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Analysis of the Genetic Diversity of Neisseria Meningitidis in South Africa

Coulson, Garry Brian

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 General Background to *Neisseria meningitidis*

1.1.1 Introduction

"Meningococcal disease is among the most feared infections of children and young adults because of the rapidity of onset, high mortality rate, devastating sequelae, and tendency to spread and cause outbreaks" (Wenger, 1999).

Neisseria meningitidis, the causative agent of meningococcal meningitis and septicemia, is a major cause of endemic and epidemic disease in both developed and developing nations. Despite substantial advances in understanding the pathogenesis of the disease, the widespread use of antibiotics and the development of vaccines (Apicella, 2000), disease caused by *N. meningitidis* continues to be a serious cause of morbidity and mortality worldwide (Caugant et al., 1986; Rosenstein et al., 1999). At least 500,000 cases and more than 50,000 deaths are caused by this organism each year (Tikhomirov et al., 1997). It is by far the commonest cause of bacterial meningitis in the world, and is a leading cause of bacterial meningitis and septicemia in children and young adults (Ala'aldeen and Cartwright, 1996; Molling et al., 2001; Rosenstein et al., 1999). *N. meningitidis* is the only bacterium capable of generating epidemics of meningitis and has been responsible for a large number of devastating epidemics in several regions of the world (Ala'aldeen and Cartwright, 1996).

1.1.2 History

Clinical meningococcal disease was first described by Vieusseux in 1805 during an outbreak near Geneva, Switzerland (Apicella, 2000). Although epidemic cerebrospinal meningitis was known since the early 1800s, the etiologic agent was not described until 1884, when Marchiofava and Celli observed the organism in meningeal exudates (Koneman et al., 1997). The causative agent was finally isolated in pure culture by

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Weichselbaum in 1887, from the spinal fluid of a patient with purulent form of meningitis (Gotschlich, 1980). Weichselbaum went further on to describe the characteristics and etiologic role of the organism in six patients with acute cerebrospinal meningitis (Koneman et al., 1997).

Since its discovery, endemic and epidemic meningococcal disease has been reported throughout the world, stretching from Asia, Australia and New Zealand in the East, to Africa and Europe, and ultimately to the Americas and Canada in the West.

1.1.3 Organism

N. meningitidis is a Gram-negative encapsulated diplococcus that is an obligate human parasite (Bethell and Pollard, 2002). Structurally, *N. meningitidis* is similar to most other Gram-negative bacteria in that the cell envelope is composed of three major elements (Fig. 1): the cytoplasmic membrane, the rigid peptidoglycan layer, and the outer membrane.

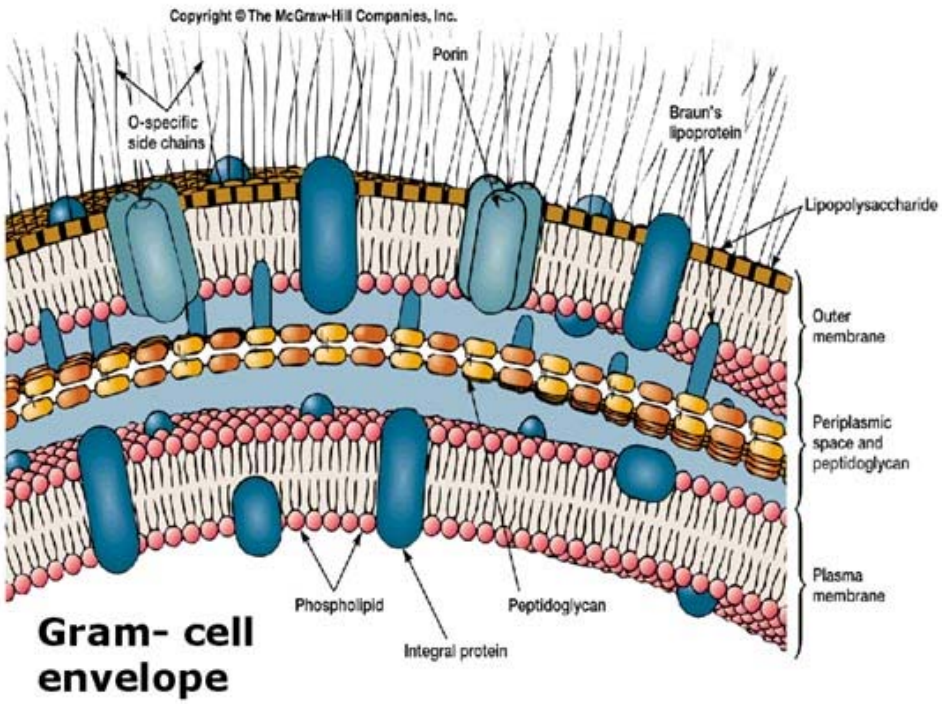


Figure 1. Diagrammatic Representation of the Classic Gram-negative Cell Envelope. (www.arches.uga.edu/ ~kristenc/cellwall.html)

