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**Effects of cigarette smoke condensate on
clarithromycin-mediated inhibition of biofilm
formation and related alterations in resistance
gene expression by *Streptococcus
pneumoniae***

By

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Declaration

I hereby declare that the work contained in this dissertation is my own original work and that I strictly followed norms in academia wherever I used other people's work and wherever I was assisted. I further declare that this body of work has not been before presented for any qualification at this institution or any other institution. This work is submitted in fulfilment of the requirements for a Master of Science degree at the University of Pretoria.

Signed: 

Date: 

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Summary

Streptococcus pneumoniae (the pneumococcus) is a Gram-positive bacterium that frequently colonises the nasopharynx of healthy humans. In susceptible hosts, especially children under 5 years with underdeveloped immune systems and the elderly, this asymptomatic colonisation can lead to development of severe disease such as pneumonia and meningitis. In many cases, disease severity is linked to the inability of the infection to be successfully treated, possibly due to formation of bacterial biofilms. In this context, cigarette smoking, which is a well-recognised risk factor for development of severe pneumococcal disease, also promotes biofilm formation by various types of bacterial pathogens. Notwithstanding poor penetration of biofilms by antibiotics, bacteria within biofilms are exposed to low levels of antibiotics, which promote gene modifications that mediate antibiotic resistance. However, little is known about the effects of exposure of the pneumococcus to cigarette smoke on the induction of pre-existing antibiotic resistance genes, specifically those that mediate resistance to macrolide antibiotics.

In addition to measuring bacterial growth and biofilm formation, the research described in this dissertation was designed primarily to investigate the effects of exposure of different strains of the pneumococcus to cigarette smoke condensate (CSC) on the expression of genes which mediate resistance to macrolide antibiotics, specifically the *erm(B)* and *mef(A)* genes. The bacterial strains used were 172 (macrolide-susceptible), 521 [macrolide-resistant, *mef(A)* efflux pump-expressing] and 2507 [macrolide-resistant, *erm(B)* ribosomal methylase-expressing], all belonging to serotype 23F. In addition, the effects of exposure of all three strains of the pneumococcus to CSC on the expression of the *SP2003* gene were also investigated. This gene encodes an ABC-type transporter, expression of which has been linked to antibiotic resistance.

All three strains of the pneumococcus were exposed to CSC (80 and 160 µg/mL) and sub-minimal concentrations of clarithromycin individually or in combination, followed by measurement of growth, biofilm formation and gene expression. Bacterial growth was measured using spectrophotometric and colony counting procedures, biofilm formation by a crystal violet-based spectrophotometric method, and gene expression [*(mef(A), erm(B) and SP2003)*] using real-time qPCR.

Exposure of all three strains of the pneumococcus to either CSC, at both concentrations used, or to clarithromycin alone, resulted in a transient inhibition of growth which persisted for several hours and was followed by a rebound. Exposure to combinations of the antibiotic and CSC resulted in prolongation of the lag phase, particularly in the case of strain 172. Augmentation of biofilm formation was observed following exposure of all three strains of the pneumococcus to CSC, while exposure of strain 172 to clarithromycin inhibited biofilm formation, which was partly attenuated by CSC. In the case of gene expression, exposure to clarithromycin alone caused significant upregulation of expression of the macrolide-resistance genes, *mef(A)* and *erm(B)*, by strains 521 and 2507 respectively, as expected. Exposure of strain 2507 to the combination of clarithromycin and CSC resulted in significant augmentation of expression of the *erm(B)* gene relative to the expression level noted with clarithromycin alone. This augmentative effect of CSC on gene expression was not, however, evident in the case of the *mef(A)* gene. In addition, and somewhat surprisingly, exposure of strain 2507 to CSC only at 160 µg/mL resulted in a significant increase in *erm(B)* gene expression. In the case of the *SP2003* gene, exposure of all three strains of the pneumococcus to CSC resulted in significant upregulation of this gene, probably as a stress response linked to elimination of smoke-derived toxicants, while exposure to clarithromycin alone resulted in modest upregulation, compatible with a role for *SP2003* in mediating macrolide resistance.

In conclusion, the pathogen-targeted effects of CSC described in this dissertation provide additional insights into the mechanisms by which cigarette smoking impacts negatively on the outcome of pneumococcal infections by undermining the therapeutic efficacy of macrolide antibiotics.

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List of abbreviation

ABC:	ATP-binding cassette
AI 2:	Type 2 autoinducer
ANOVA:	Analysis of variance
APC:	Antigen presenting cell
CAP:	Community-acquired pneumonia
CbpA:	Choline-binding protein A
cDNA:	Complementary-Deoxyribonucleic acid
ChoP :	Phosphorylcholine
Clari:	Clarithromycin
CLSI:	Clinical and laboratory standards institute
CNS:	Central nervous system
CO ₂ :	Carbon dioxide
COPD:	Obstructive pulmonary disease
Cq:	Quantification cycle
CSC:	Cigarette smoke condensate
CSF:	Cerebrospinal fluid
CSP:	Competence stimulating peptide
DMSO:	Dimethylsulfoxide
DNA:	Deoxyribonucleic acid
dNTP:	deoxynucleotide triphosphate
<i>E. coli</i> :	<i>Escherichia coli</i>
EDTA:	Ethylenediamine tetraacetic acid

<i>erm</i> (B):	Erythromycin resistance methylase B gene
GM:	Geometric mean
GOI:	Gene of interest
<i>gyr</i> :	Gyrase
HIV:	Human Immunodeficiency virus
Ig:	Immunoglobulin
IL:	Interleukin
MAC:	Membrane attack complex
MBL-MASP:	Mannose binding lectin- Mannose associated serine protease
<i>mef</i> (A):	Macrolide efflux A gene
MFS:	Major facilitator superfamily
MIC:	Minimum inhibitory concentration
MLS _B :	Macrolides-lincosamides and streptogramin B
NICD:	National Institute of Communicable Diseases
NOD:	Nucleotide oligomerization domain
NRQ:	Normalised relative quantification
NRT:	Nonreverse transcriptase
OD:	Optical density
PAF:	Platelet-activating factor
PAFr:	Platelet-activating factor receptor
PAMP:	Pathogen-associated molecular patterns
PBS:	Phosphate-buffered saline
PCR:	Polymerase chain reaction

pIgA:	Polymeric immunoglobulin A
pIgR:	Polymeric immunoglobulin receptor
Ply:	Pneumolysin
PRR:	Pattern recognition receptors
PspC:	Pneumococcal surface protein C
PTC:	Peptidyltransferase centre
qRT-PCR:	Quantitative reverse transcriptase-polymerase chain reaction
RNA:	Ribonucleic acid
RQ:	Relative quantification
rRNA:	ribosomal RNA
RT:	Reverse transcriptase
SD:	Standard deviation
sIgA/M:	Secretory immunoglobulin A/ M
TBE:	Tris Borate EDTA
TE:	Tris-EDTA
Th:	T helper cell
TLR:	Toll-like receptor
TNF- α :	Tumor necrosis factor alpha
TSB:	Tryptic soy broth

Chapter 1: Literature Review

Streptococcus pneumoniae (*S. pneumoniae*) is a Gram-positive, lancet-shaped, alpha-haemolytic facultative anaerobic organism ^{1,2}. The organism was first isolated in human saliva in 1881 and was described as “roughly lancet-shaped pairs of coccoid bacteria” by both the American and French microbiologists George M. Sternberg and Louis Pasteur, respectively ³. Since then, the microorganism has been called by many names, for instance, in 1920 it was found that pairs of cocci were the cause of pneumonia; hence it was termed *Diplococcus pneumoniae* ³. It was later, in 1974, that the organism was formally termed *Streptococcus pneumoniae*, simply because of the chains of cocci (*streptococcus*) formed in liquid media that caused pneumonia (*pneumoniae*) ^{2,3}. As shown in Figure 1.1, *S. pneumoniae* can occur as either unicellular form, or as diplococci or short chains, moreover, the different forms have also been found to coexist in a particular *S. pneumoniae* sample ⁴.

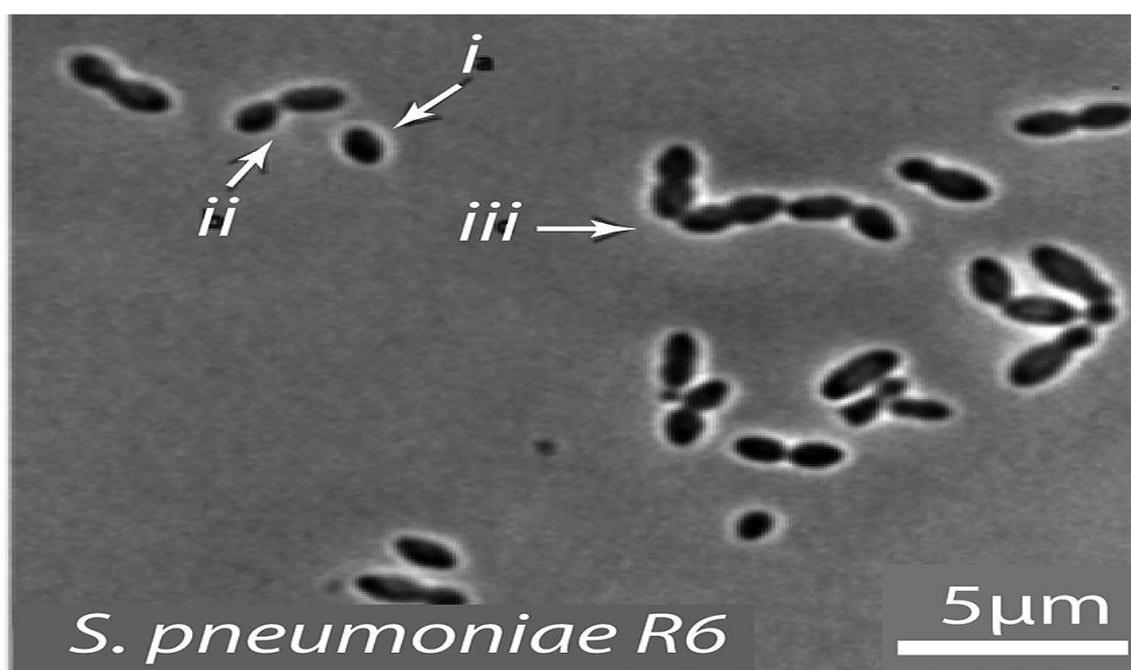


Figure 1.1: A phase-contrast micrograph of *S. pneumoniae* strain R6 showing three morphological appearances (i) single coccus (ii) diplococcus and (iii) short-chain. Used under CC BY 4.0 License ⁵ Cropped from original.

Streptococcus pneumoniae asymptotically colonises the human nasopharynx; however, in susceptible hosts such as children under 5 years, adults above 65 years of age and immune-compromised individuals ^{6,7,8}, it can cause pneumococcal diseases such as pneumonia, otitis media, meningitis, and septic shock ⁹. Pneumococcal infections have an alarming global mortality rate of more than 800 000 in children below 5 years, and, therefore remains a leading cause of morbidity and mortality worldwide ¹⁰. Cigarette smoke inhalation directly interacts with the microbiota of the upper respiratory tract and its detrimental effects on human health are well recognised ^{11,12}. Exposure of *S. pneumoniae* to cigarette smoke has been documented to promote the risk of developing pneumonia and invasive pneumococcal diseases in immune-competent adults ¹³. Smoking is associated with an increase in pneumococcal adherence by promoting upregulation of platelet-activating factor receptor (PAFr), an adhesion molecule that binds to phosphorylcholine (ChoP) expressed on the pneumococcal cell surface ^{14,15}. This interaction has been thought to play a role in disease progression and promoting chronic colonisation ¹⁵

Additionally, cigarette smoking has been linked to disease persistence by promoting biofilm formation (shown in Figure 1.2) and altering genes involved in antibiotic treatment ¹⁶. Formation of biofilms during microbial infections has been implicated in almost 80% of all pneumococcal infections, making it an important factor in preventing pneumococcal eradication ¹⁷. *In vitro* biofilm formation studies, focusing on respiratory pathogens such as *S. pneumoniae* and *Staphylococcus aureus*, have documented the upregulatory effects of bacterial biofilm formation due to exposure of cigarette smoke ^{18,19}. Bacteria encased within the extracellular matrix of the biofilm are concealed from host immune recognition and antimicrobial therapy ¹⁷. Tolerance to antimicrobial agents may be a subject of reduced efficacy of the drug by either: reacting with the fluid bathing the biofilm or reacting with the biofilm itself; or acquisition of resistance genes within the biofilm via quorum sensing mechanisms ^{20,21}.

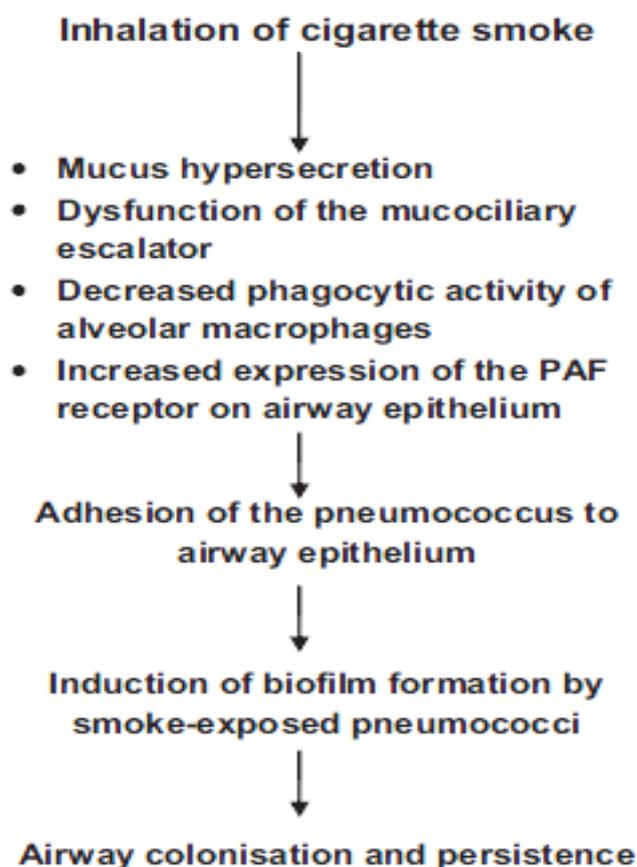


Figure 1.2: As proposed, inhalation of cigarette smoke aids in pneumococcal airway epithelium adhesion by promoting mucus hypersecretion, anti-phagocytic activities, expression of platelet-activating factor (PAF) and impaired mucociliary function. These effects of cigarette smoke on the airway epithelium leads to pneumococcal biofilm formation and disease persistence. ¹⁶ With permission.

1.1. Virulence factors

The host immune system is equipped with protective host defence mechanisms working to prevent pathogenic colonisation and invasion. These components include the mucociliary escalator, immunoglobulins and pattern recognition receptors (PRR) ¹⁶. However, the pneumococcus can avoid immune recognition, facilitate nasopharyngeal colonisation and invasion due to the activities employed by its virulence factors ⁹. The main virulence factors of the pneumococcus are: the

capsule; the pore-forming toxin pneumolysin (Ply); and a number of surface proteins²² as summarised in Table 1.1.

Table 1.1: Pneumococcal virulence factors involved in host immune diversion.

Pneumococcal virulence determinants	Function
Capsule	<ul style="list-style-type: none"> • Avoids mucus entrapment in the nasal area⁹ • Inhibits phagocytosis²³ • Aids in attachment and colonization of epithelial cells²⁴
Pneumolysin	<ul style="list-style-type: none"> • Interrupts epithelial cell integrity²⁵ • Binds to membrane cholesterol²⁶ • Cytolytic to ciliated bronchial cells²⁵
Autolysin (LytA)	<ul style="list-style-type: none"> • Mediates pneumolysin release via autolysis²⁷ • Aids in nasopharyngeal colonisation²⁸
Biofilm	<ul style="list-style-type: none"> • Decreases susceptibility to antibiotics²⁹ • Promotes pneumococcal gene transfer³⁰ • Prevents pneumococcal immune recognition²⁹
Choline bindin protein A(CbpA)	<ul style="list-style-type: none"> • Aids in host cell adhesion²⁵ • Binds to laminin³¹
Choline binding protein E (CbpE)	<ul style="list-style-type: none"> • Binds to plasminogen³²

Pneumococcal surface protein A (PspA)	<ul style="list-style-type: none"> • Interacts with lactoferrin ³³ • Interferes with complement phagocytosis ³⁴ • Promotes nasopharyngeal cell invasion ³⁵
Pneumococcal surface protein C (PspC) Neuraminidase	<ul style="list-style-type: none"> • Prevents complement deposition ³⁶ • Promotes adherence and colonisation to nasopharyngeal epithelium ³⁵. • Promotes biofilm formation ³⁷
Hyaluronidase	<ul style="list-style-type: none"> • Mediates bacterial dissemination ³⁸ • Aids in nasopharyngeal colonization ³⁸
Endonuclease A (EndA)	<ul style="list-style-type: none"> • Lyses neutrophil extracellular trap (NETs) ³⁹
ClpP protease	<ul style="list-style-type: none"> • Induces apoptosis in human neuroblastoma cells ⁴⁰

1.2. Anti-pneumococcal host defence

S. pneumoniae colonises the upper respiratory tract of healthy individuals without clinical manifestations and forms part of the normal flora ⁴¹. A Gambian study illustrated that the nasopharyngeal carriage of pneumococcus increased drastically from birth to 10 weeks, with a peak colonisation of 90% ⁴². However, if the barriers to the sterile environments such as the lower respiratory tract, lung, cerebrospinal fluid (CSF) and blood, lose their ability to prevent microbial entry, the pneumococcus can penetrate and cause an opportunistic infection ⁴³. There are several immune mechanisms that prevent pneumococcal infections; the main mechanisms are described below.

1.2.1. Immune defences against pneumococcus

Nasopharyngeal colonisation precedes invasive pneumococcal disease and is followed by breaching of the mucous membrane to the sub-epithelial tissue⁴⁴. The host defence against pneumococcus at the nasopharyngeal mucosal site is mediated by polymeric immunoglobulin (Ig) A (pIgA) and innate immune responses including the complement system⁴⁵. The pIgA, which is contained within the mucus, inhibits the binding of bacteria to the host epithelial cell by facilitating opsonised phagocytosis⁴⁶.

