads	% of multi-mapped reads	% of un-mapped reads
ST615	EEP	26,677,691	24,612,526	551,317	1,513,848	92.3	2.1	5.7
ST615	MEP	33,095,128	28,679,609	1,557,993	2,857,526	86.7	4.7	8.6
ST615	LEP	31,063,468	25,268,535	1,594,668	4,200,265	81.3	5.1	13.5
ST615	ESP	37,829,212	29,943,208	3,105,348	4,780,656	79.2	8.2	12.6
ST615	LSP	30,376,684	27,760,276	1,079,506	1,536,902	91.4	3.6	5.1
ST306	EEP	34,532,259	26,016,344	4,086,641	4,429,274	75.3	11.8	12.8
ST306	MEP	26,939,069	22,395,056	160,807	4,383,206	83.1	0.6	16.3
ST306	LEP	33,744,254	28,423,272	194,492	5,126,490	84.2	0.6	15.2
ST306	ESP	30,817,637	27,956,593	181,680	2,679,364	90.7	0.6	8.7
ST306	LSP	34,738,001	30,892,965	244,768	3,600,268	88.9	0.7	10.4

Table 3. Uniquely mapped reads are defined as "reads mapped to a single genomic location", and so do not include reads that map to paralogous genes, including most rRNA genes

4.4 Gene Expression of ST615 and ST306 at Different Points of Growth

The aim of this study is to examine the differentially expressed genes between two strains, ST615 and ST306, both of which belong to pneumococcal serotype 1, in the hope of explaining the differential properties described in Chapter 3. A pair-wise comparison of the expression of 250 virulence genes with well-known functions was carrie out by normalising the expression of ST306 in each growth phase and compared with the expression of ST615 in the same growth point (Figure 48). The data was filtered by considering TPM (transcript per million) values greater than 10, and fold-change greater than 2. It has been suggested that genes would be expressed only when TPM values are greater than 10. A TPM value of 10 was used as a threshold to define gene expression in *S. pneumoniae*, and is a conservative estimate based on the analysis of Wagner *et al* 1994 [561].

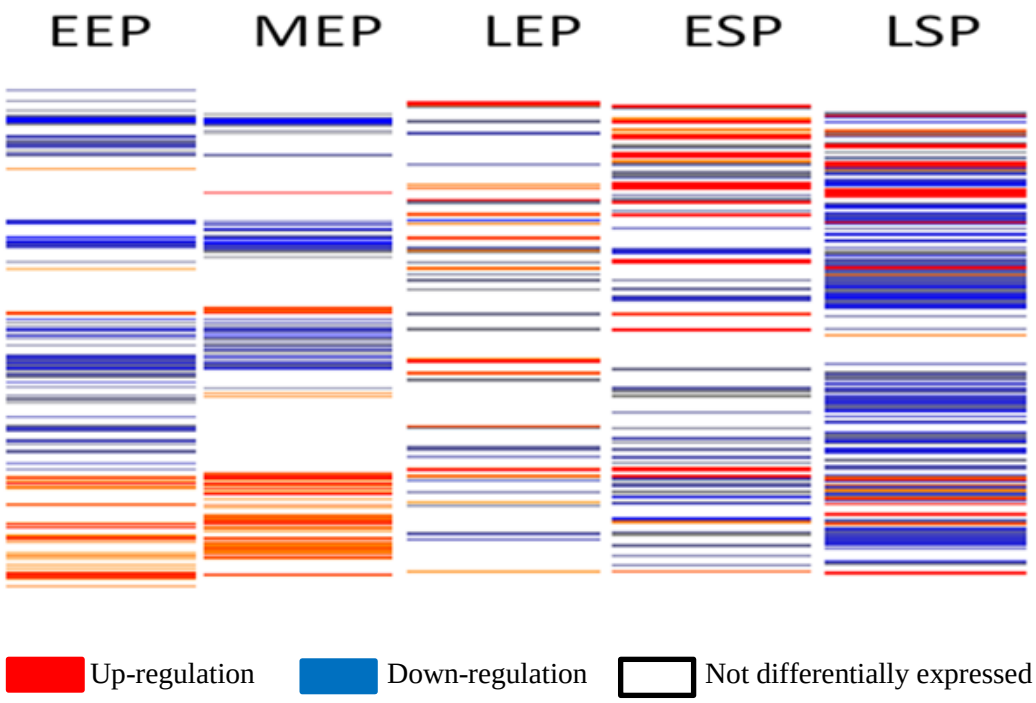


Figure 48. Pair-wise comparison of ST615 to ST306 gene expression at different time points (EEP: Early Exponential Phase, MEP: Mid Exponential Phase, LEP: Late Exponential Phase, ESP: Early Stationary Phase, LSP: Late Stationary Phase). The 250 virulence associated genes included in this analysis. TPM > 10 and > 2 fold-change. Down-regulated genes in ST615 when compared to ST306 are shown in blue, up-regulated genes ST615 when compared to ST306 are shown in red, and not differentially expressed genes between the two strains are shown in white

Orthologue genes were identified with BLASTP and the number of virulence factors were suggested to be 250 genes. As shown in Figure 49, the pair-wise comparison of the expression of 250 virulence genes between ST615 and ST306 showed similar levels of gene expression between the early and mid exponential phases, while the late exponential, early and late stationary phases had a different pattern of expression.

Growth phases	No. of genes expressed (TPM >10)	UP- Regulated genes % ST615 vs ST306	Down Regulated genes % ST615 vs ST306	Total No. of genes differentially expressed
EEP	83	10 (12.0 %)	63 (75.9 %)	73 (87.9 %)
MEP	52	16 (30.7 %)	25 (48.0 %)	41 (78.8 %)
LEP	49	21 (42.8 %)	18 (36.7 %)	39 (79.5 %)
ESP	81	30 (37.0 %)	34 (41.9 %)	64 (79.0 %)
LSP	150	30 (20%)	102 (68 %)	132 (88%)

Table 4. Number and percentage of 250 virulence genes differentially expressed in ST615 during five growth phases when compared to ST306. The percentage of differentially expressed genes is shown in parenthesis

As shown in Table 5, between (41) 78% and (132) 88% of the 250 virulence genes were differentially expressed in ST615 when compared to ST306, with a higher proportion of those being down-regulated than up-regulated.

4.5 Genes Classification Depened on their Functions During the Five Phases of Growth

4.5.1 Selection of Functional Categories

In this study, KEGG (Kyoto Encyclopedia of Gene and Genomes) was used. KEGG is a bioinformatic resource that links contemporary knowledge on molecular interaction networks such as a pathway or a complex. This resource is able to predict the cellular process and

organism behaviour by classifying genes into different functional categories. The analysis of functional categories assists for studying cellular processes that are critical for the pathogens to survive [549].

The biological functional category analysis of the two pneumococcal strains of serotype 1 was run by using information acquired from the KEGG database using DAVID Bioinformatics Resources 6.7 (https://david.ncifcrf.gov/). There was an exclusion of the hypothetical genes from the biological functional category analysis that resulted in the decrease the number of orthologue genes analysed to 250. A total of 6 biological functional categories were constructed by KEGG.

The number of orthologous genes included in the biological functional categories obtained from the KEGG database is 250 genes, representing 12% of the whole genome used in the pair-wise comparison of ST615 and ST306 (Figure 49). This focuses deeply on the difference between the two strains from the same serotype and compares them in the aspect of vital biological needs.

In this study, six biological functional categories were analysed for broad investigation, dependent on the role in the cell activities and virulence. However, the TPM values of up-regulated and down-regulated genes for all six biological functional categories for the five growth phases are shown in Appendix 2.

The category of the biological functional genes are shown in the table below. The selection was based on the investigation for critical genes that are involved in fitness and virulence of pneumococci.

Biological functional categories	Genes
Cell wall biosynthesis	*bacA*; *fibA*; *lytB*; *licC: licB: pck*; *pgdA: penA*; *dltD*; *dltA: pgm*; *cbf1*
Transport & binding protein	*malD:malC:malX:pstB:pstA:pstC:pstS:cbiO:cbio1:adcC:adcA:adcA:rafE: rafF:rafG:amiA:amiC:amiD:amiE:amiF:aliA:aliB:potA:potC:potD:ftsX:ftsE:livF: livG:livM:livH:livJ*
Protein secretion	*blpU: pspA: cbpJ: cbpG: cbpF: blpK: blpY: prtA: spxB: lmb: ply: lytA: chpA: chpD: pspaA:spoJ*
DNA replication	*recA: cinA: dprA: hexB: radC: recR: recG: DivlVA*
RNA synthesis	*prsA: marR: serS: glyO: fmt: prmA: tgt*
Metabolism	*manA: lacR: glgC: eno: lacB: ldh: nagB: atpH: galK: malA*

Table 5. Biological functional categories with genes list of orthologous genes between ST615 and ST306.

The KEGG is used to analyse few number of genes that were mentioned in the table 6. However, the genes present in this table are not the only genes involved in these categories. There are more genes not identified by the KEGG database. Because of this issue, this study was focused only on the genes in the table. Figure 50 illustrates the percentage of the five points of growth phases of differentially expressed genes for each biological functional category. In addition, each biological functional category was analysed in detail.

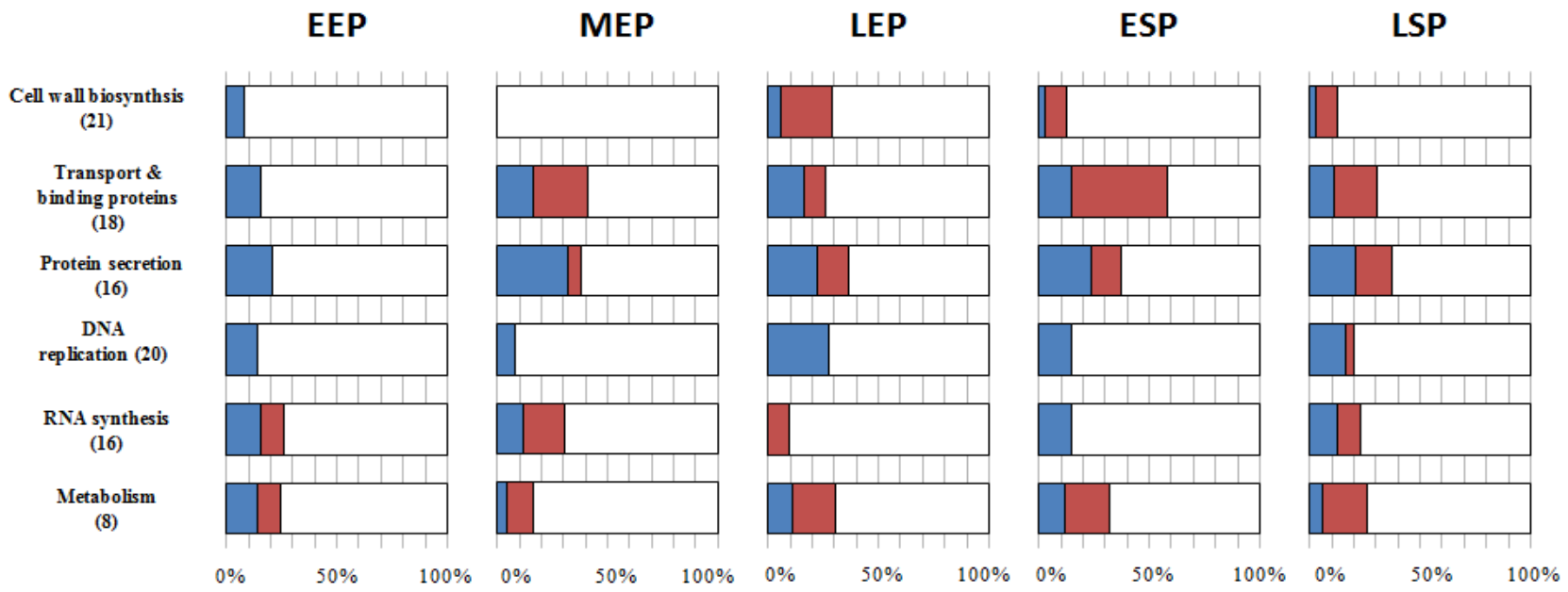


Figure 49. Pair-wise comparison of ST615 to ST306 of biological functional genes analysed at different growth phases