The outer membrane, the defining characteristic of Gram-negative bacteria, is composed of lipids, lipopolysaccharides (LPS) and outer membrane proteins (OMPs). These components enable the organism to interact with and adhere to host cells, and act as transport proteins to control the intracellular environment (Joklik et al., 1980; Van Deuren et al., 2000). Furthermore, the lipopolysaccharides (endotoxin), residing primarily in the outer membrane, help confer resistance to bactericidal mechanisms and are involved in the pathogenesis of meningococcal disease (Bethell and Pollard, 2002). During log-phase growth, the meningococcus over synthesizes its outer membrane, resulting in bleb-like structures of the outer membrane forming and being released from the outer surface into the surrounding medium. The continuous release of endotoxin may play an important role in the severe endotoxic reaction of meningococcemia (DeVoe, 1982).

Surrounding the outer membrane, virulent meningococci possess polysaccharide capsules. These capsules impart antiphagocytic and antibactericidal properties to the meningococcus, thus enhancing survival during invasion of the bloodstream or cerebrospinal fluid (CSF) (Joklik et al, 1980). Capsules also provide protective (prevent dessication) and anti-adherent properties which promote transmission, spread and survival externally (Apicella, 2000; Joklik et al., 1980; Stephens et al., 1993). Capsules produced by *N. meningitidis* are chemically and immunologically distinct for each serogroup (DeVoe, 1982)

N. meningitidis also possess pili, which are hairlike protein polymers projecting from the surface of the bacterial cell. Their role in both promoting bacterial adhesion to epithelial and endothelial cells, and mediating invasion of epithelial cells has been well-established (Koneman et al., 1997; Nassif et al., 1999; Tzeng and Stephens, 2000).

1.1.4 Classification

Meningococci are subgrouped into serologic groups (serogroups) depending on the antigenicity of their polysaccharide capsule. Currently, 13 serogroups identifying capsule

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antigens (A, B, C, D, 29E, H, I, K, L, W135, X, Y and Z) are recognized (Knapp and Rice, 1995). Further classification beyond serogroup can be made by identifying differences in the outer membrane proteins (serotype and subtype) and LPS (immunotype) (Bethell and Pollard, 2002). Unique serotypes are defined on the basis of antigenic differences in the Class 2 and 3 OMP antigens, whereas differences in the Class 1 OMP antigens determine the subtype (Riedo et al., 1995).

1.2 Clinical Features and Pathogenesis of Meningococcal Infection

1.2.1 Acquisition, Carriage and Transmission

The meningococcus usually inhabits the human naso- or oropharyngeal region without causing any symptoms. The organism survives poorly in the environment and has no other host, therefore, human carriers are the major reservoir (Gotschlich, 1980; Knapp and Rice, 1995). Asymptomatic persons can carry the organism for variable time periods, from several weeks to several months, and carriage may occur in as many as 5-15% of individuals, or more in closed communities such as military recruit camps, prisons and schools (Cartwright and Ala'aldeen, 1997). Carriage of meningococci in the nasopharynx is thought to be self-limiting (Bethell and Pollard, 2002), occurring as transient, intermittent or chronic carriage, with the length of time carrying the organism varying with the individual and the bacterial characteristics of the colonizing strain (i.e. serogroup) (Anderson et al, 1998; Koneman et al., 1997).

By a conservative estimate, 500 million people in the world carry *N. meningitidis* in the nasopharynx (Stephens, 1999). Although meningococcal carriage is common in most populations, relatively few persons actually contract the disease. In most hosts, meningococcal carriage in the upper respiratory tract is an immunizing event, resulting in the formation of serogroup-specific protective serum bactericidal antibodies within 7-10 days (Apicella, 2000; Goldschneider et al., 1969). This response does not eliminate the carriage state, but it may protect the host from overt disease (Koneman et al., 1997). The highest rates of carriage are in adolescents and young adults and lowest in children and

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infants (Stephens, 1999). Children and infants more frequently carry the nonpathogenic species, *Neisseria lactamica*, which may be an important means of acquired cross-protective immunity (Raghunathan et al., 2004). These cross-protective antibodies appear to be protective during the critical period between infection and development of a specific immune response against pathogenic meningococcal strains (Goldschneider et al., 1969).