1.2.1.1. Complement defence against pneumococcus

The complement (C) system consists of three pathways; namely the classical, lectin-binding and alternative pathways, activated respectively by C1-antibody (IgM or IgG)-antigen complexes, bacterial carbohydrate moieties (mannose) bound to mannose-binding lectin (MBL)-associated serine protease (MASP) and hydrolysed serum C3 (C3b/C3bi)⁴⁶. All of these pathways result in the production of several key complement components, including the membrane attack complex (MAC), which induces cell lysis due to osmotic imbalance⁴⁷. In the setting of a pneumococcal infection, the classical pathway plays a major role in the recognition and phagocytosis of this pathogen⁴⁸.

Pattern recognition receptors (PRR) including Toll-like receptor (TLR)-2, TLR-4, TLR-9, nucleotide oligomerisation domain (NOD)-1 and deoxyribonucleic acid (DNA) sensors on antigen-presenting cells (APC) recognise a broad range of pneumococcal antigens known as pathogen-associated molecular patterns (PAMP). The recognition of PAMPs result in APC activation, production of inflammatory cytokines (including tumour necrosis factor- α [TNF- α], interleukin [IL]-1 and IL-6) and neutrophil chemotaxis⁴⁴. The main role of IL-1 and TNF- α in this setting is to increase the permeability and vasodilation of the vascular endothelium, while IL-6 has been shown to assist in pneumococcal resistance in murine models⁴⁹. The recruited neutrophils and other leukocytes, as well as activated complement, facilitate the containment or removal of the pneumococcus⁴⁴.

1.2.1.2. Combating Immune cells against pneumococcus

During a subclinical pulmonary infection, *S. pneumoniae* is opsonised by complement molecules, with the main objective of effectively removing this pathogen through phagocytosis⁵⁰. This objective is carried out by alveolar and monocyte-

derived macrophages that mediate pneumococcal clearance through the production of nitrogen species⁵¹. In some instances, ingested bacteria may not be effectively killed by macrophages that have reached their killing capacity, it is under these conditions that the incapable macrophages will undergo apoptosis, which is enough to mask clinical manifestations of the disease and eliminate the threat. However, if the macrophages fail to eliminate the threat through apoptosis, recruitment of neutrophils and T helper (Th) cells commence that lead to production of proteases and inflammatory cytokines which effectively mediate the killing of the ingested pathogen (reviewed in⁵¹. Although the inflammatory reactions are crucial in mediating an effective bacterial clearance, exacerbated inflammatory responses can cause tissue damage which could be deleterious to the host⁵².

1.3. Mechanisms of pneumococcal colonisation

As described, the prerequisite for pneumococcal infection is based on nasopharyngeal colonisation. The first step required is the release of the quiescent pneumococcus from innocuous biofilm, a process believed to be induced by host signals responding to respiratory viral infections. The invasive pneumococcus becomes encapsulated, blocking deposition of complement proteins to its surface, resulting from the genotype and phenotypic shift that promotes its survival and pathogenicity^{53,54}. The pneumococcus can migrate through epithelial cells via two mechanisms; firstly, by taking advantage of the activated epithelial cells expressing PAFr that binds pneumococcal phosphorylcholine (ChoP), thus allowing it to migrate via the PAFr recycling pathway. Secondly, the pneumococcus can enter the polymeric-immunoglobulin receptor (plgR) recycling pathway by pneumococcal choline-binding protein A (Cbp A) that binds to plgR, a molecule that assists IgM and IgA in cellular translocation⁴⁴. *S. pneumoniae* employs various mechanisms that aid in prevention of complement C3 deposition and phagocytosis (Figure 1.3). Autolysin LytA induced autolysis mediates release of the intracellular toxin pneumolysin (Ply), a protein that is ubiquitously expressed by invasive pneumococci⁵⁵. Activation of Ply interferes with host immunity in various ways including: stimulation of apoptosis in macrophages, neutrophils and neuronal cells and activation of host complement and proinflammatory activities (reviewed by⁵⁶.

Additional virulence factors that target complement proteins include pneumococcal surface protein C (PspC) that can bind to Factor H, a molecule that

regulates the alternative complement pathway, thereby inhibiting C3b production. It has also been shown that PspC can bind to human sIgA, reducing opsonisation⁵⁴.

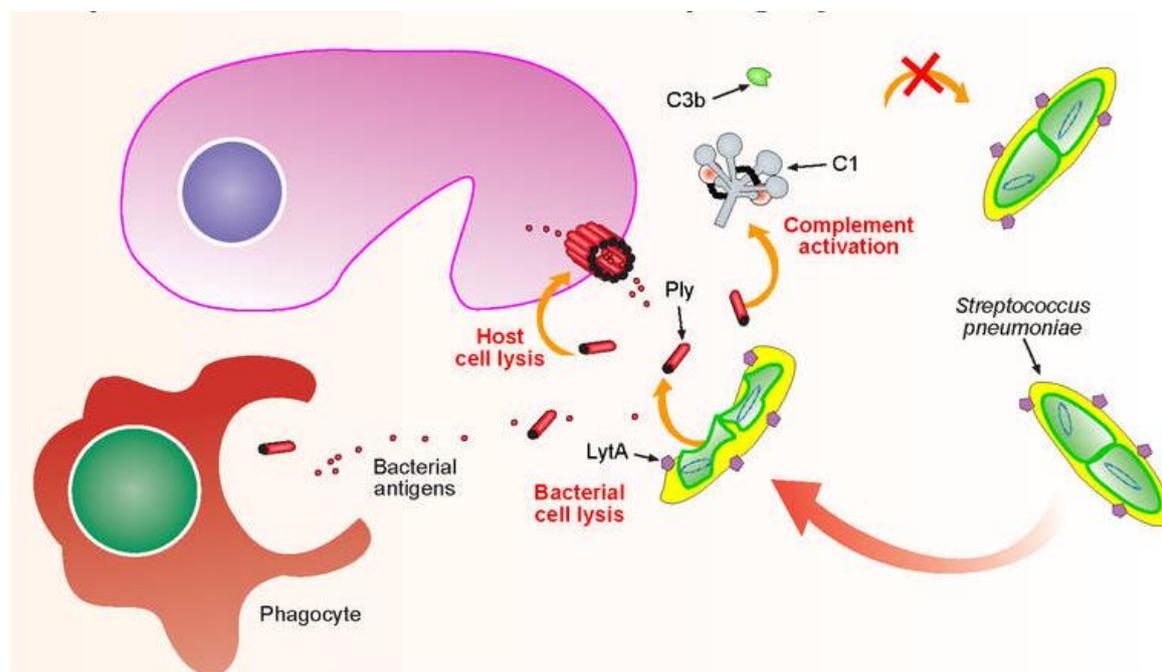


Figure 1.3: Complement evasion and inhibition of phagocytosis strategy employed by the *S. pneumoniae*. LytA released by *S. pneumoniae* mediates the release of Ply leading to complement activation, host cell lysis and inhibition of phagocytosis. [Used under terms of use⁵⁷](#).

1.4. Resistance genes of *S. pneumoniae*

Treatment of bacterial infections remains a continuous global challenge despite the development of effective vaccines and/or antimicrobial agents; mainly due to the development of antimicrobial resistance mechanisms⁵⁸. Two such mechanisms, (i) drug efflux, mediated by *mef(A)* gene and (ii) target modification, mediated by *erm(B)* gene, confer macrolide resistance in pneumococci⁵⁹ and are well described. Macrolides are bacteriostatic antibiotics that inhibit protein synthesis by binding to the 50S ribosomal subunit. They are used in severe CAP as immunomodulatory agents.

1.4.1. Efflux pump gene: *mef(A)*

Macrolide efflux pumps are proteins of the major facilitator superfamily (MFS), which comprises 12 transmembrane domains interconnected by hydrophilic loops⁶⁰. These

proteins, mainly expressed in Gram-positive bacteria, function as antiporters that actively export macrolide antibiotics to the extracellular compartment of bacteria^{61,62}. The first *mef* gene to be discovered, the *mef(A)*, was initially described in *S. pyogenes*⁶³ but has since been found to occur in other streptococcal species, including *S. pneumoniae*⁶⁴. The *mef(A)* has been found to be carried by Tn 1207.1, a genetic element of the transposon described in *S. pyogenes*⁶⁵. Another *mef* gene, *mef(E)*, that exhibits 90% homology to *mef(A)*, is carried by a macrolide efflux genetic assembly (mega) that is relatively close to that of *mef(A)*^{66,67}. Macrolide resistance due to *mef(E)* has been found to be synergistic with *mel*, a gene encoding an ATP-binding cassette (ABC) transporter protein which lacks a membrane binding domain⁶⁸. Although they are carried by distinct genetic elements, both the *mef* genes confer the 'M phenotype' resistance⁶¹. The efflux pump mechanism, encoded by the *mef(A)* gene exports antimicrobial agents to the exterior environment of the pneumococcus thus promoting multidrug intrinsic resistance⁶⁹. The expression of the *mef(A)* gene confers low level resistance in the pneumococcus to macrolides with minimum inhibitory concentrations (MIC) range of 1 - 32 µg/mL¹⁷, and the efflux pump inhibits intracellular macrolide accumulation conferring resistance to the 14- and 15-membered ring macrolides (M phenotype), but not the 16-membered ring macrolides^{70,71,72}.

1.4.2. Methylase gene: *erm(B)*

Target modification and drug efflux mechanisms are the two most common antimicrobial resistance mechanisms used by the pneumococcus^{70,73}. Erythromycin ribosomal methylase (*erm*) family genes are responsible for encoding methyltransferase proteins that modify the N6 position of nucleotide A2058 (*E. coli*) of the 23S ribosomal ribonucleic acid (rRNA) either by mono- or di-methylation⁷⁴.

The first emergence of pneumococcal macrolide-resistant strains was reported in the United Kingdom in the late 1950s⁷⁵. An example of a target modification mechanism is the *erm(B)* gene product, RNA methylase, which dimethylates a specific adenine nucleotide (A2058 in domain V) in 23S rRNA preventing the interaction of macrolides, lincosamides and streptogramin B (MLS_B) antibiotics with the ribosome, accounting for cross-resistance to the 3 classes of drugs termed MLS_B phenotype^{70,71}. In contrast to *mef(A)*, expression of *erm(B)*, carried by conjugate transposon Tn1545 along with other resistance genes such as tetracycline

resistance gene *tet(M)*, confers high level resistance in the pneumococcus to the macrolides, with erythromycin MIC > 64 µg/mL^{17,76}. The *erm(B)* gene can either be inducible or constitutively expressed; in inducible resistance, the pneumococcus produces an inactive messenger RNA (mRNA) that cannot translate RNA methylase in the absence of an inducer. However, in the presence of macrolide inducers, the mRNA sequence is rearranged and activated, enabling the synthesis of methylase in the ribosome, leading to resistance of the inducers^{77,78}. Since the *erm(B)* gene is inducible only by 14- or 15-membered macrolides, it remains susceptible to non-inducer macrolides and ketolides with 16- and 14-membered rings, respectively⁷⁵. In contrast, constitutive resistance expression produces an active mRNA that encodes for RNA methylase which acts independently of macrolide inducers⁷⁹.

1.4.3. ATP-binding cassette (ABC)-transporter gene: *SP2003*

ABC-transporters make up a superfamily of bacterial protein structures associated with transmembrane transportation of a wide range of substances, including micronutrients, sugars, amino acids and peptides, and antimicrobial peptides. To date, 68 ABC-transporters have been identified in *S. pneumoniae*. Many ABC-transporters have been found to play an important role in pathogen-host interactions. These interactions may include; aiding bacterial growth in nutrient-restricted conditions and/or reducing the sensitivity of the bacteria to host-derived antimicrobial peptides or antibiotics. The ABC-transporter gene, *SP2003* has been identified as an ABC-exporter associated with the two-component system and is reported to transport drugs and other antimicrobial peptides out of the bacterium⁸⁰. *SP2003* is also recognised as a regulator of the stress response and has been shown to be significantly upregulated after short exposures to vancomycin⁸¹.

1.5. Cigarette smoking and biofilm formation

1.5.1. Cigarette smoking

Cigarette smoking remains a major public health threat worldwide, with more than 6 million smokers in South Africa, and accounting for approximately 42 000 smoking-related deaths annually⁸². Importantly, the prevalence of cigarette smoking among adults with human immunodeficiency virus (HIV) in North America and Europe is more common (40-70%)^{83,84} compared to the general population (15-20%)⁸⁵ and these individuals are also reported to be less likely to stop smoking^{86,87}. Helleberg *et al.* reported that HIV-related and non-HIV related disorders, as well as death, are

significantly increased in HIV-positive individuals who also smoke cigarettes and that the increased mortality is associated with smoking rather than HIV-related factors⁸⁶. In addition, smoking has been well documented to be an increased risk factor for respiratory complications such as tuberculosis, and chronic obstructive pulmonary disease^{84,85,86,88,89,90,91,92}. Biofilm-associated diseases, such as community-acquired pneumonia (CAP), otitis media, vaginosis and chronic periodontitis are also found to occur more frequently amongst individuals who smoke⁹³.

Host microbiota plays a protective role in the hosts immunity, and it competes with invasive pathogens, offering colonisation resistance^{94,95}. Cigarette smoking has been linked to microbiota dysbiosis, increasing the chances of *S. pneumoniae* colonisation amongst other pathogenic bacteria^{96,97,98,99}. Recently, Shen *et al.* (2017) have shown that nasal dysbiosis in cigarette-smoke exposed mice occurs as a result of established nasal pneumococcal colonisation, which in turn increases the frequency of other pathogenic bacteria such as *Neisseria*¹⁰⁰. Moreover, this increased burden of the pneumococcus and other pathogens was further linked to a decrease in normal flora commensals, predisposing smokers to invasive pneumococcal diseases^{96,101,102}. Exposure to cigarette smoke influences pneumococcal virulence by exposing host receptors like PAFr, which are recognised by pneumococcal surface proteins, aiding in respiratory epithelium adhesion¹⁰³. This increase of adherence due to smoke exposure is required in airway colonisation¹⁶. Additionally, after successful colonisation, cigarette smoke has been associated with the development of biofilm, which is important in the persistence of bacterial infections and has been shown to occur in approximately 80% of respiratory microbial infections¹⁷. Biofilm formation by a variety of bacteria has been shown to be affected by cigarette smoke exposure^{19,104,105}. Amongst those, *S. pneumoniae* biofilm formation was shown to be upregulated by CSC *in vitro* in a concentration dependent manner¹⁹. In another study it was shown that the two-component system genes, previously reported to be involved in biofilm formation in *S. mutans*, were also up-regulated on exposure to CSC in *S. pneumoniae*¹⁰⁶. These pro-biofilm effects of cigarette smoke have directed scientists to believe smoking influences treatment failure since bacteria encased within biofilms evade immune recognition and the efficacy of antibacterial treatment is reduced, allowing bacterial infections to persist¹⁶.

1.5.2. Biofilm formation

Biofilms are specialised multicellular communities of bacteria embedded in a self-produced extracellular matrix made up of various polymeric molecules, which enhance disease persistence due to reduced susceptibility to antimicrobial agents and resistance to immune recognition²⁹. For biofilm formation to occur: the planktonic bacteria must firstly adhere to the surface of abiotic or biotic components such as medical implanted devices or host tissue, respectively¹⁰⁷. Secondly, the bacteria then form multicellular aggregates, including channels that allow oxygen to move deep into the biofilm. Lastly, at the maturation phase of biofilm formation, bacteria detach from the biofilm to infiltrate and occupy other body niches^{29,108} (shown in Figure 1.4). A study by Vidal *et al.* (2013) demonstrated that differences in epithelium tissue affect the biomass of early biofilm formation, based on the discoveries that for example, an invasive clinical isolate of the pneumococcal serotype 23F promotes robust biofilm formation on lung cells compared to pharyngeal cells²¹. Another study carried out by Mutepe *et al.* (2013), has shown that exposure of pneumococci to CSC for 16h resulted in a significant dose-related increase in biofilm formation ($p \leq 0.05$ at 80 and 160 $\mu\text{g/mL}$) with a down-regulation of expression of the *ply*, an important virulence factor of *S. pneumoniae*. This cigarette smoke augmentation of pneumococcal biofilm formation is likely to favour microbial colonisation and persistence¹⁹.

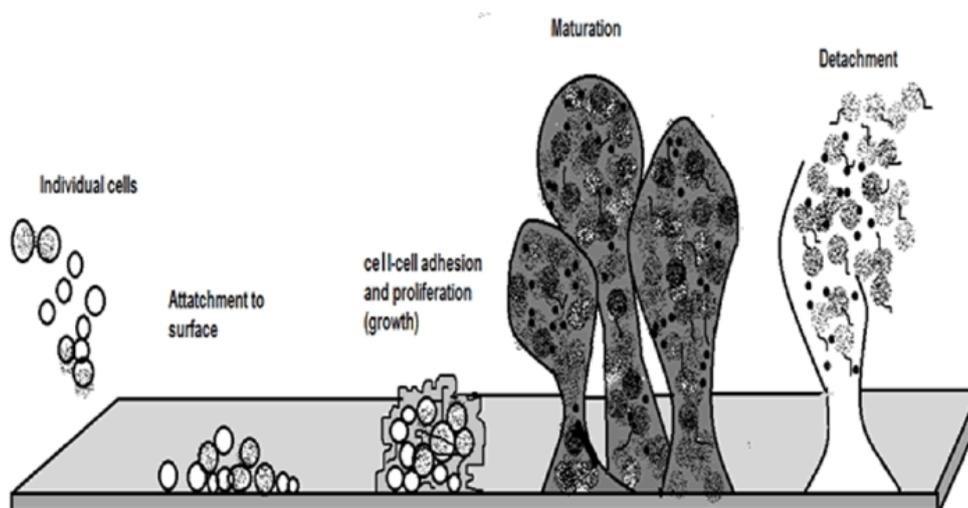


Figure 1.4: Stages involved in biofilm formation: surface attachment, growth and maturation phase, and detachment. [Used under CC BY 4.0 License.](#)¹⁰⁹

Bacteria within biofilms are more protected from environmental stresses when compared to their planktonic counterparts. Biofilm-encased bacteria have been shown to be less sensitive to antibiotics and are concealed from host immune recognition^{29,110}. Anderl *et al.* reported that a strain of β -lactamase-negative *Klebsiella pneumoniae* in biofilm resisted killing by ampicillin at concentrations as high as 5000 $\mu\text{g/mL}$, compared to the same strain grown in a planktonic growth phase with an ampicillin MIC of 2 $\mu\text{g/mL}$ ¹¹¹. In addition, bacteria within biofilm retain the capacity to divert deposition of complement component C3b, C-reactive protein and C1q on their cell surfaces, obstructing opsonisation and destruction by the immune system²⁹. In addition, activation of the alternative complement pathway is prevented by suppressing the PspC (Domenech *et al.* 2013). In general, biofilm formation is usually associated with persistent infections such as chronic lung infection¹¹².