4.6 Biological Functional Category

4.6.1 Cell Wall Biosynthesis

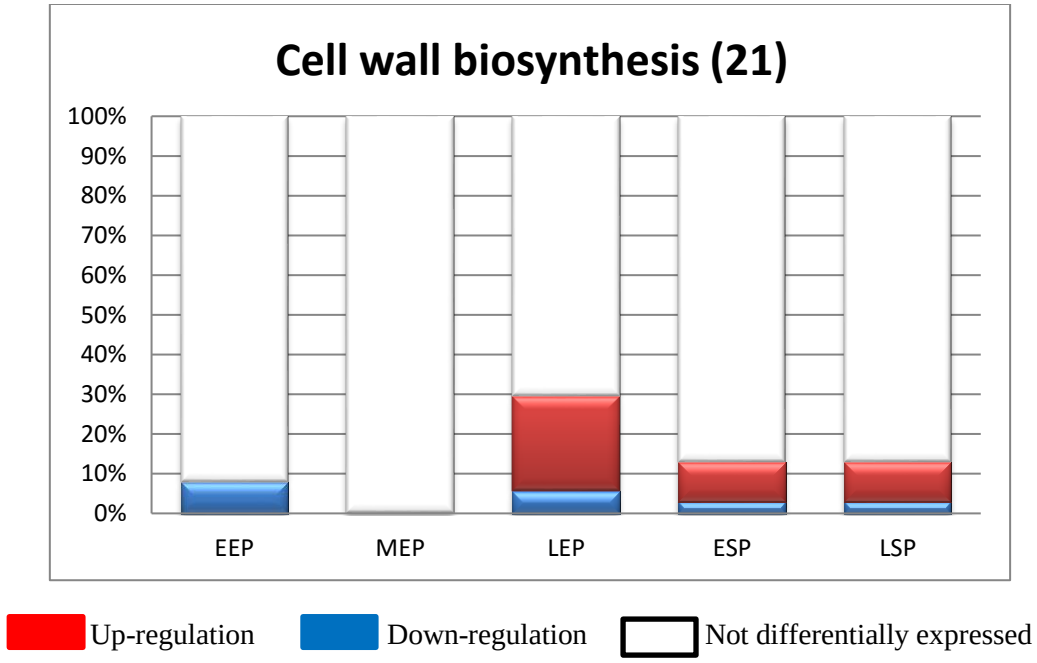


Figure 50. The percentage of pair-wise comparison of ST615 to ST306 between cell wall biosynthesis genes at different time points (EEP: Early Exponential Phase, MEP: Mid Exponential Phase, LEP: Late Exponential Phase, ESP: Early Stationary Phase, LSP: Late Stationary Phase). Down-regulated genes in ST615 when compared to ST306 are shown in blue, up-regulated genes ST615 when compared to ST306 are shown in red, and not differentially expressed genes between the two strains are shown in white. The total number of genes involved in the cell wall biosynthesis was 21 genes.

The cell wall is important for stress-bearing and shape-maintaining in bacteria, and its integrity is of critical importance to cell viability. The cell wall consists of the cross-linked polymer peptidoglycan (PG). Many studies have explained the relationship between PG synthesis and bacterial growth and cell shape by focusing at changes in cell shape in mutants that lack one or

several enzymes involved in the synthesis of PG or other cell wall components or by looking at the incorporation of labeled PG precursors into the cell wall [551]. In Gram-positive bacteria, teichoic acids (TAs) are essential and can be divided, according to their anchors, into cell wall covalently attached TA (WTA) and membrane-anchored lipoteichoic acid (LTA) [551].

As an important element in the biology of *S. pneumoniae*, the phosphorylcholine (*P*-Cho) moiety is unique to pneumococcal TAs, imparting anchors for choline-binding proteins (CBPs). The CBP family consists of several members, including LytA, -B, and -C (three autolysins); CbpA (an adhesin); and PspA (a protective antigen formerly named CbpC). In addition, specific binding of *P*-Cho to the receptor of platelet-activating factor (rPAF) has been found to contribute to pneumococcal adherence and invasion [551].

Rod-shaped bacteria always divide through the same medial plane and have two modes of cell wall synthesis [551]: the first one is responsible for the elongation of the cell and the second one is responsible for the formation of the division septum. The two modes of synthesis seem to be catalyzed by different protein complexes. *S. pneumoniae* cells are not "true" cocci, as their shape is not completely round, but instead have the shape of a rugby ball and synthesize the cell wall not only at the septum but also at the so called "equatorial rings". These differences in sites for cell wall synthesis and the mode of division reflect some of the diversity existing in bacteria - a basic aspect of bacterial cell biology.

The recent bioinformatic study found evidence for 16 genes and other hypothetical genes, primarily clustered in the *lic* locus, that were involved in the biosynthesis of pneumococcal TAs and cell membrane [552]. Penicillin-binding proteins are encoded by four genes, *pbp1a, pbp2a, pbp2x* and *pbp2b* [552]. These genes have different functions - *pbp1a* and *pbp2x* are responsible for cell division, while *pbp2a* and *pbp2b* are involved in cell wall synthesis.

Moreover, there are some enzymes are essential for cell wall synthesis, like DD-carboxypeptidase (*dacA)* which regulates cross-linking degree and coordinates of the division process, and monofunctional glycosyltransferases (Mgt) are able to catalyse only the formation of uncross-linked peptidoglycan [551].

As shown in Figure 51, cell wall biosynthesis genes in ST615 were up-regulated compared to ST306 strain especially at late exponential phase. However, these genes in ST615 were down-regulated compared to ST306 at early exponential phase.

4.6.2 Transport & Binding Protein

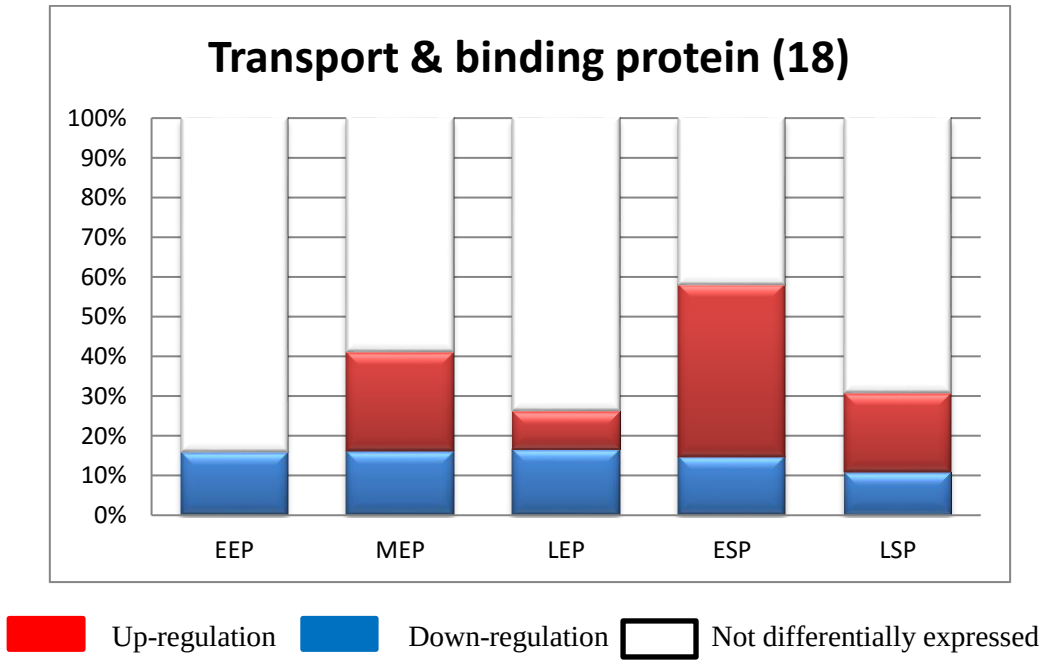


Figure 51. The percentage of pair-wise comparison of ST615 to ST306 between transport & binding protein genes at different time points (EEP: Early Exponential Phase, MEP: Mid Exponential Phase, LEP: Late Exponential Phase, ESP: Early Stationary Phase, LSP: Late Stationary Phase). Down-regulated genes in ST615 when compared to ST306 are shown in blue, up-regulated genes ST615 when compared to ST306 are shown in red, and not differentially expressed genes between the two strains are shown in white. The total number of genes involved in the transport & binding protein was 18 genes.

Few molecules are able to enter or leave cells, or cross organellar membranes, unaided by proteins. Even the transport of molecules, such as water and urea, which can diffuse across pure phospholipid bilayers, is often facilitated by transport proteins. All are integral transmembrane proteins and display a high degree of specificity for the substance transported. There are three major types of transport proteins. The first is uniporters that transport one molecule at a time down a concentration gradient - for example, moves glucose or amino acids across the plasma membrane. In contrast, the second and third are antiporters and symporters, which are responsible for the movement of one type of ion or molecule against its concentration gradient to the movement of a different ion or molecule down its concentration gradient [553].

In terms of transport proteins, polyamine transport in bacteria typically associates with the action of ABC (ATP-binding cassette) transporters or antiporters that are selective for specific molecules. The important role of polyamine is protecting the cell from physiological stress and modulating the activity of porins, transmembrane channels that allow the diffusion of hydrophilic compounds across the outer membrane. However, polyamine synthesis and the molecular function of these molecules in the pneumococcus has still unknown [554]. Moreover, *psaB* and *psaC* are involved in manganese ABC transporter, ATP-binding protein, while *psaA* is involved in manganese-binding adhesion lipoprotein. The main function of *adcC* is zinc ABC transpoter and ATP- binding protein. The genes are responsible for sugar and maltodextrin transporters are *msmE* and *malD*, respectively [543].

In Figure 52, the transport and binding protein genes in ST615 strain were up-regulated compared to ST306 at early stationary phase. There were no differences in expression of transport and binding protein genes in ST306 strain at all growth phases.

4.6.3 Protein Secretion

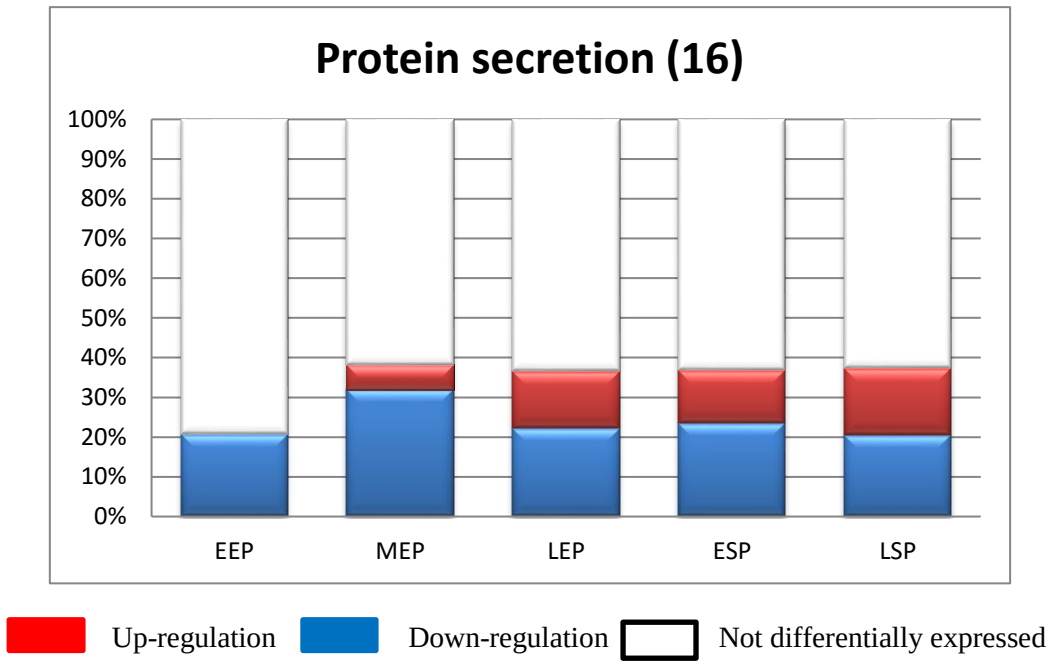


Figure 52. The percentage of pair-wise comparison of ST615 to ST306 between protein secretion genes at different time points (EEP: Early Exponential Phase, MEP: Mid Exponential Phase, LEP: Late Exponential Phase, ESP: Early Stationary Phase, LSP: Late Stationary Phase). Down-regulated genes in ST615 when compared to ST306 are shown in blue, up-regulated genes ST615 when compared to ST306 are shown in red, and not differentially expressed genes between the two strains are shown in white. The total number of genes involved in the protein secretion was 16 genes.

Protein secretion plays a key role in modulating the interactions of bacteria with their environments [555]. This is the main case when symbiotic bacteria (whether pathogenic, commensal or mutualistic) interact with larger host organisms. Large numbers of proteins are secreted by pathogens and other symbionts are able to enter inside host cells to adjust host physiology to promote colonization (toxins and effector proteins) [555].