Asymptomatic carriers are presumably the major source of transmission of pathogenic strains (Caugant et al., 1994). Person-to-person transmission occurs by direct contact with respiratory secretions (e.g. kissing, drinking from same source) or by aerosol droplets (e.g. coughing, sneezing) contaminated with the organism (Knapp and Rice, 1995). In open populations, transmission of *N. meningitidis* is relatively slow. Rates of transmission, and carriage, are much higher among populations living in confined areas (military recruits, dormitories), and are exacerbated by smoking and upper respiratory tract infections (Stephens, 1999).

When individuals without adequate levels of immunity acquire the meningococcus, usually through close contact with a healthy carrier, the resulting nasopharyngeal infection may lead rapidly to bacteremia and acute purulent meningitis (Gotschlich, 1980). Meningococci enter the body via the upper respiratory tract and establish themselves in the membranes of the nasopharynx. Nasopharyngeal acquisition of the organism precedes dissemination by an indeterminate period of time. The incubation period is a matter of days and is usually less than one week. Dissemination of meningococci then occurs via the bloodstream and resulting in invasive disease with lesions in various areas of the body, such as the skin, meninges, joints, eyes and lungs (Joklik et al., 1980).

1.2.2 Invasive Disease/Pathology/Symptoms

In a small portion of individuals, shortly after colonization, the bacterium may evade the host immune system, invade the bloodstream and cross the membranes (meninges)

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surrounding the brain and the spinal cord, to gain access to the cerebrospinal fluid to produce disease (Cartwright et al., 1997; Caugant, 1998; Moore et al., 1994). The incidence of disease is typically highest in infants between 6 months and 1 year of age and in adolescents. Disease may be mild or it may progress rapidly, resulting in death of a previously healthy person within a few hours (Morello et al., 1991). Untreated meningococcal disease is generally fatal, while prompt antibiotic treatment reduces the case fatality rate to 10% (Caugant, 1998).

The meningococcus causes a spectrum of disease, of which there are two main clinical forms: meningococcal meningitis and meningococcal septicemia. Meningococcal meningitis is the more common entity and outcome is good if appropriately treated, whereas septicemia is less common, but highly fatal, even when actively treated (WHO, 1995). Other manifestations include septic arthritis, pneumonia, purulent pericarditis, conjunctivitis, otitis, sinusitis and urethritis (Tzeng and Stephens, 2000)

Meningitis is an inflammation of the membranes surrounding the brain meninges and spinal cord (meninges), occurring either as a primary disease, or secondarily to disease in some other part of the body (Easmon, 1990). Although the pathogenesis of meningococcal disease is poorly understood and very little is known about the dynamics of disease production (Koneman et al., 1997), it is believed that bacteria invade the meninges either by direct spread from the nasal mucosa, or indirectly via the bloodstream (Christodoulides et al., 2002; Easmon, 1990). Once bacteria have penetrated the blood-brain barrier, host defenses are incapable of inhibiting growth for the first 24 hours (Easmon, 1990). During this time, bacterial products may enter the CSF causing both an increase in intracranial blood pressure, and a disruption of the blood-brain barrier. These reactions, together with the induced inflammatory host immune response mounted at the site of invasion, may lead to cerebral oedema, abnormal spinal blood flow, obstruction to spinal fluid outflow and vasodilation (Easmon, 1990). The inflammatory process may, therefore, contribute to brain damage in meningitis. The symptoms and signs of acute meningitis are variable and depend largely on the age of the patient and duration of the illness. Lethargy, anorexia, vomiting, and irritability are common and convulsions may

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occur, particularly in children. The classical symptoms are headache, photophobia and neck stiffness. In severe cases, the patient may become comatose (Cartwright and Ala'aldeen, 1997).

About a fifth of those who survive from meningococcal meningitis will suffer from significant residual neurological dysfunctions, including paralysis, deafness, palsies and seizures, as well as mental disorders (Moore, 1994; WHO, 1995).

