Chapter One

Introduction

Streptococcus pneumoniae, or the pneumococcus, is a major cause of pneumonia, bacteremia, meningitis, otitis media and sinusitis and is responsible for significant morbidity and mortality worldwide, especially in the developing world.

1.1 *Streptococcus pneumoniae*

1.1.1 Microbiology

S. pneumoniae is a Gram-positive coccus (Figure 1.1). The organism is α-hemolytic due to the production of pneumolysin, which breaks down haemoglobin and results in a green zone when grown on blood agar plates. Pneumococci are optochin-sensitive and bile soluble, and these properties are used for the identification of the organism (Musher, 2005).

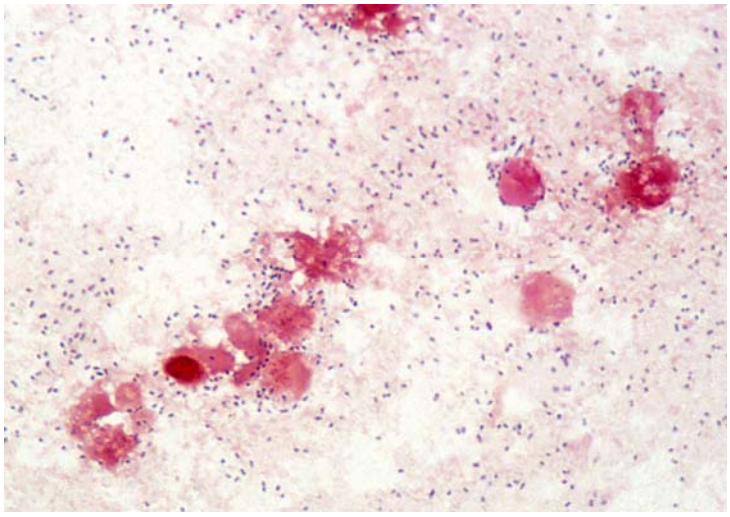

Figure 1.1 Gram-stain of cerebrospinal fluid from a patient with pneumococcal meningitis at 1000x magnification

The majority of clinical isolates contain polysaccharide capsules. Based on antigenic differences in the capsules, 90 serotypes of *S. pneumoniae* can be distinguished. The quellung reaction, based on an agglutination reaction with serum antibodies, is used for the identification of the serotype (Figure 1.2) (Musher, 2005).

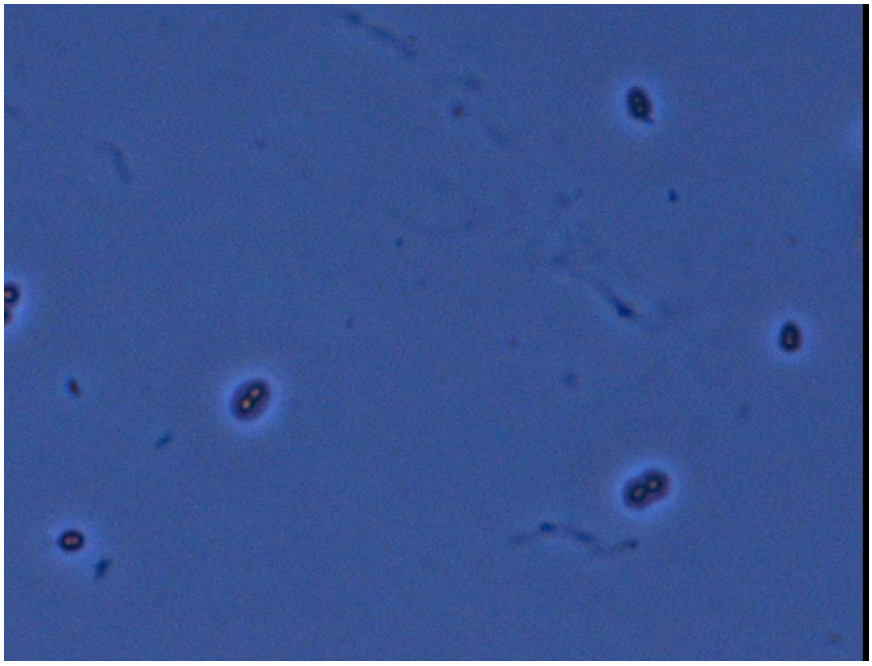

Figure 1.2 Phase-contrast microscopy of pneumococcal cells illustrating the capsular swelling of the quellung reaction

In addition to the traditional methods of pneumococcal identification, including α-hemolysis on blood agar, bile solubility, optochin sensitivity and the quellung reaction (Musher, 2005), molecular methods based on the polymerase chain reaction (PCR) are used. These include detection of the *lyt*A (autolysin), *psa*A (pneumococcal surface adhesin A) and *ply* (pneumolysin) genes (Messmer et al., 2004; Morrison et al., 2000).

1.1.2 Molecular Aspects of the Pneumococcus

The single circular chromosome of the pneumococcus is approximately 2 000 000 base pairs (bp) in size. It contains approximately 2000 protein-encoding genes and four rRNA operons (Hoskins et al., 2001). The pneumococcus undergoes natural transformation, the uptake of DNA from pneumococci or other bacterial species. During transformation, double-stranded DNA binds to the cell surface, is cleaved and a single strand enters the cell while the complementary strand is degraded. Binding proteins cover the strand inside the cell. If the strand contains homology to the chromosome, recombination will take place. If there is no significant homology the strand will be degraded (Lacks, 2004). Pneumococci become competent to take up DNA at a specific point during their growth when they reach a suitable density. At this stage of growth the bacteria synthesise competence-stimulating polypeptide (CSP) which induces a state of competency enabling transformation to take place (Lacks, 2004). Transformation plays an important role in the development of antibiotic resistance and can also result in capsular switching, in which the organism changes serotype (Musher, 2005).

1.1.3 Pathogenesis and Pneumococcal Disease

The pneumococcus commonly colonises the nasopharynx and is predominantly carried asymptomatically. However in certain cases, dependent on host and pathogen factors, the organism penetrates the mucosal membranes resulting in invasive disease, and causes significant morbidity and mortality. Invasive pneumococcal disease (IPD) is defined as the isolation of *S. pneumoniae* from a normally sterile site. The capsule plays a central role in the ability of *S.*

pneumoniae to resist phagocytosis and cause disease. Certain serotypes are associated with greater virulence than others. In addition to the capsule, the pneumococcus has noncapsular virulence factors. These include proteins such as pneumolysin, autolysin, neuraminidase, pneumococcal surface protein A (PspA), pneumococcal surface protein C (PspC) and pneumococcal surface adhesin A (PsaA) (Musher, 2005).

Spread of the pneumococcus from the nasopharynx results in a variety of different clinical syndromes. *S. pneumoniae* is one of the most prevalent pathogens causing community-acquired pneumonia (CAP), bacteremia, meningitis, acute otitis media and acute sinusitis (Winn et al., 2006). Pneumonia, or infection of the alveoli, is the most common cause of death due to an infection in the United States of America (US) (Donowitz and Mandell, 2005). Otitis media, common in children under the age of three, is an infection of the middle ear and causes fever, pain and hearing loss. The pneumococcus is the most common bacterial cause of acute otitis media (Klein, 2005). Pathogenesis of the pneumococcus from bacteremia (infection of the bloodstream) or the sinuses and middle ear to the central nervous system results in meningitis. Symptoms of meningitis include fever, headache, and confusion. Bacterial meningitis is most commonly caused by the pneumococcus, with a high mortality rate of 19 to 26% (Tunkel and Scheld, 2005).

1.1.4 Burden of Disease

Pneumococcal disease continues to result in significant illness and death worldwide. In developing countries, approximately 814 000 deaths in children less than five years of age are attributed to pneumococcal disease every year (Scott, 2006). Worldwide, pneumonia has been to shown to be the leading killer of children less than 5, resulting in more deaths than AIDS, malaria and diarrhoeal diseases. In the developing world, more than 2 million children under the age of 5 die annually as a result of this disease, of which the pneumococcus is a major cause (Wardlaw et al., 2006).

Community-based studies in Africa have shown that bacterial infections are responsible for the most deaths in children (Mulholland and Adegbola, 2005). In a study of invasive bacterial infections in children admitted to a rural hospital in Kenya from 1998 to 2002 it was found that the minimal annual incidence of community-acquired bacteremia in children less than 5 years was 505 cases per 100 000, with 26% of all hospital deaths caused by this disease. *S. pneumoniae* was the most commonly isolated organism in this study (Berkley et al., 2005). A further study, at this same Kenyan hospital, on all children visiting the outpatient facility between May and October 2003 showed the incidence of pneumococcal bacteremia to be 597 per 100 000 in children younger than 5 years (Brent et al., 2006).

The burden of pneumococcal disease has significantly increased as a result of the human immunodeficiency virus (HIV) epidemic. HIV-infected individuals have a

higher risk of developing IPD and are also more likely to be infected with an antibiotic-resistant strain. In a study conducted on South African children under the age of twelve it was found that there was a 41.7 fold increase in severe IPD in HIV-infected children, compared with HIV-uninfected children (Madhi et al., 2000). In another South African study, pneumococcal bacteremia was found to be 36.9 fold higher in HIV-infected children and 8.2 fold higher in HIV-infected adults, than in HIV-negative patients (Jones et al., 1998).

1.1.5 Prevention of Disease

Currently, two types of pneumococcal vaccines are available. The 23-valent polysaccharide vaccine provides protection against 23 pneumococcal serotypes responsible for a large proportion of disease: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F. However it is not effective in children less than 2 years of age as it only elicits a B-cell response (Winn et al., 2006).

The 7-valent pneumococcal conjugate vaccine (PCV7), by use of a carrier protein, is able to stimulate immune responses in children younger than 2. In addition, it reduces pneumococcal carriage of the serotypes included in the vaccine. The vaccine includes seven pneumococcal serotypes 4, 6B, 9V, 14, 18C, 19F, 23F (Winn et al., 2006). Since the introduction of PCV7 in the US in 2000, it has been shown to be effective in reducing IPD in vaccinated children and has also been shown to have a herd immunity effect by reducing disease due to *S. pneumoniae* in adults (Whitney et al., 2003).

The selective pressure resulting from PCV7 promotes serotype replacement in which vaccine serotypes are replaced with nonvaccine serotypes (Beall et al., 2006; Hanage et al., 2007). This presents a problem for the future effectiveness of the vaccine. Vaccines using pneumococcal surface proteins, such as PspA, are being investigated with the advantage that they provide cross-protection across all serotypes (Winn et al., 2006).

1.2 Protein Synthesis – Inhibiting Antibiotics

1.2.1 Bacterial Protein Synthesis

Bacterial gene expression takes place in two steps, namely transcription and translation. During transcription, messenger RNA (mRNA) is synthesized by RNA polymerase from the DNA template. RNA polymerase binds to DNA at the promoter region by recognition of two sequences, a 6 bp sequence situated 35 bases upstream from the transcription start site, and a Pribnow box (TATAAT) situated 10 bases upstream from the transcription start site. In the second step, translation, proteins are synthesised when mRNA is translated into an amino acid sequence. The site of protein synthesis is the ribosome. Bacterial ribosomes consist of two subunits, a large 50S subunit and a small 30S subunit. The small subunit consists of 16S ribosomal RNA (rRNA) and 21 ribosomal proteins and the large subunit consists of 23S rRNA, 5S rRNA and 34 ribosomal proteins. Protein synthesis occurs in three stages: initiation, elongation and termination. In initiation, an initiator transfer RNA (tRNA; *N*-formylmethionyl-tRNA^{fMet}) and mRNA bind to the free 30S subunit. The 50S subunit then binds to the 30S-

mRNA complex forming the 70S initiation complex. The Shine-Dalgarno sequence on the mRNA, approximately 10 nucleotides upstream of the translation start codon, specifies the translation initiation site. In each elongation cycle an amino acid is added to the growing polypeptide chain, which passes through the peptide exit tunnel of the large ribosomal subunit. The peptide bond is formed by a peptidyl transferase in the 50S subunit. Protein synthesis is terminated when the ribosome reaches a stop codon. The ribosome then releases the mRNA and separates into two subunits (Prescott et al., 2002; Voet et al., 1999).

1.2.2 Macrolides

Erythromycin is composed of a 14-membered macrolactone ring attached to two sugars, namely desosamine and L-cladinose (Figure 1.3). Azithromycin (15-membered macrolide) and clarithromycin (14-membered macrolide) are semi-synthetic derivatives of erythromycin (Sivapalasingam and Steigbigel, 2005). Rokitamycin is a 16-membered macrolide. Macrolides inhibit bacterial protein synthesis in two ways. Firstly, by binding to domain V of 23S rRNA in the peptidyl transferase region of the large ribosomal subunit, blocking the peptide exit tunnel and thereby blocking peptide chain elongation (Douthwaite and Champney, 2001; Zuckerman, 2004). Secondly, macrolides interfere with assembly of the 50S ribosomal subunit. They bind to a precursor of the 50S subunit and stall the assembly of the subunit resulting in it being defective and therefore degraded (Champney**,** 2001). The macrolides interact with the central loop of domain V of 23S rRNA, specifically residues A2058, A2059 and G2505 (*Escherichia coli* numbering) (Douthwaite and Champney, 2001).

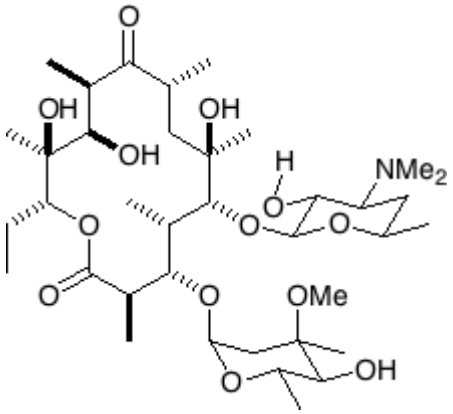

Figure 1.3 Chemical structure of erythromycin (Accessed from http://www-jmg.ch.cam.ac.uk/data/molecules/polyketides/erythromycin.html; January 2007)

1.2.3 Ketolides

Telithromycin, the first ketolide approved for clinical use, is a semi-synthetic derivative of the macrolide erythromycin A. Modifications of the 14-membered macrolactone ring include a replacement of the L-cladinose sugar at position 3 with a ketone group and the addition of a carbamate group at position C11-C12 with an alkyl-aryl extension (Figure 1.4) (Sivapalasingam and Steigbigel, 2005). Telithromycin binds to the same site on the large ribosomal subunit and has a similar mechanism of action to erythromycin however, in addition to the primary binding site of erythromycin, A2058 in domain V, the C11-C12 carbamate extension of telithromycin enables it to bind to A752 in domain II of 23S rRNA (Douthwaite and Champney, 2001). As a result, telithromycin has a stronger binding affinity for the ribosome and can therefore overcome common macrolide resistance mechanisms including target modification and drug efflux (Capobianco

et al., 2000; Kresken et al., 2004). In addition, telithromycin does not induce macrolide-lincosamide-streptogramin B (MLS_B) resistance due to the replacement of the cladinose sugar with a ketone group (Leclercq, 2001).

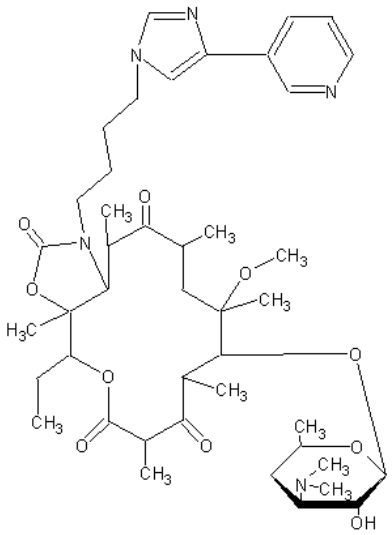

Figure 1.4 Chemical structure of telithromycin (Accessed from http://en.wikipedia.org/wiki/Telithromycin; January 2007)

1.2.4 Lincosamides

Clindamycin, a chemical modification of lincomycin, is chemically unrelated to erythromycin however has a similar binding site on the 50S ribosomal subunit (Douthwaite, 1992). It inhibits bacterial protein synthesis by interfering with the formation of the peptide bonds (Sivapalasingam and Steigbigel, 2005).

1.2.5 Tetracyclines

Tetracyclines, such as doxycycline and tetracycline HCl, bind to the small 30S ribosomal subunit and inhibit protein synthesis. They block the aminoacyl-tRNA acceptor site and therefore prevent new amino acids from being added to the polypeptide chain (Meyers and Salvatore, 2005).

1.2.6 Chloramphenicol

Chloramphenicol binds to the large ribosomal subunit preventing the binding of the aminoacyl-tRNA, and therefore preventing the formation of a peptide bond in the growing polypeptide chain (Meyers and Salvatore, 2005).

1.2.7 Streptogramins

Streptogramins consist of a combination of group A and group B streptogramins, which act synergistically. Quinupristin-dalfopristin is a mixture of the semi-synthetic streptogramins quinupristin (pristinamycin I_A) and dalfopristin (pristinamycin II_A). They bind to the large ribosomal subunit and inhibit protein synthesis. Group A streptogramins interfere with the formation of peptide bonds and group B streptogramins cause incomplete polypeptides to be released from the ribosome. The two components act synergistically because the binding of the group A streptogramins enhances the affinity of the ribosome for binding of the group B streptogramins (Yao and Moellering, 2003). Pneumococci only develop resistance against the group B streptogramins.

1.2.8 Oxazolidinones

Oxazolidinones, of which linezolid (Figure 1.5) was the first to be approved for clinical use, are synthetic molecules with unique chemical structures (Donowitz, 2005). Oxazolidinones inhibit protein synthesis by a unique mechanism of action. They bind to the P site of the 50S ribosomal subunit (Aoki et al., 2002; Lin et al., 1997) and prevent the formation of the 70S initiation complex in bacterial translation systems (Livermore, 2003; Shinabarger et al., 1997; Swaney et al., 1998).

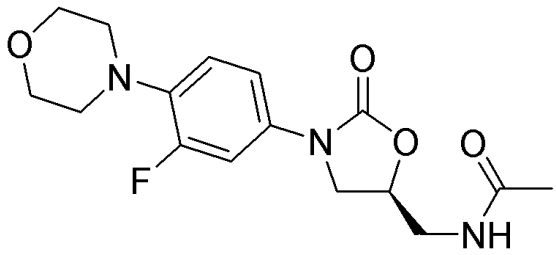

Figure 1.5 Chemical structure of linezolid (Accessed from http://en.wikipedia.org/wiki/Linezolid; January 2007)

1.3 Mechanisms of Antibiotic Resistance

1.3.1 Development of Antibiotic Resistance

An organism may develop resistance in response to selective antimicrobial pressure. The mechanism of resistance varies and can include one or more of the following: decreased membrane permeability, efflux of the antibiotic from inside

the cell, inactivation of the antibiotic by enzymes, alteration of the drug target site, and bypass of the target affected by the antibiotic (Leclercq, 2001).

Resistance to an antibiotic may be intrinsic to an organism, which is a natural inherent resistance, or it may be acquired by the organism. Acquired resistance can occur independently by the mutation of genes or the organism may acquire resistance genes from other organisms by gene exchange. Antibiotic resistance genes may be acquired in a number of ways; natural transformation by the direct uptake of DNA from the environment, and through transposons, which are small mobile genetic elements (Kaye et al., 2004). Conjugative transposons have the ability to be transferred between bacterial chromosomes (Rice et al., 2003).

The development or acquisition of antibiotic resistance by bacteria in order to survive under antibiotic selective pressure may result in a loss of fitness in the organism and therefore in most cases there is a biological cost to resistance. This often occurs when the mutations conferring resistance occur in genes essential for the survival of the organism. In order to overcome the loss of fitness and survive in a competitive environment bacteria develop compensatory mutations (Bjorkman et al., 2000; Gillespie and McHugh, 1997). Bacteria may also regulate the expression of resistance genes according to the environment, which enables them to resist antimicrobial action when necessary and to regain fitness in the absence of the antibiotic (Depardieu et al., 2007).

Antibiotic-resistant strains of pneumococci have spread worldwide by clonal dissemination and a number of global pneumococcal clones have been classified by the Pneumococcal Molecular Epidemiology Network (PMEN) using molecular characterisation techniques (McGee et al., 2001b). An example of this is the spread of the Spain^{23F}-1 pneumococcal clone that originated in Spain and subsequently has been isolated in Europe, the United Kingdom, the US, South and Central America, South Africa and Asia. This clone is resistant to penicillin, chloramphenicol, tetracycline and erythromycin (McGee et al., 2001b).

1.3.2 Macrolides, Lincosamides and Streptogramins

Macrolides, lincosamides and streptogramins (MLS_B) have similar target sites and mechanisms of action and therefore have similar mechanisms of resistance. The MLS_B ribosome-binding pocket is formed by domains II (hairpin 35) and V (peptidyltransferase region) of 23S rRNA, and ribosomal proteins L4 and L22 (Douthwaite and Champney, 2001; Hansen et al., 1999). Ribosomal proteins L4 and L22 are located in the peptide exit tunnel of the large ribosomal subunit (Gabashvili et al., 2001).

Resistance to macrolides in *S. pneumoniae* is conferred by two predominant mechanisms namely, active drug efflux and target-site modification. Drug efflux occurs due to acquisition of the *mef*(A/E) gene which encodes a transmembrane efflux pump. It confers resistance to 14- and 15-membered macrolides but not to lincosamides and streptogramins and is therefore referred to as the M-phenotype (Sutcliffe et al., 1996a). Phenotypically, expression of an efflux pump results in

low levels of macrolide resistance (MIC 1 – 32 μg/ml). The transferable *mef*(A) and *mef*(E) genes are both found in *S. pneumoniae* and have a similarity of 90%, however, occur on different conjugative genetic elements (Cochetti et al., 2005; Klaassen and Mouton, 2005). The *mef*(A) gene is located on the 7.2 kb Tn1207.1 transposon (Santagati et al., 2000) and the *mef*(E) gene on the 5.4 or 5.5 kb mega (macrolide efflux genetic assembly) element (Gay and Stephens, 2001). Downstream of the *mef* genes in both genetic elements is a closely related *mel* gene also involved in the efflux mechanism (Ambrose et al., 2005). Expression of the efflux pump is macrolide inducible and higher macrolide MICs are associated with increased expression (Wierzbowski et al., 2005). For the purposes of this thesis *mef*(A) will be used to describe *mef*(A) and *mef*(E).

Target modification most commonly occurs by the acquisition of an *erm*(B) gene (erythromycin ribosome methylase) which confers high-level macrolide resistance (MIC ≥ 64 μg/ml). This gene encodes a ribosomal methylase that methylates adenine 2058, a primary macrolide target site in the peptidyl transferase region of 23S rRNA. This modification prevents antibiotic binding and confers cross-resistance to the MLS_B antibiotics and is therefore known as the MLS_B phenotype. The *erm*(B) gene is carried by conjugative transposons, either Tn1545 or Tn917, and may be constitutively or inducibly expressed. In the case when expression is constitutive the mRNA is synthesised in an active conformation, and when it is inducible the mRNA is inactive and only becomes active in the presence of inducers (Leclercq and Courvalin, 2002; Weisblum, 1995). The inducible form of *erm*(B) is most common in pneumococci (Rosato et al., 1999) and the MLS_B

antibiotics act as inducers of the gene. In rare cases of macrolide resistance, the methylase enzyme is encoded by the *erm*(A) gene (Syrogiannopoulos et al., 2001). Macrolide-resistant pneumococci may have both the *erm*(B) and *mef*(A) genes (McGee et al., 2001a) and the prevalence of these isolates has been shown to be increasing. In the PROTEKT (Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin) US study from 2000 to 2003, isolates containing both resistance determinants increased from 9.7% to 16.4%. These strains are predominantly multidrug-resistant, highly clonal and display the MLS_B phenotype (Farrell et al., 2005).

