CHAPTER 1: INTRODUCTION

1.1 History

Streptococcus pneumoniae (the pneumococcus) is a human pathogen that colonizes the upper respiratory tract and causes disease such as acute otitis media (AOM), pneumonia, bacteraemia and meningitis. In the developing world, the incidence of pneumococcal disease is highest in young children whereas in the developed word the disease burden is carried mostly by the elderly population. Acute respiratory infections kill an estimated 2.6 million children annually (World Health Organization 2007). The pneumococcus causes over 1 million of these deaths, most of which occur in developing countries (World Health Organization 2007). In Europe and the United States, pneumococcal pneumonia is the most common community-acquired bacterial pneumonia. Even in economically developed regions, invasive pneumococcal disease carries high mortality; for adults with pneumococcal pneumonia the mortality rate averages 10%–20%, whilst it may exceed 50% in the high-risk groups (World Health Organization 2007). The pneumococcus is also an important opportunistic pathogen affecting children and adults with human immunodeficiency virus (HIV) infection (Jones *et al.* 1998).

The organism was first identified in 1881, and due to its recognition as the most common cause of lobar pneumonia, it was given the name pneumococcus (Musher 2005). Its identification has lead to the discovery of many concepts in microbiology. In the early 1890's Felix and George Klemperer showed that immunization with killed pneumococci protected animals against subsequent pneumococcal infections and that

protection could be transferred by infusing serum from immunized mice into naïve recipients. In the first decade of the 20th century Maynard and colleagues began to apply this concept of humoral immunity to the problem of epidemic lobar pneumonia that occurred in African miners (Musher 2005). The pneumococcus also played a central role in the recognition of DNA as genetic material. In the early 1920's Griffith showed that injection of mice with live, unencapsulated (mutant) pneumococci together with heat-killed encapsulated pneumococci lead to the emergence of viable, encapsulated bacteria. This observation was concluded in the 1940's by Avery and colleagues who provided conclusive evidence that these mutants had recovered the capacity to produce capsule by taking up DNA from killed virulent organisms (Avery *et al.* 1979).

1.2 Microbiology

S. pneumoniae is a Gram-positive coccus consisting of three major surface layers: a surface membrane, cell wall and capsule. The polysaccharide capsule, which is present in nearly all clinical isolates, is the thickest and constitutes the outermost layer of pneumococci. It is made up of high molecular weight polymers consisting of repeating oligosaccharide units. The capsule is believed to be the major virulence determinant and provides the basis for differentiation of pneumococci (Austrian 1976). Typing sera are used to divide pneumococci into serogroups and serotypes. These sera have been developed such that they are specific for the immunochemical differences between capsular polysaccharides. Currently, there are 46 pneumococcal serogroups, which are further divided into 90 immunologically distinct serotypes (Henrichsen 1995). Attached to the cell wall peptidoglycan macromolecules are proteins that significantly contribute

to pathogenesis and may also play a role in the disease process. These include molecules such as hyaluronate lyase, neuraminidase, autolysin and choline binding protein A (Jedrzejas 2001).

Pneumococci are identified in the laboratory by the following reactions: 1) alpha hemolysis of blood agar, 2) catalase negativity, 3) optochin sensitivity and 4) bile solubility. Gram staining of sputum, cerebrospinal fluid and other body fluids from the site of infection usually shows typical Gram-positive, lancet-shaped cocci (elongated cocci with a slightly pointed curvature). Usually they appear in pairs (diplococci), but they may also appear singly and in short chains.

Pneumococci are fastidious bacteria, growing optimally in 5% CO_2 on blood agar medium. Colonies characteristically produce a zone of alpha (green) hemolysis, which differentiate pneumococci from the group A (beta haemolytic) streptococci, but not from commensal alpha haemolytic (viridans) streptococci which are co-inhabitants of the upper respiratory tract. Specialised tests such as bile (e.g. deoxycholate) solubility and optochin sensitivity are routinely employed to differentiate pneumococci from viridans group streptococci. Typically, pneumococci are optochin sensitive and undergo lysis by bile salts.

1.3 Pathogenesis

The pneumococcus spreads from person to person through direct contact of mucous membranes or indirectly through respiratory droplets. The organism is part of the normal

flora of the upper respiratory tract and may be carried for months by healthy individuals without causing disease (Dagan *et al.* 2002). The rate of colonization is higher in children, with a peak incidence occurring in the first three years of life, followed by a gradual decrease with age (Bogaert *et al.* 2004; Hill *et al.* 2006). Most pneumococcal disease does not occur after prolonged carriage but follows the acquisition of a recently acquired organism (Gray *et al.* 1979). Following colonization, infection may be cleared by the primary defence mechanism (secretory IgA, cough reflexes, mucosal secretions, and cilliary transport). Failure of clearance by the primary defence may lead to access of pneumococci to bacteria-free sites resulting in a variety of disease manifestations such as AOM, sinusitis, pneumonia, bacteraemia and meningitis.

In AOM the colonizing bacteria gain access into the middle ear cavity through the Eustachian tubes, leading to an accumulation of fluid in the middle ear cavity. In most studies of AOM, the pneumococcus is the most commonly isolated organism, or second only to nontypable *Haemophilus influenzae* (Musher 2005). In acute infection of the sinuses the pneumococcus also dominates or is second to *H. influenzae*. Sinusitis results from congestion of the mucous membrane caused by allergy or viral infection. The resulting obstruction prevents clearance of bacteria, while the accumulation of fluid in the paranasal cavities provides a medium for bacterial proliferation and subsequent acute sinus infection.

Pneumonia occurs when organisms gain access to the alveoli. Following replication, they are carried along the alveolar septa where they activate complement, generate

cytokine production and upregulate receptors on vascular endothelial surfaces. Exudate fluid and white blood cells accumulate in the septa and alveoli and extend to uninvolved areas through pores of Kohn. This filling of the alveoli with organisms and inflammatory exudates define the presence of pneumonia.

In some cases, pneumococci also act as invasive organisms, penetrating mucosal barriers. The capsule is able to escape the immune system by preventing direct contact between phagocytes and the pneumococcus (Musher 2005). This anti-phagocytic effect enables the pneumococcus to gain access to the blood and cause bacteraemia. Expression of the capsule is important for survival in the blood and is strongly associated with the ability of the organism to cause invasive pneumococcal disease (IPD). Direct extension of organisms from sinuses or the middle ear or as a result of bacteremia can cause infection of the nervous system, including the meninges, which results in meningitis. Except during an epidemic of meningococcal infection, *S. pneumoniae* is the most common cause of bacterial meningitis in adults (Musher 2005).

1.4 Prevention

Pneumococcal infections are treated with antibiotics and penicillin has been the drug of choice since the 1940s (Ramirez JA & Raff MJ 1995). Since 1965, however, when the first clinical isolate with reduced susceptibility to penicillin was described, resistance to penicillin has become an enormous problem worldwide (Kislak *et al.* 1965). In addition resistance has now developed to almost all antimicrobial drugs currently used in therapy.

The costs associated with infection and the rising rates of drug resistance means that vaccination has emerged as a public health priority. The 23-valent pneumococcal polysaccharide vaccine (PPV), available since the 1980s, is used in many countries to prevent invasive disease in adults and contains capsular polysaccharide from 23 commonly infecting adult serotypes (Hausdorff *et al.* 2005). This vaccine is recommended for all adults older than 64 years and high risk individuals who are 2 years of age and older. Unfortunately, because the polysaccharide antigens induce T-cell independent immunity which leads to low antibody levels and poor immunologic memory, the vaccine is not effective in children younger than 2 years of age (Hausdorff *et al.* 2000b).

The recently developed pneumococcal conjugate vaccines (PCV-7, -9, and -11) hold greater promise for reducing the burden of IPD in young children. These vaccines contain purified capsular polysaccharides chemically linked to protein carriers. The protein carrier serves to increase the efficacy of the vaccine in young children, by improving the quality of the immune response elicited by these vaccines. Conjugate vaccines induce T-cell dependent immunity in young children which leads to effective antibody production and immunologic memory (Stein 1992).

PCV7 contains capsular polysaccharide from pediatric serotypes 4, 6B, 9V, 14, 18C, 19F and 23F (Hausdorff *et al.* 2000b). These serotypes are responsible for more than 80% of severe infection in young children in the United States and Canada. However, due to epidemiological differences, these serotypes cause less disease in many

developing countries (Sniadack *et al.* 1995). PCV7 has been part of the routine childhood immunization programme in the USA since 2000 (Centers for Disease Control and Prevention 2001). Studies assessing the impact of PCV7 in the United States have shown significant reductions in the number of invasive infections due to vaccine serotypes among vaccine eligible children in the USA (Whitney 2005). Reduction among older children and adults have been reported, suggesting an indirect benefit from herd immunity through reduced transmission of vaccine serotype strains from the vaccinated group (Frazao *et al.* 2005; Pai *et al.* 2005; Hausdorff *et al.* 2005). Although the benefits of PCV7 have been demonstrated in the USA, cost constraints are limiting its use in many developing countries, including South Africa where the vaccine is currently only available in the private sector. PCV9 includes additional serotypes 1 and 5, and PCV11 contains PVC9 serotypes and includes serotypes 3 and 7F (Hausdorff *et al.* 2005). Serotype 1 and 5 are more prevalent in developing countries than in industrialised countries and PVC9 is therefore expected to provide improved protection in children in developing countries (Hausdorff *et al.* 2000b). Clinical trials conducted in South Africa have demonstrated reduction in carriage and IPD caused by vaccine serotypes in both HIV-positive and HIV-negative children who received PCV9 (Mbelle *et al.* 1999; Klugman *et al.* 2003). In addition, in South Africa a significant reduction in carriage rates and IPD caused by penicillin-resistant and co-trimoxazole-resistant pneumococci were observed in vaccinated children compared to control children (Mbelle *et al.* 1999; Klugman *et al.* 2003).

1.5 Typing methods

Methods used for typing of pneumococci include both phenotypic and genotypic (molecular) procedures. Phenotypic methods used for typing of pneumococci are based on capsular reaction with antisera (Austrian 1976; Sorensen 1993). Molecular typing methods are discriminatory enough to subtype or discriminate between strains characterized by phenotypic methods such as the quellung test. This process of subtyping is important epidemiologically for the following reasons: recognizing outbreaks of disease, detecting cross transmission of nosocomial pathogens, determining the source of infection, recognizing particularly virulent strains and monitoring vaccination programs (Olive & Bean 1999).

1.5.1 Phenotypic typing methods

The method classically used for serotyping of pneumococci is the capsular reaction test known as the quellung reaction. The clinical application of the quellung reaction in serotyping pneumococci was first described in 1931 and is still the most widely used method for serotyping of pneumococci (Austrian 1976). The quellung is a serological test that relies on swelling of the capsule upon binding of homologous antibody. The test consists of mixing a loopful of colonies with an equal quantity of type specific antiserum and then examining microscopically for capsular swelling (Austrian 1976). Using this method, 46 serogroups which are further divided into 90 distinct serotypes have been identified so far (Henrichsen 1995). Although generally highly specific, cross-reactivity has been observed between certain capsular types (Austrian 1976). Modification of this

method includes the chessboard typing system that has been modified to allow typing/or grouping of capsular types commonly isolated from blood or CSF (Sorensen 1993).

1.5.2 Genotypic typing methods

Molecular characterization of pneumococci addresses two kinds of epidemiological problems. Firstly, to investigate whether isolates recovered from a localised outbreak of disease are the same or different strains (short term or local epidemiology) or whether isolates causing disease in a certain geographic area are the same as those causing disease from other areas (global epidemiology). For local epidemiology, typing methods are used that identify variations within the chromosomal DNA that accumulate rapidly within a bacterial population (Maiden *et al.* 1998). Such methods include pulsed-field gel electrophoresis (PFGE) and polymerase chain reaction (PCR) with repetitive primers or arbitrary primers. For global epidemiology, variations that accumulate very slowly within a bacterial population and are likely to be selectively neutral are used (Enright & Spratt 1998). Examples of such methods are multilocus enzyme electrophoresis (MLEE) and multilocus sequence typing (MLST).