Meningococcemia is the most severe form of infection. The condition is often fulminant, with death occurring 12 to 48 hours after presentation (Riedo, 1995). Meningococcemia is usually characterized by profound vascular effects, the most visible being a petechial or purpuric rash that occurs in 75% of patients with the disease. In fulminating infection (Waterhouse-Friderichsen syndrome), there is widespread vascular coagulation and fulminant sepsis, resulting in shock and usually death (Knapp and Rice, 1995). Patients who suffer from meningococcal septic shock may suffer a disfiguring loss of skin and parts of limbs. Shock is characterized by a loss of blood pressure, particularly in the extremities (Moore, 1994). The pathogenic mechanism of the effects is not entirely clear, but presumably this reaction is related to, or initiated by, endotoxin (LPS) in the meningococcal outer membrane (Knapp and Rice, 1995). Endotoxin causes extensive vascular damage by stimulating the production of proteins that increase the permeability of blood vessels. This change in vascular permeability can precipitate an often-lethal drop in blood pressure. The severity of meningococcal disease is, therefore, related to the level of endotoxin in plasma and CSF, which in turn determines the intensity of the host's proinflammatory response (Braun et al., 2002; Unkmeir et al., 2002).

1.2.3 Risk factors for Disease

Risk factors for invasive disease and for outbreaks are not completely understood, and may vary between developed countries and developing countries, between endemic and epidemic disease and among illnesses caused by various serogroups (Schwartz, 1989). Risk factors can be categorized into organism characteristics that promote virulence;

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environmental factors that facilitate exposure to meningococci; and host factors that increase bacterial colonization, invasion and survival in the bloodstream (van Deuren et al., 2000).

The risk of meningococcal disease differs between serogroups (Tikhomirov et al., 1997). Certain serogroups of meningococci are more virulent and more likely to cause disease. Furthermore, within serogroups, some strains are more closely associated with epidemic disease, whereas others are less likely to cause infection (Schwartz, 1989). Most cases of meningococcal disease associated with epidemics and outbreaks are generally caused by a limited number of meningococcal strains (Caugant et al., 1986). Examples of these are strains belonging to the ET-5 complex, ET-37 complex, Subgroup I and III, and the A4 cluster (Achtman, 1995; Caugant et al., 1986). Certain pathogenic meningococci may also possess pathogenicity islands or mobile genetic elements that increase their ability to cause disease (Bille et al., 2004).

Environmental factors such as low absolute humidity and high temperatures may enhance meningococcal invasion by damaging the mucosal barrier directly or by inhibiting the mucosal immune defenses (Moore, 1994). Crowding and close contact may also result in increased transmission of *N. meningitidis* and increased rates of invasive disease (Tzeng and Stephens, 2000). Active or passive exposure to tobacco smoke, which may increase the rate of meningococcal disease by 20% (Tzeng and Stephens, 2000), as well as concurrent viral infection (mycoplasma, adenovirus, parainfluenza, rhinovirus and respiratory syncytial virus) (WHO, 1995) of the upper respiratory tract, increases the risk of meningococcal disease by enhancing the formation and spread of respiratory droplets or diminishing the functional and mechanical integrity of the respiratory mucosa as a barrier to invasion (Fischer et al., 1997; Moore et al., 1990; Rosenstein et al., 2001; Young et al., 1972).

Individual host factors that predispose to invasive meningococcal disease have been well described. The most well-known are deficiencies in the compliment system, particularly deficiencies in the terminal compliment components (C5-C8) (Riedo et al., 1995). If

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properdin, a component of the alternative pathway, is absent or dysfunctional, there is an increased risk for meningococcal disease (Riedo et al., 1995). Other underlying conditions such as hepatic failure, systemic lupus erythematosus, multiple myeloma and asplenia may also predispose to serious meningococcal disease (Apicella, 2000).

Host humoral immunity is an essential factor in the prevention of meningococcal disease. The presence of serum bactericidal antibodies (immunoglobulins G and M) is probably the most important factor preventing invasive disease (Schwartz, 1989). Carriage with non-pathogenic *Neisseria* species, such as *Neisseria lactamica*, may provide immunity by stimulating the formation of cross-protective antibodies against other pathogenic strains (Gold et al., 1978; Goldschneider et al., 1969; Oliver et al., 2002)

1.3 Diagnosis and Laboratory Identification

Effective treatment of meningococcal infection requires the rapid and accurate detection and identification of meningococci from a diseased patient. Laboratory confirmation typically requires the isolation of organisms from normally sterile body sites, such as CSF and blood. Isolation of meningococci from CSF and blood can be achieved for 90% and 50% of patients, respectively, but this percentage is reduced to 5% or less following early antibiotic treatment (Cartwright et al., 1992). Although not ideal, organisms can also be isolated from the nasopharynx, skin lesions, joints and eyes (Cartwright and Ala'aldeen, 1997).