Target modification may also arise from mutations in the genes encoding domain V of 23S rRNA and the ribosomal proteins L4 and L22, although less common (Canu et al., 2002; Farrell et al., 2003; Pihlajamaki et al., 2002; Reinert et al., 2003; Tait-Kamradt et al., 2000a; Tait-Kamradt et al., 2000b). The phenotype associated with this resistance mechanism varies according to the position, nature and number of mutations. Mutations in ribosomal proteins L4 and L22 affect the conformation of 23S rRNA and therefore the binding of the antibiotic to the target site (Gregory and Dahlberg, 1999). Pneumococci have four copies of the rRNA operon and mutations must occur in at least two alleles for phenotypic resistance. Resistance increases with the number of mutant rRNA alleles (Edelstein, 2004; Tait-Kamradt et al., 2000a).

1.3.3 Ketolides

Telithromycin shares a common binding site with the MLS_B antibiotics however due to its structural modifications it forms an additional bond in domain II of 23S rRNA and therefore forms a tighter bond with the ribosome and remains active against strains expressing *mef*(A) and *erm*(B) (inducible or constitutive). Macrolide-resistant pneumococci have increased telithromycin MICs but remain susceptible to the antibiotic (Capobianco et al., 2000; Kresken et al., 2004; Low et al., 2002). In contrast to erythromycin, telithromycin is a weak inducer of inducible *erm*(B) genes (Rosato et al., 1998; Zhong et al., 1999).

Telithromycin resistance in *S. pneumoniae* remains rare (Hisanaga et al., 2005; Farrell and Felmingham, 2004). In laboratory-generated mutants with reduced susceptibility to telithromycin, mutations were observed in the genes encoding domains II (A752) and V (A2058 and C2611) of 23S rRNA, ribosomal protein L22, the upstream region of *erm*(B) and the structural *erm*(B) gene (Canu et al., 2002; Walsh et al., 2003). Mutations in the genes encoding domain V of 23S rRNA, ribosomal proteins L4 and L22, the upstream region of *erm*(B) and the structural *erm*(B) gene have been identified in clinical isolates non-susceptible to telithromycin (Farrell et al., 2004a; Perez-Trallero et al., 2003; Pihlajamaki et al., 2003; Reinert et al., 2005b; Tait-Kamradt et al., 2000b; Tait-Kamradt et al., 2001). A combination of mutations in these genes appears to result in higher levels of resistance to telithromycin than a mutation in only a single gene (Faccone et al., 2005; Perez-Trallero et al., 2003; Tait-Kamradt et al., 2001). Macrolide-resistant pneumococci containing *erm*(B) may display heterogeneous

resistance to telithromycin, indicated phenotypically by the growth of colonies inside the zone of inhibition of a telithromycin disk (Rantala et al., 2006).

1.3.4 Tetracyclines

Tetracycline resistance in pneumococci occurs by ribosomal protection proteins encoded by *tet*(M) or *tet*(O). These proteins displace tetracycline from the ribosome (Widdowson and Klugman, 1999). The *tet*(M) gene is carried on the same conjugative transposon as the *erm*(B) gene, Tn1545, and therefore these resistance determinants are often spread together (Montanari et al., 2003b). A less common efflux mechanism is encoded by the *tet*(K) and *tet*(L) genes (Chopra and Roberts, 2001).

1.3.5 Chloramphenicol

Resistance to chloramphenicol in *S. pneumoniae* occurs by production of the enzyme chloramphenicol acetyltransferase (CAT) (Dang-Van et al., 1978; Miyamura et al., 1977). The *cat* gene is carried on the conjugative transposon Tn5253 in pneumococci. Widdowson et al. (2000) showed that acquisition of the *cat* gene can occur by the linearization and integration of the *Staphylococcus aureus* plasmid pC194 into a conjugative element of the pneumococcal chromosome. CAT catalyzes the acetylation of chloramphenicol resulting in 1-acetoxy, 3-acetoxy and 1, 3-diacetoxy derivatives of chloramphenicol that are unable to bind the 50S ribosomal subunit (Widdowson and Klugman, 1999).

1.3.6 Oxazolidinones

To date, linezolid-resistant pneumococcal strains are rare. This may be due to the fact that linezolid is a synthetic compound with a different mechanism of action to the other protein synthesis-inhibiting antibiotics (Meka and Gold, 2004). As its therapeutic use is increasing, resistant isolates are starting to emerge. Bacteria develop resistance to linezolid by mutation of the antibiotic target site.

In linezolid-resistant clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA), resistance was found to be conferred by mutations in the central loop of domain V of 23S rRNA (Meka et al., 2004a; Tsiodras et al., 2001; Wilson et al., 2003). Mutations in domain V of 23S rRNA have also been responsible for linezolid resistance in enterococci (Dibo et al., 2004; Herrero et al., 2002; Marshall et al., 2002). In both the MRSA and enterococci the most prevalent mutation associated with resistance to linezolid occurs at nucleotide G2576 in domain V and a correlation is shown between the number of mutated 23S rRNA alleles and the level of resistance (Marshall et al., 2002). Results from linezolid-resistant laboratory-generated mutants and clinical isolates strongly implicate the central loop of domain V of 23S rRNA (peptidyltransferase region) in the interaction between linezolid and the bacterial ribosome (Meka and Gold, 2004).

1.4 Prevalence of Pneumococcal Antibiotic Resistance

Pneumococci were predominantly susceptible to most antibiotics until the development of penicillin resistance in the mid-1970s. The increase in penicillin resistance during the 1980s and 1990s resulted in increased use of macrolides and other non-beta-lactam antibiotics for the treatment of pneumococcal infections, with a concomitant increase in the resistance of pneumococci to these antimicrobials (Musher, 2005). Since erythromycin resistance was first detected in 1967 in the US, macrolide resistance has been increasing globally with increased use of this antibiotic (Leclercq and Courvalin, 2002). An increase in macrolide resistance is associated with an increase in macrolide use (Bergman et al., 2006). Macrolide use in the US increased 13% from 1993 to 1999 and specifically increased 320% in children less than five years of age (Hyde et al., 2001). The high prevalence of macrolide resistance is due to both the clonal spread of resistant strains as well as the horizontal gene transfer of resistance determinants (Leclercq and Courvalin, 2002).

The increase in the use of macrolides, commonly used to treat community-acquired respiratory tract infections (CARTIs), is associated with an increase in macrolide resistance (Hyde et al., 2001; Klugman and Lonks, 2005). The Alexander Project, conducting global surveillance on the antibiotic resistance of pathogens involved in adult CARTIs, has shown an increase in pneumococcal macrolide resistance from 16.5% in 1996 and 21.9% in 1997 (Felmingham and Gruneberg, 2000) to 24.6% in the 1998 to 2000 period (Jacobs et al., 2003). The

global PROTEKT surveillance study reported 32.9% of pneumococcal isolates collected from 1999 to 2002 to be macrolide resistant (Farrell et al., 2004a). In the US, where macrolides are the first-line treatment for community-acquired pneumonia (CAP) in adults, invasive isolates collected by the Centers for Disease Control and Prevention (CDC) from 1995 to 1999 showed that macrolide resistance increased from 10.6% in 1995 to 20.4% in 1999 (Hyde et al., 2001). The Canadian Bacterial Surveillance Network reported an increase in macrolide-nonsusceptible pneumococcal isolates from 11.4% in 2000 to 13.9% in 2002 (Powis et al., 2004). The PneumoWorld Study reported pneumococcal macrolide resistance in eight European countries from 2001 to 2003 to be 28% (Reinert et al., 2005c).

Mechanisms of macrolide resistance vary geographically. The PROTEKT surveillance study showed for the 1999-2000 period that globally *erm*(B) is the predominant mechanism (56.2%) followed by *mef*(A) (35.3%) (Farrell et al., 2002). In the US *mef*(A) has been found to be the predominant mechanism. Macrolide-resistant isolates, collected from patients with CARTIs in the PROTEKT US 2000-2001 study, were 70.9% *mef*(A), 17.4% *erm*(B), and 10% *mef*(A) plus *erm*(B) (Brown et al., 2004). Similarly, in sterile site isolates collected in Canada in 2002, the M phenotype (56.7%) predominated over the MLS_B phenotype (46.3%) (Powis et al., 2004). Overall *erm*(B) is the predominant genotype in Europe, however the distribution in Europe varies widely by country with *erm*(B) being the most common macrolide resistance genotype in France,

Spain, Switzerland and Poland, whereas *mef*(A) predominates in Greece (Reinert et al., 2005a).

Pneumococcal resistance to macrolides may be largely a result of inappropriate or incorrect use of antibiotics. Factors which are associated with increased risk of acquiring a resistant pneumococcal strain include age (children less than two years and adults over 65 years), prior antibiotic exposure, hospitalisation or attendance of a child care centre (Kaye et al., 2004; Klugman and Lonks, 2005). Pneumococcal macrolide resistance has been shown to be of clinical relevance (Lonks et al., 2002).

Telithromycin is highly active against most strains of the pneumococcus. The PROTEKT surveillance study for the 1999-2000 period showed that 99.9% of isolates were susceptible to telithromycin (Felmingham et al., 2002). Data collected from the PROTEKT study for the three year period from 1999 to 2003 showed similar results with 99.8% of isolates susceptible to telithromycin. All of the ten isolates that were resistant to telithromycin contained the *erm*(B) gene (Farrell and Felmingham, 2004).

The LEADER 2004 surveillance study was established in the US with the aim of conducting national surveillance on linezolid activity. All 422 *S. pneumoniae* isolates collected during 2004 were susceptible to linezolid (Draghi et al., 2005).

The high global prevalence of macrolide resistance reported by numerous studies is of serious concern, however reports of a decrease in macrolide resistance in

invasive pneumococci that have been observed since the introduction of PCV7 in the US in 2000 are promising. The incidence of invasive disease due to a resistant organism of vaccine serotype decreased in both children and adults following the introduction of PCV7 (Kyaw et al., 2006; Stephens et al., 2005). There was however an increase in the incidence of disease caused by drug-resistant nonvaccine serotypes.

1.5 Study Objectives

Protein synthesis-inhibiting antibiotics are often used as a first-line treatment for pneumococcal infections. The widespread use of these antibiotics has resulted in increased levels of resistance being reported globally. In addition, as new and modified antimicrobials are developed and used for treatment, the ability of the pneumococcus to adapt under selective pressure results in the development of novel resistance mechanisms. In order to combat the global problem of antibiotic resistance and multidrug resistance, it is essential to monitor the prevalence of resistant strains and understand the mechanisms of antibiotic resistance in the pneumococcus.

The objectives of this study were:

1. To investigate the mechanisms of resistance in two linezolid-resistant clinical isolates of *S. pneumoniae*.
2. To investigate gene conversion and heterogeneous macrolide resistance in the pneumococcus.
3. To investigate the mechanisms of resistance in two telithromycin-resistant clinical isolates of *S. pneumoniae*.
4. To investigate the prevalence and epidemiology of macrolide resistance in invasive South African isolates for the period 2000 to 2005, and to determine the mechanisms of resistance in these strains.

Chapter Two

Materials and Methods

In this chapter general methods are described. Specific methods are described in the relevant chapters.

2.1 Culture and Storage of Bacterial Isolates

Pneumococci were routinely cultured at 37˚C in 5% CO_2 on Mueller-Hinton agar (MHA; Diagnostic Media Products, Johannesburg, South Africa) supplemented with 5% horse blood. Isolates were stored at -70°C in 10% skim milk (Diagnostic Media Products).

2.2 Phenotypic Characterisation of Isolates

Serotyping was performed by the quellung reaction (Austrian, 1976) with specific antisera from the Statens Serum Institut (Copenhagen, Denmark). Minimum inhibitory concentrations (MICs) were determined by the agar dilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute, 2006a), and the Etest (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions. For the agar dilution method, MHA plates were prepared containing 5% horse blood and doubling dilutions of antibiotic (Appendix A). Overnight cultures were suspended in Brain Heart Infusion (BHI) broth (Diagnostic Media Products) to a turbidity equivalent

to that of a 0.5 McFarland standard (Diagnostic Media Products) and were inoculated onto the antibiotic-containing plates using a Multipoint Inoculator (Mast Laboratories, Merseyside, United Kingdom). Plates were incubated for 20 – 24 hrs at 37°C in ambient air. The MIC was recorded as the lowest concentration of antibiotic that completely inhibited growth. For the Etest, overnight cultures were suspended in saline solution (Diagnostic Media Products) to a turbidity equivalent to that of a 0.5 McFarland standard. The suspension was inoculated onto MHA plates containing 5% sheep blood (Diagnostic Media Products) and following the application of the Etest strip, plates were incubated for 20 – 24 hrs at 37˚C in 5% CO_2. CLSI breakpoints were used (Clinical and Laboratory Standards Institute, 2006b) to define isolates as susceptible, intermediately resistant, or resistant to the appropriate antibiotic. Isolates were defined as nonsusceptible to an antibiotic if they were intermediately resistant or resistant to the antibiotic. *S. pneumoniae* ATCC 49619 was used as a control strain.

2.3 Genomic DNA Extraction

Genomic DNA was extracted using a phenol/chloroform method (Ausubel et al., 1989). Bacterial cells from overnight plate cultures were harvested and suspended in 550 µl Tris-EDTA (TE) buffer. 30 µl of a 10% sodium dodecyl sulphate (SDS) solution was added and tubes were incubated at 37°C for 1 hr. 70 µl of 5 M NaCl and 60 µl of 10% CTAB/ 0.7 M NaCl were added and the tubes were incubated at 65°C for 10 min. 600 µl of chloroform (Merck, Darmstadt, Germany) was added and the tubes were centrifuged for 5 min. The aqueous phase (top phase) was

transferred to a new tube and the DNA was precipitated by adding 0.7 volume of isopropanol (Merck) and centrifuging for 10 min. The pellets were washed by adding 800 µl of ice-cold 70% ethanol and centrifuging for 2 min. The pellets were dried at 50°C for 30 min and were resuspended in 70 µl of TE buffer containing RNase A, resulting in an approximate DNA concentration of 50 ng/µl. DNA extracts were stored at –20°C.

All centrifugation steps were carried out at 12 000 rpm using an Eppendorf MiniSpin benchtop centrifuge (Hamburg, Germany). Preparation of the chemicals and reagents are described in Appendix B.

2.4 Detection of *erm*(B) and *mef*(A) by PCR

The common macrolide resistance genotypes, *erm*(B) and *mef*(A), were detected by a duplex PCR adapted from methods previously described (Klugman et al., 1998; Sutcliffe et al., 1996b). For each 50 µl reaction, 2 µl of chromosomal DNA was added to a mix containing 1x PCR Gold Buffer (Applied Biosystems, Foster City, CA), 2.5 mM $MgCl_2$ (Applied Biosystems), 125 µM each of dATP, dCTP, dGTP and dTTP (Invitrogen, Carlsbad, CA), 200 nM each of forward primers ermF-Int (CGAGTGAAAAAGTACTCAACC) and mefF-Int (AGTATCATTAATCACTAGTGC) and reverse primers ermR-Int (GGCGTGTTTCATTGCTTGATG) and mefR-Int (TTCTTCTGGTACTAAAAGTGG) and 1.25 U of AmpliTaq Gold (Applied Biosystems). Cycling conditions were: 94˚C for 10 min; 94˚C for 1 min, 54˚C for

1 min and 72˚C for 1 min for 30 cycles; and 72˚C for 5 min. Amplification reactions were carried out with an OmniGene Thermal Cycler (Hybaid, Middlesex, UK). The PCR products were resolved by electrophoresis through a 1% agarose gel containing 0.6 μg/ml ethidium bromide (Appendix C). Electrophoresis was performed in 1x TAE Buffer (Appendix C) using a horizontal electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA) and gels were visualised using a Spectroline TR-312A UV transilluminator (Spectronics Corporation, Westbury, NY). The *erm*(B) product is 617 bp and the *mef*(A) product is 346 bp.

2.5 PCR Amplification

The four alleles of 23S rRNA and the genes encoding ribosomal proteins L4 (*rplD*) and L22 (*rplV*) were amplified by a modification of previously described methods (Farrell et al. 2003; Tait-Kamradt et al., 2000a). For each reaction, 3 μl of chromosomal DNA was added to a mix containing 2.5 U of Super-Therm *Taq* DNA polymerase (JMR Holdings, Kent, UK), 1x Reaction Buffer (JMR Holdings), 1.5 mM $MgCl_2$ (JMR Holdings), 200 μM of each deoxynucleoside triphosphate (dNTP; dATP, dCTP, dGTP and dTTP) (Invitrogen) and 800 nM each of forward and reverse primer in a final reaction volume of 50 μl. Primers were synthesised by Inqaba Biotec, Hatfield, South Africa. Amplification reactions were carried out with an OmniGene Thermal Cycler and PCR products were resolved by electrophoresis as described in Section 2.4.

2.5.1 23S rRNA

The four alleles of the 23S rRNA gene were amplified individually using a common forward primer (23SF: GGTTAAGTTAATAAGGGCGCACGGT) and an allele-specific reverse primer (Table 2.1). The cycle parameters were: one cycle of 94˚C for 2 min; 15 cycles of 92˚C for 30 s, 60˚C for 30 s, and 70˚C for 5 min; 18 cycles of 92˚C for 30 s, 60˚C for 30 s, and 70˚C for 5 min 15 s; and one cycle of 70˚C for 10 min. Amplified products were purified with the QIAquick Gel Extraction kit (Qiagen Ltd., Surrey, UK).

2.5.2 Ribosomal Proteins L4 and L22

The gene encoding ribosomal protein L4 was amplified using primer pair L4F (AAATCAGCAGTTAAAGCTGG) and L4R (GAGCTTTCAGTGATGACAGG) resulting in a 720 bp PCR product. The gene encoding ribosomal protein L22 was amplified using primer pair L22F (GCAGACGACAAGAAAACACG) and L22R (ATTGGATGTACTTTTTGACC) resulting in a 420 bp PCR product. Cycling parameters for L4 and L22 were: one cycle of 94˚C for 2 min; 27 cycles of 94˚C for 1 min, 54˚C for 2 min and 72˚C for 3 min; and one cycle of 72˚C for 10 min. Amplified products were purified with the QIAquick PCR Purification kit (Qiagen).

2.5.3 *erm*(B)

The *erm*(B) gene was amplified using forward primer ermBF (CTTAGAAGCAAACTTAAGAG) and reverse primer ermBR (ATCGATACAAATTCCCCGTAG) resulting in a PCR product of 1196 bp.

Cycling parameters were: 94˚C for 2 min; 94˚C for 1 min, 52˚C for 1 min and 72˚C for 3 min for 30 cycles; and 72˚C for 5 min. Amplified products were purified with the QIAquick PCR Purification kit (Qiagen).

2.6 DNA Sequencing

The cycle sequencing reactions were performed using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems). The 0.5x cycle sequencing reaction was prepared according to the manufacturer's recommendations. DNA template consisted of 50 – 100 ng of purified PCR product. Cycle sequencing parameters were: 94°C for 1 min; 25 cycles of 95°C for 30 s, 50°C for 20 s and 60°C for 4 min. Excess dye terminators were removed and cycle sequencing reactions were cleaned by use of the DyeEx 2.0 Spin Kit (Qiagen). Vacuum-dried samples were resuspended in Hi-Di Formamide (Applied Biosystems), denatured at 95°C for 2 min and cooled on ice for 1 min. Sequencing was performed using an Applied Biosystems Model 310 automated DNA sequencer.

The genes encoding ribosomal proteins L4 and L22 were sequenced using primers L4F and L4R, and L22F and L22R, respectively. Each of the four alleles of 23S rRNA were sequenced in 8 fragments using primers listed in Table 2.2. To sequence hotspots of the 23S rRNA genes, in domains II and V, primers 23SF-3, 23SF-7 and 23SF-8 were used. Primers used to sequence *erm*(B), including the upstream region of *erm*(B), are listed in Table 2.2.

2.7 Transformation

Two pneumococcal strains were used as recipients for transformation studies; (1) an unencapsulated laboratory strain R6, susceptible to all antibiotics, and (2) a strain (PC13) representative of the global pneumococcal clone 13 (South Africa^{19A}) (McGee et al., 2001b). PC13 was used as an additional recipient strain for transformation studies due to the fact that R6 does not contain an *erm*(B) gene for homologous recombination. Attempts to introduce a wild-type *erm*(B) gene into R6 by means of transformation, electroporation and conjugation were unsuccessful. The PC13 strain was selected on the basis that it contained a wild-type *erm*(B) gene. The genes encoding 23S rRNA and ribosomal proteins L4 and L22 in PC13 were confirmed to be wild-type.

R6 and PC13 were made competent by culture in C-medium (Tomasz and Hotchkiss, 1964) (Appendix D). Bacteria were grown in C-medium at 37°C to an optical density (OD) of 0.17 at 600 nm (WPA CO 8000 Biowave Cell Density Meter, Biochrom Ltd., Cambridge, UK). After the addition of 10% glycerol (final concentration) (Merck), cells were aliquoted and stored at –70°C. Purified PCR products were used as transforming DNA. For transformation, 500 µl of competent cells were incubated with 200 ng of competence-stimulating peptide (amino acid sequence: H-EMRLSKFFRDFILQRKK-OH) (Håvarstein et al., 1995) and 1 µg of donor DNA at 30˚C for 45 min, followed by 37˚C for 90 min. Eighty microlitre volumes were plated onto MHA plates supplemented with 5% horse blood and containing increasing concentrations of antibiotic (Appendix A).

The plates were incubated at 37˚C for 72 hrs in ambient air. Transformants were picked from the plates containing the highest antibiotic concentration possible. The MICs of the transformants were determined by methods described in Section 2.2, and the presence of mutations in the transformants were confirmed by DNA sequencing as described in Section 2.6.

2.8 Growth Studies

Exponential-phase glycerol starter stocks of cultures were prepared by growing bacteria in Tryptone Soya Broth (TSB) (Appendix E) at 37°C in 5% CO_2 to an OD of 0.3 at 600 nm (WPA CO 8000 Biowave Cell Density Meter, Biochrom Ltd.). When cultures reached the desired OD, 15% glycerol (final concentration) (Merck) was added and 1 ml aliquots of the cells were stored at -70°C.

For each strain, 100 ml of prewarmed TSB was inoculated with 1 ml of the glycerol starter stock (1:100 dilution) in a 250 ml glass bottle and incubated at 37˚C in 5% CO_2. Growth was monitored turbidimetrically at 600 nm (WPA CO 8000 Biowave Cell Density Meter, Biochrom Ltd.) at intervals of 30 min until cultures reached the stationary phase of growth. The mass doubling time of each strain during the exponential phase was calculated as follows:

$$Td = \ln 2 / \ln X - \ln X_0 / dt$$

Where: Td = Mass doubling time of the strain (min)

X = Second OD chosen during the exponential phase

X_0 = Initial OD chosen during the exponential phase

dt = Time (min) between the initial and second ODs chosen

2.9 Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed according to an adaption of previously described methods (Lefevre et al., 1993; McEllistrem et al., 2000). Bacteria from overnight plate cultures were resuspended in 600 µl of cell suspension buffer (Appendix F) to a turbidity of approximately 1.3 (MicroScan Turbidity Meter, Dade Behring, West Sacramento, CA). 300 µl of the bacterial suspension was warmed to 45^{o}C and mixed with an equal volume of 1% SeaKem Gold agarose (Cambrex Bio Science, Rockland, ME) prepared in cell suspension buffer. The mixture was dispensed into plugs moulds and allowed to solidify for 15 min at room temperature. To lyse bacterial cells, plugs were incubated at 50^{o}C for 2 hrs in 5 ml cell lysis buffer (Appendix F). Following cell lysis, plugs were washed twice with preheated dH_2O (15 min, 50°C) and twice with Tris-EDTA (TE) buffer (15 min, 50°C) (Appendix F). Plugs were stored in TE buffer at 4°C.