1.5.3. Mechanisms within biofilm

1.5.3.1. Quorum sensing

Several mechanisms that promote bacterial antimicrobial tolerance exist within a biofilm, for example the quorum sensing mechanism (shown in Figure 1.5). Quorum sensing is the ability of cells to communicate with one another¹¹³, which allows these cells to determine population density via extracellular signalling molecules called autoinducers in their local milieu¹¹⁴. This mechanism controls processes that require bacterial co-operation such as biofilm formation, bioluminescence production and regulation of differential gene expression^{114,115}. The *ComABCDE* and the *LuxS/Autoinducer 2* (*LuxSAI 2*) genes are the most studied quorum sensing genes in *S. pneumoniae*. These genes produce the competence stimulating peptide (CSP) and type 2 autoinducer (AI 2), respectively. Both of these quorum sensing signalling molecules accumulate in the extracellular compartment and can be detected by other cells, as well as themselves¹¹⁴. A study by Vidal *et al.* used a bioreactor to mimic the microenvironment of human respiratory epithelium. In the study, they reported that the initial attachment of biofilm, whether on abiotic or biotic surfaces, was mainly regulated by the *LuxSAI 2* quorum sensing molecules while the *ComABCDE* molecules regulated early biofilm formation on biotic but not abiotic surfaces²¹.

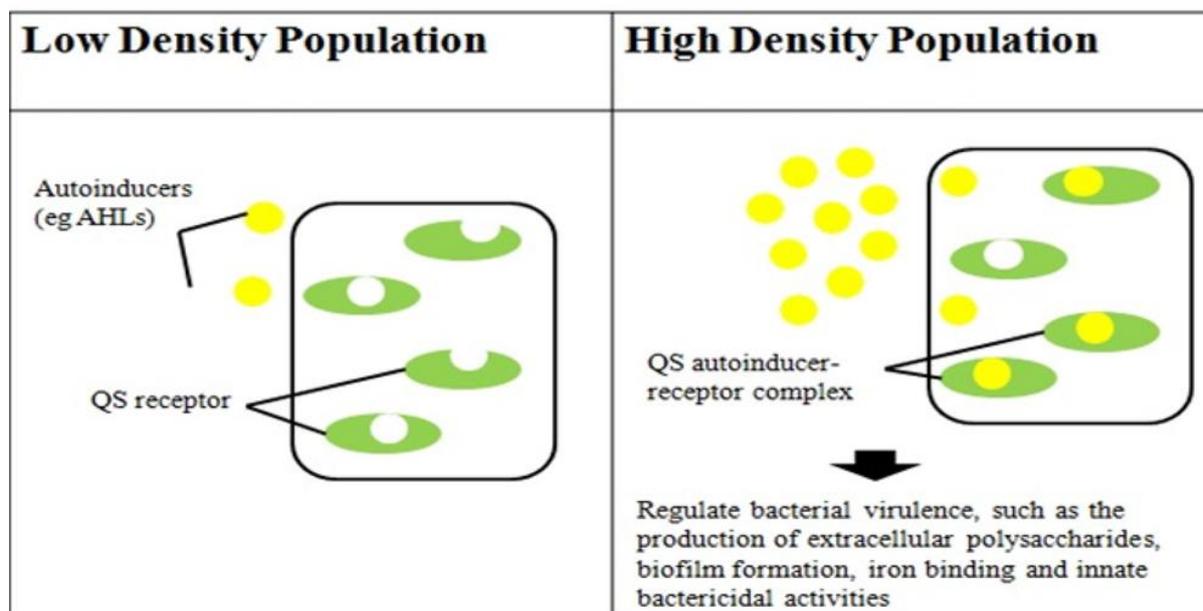


Figure 1.5: Quorum sensing mechanism. [Used under CC BY 4.0 License.](#) ¹¹⁶

1.5.3.2. Gene transfer

Formation of biofilm plays a pivotal role during pneumococcal colonisation by allowing the pneumococcus to initiate competence, enabling acquisition of extracellular genetic material by a process known as horizontal gene transfer that takes place within colonising rather than invasive pneumococci at the nasopharyngeal site ^{117,118,119}. Competence in *S. pneumoniae* is initiated when ComC-derived competence stimulating peptide (CSP, 1-10 ng/mL) is released in an autocrine manner and binds to its histidine kinase receptor (ComD). The function of the active ComD is to phosphorylate its cognate response regulator (ComE) ^{120,121,122}. The function of ComE is to induce the expression of genes that contain ComE binding sites at their promoter regions ^{123,124,125}. Since the promoter region of ComX contains the binding site of ComE, its expression marks the synthesis of the late competence genes that are involved in the uptake and incorporation of extracellular DNA ^{123,124,126}. The transformation process is crucial as it allows the pneumococcus to acquire extracellular DNA in the vicinity, aiding in bacterial acquisition of novel traits and antibiotic resistance ^{127,128,129}. It has been shown that the acquisition of the extracellular DNA is achieved when a sub-fraction of the pneumococcus population undergoes lysis ¹³⁰. Several other studies have also shown that the biofilm matrix contains elevated levels of DNA that arise as a result of autolysis ^{131,132,133,134}.

Clarithromycin interacts with the 23S rRNA forming part of the peptidyltransferase centre (PTC), a vital component of the 50S ribosomal subunit. This process leads to subsequent dissociation of peptidyl-tRNA hindering mRNA translation resulting in the inhibition of protein synthesis^{136,137}. Resistance to macrolides by *S. pneumoniae* is mainly due to target modification and efflux pumps. The *erm(B)* gene, as discussed above, confers resistance by coding for a methylase protein that methylates the target site, blocking macrolide-rRNA interaction. Secondly, the bacterial *mef(A)* gene results in the production of proteins that export the antibiotics to the exterior environment (efflux pump), allowing the bacteria to survive^{72,138}.

1.7. Treatment of pneumococcal infections

Treatment of pneumococcal infections such as CAP, a leading cause of mortality and morbidity, is based on the severity of the infection upon presentation^{139,140,141}. The majority of guidelines recommend either monotherapy of moxifloxacin or levofloxacin; or a combination therapy of β -lactam coupled with either a macrolide or ciprofloxacin to be administered to patients suspected to have CAP at non-intensive care units^{138,142,143,144,145}. Although antibiotic use often leads to resistance, the negative impact on the emergence of resistance has not yet been documented on the extensive use observed of fluoroquinolones and macrolides^{138,142}. The treatment of respiratory tract infections with antimicrobial agents¹⁴⁶ can result in the development of antibiotic resistant strains, affecting both patient care and health economics negatively^{147,148}. Cigarette smoking has been documented to promote respiratory tract infections, enhance the presence of bacteria and viruses in the lungs, and reduce local immune responses¹⁴⁹; resulting in increased antibiotic use in these patients¹⁵⁰. It has been demonstrated that the level of treatment failure of *Helicobacter pylori* infections in smokers were doubled compared to those of non-smokers¹⁵¹. Another study, by Blix *et al.* showed that increased cigarette consumption is associated with increased broad spectrum antibacterial prescription¹⁵². It is therefore important to understand the impact that smoking, alone and in conjunction with antibiotic use imposes on the survival mechanisms employed by *S. pneumoniae*.

1.8. Aim:

The aim of this study, therefore, is to determine the effects of cigarette smoke condensate on pneumococcal growth, biofilm formation and related alterations in antimicrobial resistance gene expression, in a sub-MIC antimicrobial setting.

1.9. Objectives:

The objectives of this research are:

- To determine if CSC affects planktonic pneumococcal growth and biofilm formation in a sub-MIC antimicrobial setting.
- To investigate if exposure to CSC, alone and in combination with clarithromycin (at sub-MIC concentrations), alters antimicrobial resistance gene expression in *S. pneumoniae* macrolide-susceptible strain 172 and macrolide-resistant strains expressing *mef(A)* (strain 521) and *erm(B)* (strain 2507), as well as to gain insight into the possibility that *SP2003* may be involved in treatment failure and may be linked to the development of resistant strains of *S. pneumoniae*.

Chapter 2: Pneumococcal Planktonic Growth

The present chapter focuses on the effect of CSC and clarithromycin, alone and in combination, on the *S. pneumoniae*, subtype 23F, macrolide-sensitive strain 172 following a 16h overnight incubation. In addition, the effect of CSC alone on the planktonic growth phase of two macrolide-resistant strains of the pathogen (521 and 2507) was also investigated.

2.1. Introduction

Planktonic bacteria play a pivotal role in bacterial biofilm formation, serving as precursors for surface attachment at the initial stage of formation. At the end stage of biofilm formation, mature biofilms release planktonic bacteria which disseminate to other body niches causing infections and also completing the life-cycle of the biofilm¹⁵³. It has previously been documented that dissemination of the pneumococci beyond the nasopharynx can cause invasive pneumococcal disease to otherwise sterile sites such as the central nervous system (CNS), lungs and blood^{154,155}. Therefore, factors that promote the growth of the planktonic bacteria also predispose the host to infections. Among these factors, exposure to cigarette smoke has been found to promote planktonic growth, albeit modestly, and skew the host immunity, favouring successful colonisation by the bacteria¹¹.

2.2. Materials and Methods

2.2.1. Study design

The present study investigated the effect of two concentrations of CSC (80 and 160 µg/mL) on the planktonic phase of growth of *S. pneumoniae* strains 172, 521 and 2507. The combination treatment of CSC (80 and 160 µg/mL) with clarithromycin at a sub-minimum inhibitory concentration (MIC) (0.0015 µg/mL) was further investigated on the planktonic growth phase of the macrolide-sensitive strain 172. The effect of the CSC combined with clarithromycin was not investigated in the case of strains 521 and 2507 due to the resistance of these strains to clarithromycin.

2.2.2. Bacterial strains

The wild-type pneumococcal clinical isolate strain 172 (macrolide-susceptible), as well as the two macrolide-resistant strains, 521 and 2507, all serotype 23F, were

kindly donated by the National Institute of Communicable Diseases (NICD), Johannesburg, South Africa. Seed cultures were maintained and stored at -80°C , and used as inoculum in the experiments described below.

2.2.3. Chemicals and reagents

Unless otherwise indicated, all chemicals and reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2.4. Cigarette smoke condensate

Cigarette smoke condensate (CSC) was purchased from Murty Pharmaceuticals (Lexington, KY, USA) and used at final concentrations of 80 and 160 $\mu\text{g}/\text{mL}$. Solvent control dimethyl sulfoxide (DMSO) was included in all experiments. According to a study carried out by Davies and Day, combustion of a single cigarette produces about 26.3 mg smoke condensate ¹⁵⁶.

2.2.5. Clarithromycin

Clarithromycin was solubilised in sterile distilled water with slight acidification (using 0.1N hydrochloric acid) to a stock concentration of 10 mg/mL. The clarithromycin was further diluted in tryptic soy broth (TSB) (Merck, Darmstadt, Germany) and used at a final sub-MIC concentration of 0.0015 $\mu\text{g}/\text{mL}$ for strain 172.

2.2.6. Culturing of bacteria

The pneumococcal cultures for strains 172, 521 and 2507 were grown as described by Mutepe and co-workers ¹⁹. Briefly, the pneumococcal strains (5 μL inoculum) were grown in 50 mL tubes containing approximately 20 mL TSB to the mid-logarithmic growth phase by incubating for 16h in a 37°C , 5% CO_2 environment (ESCO, Horsham, PA, USA). After incubation, the tubes were centrifuged at 2750 $\times g$ for 15 min at 25°C , the supernatant removed and the pellet re-suspended in fresh TSB. The bacterial suspension was optically standardised (OD 0.1) using the PowerWaveX spectrophotometer (Bio-Tec Instruments Inc., Winooski, VT, USA) equating to a final concentration of 1×10^6 cfu/mL.

2.2.7. Clarithromycin minimum inhibitory concentration (MIC) determination

The MIC of clarithromycin for the wild-type strain 172 was determined using a broth microdilution method as outlined in the EUCAST guidelines¹⁵⁷. The antibiotic stock solution was diluted in TSB to concentrations ranging from 32 - 0.00075 µg/mL in order to determine the MIC. The pneumococcal inoculum (100 µL, 1x10⁶ cfu/mL) was then exposed to clarithromycin in a 96-well plate, which was then incubated overnight for 16 hours (h) at 37°C, 5% CO₂ (ESCO). The MIC was considered as the lowest concentration that inhibited visible bacterial growth measured using a PowerWaveX spectrophotometer (Bio-Tech Instruments Inc.) at a wavelength of 540 nm and the identified sub-MIC concentration of 0.0015 µg/mL was then used in all subsequent experiments for strain 172.

2.2.8. Determination of bacterial growth

Following optical standardisation with the PowerWaveX spectrophotometer (Bio-Tech Instruments Inc.), the two macrolide-resistant pneumococcal strains (521 and 2507) were exposed to solvent control (DMSO) or CSC (at either 80 or 160 µg/mL) using a fixed incubation time of 16h. The effect of CSC (80 and 160 µg/mL) was also determined for the macrolide-susceptible strain, 172 in the presence and absence of clarithromycin. The different systems were then incubated for 16h at 37°C, 5% CO₂ (ESCO). The growth effects were determined spectrophotometrically at a wavelength of 540 nm (PowerWaveX).

To further investigate the time course of bacterial growth in the presence and absence of the various stressors, an additional set of experiments in which growth was monitored at 2 hourly intervals over an 18h period was performed.

2.2.9. Statistics

A total of three to six different experiments were conducted with triplicate determinations for each system for the *S. pneumoniae* planktonic growth phase. The results are expressed as the mean ± standard deviation (SD). Statistical significance was calculated using the Mann-Whitney U-test (two-tailed) and a p-value of <0.05 was considered significant.

2.3. Results

2.3.1. Effects of CSC and clarithromycin on the growth of strain 172

The effects of the sub-MIC concentration of clarithromycin (0.0015 µg/mL) and CSC (80 and 160 µg/mL), alone and in combination, on the macrolide-sensitive *S. pneumoniae* strain 172 are shown in Figure 2.1. These results demonstrate that exposure of strain 172 to clarithromycin alone at a sub-MIC concentration (0.0015 µg/mL) significantly inhibited bacterial growth ($p \leq 0.05$), while exposure to CSC alone at both concentrations tested caused statistically significant, albeit modest, increases in bacterial growth in agreement with the data for the time course studies presented in Figure 2.3 (a). However, exposure of the bacteria to the combination of clarithromycin (0.0015 µg/mL) and CSC (80 or 160 µg/mL) significantly inhibited bacterial growth to levels below those observed with clarithromycin alone ($p \leq 0.05$), possibly attributable to an augmentative effect of the antibiotic/CSC combination in delaying the lag phase to beyond the 16h incubation period of the experiment. As also observed with the time course data presented in Figure 2.3 (a), exposure of the pneumococcal strain 172 to CSC alone at either 80 or 160 µg/mL resulted in significant increases in bacterial growth ($p \leq 0.05$).

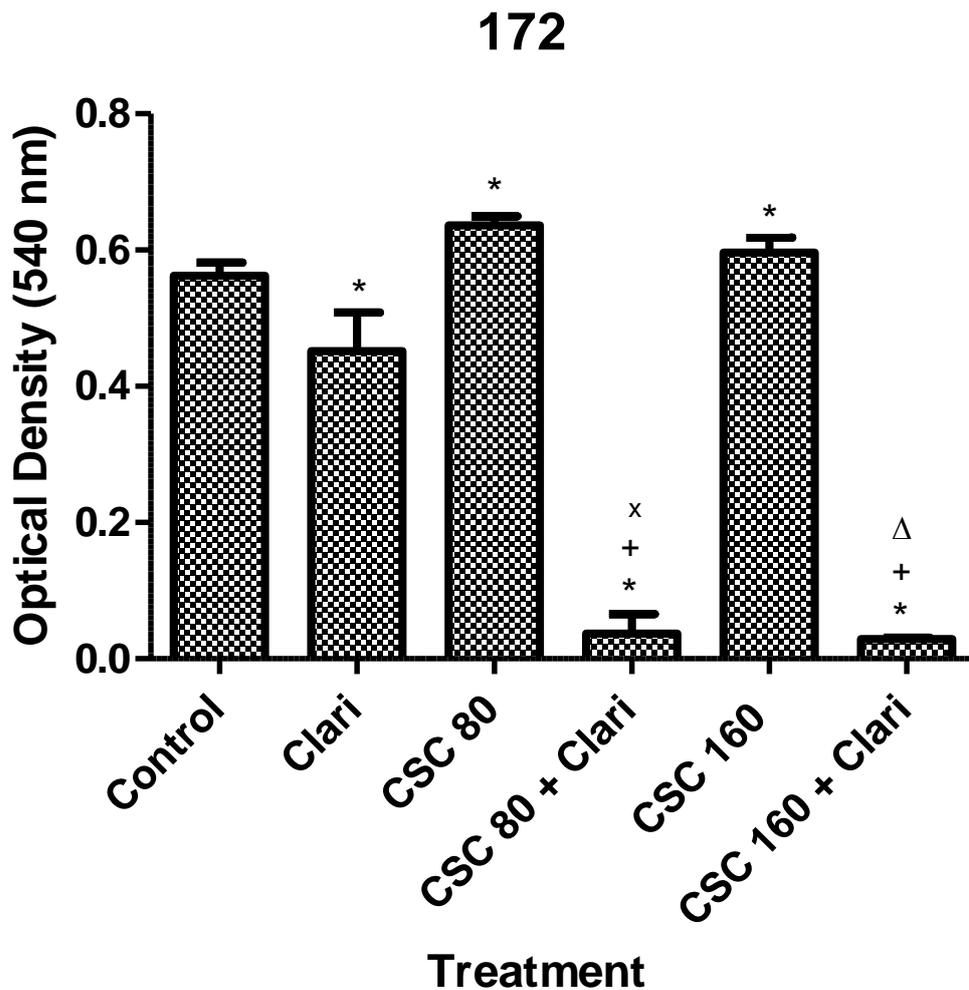
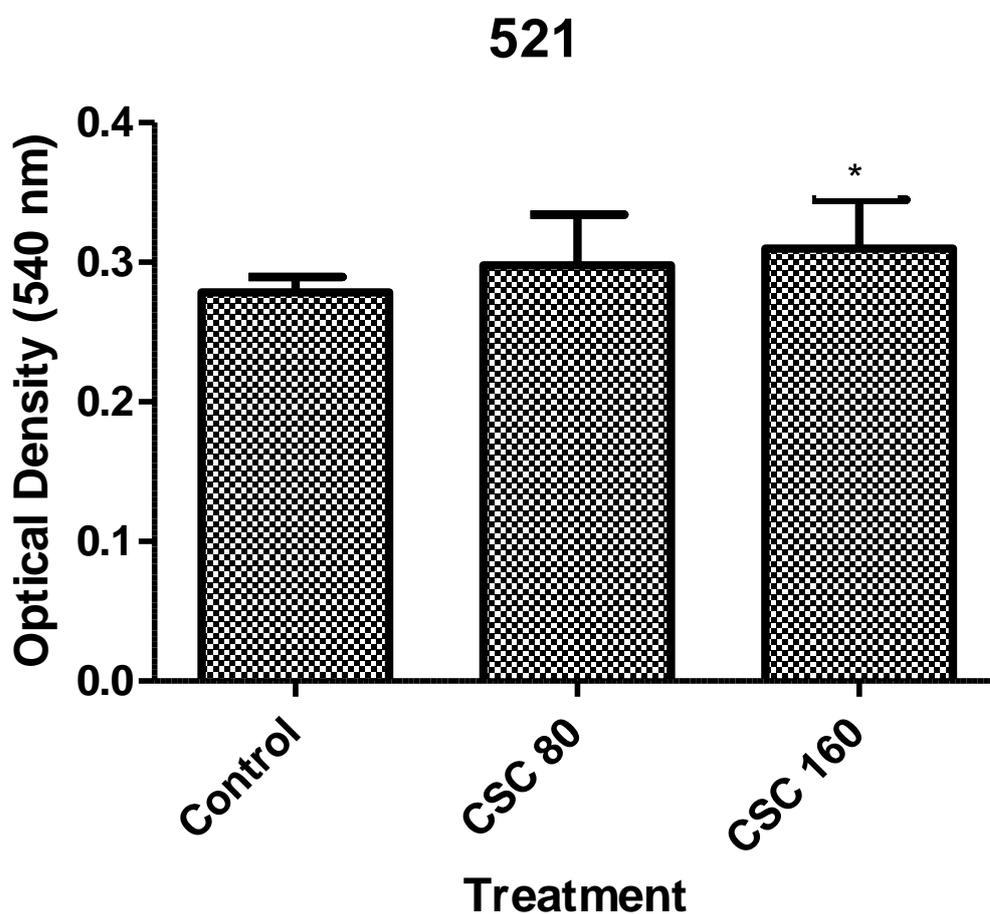