1.3.1 Culture Methods

The classic laboratory diagnosis of meningococcal disease has relied on bacteriologic culture. Culture of *N. meningitidis* from blood, CSF or a normally sterile site represents the optimal confirmation of invasive meningococcal disease. Meningococci are facultatively anaerobic organisms that are often propagated on non-selective media, such as chocolate blood agar, in 5% CO_2 (Joklik et al., 1980). On agar media, well-isolated colonies are grey, round and convex, with a smooth, moist surface and entire edge

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(Morello et al., 1991). Presumptive identification is based on the growth of organism from non-sterile sites on non-selective media, together with the results from the oxidase test, catalase test and Gram stain (Morello et al., 1991). *N. meningitidis* colonies are Gram-negative, oxidase-positive, catalase-positive (Koneman et al., 1997). Confirmatory identification is achieved through the use of carbohydrate fermentation tests (Morello et al., 1991). In carbohydrate fermentation tests, meningococci acidify glucose and maltose, but not sucrose, lactose or fructose (Koneman et al., 1997).

Although probably still the most widely used method for the identification of meningococci, culture is time-consuming and false-negative results are sometimes obtained due to prior antibiotic treatment (Olcen, 1995).

1.3.2 Non-culture Methods

1.3.2.1 Microscopy

Microscopy offers a very rapid means of identifying *N. meningitidis*. Meningococcal infection is specifically diagnosed by the identification of characteristic *N. meningitidis* Gram-negative diplococci in stained smears (Joklik et al., 1980). Visualizing Gram-negative intracellular diplococci in the CSF provides a highly specific confirmatory test (PHLS, 2002). Although rapid and fairly specific, microscopy requires a high concentration of bacteria in CSF (Olcen, 1995).

1.3.2.2 Polysaccharide Antigen Testing

The sensitivity of culture may be low, especially when performed after the initiation of antibiotic treatment. Non-culture methods, such as the use of commercially available kits to detect polysaccharide antigen in CSF, have been used to enhance the laboratory diagnosis. Latex agglutination and coagglutination tests for detecting meningococcal antigens in body fluids are now both commercially available. Latex agglutination testing can rapidly detect meningococcal polysaccharide antigens in CSF and provide serogroup

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identification. Positive results provide a rapid presumptive diagnosis and allow early administration of appropriate therapy (Morello et al., 1991).

Although commercial latex agglutination kits detect *N. meningitidis* capsular antigens with high sensitivity and specificity among culture-confirmed cases, and can produce a serogroup-specific diagnosis, these tests appear to have low sensitivity when Gram-stain and culture of CSF are negative (Camargos et al., 1995; Perkins et al., 1995; Tarafdar et al., 2001). False-positive results are also common (Rosenstein et al., 2001). Therefore, in the clinical laboratory, it is suggested that antigen detection tests should always be used with Gram-stain and culture.

1.3.2.3 Polymerase Chain Reaction

Biochemical, morphological and serologic tests, the traditional basis for the identification of *N. meningitidis*, requires the growth of the organism which is not always possible under certain conditions. Polymerase chain reaction (PCR) has become established as part of the routine diagnostic methods available in many laboratories worldwide. Using primers targeting specific genes unique to *N. meningitidis* (e.g. *ctrA*) and serogroup-specific sequences (e.g. *siaD*, *omp*), PCR analysis offers the advantages of detecting species-specific and serogroup-specific *N. meningitidis* DNA in CSF and blood, and of not requiring live organisms for a positive result (Rosenstein et al., 2001). These techniques have been adapted to fully automated real-time PCR systems such as the TaqMan (Guiver et al., 2000) and LightCycler PCR systems (Molling et al., 2002). PCR, therefore, allows the rapid, sensitive and specific confirmation of meningococcal etiology as well as the identification of the main disease-causing serogroups (Raghanuthan et al., 2004).

1.4 Treatment

The introduction of antibiotics has dramatically altered the prognosis of meningococcal disease. Today, the expected mortality rate under optimal conditions should not exceed 8

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to 10% (Apicella, 2000). Rapid sterilization of the CSF is the key objective of antibiotic therapy in bacterial meningitis, with delayed sterilization associated with an increased frequency of neurological complications (Lebel and McCracken, 1989). Consequently, antimicrobial treatment should be instituted as soon as possible, without waiting for laboratory results.