For digestion of genomic DNA, 2-3 mm slices of agarose plugs were incubated for 15 min at 30°C in 100 µl of Buffer A (Roche Diagnostics GmbH, Mannheim, Germany) as recommended for *Sma*I restriction enzyme (Roche). The plugs were then incubated at 30°C for 2 hrs in 150 µl of Buffer A containing 20 units of *Sma*I. Plugs were placed onto a comb, placed in a gel-casting tray and allowed to air-dry for 15 min. 1.1% SeaKem Gold agarose (Appendix F) was poured into the casting tray and the gel was allowed to polymerize for 30 min at room temperature. PFGE was performed in 0.5x Tris-borate EDTA (TBE) buffer (Appendix F) cooled to 14°C, for 21 hrs at 6 V/cm and an angle of 120° using the CHEF-DR III electrophoresis system (Bio-Rad Laboratories, Hercules, CA). Block 1 had an initial pulse time of 1 s increased to 30 s for 16 hrs and block 2 had an initial pulse time of 5 s increased to 9 s for 5 hrs.

The gel was stained for 20 min in 0.5x TBE buffer containing 1 mg/ml ethidium bromide and destained three times with dH_2O. Fingerprint patterns were visualised using a Spectroline UV transilluminator (Spectronics, Westbury, NY) and the image was captured using GeneSnap version 6.06 software (Syngene, Cambridge, UK). PFGE banding patterns were analysed using GelCompar II version 4.1 software (Applied Maths, Kortrijk, Belgium). Dendrograms were created using the unweighted pair group method with arithmetic averages (UPGMA). Analysis of the banding patterns was performed with the Dice-coefficient, an optimisation setting of 1.5% and a position tolerance setting of 1% for the band migration distance.

2.10 Multilocus Sequence Typing

DNA was prepared by boiling bacterial cultures. A loopful of culture was suspended in 100 µl of sterile dH_2O, heated at 95°C for 10 min and briefly centrifuged. Multilocus sequence typing (MLST) was carried out as previously described by Enright and Spratt (1998). Internal fragments of seven housekeeping genes were amplified; *aro*E*, gdh, gki, rec*P*, spi, xpt,* and *ddl*. For each reaction, 1 µl of chromosomal DNA was added to a mix containing 2 U of Super-Therm *Taq* DNA polymerase (JMR Holdings), 1x Reaction Buffer (JMR Holdings), 1 mM $MgCl_2$ (JMR Holdings), 125 µM of each deoxynucleoside triphosphate (dNTP; dATP, dCTP, dGTP and dTTP) (Invitrogen GmbH) and 400 nM each of forward and reverse primer in a final reaction volume of 25 µl. Primers were synthesised by Inqaba Biotec, Hatfield, South Africa and are listed in Table 2.3. Cycling conditions were as follows: one cycle of 94°C for 2 min, 30 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 30 s, and one cycle of 72°C for 5 min. Amplified products were purified with the QIAquick PCR Purification kit (Qiagen). The fragments were sequenced in the forward and reverse directions as described in Section 2.6 using the same primers as used for amplification (Table 2.3).

Sequences for each of the seven loci of an isolate were submitted to the MLST website (http://spneumoniae.mlst.net) and each locus was assigned an allele number. The combination of allele numbers for the seven loci (allelic profile) was used to determine the sequence type (ST) of an isolate.

Table 2.1 Allele-specific reverse primers for amplification of the four alleles of 23S rRNA

Allele	Reverse Primer	Sequence	Product size (bp)
23S-1	23SR-1	TACCAACTGAGCTATGGCGG	3119
23S-2	23SR-2	GCCAGCTGAGCTACACCGCC	3904
23S-3	23SR-3	TACACACTCACATATCTCTG	3906
23S-4	23SR-4b	CTGGATTCGAACCAACGCATGAGG	4072

Table 2.2 Primers used for the sequencing of genes encoding 23S rRNA and *erm*(B)

Gene	Primer	Sequence
23S rRNA	23SF-1b	ATGCCTTGGCACTAGGAGCCGA
	23SF-2	AGAGAGTAATAGCCTCGTAT
	23SF-3	GGCGAGTTACGTTATGATGC
	23SF-4	ATTATGGTCGGCAGTCAGAC
	23SF-5	AGACAGGTGAGAATCCTGTC
	23SF-6	AGTAGCCTCAGGTGAGCGAG
	23SF-7	CGCACGAAAGGCGTAATGAT
	23SF-8	AGTGATCCGGTGGTTCCGTA
erm(B)	ermBF	CTTAGAAGCAAACTTAAGAG
	ermBM-F1	ACGAGTGAAAAAGTACTCAA
	ermBM-F2	TTGCTCTTGCACACTCAAGTC
	ermBM-R1	CTGTAGAATATCTTGGTGAA
	ermBM-R2	CTGTCTAATTCAATAGACGT
	ermBR	ATCGATACAAATTCCCCGTAG

Table 2.3 Primers used for the amplification and sequencing of seven housekeeping genes in multilocus sequence typing of the pneumococcus

Gene	Forward Primer	Reverse Primer	Fragment Size (bp)
*aro*E	GCCTTTGAGGCGACAGC	TGCAGTTCAG/AAAACATA/TTTCTAA	405
ddl	TGCC/TCAAGTTCCTTATGTGG	CACTGGGTG/AAAACCA/TGGCAT	441
gdh	ATGGACAAACCAGCNAGC/TTT	GCTTGAGGTCCCATG/ACTNCC	459
gki	GGCATTGGAATGGGATCACC	TCTCCCGCAGCTGACAC	483
*rec*P	GCCAACTCAGGTCATCCAGG	TGCAACCGTAGCATTGTAAC	448
spi	TTATTCCTCCTGATTCTGTC	GTGATTGGCCAGAAGCGGAA	472
xpt	TTATTAGAAGAGCGCATCCT	AGATCTGCCTCCTTAAATAC	486

Chapter Three

A Novel Mechanism of Resistance to Oxazolidinones, Macrolides and Chloramphenicol in Ribosomal Protein L4 of the Pneumococcus

3.1 Introduction

Two clinical isolates of *S. pneumoniae* with elevated macrolide, linezolid and chloramphenicol MICs were identified from two separate surveillance studies. In this study the isolates were investigated in order to identify the mechanisms conferring such resistance. The initial isolate containing a 6 bp deletion in the gene encoding ribosomal protein L4, described by Farrell et al. (2004a), is further investigated and the second isolate, found to contain a similar L4 deletion, is described.

3.2 Materials and Methods

3.2.1 Clinical Isolates

Clinical isolate PU1071099 was isolated from the sputum of a 67 year-old outpatient in Boston in 2001 and submitted to the PROTEKT surveillance study (Farrell et al., 2004a). Isolate TN33388 was isolated from the blood of a 32 year-old in Tennessee in 2003 who had been exposed to long-term azithromycin prophylaxis and was submitted to the Active Bacterial Core surveillance (ABCs) program of the CDC.

3.2.2 Phenotypic and Genotypic Characterisation

MICs were determined by the agar dilution method and the Etest, and serotyping was performed by the quellung reaction as described in Section 2.2. CLSI breakpoints were used to classify the isolates as susceptible or nonsusceptible to the respective antimicrobials (Clinical and Laboratory Standards Institute, 2006b). MLST of the seven housekeeping genes was performed as described in Section 2.10.

Chromosomal DNA was extracted (Section 2.3) and PCR-based methods were used to screen for *erm*(B) and *mef*(A) (Section 2.4). PCR was used to detect the presence of the *cat* gene, commonly associated with chloramphenicol resistance in the pneumococcus. The PCR reaction was performed as described in Section 2.5. Forward primer catF (AGTATAACCACAGAAATTGA) and reverse primer catR (CATTAGGCCTATCTGACAAT) were used for amplification of the 555 bp

product. Cycling conditions were one cycle of 94°C for 2 min; 30 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min; and one cycle of 72°C for 5 min. Ribosomal protein genes *rplD* (L4) and *rplV* (L22) and the four alleles encoding 23S rRNA were amplified and sequenced as described in Chapter 2.

3.2.3 Transformation Studies

S. pneumoniae R6 was made competent by culture in C-medium and transformation performed as described in Section 2.7. The L4 gene of each isolate was used individually as donor DNA, and transformants (4 for each isolate) were selected on MHA supplemented with 5% horse blood and containing ERY (0.25 – 0.5 μg/ml). The MICs of the transformants were determined, and the L4 mutations were confirmed by DNA sequencing.

3.2.4 Growth Studies

Growth studies were performed in duplicate by inoculating glycerol stocks of pneumococci into Tryptone Soya Broth (1:100 dilution) and monitoring turbidity at 600 nm every 30 min for 9 hrs (Section 2.8).

3.3 Results

3.3.1 Phenotypic and Genotypic Characterisation

Isolate PU1071099 was found to be serotype 9N and ST66. Isolate TN33388 was found to be serotype 33F and ST100. Based on MLST analyses the isolates are clonally unrelated. The antibiotic susceptibilities of the isolates are shown in Table 3.1. The isolates had low-level resistance to macrolides and were susceptible to clindamycin. The isolates were nonsusceptible to linezolid and chloramphenicol. Both isolates were negative for the common resistance determinants associated with macrolide and chloramphenicol resistance: *erm*(B), *mef*(A) and *cat*. Each isolate was found to contain a deletion mutation in the L4 gene. Isolate PU1071099 carried a 6 bp deletion in the L4 gene resulting in the deletion of two amino acids ($_{64}$PWRQ$_{67}$ to $_{64}$P_Q$_{67}$). Isolate TN33388 carried a novel 6 bp deletion in the L4 gene ($_{67}$QKGT$_{70}$ to $_{67}$Q_T$_{70}$). For both isolates, the genes encoding 23S rRNA and ribosomal protein L22 were found to be wild-type when compared with *S. pneumoniae* R6 and *S. pneumoniae* ATCC 33400.

3.3.2 Transformation Studies

In order to investigate whether the L4 deletion mutations detected were associated with the decreased susceptibility of the isolates to protein-synthesis inhibiting antibiotics, transformation experiments were performed.

The mutated L4 gene from isolate PU1071099 was used for transformation of *S. pneumoniae* R6 and resulted in R6$^{PU1071099/L4}$ transformants. These transformants,

expressing the mutated L4 gene, showed decreased susceptibility to 14- and 15-membered macrolides, linezolid and chloramphenicol (Table 3.1) in comparison with untransformed R6. For each of these antibiotics the MICs of the R6 transformants were increased to, or within one dilution of, the MIC of the donor isolate from which the mutant gene was originally derived (ERY: 0.12 μg/ml in R6 to 1 μg/ml in the transformants; CLR: 0.06 μg/ml to 1 μg/ml; AZM: 0.12 μg/ml to 2 μg/ml; LZD: 1 μg/ml to 4 μg/ml; CHL: 4 μg/ml to 16 μg/ml). The streptogramin B MIC increased by one dilution in the transformants in comparison with untransformed R6. The susceptibility of R6 to clindamycin, quinupristin-dalfopristin, telithromycin and tetracycline was not reduced by expression of the mutant L4 gene in the transformants.

The gene encoding L4 from isolate TN33388 carrying a 6 bp deletion was used to transform R6 resulting in $R6^{TN33388/L4}$ transformants. As for the $R6^{PU1071099/L4}$ transformants, decreased susceptibility to erythromycin, clarithromycin, azithromycin, linezolid and chloramphenicol was observed for the transformants compared with untransformed R6 (ERY: 0.12 μg/ml in R6 to 2 μg/ml in the transformants; CLR: 0.06 μg/ml to 0.5 μg/ml; AZM: 0.12 μg/ml to 2 μg/ml; LZD: 1 μg/ml to 4 μg/ml; CHL: 4 μg/ml to 8 μg/ml) (Table 3.1). In addition the transformants showed reduced susceptibility to quinupristin-dalfopristin in comparison with untransformed R6 (0.5 μg/ml to 2 μg/ml). The transformants showed a one-dilution increase in the MIC of clindamycin (0.06 μg/ml to 0.12 μg/ml) and streptogramin B (4 μg/ml to 8 μg/ml) in comparison with wild-type

R6. The susceptibility of R6 to telithromycin and tetracycline was not reduced by the L4 mutation.

3.3.3 Growth Studies

Growth curves are shown in Figure 3.1. Mass doubling times (min) during the exponential phase of growth were as follows: PU1071099, 53.9; TN33388, 53.4; untransformed R6, 59.6; $R6^{PU1071099/L4}$, 88.1 and $R6^{TN33388/L4}$, 102.6.

Table 3.1 Phenotypic and genotypic data of PU1071099, TN33388, R6 and R6 transformants

Strain	Serotype	Multilocus sequence type	L4 Deletion	MIC (µg/ml) of[a]:										
				ERY	CLR	AZM	CLI	LZD	S-B	Q-D	CHL	TEL	TET	PEN
Isolates														
PU1071099	9N	ST66 (2-8-2-4-6-1-1)	$_{65}$WR$_{66}$	2	1	4	0.12	4	4	1	16	0.015	0.25	0.03
TN33388	33F	ST100 (5-12-29-12-9-39-18)	$_{68}$KG$_{69}$	2	1	4	0.12	4	4	2	16	0.015	0.25	0.03
Transformants														
R6			None	0.12	0.06	0.12	0.06	1	4	0.5	4	0.015	0.5	0.03
R6 $^{PU1071099/L4}$			$_{65}$WR$_{66}$	1	1	2	0.06	4	8	0.5	16	0.008	0.5	0.015
R6$^{TN33388/L4}$			$_{68}$KG$_{69}$	2	0.5	2	0.12	4	8	2	8	0.008	0.5	0.015

Table 3.1 Legend

[a] Abbreviations: ERY, erythromycin; CLR, clarithromycin; AZM, azithromycin; CLI, clindamycin; LZD, linezolid; S-B, streptogramin B; Q-D, quinupristin-dalfopristin; CHL, chloramphenicol; TEL, telithromycin; TET, tetracycline; PEN, penicillin

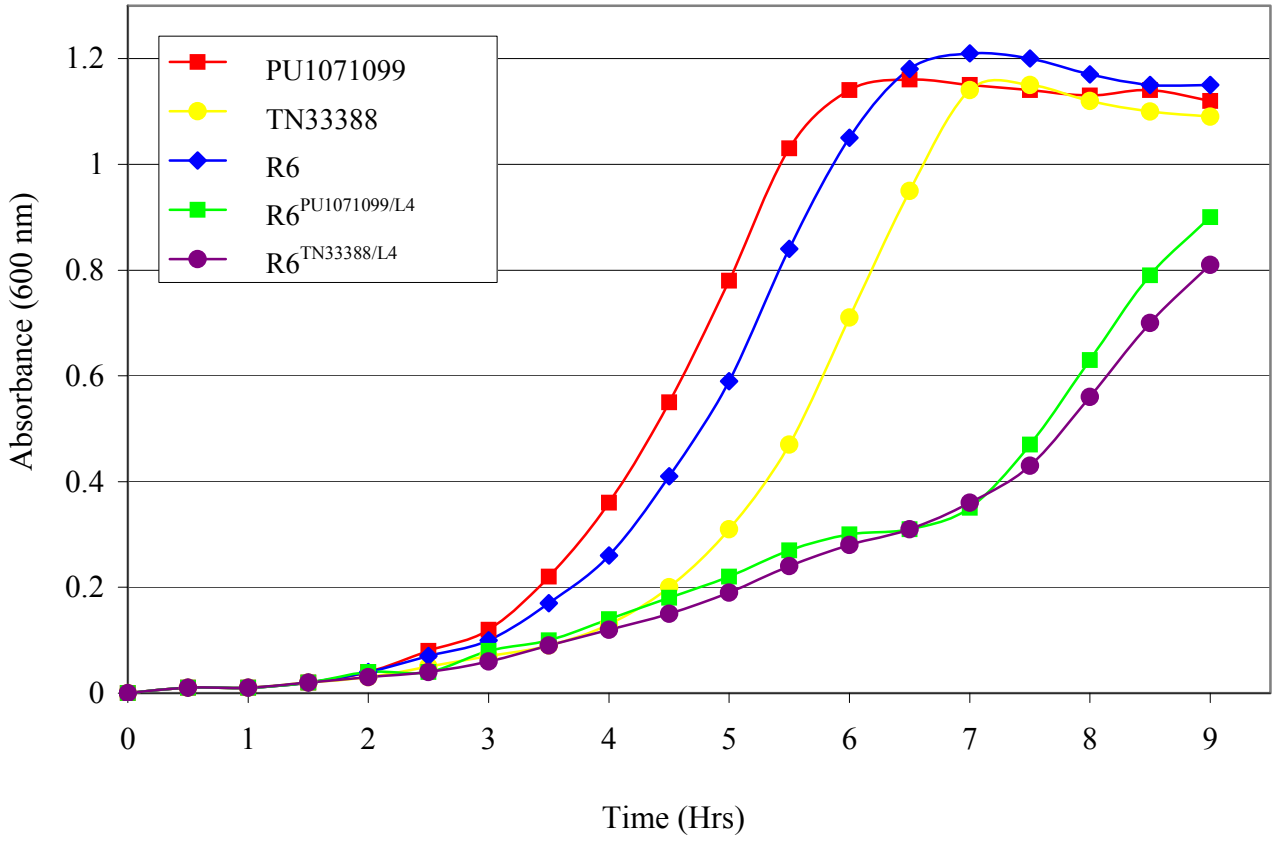

Figure 3.1 Growth curves at 37˚C of PU1071099, TN33388, R6 and R6 transformants carrying L4 deletion mutations

3.4 Discussion

The L4 mutations detected are likely to account for the macrolide resistance in the isolates as L4 mutations, most commonly in a highly conserved region ($_{63}$KPWRQKGTGRAR$_{74}$), have been shown to confer macrolide resistance in *S. pneumoniae* as well as in a number of other Gram-positive bacteria. Previously described pneumococcal L4 mutations resulting in macrolide resistance include amino acid substitutions such as $_{69}$G to C and $_{69}$GTG$_{71}$ to TPS as well as amino acid insertions $_{67}$Q**SQ**K$_{68,}$ $_{69}$GTG**REKGTG**RAR$_{74}$ and $_{67}$Q**RRQ**K$_{68}$ (Nagai et al., 2002; Pihlajamäki et al., 2002; Tait-Kamradt et al., 2000a; Tait-Kamradt et al., 2000b). The numerous mutations in ribosomal protein L4 conferring resistance to macrolides indicate that this protein forms an important component of the macrolide binding site on the large ribosomal subunit.

The L4 mutations described also accounted for the nonsusceptibility of the isolates to linezolid, with the transformants having equivalent MICs to the parent isolates. This represents a novel mechanism of linezolid resistance as previous reports have attributed resistance in Gram-positive bacteria to mutations in domain V of 23S rRNA (Marshall et al., 2002; Tsiodras et al., 2001). Mutations in the L4 gene confer macrolide resistance in *S. aureus* (Prunier et al., 2003). Should these mutations be shown to confer resistance to linezolid in *S. aureus*, they would be of particular significance as linezolid is widely used for the treatment of MRSA.

In addition to macrolides and linezolid, the L4 deletion mutations described in this study were also found to confer resistance to a third class of antibiotic, chloramphenicol. This represents a novel mechanism of resistance to chloramphenicol. A link between erythromycin, chloramphenicol and their binding to L4 has been established in studies by Suryanarayana (1983), who demonstrated that extracted *E. coli* ribosomal protein L4 binds to both erythromycin- and chloramphenicol- coupled affinity columns. The fact that chloramphenicol and oxazolidinones share a common binding site is suggested by the fact that chloramphenicol competes with the binding of the oxazolidinone, eperezolid, to the 50S ribosomal subunit (Lin et al., 1997). Mutations in domain V of 23S rRNA of *Halobacterium halobium* conferring resistance to linezolid were found to also confer resistance to chloramphenicol (Kloss et al., 1999). Therefore, although these antibiotics have different mechanisms of action, they appear to share a common binding site on the large ribosomal subunit. From the results of this study it can be concluded that ribosomal protein L4 forms an integral part of this binding site.

The ribosomal protein L4 $_{65}$WR$_{66}$ deletion detected in isolate PU1071099 has been previously described in group A streptococci (Bingen et al., 2002; Bozdogan et al., 2003). For both of the previously reported resistant *Streptococcus pyogenes* isolates, resistance developed during treatment with a macrolide antibiotic. The mutants were phenotypically characterized by decreased susceptibility to macrolides but not to clindamycin, as for isolate PU1071099. Susceptibility to linezolid and chloramphenicol were however not determined in those studies. The

results obtained for isolate PU1071099 show that this mutation confers linezolid and chloramphenicol resistance in *S. pneumoniae* and is likely therefore to also confer such resistance in *S. pyogenes*.

The reduced growth rates of the transformants suggests that the L4 mutations are associated with a fitness cost. The *rplD* gene encoding ribosomal protein L4 is essential and is regarded as one of the minimal set of genes necessary for bacterial life (Gil et al., 2004). L4 forms a part of the exit tunnel of the large ribosomal subunit and is thought to be involved in processing of the nascent polypeptide chains (Gabashvili et al., 2001). Mutations in this ribosomal protein may prevent complete binding of the antibiotics to this site, however the changes in the exit tunnel may hinder protein synthesis and affect bacterial growth. Inhibition of antibiotic binding may be due to direct structural changes resulting from the L4 mutations or may be due to indirect changes in the three-dimensional structure of 23S rRNA as a result of the L4 mutations (Gregory and Dahlberg, 1999). In contrast, the mass doubling times of the clinical isolates were shorter than for R6. Bacteria adapt to a decrease in fitness as a result of resistance mutations, by developing compensatory mutations that restore their fitness without affecting resistance (Gillespie, 2001). Our data suggest that the isolates may have acquired such compensatory mutations.

3.5 Conclusion

Two 6 bp deletion mutations, each resulting in the deletion of two amino acids from ribosomal protein L4, have been found to confer resistance to three classes of protein synthesis-inhibiting antibiotics, namely, macrolides, linezolid and chloramphenicol. These antibiotics are therefore likely to share a common binding site within the 50S ribosomal subunit. The likely selective agent for the mutation in isolate TN33388 conferring multiple resistance was long term intermittent exposure to azithromycin. Mutations conferring linezolid nonsusceptibility in clinical isolates of *S. pneumoniae* have been described for the first time, together with a novel mechanism of cross-resistance including oxazolidinone, chloramphenicol and macrolide resistance.

Chapter Four

Heterogeneous Macrolide Resistance and Gene Conversion in the Pneumococcus

4.1 Introduction

The rRNA-encoding genes differ from most prokaryotic chromosomal genes in that they are present in multiple copies in the genome. In *S. pneumoniae* there are four copies of the *rrn* operon (Klappenbach et al., 2001; Tait-Kamradt et al., 2000a). Multiple copies maintain extremely high homogeneity by gene conversion, or nonreciprocal recombination (Hashimoto et al., 2003; Liao, 2000). Gene conversion is the homologous recombination between mutant and wild-type alleles of a gene. In cases where resistance mutations occur in genes present in multiple copies, such as the 23S rRNA genes in macrolide resistance, the study of resistance mechanisms is complicated by gene conversion. In this study the implications of gene conversion for macrolide resistance due to heterologous 23S rRNA mutations were investigated.