Figure 2.1 : Effects of clarithromycin (Clari, 0.0015 $\mu\text{g}/\text{mL}$) and cigarette smoke condensate (CSC; 80 and 160 $\mu\text{g}/\text{mL}$) alone and in combination on the growth of the macrolide-susceptible *S. pneumoniae* strain 172 measured spectrophotometrically at 540 nm. The results are expressed as the mean \pm standard deviation (SD) of six different experiments with triplicate determinations for each system in each experiment. The significance in comparison to the control (*), clarithromycin (+), CSC 80 (x) and/or CSC 160 (Δ) is indicated for $p < 0.05$.

2.3.2. Effects of CSC on the growth of strains 521 and 2507

The results showing the effect of CSC (80 and 160 $\mu\text{g/mL}$) exposure alone on the planktonic growth phase of strains 521 and 2507 of the pneumococcus are shown in Figures 2.2 (a) and 2.2 (b), respectively. The effect of clarithromycin alone was not investigated for the macrolide-resistant strains 521 and 2507 due to the high MIC values reported for this antibiotic. In the case of strain 521, a significant increase in planktonic growth was observed following treatment of the pneumococcus with CSC at 160 $\mu\text{g/mL}$ ($p = 0.05$). A modest, but not significant, increase in planktonic growth was also observed in the systems treated with 80 $\mu\text{g/mL}$ of CSC. In the case of strain 2507, a significant concentration-dependent increase in planktonic growth was observed for both CSC concentrations ($p < 0.05$ for both 80 and 160 $\mu\text{g/mL}$).



a

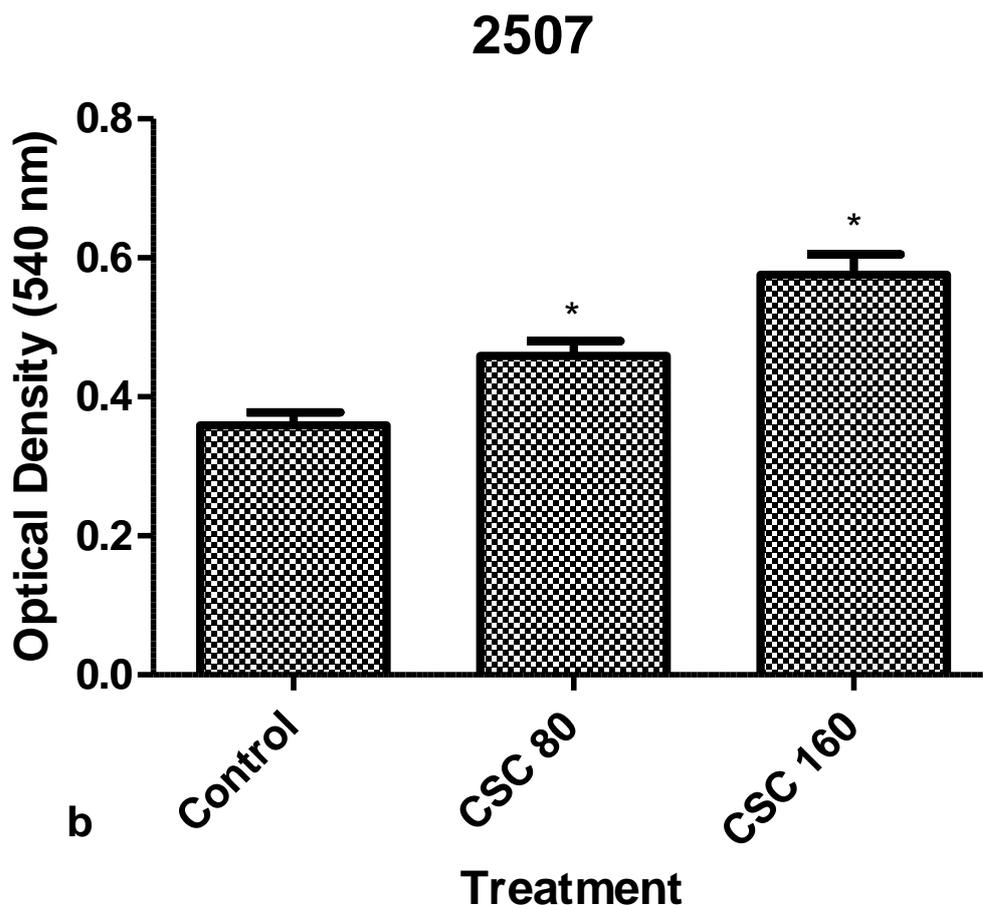


Figure 2.2: Effects of cigarette smoke condensate (CSC) on the planktonic growth phase of *S. pneumoniae* strains 521 (a) and 2507 (b). The results are presented as the optical density observed at a wavelength of 540 nm. The results are expressed as the mean \pm SD of 3 different experiments with triplicate determinations for each system. $p \leq 0.05$ compared to the solvent control is indicated with an asterisk (*).

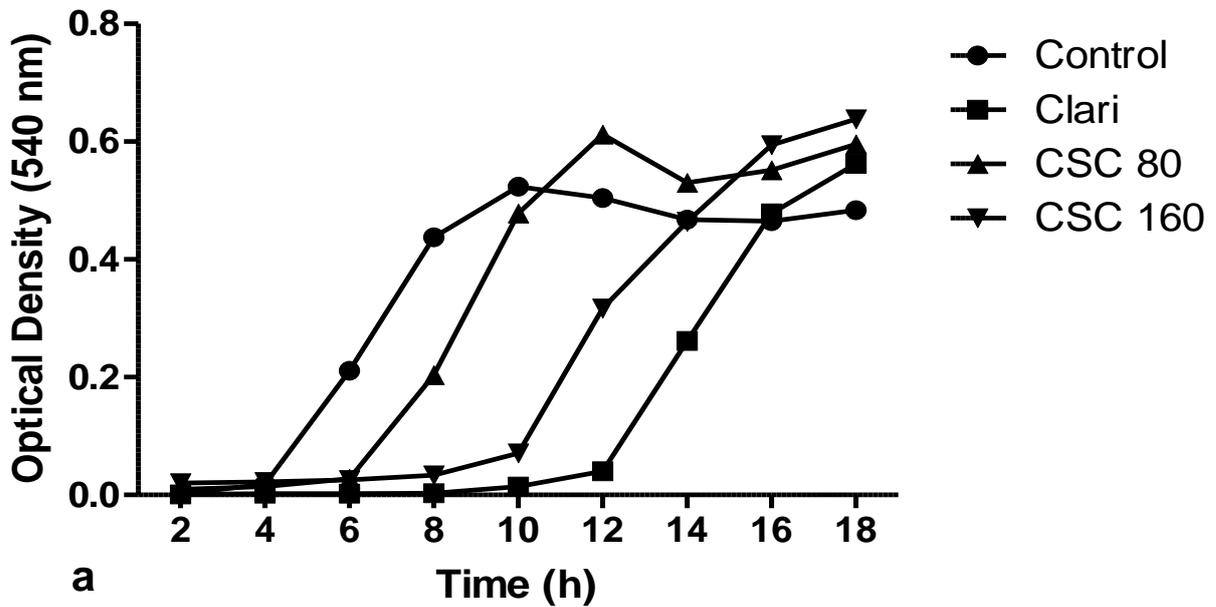
2.3.3. Time course of growth of *S. pneumoniae*

The results for the assessment of the time course of growth of strain 172 exposed to either clarithromycin (0.0015 $\mu\text{g}/\text{mL}$) or CSC (80 and 160 $\mu\text{g}/\text{mL}$) are shown in Figure 2.3 (a). The results indicate that there is an initial lag phase in the growth of *S. pneumoniae* of about 4h duration. This is followed by rapid, exponential growth until 8h, with a stationary phase in the growth of the unexposed, control bacteria being reached after 10h. In the case of CSC exposed at 80 and 160 $\mu\text{g}/\text{mL}$, the lag phase in the growth of the bacteria was prolonged, extending to 6h and 8h

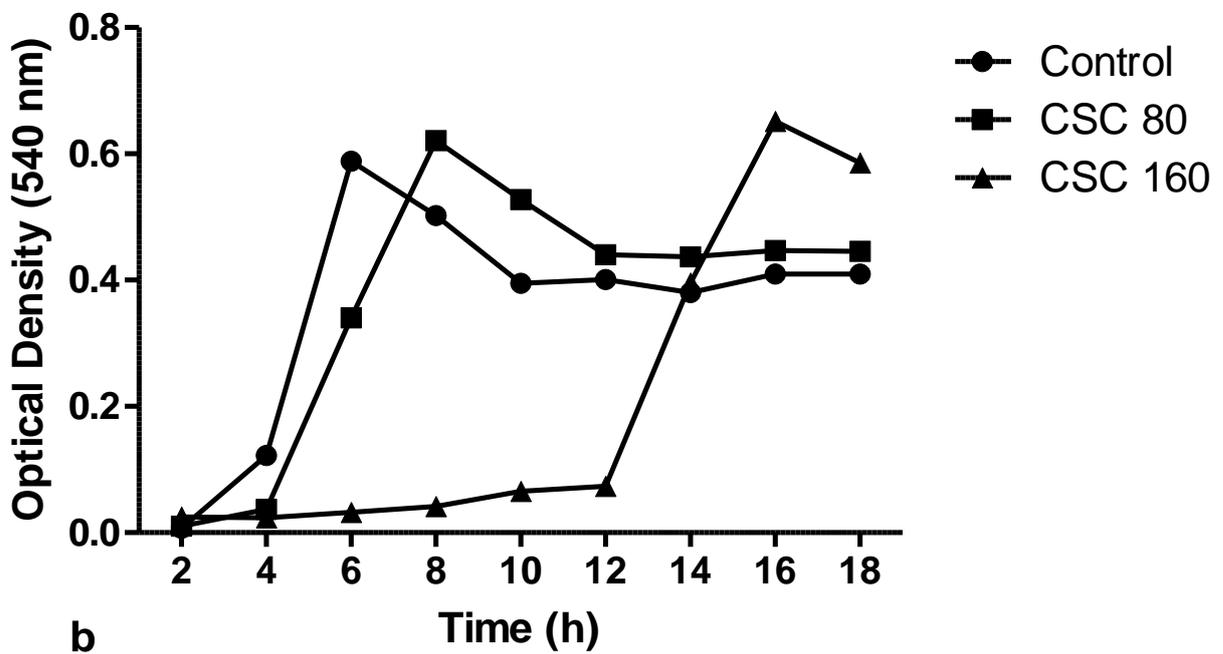
respectively. This was followed by an exponential growth phase that plateaued at 12 and 16 hours for CSC 80 and 160 $\mu\text{g/mL}$, respectively. Exposure of strain 172 to clarithromycin was also found to result in an extended lag phase of 12h. This was followed by a rapid rebound in growth that slowed at 18h, However, the experiment was terminated prior to determining whether the bacteria had indeed reached a stationary phase. Interestingly, *S. pneumoniae* exposed to either clarithromycin or CSC, at both concentrations tested, reached a stationary phase that appeared to be somewhat higher than that observed for the untreated control system.

The corresponding data for strains 521 and 2507 exposed to CSC (80 and 160 $\mu\text{g/mL}$) are shown in Figures 2.3 (b) and 2.3 (c), respectively. In contrast to strain 172, a much shorter lag phase was observed for the untreated, control strain 521, while, as with strain 172, CSC caused a concentration-dependent lag in the rate of growth of the bacteria, which was particularly evident at a concentration of 160 $\mu\text{g/mL}$ of the condensate. For the untreated, control strain 2507, the duration of the lag phase was about 4h, which was prolonged, extending to 6h and 10h in bacteria exposed to CSC of 80 and 160 $\mu\text{g/mL}$, respectively. Despite the delayed onset of growth, CSC-exposed bacteria eventually reached a stationary phase which was either equivalent to or slightly higher (only strain 521 at 160 $\mu\text{g/mL}$ CSC) than the unexposed control for both of the macrolide-resistant strains.

172



521



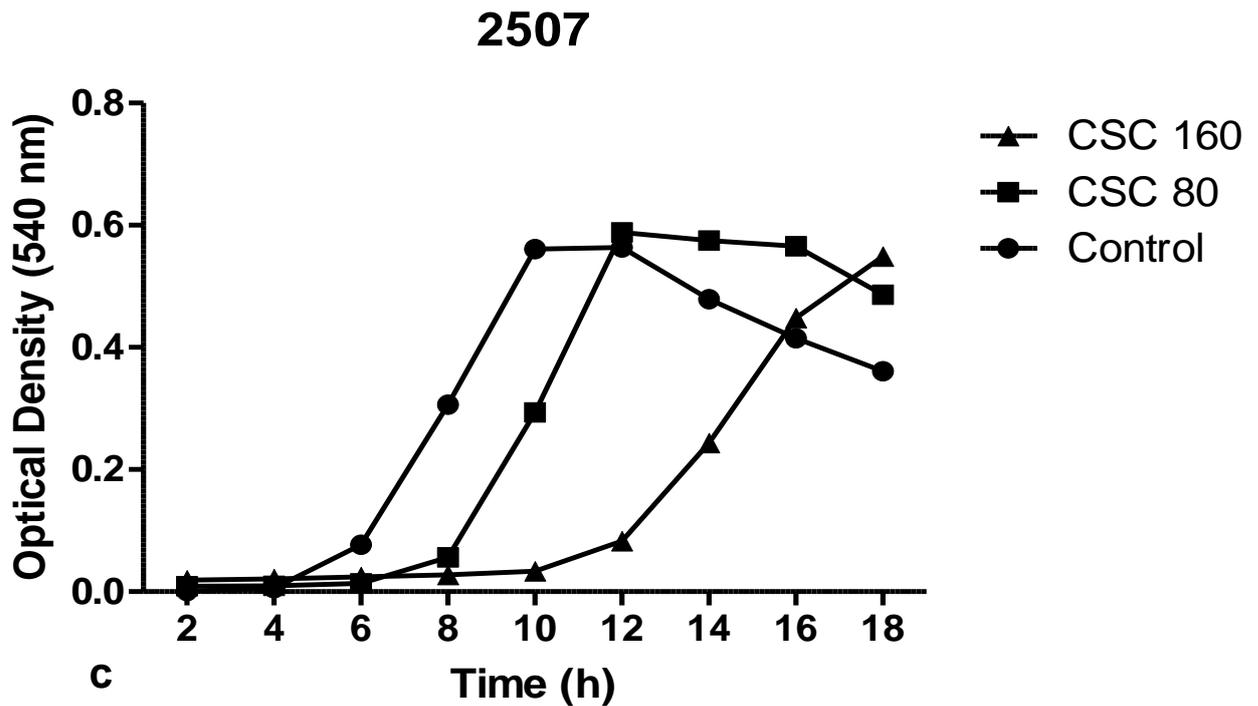


Figure 2.3: The effect of clarithromycin (0.0015 $\mu\text{g}/\text{mL}$) and CSC (80 and 160 $\mu\text{g}/\text{mL}$) on the time courses of growth of strains 172 (a), 521 (b) and 2507 (c) of *S. pneumoniae* over an 18h period. The results presented are from between three and four different experiments with triplicate determinations for each system.

2.4. Discussion

The results presented in this Chapter describe the effect of CSC (80 and 160 µg/mL) alone and in combination with a sub-MIC concentration of clarithromycin (0.0015 µg/mL) on the growth of *S. pneumoniae* (strains 172, 521 and 2507) following overnight incubation. Not surprisingly, the pneumococcal strains were found to exhibit an increase in the lag phase of their growth following the stress of exposure to either a sub-MIC concentration of clarithromycin or CSC. Bacteria commonly display a lag phase in their growth due to changes in their environment. This is a period of slowed growth while the bacteria are modified to take advantage of their new environment. These adaptations allow the bacteria to gain maximal biomass¹⁵⁸. This is reflected in the results showing that the stationary phase of the pneumococcal growth significantly exceeded that of the control system when the bacteria were exposed to either clarithromycin or CSC alone.