Several PCR based methods for serotyping pneumococci have been developed (Brito *et al.* 2003; Moreno *et al.* 2005; O'halloran & Cafferkey 2005). These multiplex PCR assays are designed to target important serotypes such as those present in the conjugate vaccines. Compared to conventional typing methods (e.g quellung reaction), these methods provide a fast and cost-effective way of analysing large numbers of samples.

The random amplified polymorphic DNA assay (RAPD), also referred to as arbitrarily primed PCR, is a DNA fingerprinting assay based on the use of short random primers (Olive & Bean 1999). The primers hybridize with sufficient affinity to chromosomal DNA sequences at low annealing temperatures such that they can be used to initiate amplification of regions of the genome. The number and location of these random regions differs for different strains within a bacterial species. Thus, separation of amplification products by agarose gel electrophoresis, results in a pattern of bands, which is specific for a given bacterial strain. A number of studies have reported success in using RAPD to distinguish bacterial strains within a species. Major limitations of this assay are its lack of reproducibility and difficulty in standardizing the assay.

Repetitive extragenic palindrome is a fingerprinting technique based on amplification of regions of repetitive DNA elements present within the bacterial genome. For *S. pneumoniae*, BOX elelements which are located within intergenic regions of the genome are amplified (van Belkum A. *et al.* 1996). BOX elements are mosaic repetitive elements composed of various combinations of three subunits referred to as BOXA, BOXB, and BOXC (van Belkum A. *et al.* 1996; Olive & Bean 1999). BOX fingerprinting is highly discriminatory, easy to perform and can therefore be used to analyse a large collection of pneumococcal strains (Hermans *et al.* 1995). This method is however, less discriminatory than pulsed-field gel electrophoresis (PFGE) (Hermans *et al.* 1995).

Restriction fragment length polymorphism (RFLP) refers to the polymorphic nature of the location of restriction enzyme sites within defined regions of the bacterial

chromosome. In this method, chromosomal DNA is digested with a restriction enzyme (e.g. *Eco*RI) and the fragments are separated by electrophoresis through polyacrylamide gels (Hermans *et al.* 1995). The differentiation of bacterial strains is based on the observation that the locations of various restriction enzyme recognition sites within a particular genetic locus of interest can be polymorphic from strain to strain, resulting in bands that differ in size between different strains. This method can provide information about evolutionary relationships of strains examined, and include methods such as PFGE. PFGE is considered to be the 'gold standard' of molecular typing methods for bacterial organisms such as *Streptococcus pneumoniae* (Tenover *et al.* 1995; Lefevre *et al.* 1993). It is an ideal and the most used method for determining relationships between outbreak isolates or isolates collected within a similar time period from the same geographic area (i.e. short term or localized epidemiology). PFGE involves endonuclease restriction of whole chromosomal DNA embedded in agarose plugs. The digested DNA is then electrophoresed in an agarose gel and the polarity of the current is changed at predetermined time intervals (pulsed times) (Lefevre *et al.* 1993). The pulsed field allows clear separation of very large DNA fragments ranging from 10 to 800 kb (Olive & Bean 1999). Although laborious, PFGE is highly discriminatory and has been proven to be superior to several other molecular typing methods (Olive & Bean 1999; Kuhn *et al.* 1995).

For long term epidemiology, MLEE is a more appropriate method. MLEE characterizes relationships between isolates by comparing the electrophoretic mobilities of enzymes encoded by multiple housekeeping genes (Selander *et al.* 1986). This method is suitable

for long term epidemiology since variation within housekeeping genes accumulate very slowly within a population and are likely to be selectively neutral (Maiden *et al.* 1998; Selander *et al.* 1986). A major limitation of MLEE is that results from different laboratories cannot be compared. To overcome this limitation, MLST was proposed in 1998 as a general approach to provide accurate, portable data that is appropriate for the global epidemiological investigation of bacterial pathogens and which also reflected their evolutionary and population biology (Maiden *et al.* 1998). MLST is based on the same principle as MLEE but characterizes bacterial isolates according to alleles present at multiple housekeeping genes, directly by nucleotide sequencing rather than indirectly from the electrophoretic mobilities of their gene product. All unique sequences for a given locus are assigned allele numbers via the MLST website (Multilocus sequence typing 2007b). The alleles present at each of the MLST loci for a given isolate are combined into an allelic profile and are assigned a sequence type (ST). Relationships among isolates are apparent by comparison of allelic profiles; closely related isolates have identical STs, or STs that differ at one or two loci, whereas unrelated isolates differ at three or more of the seven loci (Maiden *et al.* 1998). The ability to characterize isolates by comparing their allelic profiles via the internet with those available in the MLST database provides MLST with an unambiguous approach that has great advantages over MLEE and other typing methods. Since 2003 MLST has been used to characterize strains causing IPD in order to gain a better understanding of the relationship between different serotypes causing disease (Enright & Spratt 1998). Since the establishment of the pneumococcal MLST scheme and the MLST database, MLST is now widely used for the global epidemiology of *S. pneumoniae*.

1.6 Epidemiology

Although a total of 90 pneumococcal serotypes have been identified, only 15 to 20 serotypes are responsible for the majority of disease (Hausdorff *et al.* 2000a; Shouval *et al.* 2006). Moreover, the frequency of carriage and disease caused by individual serotypes varies by age, season and geographic area (Brueggemann *et al.* 2004; Scott *et al.* 1996). Serotypes 1 and 5 are infrequently carried and are both significant causes of disease in developing countries, more so than in industrialized countries (Sniadack *et al.* 1995). Serotypes included in the PCV7 are significantly associated with carriage and antimicrobial resistance (Doern *et al.* 1998; Hausdorff *et al.* 2005; Koornhof *et al.* 1992). These serotypes also cause the majority of invasive disease in young children, particularly in the United States (Hausdorff *et al.* 2000b).

Pneumococcal resistance to antimicrobials, including the commonly used penicillins, macrolides and co-trimoxazole, has been increasing in many countries since the late 1960's, with many isolates now resistant to multiple antibiotics (Memish *et al.* 2004; Breiman *et al.* 1994; Ridgway *et al.* 1995). Nasopharyngeal carriage has been shown to be an important reservoir for antibiotic resistance (Lauderdale *et al.* 2006). Serogroups 6, 9, 14, 19 and 23 are often associated with antimicrobial resistance and are also frequently isolated during carriage studies (Breiman *et al.* 1994; Hausdorff *et al.* 2000b; Memish *et al.* 2004). Resistant strains are easily transmitted from person to person in environments where antibiotic use is frequent e.g. day care centres, and this often leads to the selection of resistant strains. Several studies have demonstrated the association between day care attendance and increased carriage of resistant strains (Chiu *et al.* 2001;

Frazao *et al.* 2005; Yagupsky *et al.* 1998). Higher rates of resistant pneumococci have also been reported among HIV-positive adult patients compared to HIV-negative adult patients (Crewe-Brown *et al.* 1997; Karstaedt *et al.* 2001).

In 1997 the Pneumococcal Molecular Epidemiology Network (PMEN) was established under the auspices of the International Union of Microbiological Societies (IUMS) (McGee *et al.* 2001). The Network was established with the aim of global surveillance of antibiotic resistant *S. pneumoniae* and the standardization of nomenclature and classification of resistant clones. There are currently 26 clones described by the PMEN, including two clones that were first identified in South Africa (clones South Africa^{19A}-7 and South Africa^{19A}-13) (Multilocus Sequence Typing, 2007e). These are important national and international clones that have contributed to the rapid increase in resistance in pneumococci worldwide.

S. pneumoniae is naturally transformable and a single serotype can include a number of genetically divergent clones due to horizontal gene transfer. Previous studies have demonstrated that different clones within a given pneumococcal serotype have different abilities to cause disease (Brueggemann *et al.* 2003; Sjostrom *et al.* 2006). Also, certain clones such as the multidrug-resistant serotype 23F clone (Spain ^{23F}-1), are important in antimicrobial resistance and have contributed to the global increase of antimicrobial resistance (McGee *et al.* 2001). Discriminating between different serotypes therefore provides limited information on different clones causing pneumococcal disease.

1.6.1 Serotype 3 disease

Serotype 3 is known to be one of the more virulent pneumococcal serotypes commonly associated with invasive disease in older children and adults (Hausdorff *et al.* 2000a), however this serotype has also been shown to cause AOM in young children (Hausdorff *et al.* 2002; Gray *et al.* 1979). In a study by Hausdorff and colleagues, which included data from different geographical areas such as the United States, Canada and Europe, serotype 3 was more often isolated from middle ear fluid of young children with AOM than from specimens from normally sterile sites (Hausdorff *et al.* 2000a). Also, in a study conducted in the Czech republic, serotype 3 was responsible for 15% of AOM in children younger than 2 years of age and was the most frequently isolated serotype (Prymula *et al.* 2004). In the same study, serotype 3 only ranked fifth, accounting for 7.4% of IPD cases. In another study involving analysis of datasets from several countries including South Africa, serotype 3 was an important cause of AOM, more significantly in the <6- and ≥60- months age groups (Hausdorff *et al.* 2002). In a study conducted at Tumeco, Southern Chile, serotype 3 was responsible for 15% of IPD in children 5 years of age and older, but it was never found in IPD cases of children less than 5 years of age (Inostroza *et al.* 2001).

Data from several geographical areas including Spain, Canada, Britain and the United States have demonstrated that IPD due to serotype 3 increased progressively with age, up to a peak in the seventh decade of life (Scott *et al.* 1996). Also, according to a study in the 1950's, in one Hospital in New York, this serotype was responsible for two thirds of all pneumococcal infections in persons 50 years of age or older (Austrian & GOLD

1964). Serotype 3 pneumococci have been shown to be associated with higher case fatality. In a multicenter study that included 5 centres located in Canada, Spain, Sweden, United Kingdom and United States, not only was this serotype found to be one of the most common causes of invasive disease, but it was also associated with high fatality case rate (Henriques *et al.* 2000). A South African study conducted from January 1984 to December 1985, reported serotype 3 as the major cause of intensive care admissions of patients admitted with bacterial pneumonia to Hillbrow Hospital, Johannesburg and these patients had the highest complication rate and mortality (Feldman *et al.* 1988).

A recent study carried out in Stockholm, showed that serotype 3 was more prevalent in cases of invasive disease than in carriage (Sandgren *et al.* 2004). Nasopharyngeal colonization by serotype 3 has been reported in children (Austrian & GOLD 1964; Kronenberg *et al.* 2006; Sandgren *et al.* 2004; Gray *et al.* 1979). Although serotype 3 has been isolated from nasopharyngeal carriage isolates, it has demonstrated low levels of antibiotic resistance over the years (Kasahara *et al.* 2005; Kronenberg *et al.* 2006; Lauderdale *et al.* 2006; Lauderdale *et al.* 2006; Crewe-Brown *et al.* 1997). However, a fatal infection caused by a multidug-resistant serotype 3 isolate from a South African 17-year-old school boy in the mid 1980's highlights the importance of this serotype in South Africa (Lawrenson *et al.* 1988). In the United States, where PCV7 has been introduced, the proportion of AOM cases caused by serotype 3 increased from 3% per year in 1999 before the introduction of PCV7 to 11% per year in 2002, after the introduction of the vaccine (McEllistrem *et al.* 2005).

Like other invasive serotypes, serotype 3 has been found to be clonal, with one clone, ST180 being the global clone that predominates among invasive serotype 3 isolates from a number of places such as United States and Scotland (Beall *et al.* 2006; Clarke *et al.* 2004). However, this major clone was found to be more abundant among carriage isolates than invasive isolates recovered from children in Oxford in the UK (Brueggemann *et al.* 2003).