There are seven types of secretion systems used by bacteria that depend on bacteria category. The major differences between gram-positive and gram-negative bacteria are the thickness of

cell wall and the number of membrane surrounding the cell. The genomes of gram-positive species encode a family of specialized secretion systems generally called type VII section systems (T7SS). Recently, a study has identified genes for membrane proteins with abortive-infectivity domains, designated *spdABC* (surface protein display) that are required for the trafficking of YSIRK/GS proteins into the cross wall compartment [556].

With regard to secreted proteins, Autolysin (LytA) is an important protein to lyse the cell and release other virulence factors such as pneumlysin. Moreover, LytA mediates lysis to release proteins participated in immune evasion or cell wall components that may interfere with the host immune response [557]. Pneumolysin (Ply) is intracellular protein which exerts its impact when released in the environment. Ply causes death to wide range of cells and is responsible for immunomodulatory effects [248]. Sortase A (SrtA) is a membrane-anchored transpeptidase expressed by gram-positive bacteria. SrtA enzymes are responsible for the covalent attachment of specific proteins to the cell wall and are crucial for adherence and pathogenicity for pneumococcal [558].

In Figure 53, protein secretion genes in ST306 strain were up-regulated at all growth phases particularly in mid exponential phase, while these genes in the ST615 strain were up-regulated in late stationary phase.

4.6.4 DNA Replication

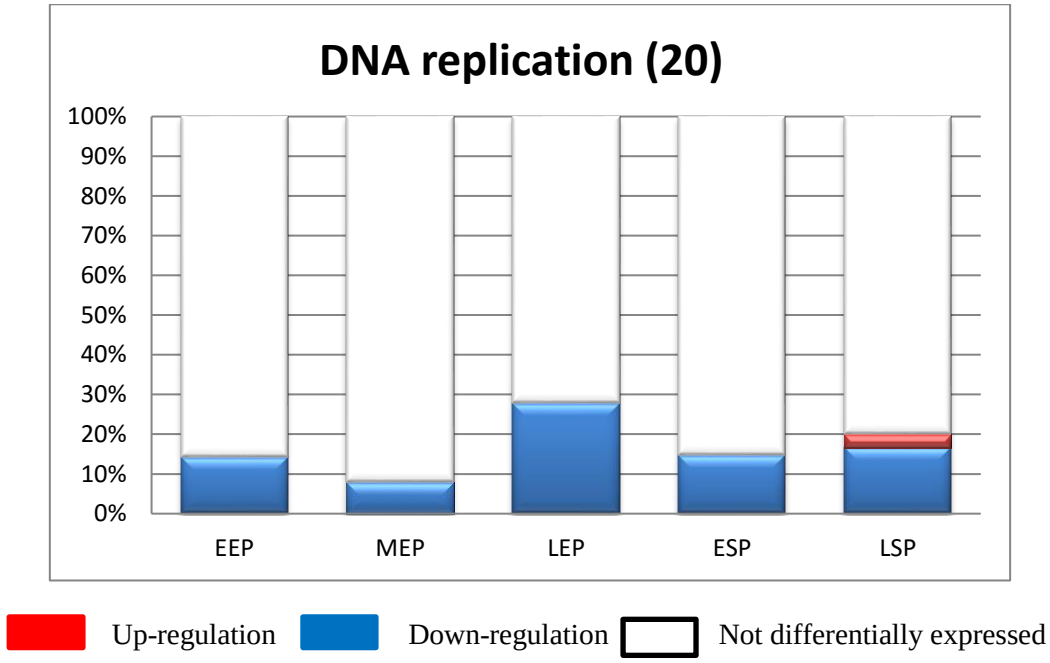


Figure 53. The percentage of pair-wise comparison of ST615 to ST306 between DNA replication genes at different time points (EEP: Early Exponential Phase, MEP: Mid Exponential Phase, LEP: Late Exponential Phase, ESP: Early Stationary Phase, LSP: Late Stationary Phase). Down-regulated genes in ST615 when compared to ST306 are shown in blue, up-regulated genes ST615 when compared to ST306 are shown in red, and not differentially expressed genes between the two strains are shown in white. The total number of genes involved in the DNA replication was 20 genes.

The genetic information within the cells is stored in the double helix of DNA that is able to replicate to produce a new cell. The process of DNA replication is beginning by special initiator proteins that bind to double-stranded DNA and separate the two strands apart, breaking the hydrogen bonds between the bases. The replication origin (*ori*) contains two binding boxes for host-encoded proteins, IHF and DnaA, respectively, separated by an AT-rich region, followed by three repeats region DR-1, DR-2 and DR-3. Moreover, *ori* is a critical region due to its ability to enhance or repress the replication [559].

There are several proteins required for DNA replication. These include DNA polymerase, single-strand DNA binding proteins, helicases, primase, topoisomerases, polymerases and others.

Helicase cleaves the hydrogen bonds that linking the pairs together; DnaB is a principal helicase in DNA replication. Single-stranded DNA binding protein (SSB) binds to the single-stranded of each DNA strand to prevent the strands from reassociating and to protect them from denaturation by nucleases. Primase is another enzyme that copies a DNA template strand by generating an RNA strand complementary to that DNA template. DNA polymerase I is involved in removing RNA primers in the processing of DNA after replication, whereas DNA polymerase III initiates synthesizing DNA from 5' to 3' direction, beginning at 3' end of each RNA primer [477].

In Figure 54, DNA replication genes in ST615 strain were down-regulated compared to ST306 strain. However, the expression of DNA replication genes in ST615 were up-regulated compared to ST306 in the late stationary phase.

4.6.5 RNA

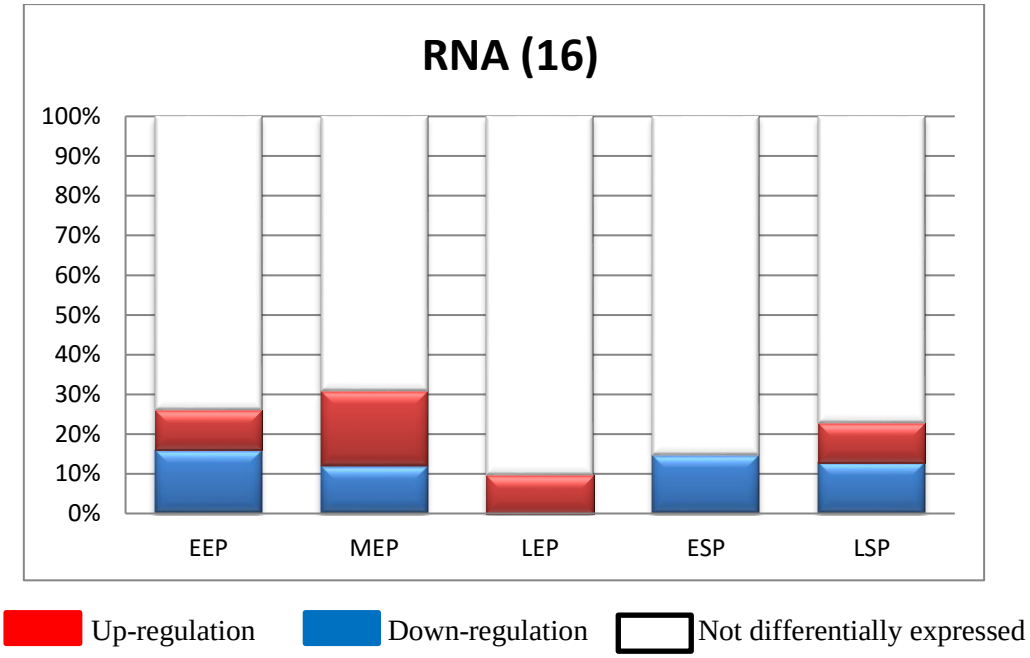


Figure 54. The percentage of pairwise comparison of ST615 to ST306 between RNA genes at different time points (EEP: Early Exponential Phase, MEP: Mid Exponential Phase, LEP: Late Exponential Phase, ESP: Early Stationary Phase, LSP: Late Stationary Phase). Down-regulated genes in ST615 when compared to ST306 are shown in blue, up-regulated genes ST615 when compared to ST306 are shown in red, and not differentially expressed genes between the two strains are shown in white. The total number of genes involved in the RNA was 16 genes.

There are three major classes of RNA - mRNA, tRNA and rRNA - that are synthesised by a single DNA-dependent RNA polymerase in prokaryotic [552]. A small number of additional RNA molecules have been identified producing in the cells, known as small RNA (sRNA). Small RNA has several functions, including splicing, and is also required for the subcellular localization and extracellular transport proteins [560].

In order to synthesise RNA, there are three main steps: specific initiation at a promoter, elongation and termination at a specific site. RNA polymerase must locate and recognise the promoter signal on DNA double strand, denature the double helix at the start region (formation

of open complex). The polymerase has to stay tightly bound to the DNA in the ternary transcription complex to synthesise mRNA. This enzyme must recognise a termination signal to release the RNA and to start generating other new RNA [561].

The genes involved in transcription and regulation are: *ciaR* DNA-binding response regulator, *vncS* sensor histidine kinase, *prsA* ribose-phosphate pyrophosphokinase, ATP-dependent RNA helicase, ABC transporter-ATP binding protein and *marR* transcriptional regulator. In addition, the translation genes are: all ribosomal proteins, *serS* seryl-tRNA synthetase, *glyO* glyl-tRNA synthetase, *fmt* methionyl-tRNA formyltransferase, *prmA* ribosomal protein methyltransferase and *tgt* tRNA- ribosyltransferase.

In Figure 55, RNA genes in ST615 strain were up-regulated compared to ST306 strain at mid exponential phase and were down-regulated at early stationary phase. However, these genes in the ST615 strain were down-regulated compared to ST306 at early stationary phase and up-regulated in the late exponential phase.

4.6.6 Metabolism

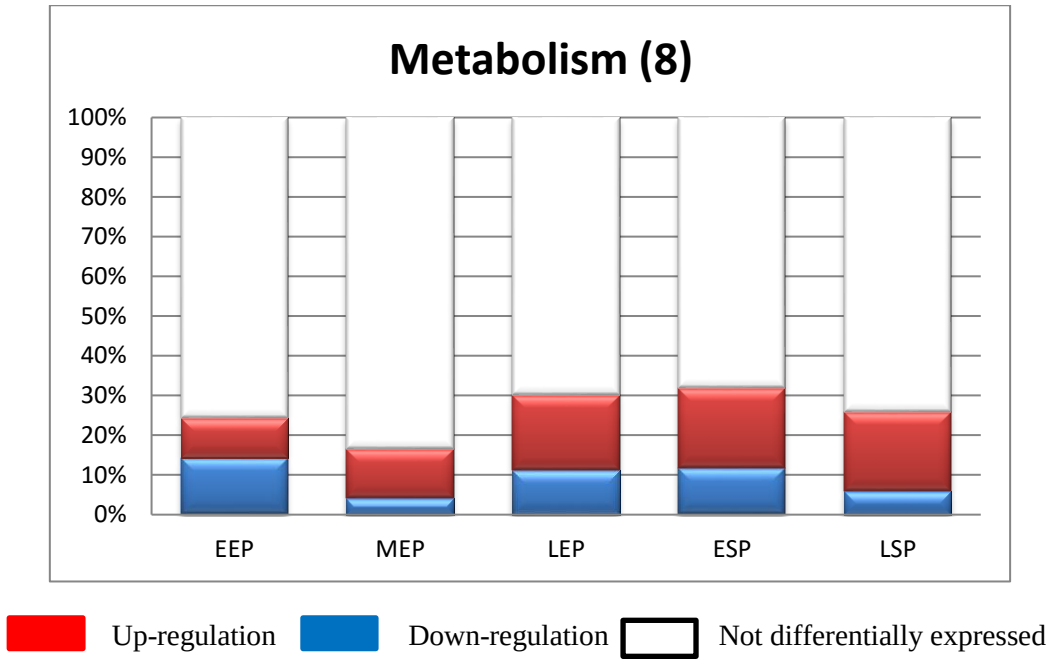


Figure 55. The percentage of pairwise comparison of ST615 to ST306 between metabolic genes at different time points (EEP: Early Exponential Phase, MEP: Mid Exponential Phase, LEP: Late Exponential Phase, ESP: Early Stationary Phase, LSP: Late Stationary Phase). Down-regulated genes in ST615 when compared to ST306 are shown in blue, up-regulated genes ST615 when compared to ST306 are shown in red, and not differentially expressed genes between the two strains are shown in white. The total number of genes involved in the metabolism was 8 genes.