Until recently, the drug of choice for the treatment of meningococcal disease was penicillin, administered intravenously. Although it typically does not penetrate the normal blood-brain barrier, it does so readily when the meninges are inflamed (Gotschlich, 1980). Though most meningococci are highly sensitive to benzylpenicillin, penicillin-resistant strains have been documented in many countries, including Spain, Italy and the UK (Canica et al., 2004; Kyaw et al., 2002; Stefanelli et al., 2004). In addition to penicillin, third-generation cephalosporins may also be given for therapy in all age groups. These antibiotics are safe, have greater intrinsic activity than penicillin, and a better CSF to MIC concentration ratio, which ensures activity against strains less sensitive to penicillin. They are also active against β-lactamase producing meningococci (Wall, 2001). Third-generation cephalosporins such as cefotaxime and ceftriaxone are widely used in developed countries for initial hospital treatment of bacterial meningitis and are effective against almost all common meningeal pathogens (Cartwright and Ala'aldeen, 1997).

1.5 Prevention

1.5.1 Chemophrophylaxis

An important aspect of controlling and preventing meningococcal disease is to break the cycle of acquisition and transmission by eradicating nasopharyngeal carriage. "If there are no carriers, there are no cases" (Aycock and Mueller, 1950). Systemic antibiotics can eradicate nasopharyngeal carriage of meningococci among contacts of sporadic cases and thus prevent secondary disease. Antimicrobial chemoprophylaxis is therefore the primary means of preventing the spread of meningococcal disease (Rosenstein et al.,

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2001). It should be given to persons who are at elevated risk for contracting disease, such as household contacts, day-care center and school contacts, and close personal contacts where respiratory secretions may be transmitted (coughing, sneezing, kissing and sharing drinks etc.) (De Wals et al., 1981; Memish and Alrajhi., 2002; Zangwill et al., 1997)

In the past, rifampin and the tetracycline, minocycline were used extensively. Both of these antibiotics have been shown to eradicate the carrier state rapidly, and that eradication can persist for up to 6-10 weeks after treatment (Devine et al., 1971; Guttler et al., 1971). Rifampin is highly effective, but surviving meningococci are often highly resistant (Easmon, 1990), with the emergence of rifampin-resistant meningococci in 10-27% of cases treated being reported (Weidner et al., 1971). Minocycline is equally effective, but there have been adverse reactions at higher doses (Easmon, 1990). Currently, the drugs of choice for chemoprophylaxis campaigns are ciprofloxacin and ceftriaxone, which have been shown to eradicate nasopharyngeal carriage of meningococci for up to 2 weeks (Pugsley et al., 1987; Schwartz, 1991).

1.5.2 Immunoprophylaxis (Vaccines)

1.5.2.1 Introduction

Neisseria meningitidis is by far the commonest cause of bacterial meningitis in the world and the only bacterium capable of generating epidemics of meningitis (Ala'aldeen and Cartwright, 1996). Therefore, the development of protective meningococcal vaccine should remain a research priority. An ideal vaccine is one that is safe, offers long-lasting immunity to all age groups, and cross-protects against all meningococcal serogroups, serotypes and serosubtypes (Ala'Aldeen and Cartwright, 1996). Unfortunately, it is now more than a century since the meningococcus was first discovered, and despite extensive studies, no such vaccine exists.

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1.5.2.2 Polysaccharide Vaccines

Because the virulence of *N. meningitidis* is closely associated with the group-specific capsular polysaccharides of the organism, it has been possible to develop vaccines to protect against meningococcal disease. Univalent and bivalent group A and group C polysaccharide vaccines have been developed, as has a quadrivalent group A, C, W135 and Y polysaccharide vaccine (Frasch, 1989). The antibody responses to each of the four polysaccharides in the quadrivalent vaccine are serogroup-specific and independent. The serogroup A and C vaccines have good immunogenicity, with clinical efficacy rates of >85% among children of five years of age or older and adults (Rosenstein et al., 1998; Rosenstein et al., 2001). Serogroup Y and W135 polysaccharides are safe and immunogenic in older children and adults, and although clinical protection has not been documented, vaccination with these polysaccharides induces bactericidal antibody (Lepow and Hughes, 2003; Rosenstein et al., 2001).

Although these vaccines are able to elicit an immune response in both children and adults, these capsular polysaccharide vaccines are non-boostable (T-cell independent), poorly immunogenic in infants and children < 2 years, and stimulate an immune response for only approximately 3 years (Poolman, 1995). It has been noted that, particularly in younger children, the elicited antibody titre falls rapidly over time (Kayhty et al., 1980). Whilst the A, C, Y and W135 polysaccharide vaccine has had an important place in the control of outbreaks and for special high-risk groups, they have not been used in routine immunization programs for the reasons above (Bethell and Pollard, 2002). Currently the quadrivalent vaccine is recommended only for individuals at high risk, such as those with complement deficiencies, travelers to areas that are highly endemic for meningococcal disease, military recruits, and those individuals living in an outbreak situation (Koneman et al. 1997).