These delays in initiation of the log phase of pneumococcal growth following exposure of strain 172 to either clarithromycin or CSC, or of strains 521 and 2507 to CSC, were followed by a rebound in growth, presumably associated with recovery from stress, reaching stationary phase levels, which in some cases exceeded those of the untreated control systems. Similar increases in the planktonic growth of *S. pneumoniae* following exposure to CSC have been observed previously¹⁹. Interestingly, but not surprisingly, treatment of strain 172 with a combination of CSC with clarithromycin resulted in a significant, augmentative reduction in planktonic growth measured after 16h of incubation. This apparent inhibition of bacterial growth may be attributed to several possible mechanisms. These include: i) an augmentative prolongation of the lag phase by the antibiotic/CSC combination, extending beyond the 16h incubation period; ii) an exaggerated stress response resulting in preferential, intense initiation of biofilm formation involving activation of quorum-sensing mechanisms¹⁵³; and iii) possibly, but less likely, a bactericidal effect of the combination. Clearly, further studies are necessary to investigate which of these potential mechanisms underpin the augmentative inhibitory effects of the antibiotic/CSC combination on bacterial growth.

Based on earlier studies by Mutepe *et al.*¹⁹, induction of biofilm formation seems the most probable mechanism underpinning the augmentative inhibitory effect of the clarithromycin/CSC combination on the growth of strain 172 of the

pneumococcus. As discussed in Chapter 1, biofilms are specialised multicellular communities of bacteria embedded in a self-produced extracellular matrix made up of various polymeric molecules, which enhance disease persistence due to reduced susceptibility to antimicrobial agents as well as other environmental factors ^{29,159}. Biofilms enable the pneumococcus to enter a quiescent, persistent phase from which they can emerge following host defences being compromised or the removal of harsh environmental conditions ^{153,160}. In the context of the current study, however, interactive effects of antimicrobial agents and CSC on the growth of, and induction of biofilm formation by *S. pneumoniae* is a largely unexplored field of research.

In conclusion, the major findings of the experiments described in this Chapter are firstly, transient inhibition of the growth of all three strains of the pneumococcus following exposure to CSC. Secondly, and most importantly, the profound inhibition of the growth of strain 172 of the pneumococcus following exposure to the combination of clarithromycin and CSC. These experiments are a necessary prerequisite with respect to those described in the following Chapter, which are focused on biofilm formation.

Chapter 3: Total Biofilm Formation

Chapter 3 outlines the effects of CSC (80 and 160 µg/mL) and clarithromycin on total biofilm formation by the three clinical subtype 23F *S. pneumoniae* strains. These strains include the macrolide-sensitive strain 172 and the two macrolide-resistant strains (521 and 2507).

3.1. Introduction

Bacterial biofilms are highly organised structures that are encased within an extracellular polymeric matrix attached to either a biotic or abiotic surface¹⁶¹. The initial step to biofilm formation requires the reversible attachment of free floating planktonic components to a surface¹⁶². This is followed by a non-reversible attachment that includes factors such as adhesion molecules and several other processes that will eventually lead to the formation of a mature biofilm consisting of channels that allow distribution of nutrients and signalling molecules¹⁶³. At the end stage of biofilm formation, the concealed bacteria bud out to a planktonic state and are able to disseminate and infect other body niches¹⁶⁴. Biofilms enhance microbial survival and pathogenicity by providing an environment that is tolerant to antimicrobial treatment and host immunity¹⁶². These structures have been found to occur in almost 80% of all infections and are drivers of disease persistence which impose a negative impact on the public health sector¹⁷.

3.2. Materials and Methods

3.2.1. Study design

The present study was designed to investigate the effects of CSC and the macrolide antibiotic, clarithromycin, on *S. pneumoniae* biofilm formation. The effects of CSC (80 and 160 µg/mL) alone and in combination with clarithromycin were investigated on strain 172, whereas strains 521 and 2507 were exposed only to CSC (80 and 160 µg/mL). The effect of clarithromycin on the biofilm formation by strains 521 and 2507 was not investigated due to the resistance of these strains to this antibiotic. The study was performed in parallel with the planktonic study described in Chapter 2, section 2.2.6 and the same standardised bacterial cultures were used.

3.2.2. Bacterial strains

As described in Chapter 2, section 2.2.2

3.2.3. Chemicals and reagents

As described in Chapter 2, section 2.2.3

3.2.4. Cigarette smoke condensate

As described in Chapter 2, section 2.2.4

3.2.5. Clarithromycin

As described in Chapter 2, section 2.2.5

3.2.6. Total biofilm assay

Total biofilm formation was determined as described by Mutepe and co-workers¹⁹. The three clinical strains investigated were standardised to an OD (0.1) representing a final concentration of 1×10^6 cfu/mL. The wild-type strain 172 was exposed to either a sub-MIC concentration of clarithromycin (0.0015 $\mu\text{g/mL}$) or CSC (80 or 160 $\mu\text{g/mL}$) alone and in combination. The two macrolide-resistant strains 521 and 2507 were exposed to CSC (80 or 160 $\mu\text{g/mL}$) alone or the DMSO solvent control in TSB. A maximum volume of 2.5 mL per well (in duplicate) of the treated bacteria were dispensed into a six-well tissue culture plate and incubated for 16h at 37°C, in a 5% CO₂ environment (ESCO) to allow adherence and biofilm formation. After incubation, the non-adherent bacteria and medium were removed and the plate was washed 3 times with 2.5 mL of phosphate-buffered saline (PBS) solution (pH 7.4, 0.15 M) and air dried for 15 min. Following removal of the unbound bacteria, the biofilm was stained with 2 mL 0.1% crystal violet for 15 min, the excess dye was removed and the wells were again washed 5 times with 2.5 mL PBS and air dried for another 15 min. The bound crystal violet dye was released from the biofilm by the addition of 1 mL 96% ethanol. The amount of total biofilm formed is directly associated with the intensity of the crystal violet/ ethanol colour and determined spectrophotometrically at a wavelength of 570 nm using the PowerWaveX plate reader (Bio-Tech Instruments Inc.).

3.2.7. Statistics

The pneumococcal biofilm experiments were repeated between three and six times for each pneumococcal strain with triplicate determinations for each system. The results are expressed as mean \pm standard deviation (SD) and statistical significance was calculated using the Mann-Whitney U-test (two-tailed) and a p-value of <0.05 was considered significant.

3.3. Results

3.3.1. Effects of clarithromycin and CSC on biofilm formation by strain 172

The results for biofilm formation by wild-type strain 172 exposed to clarithromycin (0.0015 $\mu\text{g/mL}$) or CSC (80 and 160 $\mu\text{g/mL}$) alone or in combination are shown in Figure 3.1. Treatment of the pneumococcus with clarithromycin (0.0015 $\mu\text{g/mL}$) alone showed significant inhibition of biofilm growth after 16h incubation ($p = 0.0056$). In contrast, treatment of the wild-type strain with both concentrations of CSC alone showed a concentration-dependent, significant increase in biofilm formation ($p < 0.05$). Interestingly, when strain 172 was exposed to a combination of clarithromycin and CSC (80 and 160 $\mu\text{g/mL}$), a significant increase in biofilm formation of similar magnitude was also observed ($p < 0.05$).

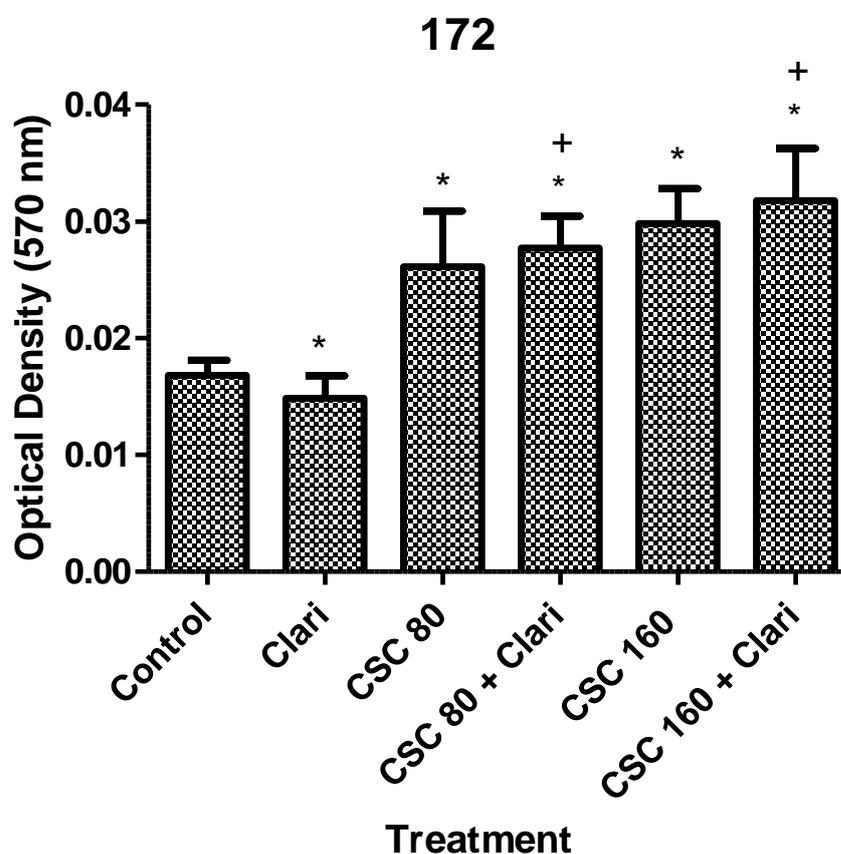
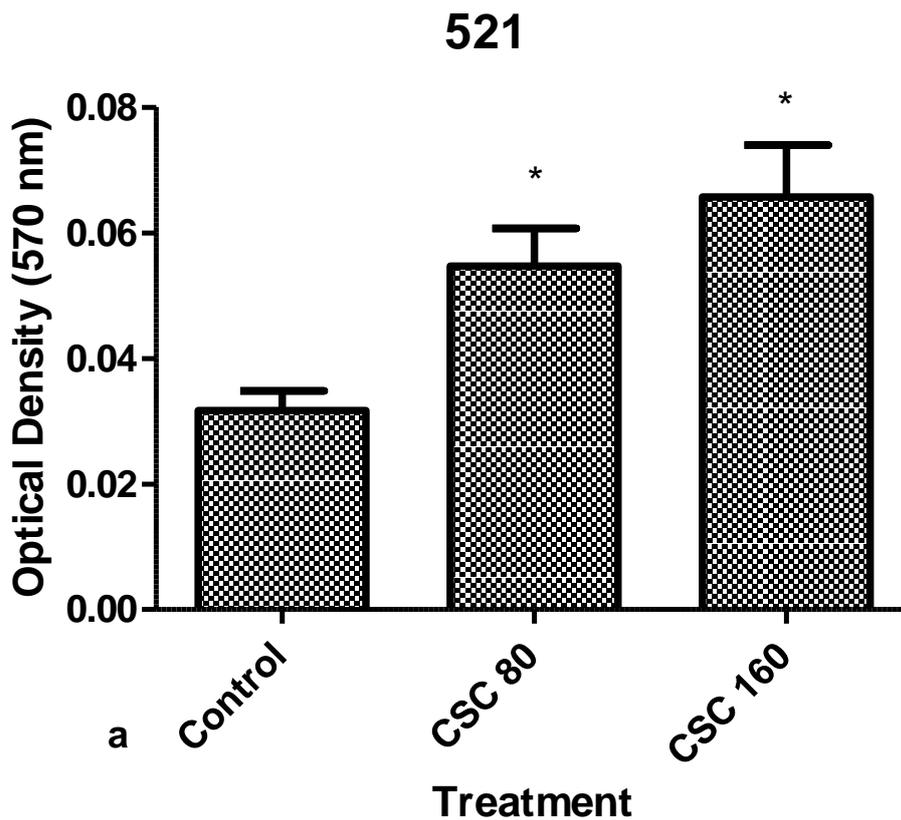


Figure 3.1: Effects of clarithromycin (Clari; 0.0015 $\mu\text{g/mL}$) on the macrolide-susceptible *S. pneumoniae* strain 172 alone and in combination with cigarette smoke condensate (CSC) at 80 and 160 $\mu\text{g/mL}$ on biofilm formation. The results are expressed as mean \pm standard deviation (SD) of six different experiments performed in triplicate for each system. The significance in comparison to the control (*) and clarithromycin (+) is indicated for $p < 0.05$.

3.3.2. Effects of CSC on biofilm formation by strains 521 and 2507 of the pneumococcus

The effect of CSC (80 and 160 $\mu\text{g/mL}$) on *S. pneumoniae* biofilm formation by the two macrolide-resistant strains, 521 and 2507, are shown in Figures 3.2 (a) and 3.2 (b), respectively. The results indicate that CSC potentiates pneumococcal biofilm formation in a concentration dependent manner by both macrolide-resistant strains investigated ($p < 0.05$).



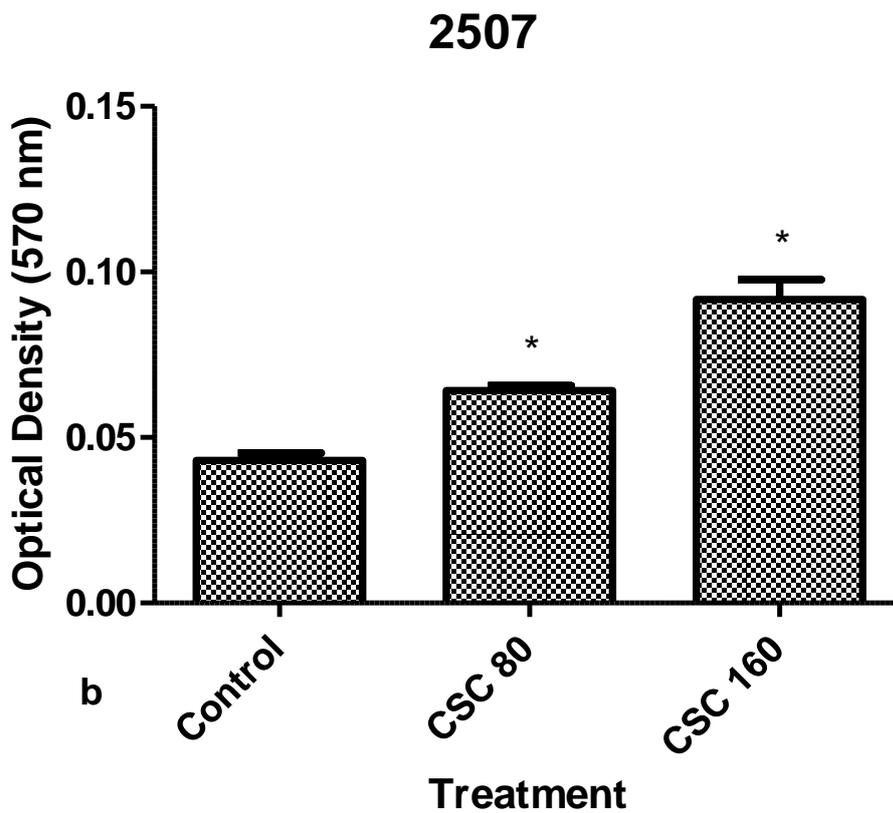


Figure 3.2: Effects of cigarette smoke condensate (CSC) on biofilm formation by *S. pneumoniae* strains 521 (a) and 2507 (b). The results are expressed as mean \pm SD of three different experiments performed in triplicate for each system. The significance in comparison to the solvent control (*) is indicated for $p < 0.05$.

3.4. Discussion

Treatment of biofilm-associated infections such as those caused by *S. pneumoniae* has always been problematic due to the inability of antimicrobial agents to penetrate the formed biofilm, thereby supporting bacterial persistence and acting as pathogen reservoirs^{118,165}. Moreover, microbial biofilms provide an environment suitable for acquisition of resistance genes that aid in antimicrobial tolerance¹⁶⁶. It has previously been reported that CSC acts as a stressor and, as such, may initiate quorum-sensing mechanisms within the bacteria. This, in turn, may result in biofilm formation allowing the pneumococcus to enter a quiescent, persistent state until the host defences are transiently compromised or environmental conditions return to favour bacterial growth^{19,160}]. Although the effects of the macrolides and CSC on biofilm formation have previously been reported, their effect in combination, do not appear to have been investigated.

As has been reported for *S. pneumoniae*, clarithromycin, at a sub-MIC concentration, inhibited biofilm formation by *S. pneumoniae* strain 172. Similarly, these inhibitory effects of clarithromycin at sub-MICs have been observed in other microorganisms such as *Mycobacterium avium*, *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis*^{167,168,169}. In the current study, however, exposing strain 172 of the pneumococcus to a combination of clarithromycin and CSC negated the modest, albeit significant, inhibitory effect of the antibiotic on biofilm formation. These findings suggest that smoking may counteract the potentially beneficial inhibitory effects of macrolide antibacterial agents on biofilm formation by the pneumococcus. This may lead to augmentation of virulence and persistence of this dangerous respiratory pathogen, even in the setting of seemingly appropriate antimicrobial chemotherapy. This contention may seem somewhat counterintuitive, however, based on the results presented in the previous Chapter, which demonstrated augmentative inhibition of bacterial growth in the presence of the combination. In this context it is noteworthy that exposure of strain 172 of the pneumococcus to CSC has previously been reported to upregulate expression of the gene *SP2003*, which encodes an ATP-binding cassette transporter (ABC-transporter) that mediates efflux of xenobiotics (Cockeran et al., 2014). It is therefore possible that following initial induction of stress-associated inhibition of bacterial growth by the antibiotic/CSC combination, ABC-transporter-mediated efflux of clarithromycin may underpin

attenuation of antibiotic-mediated inhibition of biofilm formation. This contention is supported by the findings described in the following Chapter.

For all three *S. pneumoniae* clinical strains, exposure to CSC at both concentrations investigated increased the formation of biofilm. These findings are in agreement with those reported earlier by Mutepe *et al*, which showed a CSC-induced concentration-dependent increase in biofilm formation by the wild-type strain 172¹⁹. Cigarette smoke-mediated induction of biofilm formation has also been documented for other streptococcal species^{170,171}. Moreover, cigarette smoking has been associated with an increase in biofilm formation by *S. aureus* in which it was found to induce expression of fibronectin-binding protein A, which enhances adherence to human cells, which also promotes formation of biofilm¹⁸. The impact of cigarette smoking on human health is well documented, with cigarette smoking identified as the strongest risk factor for developing invasive pneumococcal disease, as well as COPD¹⁷².