Metabolism refers to all the biochemical reactions that occur in a cell. The study of bacterial metabolism concentrates on the chemical diversity of substrate oxidations and dissimilation reactions (reactions by which substrate molecules are broken down), which typically function in bacteria to generate energy [562].

The bacterial metabolism studies also focus on the uptake and utilization of the inorganic or organic compounds required for growth and maintenance of a cellular steady state. These respective endergonic (energy-requiring) and exergonic (energy-yielding) reactions are then catalyzed within the living bacterial cell by integrated enzyme systems, the end result being

self-replication of the cell. The microbial cells are capable to live, function, and replicate in an appropriate chemical milieu and the chemical changes rely on bacterial metabolism [562].

In terms of pneumococcal metabolism, the efficient utilisation of nutrients in order to generation of metabolic energy is crucial for these bacteria to survive *in vivo*. Many studies have shown central metabolic pathways in pneumococcal virulence. For instance, NADH oxidase, which reoxidizes NADH and decreases molecular oxygen to water, was observed to be required for the development of competence for genetic transformation and for virulence and surviving in mice. The other protein deeply studied that links pneumococcal central metabolism to virulence is the glycolytic enzyme α-enolase, which is responsible for binding to plasminogen [563].

The metabolic genes that were analysed were: *manA* mannose-6-phosphate isomerase, *lacR* lactose system repressor, *glgC* glucose-1-phosphate edenylyltransferase, *eno* enolase, *lacB* galactose-6-phosphate isomerase, *ldh* L-lactate dehydrogenase, *nagB* glucosamine-6-phosphate isomerase, *atpH* ATP synthase F1, delta subunit, *galK* galactokinase, *malA* maltose metabolism.

In Figure 56, this set of metabolic genes in the ST615 strain were up-regulated compared to ST306 strain at early exponential phase and the expression pattern was stable in late exponential and early stationary phase. Overall, metabolic genes in ST615 strain were up-regulated at all growth phases, regardless to the percentage of expression.

Gene name	ST306 Identifier	ST615 Identifier	Gene product
bgaA	SPZ_01881	SPE_00804	Beta-galactosidase
cbpA	SPZ_00952	SPE_01492	Choline binding protein A
cbpC	SPZ_01637	SPE_01724	Choline binding protein C
cbpD	SPZ_00940	SPE_01502	Choline binding protein D
cbpE	SPZ_02187	SPE_00629	Choline binding protein E
cbpF	SPZ_01653	SPE_01766	Choline binding protein F
cbpG	SPZ_01652	SPE_01765	Choline binding protein G
cps2A	SPZ_01606	SPE_02053	Integral membrane regulatory protein
cps2B	SPZ_01607	SPE_02054	Tyrosine-protein phosphatase
eno	SPZ_00106	SPE_00808	Binds to plasminogen/phosphopyruvate hydratase
hyl	SPZ_01559	SPE_00265	Breaks down hyaluronan-containing extracellular matrix components
igA	SPZ_00128	SPE_01659	Cleaves human IgA1
lytA	SPZ_01233	SPE_01854	Autolysin - digests the cell wall
nanA	SPZ_00639	SPE_01261	Sialidase A
nanB	SPZ_00634	SPE_01256	Sialidase B
pavA	SPZ_02221	SPE_00661	Binds to fibronectin /adherence and virulence protein A
pcpA	SPZ_01005	SPE_01471	Choline binding protein PcpA
ply	SPZ_01247	SPE_01841	Pneumolysin
psaA	SPZ_00597	SPE_01219	Component of the ABC transport system
psaB	SPZ_00595	SPE_01217	Manganese ABC transporter ATP-binding protein
psaC	SPZ_00596	SPE_01218	Manganese ABC transporter permease
pspA	SPZ_01373	SPE_00088	Surface protein - prevents binding of C3 onto pneumococcal surface
spxA	SPZ_01444	SPE_00151	Transcriptional regulator Spx
spxB	SPZ_01962	SPE_02056	Pyruvate oxidase
srtA	SPZ_00187	SPE_00844	Sortase
strH	SPZ_01314	SPE_00029	Beta-N-acetylhexosaminidase - adherence

Table 6. The list of well-charactrised genes selected from 250 virulence factors

4.7 Differential Expression of the Virulence Factors Genes Between ST615 and ST306

The level of expression for each virulence factors at different points of growth phases is presented in Figure 57.

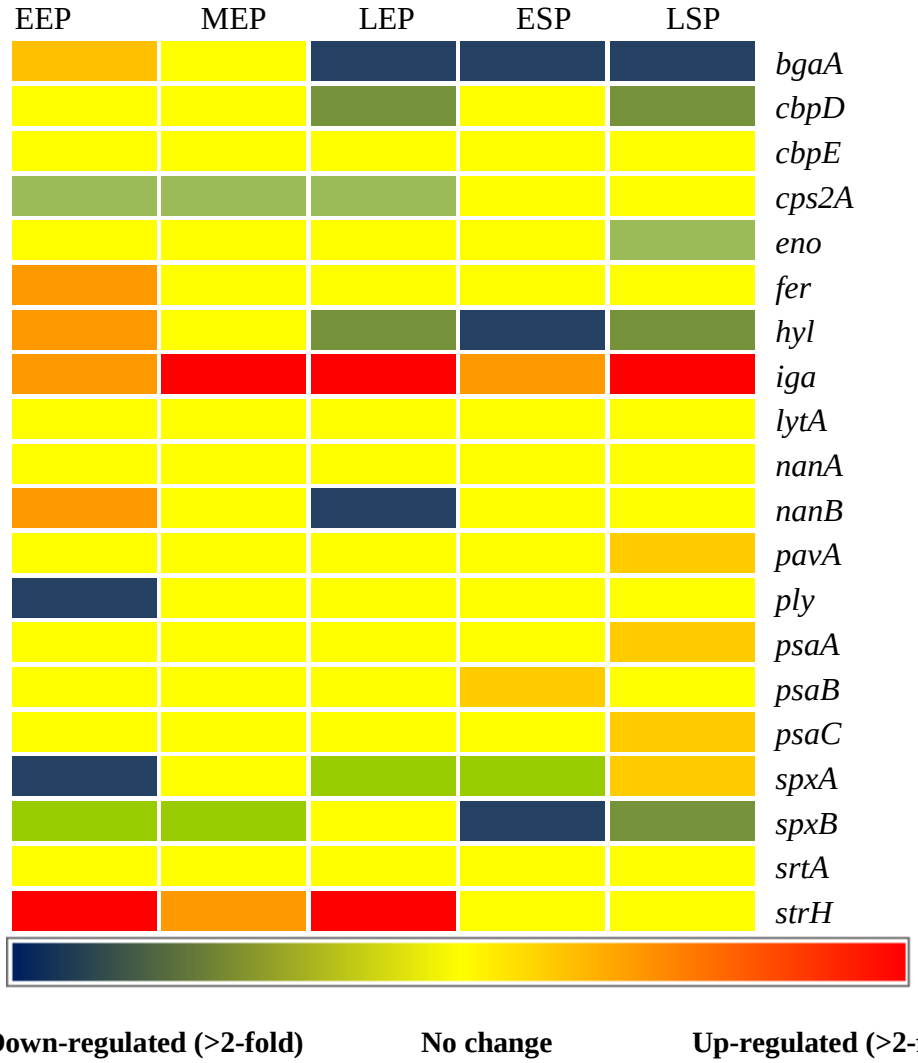


Figure 56. Heat map of Virulence genes that are differentially expressed in ST615 compared to ST306. TPM>10 and 2 fold-change. Red is >2 fold up-regulation, yellow is no differential expression and blue is >2 fold down-regulation.

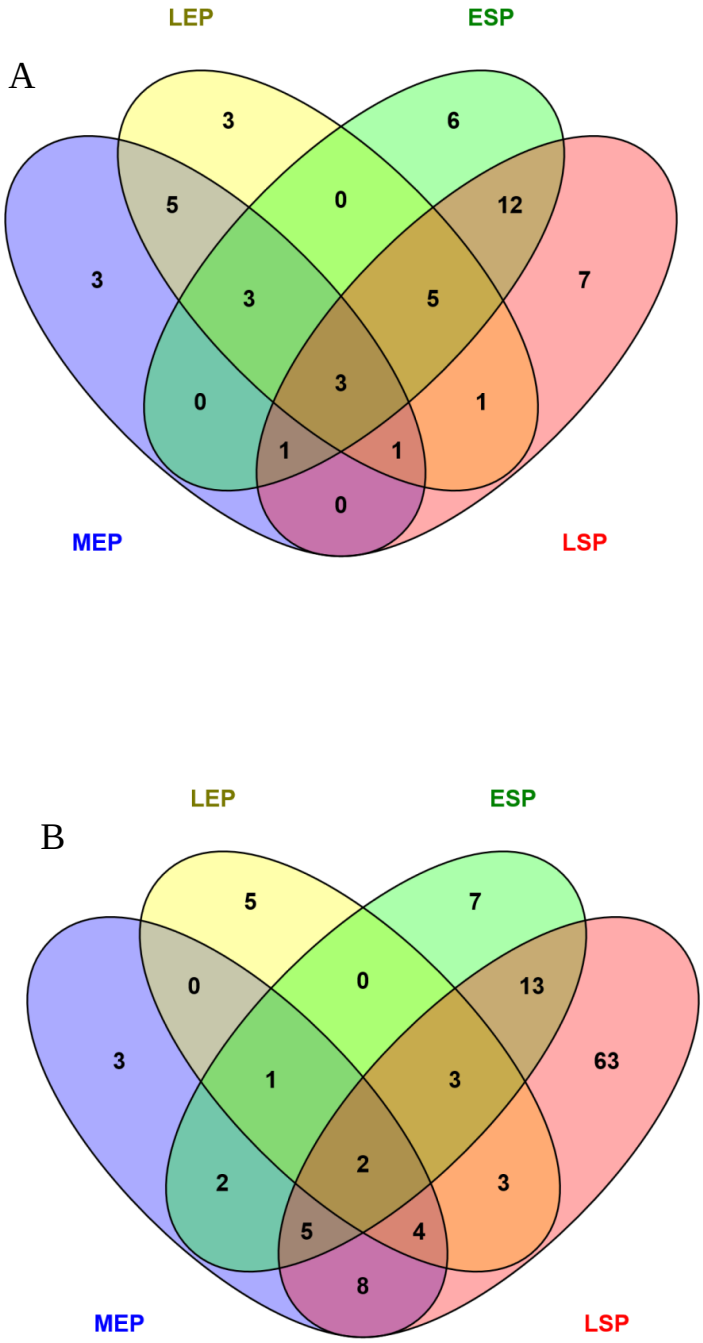


Figure 57. Venn diagram of pair-wise comparison of ST615 to ST306 of the 250 virulence genes that are different between mid and late exponential phases, and early and late stationary phases (A) upregulation and (B) down-regulation.

The virulence genes would be the most interesting to show because they are expressed across all pneumococcal strains. As observed in Table 8, the number of the down-regulated genes was higher than up-regulated genes in ST615 compared to ST306. Overall, the growth phases with a greater number of differentially expressed genes were in the early stationary phase and the late stationary phase. The gene expression for all virulence factors was analysed in detail.