4.2 Materials and Methods

4.2.1 Phenotypic and Genotypic Characterisation

A clinical isolate of *S. pneumoniae*, PU1004017, was obtained from the PROTEKT surveillance study. MICs were determined by the agar dilution method and the Etest as described in Section 2.2. Chromosomal DNA was extracted from the isolate and PCR-based methods were used to screen for *erm*(B) and *mef*(A). The genes encoding ribosomal proteins L4 and L22 and all four alleles encoding 23S rRNA were amplified and sequenced as described in Chapter 2.

4.2.2 Transformation Studies

S. pneumoniae R6 was made competent by culture in C-medium and transformation performed as described in Section 2.7. The 23S-1 allele of isolate PU1004017 (G2057A and A2059G) was used as donor DNA and a transformant (T) was selected on a MHA plate supplemented with 5% horse blood and containing ERY (16 μg/ml). The MICs of the transformant were determined and the 23S rRNA alleles were sequenced.

4.2.3 Gene Conversion Studies

The transformant was initially subcultured 3 times over a 1-week period on ERY plates (64 μg/ml) to obtain a uniform culture, which showed all four 23S rRNA alleles mutated ($_{2057}$**AAG**$_{2059}$). At this point two single colonies (TA and TB) were selected for further analysis. TA and TB were serially passaged 10 times over a 3-

week period on MHA supplemented with 5% horse blood with and without ERY (64 μg/ml). On days 0, 7, 14 and 21, MICs were determined by the agar dilution method and the 23S rRNA genes were sequenced (Table 4.1).

4.2.4 Growth Studies

Growth studies were performed on TA at 21 days of subculture either in the absence (TA-) or presence (TA+) of ERY. Glycerol stocks were inoculated into Tryptone Soya Broth (1:50 dilution) and turbidity was monitored at 600 nm every 30 min for 12 hrs as described in Section 2.8. Sequencing of the 23S rRNA alleles at 0 hrs and 12 hrs confirmed that gene conversion did not occur for the duration of the growth curve.

4.3 Results

4.3.1 Phenotypic and Genotypic Characterisation

PU1004017 was found to be highly resistant to macrolides (MICs (μg/ml): erythromycin 128, clarithromycin >256; azithromycin >256) and susceptible to clindamycin and telithromycin. Erythromycin Etests showed a heterogeneous phenotype. A zone of inhibition was observed for isolate PU1004017 as is typical of a susceptible strain, however satellite colonies occurred to an MIC of 256 μg/ml.

PU1004017 tested negative for *erm*(B) and *mef*(A) and the genes encoding L4 and L22 were wild-type. Sequencing of 23S rDNA revealed the mutation G2057A (*E. coli* numbering) in all four alleles, while alleles 23S-1 and 23S-4 showed mixed bases at position 2059 (A/G). The heterogeneous phenotypic and genotypic data indicated that gene conversion may be taking place between the 23S rRNA alleles. Following subculture of the isolate in the presence of antibiotic (ERY 64 μg/ml), the 23S rRNA genes were re-analysed and homogeneous results were obtained at both loci (G2057A (4/4) and A2059G (3/4)).

4.3.2 Transformation Studies

To confirm the role of the 23S rRNA mutations in the macrolide resistance of the isolate, transformations of susceptible strain R6 were performed. Sequencing of the transformant 23S rRNA alleles revealed 3 fully mutated ($_{2057}$**AAG**$_{2059}$) and

one wild-type ($_{2057}$**G**A**A**$_{2059}$). As for isolate PU1004017, erythromycin Etests were inconsistent for the R6 transformant. Initial Etests showed a fully resistant phenotype, however after one week of subculture on antibiotic-free media the transformant showed a heterogeneous phenotype with satellite colonies within the sensitive zone of inhibition and after 10 days the transformant showed a sensitive phenotype.

4.3.3 Gene Conversion Studies

The conversion of the 23S rRNA alleles between the wild-type and mutated forms was further investigated. Subculturing of both TA and TB in the presence of antibiotic maintained all four alleles in the mutated form over the 3-week period and both cultures remained macrolide-resistant (ERY MIC >128 μg/ml). However, after one week of subculturing TA in the absence of antibiotic, one allele had completely reverted to the wild-type and the three remaining alleles showed heterogeneous sequences at locus 2057 (G/A) and locus 2059 (A/G). The strain still maintained its MIC of >128 μg/ml. After two weeks in the absence of antibiotic, all alleles had reverted to wild-type and the MIC decreased to 0.03 μg/ml. The 23S rRNA alleles and MICs remained unchanged after the third week in the absence of the antibiotic (Table 4.1). For TB, three weeks of subculture in the absence of erythromycin did not result in any change in the four mutated 23S rRNA alleles or the ERY MIC (>128 μg/ml).

4.3.4 Growth Studies

Mass doubling times (min) during the exponential phase of duplicate growth curves were as follows: R6, 68.3; TA-, 84.9; TA+, 114.1. Growth curves are shown in Figure 4.1.

Table 4.1 Erythromycin MICs (μg/ml) and 23S rRNA sequence data of R6 populations following continuous culture in the absence or presence of erythromycin

Transformant population	Presence/Absence of antibiotic	Day 0					Day 7					Day 14					Day 21				
		23S rRNA gene[1]				MIC	23S rRNA gene[1, 2, 3]				MIC	23S rRNA gene[1, 3]				MIC	23S rRNA gene[1, 3]				MIC
		1	2	3	4		1	2	3	4		1	2	3	4		1	2	3	4	
TA	- ERY	M	M	M	M	>128	H	H	H	W	>128	W	W	W	W	0.03	W	W	W	W	0.03
	+ ERY						M	M	M	M	>128	M	M	M	M	128	M	M	M	M	>128
TB	- ERY	M	M	M	M	>128	M	M	M	M	>128	M	M	M	M	>128	M	M	M	M	>128
	+ ERY						M	M	M	M	>128	M	M	M	M	>128	M	M	M	M	>128

[1]M: Fully mutated at loci 2057 and 2059 ($_{2057}$AAG$_{2059}$)

[2]H: Heterogeneous at loci 2057 and 2059 ($_{2057}$GAA$_{2059}$ and $_{2057}$AAG$_{2059}$)

[3]W: Wild-type at loci 2057 and 2059 ($_{2057}$GAA$_{2059}$)

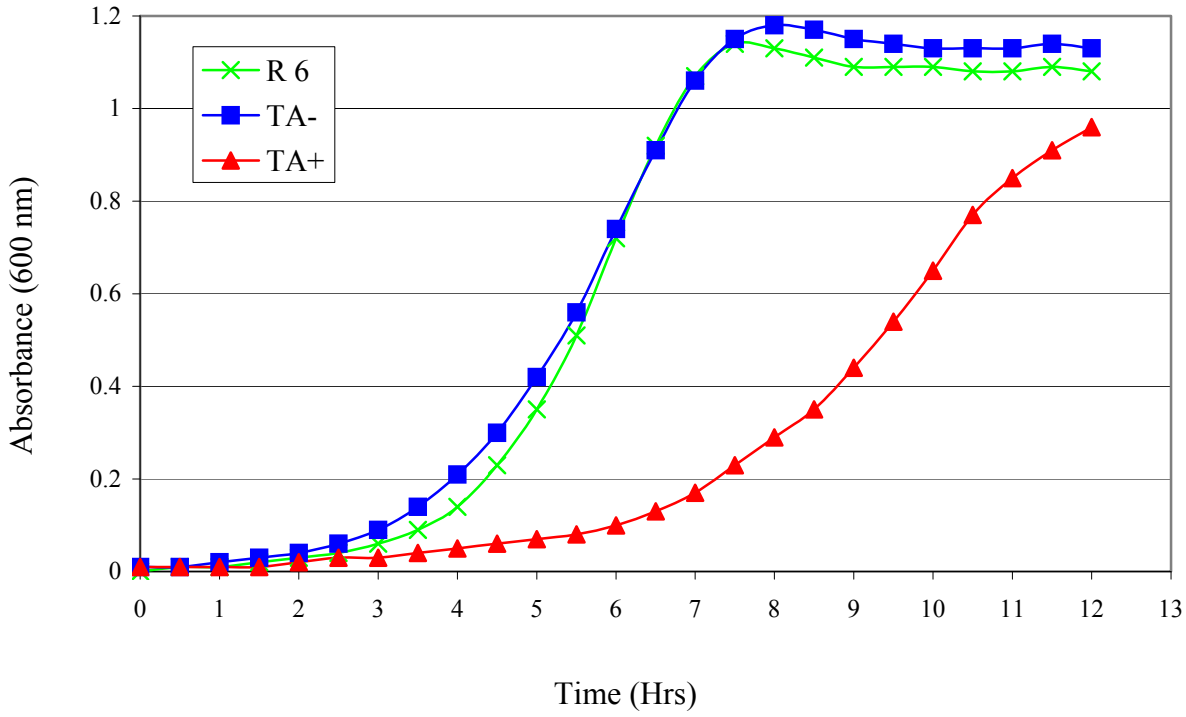

Figure 4.1 Growth curves at 37˚C of R6, compared to R6 transformants that had been subcultured for 21 days in the absence (TA-; $_{2057}$GAA$_{2059}$ (4/4)) or presence (TA+; $_{2057}$AAG$_{2059}$ (4/4)) of erythromycin (64 µg/ml)

4.4 Discussion

Bacterial gene conversion has been shown to be involved in the development of antibiotic resistance (Lobritz et al., 2003). Under antibiotic pressure wild-type alleles convert to the mutated forms by homologous recombination with mutated alleles (Adrian et al., 2000; Marshall et al., 2002). This process is mediated by RecA (Prammananan et al., 1999). Pillai et al. (2002) described a linezolid-resistant *Staphylococcus aureus* strain with a G2576U mutation in all five copies of 23S rDNA that displayed a stable resistant phenotype. However, Meka et al. (2004b) documented a linezolid-resistant *S. aureus* strain with one wild-type and four mutant alleles that reverted to susceptibility in the absence of antibiotic pressure.

In this study gene conversion complicated the study of macrolide resistance in isolate PU1004017. Both the original isolate and resulting transformant showed a heterogeneous phenotype and genotype. Culture of the transformant containing 3/4 mutated alleles in the presence of erythromycin for one week resulted in conversion of all four alleles to the mutated form. Continuous culture in the presence of antibiotic maintained the mutations and macrolide resistance. However when cultured in the absence of antibiotic, gene conversion started within one week (3 passages) and complete reversion to susceptibility was observed within two weeks (6 passages). This occurred in a far shorter period than that reported for a *S. aureus* isolate that reverted to susceptibility (maintaining one mutated copy) after 60 passages in antibiotic-free media (Meka et al., 2004b).

Intragenomic allelic exchange therefore appears to occur far more rapidly in *S. pneumoniae.* Although all four alleles of the transformant were mutated following culture in the presence of erythromycin, subculture of TA in the absence of antibiotic resulted in complete reversion of all four alleles to wild-type apparently without a wild-type allele present for homologous recombination. In contrast TB, after three weeks of culture in antibiotic-free media, maintained the resistant phenotype. Complete conversion has been previously shown to result in stable resistance (Adrian et al., 2000; Pillai et al., 2002), as was observed for TB. We therefore propose that TA may not have reached complete conversion and wild-type copies of 23S rDNA may have been present in a small proportion of cells in comparison with the mutant alleles, such that they were not detected by sequencing.

The reduced growth rate of TA+ (all four mutated genes) in comparison with TA- (wild-type) suggests that the mutations are associated with a fitness cost. We therefore hypothesize that in the absence of antibiotic pressure, and provided that the strain has not reached complete conversion, pneumococci will revert to susceptibility in order to regain fitness, by means of gene conversion.

4.5 Conclusion

Gene conversion in the absence or presence of antibiotics has implications for susceptibility testing as well as for the study of resistance mechanisms. In this study it has been shown that routine subculture on antibiotic-free media can result in reversion to susceptibility. A resistant strain may therefore be inconsistently and incorrectly classified with resulting implications for patient treatment. This study has shown the ability of pneumococci to rapidly alter their resistance phenotype and genotype by means of gene conversion.

Chapter Five

High-Level Telithromycin Resistance in a Clinical Isolate of *Streptococcus pneumoniae*

5.1 Introduction

In 1996 a clinical isolate of *S. pneumoniae*, BSF11524, was submitted to the Canadian Bacterial Surveillance Network as part of an ongoing pneumococcal resistance surveillance program. BSF11524 was isolated from the conjunctiva of a 1-year old boy in Canada and was identified as highly resistant to telithromycin. This isolate occurred several years prior to the approval of telithromycin in Canada and no further telithromycin-resistant isolates have been detected by the same network (Low et al., 2002; Powis et al., 2004). The occurrence of this isolate was described by Tait-Kamradt et al. (2001). In this study BSF11524 was investigated further, to establish the mechanism of telithromycin resistance in this rare isolate.

5.2 Materials and Methods

5.2.1 Phenotypic and Genotypic Characterisation

MICs were determined by the agar dilution method and the Etest, and serotyping was performed by the quellung reaction as described in Section 2.2. For telithromycin, breakpoints used were: ≤ 1 μg/ml for susceptible, 2 μg/ml for intermediate and ≥ 4 μg/ml for resistant (Clinical and Laboratory Standards Institute, 2006b). Chromosomal DNA was extracted from the isolate and PCR was used to screen for the common macrolide resistance determinants, *erm*(B) and *mef*(A). The genes encoding ribosomal proteins L4 and L22, *erm*(B) and all four alleles encoding 23S rRNA were amplified and sequenced. A detailed methodology is provided in Chapter 2.

5.2.2 Transformation Studies

Transformation was performed using two recipient strains: R6 (telithromycin MIC 0.015 μg/ml), and PC13 (telithromycin MIC 0.06 μg/ml). The elevated telithromycin MIC of PC13 compared with R6 and other fully susceptible strains is due to the presence of the *erm*(B) gene (Davies et al., 2000; Jalava et al., 2001). R6 and PC13 were made competent by culture in C-medium and transformation performed as described in Section 2.7. Transformants were selected on MHA plates supplemented with 5% horse blood and containing erythromycin (1 μg/ml) for R6 and telithromycin (0.5 μg/ml) for PC13. The MICs of the transformants were determined and the presence of mutations confirmed by DNA sequencing.

5.2.3 Growth Studies

Growth studies were performed by inoculating Tryptone Soya Broth with glycerol stocks (1:100 dilution) and monitoring turbidity at 600 nm every 30 min as described in Section 2.8. Mass doubling times were calculated for each strain during the exponential phase of growth. Statistical differences between mass doubling times were calculated using the unpaired *t* test with *P* values interpreted at the 95% confidence level.

5.3 Results

5.3.1 Phenotypic and Genotypic Characterisation

BSF11524 is a serotype 19A strain and is highly resistant to erythromycin (MIC >256 μg/ml), clindamycin (MIC >256 μg/ml) and telithromycin (MIC >256 μg/ml). It is resistant to tetracycline (MIC 12 μg/ml) and penicillin (MIC 16 μg/ml) but susceptible to chloramphenicol (MIC 2 μg/ml). It is *erm*(B) positive and *mef*(A) negative. The *erm*(B) gene contains an adenine base insertion in the control peptide creating a stop codon and resulting in the truncation of the control peptide to 10 amino acids (Figure 5.1). In addition, three mutations were found in *erm*(B); I75T, S100N and H118R. Ribosomal protein L4 was found to contain the following mutations E13Q, S20N, E30Q, $_{69}$GTG$_{71}$ to TPS, V88I, G98A, A128S and S130E. Ribosomal protein L22 and all four alleles of 23S rRNA were wild-type.

5.3.2 Transformation Studies

In order to confirm the role of the mutations in conferring telithromycin resistance on the isolate, transformations were carried out. PC13 was transformed with the mutant *erm*(B) gene of BSF11524. PC13^{ermB} transformants had a telithromycin MIC of 1 μg/ml (Table 5.1). PC13 was transformed with the mutant L4 gene of BSF11524. PC13^{L4} transformants had a telithromycin MIC of >256 μg/ml (Table 5.1). The L4 gene of ten PC13^{L4} transformants were sequenced and were found to contain various combinations of the mutations in the L4 gene of BSF11524

however only the $_{69}$GTG$_{71}$ to TPS mutation occurred in all ten transformants. This mutation occurs in a highly conserved region ($_{63}$KPWRQKGTGRAR$_{74}$) of L4. In order to confirm that this mutation alone confers telithromycin resistance a fragment of the L4 gene (L4Fr) containing only this mutation was used to transform PC13. PC13^{L4Fr} transformants had a telithromycin MIC of >256 μg/ml. The S20N mutation in L4 of BSF11524 has been associated with resistance to macrolides (Reinert et al., 2003) and has been identified in telithromycin-nonsusceptible strains (Al-Lahham et al., 2006). Therefore PC13 was transformed with a mutated L4 gene containing only the S20N mutation. Transformants were not selected in the presence of telithromycin. The role of the L4 mutations in the absence of an *erm*(B) gene was investigated by transforming R6 with the full-length L4 gene of BSF11524 and the L4Fr fragment containing only the $_{69}$GTG$_{71}$ to TPS mutation. R6^{L4} and R6^{L4Fr} transformants had telithromycin MICs of 0.12 μg/ml and erythromycin MICs of >256 μg/ml (Table 5.1).

5.3.3 Growth Studies

The growth curves are shown in Figure 5.2. Mass doubling times (min, mean ± SEM) during the exponential phase of growth, were as follows: BSF11524, 40.7 ± 2.05; PC13, 42.05 ± 0.45; PC13^{ermB}, 41.6 ± 0.9; PC13^{L4Fr}, 40.7 ± 0.4; R6, 48.3 ± 0.05 and R6^{L4Fr}, 47.3 ± 0.65. There was no significant difference between the doubling times of PC13 and PC13^{ermB} (P = 0.71), or PC13 and PC13^{L4Fr} (P = 0.16). The doubling times of R6 and R6^{L4Fr} were not significantly different (P = 0.37).

A) Wild-type

atgttggtattccaaatgcgtaatgtagataaaacatctactgttttgaaacagactaaaaacagtgattacgcagataaa
taa

M L V F Q M R N V D K T S T V L K Q T K N S D Y A D K -

B) Isolate BSF11524

atgttggtattccaaatgcgtaaatgtaga

M L V F Q M R K C R -

Figure 5.1 Nucleotide and corresponding amino acid sequences of the *erm*(B) control peptide of wild-type strain R6 (A) and isolate BSF11524 (B). The adenine insertion mutation is highlighted.

Table 5.1 Phenotypic and genotypic data of BSF11524, PC13, R6 and transformants

Strain	*erm*(B)[a]	L4[b]	MIC (µg/ml) of[c]:						
			TEL	**ERY**	**AZM**	**CLR**	**CLI**	**TET**	**CHL**
BSF11524	mt	mt	> 256	> 256	> 256	>256	> 256	12	2
PC13 Transformations[d]									
PC13	wt	wt	0.06	> 256	> 256	> 256	> 256	12	12
PC13^{ermB} transformant	mt	wt	1	> 256	> 256	> 256	> 256	8	12
PC13^{L4} transformant	wt	mt	> 256	> 256	> 256	> 256	> 256	12	12
PC13^{L4Fr} transformant	wt	$_{69}$GTG$_{71}$ to TPS	> 256	> 256	> 256	> 256	> 256	8	12
R6 Transformations[e]									
R6	–	wt	0.015	0.094	0.25	0.06	0.06	0.094	1.5
R6 ^{L4} transformant	–	mt	0.12	> 256	> 256	24	0.125	0.094	2
R6 ^{L4Fr} transformant	–	$_{69}$GTG$_{71}$ to TPS	0.12	> 256	> 256	24	0.094	0.06	2

Table 5.1 Legend

[a] wt, wild-type *erm*(B) gene; mt, mutant *erm*(B) gene of BSF1124

[b] wt, wild-type L4 gene; mt, mutant L4 gene of BSF11524

[c] TEL, telithromycin; ERY, erythromycin; AZM, azithromycin; CLR, clarithromycin; CLI, clindamycin; TET, tetracycline; CHL, chloramphenicol

[d] PC13 transformed with the mutant *erm*(B) gene, the mutant L4 gene or with a gene fragment (L4Fr) housing the $_{69}$GTG$_{71}$ to TPS mutation of L4 from BSF11524

[e] R6 transformed with the mutant L4 from BSF11524 or the L4Fr gene fragment

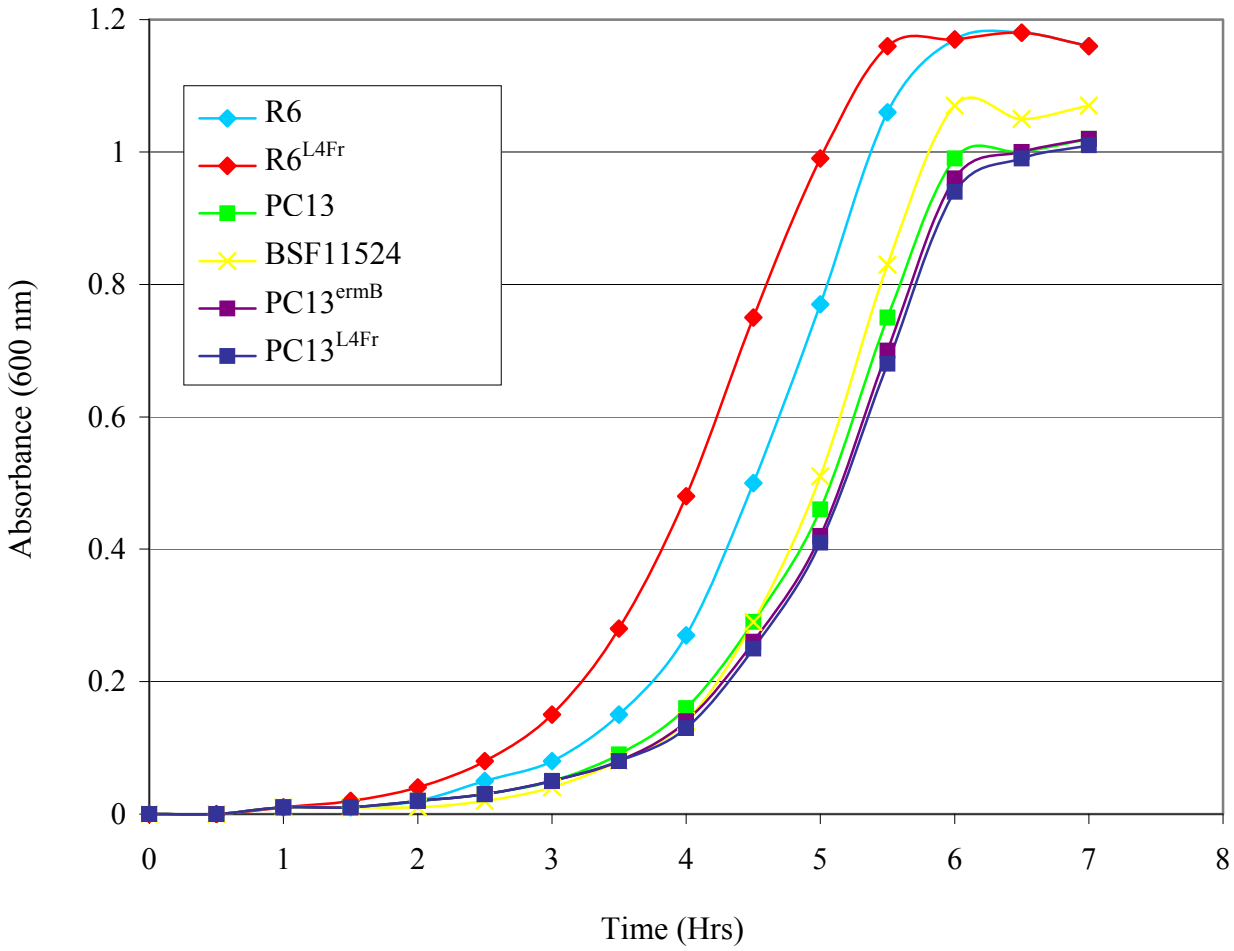

Figure 5.2 Growth curves at 37˚C of BSF11524, PC13, PC13^{ermB}, PC13^{L4Fr}, R6, and R6^{L4Fr}

5.4 Discussion

The mutant *erm*(B) gene of BSF11524 reduces the susceptibility of PC13 to telithromycin, however does not confer the high-level resistance observed in the isolate. The reduced telithromycin susceptibility of the PC13^{ermB} transformants in comparison with PC13 may be due to increased dimethylation of A2058 in 23S rRNA as a result of the truncated control peptide in *erm*(B) (Douthwaite et al., 2005; Liu and Douthwaite, 2002). The full-length L4 gene and the fragment of L4 containing the $_{69}$GTG$_{71}$ to TPS mutation (L4Fr) conferred high-level telithromycin resistance on PC13. The L4 gene and L4Fr fragment reduced the susceptibility of R6 to telithromycin however did not confer resistance as for PC13. It is therefore highly likely to be the combination of *erm*(B), wild-type or mutant, and the $_{69}$GTG$_{71}$ to TPS mutation of L4 that confers high-level telithromycin resistance. The mutations in *erm*(B) of BSF11524 and the $_{69}$GTG$_{71}$ to TPS mutation of ribosomal protein L4 do not appear to be associated with a fitness cost.