1.6.2 Serotype 19A disease

In South Africa, the first appearance of pneumococcal resistance to penicillin was reported from Durban in 1977 among patients with bacteremia, meningitis, pneumonia and empyema (Appelbaum *et al.* 1977). During that same year, a pneumococcus that was resistant to multiple antimicrobial agents (including penicillin) was detected at Baragwanath Hospital in Johannesburg (Jacobs *et al.* 1978). The isolate was resistant to penicillin, clindamycin, erythromycin, tetracycline, chloramphenicol and trimethoprim-sulfamethoxazole. In both cases, isolates were identified as serotype 19A strains. In 1997 Smith and Klugman described two clones of serotype 19A that were predominant within South Africa's penicillin-resistant pneumococcal population (Smith & Klugman 1997). Subsequently, serotype 19A continues to be prevalent in South Africa and other parts of the world and is often associated with multidrug resistance (Pai *et al.* 2005). Since the introduction of PCV7 in the USA serotype 19A appears to be emerging as a replacement serotype (Pai *et al.* 2005). The rate of serotype 19A IPD in children less than 5 years old increased significantly from 1999-2000 to 2003-2004 and this was accompanied by significant increases in penicillin nonsusceptibility and multidrug

resistance among serotype 19A isolates (Pai *et al.* 2005). In another study, an increase in proportion of serotype 19A disease was also observed among HIV-positive adults from the USA (Fry *et al.* 2003).

Serotype 19A has also been associated with invasive disease in adults from Denmark, and in children and adults from Egypt (Clarke *et al.* 2004; Martens *et al.* 2004; Wasfy *et al.* 2005). In Italy, together with serotype 19F, serotype 19A was the most common serotype in noninvasive pneumococci with erythromycin and/or penicillin resistance isolated from young children (Marchese *et al.* 2000). In the same study, serotype 19A was the second most common serotype with erythromycin and/or penicillin resistance isolated from invasive specimens of adults. In a study conducted in Portugal this serotype was one of the top ten causes of invasive disease in children and adults (Serrano *et al.* 2005).

Serotype 19A is also a common cause of AOM in children. A multinational study showed that serotype 19A was isolated from up to 11.4% of middle ear fluid specimens of children from several countries including Argentina, France and the United States (Hausdorff *et al.* 2002). An Israeli study also demonstrated a positive association between AOM and serotype 19A (Shouval *et al.* 2006).

1.7 Study objectives

1. To investigate the clonality of South African serotype 3 invasive isolates from January 2000 to December 2003, focusing on Gauteng Province; and to compare their molecular epidemiology to serotype 3 strains described in other parts of the world.
2. To review case fatality rates (cfr) and HIV-serostatus in cases of serotype 3 reported in South Africa from January 2003 to December 2005.
3. To determine if the original 1977 antimicrobial resistant serotype 19A clone is currently circulating among multidrug-resistant serotype 19A strains isolated in South Africa from June 1999 to December 2004.
4. To review cfr and HIV-serostatus in cases of serotype 19A reported in South Africa from January 2003 to December 2005.

CHAPTER 2: MATERIALS AND METHODS

2.1 Bacterial isolates and invasive pneumococcal disease (IPD) cases

Ethical clearance for this project was obtained through the Human Research Ethics Committee (medical), Wits University, Johannesburg (Protocol number M040703).

IPD case data and pneumococcal isolates were selected from those submitted for the surveillance of IPD in South Africa. The South African surveillance for IPD is part of an ongoing laboratory-based surveillance system for invasive disease, coordinated by the Respiratory and Meningeal Pathogens Research Unit (RMPRU) of the National Institute for Communicable Diseases (NICD). From 2003 onwards surveillance was enhanced to include clinical case report forms from all surveillance sites. A case of IPD was defined as the isolation of *S. pneumoniae* from a normally sterile site specimen (e.g. blood culture, cerebrospinal fluid (CSF), pleural fluid or joint fluid) of adult and paediatric patients admitted to hospitals throughout South Africa.

Microbiological analysis was performed by RMPRU staff as part of routine laboratory work. Serotypes of isolates were confirmed by the quellung method using type specific antisera (Statens Serum Institut, Copenhagen) and then stored in 10% skim milk at –70°C. All strains were initially screened for susceptibility to penicillin (using oxacillin disks), tetracycline, chloramphenicol, erythromycin, clindamycin, rifampicin and trimethoprim-sulfamethoxazole (co-trimoxazole) using antibiotic disks supplied by Mast Diagnostics (Mast Group Ltd., Merseyside, UK) and using Clinical and Laboratory

Institute (CLSI) guidelines and break-points (Clinical and Laboratory Institute 2006). Isolates that were non-susceptible on disc screening had minimum inhibitory concentration (MIC) determined using Etest methodology or agar dilutions. Isolates were classified as susceptible or intermediately susceptible (intermediate) or resistant according to CLSI break-points (Clinical and Laboratory Institute 2006). Non-susceptible isolates included those defined as intermediately resistant or resistant. Multdrug-resistant isolates included those that were non-susceptible to penicillin and at least two other agents.

2.1.1 Serotype 3 isolates

A random sample of 102 isolates, selected from those submitted for the surveillance of IPD in Gauteng Province, South Africa, was analysed. Isolates were selected from those recovered from specimens collected from January 2000 to December 2003.

2.1.2 Serotype 19A isolates

Multidrug-resistant serotype 19A isolates submitted from June 1999 to December 2004 were analyzed. The original South African 1977 multidrug-resistant serotype 19A isolate was kindly donated by Linda McDougal of the Centers for Disease Control and Prevention, Atlanta, USA.

2.2 Pulsed-field gel electrophoresis (PFGE)

S. pneumoniae embedded in agarose blocks were prepared using a previously described procedure with some adaptations (Lefevre *et al.* 1993). Bacterial cultures were grown overnight on 5% Columbia blood agar plates (Diagnostic Media Products, National Health Laboratory Service, Johannesburg, South Africa) and incubated at 37^{o}C with 5% CO_2. Half of the bacterial culture was resuspended into 600 µl of cell suspension buffer (appendix A). The bacterial suspension was warmed to 45^{o}C and mixed with an equal volume of 1% SeaKem Gold agarose (SeaKem GTG agarose, Cambrex Bio Science Rockland, Inc. Rockland, ME, USA) melted in cell lysis buffer and cooled to 45^{o}C. The mixture (300 µl) was pipetted into moulds (Bio-Rad Laboratories, California, USA) and allowed to solidify for 10 to 15 minutes. To lyse bacterial cells, plugs were incubated overnight in a water bath at 50^{o}C, in 50 ml tubes containing cell lysis buffer (appendix A). Following cell lysis, plugs were washed twice with distilled water (15 minutes each) and then twice with Tris-EDTA (15 minutes) (appendix C). For bacterial DNA digestion, 2-3 mm slices of agarose plugs were incubated for 15 minutes in 1X buffer A (Roche Diagnostics GmbH, Mannheim, Germany) recommended for Roche *Sma*I restriction enzyme. Each slice was incubated overnight at 37^{o}C, in 200 µl of 1X buffer A with 30 units of *Sma*I. Plugs were loaded onto a comb, placed in a gel casting tray and allowed to air-dry for 15 minutes before pouring 1.1% SeaKem Gold agarose (Appendix A) into the casting tray. PFGE was performed for 21 hours at 6 V/cm and an angle of 120^{o}C using the CHEF DR III electrophoresis system (Bio-Rad Laboratories, California, USA). The following pulse times were used: 1-30 sec for 16 hours and 5-9 sec for 5

hours. The gel was stained for 30 minutes in 0.5X TBE containing 10mg/ml of ethidium bromide. The gel was destained in distilled water for 10-15 minutes. The DNA fingerprint patterns were visualised using the Spectroline UV transluminator (UVP Ltd., California, USA) and the image was captured using the Vacutec gel documentation system (Vacutec, Rooseveld Park, South Africa). PFGE banding patterns were analyzed using the Windows version of GelCompar, version 4.1 (Applied Maths, Kortrijk, Belgium). GelCompar II software includes the cluster analysis functions which involve the calculation of a hierarchical tree-like structure (dendrogram). The dendrogram allows samples to be compared and classified based on clusters produced by comparison of their banding patterns. Comparisons of the banding patterns were performed using the Jaccard similarity coefficient applied to peaks as well as by the use of unweighted pair group method using arithmetic averages (UPGMA) with a position tolerance of 1.5. A cluster was defined as three or more isolates sharing ≥80% similarity on the dendrogram (Gertz, Jr. *et al.* 2003).

2.3 DNA extraction

The method for DNA extraction was modified from a previously described procedure (Smith *et al.* 1993). Pneumococci were cultured on 5% Columbia blood agar plates overnight at 37^{o}C in 5% CO_2. Following re-suspension in 550 µl of Tris-EDTA (TE) (appendix C), bacterial cells were lysed by the addition of 30 µl of 10% sodium dodecyl sulfate (SDS). The lysate was mixed and incubated at 37^{o}C for 60 minutes. This was followed by the addition of 70 µl of 5M NaCl (appendix C) and 60 µl of 10%

CTAB/NaCl (appendix C). The lysate was mixed and incubated at 65^{o}C for 10 minutes. Following the addition of 600 µl of chloroform (Merck Chemicals, Darmstadt, Germany), the tubes were centrifuged at 11269 x gravity (*g*) for 5 minutes. The clear aqueous (top) phase was then transferred to a clean tube and DNA was precipitated by the addition of 0.7 volume of isopropanol (Merck Chemicals, Darmstadt, Germany). DNA was pelleted by centrifugation at 11269 x *g* for 5 minutes, washed with ice-cold 70% ethanol and dried on a 37^{o}C heating block for 30 minutes. The pellet was re-suspended in 70 µl of TE/RNAse (appendix C).

2.4 Polymerase Chain Reaction (PCR)

PCR reagents and primers (Inqaba Biotech, Johannesburg, South Africa) are listed in appendix B. DNA amplification was carried out in a Hybraid OmniGene thermal cycler (Hybraid OmniGene, UK) using the following cycle conditions: 30 cycles of denaturation (94^{o}C for 60 seconds (s)), annealing (50^{o}C for 60s) and extension (72^{o}C for 30s). PCR amplicons were purified using the QIAquick purification kit (Qiagen Inc., CA, USA), according to the manufacturer's instructions.

2.5 Multilocus sequence typing (MLST)

The procedure for MLST was carried out as previously described by Enright and Spratt (Enright & Spratt 1998). Briefly, the nucleotide sequences of ~450-bp internal regions of seven of pneumococcal housekeeping genes were amplified by PCR as described above. DNA strands of the PCR products were then sequenced in the forward and reverse

directions using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, USA), as described in appendix B. Cycle sequencing was carried out in a Hybraid OmniGene thermal cycler using the following cycling conditions: 25 cycles of denaturation (96^{o}C for 60s), annealing (50^{o}C for 60s) and extension (72^{o}C for 30s) followed by a final step of denaturation (95^{o}C for 5 minutes). Following cycle sequencing, the samples were cleaned using the DyeEx 2.0 Spin Kit (Qiagen Inc., CA, USA), according to the manufacturer's instructions. The recovered DNA samples were dried in a speed vacuum concentrator (Lasec Laboratory & Scientific, Johannesburg, South Africa) for 45 minutes. The dried samples were re-suspended in 15 µl of Template Suppression Reagent (TSR) or Hi-Di Formamide (Applied Biosystems, Foster City, CA). The samples were placed in a heating block at 95°C for 2 minutes, chilled on ice for 1 minute. The samples were then analysed on an ABI PRISM 310 automated DNA sequencer (Applied Biosystems, Foster City, CA).

A sequence type (ST) for each isolate was determined via the internet using software available at the MLST website (Multilocus sequence typing 2007b). Sequences were submitted to the MLST website to assign alleles for each of the seven loci. The seven allele numbers (in the following order *aroE, gdh, gki, recP, spi, xpt, and ddl*) were used to define the allelic profile or ST.

2.6 Phylogenetic analysis

Serotype 19A MLSTs were analyzed using the program eBURST. The BURST algorithm first identifies mutually exclusive groups of related genotypes in the population (typically MLST database), and attempts to identify the founding genotype (ST) of each group. The algorithm then predicts the descent from the predicted founding genotype to the other genotypes in the group, displaying the output as a radial diagram, centred on the predicted founding genotype (Multilocus sequence typing 2007d). Analysis was done for single locus variants (SLVs) and double locus variants (DLVs), i.e. isolates that differ from each other by one or two MLST alleles.