	EEP	MEP	LEP	ESP	LSP
Number of Up-regulated genes	**0**	**3**	**3**	**6**	**7**
Number of Down-regulated genes	**4**	**3**	**5**	**7**	**10**
Number of differentially expressed genes	**4**	**6**	**8**	**13**	**17**

Table 7. Number of virulence genes that are up-regulated and down-regulated in ST615 when compared to ST306 in the different stages of growth

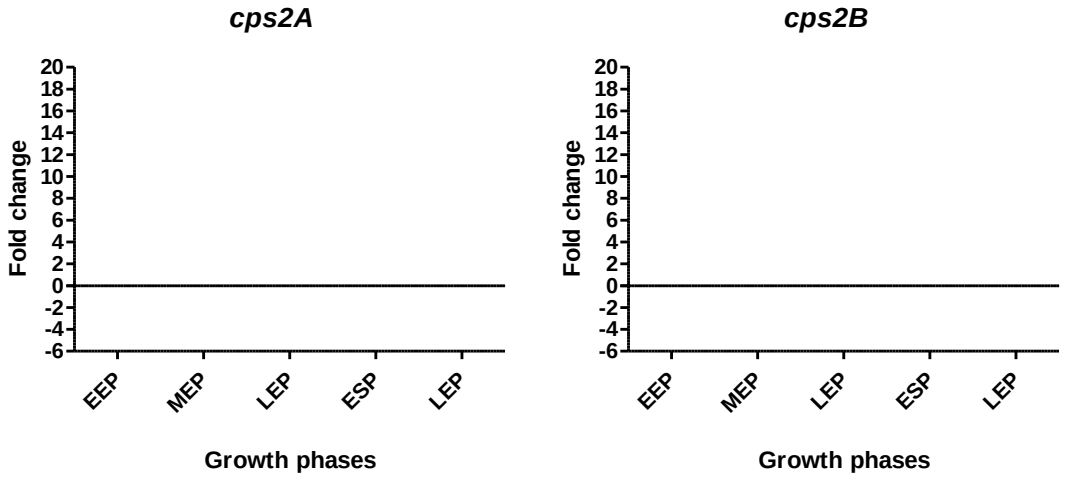
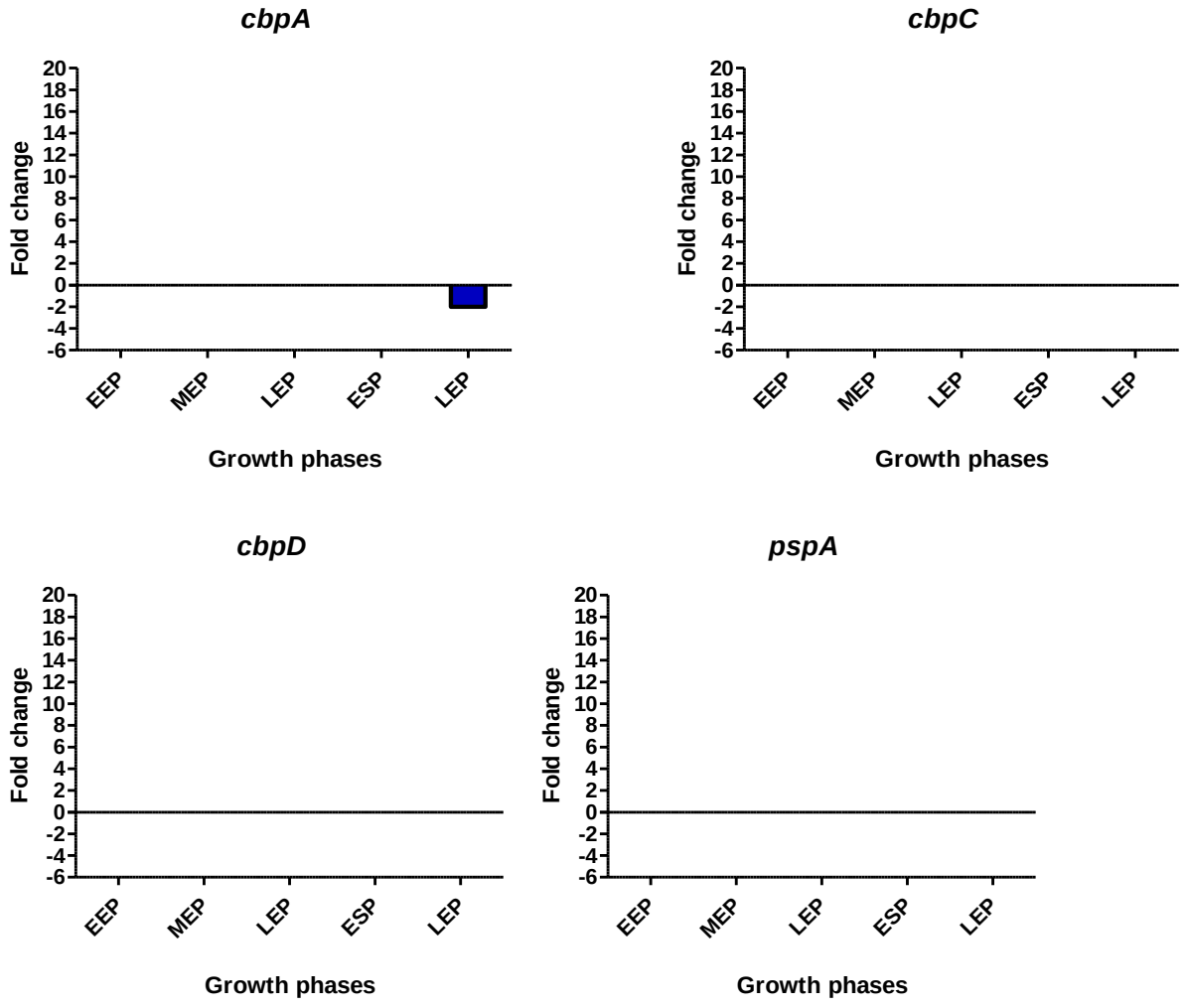
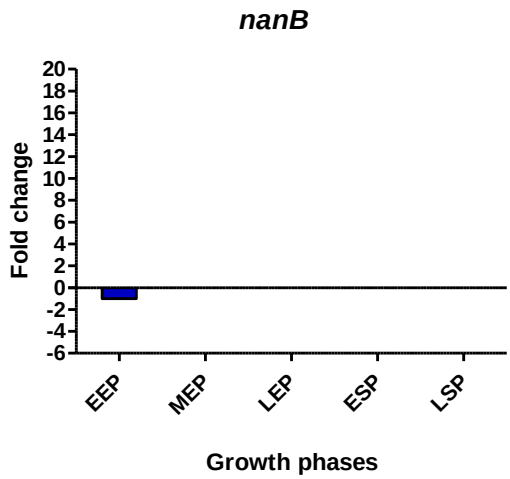


Figure 58. Capsule genes are not differentially expressed between strains ST615 and ST306. Data are shown as fold change after filtering TPM values greater than 10




nanB
Fold change
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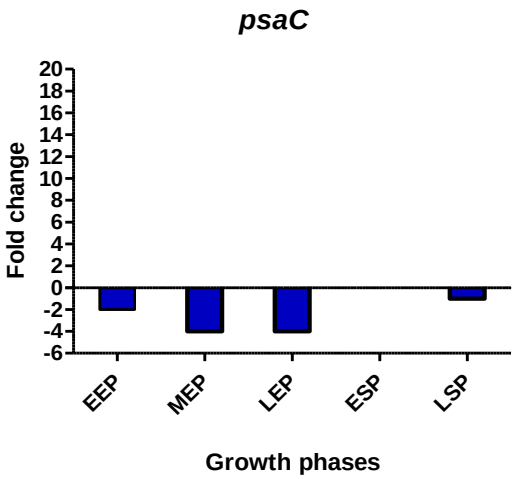
EEP
MEP
LEP
ESP
LSP
Growth phases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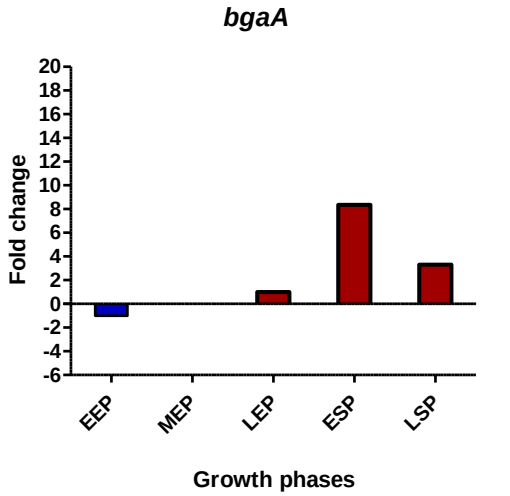
Growth phases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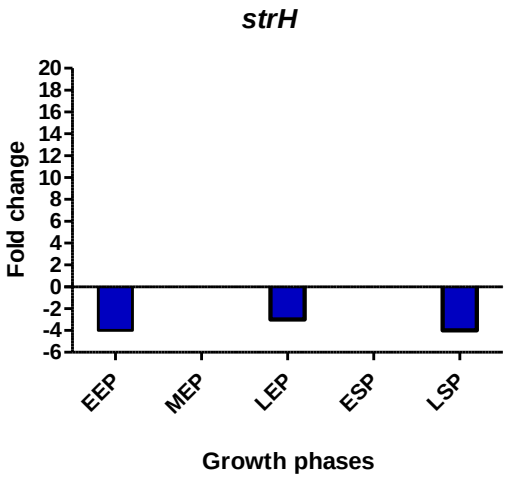
Growth phases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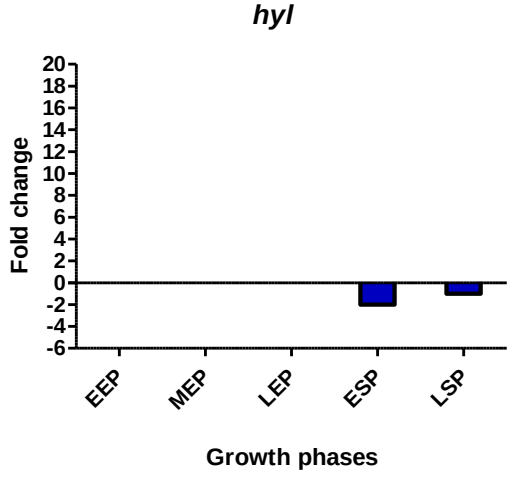
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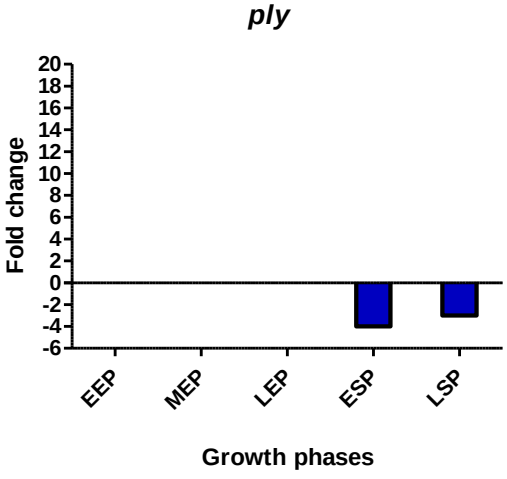
Growth phases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Growth phases

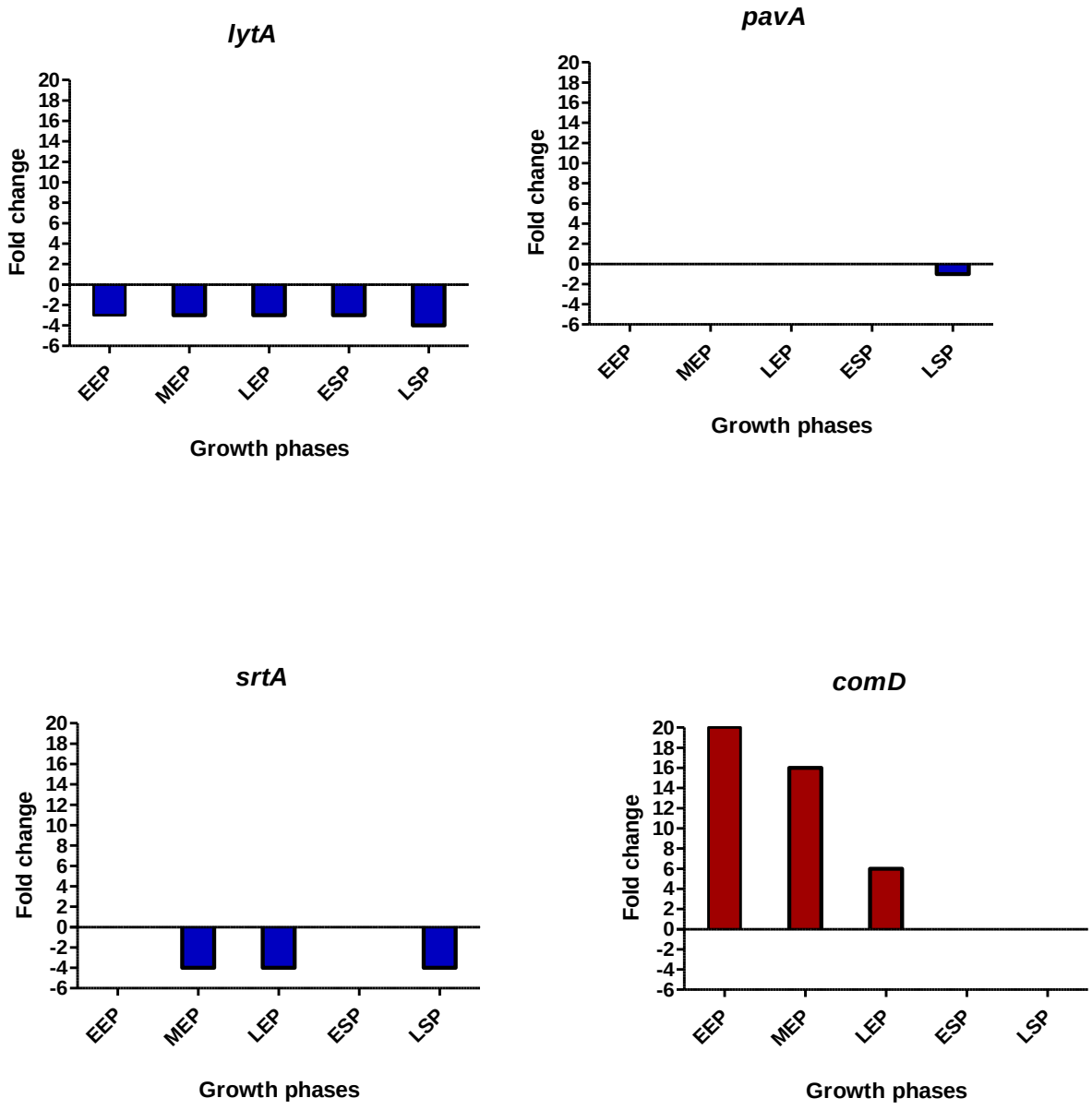

Figure 59. Differential expression of the choline binding proteins genes *cbpA*, *cbpC*, *cbpD* and *pspA*, the pneumococcal surface adhesion gene *psaC* , the surface exoglycosidases genes *nanB*, *bgaA* and *strH*, the autolysin and pneumolysin genes *lytA* and *ply*, the hyaluronidase genes *hyl*, the pneumococcal adherence and virulence factor A genes *pavA*, the sortase genes *srtA* and the competence genes *comD* in ST615 compared to ST306. Data are shown as fold change after filtering TPM values greater than 10