The present study, therefore, confirms and extends previous reports showing that exposure of the pneumococcus to cigarette smoke results in augmentation of biofilm formation, which, in turn, may contribute to microbial colonisation and persistence. In addition, the findings of this study suggest that smoking may also attenuate the inhibitory effects of clarithromycin, albeit modest at the sub-inhibitory concentration of used, on biofilm formation, which may also negate the efficacy of macrolide treatment.

Chapter 4: Gene Expression Studies

The effect of clarithromycin and cigarette smoke condensate (CSC) exposure on the expression of *S. pneumoniae* genes encoding for the macrolide efflux pump [*mef(A)*], erythromycin resistance methylase [*erm(B)*] and the ATP-binding cassette (ABC)-transporter (*SP2003*) are described in this chapter.

4.1. Introduction

Treatment of bacterial infections with antibiotics has been a life-saving strategy for decades, but is now complicated by the emergence of resistant strains as a result of overuse and incorrect prescribing of antibiotics, as well as antimicrobial selective pressure of the environment and the ever evolving bacterial genome^{173,174}. The transition from susceptible to resistant strains is a result of resistance gene expression that modifies the target sites, interfering with antimicrobial activity or exportation of antimicrobial agents to the extracellular compartment of the bacteria^{73,175}. The present study focuses on the effects of exposure of the pneumococcus to CSC on the expression levels of the macrolide efflux pump [*mef(A)*], ribosomal methylase [*erm(B)*] and ABC-transporter (*SP2003*) genes that are associated with antimicrobial resistance mechanisms. The effect of the macrolide antimicrobial agent, clarithromycin, alone and in combination with CSC, on the expression of these resistance genes was investigated.

4.2. Materials and Methods

4.2.1. Study design

This laboratory based study was designed to investigate the effects of clarithromycin, a first line antimicrobial agent prescribed for the treatment of pneumococcal infections, alone and in combination with CSC, on the expression of the resistance genes *mef(A)*, *erm(B)* and *SP2003* by three different pneumococcal clinical strains. The *S. pneumoniae* strains tested were all serotype 23F and included the macrolide-susceptible strain, 172 (wildtype), and the resistant strains 521 [expressing *mef(A)*] and 2507 [expressing *erm(B)*]. The two reference genes [*gyr(A)* and *gyr(B)*] were chosen because their expression was neither affected by CSC nor Clarithromycin. The *mef(A)* gene has been shown to encode an efflux pump that exports antimicrobial agents to the extracellular compartment of the bacteria, while *erm(B)* encodes the ribosomal methylase enzyme that modulates the 23S subunit 50S rRNA

⁷³. The ABC-transporter *SP2003* gene has been included to gain insight into its possible involvement in treatment failure, particularly in the context of cigarette smoke exposure ⁸¹.

4.2.2. Chemicals and reagents

As described in Chapter 2, section 2.2.3.

4.2.3. Cigarette smoke condensate

As described in Chapter 2, section 2.2.4.

4.2.4. Clarithromycin

The antibiotic was prepared as described in Chapter 2, section 2.2.5 and further diluted in TSB and used at final sub-MICs of 0.125 µg/mL (strain 172), 2 µg/mL (strain 521) and 8 µg/mL (strain 2507). The sub-MIC concentrations used were determined according to the EUCAST guidelines as described in Chapter 2.

4.2.5. Antimicrobial culture and exposure

The three pneumococcal strains, 172, 521 and 2507, were cultured overnight (16 hours) at 37°C in a 5% CO₂ incubator in TSB to the mid-log growth phase. The cultures were centrifuged for 15 min at 1200 x g in a GPR centrifuge (Beckman, Darmstadt, Germany) and the supernatant was discarded followed by optical standardisation of the bacteria at 540 nm wavelength to 2.0 x 10⁸ cfu/mL using the PowerWaveX spectrophotometer (Bio-Tec Instruments Inc.), as described in Chapter 2.

The bacterial suspensions were subjected to a pulsed exposure to either DMSO (solvent control), or CSC (80 or 160 µg/mL, final, as two successive 90 min exposures) at 37°C in a 5% CO₂ incubator. After the CSC exposure, the bacteria were centrifuged at 1200 x g for 15 min to remove any remaining CSC. The pellet was resuspended in fresh TSB and each strain was then treated with a sub-MIC of clarithromycin as follows: 172 (0.125 µg/mL), 521 (2 µg/mL) and 2507 (8 µg/mL) and incubated for 15 min at 37°C in a 5% CO₂ incubator. Following incubation, the tubes were centrifuged and the pellet was snap frozen in liquid nitrogen before storing at -80 °C until required for RNA extraction.

4.2.6. RNA extraction

The treated bacteria were thawed on ice before undergoing a two-way RNA extraction process¹⁷⁶. Firstly, bacterial cells were disintegrated by adding 200 μL fresh lysozyme (15mg/mL) in Tris-EDTA (TE) buffer (Roche, Basel, Switzerland) to the thawed pellets. The pellets were vortexed for 10 seconds (s) and incubated at room temperature for 15 min on a shaker. During the incubation period, the pellets were vortexed for 10s every 5 min. Using the RNeasy mini kit (Qiagen, Hilden, Germany), 700 μL of lysis buffer (RLT buffer) was added to the lysate and vortexed for 10s before transferring the lysate to 2 mL microcentrifuge tubes containing 25-50 mg of 100 μm glass beads (Sigma). The cell membranes were disrupted on a TissueLyser (Retsch, Haan, Germany) using 2 cycles of 2 min at a frequency of 20/s, and then centrifuged using a Scispin micro-centrifuge (Sigma) for 10s at 15 500 x g. The supernatant was carefully recovered to avoid collecting glass beads and transferred to an unused 2 mL microcentrifuge tube containing 100% ethanol and mixed thoroughly by pipetting. A maximum of 700 μL of the solution was then transferred onto the RNeasy mini column and centrifuged at 15 500 x g for 30s. The flow-through was discarded and the remaining solution was added to the column and the column was again centrifuged at 15 500 x g for 30s.

To the same column, 350 μL of wash buffer (RW1 buffer) was added and the column centrifuged at 13 500 x g for 5 min, with the subsequent addition of 80 μL RNase-free DNase1 incubation mix (prepared by adding 10 μL DNase1 stock to 70 μL RDD buffer; Qiagen) directly onto the RNeasy silica-gel membrane. The RNeasy silica-gel membrane was then incubated at room temperature for 15 min. Thereafter, 350 μL RW1 was added to the RNeasy column, which was then centrifuged for 30s at 13 500 x g, and the flow-through discarded. A further 700 μL of the RW1 buffer was added and the columns centrifuged at the same conditions. Five hundred microliters of ethanol-containing RPE buffer was added onto the mini column and centrifuged for 30s at 13 500 x g. A further 500 μL was added to the column and spun for 2 min at 13 500 x g. For RNA elution, the RNeasy column was transferred to a new 1.5 mL microcentrifuge tube with the subsequent addition of 50 μL nuclease-free water directly onto the RNeasy silica-gel membrane. The tubes were gently closed and allowed to stand for 3 min before centrifuging for 1 min at 13 500 x g. This was followed by a re-elution step with the same RNA-containing water and

centrifuged for an additional 1 min at 13 500 x g. From the 50 µL RNA-containing water, an aliquot of 10 µL was used for a second DNase treatment using DNase1 (New England Biolabs, Ipswich, MA). This step was achieved by adding 78 µL nuclease-free water, 10 µL of 10x DNase buffer and 2 µL DNase to the 10 µL aliquot of RNA and mixed thoroughly by pipetting. After the addition of 1 µL of 0.5 M EDTA (final concentration of 5 mM), the solution was incubated at 37°C for 10 min using a dry-block heater (Hägar Designs, Germany), followed by a heat inactivation step of the DNase at 75°C for 10 min using a CH 3-150 combitherm-2 (Biosan, Riga, Latvia).

For the second column purification, 350 µL RLT buffer and 250 µL absolute ethanol was added to the RNA-containing tube, then transferred to a new RNeasy mini-column and centrifuged for 15s at 15 500 x g. Five hundred microliters of wash buffer, for washing membrane-bound RNA (RPE buffer), was added to the column and centrifuged at 15 500 x g for 15s. Thereafter, a further 500 µL RPE buffer was added and centrifuged for a further 2 min at 15 500 x g. The column was then placed in a new 2 mL collection tube and again centrifuged for 1 min at 15 500 x g. For the second RNA elution, the RNeasy column was transferred to a new 1.5 mL microcentrifuge tube, with the subsequent addition of 30 µL RNA-free water directly onto the silica-gel membrane and incubation at room temperature for 2 min. Post incubation, the tubes were then centrifuged at 15 500 x g for 1 min. The same RNA-containing water was re-eluted and again spun for 1 min at 15 500 x g. The RNA was aliquoted and stored at -80°C with the exception of 1.5 µL RNA that was used to determine the RNA concentration spectrophotometrically at A260/280 using a Nanodrop 2000™ (ThermoScientific Inc, Waltham, MA, USA).

4.2.6.1. Agarose gel electrophoresis

The integrity of the extracted RNA was determined by electrophoresis (shown in Figure 4.1). The RNA electrophoresis 10x TBE (Tris-borate EDTA) buffer stock mixture was prepared by dissolving 108 g Tris and 55 g boric acid in 900 mL distilled water, followed by addition of 40 mL [0.5M Na₂EDTA (pH 8.0)]. The mixture was then adjusted to a final volume of 1000 mL by adding distilled water, and then stored at room temperature. One percent agarose gel was prepared by the addition of 1 g of agarose to 100 mL of 1x TBE buffer (from 10x TBE stock). The mixture was heated in a microwave at maximum power output for about one minute until the

agarose fully dissolved. The agarose mixture was then cooled down under running tap water. Using nitrile gloves, 10 μ L of ethidium bromide was added to the dissolved agarose and mixed thoroughly, thereafter the mixture was carefully transferred to an electrophoresis cassette with the comb inserted proximal to the cathode ensuring no bubbles formed. The gel was then allowed to set for 20 min at room temperature followed by the addition of approximately 250 mL of 1x TBE buffer. Five microliters of each sample (1 μ L RNA and 4 μ L of loading dye) was added to the respective wells. In addition, 5 μ L of 100bp DNA Ladder (RTU 5 μ g/50 μ L, GeneDireX) was added to the first well, serving as a control. The gel was then electrophoresed at \pm 100 V for 30 minutes using the Thermo EC Midicell Primo EC-330 Horizontal Gel System (ThermoScientific. Inc). Post electrophoresis, the gel was removed and viewed on a UV light box (Bio-Rad Laboratories Inc, Hercules, CA, USA) and a snapshot of the bands was taken. RNA integrity was estimated by determining the ratio of the 16S and 23S rRNA bands.

4.2.7. Complementary DNA (cDNA) synthesis

The High-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) was used according to the manufacturer's instructions for the generation of the cDNA. Briefly, 10 μ L of reverse transcription (RT) reaction mixture was prepared by adding 2.1 μ L Best Quality Water SABAX (Adcock, Johannesburg, SA), 1 μ L RT buffer, 0.4 μ L 25x deoxynucleotide (dNTP) mix (100 mM), 1 μ L 10x random primers, 0.5 μ L reverse transcriptase and 5 μ L of total RNA (100 ng/ μ L). For non-reverse transcribed (NRT) negative controls, nuclease-free water was added as a substitute for the reverse transcriptase. Samples were incubated at 25°C for 10 min, followed by incubation at 37°C for 120 min, enzyme inactivation at 85°C for 5s and a cooling step at 4°C using an MJ Mini Personal Thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA).

4.2.8. PCR Primers

Antimicrobial resistance gene expression was determined using real-time reverse transcription polymerase chain reaction (qRT-PCR). The forward and reverse primers for the macrolide-resistance genes [*mef(A)* and *erm(B)*], the ABC-transporter (*SP2003*) and the house-keeping genes [*gyr(A)* and *gyr(B)*] are described in Table 4.1 below.

Table 4.1: PCR primer

Gene	Strain type	Primers
<i>gyr(A)</i>	172, 521 & 2507	5' - AATCTTGCTCATACGTGCCTCGGT - 3'
		5' - ATGGTGGAGCTACCGTTACATGCT - 3'
<i>gyr(B)</i>	172, 521 & 2507	5' - TCAGCCAAATCTGGTCGTAACCGT - 3'
		5' - AATTCTGCGCCAAATCCTGTTCCC - 3'
SP2003	172 & 2507	5' - ATCGTGACTCCTTGCGCTTT - 3'
		5' - TCTTGACCTTGATTGGGCGA - 3'
SP2003	521	5' - CGCCCAATCAAGGTCAAAC - 3'
		5' - CCAGCAACAAAAAGCCCAG - 3'
<i>mef(A)</i>	521	5' - CTTTTCATACCCCAGCACTC - 3'
		5' - GCAATCACAGCACCCAATAC - 3'
<i>erm(B)</i>	2507	5' - AGGGCATTTAACGACGAAAC - 3'
		5' - GACGCATGGCTTTCAAAAAC - 3'

4.2.9. Real-Time qPCR

Stratagene Brilliant II SYBR® Green QPCR low ROX master mix (Agilent, Santa Clara, CA, USA) and PIKOREAL 96 well plates (ThermoScientific Inc.) on a PIKOREAL 96 real-time detection system (ThermoScientific Inc.) were used for real-time qPCR determinations. Each 10 µL of reaction mixture contained 4 µL of 2.5 ng/µL cDNA, 0.6 µL Best Quality Water (SABAX), 0.2 µL of 200 nM of each primer and 5 µL of 1x Brilliant II SYBR® Green QPCR low ROX master mix. The PCR conditions for the thermocycler consisted of an initial denaturation step at 95°C for 10 min, 40 amplification cycles at 95°C for 30s and 60°C for 1 min, and a melt-curve step at 60°C to 95°C with a hold time of 1s, and temperature increments of 0.2°C. Samples were assayed in duplicate.

Gene expression calculations were done according to the method described by Hellemans and colleagues¹⁷⁷. To determine the relative change in gene expression, the following steps shown below were followed.

Step 1: $\Delta Cq = \text{control} - \text{treatment}$

Step 2: RQ (relative quantification) = $E^{\Delta Cq} = 2^{\Delta Cq}$

Step 3: $RQ_{GM} = \sqrt{[RQ_{gyr(A)} \times RQ_{gyr(B)}]}$

Step 4: NRQ = RQ_{GOI}/RQ_{GM}

Step 5: Log Transformation = $\log_{10}(\text{NRQ})$

Briefly, to obtain the change in quantification cycle (ΔCq) values of the genes of interest [*mef(A)*, *erm(B)* and *SP2003*] and the reference genes [*gyr(A)* and *gyr(B)*], Cq values of the treated samples were subtracted from the Cq values of the untreated samples. The relative quantification ($2^{\Delta Cq}$) values of the interest and reference genes were calculated. The geometric mean (GM) value of the two reference genes was calculated from the $2^{\Delta Cq}$ of the reference genes. For normalization (NRQ), the relative quantity of the gene of interest (GOI) was divided by the calculated GM of the reference genes. The normalised Cq values of these genes were log transformed and plotted using Microsoft excel.