2.7 Statistical analysis

Statistical analysis was used to compare IPD cases with available case report forms submitted to RMPRU from 2003 through 2005. Data were analysed by year of specimen collection and age category, namely <15 years of age and ≥15 years of age. HIV association was compared among patients with serotype 3 disease and those with non-serotype 3 disease using the Mantel-Haenszel test. In addition, the case fatality rate was compared among serotype 3 and non-serotype 3 cases. The same analyses were also performed for serotype 19A cases.

CHAPTER 3: RESULTS

3.1 Serotype 3

3.1.1 Demographic results

A total of 9599 cases of IPD were reported during January 2000 to December 2003 from all laboratories participating in the national surveillance and of these, 8851 (92%) had viable isolates available for further testing. The majority of all IPD cases with known HIV results were HIV-positive (1910/2227, 86%). Four hundred and sixteen (4.7%) of the 8851 isolates were identified as serotype 3 and were distributed as follows: 87/1828 (4.6%), 120/2120 (5.7%), 101/2009 (5.0%), and 108/2894 (4.9%) for the years 2000, 2001, 2002 and 2003, respectively. The majority of serotype 3 isolates were recovered from blood cultures (331/416, 80%), followed by CSF (71/416, 17%), pleural fluid (12/416, 3%), and joint fluid (1/416, 0.2%). The ages of the patients were known for 341/416 isolates, and the majority of the isolates with known age (300/341, 88%) were from older children and adults (≥5yrs years of age). HIV status was known for 122 of the 416 cases; the majority (98/122, 80%) of which were HIV-positive.

3.1.2 Antimicrobial susceptibility

The majority of the serotype 3 isolates were susceptible to all of the antimicrobial agents tested (Table 3.1). The highest rate of non-susceptibility was detected for trimethoprim-sulfamethoxazole (19/416, 4.6% isolates), followed by rifampicin (6/416, 1.4% isolates), penicillin (4/416, 1.0% isolates), tetracycline (2/416, 0.5% isolates) and erythromycin (1/416, 0.2% isolates). Resistance to more than one agent was detected in three isolates,

all of which were resistant to penicillin and trimethoprim-sulfamethoxazole. Two of these isolates were also resistant to rifampicin and tetracycline, respectively.

3.1.3 Description of selected isolates and cases

The majority of serotype 3 isolates (303/416, 73%) originated from laboratories within Gauteng Province, of which a random sample of 102 isolates (34%) was selected. Fourteen, 29, 29 and 30 of these isolates were isolated in 2000, 2001, 2002 and 2003, respectively. The majority of the isolates were recovered from blood cultures (99/102, 97%), two were recovered from CSF and one from pleural fluid. Isolates were recovered mainly from specimens of older children and adults (≥5 years of age) (87/102, 85%), and relatively few were from specimens of infants and young children less than 5 years of age (14/102, 14%). The age of one patient was not known. No selection on the basis of antimicrobial susceptibility was made. Only one of the selected isolates showed reduced antimicrobial susceptibility. This isolate was non-susceptible to penicillin (MIC=0.12µg/ml) and trimethoprim-sulfamethoxazole (MIC=4µg/ml).

3.1.4 Molecular Characterization

PFGE analysis of isolates randomly selected from Gauteng Province revealed that the vast majority of these isolates (79/102, 78%) were grouped into four clusters (Cluster-A, -B, -C and -D) (Fig 3.1). The largest, Cluster-A comprised 36/102 (35%) isolates, Cluster-B comprised 16/102 (16%) isolates, Cluster-C comprised 14/102 (14%) isolates and Cluster-D comprised 13/102 (13%) isolates. The proportion of isolates within Cluster-A was 7/14 (50%), 9/29 (31%), 10/29 (34%), 10/30 (33%) for the years 2000,

2001, 2002, and 2003, respectively (Figures 3.2a to 3.2d). The remaining 23 isolates were represented by four small clusters: three of which consisted of three isolates each and one cluster of four isolates; and ten unrelated isolates.

MLST of seven isolates randomly selected from Cluster-A revealed that these isolates belonged to ST458. An isolate from Cluster-B was characterized as novel ST1765. Four isolates randomly selected from Cluster-C were characterized as ST180. Three isolates from Cluster-D were characterized as ST378. Three isolates, each selected from the smaller clusters, were characterised as ST1024 (n=1) and ST458 (n=2). One of the unrelated isolates was characterized as novel ST1799.

3.1.5 Statistical analysis

The observed case fatality rate (cfr) among serotype 3 cases from South Africa did not decrease significantly from 2003 (38%) to 2005 (29%) (P=0.5) (Table 3.2a). Overall, the cfr did not differ significantly for serotype 3 cases (35%) compared with non-serotype 3 cases (28%) (P=0.8) (Table 3.2b). The proportion of HIV-positive serotype 3 cases did not change significantly over the three year period (~92%) (P=0.36) (Table 3.3a). The proportion of HIV-positive cases was similar in both serotype 3 (92%) and non-serotype 3 cases (86%) (P=0.1) (Table 3.3b)

Table 3.1. Antimicrobial susceptibilities for invasive pneumococcal serotype 3 isolates from South Africa, January 2000 to December 2003 (n = 416)

Antimicrobial Agent	**No (%) of isolates**		
	Sensitive	**Intermediate**	**Resistant**
Penicillin	412 (99)	4 (1)	0
Trimethoprim-sulfamethoxazole	397 (95)	12 (3)	7 (2)
Erythromycin	415 (99.8)	0	1 (0.2)
Chloramphenicol	416 (100)	0	0
Tetracycline	414 (99.5)	0	2 (0.5)
Rifampicin	410 (98.6)	1(0.2)	5 (1.2)

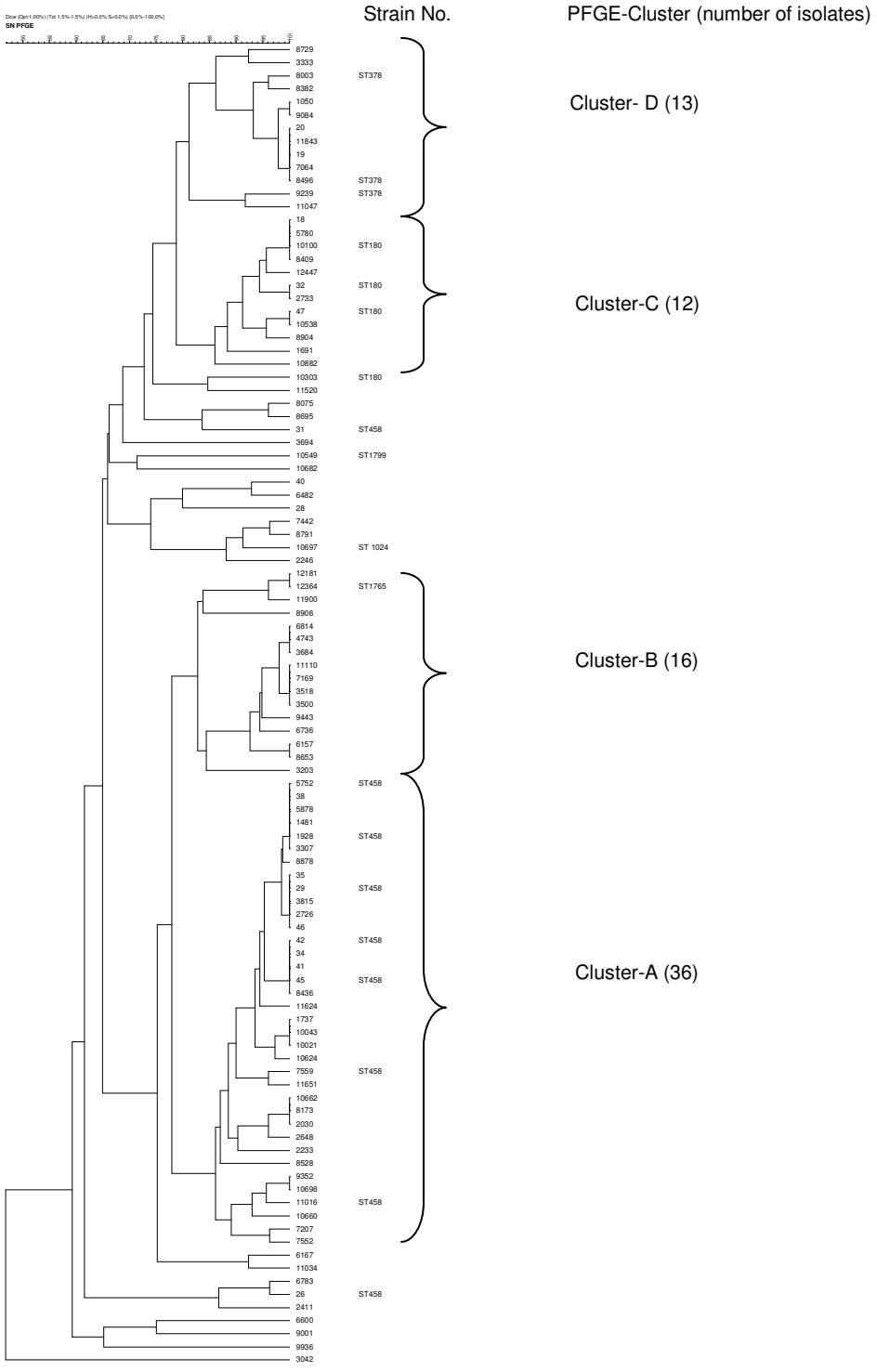

Figure 3.1. PFGE dendrogram of pneumococcal serotype 3 isolates causing invasive disease in Gauteng Province, January 2000 to December 2003

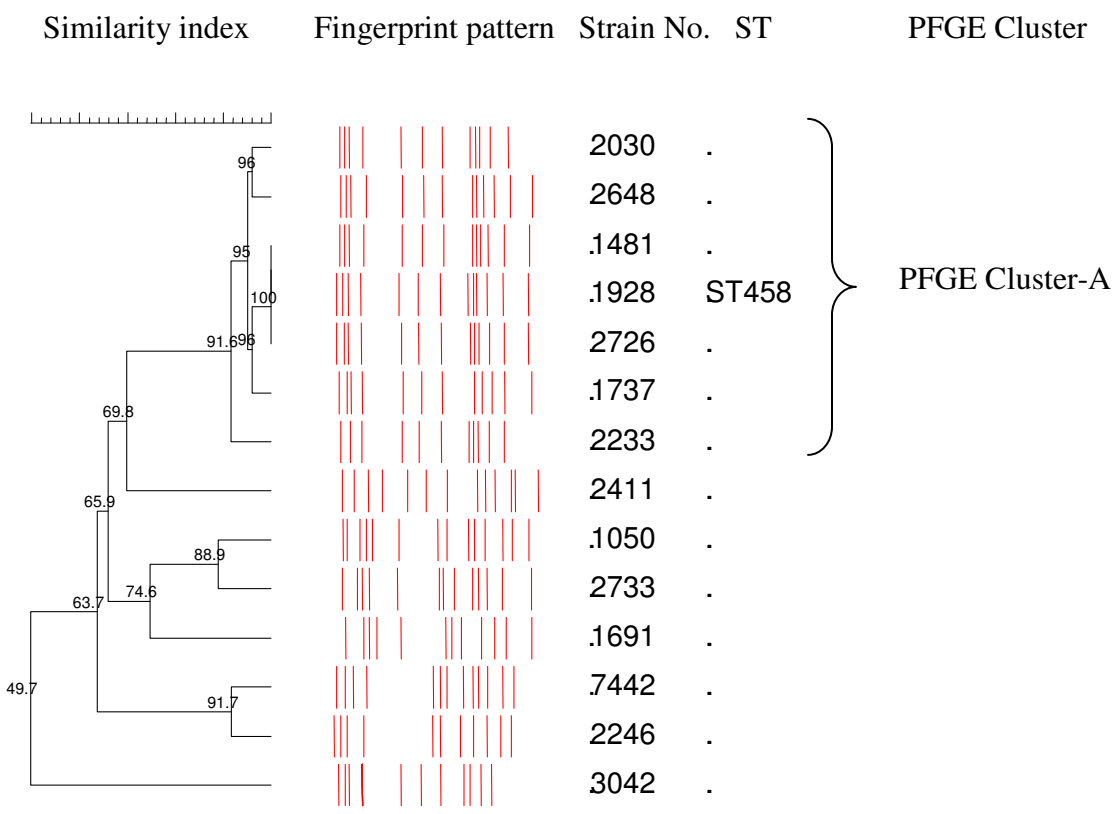

Figure 3.2a. PFGE dendrogram of pneumococcal serotype 3 isolates causing invasive disease in Gauteng Province, January to December 2000, showing MLST associations