4.8 Capsule Genes: *cps2A* and *cps2B*

There are more than 90 pneumococcal stains that are classified depending on the polysaccharide capsule. The pneumococcal capsule is one of the main pathogenic factors and all virulent strains are able to express the capsule. The *cps2* is a series capsule gene in serotype 1. There is a great similarity between all serotype in the genes *cps2A* to *cps2J*. The similarity between the strains of serotype 1 in these genes is around 89% [564].

As shown in Figure 59, the *cps2A* and *cps2B* genes were analysed in this study in order to observe any differences between ST615 and ST306. However, there was no difference in expression when ST615 was compared to ST306 in all points of growth phases because of both ST615 and ST306 strains belong to serotype 1.

4.9 Surface Protein

4.9.1 Choline Binding Protein: *cbpA, cbpC, cbpD* and *pspA*

The choline binding proteins are a family consisting of 15 surface proteins that are able to bind to choline residues of teichoic acid and lipoteichoic acid existing in the cell wall. The presence of choline binding proteins allows pneumococcal to interact with host cellular receptors [565]

As shown in Figure 60, all choline-binding proteins were similar in expression for both strains. However, a slight difference was detected in the late stationary phase for the *cbpA* gene when ST615 compared to ST306.

CbpA plays a critical role in complement activities by binding to factor H that prevents C3b deposition and leads to inhibit opsonising the bacteria [566]. CbpA has another role in immunity system by binding to polymeric immunoglobulin receptor, mostly involved in IgA secretion. Thus, *cbpA* is probably responsible for the translocation of pneumococcal through tissues [567].

cbpA showed the same level of the expression during all growth phases except in the late stationary phase where the gene is down-regulated in ST615 compared to ST306. This similarity in the expression is because of using the same growth condition, media, PH and temperature.

Although *cbpC, cbpD* and *pspA* were similar in the expression, all of these genes did not show any differences could be detected. In addition, these genes have been observed to be a key for adherence by their ability to adhere directly to nasopharyngeal carriage and virulence [566].

4.9.2 Pneumococcal Surface Adhesion: *psaC*

The pneumococcal surface adhesion *psaC* and *psaB* are members of the ABC transport family and are participated in adhesion that may associate with acquiring the nutrients through the ABC transporter system [568]. In this study, the expression of *psaC* in ST615 is down-regulated, especially in the mid and late exponential phases, when compared to ST306. The low expression of *psaC* has a negative impact on pneumococcal growth and virulence.

4.9.3 Surface Exoglycosidases: *NanB, BgaA and StrH*

The nasopharynx is normally the first host niche that the pneumococcus faces during infection. The concentration of sugars, the major nutrient for the pneumococcus, is low in the nasopharynx. Therefore, pneumococcal requires an efficient mechanism to use the available sugars existing in the environment [569]. Pneumococcus possesses special proteins that are responsible for cleaving extracellular sugars and modifying the sugars to be more conveniently to use by bacteria. These proteins are known by exoglycosidases [189]

The expression of *nanB* in ST615 is down-regulated compared to ST306 in the early exponential phase and there was no expression during other phases. Moreover, *bgaA* down-regulated in ST615 compared to ST306 at early exponential phase. However, *bgaA* was up-regulated in the late exponential phase, early and late stationary phases. The expression of *bgaA* reached a peak in the early stationary phase in ST615 as compared to ST306. For *strH* is down-regulated in the early, late exponential and late stationary phase in ST615 compared to ST306.

4.10 Autolysin and Pneumolysin: *lytA* and *ply*

LytA is an amidase that is principle for cleaving the N-acetylmuramoyl-L-alanine bond of pneumococcal peptidoglycan, leading to the autolysis of pneumococcus [570]

Ply is a major virulence factor that has been broadly studied. Pneumolysin is a cholesterol-dependent cytolysin that is indirectly secreted from pneumococcus. The release of pneumolysin mostly occurs after the cell lysis by either autolysis or lysis caused by external components such as antibiotics that lyse the cell by forming pores on their membrane [571]. Moreover, ply contributes for pneumococcal survived and dissemination by inducing inflammation in the host system [572].

As Figure 60 shown, the expression of *lytA* is down-regulated in all growth phases and especially at the late stationary phase in ST615 when compared to ST306. In the pneumolysin case, no differential expression was seen in the early, mid and late exponential phases but there was a down-regulation of *ply* gene in the early and late stationary phases in ST615 when compared to ST306.

4.11 Hyaluronidase

Hyaluronidase is a surface enzyme that dislocates hyaluronic acid, which is present in mammalian connective tissue and in the extracellular matrix [573]. The degradation occurs by hyaluronidase in hyaluronic acid increase the permeability of mammalian tissues. It is,

therefore, a key virulence factor in the translocation of the pneumococcus between tissues [573].

As shown in Figure 60, no differential expression was seen during the early, mid and late exponential phases of growth. The hyaluronidase *hyl* was down-regulated in ST615 when compared to ST306 in the late stationary phase and that was more obvious during the early stationary phase.

4.12 Pneumococcal Adherence and Virulence Factor A: *pavA*

PavA is a pneumococcal protein that binds to fibronectin and is responsible for adherence and virulence in the pneumococcus. That was proved after using PavA-deficient pneumococci were still able to bind to fibronectin by approximately 50% [169].

The pathogen-fibronectin interaction facilitates the attachment of the pneumococcus to host cells and it has been observed that the ability to adhere and cause invasive disease diminished in *pavA* mutants strains.

As shown in Figure 60, the *pavA* gene was down-regulated in the late stationary phase and no expression was seen during all exponential phases and in the early stationary phase in ST615 when compared to ST306. The deficiency of *pavA* gene in pneumococcus has increased the ability to be invasive. Moreover, the external host factors could indirectly affect the expression of the *pavA* gene.

4.13 Sortase A

Sortase A is highly conserved pneumococcal transpeptidase enzyme and is involved for connecting the pneumococcal pili and other specific proteins to the cell wall [574]. The impact of SrtA pneumococcal in colonization and pathogenesis has been studied, observing that

SrtA is responsible for pneumococcal adherence to human cells. However, no important impact of SrtA on pathogenicity of pneumococcal has been reported [575].

As shown in Figure 60, sortase A was down-regulated in the mid, late exponential phases and in the late stationary phase. However, no differential expression was seen in the early exponential and stationary phases in ST615 when compared to ST306.

4.14 Competence *comD*

A previous study showed, the capability of pneumococcus to undergo genetic transformation was identified in this bacterium and was highly competent. Competence is a multicellular bacterial response which depends on a diffusible signal. This mechanism is known as quorum-sensing and the mechanism was observed in gram positive and gram negative. Pneumococci produced a 17 amino acid peptide pheromone, namely competence-stimulating peptide, which is responsible, for quorum sensing. There are two important components for sensing the peptide and activation transcription of competence genes: the histidine kinase receptor (ComD) and its cognate response regulator (ComE) [576].

As Figure 60 shown, *comD* gene was up-regulated in all exponential phases, especially in the early exponential phase, which reached a peak. However, no differential expression was seen in the early and late stationary phases in ST615 when compared to ST306.

Gene regulation	Compared ST306 to ST615	Other studies	Description	Strain
ply			Was down-regulated in CSF [544]	TIGR4
psaC			Was up-regulated in blood and CSF [544]	TIGR4
pspA			Was down-regulated in early growth in THY broth [542]	D39
bgaA			Was up-regulated after one hour incubation with THP-1 [543]	TIGR4
nanA			Was up-regulated after one hour incubation with THP-1 [543]	TIGR4
cbpA			Was up-regulated in the blood of infected mice [496]	WCH43
cpsA and *cpsE*			No production of capsule gene was detected in mid-exponential phase in THY [545]	TIGR4
lytA			Was down-regulated in murine nasopharyngeal [546]	TCSTS (0100993 mutant strain)
htrA			Was down-regulated in murine nasopharyngeal [546]	TCSTS (0100993 mutant strain)
lytC			Was up-regulated in children nasopharyngeal [495]	Normal flora strains
comD			Moderate regulation in children nasopharyngeal [495]	Normal flora strains

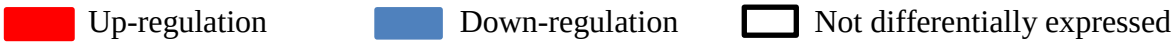

Table 8. Pair-wise comparison of ST306 to ST615 of virulence genes and comparing to previous studies.

4.15 Conclusion

The analysis of gene expression *in vitro* for the ST615 strain in comparison of ST306 strain showed that 41-132 genes of virulence genes were differentially expressed between the two strains. There was a different in level of the expression between the growth phases. However, the difference was between the exponential and stationary phases, while this difference was less obvious between the three subclasses of the exponential phases and the two subclasses of the stationary phases.

This study showed the differential expression of several biological function categories in order to illustrate the possible differences in the cellular function, resulting in differences in virulence and the survival of the two strains used.

The differential expressions of 16 critical pneumococcal virulence factors were analysed in the ST615 and the ST306 strains. The number of differentially-expressed genes varied between growth phases, ranging between 4 and 17 differentially expressed genes. The majority of differentially expressed genes were down-regulated in ST615 when compared to ST306.

However, the number of up-regulated and down-regulated genes was the same at mid exponential phase.

The expression of genes involved in cell wall biosynthesis in ST615 strain was up-regulated compared to ST306 strain. Moreover, many genes involved in metabolism were up-regulated in ST615 when compared to ST306. The metabolism genes were up-regulated in ST615 as this bacterium grew faster and consumed a greater amount of nutrients in the media. In addition, many genes involved in transport and binding protein genes were up-regulated in ST615 as compared to ST306 at mid exponential phase, early and late stationary phases. Metabolism and transport and binding protein genes will directly contribute to growth and survival of the ST615.

On the other hand, many genes involved in protein secretion were down-regulated in ST615 compared to ST306 at all phases except late stationary phase. The environment plays indirect role in the expression levels of secreted protein genes, for instance, a broth or a tissue. Twenty genes involved DNA synthesis were down-regulated in ST615 when compared to ST306. Additionally, there was a difference in the expression levels of RNA synthesis at different growth phases in ST615 compared to ST306.

This study analysed the differentially expressed genes in the ST615 and ST306 strains and observed that the expression of some genes involved in invasive disease - for instance, pneumolysin and hyaluronidase lyase – were down-regulated in ST615 when compared to ST306. In addition, some genes involved in adherence and colonization, for example, pneumococcal surface adhesins, pneumococcal adherence and enolases were also down-regulated in ST615 when compared to ST306. In other words, ST306 showed highly expressed genes of pneumococcal adherence than ST615.

However, there was a similarity of expression between ST615 and ST306 in genes responsible for the expression of choline-binding proteins. Interestingly, the *comD* gene was up-regulated in ST615 when compared to ST306. In this study, the adherence and colonisation-related genes were down-regulated, whereas the genes involved in invasive in the pneumococcus were up-regulated regardless of sequencing types. This could explain why that serotype 1 tends to cause invasive disease than nasopharyngeal carriage.