The $_{69}$GTG$_{71}$ to TPS mutation of L4 has been previously described (Pihlajamäki et al., 2002; Tait-Kamradt et al., 2000b) and, as shown here, when not combined with *erm*(B) it confers high-level erythromycin resistance but only reduced susceptibility to telithromycin. Erythromycin and telithromycin share a common binding site, however telithromycin forms a tighter bond with the ribosome due to an additional interaction with A752 in domain II of 23S rRNA (Douthwaite and Champney, 2001). The $_{69}$GTG$_{71}$ to TPS mutation in L4 may therefore destabilize

the binding of telithromycin but does not block it completely, as is the case for erythromycin. However, the combined effect of a methylated A2058 in 23S rRNA, due to *erm*(B), and the $_{69}$GTG$_{71}$ to TPS mutation in L4 is sufficient to block the binding of telithromycin to the ribosome.

High levels of resistance to telithromycin appear to be a result of a combination of mutations. Faccone et al. (2005) described a clinical isolate with a telithromycin MIC of 256 μg/ml with an A2058T mutation in 23S rRNA and a deletion in L22. A combination of an A2058G mutation in 23S rRNA and a RTAHIT insertion in L22 resulted in a telithromycin MIC of 16 μg/ml (Pérez-Trallero et al., 2003). In addition, a telithromycin-resistant isolate with a MIC of 8 μg/ml was found to contain an *erm*(B) gene, a S20N mutation in L4, and a number of mutations in 23S rRNA (Reinert et al., 2005b). A highly-resistant laboratory-generated strain (MIC >32 μg/ml) contained a 210 bp deletion in the *erm*(B) upstream region together with a K94Q mutation in riboprotein L22 (Walsh et al., 2003).

5.5 Conclusion

In this study, high-level telithromycin resistance was shown to be conferred by an *erm*(B) gene in combination with a $_{69}$GTG$_{71}$ to TPS mutation in a highly conserved region of ribosomal protein L4. The rarity of the emergence of such a resistant phenotype, despite the availability and worldwide use of telithromycin since 2002, may be because of the need for both the *erm* gene and mutations in ribosomal genes. Its failure to disseminate may be, in part, due to the lack of selective pressure since its appearance predated the approval and use of telithromycin in Canada by several years.

Chapter Six

Telithromycin Resistance in *Streptococcus pneumoniae* conferred by a Deletion in the Promoter Region of *erm*(B)

6.1 Introduction

A common mechanism of macrolide resistance in the pneumococcus is ribosomal target modification due to the acquisition of the *erm*(B) gene. The ribosomal methylase encoded by this gene methylates A2058 in domain V of 23S rRNA. As this is a primary binding site of macrolides, modification of the site prevents macrolide binding and confers high-level macrolide resistance, as well as resistance to lincosamides and streptogramin B (Leclercq and Courvalin, 2002). The *erm*(B) gene can be constitutively or inducibly expressed. In the pneumococcus the *erm*(B) gene is predominantly inducibly expressed (Rosato et al., 1999). Control of expression of the gene occurs on the post-transcriptional level by a control peptide upstream of the structural *erm*(B) gene. A translational attenuation model has been studied in *erm*(C) of *Staphylococcus aureus* (Weisblum, 1995). The 5' end of *erm*(B) contains a number of inverted repeats which form various stem-loop structures resulting in alternative conformations of mRNA. In the inactive conformation, the secondary structures block the ribosome-binding site and initiation codon and therefore prevent translation. In the presence of an inducer, such as erythromycin, the binding of erythromycin to the ribosome-mRNA complex causes the ribosome to stall and destabilizes the

stem-loop structures, unblocking the necessary sites for translation to take place. Basal levels of ribosomal methylation vary between pneumococcal strains and the higher the concentration of an inducer, the higher the level of ribosomal methylation (Zhong et al., 1999).

Due to structural differences between macrolides and ketolides, telithromycin remains active against strains expressing *erm*(B) (Capobianco et al., 2000). This is due to the weak inducing capacity of ketolides (Rosato et al., 1998; Zhong et al., 1999) as well as their increased affinity for the ribosome due to an additional binding site in domain II (A752) of 23S rRNA (Capobianco et al., 2000; Douthwaite et al., 2000).

Telithromycin-resistant pneumococcal clinical isolates are rare (Farrell and Felmingham, 2004; Hisanaga et al., 2005). In this study, a clinical isolate of *S. pneumoniae* submitted to the PROTEKT surveillance study and identified as resistant to telithromycin was investigated in order to establish the mechanism of telithromycin resistance.

6.2 Materials and Methods

6.2.1 Phenotypic and Genotypic Characterisation

A telithromycin-resistant clinical isolate of *S. pneumoniae,* P1501016, was obtained from the PROTEKT surveillance study (Farrell et al., 2002). MICs were determined by the agar dilution method and the Etest, and serotyping was performed by the quellung reaction as described in Section 2.2. For telithromycin, breakpoints used were: ≤ 1 μg/ml for susceptible, 2 μg/ml for intermediate and ≥ 4 μg/ml for resistant (Clinical and Laboratory Standards Institute, 2006b). Chromosomal DNA was extracted from the isolate and PCR was used to screen for the common macrolide resistance determinants, *erm*(B) and *mef*(A). The genes encoding ribosomal proteins L4 and L22, *erm*(B) and all four alleles encoding 23S rRNA were amplified and sequenced. A detailed methodology is provided in Chapter 2. The transposon (Tn1545 or Tn917) carrying *erm*(B) was identified as previously described (Okitsu et al., 2005) using transposon specific forward primers (Tn1545 primer ermBF: CTTAGAAGCAAACTTAAGAG and Tn917 primer Tn917F: TGACGGTGACATCTCTC) and a common reverse primer (ermBM-R2: CTGTCTAATTCAATAGACGT).

6.2.2 Transformation Studies

Susceptible strain PC13, containing a wild-type *erm*(B) gene, was made competent by culture in C-medium and transformed with the *erm*(B) gene of P1501016 as described in Section 2.7. Transformants were selected on MHA plates supplemented with 5% horse blood and containing telithromycin

(0.5 μg/ml). The MICs of the transformants were determined and the presence of mutations confirmed by DNA sequencing.

6.2.3 Growth Studies

P1501016, PC13 and a PC13 transformant were inoculated from glycerol stocks into Tryptone Soya Broth (TSB) and TSB containing erythromycin (1 μg/ml) (1:100 dilution), as described in Section 2.8. Growth was monitored turbidimetrically at 600 nm at intervals of 30 min. Mass doubling times during the exponential phase of growth were calculated.

6.2.4 Relative Quantification of *erm*(B) Expression

Isolate P1501016, PC13 and a PC13 transformant were grown in TSB at 37°C 5% CO_2 to an OD at 600 nm of 0.2. Cultures were grown in: 1. absence of antibiotic, 2. presence of telithromycin (0.008 μg/ml) or 3. presence of erythromycin (0.25 μg/ml). Once cultures reached the required OD, 400 μl of culture was mixed with 800 μl of RNAprotect Bacteria Reagent (Qiagen). Following a 5 min incubation at room temperature, cultures were centrifuged at 6000 rpm for 10 min and the pellet was stored at -70°C. Total RNA was extracted from cultures using the RNeasy Mini Kit for bacteria (Qiagen). The RNA extracts were then processed by the RNase-Free DNase set (Qiagen) in order to digest contaminating DNA and were cleaned using the RNeasy Mini protocol for RNA cleanup (Qiagen). The RNA was diluted 10x in RNase-free H_2O, and converted to complementary DNA (cDNA) by reverse transcription using the High-Capacity

cDNA Archive Kit (Applied Biosystems), according to the manufacturer's instructions. For each sample, a control was prepared containing all reaction components except for the reverse transcriptase enzyme in order to control for DNA contamination. For each PCR reaction, 5 μl of cDNA was added to a 45 μl mix containing 1x SYBR Green PCR Mastermix (Applied Biosystems) and 12.5 pmol each of forward primer and reverse primer (Applied Biosystems). Real-time PCR was performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Two PCR reactions, to amplify the target gene and endogenous reference gene, were performed separately from the same total RNA for each sample. The *erm*(B) cDNA was amplified using primers ermB747F (AGGGTTGCTCTTGCACACTCA) and ermB806R (CATTCCGCTGGCAGCTTAAG). The cDNA of *gdh*, a housekeeping gene, was amplified using primers SPgdhF1 (ATTCCGTGGTGTTCCTTTCTTTT) and SPgdhR1 (TTCCTTTTTCAGTCAGTCGTTTAC). The relative expression of *erm*(B) compared with an endogenous control housekeeping gene, *gdh* was determined. Relative standard curves were used to calculate the quantities of *erm*(B) and *gdh*, and the ratio of *erm*(B) to *gdh* was calculated for each sample. The endogenous control, *gdh*, was used to control for the amount of cDNA added to a reaction. Statistical differences in *erm*(B) expression between strains were calculated using the paired *t* test with *P* values interpreted at the 95% confidence level (n=4).

6.3 Results

6.3.1 Phenotypic and Genotypic Characterisation

Isolate P1501016 was found to be highly resistant to macrolides (MICs (μg/ml): erythromycin >256, clarithromycin >256) and clindamycin >256 μg/ml. More significantly this isolate was identified as resistant to telithromycin (8 μg/ml). The isolate was serotype 23F. Genotypic analysis of the isolate showed it to be *erm*(B) positive and *mef*(A) negative. Sequencing of the four alleles encoding 23S rRNA, and ribosomal proteins L4 and L22 showed them to be wild-type. The *erm*(B) gene was located in the transposon Tn1545. The *erm*(B) gene contained a 136 bp in-frame deletion in the promoter region, resulting in a 3 amino acid truncation of the control peptide as well as removal of the second Shine-Dalgarno (SD2) sequence. The mutant Erm(B) protein therefore consisted of the remaining portion of the control peptide fused with the *erm*(B) gene. The mutant protein was longer than the wild-type and was under the control of SD1 (Figure 6.1).

6.3.2 Transformation Studies

The role of the *erm*(B) deletion in conferring telithromycin resistance on the isolate was investigated by transformation of a telithromycin-susceptible strain, PC13 (telithromycin MIC 0.06 μg/ml). Transformants had a telithromycin MIC of 16 μg/ml and contained the 136 bp deletion in *erm*(B) as for P1501016 (Table 6.1).

6.3.3 Growth Studies

Growth curves are shown in Figure 6.2 (absence of erythromycin) and Figure 6.3 (presence of erythromycin (1 μg/ml)). Mass doubling times (min) during the exponential phase of growth in the absence of erythromycin were as follows: P1501016, 35.5; PC 13, 47.9 and PC13 transformant, 48.9. In the presence of erythromycin mass doubling times were as follows: P1501016, 43.3; PC 13, 53; PC13 transformant, 52.5.

6.3.4 Relative Quantification of *erm*(B) Expression

The relative expression of *erm*(B) is shown in Figure 6.4. In the absence of antibiotic, there was no significant difference in the expression of *erm*(B) between PC13 and the PC13 transformant (P=0.24). Expression was significantly higher in the PC13 transformant than in PC13 in the presence of telithromycin (P=0.02) and in the presence of erythromycin (P=0.04). For all strains, expression was increased in the presence of erythromycin in comparison with no antibiotic (P<0.05).

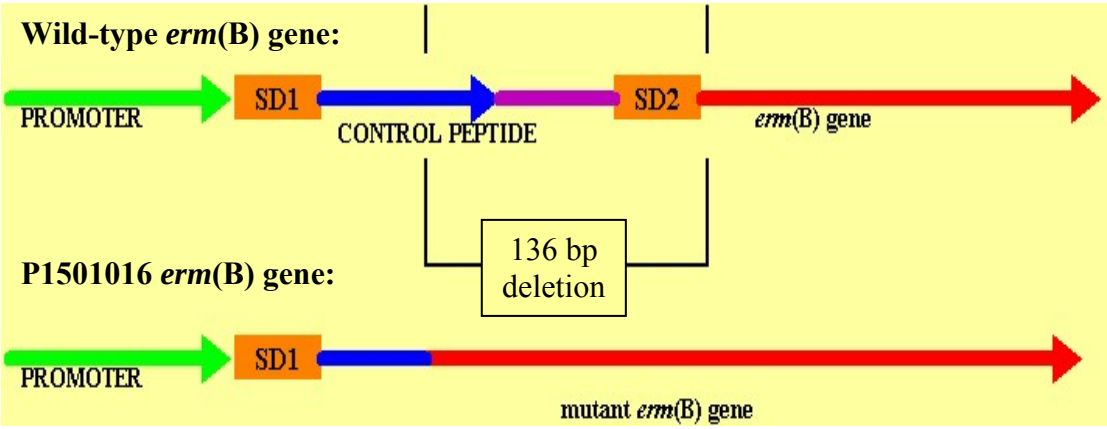

Figure 6.1 Diagram representing a wild-type *erm*(B) gene and the mutant *erm*(B) gene of isolate P1501016 (SD; Shine-Dalgarno sequence)

Table 6.1 Phenotypic and genotypic data of P1501016, PC13 and a PC13 transformant

Strain	*erm*(B) sequence	MIC (µg/ml) of[a]:				
		ERY	**CLR**	**CLI**	**TET**	**TEL**
P1501016	136 bp deletion in promoter region	>256	>256	>256	4	8
PC13	Wild-type	>256	>256	>256	16	0.06
PC13 transformant	136 bp deletion in promoter region	>256	>256	>256	16	16

[a] Abbreviations: ERY, erythromycin; CLR, clarithromycin; CLI, clindamycin; TET, tetracycline; TEL, telithromycin

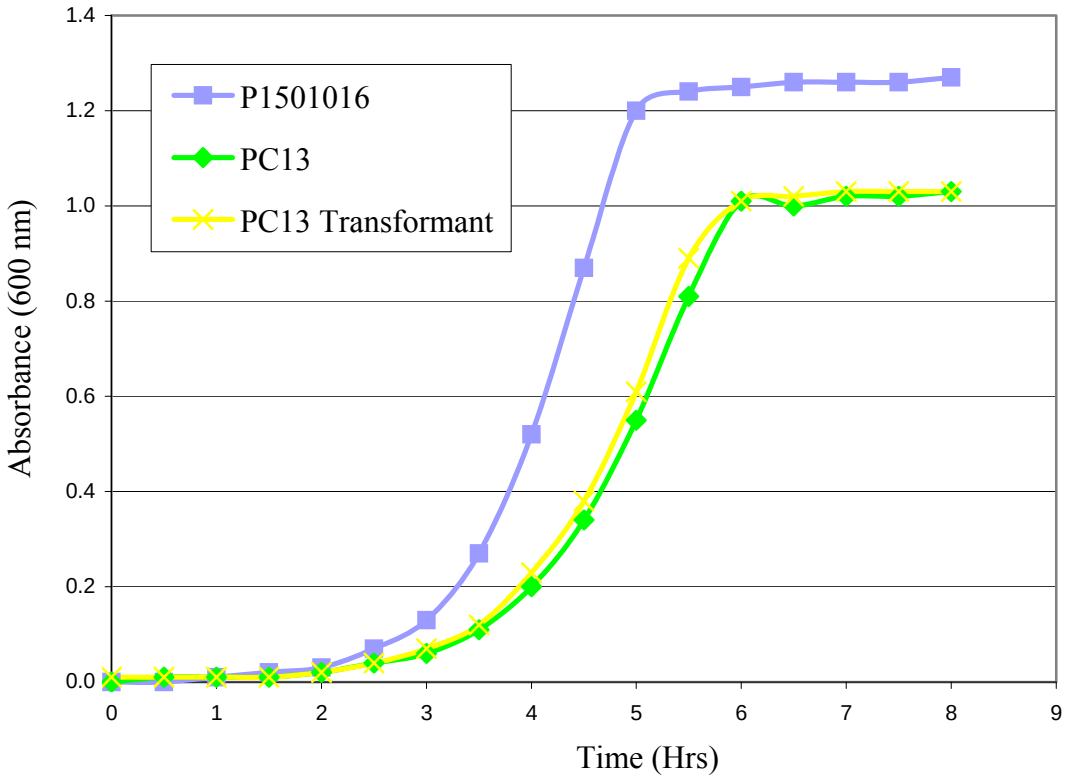

Figure 6.2 Growth curves at 37˚C of isolate P1501016, PC13 (wild-type *erm*(B)), and a PC13 transformant (mutant *erm*(B) of P1501016), in the absence of erythromycin

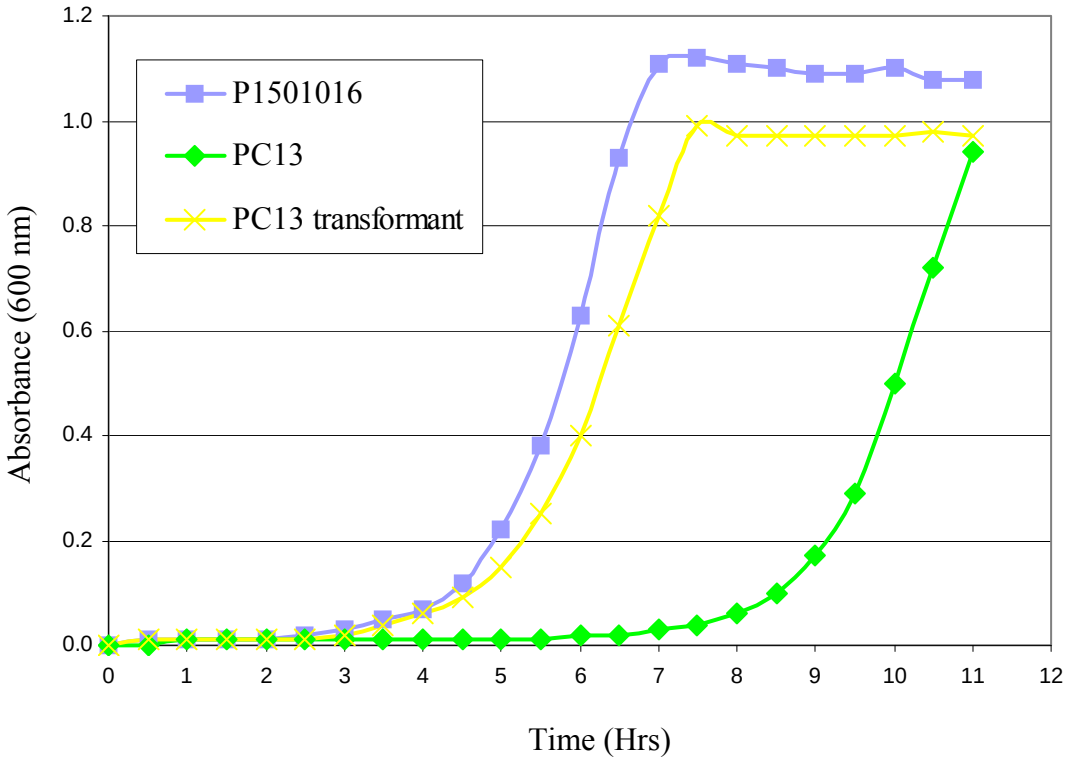

Figure 6.3 Growth curves at 37˚C of isolate P1501016, PC13 (wild-type *erm*(B)) and a PC13 transformant (mutant *erm*(B) of P1501016), in the presence of erythromycin (1 μg/ml)

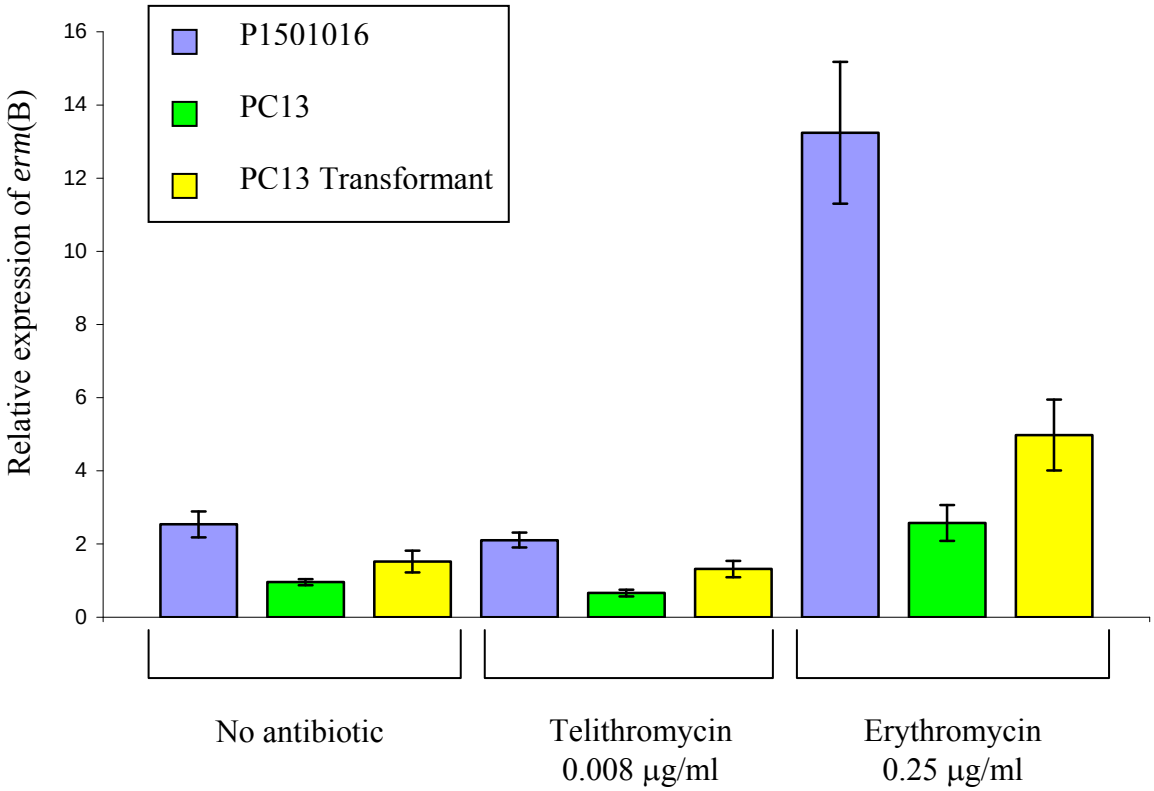

Figure 6.4 Relative expression of *erm*(B), determined by quantitative real-time reverse transcription PCR, in isolate P1501016, PC13 and a PC13 transformant. Cultures were grown either in the absence of antibiotic, presence of telithromycin (0.008 µg/ml) or presence of erythromycin (0.25 µg/ml). Error bars represent the standard error of the mean (n=4)

6.4 Discussion

In this study, a rare telithromycin-resistant clinical isolate of *S. pneumoniae* was identified and was found to contain a 136 bp deletion in the promoter region of the *erm*(B) gene resulting in a mutant Erm(B) protein that was longer than the wild-type and under the control of SD1. Pneumococcal genes encoding 23S rRNA, and ribosomal proteins L4 and L22 that have been associated with telithromycin resistance (Hisanaga et al., 2005) were wild-type. By transformation of telithromycin-susceptible PC13 with the mutant *erm*(B) gene from isolate P1501016, the mutant gene was shown to confer telithromycin resistance.