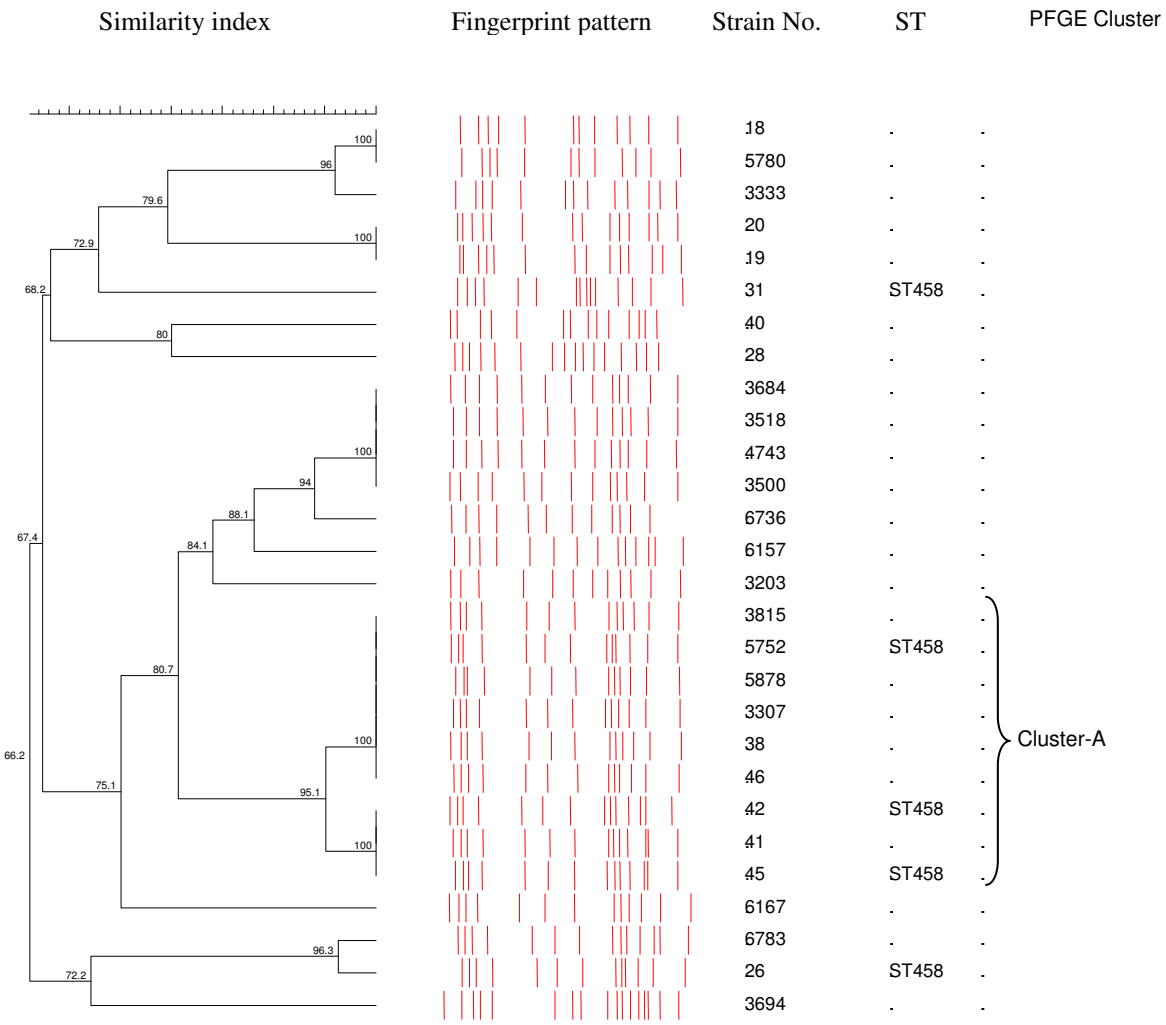

Figure 3.2b. PFGE dendrogram of pneumococcal serotype 3 isolates causing invasive disease in Gauteng Province, January to December 2001, showing MLST associations

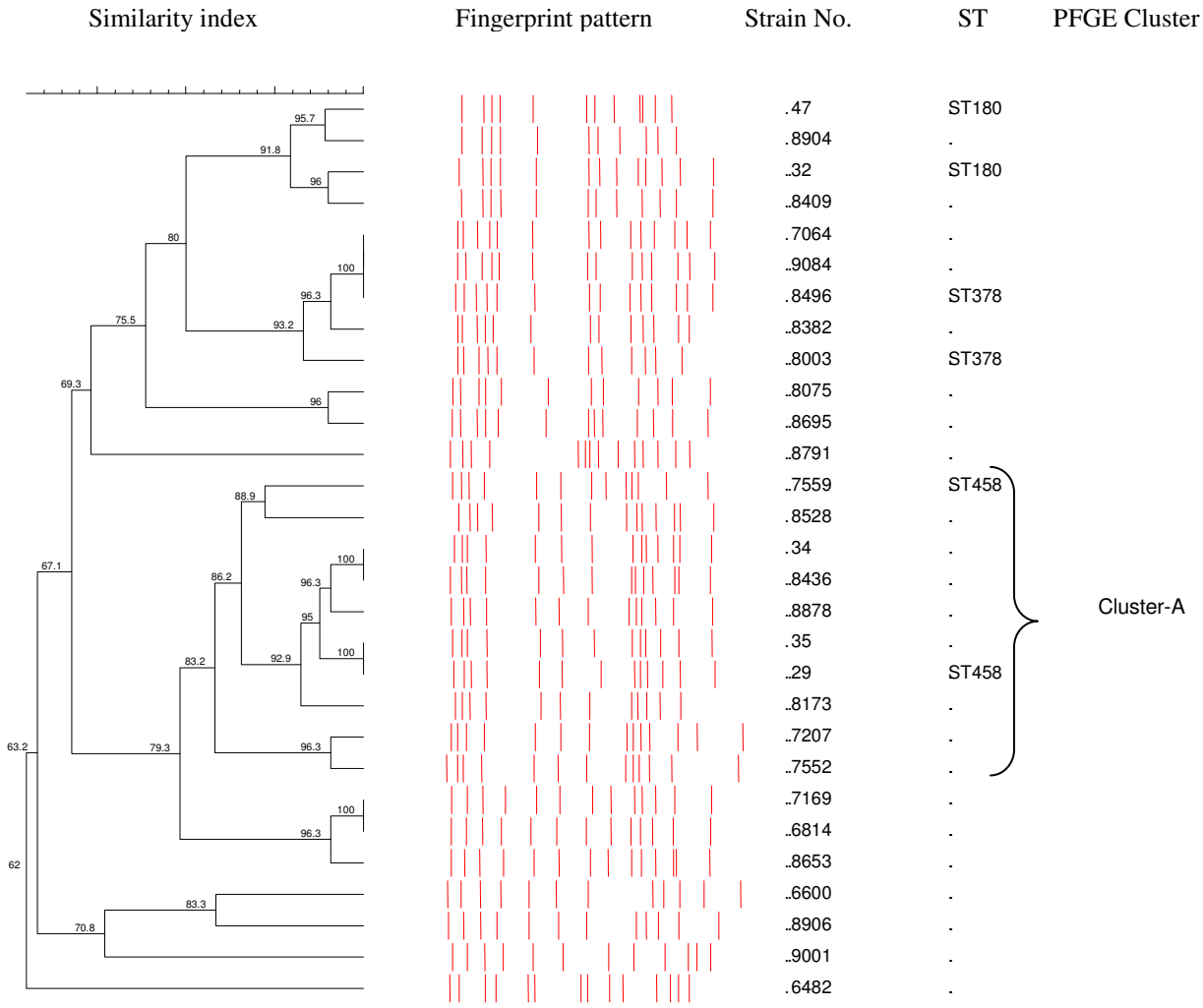

Figure 3.2c. PFGE dendrogram of pneumococcal serotype 3 isolates causing invasive disease in Gauteng Province, January to December 2002, showing MLST associations

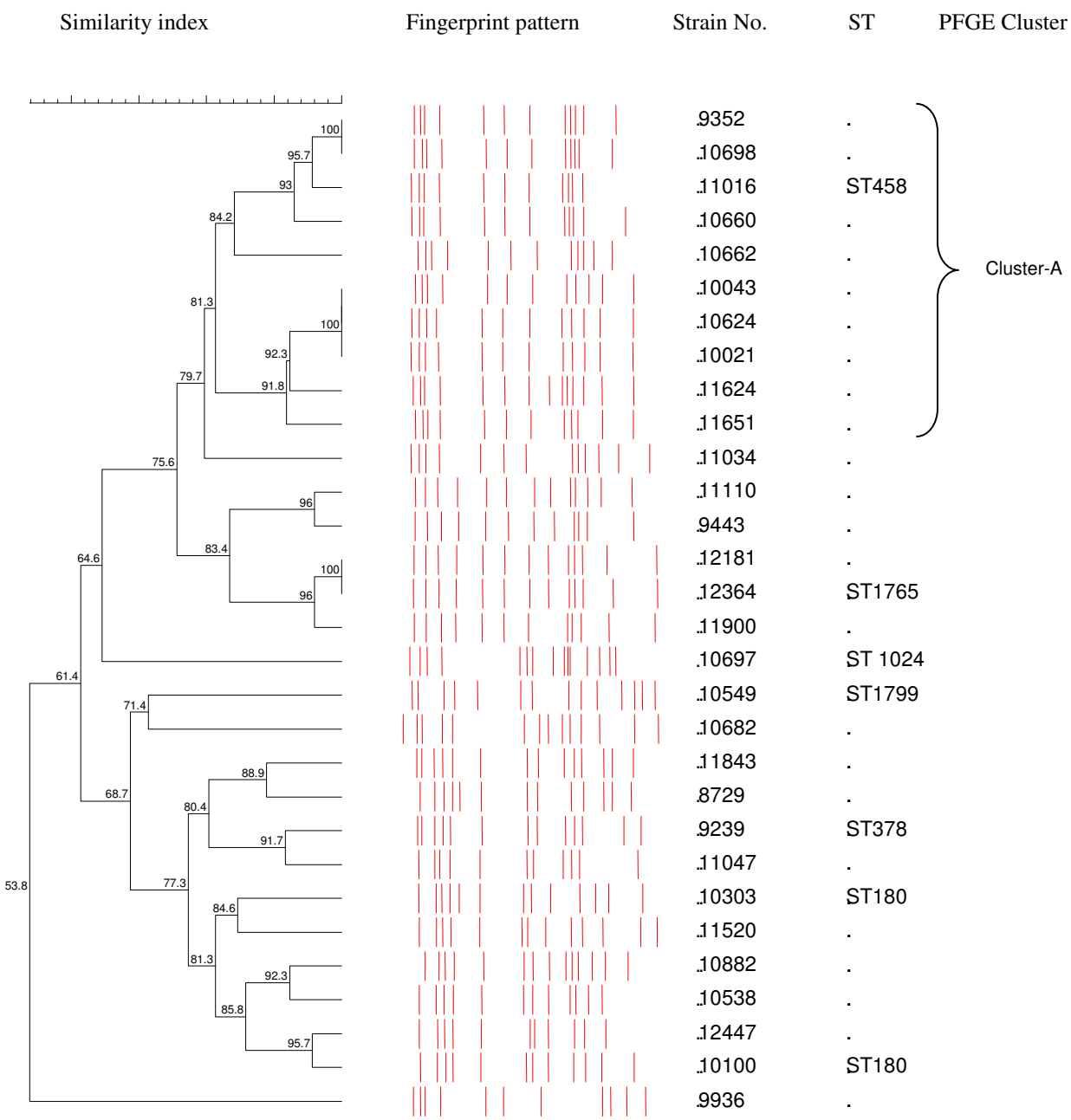

Figure 3.2d. PFGE dendrogram of pneumococcal serotype 3 isolates causing invasive disease in Gauteng Province, January to December 2003, showing MLST associations