However, there is a pattern of genes involved in nasopharyngeal colonisation and invasiveness that showed similarities in expression. Moreover, it is important to know that some of the proteins mentioned above are frequently involved in both carriage and invasive disease. There were few differences in virulence genes expression between ST615 and ST306 as both strains belong to serotype 1.

General discussion

5.1 Introduction

Streptococcus pneumoniae is a commensal bacterium commonly found in the human nasopharynx. It is a prominent cause of morbidity and mortality worldwide, responsible for a wide range of infections, from sinusitis and acute otitis media to meningitis, septicaemia and pneumonia, with the highest disease incidence reported in developing countries. Of the nearly 100 different serotypes that were reported so far, serotype 1 ranks among the most prevalent pneumococcal serotypes causing invasive pneumococcal disease (IPD) worldwide.

Serotype 1 (S1) pneumococci exhibit specific characteristics compared to other pneumococcal serotypes as it is often associated with outbreaks in closed communities and was found not to be associated with antimicrobial resistance [577]. The short duration and low bacterial density associated with serotype 1 nasopharyngeal colonisation may explain low antimicrobial resistance levels.

Additionally, ST306 pneumolysin was documented for its ability to bind to erythrocytes; however, this pneumolysin variant was found not to be able to form pores on erythrocyte membrane [361]. Simultaneously, ST615 was a representive sequencing type for lineage C and was compared to ST306. This project was focused on the understanding of ST306 pneumococcal variant in its ability to cause invasive disease despite in low haemolytic activity. In this study, we demonstrated that ST306 was able to colonise mouse nasopharyngeal niche for a longer period of time compared to ST615. Our results found that the European ST306 used in this project was unable to cause invasive disease in mice unless supplemented with purified PLY, in which case systemic dissemination to the lung and blood could occur.

5.2 Pneumococci ST615 behave as an aggressive pathogen than ST306 in *vitro*

To shed light on the mechanisms of pathogenicity specific to ST306 pneumococci, their properties were assessed *in vitro*: haemolytic activity, ability to adhere and invade epithelial cells, ability to evade phagocytosis and complement deposition.

The haemolytic activity of the European ST306 and South American ST615 serotype 1 isolates were assayed to determine the levels of haemolytic activity in these sequence types in comparison to ST304 and ST128. Among the 16 isolates tested (Figure 16 A), both ST306 and ST615 were found to have a significantly lower haemolytic activity than serotype 2, D39 strain, used as the comparator strain. Interestingly, the haemolytic activity of all ST306 strains tested was the lowest, while the activity of ST615 was only slightly lower than that of D39. These findings are in line with current literature, describing that Ply expressed by ST306 serotype 1 isolates has a defective haemolytic activity [119]. The differential levels of haemolysis observed between D39 strain and serotype 1 (Figure 16 B) used in this study suggest that pneumolysin alone may have a significant impact on the reduced virulence of the ST306 serotype 1 isolates. Pneumolysin was indeed found to be an essential and sufficient factor in

inducing an invasive disease [119]. However, these previous studies were focused on strains presenting fully-active pneumolysin or strains carrying pneumolysin mutations.

The pneumococcus is known for its ability to adhere and invade epithelial and endothelial cells [56]. Several pneumococcal virulence factors have been reported to be involved in host cell adherence and invasion: CbpA (binds to glycoconjugates from human cells and is involved in translocation between tissues by binding to the IgA receptor), neuraminidases (cleaves terminal sugars from host surfaces revealing receptors for adherence), PavA (binds to fibronectin in the extracellular matrix), hyaluronate lyase (breaks down the extracellular matrix increasing tissue permeabilisation), enolase (binds to human plasminogen), PsaA (acts as an adhesion) and pili (adheres to human cells). The deficient expression of any of these virulence factors has been shown to reduce the ability of the pneumococcus to adhere and decrease invasive properties [581, 582]. The ability to adhere promotes successful colonisation of the nasopharyngeal tissue and may be a precursor event to the onset of invasive disease. Here, we compared the adhesion properties of ST306, ST304 and ST615 serotype 1 isolates (an isolate from a pneumonia patient, an isolate form the nasopharynx of a healthy individual and an isolate from bacteremia patient, respectively) to those of the well-characterised serotype 2, D39 strain.

Previous authors suggested that PLY-producing pneumococcal strains are able to activate the complement cascade, leading to depletion of complement in the host and reducing opsonic activity [535]. In Figure 20, different sequence types were subjected to opsonophagocytic killing assays using IVIg. Serotype 1 strains belonging to ST306 showed more killing at low dilution of IVIg anticapsular antibodies than other strains belonging to ST304 and ST615. Opsonophagocytic killing of pneumococcal isolate strains appears to be serotype dependent, with higher opsonophagocytic activity found for serotype 2 D39 compared to serotype 1 (ST615, ST306 and ST304). While this may be due to a higher binding ability of D39 to C3b

hence increasing opsonic activity, the results did not appear to show an association between opsonic activity of serotype 1 sequence types and PLY content, invasion or adherence ability.

Human nasopharyngeal epithelial cells and human broncho-alveolar epithelial cells were used to assess the ability of the serotype 1 isolates to adhere to tissue, in comparison to serotype 2 and to examine the differences between the different serotype 1 isolates. Our results revealed a significantly lower ability of serotype 1 isolates to adhere to nasopharyngeal epithelial cells compared to D39. The CFU counts were lower in ST306 than D39 at 30 min post-infection.

This suggests that the ST615 isolate may be more potent in host cells invasion and more prone to translocate to other tissues. Despite the reported [513] ability of D39 to invade human epithelial cells, the number of invading bacteria for this strain was very low compared to the number of adherent bacteria. The invasion ratios of serotype 1 ST615 was higher than D39. There were differences in percentages of adhesion between sequencing types of serotype-1, the ability of ST615 isolate to invade is probably a property to this strain and clearly associated with enhanced invasiveness, a potential key factor in its ability to translocate through lung tissues. In broncho-alveolar epithelial cells no adhesion or invasion was observed for any of the encapsulated isolates. In contrast to our results, previous studies have shown that the pneumococcus was capable to adhere to broncho-epithelial cells; this may be due to the different bacteria:cell ratio used in these published studies, a variable that has previously been show to influence the adhesion and invasion ratios in vitro [513, 583].

An important virulence factor that has been shown to have an impact on the adherence properties of the pneumococcus is the capsule. The exposure of adhesins and other surface molecules is increased in the absence of capsule [7, 582]. In encapsulated isolates, the ability to modulate the thickness of capsule was described as an adaptation mechanism of the pneumococcus: a reduction in capsule thickness promotes adherence to host surfaces while an

increased capsule allows evasion from the host immune system [163, 585, 7, 584]. The capsular composition also plays a prominent role due to the interaction of pneumococcal surface proteins and adhesins with host surfaces [586]. In addition, the capsular serotype affects the levels of C3 deposition on the bacterial surface which may in turn impair the ability of the immune system to phagocytose the bacteria [586]. It was shown that the biochemical structure of the different capsular types controls the accessibility and functionality of surface proteins; and as a result, a differential expression of surface proteins is probably in place to overcome these differences [587, 588].The capsule thickness would impair phagocytosis and the number of complement deposition were assessed in all isolates of serotype 1 and compared to the serotype 2 strain D39. A previous study showed that carriage isolates reduce the expression of their capsule in order to increase their ability to adhere to host surfaces [419]. The variation of capsule thickness is not a permanent adaptation to specific serotype, it is reasonable that the differences in capsule thickness are only temporary and not reproducible in vitro. OPKA experiment was performed in order to study the role of capsule as well. During an opsonophagocytic killing assay where the bacteria were opsonised using intra-venous IgG, it was shown that the serotype 2 D39 strain was more susceptible to phagocytosis by HL-60 neutrophils than the serotype 1 strains. Moreover, there was also difference between the two serotype 1 isolates used in the OPKA experiments; European and South American isolates. The capsule thickness of the ST306 did not differ from serotype 1 ST615 isolates or serotype 2 strain.

The levels of C3 deposition were determined to clarify the mechanisms through which the serotype 1 isolates were more resistant to phagocytosis by HL-60-derived neutrophils than the serotype 2 strain. The C3 deposition on the surface of the different isolates showed that ST306 and ST615 had a lower complement-binding ability compared to the serotype 2 strain. There were no differences within the serotype 1 isolates suggesting that in general the biochemical

structure of the type 1 capsule might play a determining factor in the binding of complement proteins to the bacterial surface. The capsule of most pneumococcal serotypes has a neutral or a negative charge, whereas the serotype 1 capsule was described to hold both negative and positive charges [589]. The zwitterionic nature of serotype 1 capsule, which displays both negative and positive charges, suggests that the positive charges may inhibit the deposition of the complement protein C3b on the surface of the bacteria leading to reduced phagocytosis, immune evasion and therefore increased virulence. Moreover, previous authors reported that the presence of an amino group (NH3+) on the capsule of serotype 1 could explain a reduction in complement deposition [590,591]. Altogether, these observations might explain the differences described in our investigations.

To conclude, in vitro characterisation of serotype 1 isolates suggest that pneumococci serotype 1 are less prone to surface binding by complement proteins leading to impaired phagocytosis by the host, thereby rendering this serotype more resistant to host immune defense, which may thus explain its increased virulence properties. More particularly, it was also observed that the South American ST615 isolate was the only encapsulated isolate able to invade nasopharyngeal epithelial cells, and complement deposition was very low. This observation suggests that this particular isolate might express yet-to-identify virulence determinants permitting ST615 to translocate into deeper tissues.

5.3 Pneumococci ST615 are more virulent than ST306 in mouse models of pneumococcal pneumonia

In view of the results obtained in vitro, we further investigated the invasive properties of ST306 and ST615 using in vivo models of nasopharyngeal carriage and pneumococcal invasive disease.

The virulence of ST306 and ST615 isolates were compared using a well-established *in vivo* model of invasive pneumonia in mice [120]. Upon infection with serotype 2 D39, MF1 infected mice started showing disease symptoms as early as 24h post-infection and became moribund by 48h post-infection with only up to 10-20% surviving the infection [120]. A significant difference was observed between ST306 and ST615 with a mouse survival rate of 10-20% for ST615 and 100% for ST306. However, the disease progression – as measured by the onset of clinical symptoms - in ST615 and ST306 serotype 1-infected mice was considerably faster than that in mice infected with the D39 strain; approximately 80-100% of the mice (Figure 25) succumbed to the infection between 24h and 48h post-infection [120].

Overall, our results showed that the South American ST615 isolate was significantly more virulent than the European ST306. A time-course analysis was performed in order to monitor the infection kinetics and dissemination into peripheral blood. There were significant differences in the bacterial densities of the tissues analysed in mice infected with the ST615 compared to those infected with ST306.

The exact mechanisms underlying the breach of the nasopharyngeal epithelium and subsequent dissemination to the lungs or blood remain unclear [581]. Our results showed that serotype 1 ST615 tended to disseminate into blood very early (Figure 30). On the other hand, ST306 was not able to translocate into blood at any of the experimental time points, while the bacterial density in the lungs of mice were very similar between both sequence types. These observations may be correlated with the invasion patterns observed in vitro upon infection of nasopharyngeal epithelial cells, whereby the ST615 isolate was the only isolate detected intracellularly. For this reason, it could be helpful to investigate genetic differences, for example, possible single nucleotide polymorphisms (SNPs) differences, between the ST615 and the ST306 isolate, as

well as possible differences in the mRNA expression of critical virulence factors in vitro as well as in vivo.

We also sought to determine whether increasing the bacterial load in the inoculum was permissive to disseminate ST306 into peripheral blood from lung or nasopharynx. Hence mice were infected with a one-log excess dose compared to our standard model, at 10^7 CFU/mouse, but the mice failed to show any detectable levels in whole blood samples. This observation indicates that even upon administration of high dose, ST306 is not capable of invading the blood systemic circulation to cause bacteremia.

In view of the differential properties observed between ST306 and ST615, we expected to see significant differences in the host immune response generated against these 2 sequence types, however, our results failed to detect any significant disparities. These results warrant further investigations, for example, the focus could be placed on earlier markers i.e., earlier experimental time points, and on the analysis of immune cell populations in other tissues such as the spleen or blood.

In a pulse experiment, LPS-free recombinant pneumolysin was co-administered with serotype 1 ST306. We made the surprising findings that the presence of haemolitically active pneumlysin significantly promoted the translocation of pneumococci into peripheral blood as early as 12 hour post-infection. This was accompanied by signs of disease starting to show at approximately 6 hour post-infection. Most interestingly, all symptoms resolved by 72 hours.