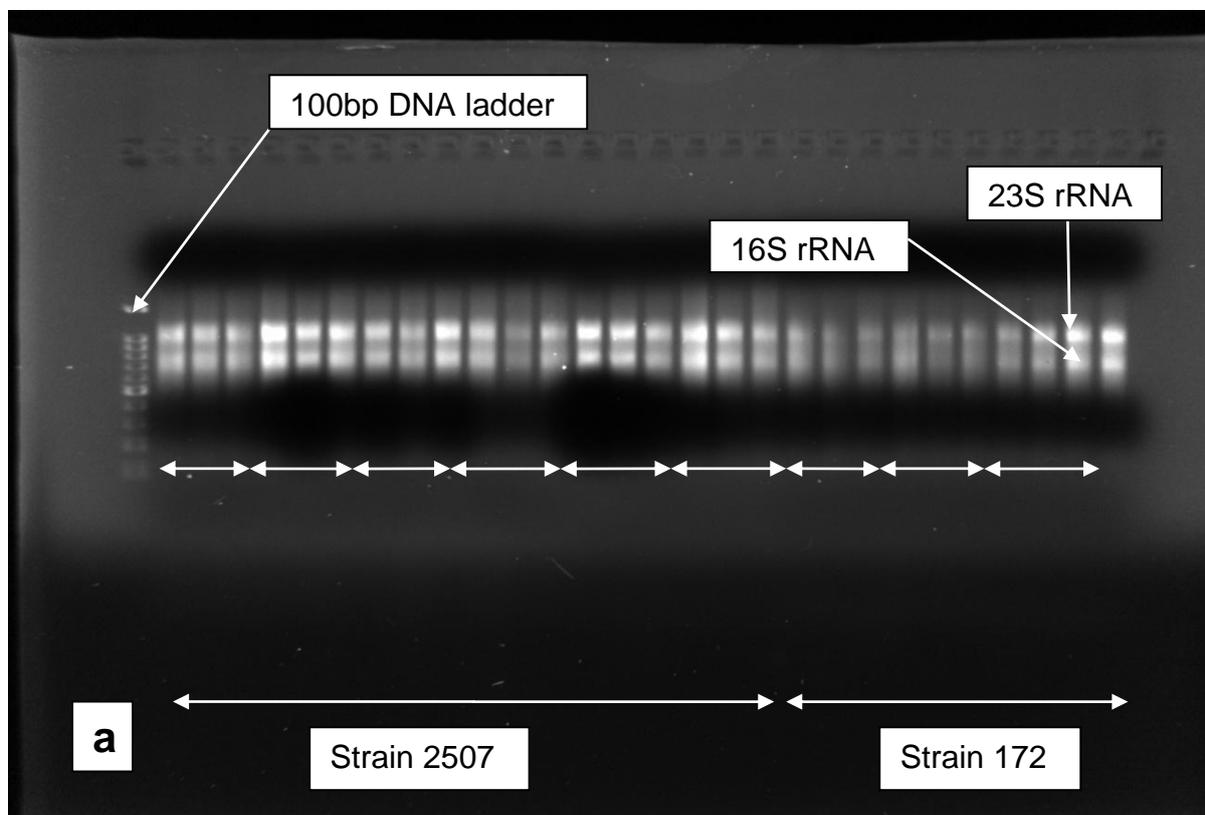
4.2.10. Statistics

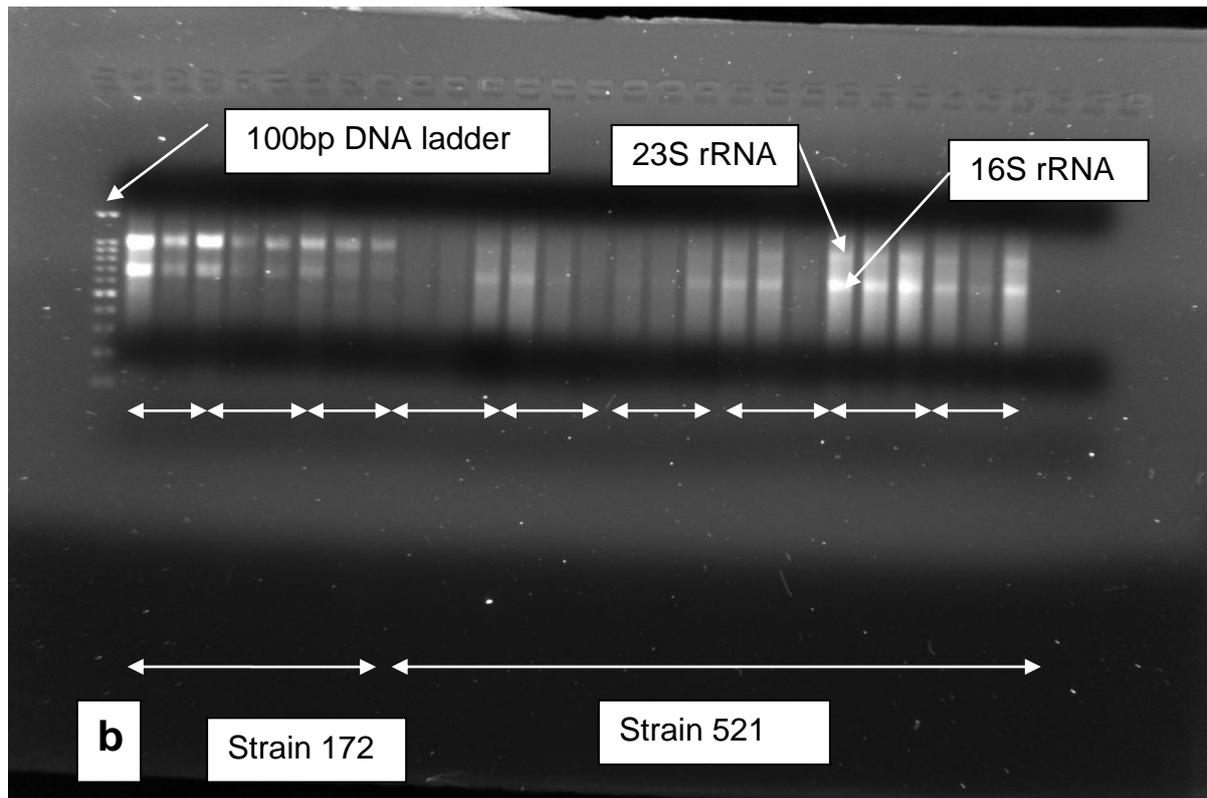
Descriptive and inferential statistic techniques were used in the analyses. The results are expressed as mean \pm standard deviation (SD) of three different experiments with duplicate measurements for each experiment. Analysis of variance was measured using repeated-measures ANOVA with Tukey-Kramer multiple comparisons test as post examination. Statistical significance determination was performed using GraphPad InStat 3 (GraphPad Software Inc., San Diego, CA, USA), and set at a p-value <0.05 .

4.3. Results

4.3.1. Agarose gel electrophoresis

A high quality RNA extraction is crucial to obtain meaningful data. Gel electrophoresis allows visual inspection of the extracted RNA and separates the RNA according to size. The clearer the bands, the better the indication that the RNA remained intact. Inclusion of a DNA ladder also allows the size of the bands to be determined. The quality of the extracted RNA, shown in Figures 4.1(a) and 4.1(b) below, was assessed from the two bands generated. The clear separation of the two bands represents the quality of the extracted RNA and is indicative that the gel was run correctly.





Figures 4.1: Analysis of extracted total RNA integrity by ethidium bromide-stained 1% agarose gel electrophoresis of three clinical isolates of *S. pneumoniae* (strains 172, 521 and 2507). The double-arrow clipart shown above represents the treatments (control; clari; CSC 80; CSC 80 + clari; CSC 160 and CSC 160 + clari) from left to right in triplicates for strain 2507, 172 and 521. The bands representing the 23S and 16S subunits of the extracted RNA are shown in Figure 4.1 (a) for strains 2507 and 172; and in Figure 4.1 (b) for strains 521 and 172. Equivalent 23S:16S ratios were observed for all three strains studied.

4.3.2. Real-Time qPCR

The results for expression of the ABC-transporter gene *SP2003* by strain 172 treated with clarithromycin (0.125 $\mu\text{g}/\text{mL}$) and CSC (80 and 160 $\mu\text{g}/\text{mL}$) alone and in combination are shown in Figure 4.2 (a). Slight, albeit insignificant, upregulation of the *SP2003* gene was observed in those bacteria exposed to either clarithromycin or CSC at 80 $\mu\text{g}/\text{mL}$. Notably, the upregulation of this gene by clarithromycin alone may be underestimated due to the low clarithromycin concentration used in this study. However, the fold increase of the *SP2003* gene reached statistical significance following exposure of strain 172 of the pneumococcus to CSC at a concentration of 160 $\mu\text{g}/\text{mL}$ in both the absence and presence of clarithromycin ($p \leq 0.05$), with the

combination of CSC and clarithromycin demonstrating additive effects on gene expression.

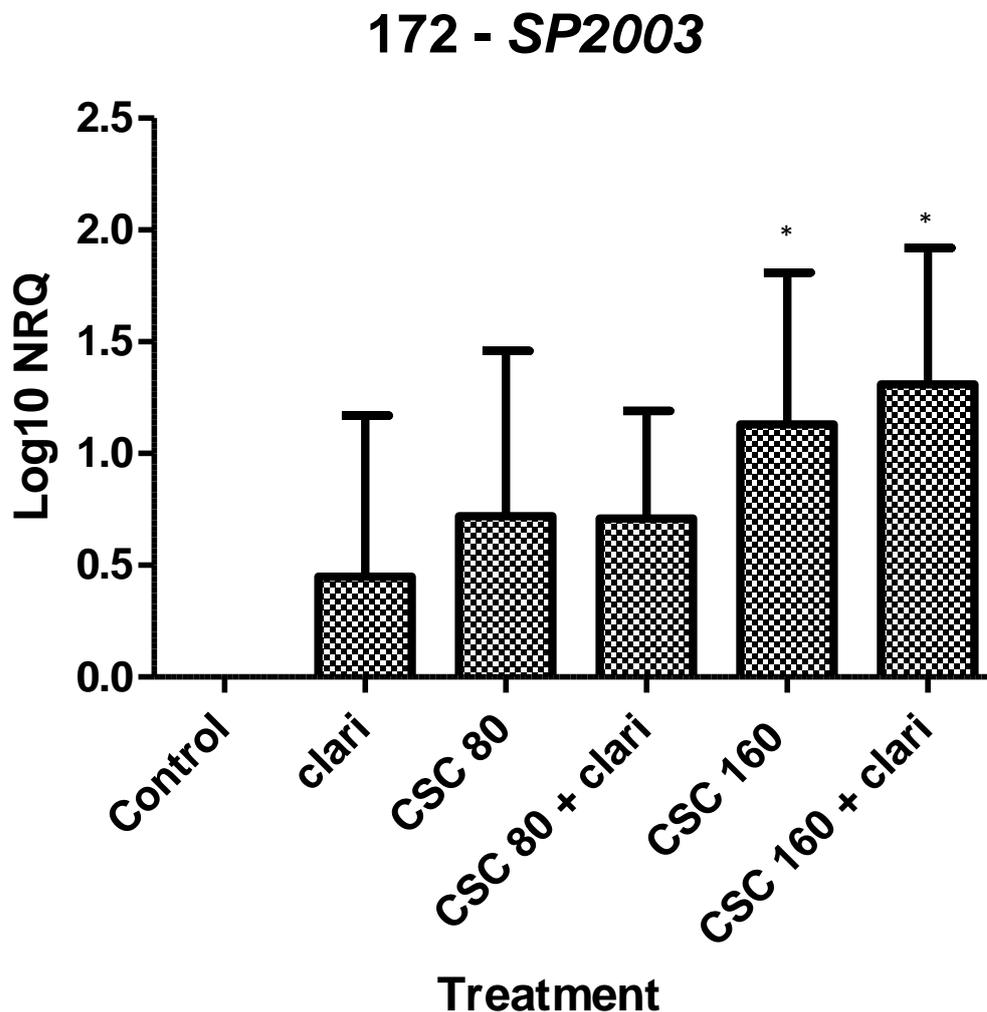


Figure 4.2 (a): Effects of exposure of *S. pneumoniae* strain 172, to clarithromycin (clari, 0.0125 µg/mL) and CSC (80 and 160 µg/mL), either alone or in combination, on the log₁₀ relative gene expression of the ABC-transporter gene *SP2003*. The results are expressed as the mean ± SD of three different experiments, with duplicate measurement for each system. Significance ($p \leq 0.05$) is indicated in comparison to the untreated control system (*).

The corresponding data for expression of the *SP2003* gene by strain 521 is shown in Figure 4.2 (b). In the case of strain 521, gene expression was significantly ($p \leq 0.05$) upregulated to an essentially comparable extent following treatment with either clarithromycin or CSC alone, as well as in combination.

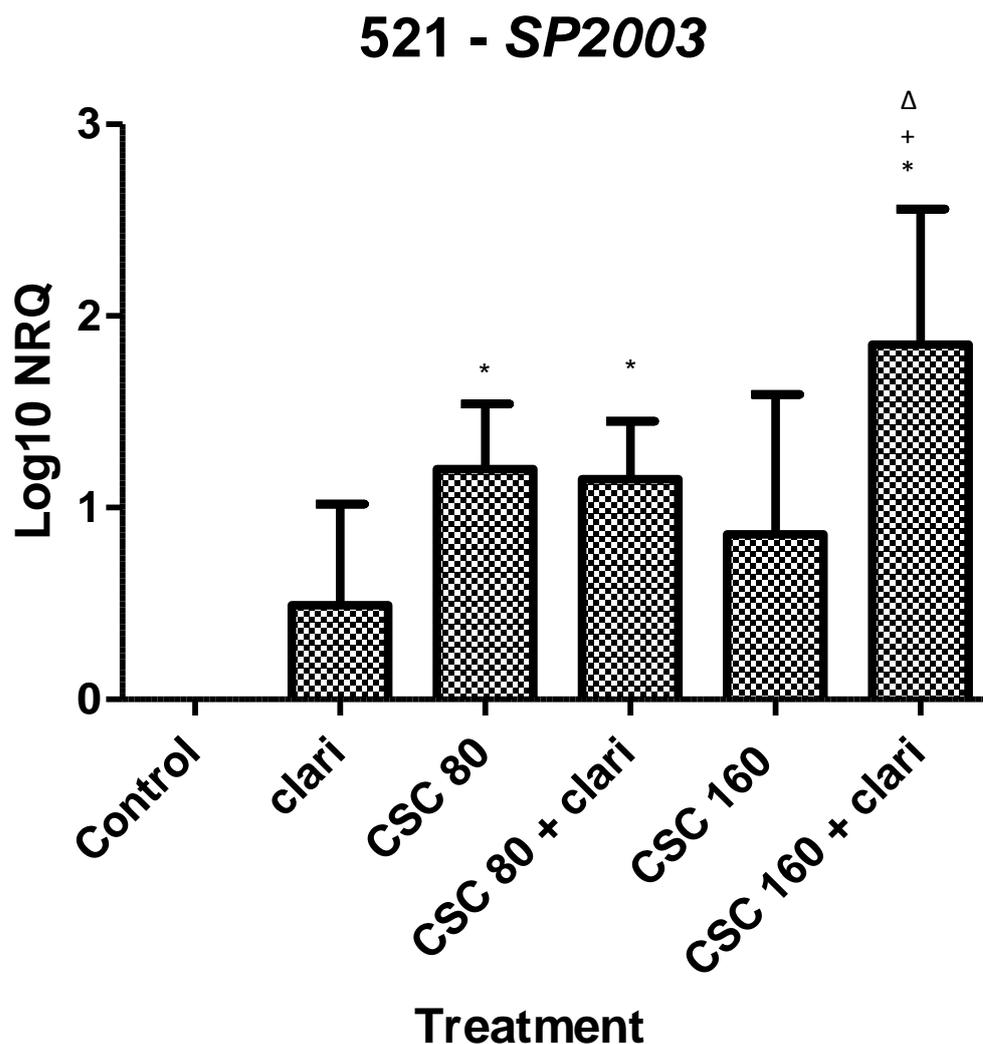


Figure 4.1 (b): Effects of exposure of *S. pneumoniae* strain 521, to clarithromycin (clari, 2 $\mu\text{g}/\text{mL}$) and CSC (80 and 160 $\mu\text{g}/\text{mL}$), either alone or in combination, on the \log_{10} relative gene expression of the ABC-transporter gene *SP2003*. The results are expressed as the mean \pm SD of three different experiments, with duplicate measurement for each system. Significance ($p \leq 0.05$) is indicated in comparison to the untreated control system (*), CSC combined with clarithromycin compared to clarithromycin alone (+) or clarithromycin combined with CSC 160 (Δ) in comparison with CSC alone.

As shown in Figure 4.2(c), modest, albeit statistically insignificant upregulation of expression of *SP2003* was also observed following exposure of strain 2507 to clarithromycin alone, while exposure to CSC alone, at both concentrations tested, resulted in significantly increased gene expression. As with strain 172, additive effects on gene expression were observed following exposure of strain 2507 to CSC (both concentrations) and clarithromycin.

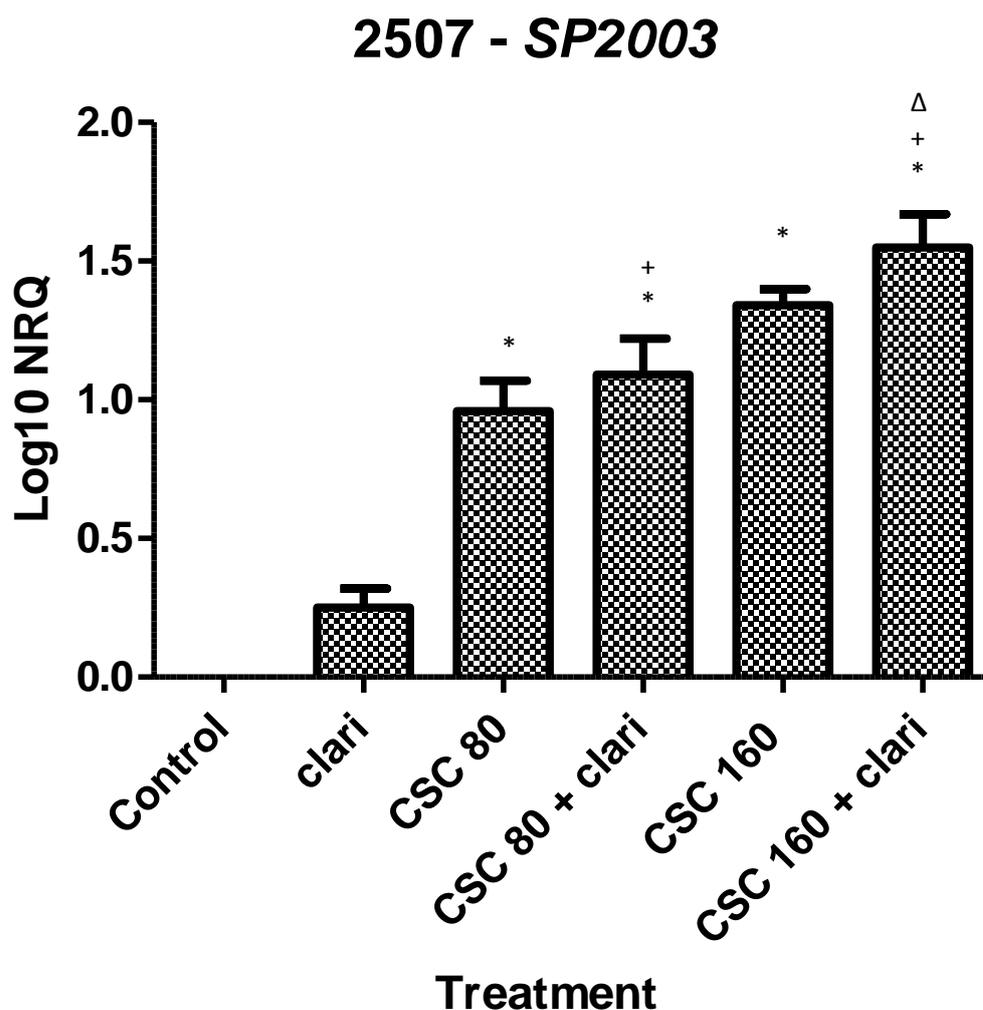


Figure 4.2(c): Effects of exposure of *S. pneumoniae* strain 2507, to clarithromycin (clari, 8 $\mu\text{g}/\text{mL}$) and CSC (80 and 160 $\mu\text{g}/\text{mL}$), either alone or in combination, on the \log_{10} relative gene expression of the ABC-transporter gene *SP2003*. The results are expressed as the mean \pm SD of three different experiments, with duplicate measurements for each system. Significance ($p \leq 0.05$) is indicated in comparison to the untreated control system (*), CSC combined with clarithromycin compared to clarithromycin alone (+) or clarithromycin combined with CSC 160 (Δ) in comparison with CSC alone.

The effects of exposure of strains 2507 and 521 of the pneumococcus on expression of the *erm(B)* and *mef(A)* genes respectively following exposure to clarithromycin and CSC individually and in combination are shown in Figures 4.3 and 4.4. Although expression of neither test gene was detected in the absence of either CSC or clarithromycin, the results shown in Figure 4.3, demonstrate that exposure of strain 2507 of the pneumococcus to CSC alone at 160 µg/mL resulted in statistically significant induction of the *erm(B)* gene ($p < 0.05$). Furthermore, exposure of the pathogen to CSC at a concentration of 160 µg/mL followed by clarithromycin resulted in increased, augmentative expression of *erm(B)*. The magnitude of gene expression for the system treated with clarithromycin and CSC at 160 µg/mL in combination, was 27% higher than that of the sum of the systems treated with the antibiotic and CSC individually, consistent with an augmentative interaction. Although not statistically significant, the corresponding increase for the system treated with clarithromycin and CSC at 80 µg/mL was 25%.