Table 3.2a. Case fatality rates for pneumococcal serotype 3 isolates causing invasive disease in South Africa by year of specimen collection (2003-2005) and age category

Collection Year	Case fatality rate (%)	P value
All ages		0.555
2003	18/47 (38.3)	
2004	18/48 (37.5)	
2005	17/58 (29.3)	
<15 years of age		0.571
2003	3/9 (33.3)	
2004	1/7 (14.3)	
2005	2/5 (40.0)	
≥15 years of age		0.351
2003	15/38 (39.5)	
2004	17/41 (41.5)	
2005	15/53 (28.3)	

Table 3.2b. Case fatality rates for pneumococcal serotype 3 and non-serotype 3 isolates causing invasive disease in South Africa, by year of specimen collection (2003-2005) and age category

	Case fatality rate (%)	**P value**
All ages		0.844
Serotype 3	53/153 (34.6)	
non-serotype 3	1188/4208 (28.2)	
<15 years of age		0.595
Serotype 3	6/27 (28.6)	
non-serotype 3	490/2075 (23.6)	
≥15 years of age		0.484
Serotype 3	47/132 (35.6)	
non-serotype 3	694/2125 (32.7)	

Table 3.3a. Proportion of pneumococcal serotype 3 isolates causing invasive disease among HIV-positive patients from South Africa, 2003-2005, by year of specimen collection and age category

Collection Year	Proportion of HIV-positive cases (%)	P-value
All ages		0.357
2003	24/26 (92.3)	
2004	18/21 (85.7)	
2005	29/30 (96.7)	
<15 years of age		0.517
2003	3/3 (100)	
2004	4/5 (80)	
2005	3 /3(100)	
≥15 years of age		0.556
2003	21/23 (91.3)	
2004	14/16 (87.5)	
2005	26/27 (96.3)	

Table 3.3b. Proportion of pneumococcal serotype 3 and non-serotype 3 isolates causing invasive disease among HIV-positive patients from South Africa, 2003-2005, by age category

	Proportion of HIV-positive cases (%)	**P value**
All ages		0.125
Serotype 3	71/77 (92.2)	
non-serotype 3	2211/2925 (86.1)	
<15 years of age		0.397
Serotype 3	10/11 (90.9)	
non-serotype 3	1053/1305 (80.7)	
≥15 years of age		0.828
Serotype 3	61/66 (92.4)	
non-serotype 3	1155/1260 (91.7)	

3.2 Serotype 19A

3.2.1 Demographic characteristics

During the period June 1999 to December 2004, a total of 14 177 cases of invasive pneumococcal disease were reported from all laboratories participating in the national surveillance. Of these cases, 13 064 (92%) viable isolates were available for further testing. Seven of the isolates were nontypable and 885 (7%) were identified as serotype 19A. Age was known for 763 of the 885 serotype 19A cases and the majority of the isolates (450/763, 60%) were from specimens of older children and adults (≥5 years of age). HIV-serostatus was known for 301 of the serotype 19A cases, the majority of which (90%, 270/301), were positive. The majority of the serotype 19A strains (668/885, 75%) were non-susceptible to at least one of the antimicrobial agents tested.

Penicillin-nonsusceptibility was detected in 204 (27%) of the 763 serotype 19A cases with known ages. These penicillin non-susceptible serotype 19A cases were distributed according to age as follows: 120 (27%) of 450 older children and adults (≥5yrs) and 84 (27%) of 313 infants and young children (<5yrs). Twenty five penicillin-nonsusceptible isolates were also nonsusceptible to at least two other agents and were therefore regarded as multidrug-resistant. Trimethoprim-sulfamethoxazole non-susceptibility was detected in 18 of the 25 multidrug-resistant isolates. Age was known for 23/25 multidrug-resistant isolates, 15 of which were from older children and adults (≥5yrs) (Table 3.4). The majority of the multidrug-resistant serotype 19A isolates were from Gauteng Province (12/25, 48%) and KwaZulu-Natal (7/25, 28%). Four isolates were

from the Western Cape and the remaining two isolates were from Limpopo and Eastern Cape, respectively.

3.2.2 Molecular Characterization

PFGE was performed on all 25 multidrug-resistant serotype 19A strains as well as the original 1977 multidrug-resistant strain. These isolates were grouped into three PFGE clusters (Cluster-1, -2 and -3) and 13 outliers (unrelated isolates) (Fig. 3.3). Two of the clusters (-1 and -2) comprised five isolates each and Cluster-3 comprised three isolates. Isolates belonging to the same cluster displayed similar antibiotic resistance profiles. Thirteen of the 25 isolates and the original 1977 strain (BAA-475) did not fall within any of these clusters and were unrelated. A closer look at the DNA fingerprint patterns of all isolates revealed that for two isolates in cluster-3 (isolates 11364 and 10023), some of the smaller DNA fragments were not visible towards the bottom end of the gel. The two isolates had almost identical DNA fingerprint patterns (Fig 3.3). Therefore, it is highly likely that these isolates are more closely related than is depicted on the dendrogram. This is supported by their identical MLST profiles and antibiograms.

MLST was performed on 16 randomly selected isolates from the three clusters and the 13 outliers. A total of 13 sequence types (STs) were identified for these isolates (Table 3.5). Two isolates from Cluster-1 were both identified as novel sequence type ST2062. One isolate selected from Cluster-2 was characterized as ST172. Two isolates from Cluster-3 were characterized as the same novel ST2058. Isolates selected from the outliers were characterized as ST361 (one isolate), ST81 (two isolates), ST1467 (one

isolate) and six novel ST's (one isolate each): ST2074, ST2060, ST2057, ST2059, ST2073, and ST2061. None of the isolates that were characterized by MLST shared the same MLST profile as the original 1977 serotype 19A strain, which was characterized as ST1608.

The eBURST analysis showed that ST2057 and ST2062 are SLVs of each other, forming a group of four STs whose predicted founder is ST2062. ST1608, ST2059, ST2060, and ST2061 each have one SLV in the MLST database. Both ST1467 and ST2058 have four SLVs. ST2073 and ST2074 have neither SLVs nor DLVs. ST81 is a predicted founder of a group of 43 STs. ST172 is part of a group of 124 STs and a predicted founder of a subgroup of eight STs, all connected by SLVs (figure 3.4). The SLVs in this subgroup include ST361 which was identified among isolates from the current study (Multilocus sequence typing 2007a). PFGE analysis showed that although this isolate did not fall in any of the groups, it was more closely related to cluster-2 isolates (one of which was ST172) than to any other isolates (Fig 3.3). Moreover, isolates 15660 (ST172) and 17229 (ST361) share the same antibiogram and were both isolated in the same year (2004).

3.2.3 Statistical analysis

Statistical analysis was used to compare IPD cases reported from 2003 through 2005 throughout South Africa irrespective of antibiogram and with available case report forms. The observed cfr among serotype 19A cases remained constant (~26%) from 2003 to 2005 (P= 0.9) (Table 3.6a). The cfr among serotype 19A cases (26%) was not

significantly higher compared with non-serotype19A cases (21%) (P=0.3) (Table 3.6b). Similarly, the proportion of HIV-positive serotype 19A cases remained constant over the three year period (~89%) (Table 3.7a). The cfr for HIV-infected serotype 19A cases (88%) did not differ significantly from HIV-positive non-serotype19A cases (86%) (P=0.5) (Table 3.7b).

Table 3.4 Antimicrobial profile and age distribution of multidrug-resistant serotype 19A strains causing invasive disease in South Africa, June 1999-December 2004

Antimicrobial profile	**No. of isolates showing reduced antimicrobial susceptibility**	
	<5yrs	**≥5yrs**
PRM	4	6
PEM		1
PTM		1
PTEM		1
PTEL[a]	2	4
PTELM	1	2
PCTRM[a]	1	
Total	8	15

Abbreviations: P, penicillin G; R, rifampicin; M, trimethoprim-sulfamethoxazole; T, tetracycline; E, erythromycin; L, clindamycin; C, chloramphenicol

[a] age of cases with isolates showing this profile was not known

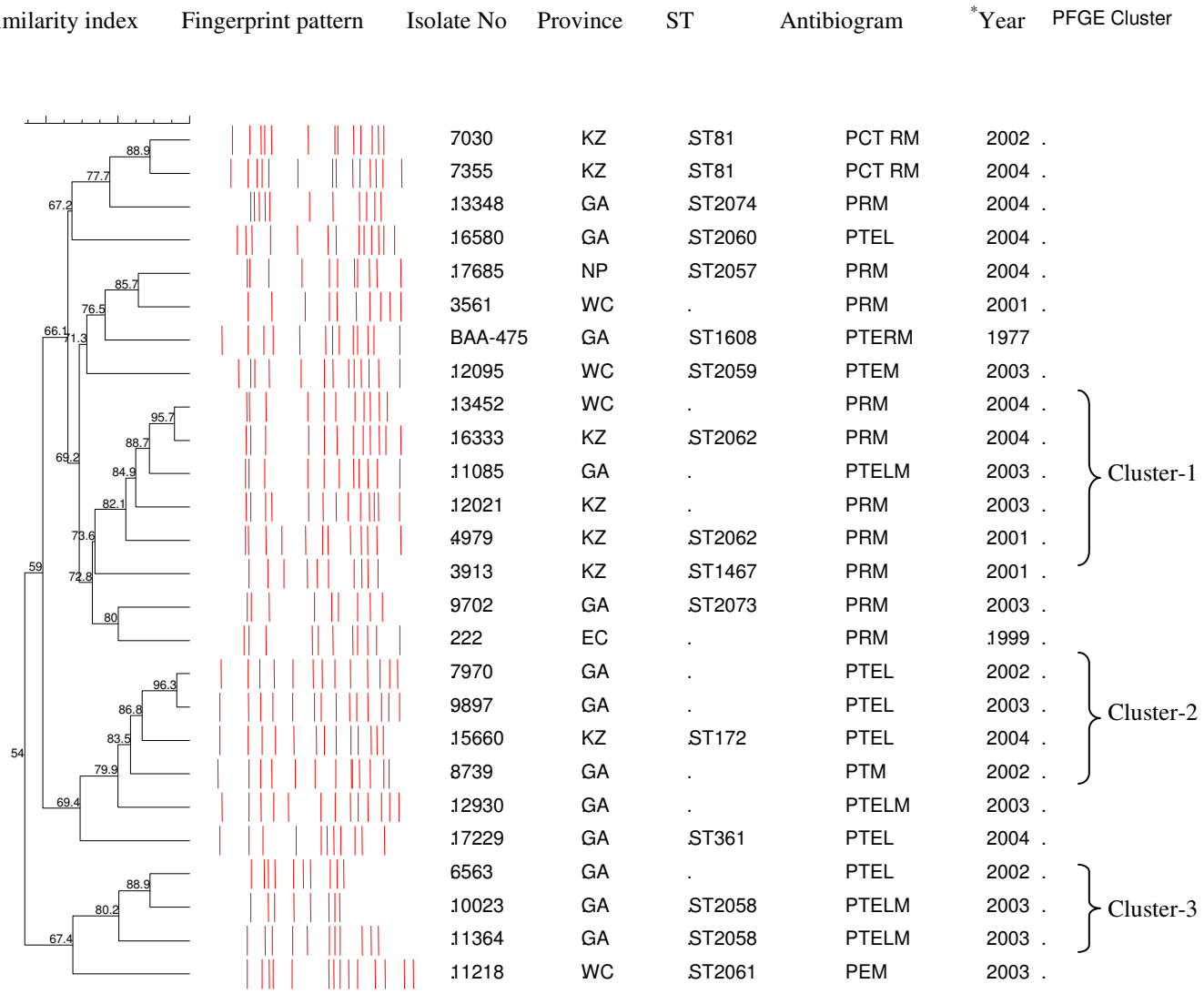

Figure 3.3 PFGE dendrogram of multidrug-resistant pneumococcal serotype 19A strains causing invasive disease in South Africa, June 1999-December 2004

*Year of specimen collection

Abbreviations: WC, Western Cape; KZ, KwaZulu-Natal; GA, Gauteng; EC, Eastern Cape; NP, Limpopo (previously called Northern Province).