Studies have shown that low levels of ribosomal methylation by *erm* genes do not confer telithromycin resistance, however higher levels do confer resistance. In pneumococcal strains with varying levels of methylation, strains with higher methylation levels had higher ketolide MICs (Zhong et al., 1999). Monomethylation of A2058 in *E. coli* ribosomes confers resistance to erythromycin but only slightly decreases susceptibility to telithromycin, however dimethylation of this site confers high-level resistance to erythromycin and telithromycin (Liu and Douthwaite, 2002). In *Streptococcus pyogenes* the degree of ribosomal methylation was found to correlate with resistance to ketolides, with a reduction in ketolide susceptibility being observed with an increase in the proportion of dimethylated ribosomes (Douthwaite et al., 2005). In this study, it was shown that the mutant *erm*(B) gene was expressed at higher levels than the wild-type gene in the presence of telithromycin and erythromycin. Therefore it is

likely that the deletion in the *erm*(B) gene of P1501016 altered the regulation of *erm*(B) expression, resulting in increased levels of Erm methylase. This, in turn, resulted in increased A2058 dimethylation levels and conferred resistance to telithromycin. This is supported by preliminary studies that have shown that *erm*(B)-containing pneumococcal strains that are resistant to telithromycin had higher levels of A2058 dimethylation than telithromycin-susceptible strains (Farrell, 2004). In addition, Rosato et al. (1999) described a pneumococcal strain with a large deletion in the *erm* gene that resulted in constitutive MLS_B resistance.

This hypothesis would account for the trend observed in the growth curves. In the absence of erythromycin, the growth curves of PC13 and the PC13 transformant were almost identical. However in the presence of erythromycin, the lag phase was shorter for the PC13 transformant than for PC13. This could be explained by the increased levels of erm methylase and methylation in the mutant strain, as it would require a shorter period of time to achieve sufficiently high levels of methylation to overcome the effects of the antibiotic. PC13, containing a wild-type *erm*(B) gene, would require a longer period of time to achieve sufficiently high levels of ribosomal methylation to be able to resist erythromycin action. However once this proportion has been reached, the culture can enter the exponential phase and replicate as per normal, reflected by the similar mass doubling times of PC13 and the PC13 transformant during the exponential phase of growth. In the absence of erythromycin, both cultures can grow equally well as ribosomal methylation is not required and therefore the level at which it occurs does not influence growth.

A model that has been proposed for the regulation of *erm* expression has postulated that it occurs on the post-transcriptional level by means of translational attenuation (Weisblum, 1995). In this study, it has been shown that regulation of expression also occurs on the transcriptional level. All strains studied showed increased levels of mRNA in the presence of erythromycin, indicating that the genes are inducible. The overall increased expression of the mutant gene was likely due to an additive effect of increased transcriptional and translational expression.

6.5 Conclusion

In this study, telithromycin resistance in a clinical pneumococcal isolate was found to be conferred by a mutant *erm*(B) gene containing a large deletion mutation in the regulatory region which affected the regulation of the expression of the gene. A wild-type *erm*(B) gene methylates the ribosome at a low level, which does not confer resistance to telithromycin. However, increased expression of the gene due to the deletion mutation results in increased ribosomal methylation levels and confers resistance to telithromycin.

Chapter Seven

Macrolide Resistance in *Streptococcus pneumoniae* causing Invasive Disease in South Africa: 2000 – 2005

7.1 Introduction

Macrolide resistance in *S. pneumoniae* is a global problem (Farrell et al., 2004a; Jacobs et al., 2003). Resistance to macrolides in South Africa was first reported in 1978 (Jacobs et al., 1978). A study by Widdowson and Klugman (1998) showed that in South African invasive isolates collected from 1987 to 1996, the prevalence of erythromycin resistance was 2.7%. The MLS_B phenotype occurred in 89% of macrolide-resistant strains and the M phenotype, although less common, increased from 0.8% of macrolide-resistant strains in the 1987-1991 period to 19.7% in the 1992 to 1996 period. Huebner et al. (2000) showed similar trends for blood and cerebrospinal fluid (CSF) isolates collected between 1991 and 1998 in South Africa. Resistance to erythromycin increased from 1.6% in the 1991-1994 period to 2.6% in the 1995 to 1998 period. The MLS_B phenotype was predominant but a significant increase in the M phenotype from 10.6% of macrolide-resistant isolates to 28.7% was reported. The aim of this study was to investigate the current prevalence and epidemiology of macrolide resistance in South Africa.

7.2 Materials and Methods

7.2.1 Bacterial Isolates

Isolates were collected as part of a national laboratory-based surveillance system for invasive pneumococcal disease (IPD). Clinical isolates together with clinical and demographic data were sent to the Respiratory and Meningeal Pathogens Research Unit (RMPRU), National Institute for Communicable Diseases in Johannesburg from laboratories throughout South Africa. A case of IPD was defined as the isolation of *S. pneumoniae* from a normally sterile site (eg. CSF, blood, joint fluid) from January 2000 to December 2005. In addition, cases included patients with specimens testing positive by latex agglutination and supported by either Gram stain microscopy or PCR. If a repeat isolate was received from the same patient within 21 days of the initial isolate, it was excluded.

7.2.2 Phenotypic Characterisation of Isolates

Confirmation of the identification of the organism was performed using standardised methodologies including optochin-susceptibility, bile-solubility and the quellung reaction (Ruoff et al., 2003). For the optochin-susceptibility test, a 0.5 McFarland standard suspension of the culture was inoculated onto MHA plates containing 5% sheep blood (Diagnostic Media Products). An optochin disc (Mast Diagnostics) was applied to the plate, and following overnight incubation at 37°C in 5% CO_2 the zone of inhibition was measured. A zone of >14 mm indicated optochin susceptibility. For the bile solubility test, two 0.5 ml volumes

of culture suspensions (0.5 McFarland standard) were aliquoted into tubes. An equal volume of 2% sodium deoxycholate (bile; Diagnostic Media Products) or saline was added to each tube, respectively. Following incubation of the tubes at 37°C for up to 2 hrs, a clearing in the presence of bile but not saline indicated a positive result for the bile solubility of the organism. MICs of all viable isolates were determined by the Etest as described in Section 2.2 and interpreted as susceptible, intermediately resistant or resistant using breakpoints recommended by Clinical and Laboratory Standards Institute guidelines (Clinical and Laboratory Standards Institute, 2006b). Pneumococcal isolates were defined as nonsusceptible to erythromycin if they were intermediately resistant or resistant (MIC ≥0.5 μg/ml). Erythromycin-nonsusceptible isolates were classified as the M phenotype if they were nonsusceptible to erythromycin, but susceptible to clindamycin; and the MLS_B phenotype if they were nonsusceptible to both erythromycin and clindamycin. Isolates were defined as nonsusceptible to an antibiotic if they were intermediately resistant or resistant to the antibiotic, and were classified as multidrug-resistant if they were nonsusceptible to at least three different antibiotic classes. Pneumococci were serotyped using the quellung method as described in Section 2.2.

7.2.3 Genotypic Characterisation of Isolates

Two hundred and sixty randomly selected erythromycin-nonsusceptible isolates from 2005 were screened for their resistance mechanism. DNA was prepared by boiling bacterial cultures. A loopful of culture was suspended in 100 μl of sterile dH_2O, heated at 95°C for 10 min and briefly centrifuged. The presence of *erm*(B)

and / or *mef*(A) was detected by a duplex PCR described in Section 2.4. The *erm*(B) and *mef*(A) primers amplify products of 617 bp and 346 bp, respectively.

Isolates negative for both *erm*(B) and *mef*(A) were further investigated to determine their resistance mechanism. Genomic DNA was extracted as described in Section 2.3. The 23S rRNA alleles and genes encoding ribosomal proteins L4 and L22 were amplified and sequenced as described in Sections 2.5 and 2.6. Hotspots of the 23S rRNA alleles were sequenced using sequencing primers 23SF-3, 23SF-7 and 23SF-8.

7.2.4 Pulsed-Field Gel Electrophoresis

A random selection of isolates positive for both *erm*(B) and *mef*(A) were characterised by PFGE, as described in Section 2.9. Isolates were defined as related if they shared ≥80% similarity on the dendrogram. Fingerprint patterns were compared with those of the available global pneumococcal clones (McGee et al., 2001b; http://www.sph.emory.edu/PMEN).

7.2.5 Multilocus Sequence Typing

MLST was performed, as described in Section 2.10, on a selection of isolates containing both *erm*(B) and *mef*(A), representative of various clusters generated by PFGE. Isolates with one or two allele differences were defined as related.

7.2.6 Statistical Analysis

Statistical analyses were performed using Epi Info software version 6.04d (Dean et al., 1996). The chi-square (χ^2) test for trend was used to assess the prevalence of macrolide nonsusceptibility over the six-year period. The χ^2-test, using the Mantel-Haenszel test, was used to assess differences in proportions. *P* values less than 0.05 were considered to be statistically significant.

7.3 Results

7.3.1 2000 - 2005

For the period January 2000 to December 2005, 17488 cases of IPD were reported to the RMPRU. Of these, 91% (15982) were viable and therefore available for further testing. Twelve percent (1921/15982) of isolates were nonsusceptible to erythromycin, of which 3 isolates were intermediately resistant and 1918 isolates were resistant. There was a linear increase in macrolide nonsusceptibility between 2000 and 2005 ($P<0.001$, χ^2- test for trend) (Figure 7.1). The proportion of erythromycin-nonsusceptible isolates increased from 8.8% (160/1828) in 2000 to 13.9% (508/3656) in 2005 ($P<0.001$).

The highest numbers of viable isolates were received from Gauteng (9590), the Western Cape (2208) and KwaZulu-Natal (1531). Of 9590 viable isolates received from Gauteng for the 2000 to 2005 period, 13.3% (1273) were nonsusceptible to erythromycin and of isolates received from the Western Cape 12.6% (278/2208) were nonsusceptible to erythromycin ($P=0.39$) (Figure 7.2). Erythromycin nonsusceptibility was 8.6% (131/1531) in KwaZulu-Natal and this was significantly lower than in Gauteng ($P<0.001$) and the Western Cape ($P<0.001$).

Age was known for 90% (15792/17488) of cases reported from 2000 to 2005. Of viable isolates received, age was known for 90% (14412/15982). For the six-year period, 18.9% (999/5284) of isolates from children <5 years of age and 8.4%

(767/9128) of isolates from individuals five years and older were nonsusceptible to erythromycin (P<0.001).

Serotypes included in PCV7 and serotype 6A accounted for 94% (1798/1921) of erythromycin-nonsusceptible isolates (Figure 7.3). Serotype 14 constituted 40% (760/1921) and serotype 23F constituted 18% (341/1921) of erythromycin-nonsusceptible isolates from all ages.

Overall 75% (1437/1921) of erythromycin-nonsusceptible isolates displayed the MLS_B phenotype and 25% (484/1921) displayed the M phenotype. The proportion of MLS_B vs. M phenotype did not vary significantly during the six-year period (P=0.83) (Figure 7.4).

7.3.2 2005

From January to December 2005, 4107 cases of IPD were reported to the RMPRU. Of these 3656 (89%) were viable for further testing. Fourteen percent (508/3656) of isolates were resistant to erythromycin. No isolates were intermediately resistant. The highest numbers of viable isolates were received from Gauteng (1979), the Western Cape (447) and KwaZulu-Natal (431). Similar trends were observed in 2005 to those occurring during the 2000 to 2005 period. Erythromycin nonsusceptibility was 15% in isolates from Gauteng (301/1979) and the Western Cape (66/447) (Figure 7.5). Of viable isolates received from the Northern Cape in 2005, 17% (5/29) were erythromycin-nonsusceptible. This was not significantly higher than Gauteng (P=0.76) or the Western Cape (P=0.71).

Of isolates nonsusceptible to erythromycin, 96% (485/508) were PCV7 serotypes and 6A (Figure 7.6). Serotype 14 constituted the majority of isolates (41%, 207/508), followed by serotype 23F (23%, 118/508).

For 95% (3465/3656) of cases reported in 2005 with viable isolates, age was known. For children <5 years, 21% (254/1195) were erythromycin-nonsusceptible and for individuals five years and older, 10% (230/2270) were erythromycin-nonsusceptible (P<0.001) (Figure 7.7). In children <5 years, 22% (55/254) of erythromycin-nonsusceptible isolates displayed the M phenotype and 78% (199/254) displayed the MLS_B phenotype. This proportion was significantly different in individuals five years and older, of which 33% (76/230) displayed the M phenotype and 67% (154/230) displayed the MLS_B phenotype (P=0.005).

In 2005, of the 3656 isolates that were available for further testing, 32% (1160/3656) were isolated from CSF specimens and 12% (143/1160) of these were nonsusceptible to erythromycin. Isolates from blood constituted 62% (2270/3656) of the total isolates, and 15% (341/2270) of these were erythromycin-nonsusceptible. The remaining isolates (6%, 226/3656) were isolates from specimens such as pleural or joint fluid, and of these isolates 11% (24/226) were nonsusceptible to erythromycin. Erythromycin nonsusceptibility was significantly higher in isolates from blood specimens than from CSF specimens (P=0.03).

Two hundred and sixty (51%, 260/508) erythromycin-nonsusceptible isolates from 2005 were randomly selected and screened for the presence of the *erm*(B)

and / or *mef(*A) genes. Of these, 56.5% (147/260) were positive for *erm*(B), 27.3% (71/260) were positive for *mef(*A), 15.4% (40/260) contained both *erm*(B) and *mef(*A) and 0.8% (2/260) were negative for both resistance determinants. Serotype 14 constituted 65% (95/147) of strains containing *erm*(B), serotype 23F constituted 46% (33/71) of strains containing *mef(*A) and serotype 19F constituted 80% (32/40) of strains containing *erm*(B) and *mef(*A) (Figure 7.8).

The erythromycin MICs of all isolates containing *erm*(B) were >256 μg/ml. Isolates containing both *erm*(B) and *mef*(A) showed the same phenotype as isolates containing *erm*(B) with erythromycin MICs of >256 μg/ml. Isolates containing *mef*(A) had erythromycin MICs of 2 to 64 μg/ml, with a MIC_{50} of 8 μg/ml and a MIC_{90} of 32 μg/ml.

The two isolates negative for both *erm*(B) and *mef(*A) were further investigated. Isolate 3695, isolated in Gauteng, was serotype 6A and had erythromycin and clindamycin MICs of >256 and 2 μg/ml, respectively. All four 23S rRNA alleles contained an A2059G mutation and ribosomal protein L4 contained a S20N mutation. Ribosomal protein L22 was wild-type. Isolate 4468 was isolated in KwaZulu-Natal and was serotype 6B. The isolate had an erythromycin MIC of 2 μg/ml and a clindamycin MIC of 0.5 μg/ml. Three of the four 23S rRNA alleles contained a C2611A mutation. The genes encoding ribosomal proteins L4 and L22 were wild-type.

Of 260 erythromycin-nonsusceptible isolates that were screened for *erm*(B) and *mef*(A), 15.4% (40/260) were found to contain both *erm*(B) and *mef*(A). Eighty percent (32/40) of these isolates were serotype 19F. All (32/32) of the serotype 19F isolates were nonsusceptible to penicillin, erythromycin, clindamycin and trimethoprim-sulfamethoxazole, and 81% (26/32) were nonsusceptible to tetracycline. The genetic relatedness of isolates containing both resistance determinants was determined by PFGE. Fourteen randomly selected serotype 19F isolates (44%, 14/32) and all isolates with serotypes other than 19F (n=8) were selected for characterisation by PFGE. PFGE fingerprint patterns were generated for 91% (20/22) of the isolates selected. The PFGE fingerprint patterns are shown in Figure 7.9. A dendrogram showing the genetic relatedness of the isolates according to PFGE, as well as STs of selected isolates, are shown in Figure 7.10. The serotype 19F isolates were clonal, and related to the Taiwan^{19F}-14 global clone. Two isolates selected from the 19F cluster were found to be ST271 and ST1412, and differed from each other by one allele. Isolate 368 (ST271) and isolate 2255 (ST1412) differed from the Taiwan^{19F}-14 global clone by one and two alleles, respectively. Three of the four serotype 23F isolates were related. Two serotype 23F isolates, analysed by MLST, were found to be ST1444 and ST2651, with three different alleles. On the basis of MLST, isolate 3600 (ST1444) was related to the Taiwan^{23F}-15 global clone, with one allele difference. The two serotype 14 isolates were identical by PFGE, and related to the England^{14}-9 global clone. Isolate 5234 was ST2652 and differed from the global clone by 2 alleles. Isolate 4881 was serotype 25 and ST2650. Isolate 315, for which no PFGE fingerprint was obtained, was serotype 3 and ST700.

All of the isolates selected for MLST analyses were nonsusceptible to penicillin, tetracycline, erythromycin, clindamycin and trimethoprim-sulfamethoxazole.

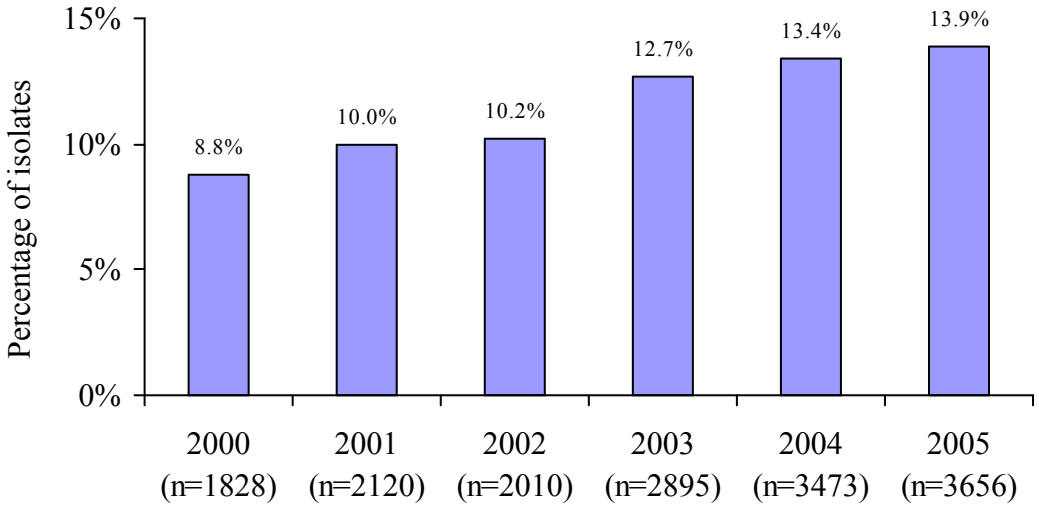

Figure 7.1 Percentage of erythromycin-nonsusceptible isolates causing invasive disease in South Africa by year (all ages, n represents total number of viable isolates by year, $P<0.001$, χ^2- test for trend)

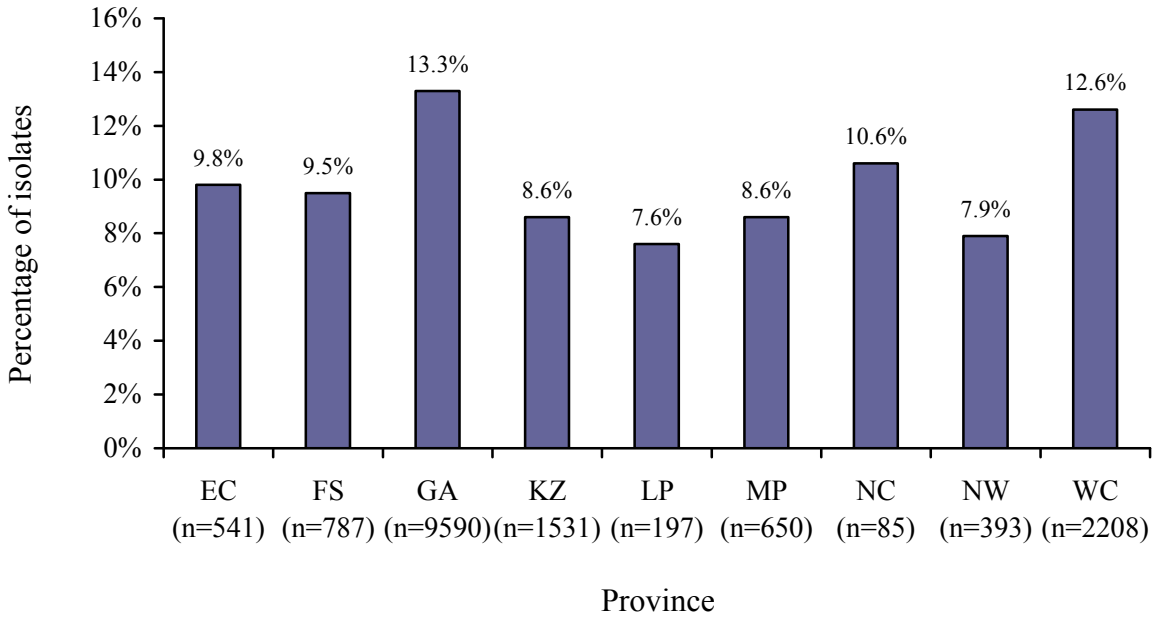

Figure 7.2 Percentage of erythromycin-nonsusceptible isolates causing invasive disease in South Africa by province (2000-2005, all ages, n represents total viable isolates received by province). EC, Eastern Cape; FS, Free State; GA, Gauteng; KZ, KwaZulu-Natal; LP, Limpopo; MP, Mpumalanga; NC, Northern Cape; NW, North-West; WC, Western Cape.