Unlike serogroup A and C polysaccharides, the serogroup B polysaccharide has a capsule that is identical in structure to the polysialic acid in human neural tissue and is, therefore, poorly immunogenic in humans. Use of serogroup B polysaccharide vaccines in humans

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has, therefore, been limited because of the theoretical risk that these vaccines will overcome immune tolerance and induce autoimmunity (Rosenstein et al., 2001). As a result, strategies for developing vaccines against serogroup B disease have focused primarily on non-capsular antigens (e.g. outer membrane proteins). Several of these vaccines have proven to be safe, immunogenic, and effective in older children and adults, and have been used successfully to control outbreaks (Bjune et al., 1991; Frasch, 1989; Sierra et al., 1991; Zollinger et al., 1991). However, the considerable diversity of outer membrane proteins that cause sporadic serogroup B disease, as well as geographic and possible temporal variations, may limit this approach (Sacchi et al., 2000; Tondella et al., 2000).

1.5.2.3 Conjugate Vaccines

Ways to increase the immunogenicity of capsular polysaccharide antigens of meningococci are under development. These efforts involve conjugating the polysaccharide material to protein carriers to provide an "adjuvant" for enhancement of the native antigenicity of the molecules (Beuvery et al., 1983; Frasch et al., 1988).

Conjugate vaccines can overcome the immunologic limitations of meningococcal polysaccharide vaccines, which provoke T-cell-independent responses. This technique has been successfully employed with the *Haemophilus influenzae* type b vaccine where it has been introduced in several countries into the infant immunization schedule (Bethell and Pollard, 2002). This technique is now being employed with meningococcal vaccine development. Using the same technology, serogroup A, C, Y and W135 polysaccharides have been conjugated to tetanus toxoid proteins (Raghanuthan et al., 2004). When the capsular polysaccharide antigen is conjugated to a protein carrier, a T-cell-dependent host immune response develops, resulting in long-lasting protection and immunologic memory even in infants (Raghunathan et al., 2004). Since November 1999, a serogroup C conjugate vaccine has been adopted into the infant immunization program in the UK. This conjugate vaccine has appeared to be highly efficacious with high levels of high avidity bactericidal antibodies elicited in infants vaccinated at 2-4 months of age (Bethell

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and Pollard, 2002). There are concerns, however, on the long-term effectiveness of this vaccine with reports suggesting a rapid waning of efficacy following routine infant vaccination (Trotter et al., 2004; Snape et al., 2005). One of the options currently being explored to potentially counter this undesirable effect is to employ a booster dose at 12-18 months, or another booster dose at 12 years old around the age when adolescents are at increased risk of meningococcal disease (Snape and Pollard, 2005).

Recently, the first quadrivalent conjugate meningococcal vaccine, Menactra® (Meningococcal [Serogroups A, C, Y and W-135] Polysaccharide Diphtheria Toxoid Conjugate Vaccine – Sanofi Aventis Group) has been licensed in the U.S. for protection against meningococcal disease in adolescents and adults aged 11-55 years (http://www.news-medical.net/?id=7313). This vaccine was found to be safe and strongly immunogenic against all four of the serogroups found in the vaccine.

1.6 Methods for Typing *N. meningitidis*

1.6.1 Introduction

Typing methods have been widely applied in epidemiologic studies of transmissible bacterial infections. Meningococcal typing is required to identify outbreaks associated with particular serogroups, to demonstrate epidemiological links between cases or between cases and carriers in an outbreak, to monitor the changing epidemiology of disease and to evaluate new vaccines (Yakubu et al., 1999). Most epidemic and endemic cases of meningococcal disease are caused by a limited number of genetically defined clonal groups (Maiden et al., 1998), therefore, the ability to accurately identify the strains of infectious agents that cause disease is central to epidemiological surveillance and public health decision (Maiden et al., 1998), particularly with regards to understanding and controlling the spread of disease in both hospitals and communities (Tenover et al., 1995).

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Not only can the ability to type isolates of *N. meningitidis* with sensitivity and reliability be used to determine whether independent disease and carrier isolates during an outbreak reflect dissemination of a single clone, but they can also be used to elucidate the population genetic structure of the species, and to examine genetic relatedness among strains that may differ in virulence (Woods et al., 1994)

Much effort has, therefore, gone into the development and application of reliable methods for the typing of bacteria isolated from patients with disease. Presently, a variety of molecular typing techniques are available to assist investigations into meningococcal epidemiology. They vary in utility for determining genetic relatedness among strains, depending on the criteria for evaluating typing systems: typeability, reproducibility, discriminatory power, ease of interpretation, ease of performance.