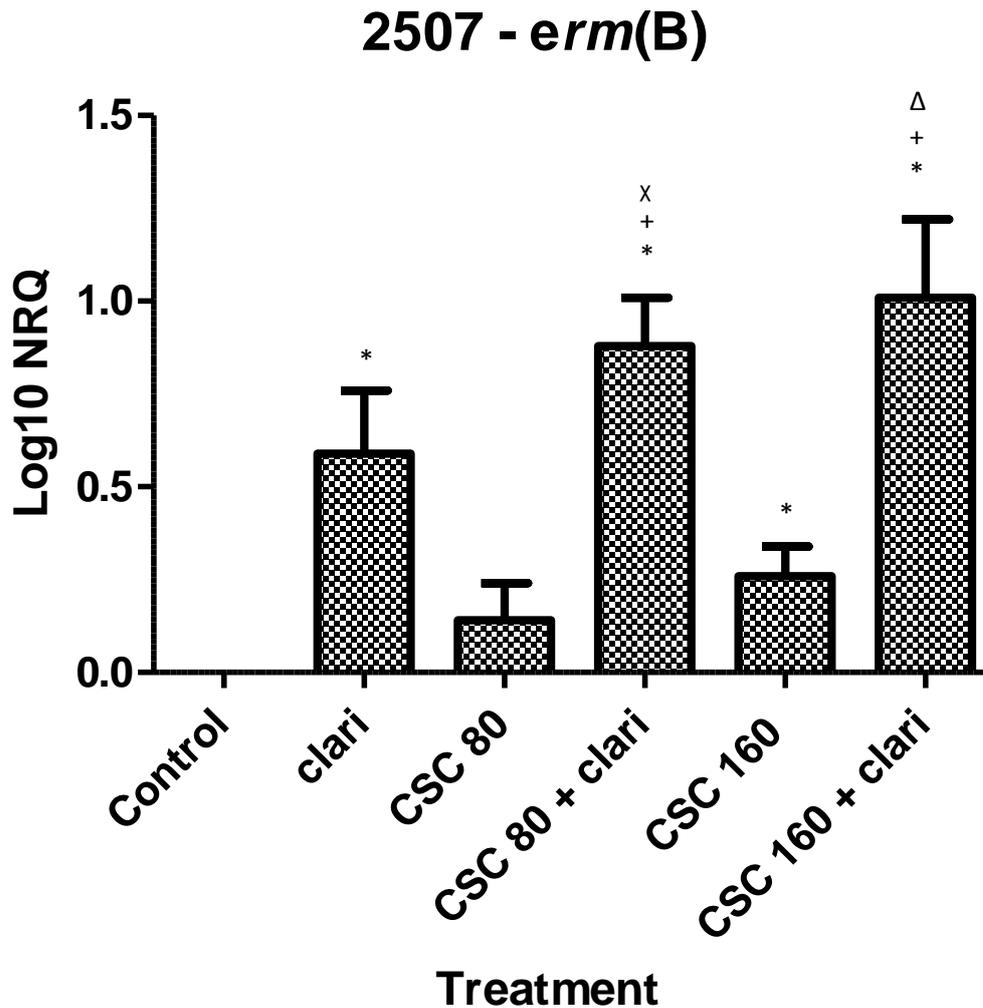


Figure 4.3: Effects of exposure of strain 2507 of *S. pneumoniae* to clarithromycin (8 $\mu\text{g}/\text{mL}$) and CSC (80 and 160 $\mu\text{g}/\text{mL}$), individually and combination, on the \log_{10} relative gene expression of the *erm(B)* gene. The results are expressed as mean \pm SD of three different experiments, with duplicate measurements for each treatment. Statistical significance ($p \leq 0.05$) is indicated by the various symbols, specifically in comparison to the untreated control system (*); system treated with clarithromycin only (+); CSC 80 $\mu\text{g}/\text{mL}$ only (x) and CSC 160 $\mu\text{g}/\text{mL}$ only (Δ).

In the case of strain 521 (Figure 4.4), expression of the *mef(A)* gene was significantly increased in the presence of clarithromycin (2 µg/mL, $p < 0.05$), but was unaffected by CSC either alone or in combination with the antibiotic.

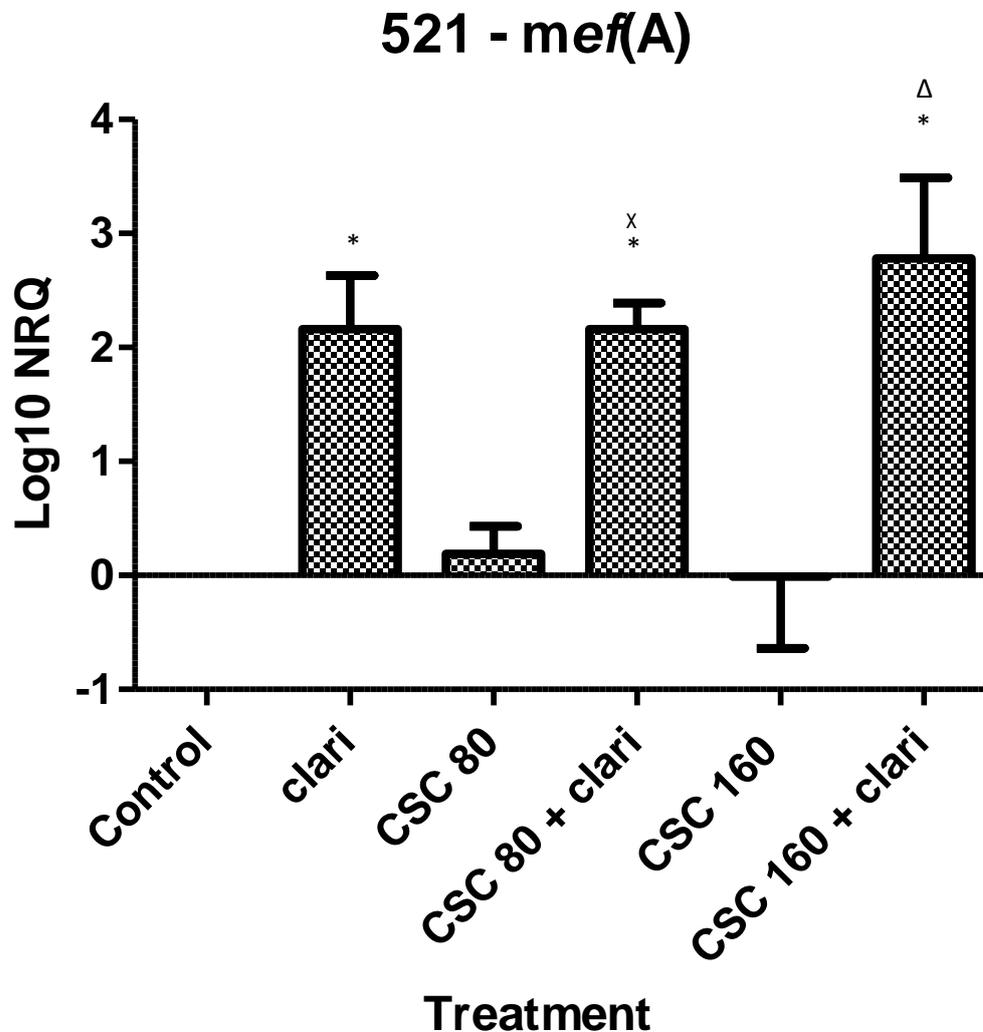


Figure 4.4: Effects of exposure of strain 521 of *S. pneumoniae* to clarithromycin (clari, 2 µg/mL) and CSC (80 and 160 µg/mL), individually and in combination, on the log₁₀ relative gene expression of the *mef(A)* gene. The results are expressed as mean ± SD of three different experiments, with duplicate measurements for each treatment. Statistical significance ($p \leq 0.05$) is indicated in comparison with the control (*), CSC 80 (x) and/or CSC 160 (Δ).

4.4. Discussion

In the present study, the possible impact of smoking on the expression of macrolide resistance genes by *S. pneumoniae* was investigated. The two predominant mechanisms of macrolide resistance operative in *S. pneumoniae* are firstly, induction of the *erm(B)* gene which encodes ribosomal methylase, resulting in target modification of macrolide binding sites on the 23S rRNA^{70,71}. This resistance mechanism is operative in strain 2507 of the pathogen. Secondly, induction of a macrolide efflux pump encoded by the *mef(A)* gene, which expels this type of antibiotic from the intracellular environment of the bacteria^{178,179}, is operative in strain 521 of the pathogen. Efflux of macrolide drugs from *S. pneumoniae* is considered to be the most prevalent mechanism of resistance in many parts of the world. Macrolide efflux is largely linked to the gene product of *mef(A)* which carries a proton motive force pump that is specific for 14- and 15-membered macrolides (M phenotype)^{68,69}.

Strain 172 of the pneumococcus, which is a macrolide-susceptible strain, was also included to monitor and compare the effects of exposure to CSC *per se*. In addition to effects on expression of the *erm(B)* and *mef(A)* genes, the effects of exposure of all three strains of the pneumococcus to CSC on expression of the ABC-transporter gene, *SP2003*, were also investigated. Importantly, this class of ABC-type transporter also appears to contribute to multidrug resistance in bacteria¹⁸⁰.

With respect to *SP2003*, expression of this gene was significantly and substantially upregulated following exposure of all three *S. pneumoniae* strains to CSC. It is noteworthy that exposure of strain 172 of the pneumococcus to CSC has previously been reported to cause upregulation of the expression of the *SP2003* gene¹⁷⁶, expression of which is linked to efflux of xenobiotics^{176,181}. In the context of the current study, increased expression of *SP2003* is most likely a stress response coupled to elimination of toxicants present in CSC. Interestingly, however, exposure of all three of the pneumococcal strains to clarithromycin alone was found to cause modest upregulation, albeit not statistically significant, of the expression of this gene. It is, however, noteworthy that increased expression of *SP2003* by *S. pneumoniae* has been reported following exposure of the pathogen to vancomycin⁸¹, which, taken together with the aforementioned findings, appears to be consistent with the involvement of the ABC-transporter encoded by *SP2003* in promoting antibiotic

resistance. In this context, future studies should focus on the effects of higher concentrations of clarithromycin and other macrolides on the expression of *SP2003* by a range of macrolide-susceptible strains of the pneumococcus, combining this with measurement of decreased sensitivity to these agents.

Relative to the effects of CSC alone, the combination of clarithromycin and CSC demonstrated little or no augmentative interactions with respect to expression of *SP2003* by strains 172 and 521. However, additive upregulation of *SP2003* expression was observed following exposure of strain 2507 to the combination of clarithromycin and CSC, which may be indicative of increased ABC-transporter-mediated efflux of antibiotics in certain strains of the pneumococcus following exposure to both the antibiotic and cigarette smoke.

With respect to *erm(B)* and *mef(A)*, neither of these genes was expressed by the pneumococcus clarithromycin- or CSC-untreated control systems. However, exposure of strain 2507 of the pathogen to CSC at 160 µg/mL, followed by clarithromycin, resulted in statistically significant, augmentative induction of the *erm(B)* gene compared to the level of expression following treatment with the macrolide alone. As mentioned earlier, the magnitude of *erm(B)* gene expression for the system treated with clarithromycin and CSC in combination was 27% higher than that of the sum of the systems treated with the antibiotic or CSC individually, consistent with an augmentative interaction. Although not statistically significant, the corresponding increase for the system treated with the combination of clarithromycin and CSC at a concentration of 80 µg/mL was 25%.

Surprisingly, exposure of the pneumococcus to CSC in the absence of clarithromycin, resulted in a moderate dose-related induction of the *erm(B)* gene, which attained statistical significance at 160 µg/mL CSC. Seemingly, spontaneous induction of the *erm(B)* gene following exposure of the pneumococcus to CSC in the absence of a macrolide has not been described previously and may be the consequence of a general stress response to CSC-mediated oxidative stress. In this context, cigarette smoke contains an abundance of pro-oxidative toxicants, including organic and inorganic highly reactive free radicals and heavy metals, which trigger, directly or indirectly, the induction of various stress response genes to counter oxidative damage. Furthermore, exposure of the pneumococcus to CSC has

previously been reported to cause significant upregulation of the two-component regulatory system 11 (TCS11)¹⁷⁶, which is involved in the induction of genes associated with, among others, biofilm formation, vancomycin resistance and the efflux of various chemical and heavy metal toxicants^{176,182,183}. However, neither the transcriptional mechanisms involved in the induction of the pneumococcal *erm(B)* gene, nor the possible involvement of its product, ribosomal methylase, in attenuating oxidative stress appear to have been described. Nevertheless, it is noteworthy that ribosomal RNA methylation as a protective response against environmental/oxidative stress has been described in both *Escherichia coli* and *Staphylococcus aureus*^{184,185}.

As expected the level of expression of the *mef(A)* gene was significantly increased following exposure of the pneumococcus to clarithromycin with no further increase in the presence of CSC or detectable effects of the condensate alone.

Although unexplored in the clinical setting, the findings reported in this dissertation indicate that smoking may sensitise macrolide-resistant pneumococci in the airways for increased expression of the *erm(B)* gene, predisposing to development of a more aggressive resistance phenotype, characterised by more rapid onset and greater magnitude. This may be of particular significance in the case of *erm(B)* gene-mediated macrolide resistance in which the acquisition of the fully resistant phenotype is delayed for up to 12 hours following exposure to the antibiotic^{186,187}. This lag period may be significantly shortened due to prior smoke-mediated induction of *erm(B)*.

The findings of the current study have therefore identified mechanisms, in addition to induction of biofilm formation, by which smoking may compromise the efficacy of the antimicrobial therapy of severe pneumococcal disease. These mechanisms involve: i) enhancement of clarithromycin-induced *erm(B)* gene expression; and ii) a macrolide-independent mechanism of gene expression.

Chapter 5: General Discussion

Streptococcus pneumoniae is a leading cause of morbidity and mortality worldwide, with an increasing prevalence of antibiotic-resistance representing a particular concern. Cigarette smoking, which is highly prevalent throughout the world (>1 billion smokers), is a major risk factor for pneumococcal infection and also affects the severity and outcome of pneumococcal infections. Furthermore, smoking has been linked to bacterial mutagenesis, which could lead to the expression of antibiotic resistance. The interactive effects of antimicrobial agents and CSC on the growth of the pneumococcus and induction of antibiotic resistance is a largely unexplored field of research. In the present study, the impact of exposure of macrolide-susceptible and macrolide-resistant strains of the pathogen to CSC alone and in combination with sub-MIC concentrations of the macrolide, clarithromycin, on the growth, formation of biofilm and expression of the macrolide resistance genes *erm(B)* and *mef(A)*, as well as the *SP2003* gene, were investigated.

With respect to the effects of the stressors on the growth of the three test strains of *S. pneumoniae*, a transient inhibition of the growth of all three strains was observed following exposure to CSC, probably as the consequence of a stress response associated with biofilm formation. As expected, exposure of strain 172 of the pathogen to a sub-MIC concentration of clarithromycin was also associated with a transient inhibition of growth, which was also evident with the two macrolide-resistant strains of the bacterial pathogen, probably as a consequence of the time required for induction of the resistance genes. Exposure of strain 172 of the pneumococcus to the combination of clarithromycin and CSC resulted in the most prolonged inhibition of growth, most likely as a consequence of intense stress caused by exposure to both stressors.

The findings of the present study also confirm and extend previous reports that exposure of the pneumococcus to cigarette smoke results in augmentation of biofilm formation, which, in turn, may contribute to microbial colonisation, persistence and antibiotic resistance. In addition, the findings of this study suggest that smoking may also attenuate the inhibitory effects of clarithromycin, albeit modest at the sub-inhibitory concentration used, on biofilm formation, which may also negate the efficacy of macrolide treatment.

Previous studies have reported on the positive effects of cigarette smoking on the survival mechanisms deployed by *S. pneumoniae*, which are seemingly mediated via enhancement of microbial virulence and antibiotic resistance^{16,19,176}. In this context, the findings of the current study have identified apparently novel mechanisms by which exposure to cigarette smoke may sensitise macrolide-resistant pneumococci in the airways for increased expression of the *erm(B)* gene, possibly predisposing to the development of a more aggressive phenotype. These include enhancement of clarithromycin-mediated induction of gene expression, as well as activation of *erm(B)* gene independently of the antibiotic. In addition, the stress-related ABC-transporter gene, *SP2003*, the role of which has not fully been elucidated, has also been shown to be induced on exposure of the pneumococcus to CSC, representing another potential mechanism that may contribute to the development of resistance to macrolides, as has previously been found with vancomycin. In this context, it is of particular note that exposure to clarithromycin alone caused modest upregulation of the *SP2003* gene in all three test strains of the pneumococcus, albeit not achieving statistical significance.

With respect to future initiatives resulting from the current study, these should address: i) inclusion of additional *erm(B)*-expressing strains of the pneumococcus; ii) more intensive investigation of the possible involvement of the ABC transporter-encoding gene, *SP2003* in promoting resistance to macrolide antibiotics; iii) unravelling the mechanism by which CSC activates *erm(B)* gene expression in the absence of a macrolide; and iv) investigating the possible involvement of CSC in attenuating the sensitivity of both macrolide-susceptible and-resistant strains of the pneumococcus to this class of antibiotics by the mechanisms described in this dissertation.

In conclusion, the pathogen-targeted effects of CSC described in this dissertation provide additional insights into the mechanisms by which cigarette smoking impacts negatively on the outcome of pneumococcal infections, apparently by undermining the therapeutic efficacy of macrolide antibiotics

Chapter 6: References

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The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.



UNIVERSITEIT VAN PRETORIA
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Faculty of Health Sciences Research Ethics Committee

27/07/2017

Approval Certificate
New Application

Ethics Reference No: 314/2017

Title: Effects of cigarette smoke condensate on clarithromycin-mediated inhibition of biofilm formation and related alterations in resistance gene expression by *Streptococcus pneumoniae*

Dear Mr Kgashane Given Matapa

The **New Application** as supported by documents specified in your cover letter dated 27/06/2017 for your research received on the 26/06/2017, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 26/07/2017.

Please note the following about your ethics approval:

- Ethics Approval is valid for 1 year
- Please remember to use your protocol number (**314/2017**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:

- The ethics approval is conditional on the receipt of **6 monthly written Progress Reports**, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

A handwritten signature in black ink that reads 'R Sommers'.

Dr R Sommers; MBChB; MMed (Int); MPharMed, PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).