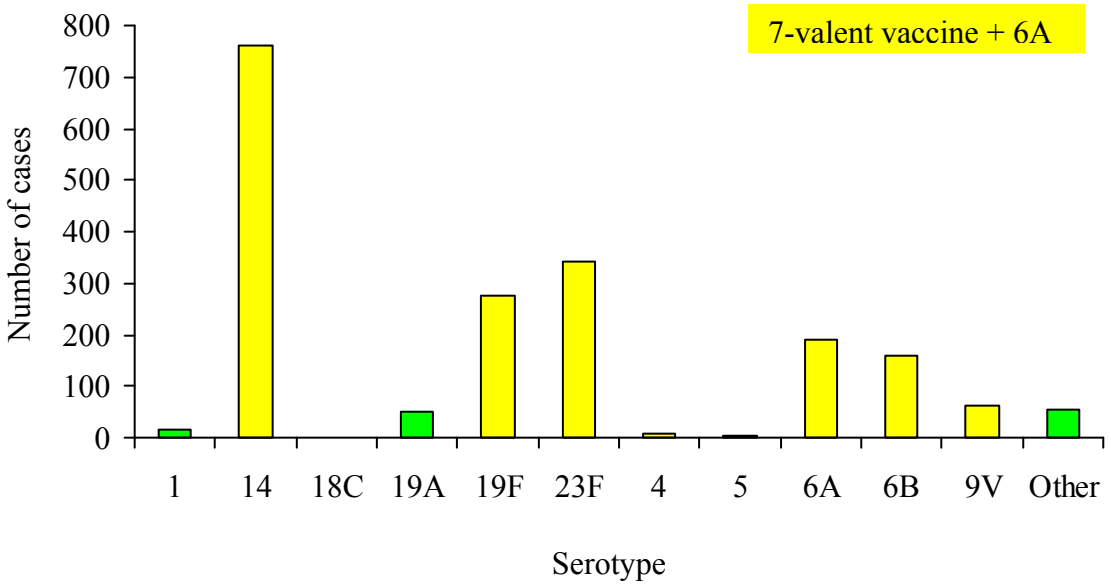

Figure 7.3 Erythromycin-nonsusceptible isolates (n=1921) causing invasive disease in South Africa by serotype (2000-2005, all ages).

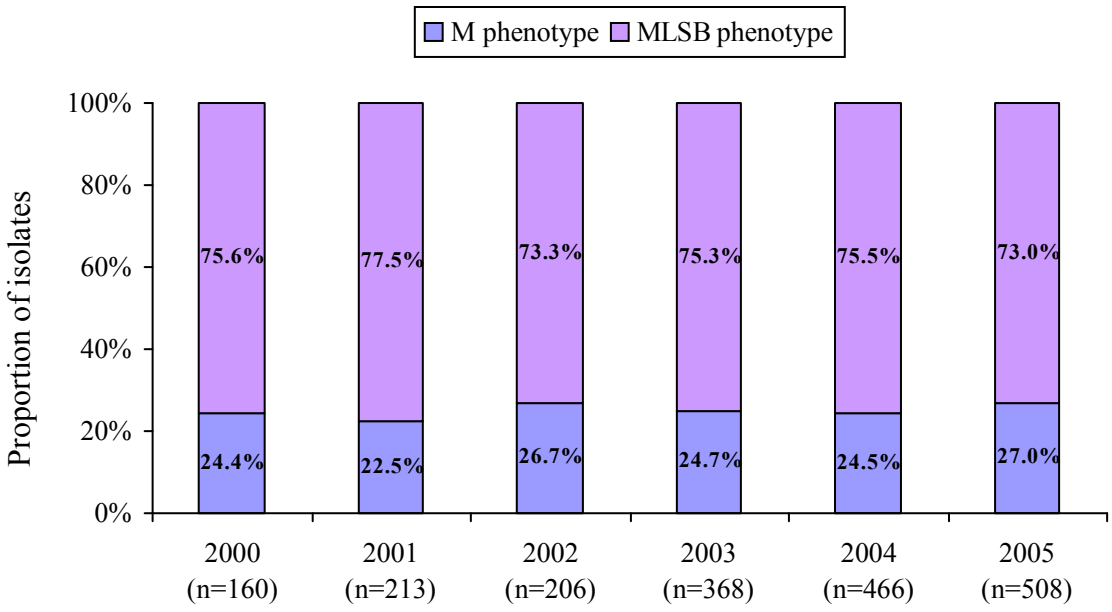

Figure 7.4 Proportion of M and MLS_B phenotypes in erythromycin-nonsusceptible isolates causing invasive disease in South Africa by year (all ages, n=1921, *P*=0.83)

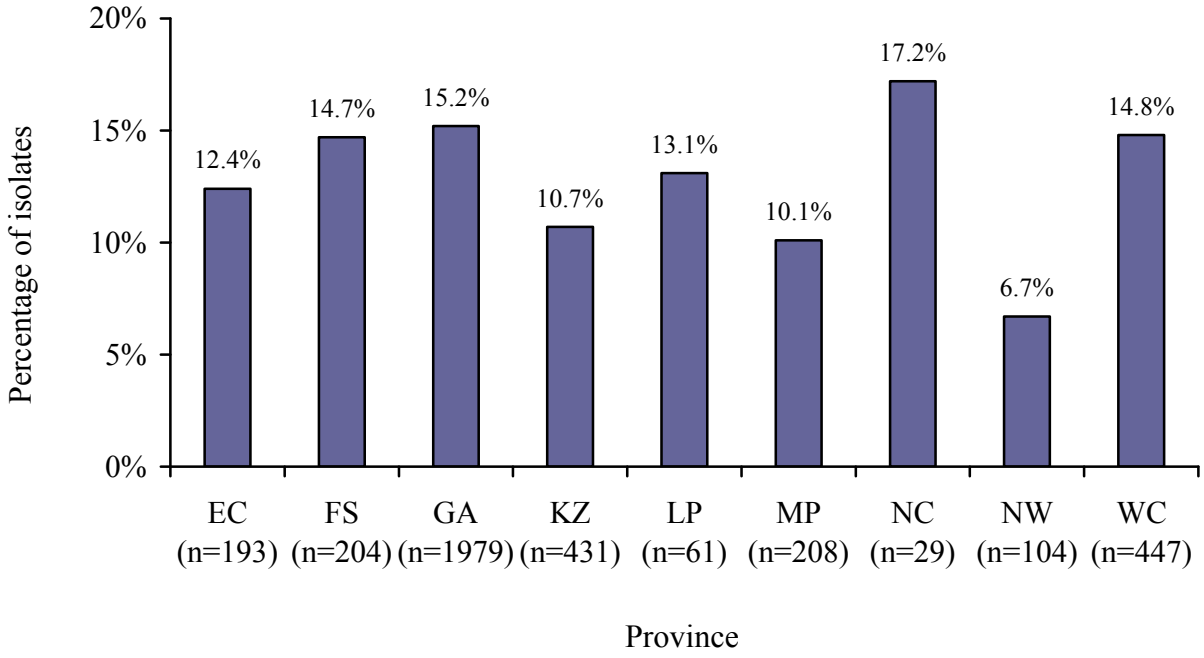

Figure 7.5 Percentage of erythromycin-nonsusceptible isolates causing invasive disease in South Africa in 2005 by province (all ages, n represents total viable isolates received by province in 2005). EC, Eastern Cape; FS, Free State; GA, Gauteng; KZ, KwaZulu-Natal; LP, Limpopo; MP, Mpumalanga; NC, Northern Cape; NW, North-West; WC, Western Cape.

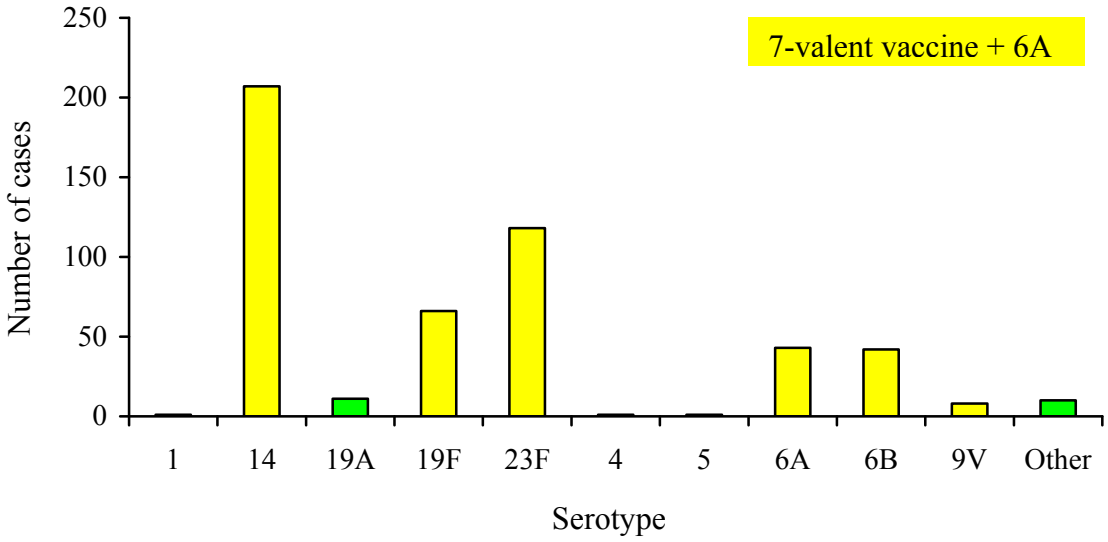

Figure 7.6 Erythromycin-nonsusceptible isolates (n=508) causing invasive disease in South Africa in 2005 by serotype (all ages)

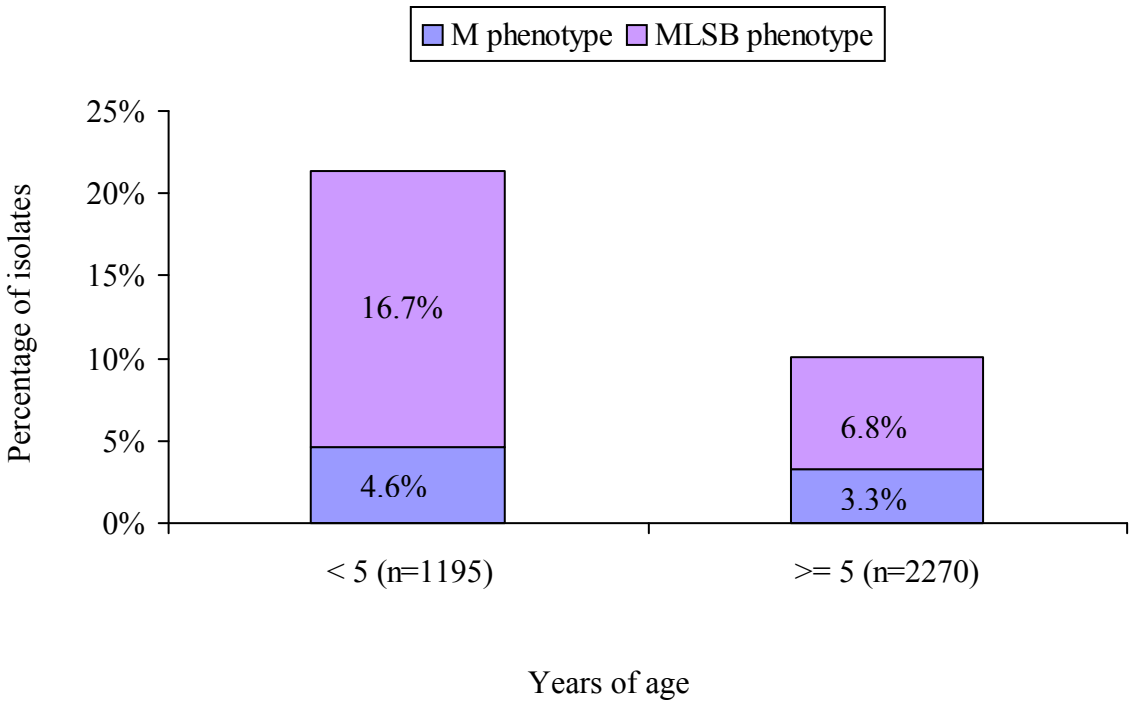

Figure 7.7 Percentage of erythromycin-nonsusceptible isolates and phenotype, causing invasive disease in South Africa in 2005 by age (n represents the total isolates received per age group, $P<0.001$)

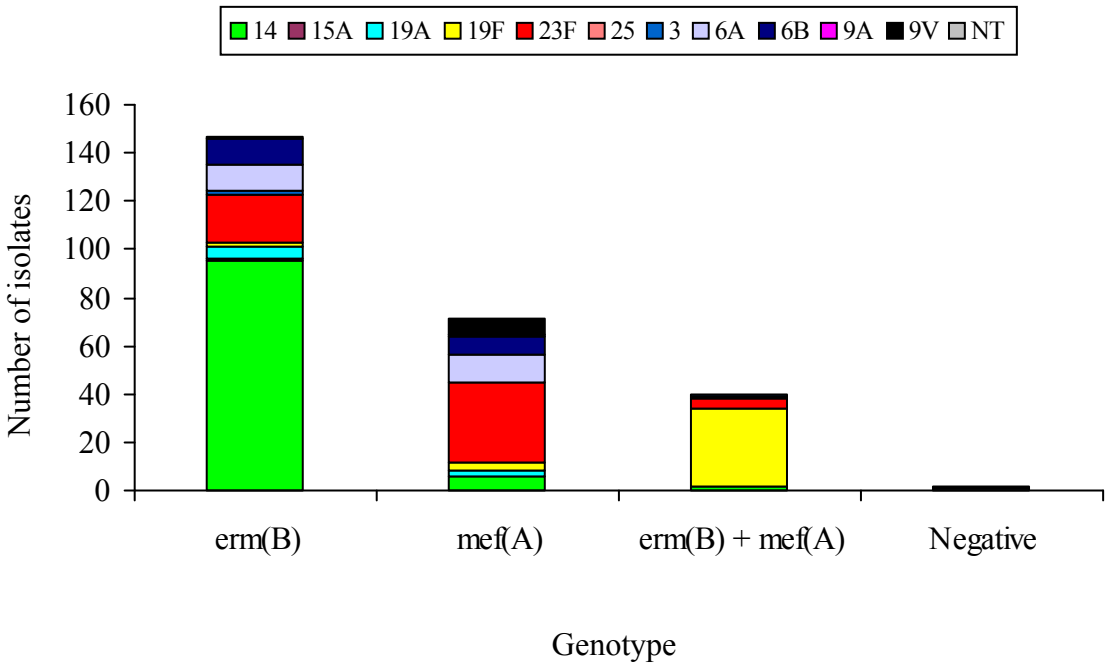

Figure 7.8 Distribution of serotypes according to macrolide resistance genotypes in 260 randomly selected isolates screened for *erm*(B) and *mef*(A) (2005, all ages)

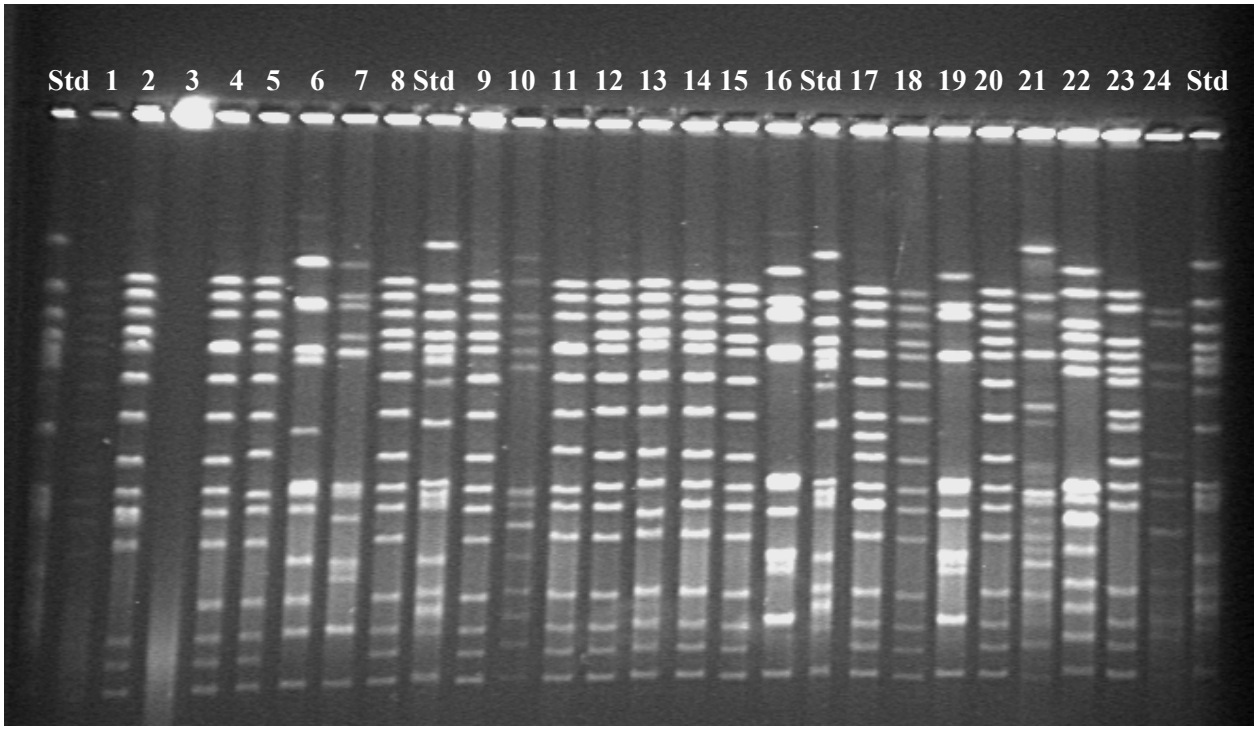

Figure 7.9 Pulsed-field gel electrophoresis of erythromycin-nonsusceptible isolates from 2005 containing *erm*(B) and *mef*(A). Lanes Std, PFGE standard pneumococcal strain (CDC#729); Lane 1, isolate 83 (serotype 19F); Lane 2, 171 (19F); Lane 3, 315 (3); Lane 4, 368 (19F); Lane 5, 517 (19F); Lane 6, 549 (23F); Lane 7, 787 (23F); Lane 8, 919 (19F); Lane 9, 1754 (19F); Lane 10, 1951 (14); Lane 11, 2181 (19F); Lane 12, 2255 (19F); Lane 13, 2316 (19F); Lane 14, 2691 (19F); Lane 15, 2801 (19F); Lane 16, 2994 (23F); Lane 17, 3136 (19F); Lane 18, 3241 (19F); Lane 19, 3600 (23F); Lane 20, 4433 (19F); Lane 21, 4881 (25); Lane 22, 5234 (14), Lane 23, Taiwan^{19F}-14; Lane 24, Portugal^{19F}-21.

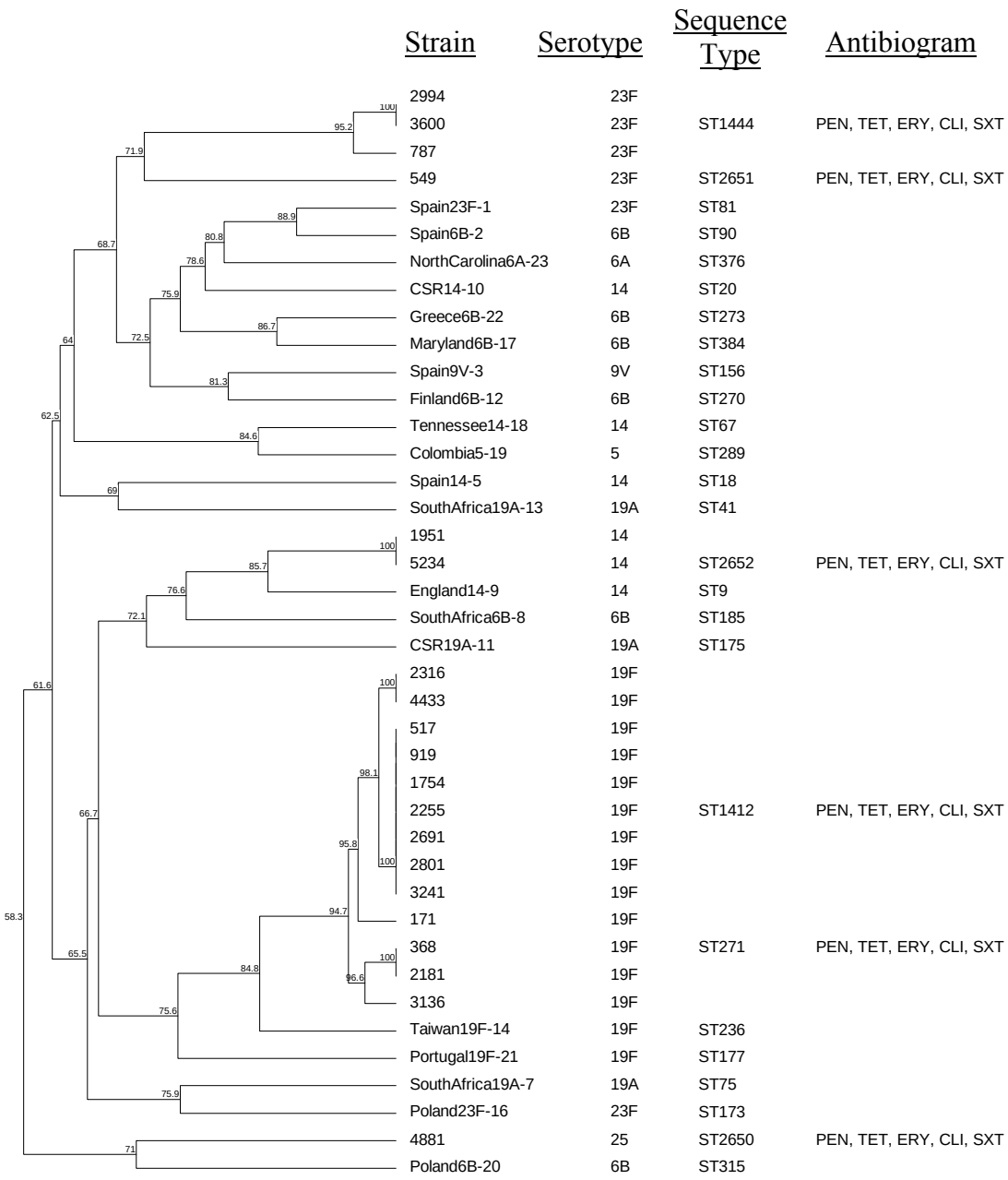

Figure 7.10 PFGE dendrogram showing the genetic relationship between South African erythromycin-nonsusceptible strains from 2005 containing *erm*(B) and *mef*(A), and available global pneumococcal clones. Similarity values are indicated on dendrogram branches. PEN, penicillin; TET, tetracycline; ERY, erythromycin; CLI, clindamycin; SXT, trimethoprim-sulfamethoxazole.

7.4 Discussion

There has been a significant increase in macrolide resistance in South Africa from 2000 to 2005. Previous reports of macrolide resistance in South Africa (Huebner et al., 2000) showed macrolide resistance to be approximately 3% in the late 1990s. Macrolide resistance therefore appears to be continually increasing and following global trends (Felmingham and Gruneberg, 2000; Jacobs et al., 2003). The Alexander Project, conducting global surveillance on pathogens involved in adult CARTIs, reported macrolide resistance to be 24.6% for the 1998-2000 period (Jacobs et al., 2003). The PROTEKT global surveillance study reported macrolide resistance in invasive and non-invasive isolates to be 32.9% for the 1999 to 2002 period (Farrell et al., 2004a). Although lower than global macrolide resistance rates, the gradual and consistent increase in macrolide resistance in South Africa is of concern.

The increase in macrolide resistance in South Africa is most likely due to an increase in macrolide usage in the country as an association between macrolide usage and resistance has been established (Bergman et al., 2006). Macrolide resistance has been reported to be higher in the private sector than in the public sector in South Africa (Feldman et al., 2006a). This is likely due to increased macrolide and antibiotic use in the private sector in comparison with the public sector. Macrolide resistance also appears to be more common in nosocomial IPD compared with community-acquired infections (Feldman et al., 2006b). Macrolide

resistance is clinically significant, and failure of macrolide therapy is more likely when infection is due to a macrolide-resistant strain (Daneman et al., 2006).