P, penicillin G; R, rifampicin; M, trimethoprim-sulfamethoxazole; T, tetracycline; E, erythromycin; L, clindamycin; C, chloramphenicol

Table 3.5 Allelic Profiles and Sequence Types (STs) of the original 1977 strain and isolates randomly selected from multidrug-resistant serotype 19A isolates causing invasive disease in South Africa, June 1999 to December 2004

Isolate	PFGE cluster	Allelic profile							ST
		aroE	gdh	gki	recP	spi	xpt	ddl	
15660	Cluster-2	7	13	8	6	25	6	8	172
17229	unrelated	7	13	8	6	6	6	8	361
7030	unrelated	4	4	2	4	4	1	1	81
7355	unrelated	4	4	2	4	4	1	1	81
3913	unrelated	4	16	19	15	14	20	26	1467
BAA-475 *	unrelated	10	11	4	1	6	149	5	1608
3561	unrelated	1	5	53	32	14	20	5	2057
10023	Cluster-3	12	19	2	17	6	22	199	2058
11364	Cluster-3	12	19	2	17	6	22	199	2058
12095	unrelated	15	29	4	21	30	27	1	2059
16580	unrelated	2	5	36	32	6	21	14	2060
11218	unrelated	8	13	14	32	4	4	14	2061
4979	Cluster-1	1	5	53	32	14	20	199	2062
16333	Cluster-1	1	5	53	32	14	20	199	2062
9702	unrelated	1	10	53	32	6	20	39	2073
13348	unrelated	1	5	14	1	6	20	15	2074

* The 1977 multidrug-resistant strain. **Abbreviations:** *aro E*, shikimate dehydrogenase; *gdh*, glucose-6-phospate dehydrogenase; *gki*, glucose kinase; *recP*, transketolase; *spi*, signal peptidase I; *xpt*, xanthine phosphoribosyltransferase; *ddl*, D-alanine-Dalanine ligase.

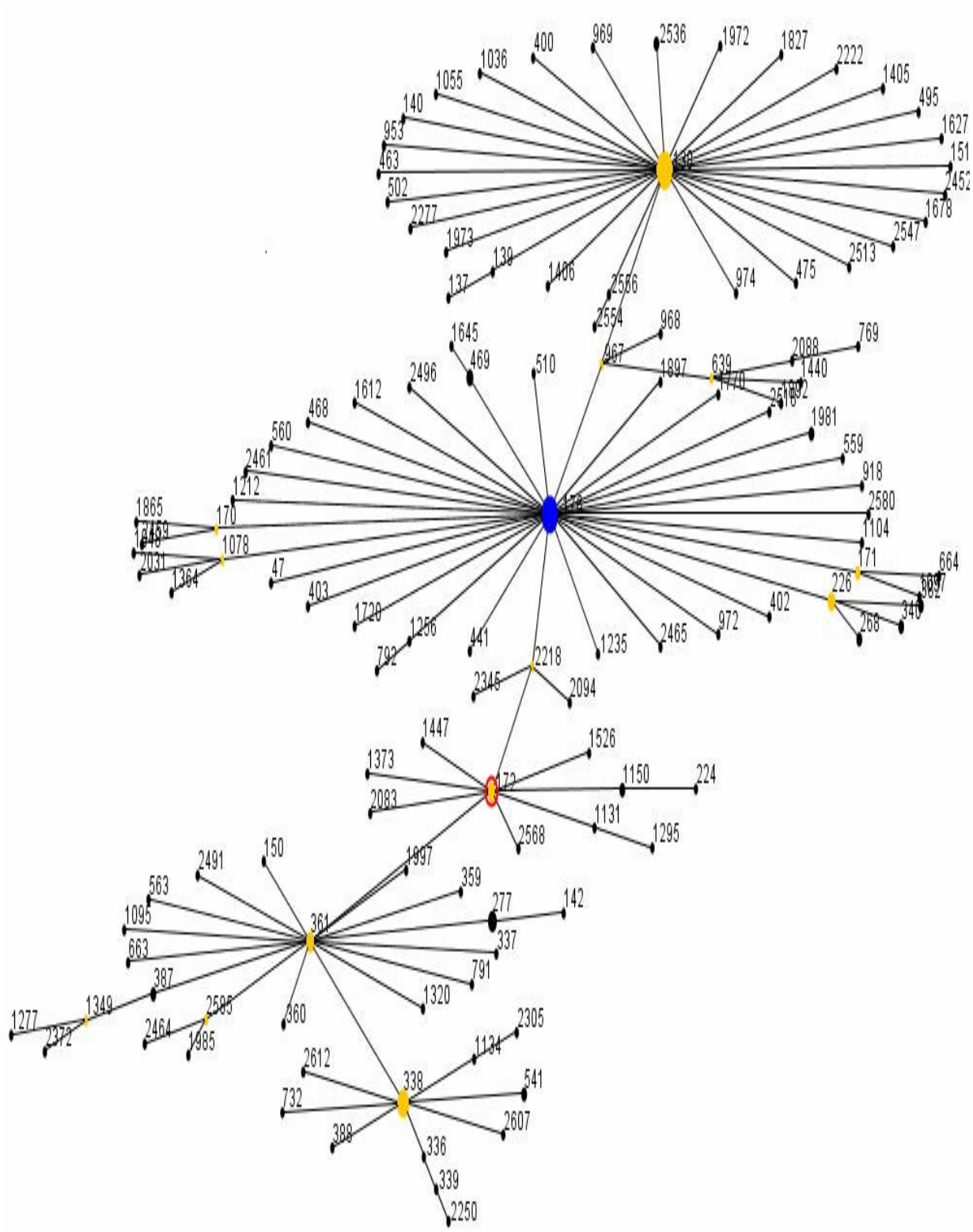

Figure 3.4. eBURST diagram showing relationship between pneumococcal ST361, ST172 and other STs (Multilocus sequence typing 2007a)

Table 3.6a. Case fatality rates for pneumococcal serotype 19A isolates causing invasive disease in South Africa, by year of specimen collection (2003 - 2005) and age category

Collection Year	Case fatality rate (%)	P-value
All ages		0.929
2003	21/78 (26.9)	
2004	29/108 (26.9)	
2005	35/140 (25.0)	
<15 years of age		0.623
2003	10/41 (24.4)	
2004	8/48 (16.7)	
2005	12/66 (18.2)	
≥ 15 years of age		0.760
2003	11/37 (29.7)	
2004	22/61 (36.1)	
2005	23/54 (31.1)	

Table 3.6b. Case fatality rates for pneumococcal serotype 19A and non-serotype19A isolates causing invasive disease in South Africa (2003-2005), by age category

Age Category	Case fatality rate (%)	P value
All ages		0.322
19A	85/326 (26.1)	
non-serotype19A	1156/4035 (21.6)	
<15 years of age		0.143
19A	29/154 (18.8)	
non-serotype19A	467/3346 (24.0)	
≥15 years of age		0.847
19A	55/171 (32.2)	
non-serotype19A	686/2086 (32.9)	

Table 3.7a. Proportion of pneumococcal serotype 19A isolates causing invasive disease among HIV-positive patients from South Africa, by year of specimen collection (2003-2005) and age category

Collection Year	Proportion of HIV-positive cases (%)	P value
All ages		0.229
2003	50/53 (94.3)	
2004	67/77 (87.0)	
2005	78/92 (84.8)	
<15 years of age		0.404
2003	24/27 (88.9)	
2004	32/39 (82.1)	
2005	39/51 (76.5)	
≥15 years of age		0.346
2003	26/26 (100)	
2004	35/38 (92.1)	
2005	39/41 (95.1)	

Table 3.7b. Proportion of pneumococcal serotype 19A and non-serotype19A isolates causing invasive disease among HIV-positive patients from South Africa (2003 – 2005), by age category

Age Category	Proportion of HIV-positive cases (%)	P value
All ages		0.480
19A	195/222 (87.8)	
non-serotype19A	2087/2423 (86.1)	
<15 years of age		0.904
19A	95/117 (81.2)	
non-serotype19A	968/1199 (80.7)	
≥15 years of age		0.171
19A	100105 (95.2)	
non-serotype19A	1116/1131 (91.4)	

CHAPTER 4: DISCUSSION

4.1 Serotype 3

Globally, pneumococcal serotype 3 is more frequently associated with invasive disease in older children and adults and less commonly isolated from invasive specimens of infants and young children (Gray *et al.* 1979; Hausdorff *et al.* 2005; Scott *et al.* 1996). Although not always the case, some studies have shown that serotype 3 is associated with increased mortality (Gransden *et al.* 1985; Hausdorff *et al.* 2002; Martens *et al.* 2004). Serotype 3 isolates were associated with a mortality rate of 50% in adults with bacteremic pneumonia in Denmark (Martens *et al.* 2004). Similarly, a 15-year study of patients diagnosed with pneumococcal bacteremia at a hospital in the UK described serotype 3 as being the most common invasive serotype in adults, and 44% (17/39) of these adults died (Gransden *et al.* 1985). A study of 354 blood isolates from five countries noted a higher case fatality rate in Spain and the USA and showed a predominance of serotype 3 isolates among patients (Henriques *et al.* 2000). Statistical analysis of IPD case data submitted to RMPRU from 2003 to 2005 showed that serotype 3 was not associated with a significantly higher case fatality rate.

Two independent studies in South Africa have demonstrated that the burden of pneumococcal disease in South Africa is higher among HIV-positive children and adults compared to their HIV-negative counterparts (Feldman *et al.* 1988; Jones *et al.* 1998). HIV-positive adult patients from both studies were more likely to be infected with serotypes that are more commonly found in children. In addition, infection due to serotype 3 was not increased in HIV-positive adults compared to

HIV-negative patients. Similarly, the current study showed that for the years 2003 through 2005 serotype 3 was not significantly more associated with HIV than other serotypes. Previous studies have showed that invasive serotypes (including serotype 3) usually have lower rates of antimicrobial resistance (Byington *et al.* 2005; Hausdorff *et al.* 2005; Mantese *et al.* 2003). The majority of South African isolates displayed a similar trend by showing susceptibility to the antimicrobial agents tested.

Although serotype 3 is frequently isolated from invasive specimens, it has also been identified among carriage isolates (Brueggemann *et al.* 2003). In addition to capsular serotype, clonal properties may be an important factor in the ability of pneumococci to cause disease (Feldman *et al.* 1988; Gertz, Jr. *et al.* 2003). It has however, previously been suggested that most invasive isolates belong to genotypes that are common to both carriage and disease (Gertz, Jr. *et al.* 2003). ST180 is an example of a serotype 3 clone that has been reported to be one of the major clones associated with invasive serotype 3 disease and is also the major clone in carriage isolates (Beall *et al.* 2006; Henriques *et al.* 2000; Jones *et al.* 1998). A study in the USA demonstrated that nearly all invasive serotype 3 isolates collected in 1999 to 2002 from that country were ST180 (Beall *et al.* 2006).