In mice infected by serotype 2, D39 strain, an increase in the number of T regulatory cells was described during invasive disease [530]. In our investigations, results showed that serotype 1 failed to induce activation of T regulatory cells, which is in line with previous reports

suggesting that the activation of Treg may be a necessary mechanism to control local inflammatory responses and systemic spread of pneumococci [530].

In summary, intra-serotype virulence differences were observed, with ST615 isolate found to be significantly more invasive than ST306 isolate. This was translated in vitro by the invasion properties of ST615 in Detroit 562 nasopharyngeal cells (Figure 18) and the highest incidence of clincal symptoms and death in mice (Figure 25). Mice infected with pneumococci ST615 pneumococci failed to combat or clear the infection and ultimately succumbed. The nature of the immune inflammatory response generated against ST615 failed to be characterised (Figure 44, 45), however current literature suggests that innate cells such as neutrophils and macrophages are actively involved in the very early stages of infection.

In our hands, low haemolytic ST306 showed the inability to translocate from lungs or nasopharynx to the blood, and mice infected with ST306 did not exhibit any visual sign of disease symptoms, and all mice exposed to ST306 ultimately survived. In line with previous studies, our results suggest that the haemolytic activity of pneumolysin is critical to the dissemination of pneumococci to blood, likely due to its tissue damaging and inflammatory properties [251]. This observation, however, does not rule out that other virulence factors maye also be involved, and this was explored through our transcriptomics analysis.

5.4 Serotype 1 during nasopharyngeal carriage

Current literature has documented pneumococci serotype 1 as a rarely detected capsular type in nasopharyngeal swabs [592, 577]. This raises the question as to whether nasopharyngeal carriage is a pre-requisite for invasive disease. A clearer understanding of serotype 1 pathogenesis woud help design preventive strategies to reduce the burden of disease caused by pneumococcal serotypes exhibiting a similar phenotype, e.g. serotype 5 and 7F.

The two sequence types ST306 and ST615 were assessed in a well-characterised in vivo model of long-term nasopharyngeal carriage in mice to understand their pattern of colonisation and identify possible differences. The serotype 2 strain D39 was documented for its ability to colonise the nasopharynx of mice at a stable and constant density for a period of at least 28 days [593]. Here our results showed that serotype 1 ST615 was able to establish colonisation for a period of up to 14 days (Figure 21). Interestingly, ST306-infected mice showed a decline in bacterial density at day 7 in the nasopharync, rose on day 14 and gradually decreased with pneumococci still present, though at lower density, on day 28. The bacterial nasopharyngeal density between ST306 and ST615 were variable during the observation period ascertaining our previous observations that the 2 strains present very distinct phenotypes and/or protein expression patterns. Overall, and in line with current reports, serotype 1 is able to establish nasopharyngeal colonisation, but for a shorter period of time and at lower bacterial density compared to D39. On the other hand, we showed that colonisaton of mice with a haemolitically-deficient ST306 was able to remain longer than those mice inoculated with a haemolitically-sufficient ST615. Our results are in contradiction with previous finding reporting that a low haemolitically- active pneumolysin promoted clearance of colonisation [97,98].

Host immune responses during nasopharyngeal carriage have been investigated in previous studies, describing the production of immunomodulatory cytokines such as IL-10 and TGF-β1, as well as the recruitment of T regulatory cells, as contributing towards the long-term persistence of pneumococcal carriage [468]. This implies that in the absence of immune-regulatory responses, pneumococcal carriage may fail to establish, potentially due to an overt pro-inflammatory response. In this project, results showed that serotype 1 failed to induce recruitment of Foxp3+ cells (Figure 46). Unexpectedly however, active TGF-β1 concentration

was enhanced in the nasopharyngeal homogenates of mice infected with ST306 compared to mice infected with ST615, particularly at day 7 post-infection (Figure 47). In our models, serotype 1 failed to induce any detectable production of immune-modulatory cytokines or T regulatory cells. Previous reports have shown that serotype 1 purified capsule was able to trigger ctivation of CD4+ T cells upon intraperitoneal injection accompanied by the induction of IL-10 and TGF-β, leading to immune tolerance [594, 595]. This differing result can be interpreted by the different experimental protocols used. Many studies suggesting induction of T regulatory cells and the production of IL-10 and TGF-β1 cytokines were performed using injected purified capsule [547]. Although capsular serotypes have emerged as important mediators of host immunity to pneumococci, the interaction of the capsule with bacterial surface proteins anchored to the cell wall can amend the host immune response [587, 586].

5.5 Co-administration of ST306 and ST615 serotype 1 in mouse model of nasopharyngeal colonisation

Co-colonisation with multiple pneumococcal serotypes was described as a contributing factor in the progression into invasive disease, as well as horizontal gene transfer and host-to-host transmission [599, 600]. Previous studies have examined the effect of inter-species nasopharyngeal co-colonisation on the density, duration and serotype distribution of pneumococcal carriage in mice [596, 582, 597, 598].

In the first few years of life, the prevalence of pneumococcal carriage increases and reaches a peak at approximately 50% to >70% in hosts aged 2–3 years, and then decrease until stabilising at an incidence of 5–10% in hosts aged over 10 years in developed countries, while the incidence reported in developing countries can be as high as 25–60%. The estimation of the median duration of carriage is approximately 31 days in adults and 60.5 days in children, with variable length of carriage based on type of capsule and previous immunologic exposure, as well as immunocompetence and age of the host [601]. In addition, serotype 1 is known to cause

invasive disease in young adults (9-15 yr) which is the age group with lower pneumococcal carriage rates. This may be due to the fact that there is no competition occurring in the nasopharynx of young adults hence leading to better colonising ability and better chances of spreading and causing invasive disease [538].

Co-colonisation is believed to promote acquisition of new serotypes, rather than leading to clearance [602]. In our study, the co-administration of ST306 and ST615 failed to show any change in the carriage patterns of each individual sequence type (Figure 21 compared to Figure 24). The co-administration of strains belonged to serotype 1 had no impact on the density of either strain ST306 and ST615. In other words, there was no evident sign of competitive interaction between the two sequence types.

There is generally a high incidence of pneumococcal carriage in infants and in the elderly, however in the particular case of serotype 1, the incidence of invasive pneumococcal disease caused by serotype 1 is higher in infants [577] suggesting that serotype 1 is more efficient at establishing nasopharyngeal carriage in individuals that have had very little exposure to the pneumococcus or that have a less mature immune system [577, 90, 603]. Hence, it would be of interest to carry a co-colonization experiment using neonate mouse models for example.

5.6 Pneumococci ST306 and ST615 differentially expressed genes during *in vitro* growth

Sequencing is a revolutionary approach that has significantly facilitated the analysis and identification of genes that are pivotal for the virulence or metabolism of bacteria [604]. We previously discussed that, using *in vivo* mouse models, ST306 of serotype 1 is less virulent than ST615, and is also less efficient at establishing nasopharyngeal carriage. Differential gene expression analysis between these two strains was performed using pure culture samples obtained *in vitro* at key stages of their growth. One previous study [605] has been performed

on the differential gene expression of specific virulence factors in serotype 1 compared to serotype 2; however, our study is the first to carry out such an in depth analysis. The focus was placed on the expression of 250 pivotal pneumococcal virulence factors and 6 groups of biological functions.

Of the 250 virulence genes, between 78% and 88% of the orthologous genes were differentially expressed between the two strains, with a general down-regulation of biological functions related to bacterial activity such as the metabolisms (*lacB, malA, nagB, glgC, manA, lacR and ldh*) protein secretion and DNA replication. It has previsouly been reported that faster growing pneumococci are better colonisers; thus, the down-regulation of mechanisms involved in growth may explain the poor ability of serotype 1 to colonise the nasopharynx [605].

One key finding was the higher expression of genes involved in cell wall biosynthesis (*bacA, fibA, lytB, licC, licB, pck, pgdA, penA, dltD, dltA, pgm* and *cbf1*) found in ST615, compared to ST306. Therefore, genes involved in metabolism (*lacB, malA, nagB, glgC, manA, lacR and ldh*) showed up-regulation in ST615 vs. ST306 during all growth phases except during the EEP. Moreover, transport and binding protein genes (*rafF,rafG,amiA,amiC,amiD,amiE,amiF,aliA,aliB,potA,potC* and *potD*) showed up-regulated in ST615 compared to ST306.

Analysis was carried out on the differential expression of 16 virulence factors and showed that virulence factors associated with carriage (i.e. choline binding proteins, pneumococcal surface adhesins, pneumococcal adherence and enolases) and invasive potential (pneumolysin and hyaluronidase lyate) are down-regulated in ST615 compared to ST306. In contrast, some factors, such as β-galactosidase and competence system, were shown to be up-regulated in ST615 when compared to ST306 of serotype 1.

5.7 Summary

In this project, we provided evidence that serotype 1 ST615 was a highly invasive sequence type capable to colonise the nasopharyngeal niche but for a shorter period of time and at a lower density than ST306.

The differential gene expression observed between ST615 and ST306 was mainly reflected in virulence factors involved in nasopharyngeal carriage and invasive properties. These observations suggest that it was reported that pneumococcal transmission is also possible during invasive episodes [606]. In this project, we sought to elucidate mechanisms inherent to serotype 1 that may explain their highly invasive nature in contrast with their poor colonising abilities. Pervious studies have demonstrated that pneumonic spread was more virulent than carriage spread [606]. However, the mechanisms leading to the increased virulence remain unknown. A possibility is the increased expression of important virulence factors during pneumonia; it is legitimate to postulate that, when pneumococci are transmitted via coughing or upon contact with the sputum, pneumococci are highly virulent and are thus more prone to becoming invasive. This may explain why serotype 1 is a major cause of disease outbreaks and is frequently isolated from communities where a recent outbreak has occurred [592].

In addition, it was observed that isolates from same sequencing type were similar to each other during adherence in vitro and during nasopharyngeal carriage in vivo. The results obtained *in vitro* seem to corroborate with our *in vivo* observations. Transcriptomics analysis suggests that the ability of serotype 1 to cause nasopharyngeal carriage might be attributed to the expression of virulence factors such as pneumolysin (*ply*), neuraminidases, hyaluronidase or the pneumococcal adherence and virulence factor (*pavA*) rather than the capsule.

In summary, not only the capsule of serotype 1 is likely to affect on its ability to carry long time during nasopharyngeal colonization, but also, serotype 1 could use unknown mechanisms

in order to survive and cause invasive diseases, with the progression into pneumonia acting as an alternative method of transmission for this serotype.

Additionally, another potential reason behind the low virulence properties of ST306 resides in the loss of the variable region of pneumococcal pathogenicity island 1 (vPPI1). According to a recently published study, ST3018 strain encoding (vPPI1) was able to survive and proliferate in the lungs and then invade the pleural cavity and blood [609]. A number of the genes present within vPPI1 of a ST3018 virulent strain exhibit sequence similarity to metabolic pathway genes and could have a secondary effect on the expression of other virulence factors. Therefore, total levels of choline-binding protein A (CbpA), neuraminidase A (NanA), and pneumolysin (Ply) were compared between the ST3018 virulent strain and its ΔPPI1 mutant strain by quantitative Western blot analysis. However, the findings revealed that no significant differences between the ST3018 virulent and its ΔPPI1 strains in the relative expression levels of CbpA, NanA, and Ply were detected [609].

5.8 Future work

In this project, there was no detectable difference in the host immune responses between serotype 1 ST306 and ST615. Hence, the immunological mechanisms resulting in the differential colonization and invasive properties of ST306 and ST615 remain to be determined, for example, using different mouse strains.

The purpose of the co-colonisation experiment was to determine whether the two strains could complement each other or instead compete with each other. The findings showed that nasopharyngeal co-colonisation had no impact on strains from the same serotype. More investigation is required to study the interaction between carriage and invasive serotypes, for example, several serotypes could be investigated in a co-colonisation experiment to further understand the mechanisms driving one serotype to enhance the density of another serotype.

The analysis of differentially expressed genes in ST615 compared to ST306 showed that there were several differences in the biological functions and virulence factors expression *in vitro*. It would be useful to investigate the differential genes expression of ST615 and ST306 *in viv*o. There is a need to develop a robust method for the extraction of good quality and yield of RNA from tissue in order to obtain confident RNAseq analysis. It would be of interest to perform the analysis of gene expression of serotype 1 *in vivo* at different points of infection and using bacteria collected from different tissue niches e.g., nasopharynx, lung, blood and brain. Finally, the results obtained in this study maybe be used as a ground to study the gene expression of various serotype 1 isolates in order to understand the mechanisms involved in carriage and invasive.

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