Macrolide nonsusceptibility occurred in isolates from all nine provinces of South Africa ranging from 7.6% in Limpopo to 13.3% in Gauteng for the six years investigated. In 2005, isolates from Gauteng and the Western Cape, from which the highest numbers of viable isolates were received, had a macrolide nonsusceptibility of 15%. In 2005 the Northern Cape had a prevalence of 17%, however the numbers of viable isolates received from the province were low. As observed in studies conducted by the CDC in the US (Hyde et al., 2001), in South Africa macrolide nonsusceptibility is significantly higher in children less than five years of age than in individuals five years and older. Isolates from blood specimens have a significantly higher macrolide nonsusceptibility than isolates from CSF specimens. This may be due to the fact that serotypes associated with bloodstream infections are carried in the nasopharynx for longer periods of time than those associated with meningitis, and therefore are more likely to be exposed to antibiotics and develop antibiotic resistance.

Serotype 14 was the most common macrolide-nonsusceptible serotype and in 2005 comprised 41% of all isolates. Serotype 23F was the second most prevalent serotype in 2005 comprising 23% of all isolates. In 2005, 96% of macrolide-nonsusceptible isolates received were serotypes included in the 7-valent pneumococcal conjugate vaccine and serotype 6A. A decrease in macrolide resistance in the US has been reported since the introduction of PCV7 in 2000

(Kyaw et al., 2006; Stephens et al., 2005). It has been shown to provide protection to vaccinated children as well as non-vaccinated individuals by means of a herd immunity effect (Whitney et al., 2003). PCV7 has been available in South Africa since late 2005. The results of this study show that PCV7 is likely to have similar positive effects in the South African population. A study has been conducted in South Africa on the efficacy of a 9-valent pneumococcal conjugate vaccine, containing serotypes 1 and 5 in addition to the PCV7 serotypes (Klugman et al., 2003). The vaccine was shown to have a high efficacy in HIV-infected and uninfected children. These studies support the routine use of PCV7 in South African children. However, due to the high cost of the vaccine it is not likely to have widespread coverage, reaching only the private sector, and therefore not impacting on the major burden of disease in the country.

As reported in previous South African studies (Huebner et al., 2000; Widdowson and Klugman, 1998) the MLS_B phenotype is the predominant phenotype in South Africa. For the 2000 to 2005 period, 75% of isolates displayed this phenotype and 25% displayed the M phenotype. The distribution of the two phenotypes did not vary significantly over the six-year period. Widdowson and Klugman (1998) and Huebner et al. (2000) reported large increases in the proportion of isolates displaying the M phenotype in the 1990s however this appears to have stabilised at 25%. The distribution of phenotypes differs between children under the age of five and individuals five years and older. In the younger group the M phenotype comprised 22% of isolates, whereas in the older group this proportion increased to

33%. The M phenotype is therefore more common in individuals five years and older than in children under the age of five.

A random sample of isolates from 2005 were selected for genotypic analysis. The most common genotype in macrolide-nonsusceptible strains was *erm*(B), which constituted 57% of isolates, followed by *mef*(A) at 27%. 15% of isolates contained both resistance determinants and 1% of isolates were negative for both. Serotype 14 was predominant in strains containing *erm*(B) whereas serotype 23F was predominant in strains containing *mef*(A). Eighty percent of isolates containing *erm*(B) and *mef*(A) were serotype 19F. The distribution of genotypes in South African macrolide-resistant isolates is similar to most parts of Europe where *erm*(B) is the most common genotype (Reinert et al., 2005a). This differs from the US where *mef*(A) is the predominant macrolide resistance genotype (Brown et al., 2004).

Isolates containing *erm*(B) and isolates containing *erm*(B) and *mef*(A) displayed the MLS_B phenotype with high-level resistance to erythromycin (MICs >256 μg/ml), as is typical for these strains. The isolates containing *mef*(A) displayed the M phenotype and had lower levels of macrolide resistance with a MIC_{90} of 32 μg/ml. The MICs of strains containing *mef*(A), historically associated with lower levels of macrolide resistance, are increasing (Farrell et al., 2007). The PROTEKT US study from 2000 to 2004 showed *mef*(A) isolates to have a MIC_{90} of 16 μg/ml (Farrell et al., 2007). The South African isolates had higher levels of resistance however the MICs may be overestimated due to the fact that, in the determination

of MICs by the Etest, cultures were incubated in an atmosphere of 5% CO_2. The activity of macrolides has been shown to be affected by incubation in CO_2 (Clark et al., 1998; Johnson et al., 1999).

In 2005, 27% of isolates displayed the M phenotype and 73% displayed the MLS_B phenotype. The phenotypic classification of isolates therefore corresponded well with the genotypic classification. The advantage of genotyping however is the ability to detect strains containing both *erm*(B) and *mef*(A), which display the MLS_B phenotype and can therefore not be distinguished phenotypically from isolates containing only *erm*(B). In addition, genotyping enables the detection of isolates containing neither *erm*(B) nor *mef*(A). Two isolates, negative for *erm*(B) and *mef*(A), were found to contain ribosomal mutations. One isolate, with high-level erythromycin resistance (MIC >256 μg/ml), contained an A2059G mutation in all four alleles of 23S rRNA as well as a S20N mutation in ribosomal protein L4. The second isolate, with low-level erythromycin resistance (MIC 2 μg/ml), contained a C2611A mutation in three of the four 23S rRNA alleles. Ribosomal mutations are common in macrolide-resistant isolates negative for *erm*(B) and *mef*(A) (Farrell et al., 2003; Tait-Kamradt et al., 2000b).

Fifteen percent of macrolide-nonsusceptible isolates genotypically screened were found to contain *erm*(B) and *mef*(A). Serotype 19F constituted 80% of the isolates, and these isolates were multidrug-resistant. The serotype 19F isolates were clonal and related to the Taiwan^{19F}-14 global clone. Isolates containing both *erm*(B) and *mef*(A) were first described in a South African study (McGee et al.,

2001a), which showed that the majority of the isolates belonged to a multi-resistant 19F clone. Based on an analysis of PFGE banding patterns, the serotype 19F isolates in this study are related to those described by McGee et al. (2001a). Since they were first described, isolates containing both resistance determinants have been detected around the world (Amezaga et al., 2002; Bean and Klena, 2002; Farrell et al., 2007; Montanari et al., 2003a; Weiss et al., 2002). Isolates containing both *erm*(B) and *mef*(A) appear to be clonal worldwide, and are predominantly serotype 19F and multidrug-resistant (Bean and Klena, 2002; Farrell et al., 2004b; Farrell et al., 2005; McGee et al., 2001a). The PROTEKT surveillance study has shown an increase in the prevalence of isolates containing both genes with the majority of isolates being related to the global multi-resistant Taiwan^{19F} –14 clonal complex 271 (Farrell et al., 2005). The epidemiology of South African isolates was similar to trends described globally, and the ST271 clonal complex was detected. However, there appears to be greater diversity in South African isolates with the ST1412 clonal complex being detected in the serotype 19F isolates, which differed from ST271 by one allele. In addition, isolates of serotypes 23F, 14, 25 and 3 constituted 20% of the isolates.

7.5 Conclusion

Macrolide resistance is increasing in South Africa and 14% of isolates received in 2005 were nonsusceptible to erythromycin. In 2005, 96% of isolates received were serotypes included in PCV7 and serotype 6A. The MLS_B phenotype was predominant and the proportion of isolates displaying the MLS_B and M phenotypes did not change significantly from 2000 to 2005. *erm*(B) (57%) was the predominant genotype followed by *mef*(A) (27%). Two isolates were negative for both resistance determinants and were found to have ribosomal mutations. Fifteen percent of isolates genotypically screened contained both resistance determinants. Of these isolates, 80% were serotype 19F. The serotype 19F isolates were clonal, multidrug-resistant and related to the Taiwan^{19F}-14 global clone.

Chapter Eight

Conclusion

Macrolides are commonly used for the treatment of pneumococcal infections and as a result, resistance to macrolides has developed and continues to increase globally. Initially, the pneumococcus developed resistance to macrolides by two predominant mechanisms, target modification and active drug efflux due to acquisition of the *erm*(B) (MLS_B phenotype) (Leclercq and Courvalin, 2002) and *mef(*A) (M phenotype) (Sutcliffe et al., 1996a) genes, respectively. More recently, the pneumococcus has developed ribosomal mutations as a form of target modification. These mutations occur in 23 rRNA or ribosomal proteins L4 and L22 (Canu et al., 2002; Farrell et al, 2003; Pihlajamaki et al., 2002; Reinert et al., 2003; Tait-Kamradt et al., 2000a; Tait-Kamradt et al., 2000b). By modifying the binding pocket on the large ribosomal subunit, macrolide binding is prohibited. Many protein synthesis-inhibiting antibiotics share overlapping binding sites on the large ribosomal subunit and therefore modification of the binding site often confers cross-resistance to a number of antibiotics.

In response to the increase in antibiotic resistance, new antibiotics such as linezolid and telithromycin have been developed. Linezolid is an oxazolidinone antibiotic that has a unique mechanism of action. It inhibits protein synthesis by preventing the formation of the 70S initiation complex in bacterial translation systems (Livermore, 2003). Two linezolid-resistant clinical isolates were found to contain 6 bp deletions, resulting in the deletion of two amino acids from a highly

conserved region of the ribosomal protein L4 ($_{64}$PWRQ$_{67}$ to $_{64}$P_Q$_{67}$ and $_{67}$QKGT$_{70}$ to $_{67}$Q_T$_{70}$). Transformation studies showed that these mutations conferred cross-resistance to three classes of antibiotics: macrolides, linezolid and chloramphenicol. The cross-resistance conferred by these mutations confirms that these antibiotics share a common binding site. The mutations described in these isolates represent novel mechanisms of resistance to linezolid and chloramphenicol.

There are four copies of the rRNA operon in the pneumococcus (Tait-Kamradt et al., 2000a) and these alleles maintain extremely high homogeneity by gene conversion (Hashimoto et al., 2003; Liao, 2000). A macrolide-resistant clinical isolate of *S. pneumoniae* with 23S rRNA mutations showed a heterogeneous phenotype and genotype. By transformation of susceptible strain R6, the 23S rRNA mutations were shown to confer macrolide resistance. The transformants showed similar heterogeneity to the original isolate. Culture of a resistant transformant in the absence of antibiotic pressure showed gene conversion to occur between the 23S rRNA alleles resulting in reversion to wild-type and susceptibility. The resistant phenotype was associated with a fitness cost. Therefore the pneumococcus can switch alleles between wild-type and mutant as an adaption to its environment. Provided that complete conversion has not been reached, alleles will convert to the mutant form under antibiotic selective pressure, and in the absence of antibiotic selective pressure alleles will revert to the wild-type in order to regain fitness. The ability of pneumococci to rapidly alter their resistance phenotype and genotype by means of gene conversion has

implications for susceptibility testing as well as for the study of resistance mechanisms.

Telithromycin is a semi-synthetic derivative of the macrolide, erythromycin. The structural modifications of this antibiotic enable it to form a tighter bond with the ribosome, and decrease the inducing effect of the antibiotic. For these reasons telithromycin remains active against macrolide-resistant strains expressing *erm*(B) and *mef*(A) (Douthwaite and Champney, 2001). To date, telithromycin-resistant clinical pneumococcal isolates are rare (Farrell and Felmingham, 2004). A clinical isolate highly resistant to telithromycin was found to contain an *erm*(B) gene with a truncated leader peptide, and a mutant ribosomal protein L4 containing a $_{69}$GTG$_{71}$ to TPS mutation in a highly conserved region in addition to other mutations. By transformation of susceptible strains, it was shown that the mutant *erm*(B) gene reduced the susceptibility of the recipient strain to telithromycin but did not confer high-level resistance. Similarly, the $_{69}$GTG$_{71}$ to TPS L4 mutation alone did not confer telithromycin resistance. However when the $_{69}$GTG$_{71}$ to TPS mutation was combined with a wild-type *erm*(B) gene, high-level resistance was achieved. Therefore methylation of A2058 in 23S rRNA together with mutations in ribosomal protein L4 altered the conformation of the target site to block telithromycin binding. Neither mechanism individually was sufficient. High levels of telithromycin resistance appear to occur when an isolate has multiple mutations in the ribosomal binding pocket.

A second telithromycin-resistant clinical isolate, containing a mutant *erm*(B) gene, was investigated for the resistance mechanism. Resistance was shown to be conferred by a 136 bp deletion mutation in the regulatory region of *erm*(B). The likely effect of this mutation was increased expression of the *erm*(B) gene and therefore increased ribosomal methylation levels. Telithromycin resistance is conferred by high levels of ribosomal methylation (Zhong et al., 1999), which are not achieved by wild-type *erm*(B) genes but may be achieved by mutated genes which result in an increase in gene expression. Although the development of resistance to telithromycin is more complicated than for macrolides and therefore less likely to occur, resistant isolates are emerging and resistance levels should be monitored.

Macrolide resistance is increasing in IPD isolates in South Africa, from 9% in 2000 to 14% in 2005. The MLS_B phenotype is predominant in South Africa, accounting for 75% of macrolide-resistant strains over the six-year period 2000-2005. 96% of macrolide-resistant strains received in 2005 were serotypes included in the 7-valent pneumococcal conjugate vaccine and serotype 6A. In a genotypic analysis of random isolates from 2005, 57% contained *erm*(B), 27% contained *mef*(A), 15% contained both *erm*(B) and *mef*(A) and 1% were negative for both resistance determinants. The two isolates negative for *erm*(B) and *mef*(A) were found to contain ribosomal mutations. Isolates carrying both *erm*(B) and *mef*(A) were clonal, with 80% of isolates being serotype 19F. These isolates were related to the Taiwan^{19F}-14 global clone and were multidrug-resistant.

Protein synthesis-inhibiting antibiotics are an essential tool for the treatment of pneumococcal infections, especially in the case of penicillin resistance. It is therefore essential to monitor resistance to these antibiotics in order to continually assess their effectiveness as a treatment option. The common binding site on the ribosome that many of these agents share is detrimental, as resistance mechanisms often confer cross-resistance against multiple classes of antibiotics. The pneumococcus will continue to evolve and develop strategies to resist antibiotic action, with implications for treatment. Investigating and understanding the mechanisms of pneumococcal resistance is critical for treatment to remain one step ahead of the pneumococcus.

Appendix A

Preparation of Antibiotic-Containing Mueller-Hinton Agar Plates

2 g	Mueller-Hinton agar
	(Oxoid Ltd., Basingstoke, Hampshire, England)
56 ml	dH_2O

Autoclave at 15 psi, 121°C for 15 min.

Allow to cool to 48°C in a water-bath.

Add 3 ml horse blood (final concentration 5%).

Add 1 ml antibiotic solution*.

Mix and pour 20 ml into a petri dish.

Allow to solidify at room temperature.

Plates can be stored at 4°C for up to one week.

* Prepare antibiotics according to CLSI recommendations (Clinical and Laboratory Standards Institute, 2006a). Prepare serial dilutions of antibiotics in the required range to a final volume of 1 ml. Erythromycin, chloramphenicol, tetracycline and clindamycin were supplied by Sigma-Aldrich, Steinheim, Germany. All other antibiotic powders were supplied by Mast Diagnostics, Merseyside, UK.

Appendix B

Preparation of Reagents for Genomic DNA Extraction

Tris-EDTA (TE) Buffer

2 ml 1 M Tris-HCl (pH 8.0)

400 µl 0.5 M EDTA (pH 8.0)

Make up to 200 ml with deionised H_2O (dH_2O).

Adjust pH to 8.0.

Autoclave.

1 M Tris-HCl (pH 8.0)

60.55 g Tris (Sigma)

400 ml dH_2O

Adjust pH to 8.0 using concentrated HCl.

Adjust volume to 500 ml with dH_2O.

Autoclave.

0.5 M EDTA (pH 8.0)

18.61 g EDTA (Merck, Darmstadt, Germany)

70 ml dH_2O

Adjust pH to 8.0 using 10 M NaOH.

Adjust volume to 100 ml with dH_2O.

Autoclave.

10 M NaOH

20 g NaOH (Merck)

50 ml dH_2O

Autoclave.

10 % Sodium Dodecyl Sulphate (SDS)

10 g SDS (Merck)

100 ml autoclaved dH_2O

5 M NaCl

29.22 g NaCl (Merck)

100 ml dH_2O

Autoclave.

10 % CTAB (hexadecyl trimethyl-ammonium bromide) / 0.7 M NaCl

2.05g NaCl (Merck)

5 g CTAB (Sigma)

40 ml autoclaved dH_2O

Heat to dissolve.

Adjust volume to 50 ml with dH_2O.

70 % Ethanol

175 ml 100 % Ethanol (Merck)

75 ml dH_2O

RNase A Stock (2 mg/ml)

4 mg	RNase A (Roche Diagnostics GmbH, Mannheim, Germany)
2 ml	TE Buffer

Boil for 10 min.

TE Buffer containing RNase

150 µl	TE Buffer
4 µl	RNase A stock

Appendix C

Agarose Gel Electrophoresis

1 % Agarose Gel

0.3 g Molecular Grade Agarose (Hispanagar, Burgos, Spain)

30 ml 1x TAE Buffer

Boil to dissolve, allow to cool slightly and add 2 µl of 10 mg/ml ethidium bromide. Pour into casting tray and allow to set for 30 min. Add 5 µl of bromophenol blue to 50 µl PCR products and load 5 µl into each well. Perform electrophoresis in 1x TAE Buffer for 30 min at 120 volts.

TAE Buffer (10x)

48.4 g Tris (Sigma)

11.42 ml Glacial Acetic Acid (Merck)

7.44 g EDTA (Merck)

Make up to 1 L with dH_2O.

Ethidium Bromide (10 mg/ml)

1 g Ethidium Bromide (Sigma)

100 ml dH_2O

Stir for several hours and store at 4°C.

Bromophenol Blue

0.25 g Bromophenol Blue (Sigma)

40 g Sucrose (Sigma)

Dissolve in 100 ml dH_2O.

Store at 4°C.

Appendix D

Preparation of C-Medium

Prepare C- medium fresh before use:

160 ml	Pre-C
5.2 ml	Supplement
4 ml	Glutamine
4 ml	Adams III
2 ml	Pyruvate
5.8 ml	1M Phosphate buffer
3.6 ml	Yeast extract (5%)

Add components to autoclaved Pre-C, filter sterilise and store in the dark at 4°C.

Check pH of C-medium is 7.7 – 8.

Pre-C (Prepare fresh)

0.6 g	Sodium acetate (anhydrous) (Merck)
2.5 g	Casein Hydrolysate (Casamino acids) (Oxoid)
2.5 mg	L-tryptophan (Sigma)
25 mg	L-cysteine (Sigma)

Make up to 500 ml with dH_2O.

Titrate pH to 7.4 - 7.6 with 10 M NaOH (Merck).

Dispense into 160 ml quantities, autoclave and store at room temperature.

Supplement (Prepare fresh)

15 ml	3 in 1 salts
6 g	glucose (Merck)
0.75 g	sucrose (Sigma)
30 mg	adenosine (Merck)
30 mg	uridine (Sigma)

Add 61.5 ml dH_2O, filter sterilise and store at 4°C.

3 in 1 salts (Store)

25 g	$MgCl_2.6H_2O$ (Merck)
0.125 g	$CaCl_2$ (anhydrous) (Merck)
50 µl	0.1 M $MnSO_4.4H_2O$ (1.12 g in 50 ml dH_2O) (Merck)

Make up to 250 ml with dH_2O and autoclave. Store at 4°C.

Adams III (Store)

24 ml	Adams I
6 ml	Adams II
0.3 g	Asparagine (Sigma)
30 mg	Choline chloride (Merck)
3.5 mg	$CaCl_2.2\ H_2O$ (Merck)

Make up to 150 ml with dH_2O. Filter sterilise. Store in a dark bottle at 4°C.

Adams I (Store)

7.5 µl	Biotin (2 mg/ml in dH_2O - filter sterilize - store at 4°C) (Roche)
15 mg	Nicotinic acid (Merck)
17.5 mg	Pyridoxal hydrochloride (Merck)
60 mg	Calcium pantothenate (Merck)
16 mg	Thiamine hydrochloride (Sigma)
7 mg	Riboflavin (Sigma)

Make up to 100 ml with dH_2O.

Filter sterilise and store in a dark bottle at 4°C.

Adams II (Store)

25 mg	$FeSO_4.7H_2O$ (Merck)
25 mg	$CuSO_4.5H_2O$ (Merck)
25 mg	$ZnSO_4.7H_2O$ (Merck)
10 mg	$MnCl_2.4H_2O$ (Merck)

Make up to 50 ml with dH_2O. Add 500 µl of concentrated HCl (Merck).

Filter sterilise and store at 4°C.

Glutamine (Prepare fresh)

40 mg glutamine (Merck) in 4 ml of dH_2O (10 mg/ml).

Filter sterilise and store at 4°C.

Pyruvate (Store)

0.2 g pyruvate (Sigma) in 10 ml dH_2O.

Filter sterilise and store in 2 ml aliquots at 4°C.

Yeast extract (5%) (Prepare fresh)

4 g yeast extract (Oxoid) in 80 ml dH_2O.

Filter sterilise or autoclave and store at 4°C.

1M Phosphate buffer (Store)

0.9 g KH_2PO_4 (Sigma)

27 g $K_2HPO_4.3H_20$ (Sigma)

Make up to 125 ml with dH_2O.

Autoclave and store at 4°C.

This is not pH 8, but makes the C-medium pH 8.

Appendix E

Preparation of Tryptone Soya Broth

TSB

For each strain prepare one bottle:

3 g	Tryptone Soya Broth (Oxoid)
100 ml	dH_2O

Stir to dissolve, autoclave and store at 4°C.

Appendix F

Preparation of Reagents for Pulsed-Field Gel Electrophoresis

5 M NaCl

58.44 g NaCl (Merck)

200 ml dH_2O

Autoclave.

1 M Tris-HCl (pH 8.0)

24.22 g Tris (Sigma)

150 ml dH_2O

Adjust pH to 8.0 using concentrated HCl.

Adjust volume to 200 ml with dH_2O.

Autoclave.

0.5 M EDTA (pH 8.0)

93.06 g EDTA (Merck)

400 ml dH_2O

Adjust pH to 8.0 using NaOH pellets.

Adjust volume to 500 ml with dH_2O.

Autoclave.

Cell Suspension Buffer

50 ml 5 M NaCl

2.5 ml 1 M Tris-HCl (pH 8.0)

Make up to 250 ml with dH_2O.

Autoclave.

Cell Lysis Buffer

125 ml 0.5 M EDTA (pH 8.0)

2.5 g N-Lauroylsarcosine (Sigma)

Make up to 250 ml with dH_2O.

Autoclave.

On day of use add 0.1 mg/ml Proteinase K (Roche).

Tris-EDTA (TE) Buffer

10 ml 1 M Tris-HCl (pH 8.0)

2 ml 0.5 M EDTA (pH 8.0)

Make up to 1 L with dH_2O.

Autoclave.

5x Tris-Borate-EDTA (TBE) Buffer

54 g	Tris (Sigma)
27.5 g	Boric Acid (Sigma)
20 ml	0.5 M EDTA (pH 8.0)

Make up to 1 L with dH_2O.

0.5x TBE Buffer

100 ml	5x TBE Buffer
900 ml	dH_2O

1.1% SeaKem Gold Agarose Gel

1.76 g	SeaKem Gold Agarose (Cambrex Bio Science, Rockland, ME)
160 ml	0.5x TBE Buffer

Boil to dissolve.

Cool to 50-55°C.

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