Although the importance of ST180 in IPD has been established, little is known about the molecular epidemiology of pneumococcal isolates from developing countries. Since only 12% (12/102) of the Gauteng isolates examined in this study

were included in PFGE Cluster-C, which also included four isolates that were characterized as ST180, the data suggest that the majority of the invasive serotype 3 isolates from Gauteng are not related to the global ST180. The majority (36/102, 35%) of invasive serotype 3 isolates from Gauteng (cluster-A) included 7 isolates that were characterized as ST458, a clone that is not common in other parts of the world. The strains in Cluster-A are well distributed throughout the four years, suggesting that the higher prevalence of ST458 among the Gauteng isolates is not due to an outbreak caused by this clone during one particular year. Henriques and colleagues previously illustrated the ability of virulent penicillin-susceptible pneumococcal clones to emerge and spread rapidly within a country (Henriques *et al.* 2001). The predominance of isolates within Cluster-A among Gauteng isolates may be a demonstration of the expansion of an antibiotic-susceptible virulent clone of serotype 3. A Ghanaian study showed that all seven serotype 3 isolates collected between 1998 and 2003 from specimens of meningitis patients from two different Ghanaian districts were characterized as ST458 (Leimkugel *et al.* 2005). ST458 has also been found among serotype 3 isolates from Egypt (Multilocus sequence typing 2007c). Together with the findings from the current study, these findings suggest that isolates belonging to ST458 may be more prevalent among serotype 3 isolates from African countries than from other parts of the world.

4.2 Serotype 19A

Childhood serogroups and serotypes such as serogroup 19 are often associated with antibiotic resistance (Hausdorff *et al.* 2000b; Koornhof *et al.* 1992). Karstaedt *et al*. observed an increase in penicillin resistant pneumococci among adult patients admitted to Chris Hani Baragwath Hospital during a decade (July 1986 to June 1987 compared to July 1996 to June 1997) (Karstaedt *et al.* 2001). This increase occurred exclusively among HIV-infected patients and was accompanied by an increase in childhood serotypes and serogroups including serogroup 19. During June 1999 to December 2004 equal percentage (27%) of serotype 19A penicillin-nonsusceptible cases were reported for children and adults patients admitted with IPD in South Africa.

Pneumococci resistant to trimethoprim-sulfamethoxazole are frequently associated with multidrug-resistance (Klugman 1990). Similarly, the majority (72%) of the serotype 19A multidrug-resistant isolates reported to RMPRU from June 1999 to December 2004 were resistant to trimethoprim-sulfamethoxazole. This may, in part, be explained by the increased use of trimethoprim-sulfamethoxazole as a prophylactic to prevent bacterial infections (including those caused by pneumococci) in HIV-positive individuals (Crewe-Brown *et al.* 2004).

Antimicrobial-resistant pneumococci, including serotype 19A strains have been reported to be highly clonal and to consist of a few predominant clones (Bogaert *et al.* 2000; Corso *et al.* 1998; Hermans *et al.* 1997; Marchese *et al.* 1998). In contrast, the multidrug-resistant serotype 19A isolates from this study were

clonally diverse. Similar results, where a number of clusters have been found among serotype 19A isolates have been reported from Portugal and Scotland (Clarke *et al.* 2004; Serrano *et al.* 2005).

Following the isolation of the first multidrug-resistant serotype 19A isolate from a specimen of a three-year-old boy from Johannesburg in 1977, staff and patients at two hospitals where the patient was admitted and treated were surveyed for nasopharyngeal carriage of resistant pneumococci. Serotype 19A was found to be the predominant serotype among the multidrug-resistant strains, most of which were from children under the age of three years. Recently, Reinert and colleagues characterized 26 representative isolates from initial outbreaks in South Africa in 1977-78 and found that the majority of multidrug-resistant strains belonged to closely related STs, namely ST41, ST1605 and ST1656 (Reinert *et al.* 2005). The study indicated that most of the pre-existing clones from the 1970's have not spread to other countries with the exception of ST172 and ST124. Likewise, none of the STs that were circulating 30 years ago, with the exception of ST172, were identified among isolates from the current study. ST172 has previously been described for an intermediately penicillin-resistant serotype 19A isolate from the United States (Gertz, Jr. *et al.* 2003) and also for serotype 15B, 19F, and 6A isolates from Portugal, Greece and Egypt, respectively (Multilocus sequence typing 2007c). ST172 is a predicted founder of a subgroup of eight STs that include ST361. In the current study isolates of clonal types ST172 and ST361 had the same antimicrobial profile. ST361 has also been described for pneumococcal serotypes 23F, 6B and 6A isolates (Multilocus sequence typing 2007c).

Current data show that the original 1977 multidrug-resistant serotype 19A strain included in this study was identified as ST1608, an ST that was described in two other penicillin-resistant serotype 19A strains isolated from South Africa in 1977 (Multilocus sequence typing 2007c), however it is no longer circulating among recent serotype 19A strains. In addition ST1608 differs by more than three alleles from the isolates that were characterized by MLST, indicating that it is not related to any of the currently circulating strains.

ST81 was identified for two isolates from this study and has previously been identified for multidrug-resistant serotype 23F isolates, including the widely disseminated Spain^{23F} clone that was first identified in Spain (Gertz, Jr. *et al.* 2003; Serrano *et al.* 2005). In a study conducted in Portugal, serotype 19A isolates with almost the same antimicrobial profile as the two isolates from this current study, were also characterized as ST81 (Serrano *et al.* 2005). The eight novel STs that were identified in this study were not related to each other or any other STs identified in this study.

CHAPTER 5: CONLUSIONS

Discriminatory power and reproducibility are important attributes in any typing system, and although PFGE is cost effective and useful for screening large numbers of isolates, comparison of fragments on the gel is hindered by variation between gels and hence comparison between different gels is often difficult. Although the GelCompar software makes allowance for this variation, analysis can still be ambiguous and subject to individual interpretation. MLST, although more expensive, provides unambiguous results. It is therefore advisable to use both methods when conducting molecular epidemiology studies. Data presented here provide information regarding the circulating clones within both serotype 3 isolates from Gauteng Province and multidrug-resistant serotype 19A isolates from South Africa. The MLST data presented here can be compared with similar data from around the world via the MLST website. Although none of the current serotype 19A strains are closely related to one of the original outbreak strains isolated in 1977, these results together with recent work by Reinert and colleagues has demonstrated the continued existence of one of the original multidrug-resistant clones, ST172, circulating among current serotype 19A strains from South Africa. Ongoing surveillance and molecular characterization of pneumococcal isolates is necessary to further our understanding of pneumococcal population genetics and to monitor the burden of disease due to vaccine serotypes. Furthermore, the predominance of serotype 3 isolates characterised as ST458 among isolates from Gauteng Province compared to the global ST180 predominating among serotype 3 isolates from many developed countries emphasizes the need for surveillance of isolates from South Africa and other

African countries. Limitations of this study include the fact that only a sample of serotype 3 isolates from Gauteng Province was characterized. Therefore, these results may not necessarily be a true reflection of the molecular epidemiology of all serotype 3 isolates from South Africa. Additional studies are required to determine the invasive potential of both ST458 and ST180.

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APPENDICES

Unless otherwise stated, all chemicals were supplied by Sigma Chemical Co., St. Louis, MO, USA or Merck Ltd, Johannesburg, South Africa and all culture media was supplied by Diagnostic Media Products, Johannesburg, South Africa.

Appendix A

Pulsed Field Gel Electrophoresis (PFGE)

1. Cell suspension buffer:
    - 1 ml of 5M NaCl
    - 50 ml of lM Tris
    - Make up to 50 ml with dH_2O

2. Cell lysis buffer:
    - 125 ml EDTA (0.25 M) (pH 8.0)
    - 2.5g N-Lauroylsarcosine (sarkosyl) (Sigma Aldrich, Germany)
    - Make up to 125 ml with dH_2O

3. 1.1% Seakem Gold® Agarose
    - 1.65g Seakem Gold® Agarose (Cambrex Bio Science, Rockland, USA)
    - 150ml 0.5X TBE Buffer
    - Dissolve agarose completely in the TBE buffer and and cool to 55°C

Appendix B

Multilocus sequence typing (MLST)

All Deoxynucleoside triphosphate were supplied by Invitrogen, Carlsbad, CA

1. Deoxynucleoside triphosphate (dNTPs) (125 µM)

    - 10µl dTTP (100mM)
    - 10µl dATP (100mM)
    - 10µl dGTP (100mM)
    - 10µl dCTP (100mM)
    - Make up to 800µl with dH_2O

2. PCR and sequencing primers

All primers were supplied by Inqaba Biotechnical Industries, Johannesburg, South Africa.

1. *Shikimate dehydrogenase* (*aroE*), gene size = 405 bp

   Forward primer: 5′ GCCTTTGAGGCGACAGC

   Reverse primer: 5′ TGCAGTTCAG(or A) AAACATA(or T)TTCTAA

2. *D-alanine-D-alanine ligase* (*ddl*), gene size = 440 bp

   Forward primer: 5′ TGCC/TCAAGTTCCTTATGTGG

   Reverse primer: 5′ CACTGGGTG(or A)AAACCA(or T)GGCAT

3. *glucose-6-phosphate dehydrogenase* (*gdh*), gene size = 460 bp

   Forward primer: 5′ ATGGACAAACCAGCNAGC(or T) TT

Reverse primer: 5′ GCTTGAGGTCCCATG(or A)CTNCC

4. *Glucose kinase* (*gki*), gene size = 483 bp

   Forward primer: 5′ GGCATTGGAATGGGATCACC

   Reverse primer: 5′ TCTCCCGCAGCTGACAC

5. *Transketolase* (*rec*), gene size = 450 bp

   Forward primer: 5′ GCCAACTCAGGTCATCCAGG

   Reverse primer: 5′ TGCAACCGTAGCATTGTAAC

6. *Signal peptidase I* (*spi*), gene size = 474 bp

   Forward primer: 5′ TTATTCCTCCTGATTCTGTC

   Reverse primer: 5′ GTGATTGGCCAGAAGCGGA

7. *Xanthine phosphoribosyltransferase* (*xpt*), gene size = 486 bp

   Forward primer: 5′ TTATTAGAAGAGCGCCATCCT

   Reverse primer: 5′ AGATCTGCCTCCTTAAATAC

3. PCR reaction mix

- 10× buffer (with no $MgCl_2$) 2.5 µl
- $MgCl_2$ stock (25 mM) 1 µl
- dNTP mix (125 µM) 2.5 µl
- Forward primer (20 µM stock) 0.5 µl
- Reverse primer (20 µM stock) 0.5 µl
- Genomic DNA 1 µl
- SuperTherm Taq DNA polymerase (5 U/µl) 0.4 µl

- Make up to 25 µl with sterile dH_2O

4. Cycle sequence reaction mix

    - Terminutesator ready reaction mix 4µl
    - 5x buffer 2µl
    - DNA 1µl
    - Single primer (forward/reverse) 1µl
    - Make up to 20µl with sterile dH_2O

Appendix C

Reagents and solutions

1. 0.5M Ethylenediaminetetra-acetate (EDTA)

    - Dissolve 186.1 g EDTA (BDH) in 700ml dH_2O
    - Adjust pH to 8 with NaOH (Merck)
    - Make up to 1L with dH_2O

2. 10 X Tris-Borate EDTA (TBE) buffer stock:

    - 108 g Trizma base
    - 55 g Boric acid
    - 7.5g EDTA powder (BDH) or 40 ml of 0.5 M EDTA (pH 8.0) stock
    - Dissolve in 1L dH_2O

3. Tris-EDTA (TE) Buffer

- 10ml of 1 M Tris (Merck)
- 2ml of 0.5 M EDTA, pH 8
- Make up to 1L with dH_2O

4. Ethidium bromide staining solution:
    - 250 ml of 0.5× TBE
    - 25 µl of 10 mg/ml ethidium bromide stock

5. 5M NaCl
    - Dissolve 292g NaCl (BDH) in 1L dH_2O

6. 1M Tris
    - 121g of Trizmabase (Sigma)
    - 800 ml dH_2O
    - Adjust pH to 8 with concentrated hydrochloric acid
    - Make up to 1L with dH_2O

7. 10% CTAB/NaCl
    - 4.1g NaCl
    - 10 g hexadodecyltrimethyl-ammonium bromide (CTAB)
    - Make up to 100ml with dH_2O

8. 2mg/ml RNAse A stock

    - 2 mg RNAse A (Roche, Johannesburg, South Africa)
    - Dissolve in 1 ml dH_2O

9. TE/RNAse

    - 150 µl TE buffer
    - 4µl 2mg/ml RNAse A