1.6.2 Phenotypic Methods

1.6.2.1 Serogrouping and Serotyping

Traditional methods of typing organisms in clinical settings have been based on serogrouping, serotyping and subtyping. These methods are based on the long-standing observation that microorganisms of the same species can differ in the antigenic determinants expressed on the cell surface. These antigenic determinants include capsular polysaccharides, membrane proteins, lipopolysaccharides, and extracellular organelles (e.g. flagella and fimbriae).

Variations in the capsular polysaccharide permits differentiation of *N. meningitidis* into 13 recognized serogroups (A, B, C, D, 29E, H, I, K, L, W135, X, Y and Z). In addition to serogrouping of meningococci using antigenic variations in the capsule, meningococci can be further classified by serotyping (using variations in the class 2/3 outer membrane proteins) and subtyping (using variations in the class 1 outer membrane protein). A combination of these characteristics defines the phenotype e.g. serogroup: serotype: subtype (Cartwright, 2002; Frasch et al., 1985).

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Meningococcal serogrouping alone is of limited value in epidemiological studies. On a global basis, serogrouping correlates poorly with genetic relatedness because one or two groups usually predominate at any one time or place (Yakubu et al., 1999).

The serotyping method is more discriminatory and has been applied successfully to numerous species, including both Gram-negative (e.g. *Haemophilus influenzae*, *Neisseria meningitidis* and *E. oli*) and Gram-positive (e.g. *Streptococcus pneumoniae*) organisms. These phenotypic methods, however, suffer from problems of antigenic variability, poor discriminatory power, inability to subtype all isolates and the need to constantly enlarge the reagent panel (Tondella et al., 1994; Yakubu et al., 1999), therefore to reveal close genetic relatedness, serotyping is not suitable for modern epidemiologic purposes (Van Deuren et al., 2000)

1.6.2.2 Multi-Locus Enzyme Electrophoresis (MLEE)

In MLEE, isolates are analyzed for differences in the electrophoretic mobilities of a set of metabolic enzymes (Selander et al., 1986). Cell extracts containing the soluble metabolic enzymes are electrophoresed in non-denaturing starch gels. Variations in the electrophoretic mobility of an enzyme, referred to as electromorphs, typically reflect amino acid substitution that alter the charge of the protein and thereby identify allelic variations in the chromosomal genes encoding the enzyme. Combinations of electromorphs are designated electrophoretic types (ETs). MLEE is a very powerful tool for population studies, but it is only moderately discriminatory for the epidemiologic analysis of clinical isolates. Moreover, MLEE requires techniques and equipment that are available in relatively few laboratories. Consequently the method has had limited application to epidemiologic studies.

1.6.3 Genotypic (Molecular) Methods

Until recently, epidemiologic identification was based on the study of phenotypic traits such as serotype and subtyping among others. Classical typing schemes for

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meningococci, although contributing to the understanding of the epidemiology of meningococcal disease, suffer from problems of antigenic variability, inability to subtype all isolates and poor expression of surface antigens, among others (Yakubu et al., 1999). Consequently, these methods suffer from a lack of sensitivity in distinguishing between strains which has become a vital part of epidemiological investigation (Caugant et al., 1987).

The problems associated with the phenotypic techniques have stimulated interest in DNA-based typing methods. In the past few years, genetic classification has become more widely available (Cartwright, 2002). Sensitive and reproducible molecular typing methods are necessary, not only for epidemiological and population genetic investigations, but also for vaccine-related studies (Yakubu et al., 1999).

1.6.3.1 Ribotyping

Ribotyping is a method that probes restriction fragments of genomic DNA with cloned probes that contain all, or part of, the 16S and 23S rRNA genes. Patterns are easy to interpret because of a limited number of hybridized fragments, and the ribotypes are stable and reproducible. Genes coding for rRNA are highly conserved and most bacteria have multiple ribosomal operons. Thus, probing permits inter- and intra-species discrimination (Yakubu et al., 1999; Stull et al., 1988). Ribotyping has been used successfully in epidemiologic studies of *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* among others (De Buyser et al., 1989; Grattard et al., 1993; Markowitz et al., 1980). Woods et al. (1992) demonstrated the effectiveness of ribotyping for subtyping of meningococci in a study conducted on serogroup C isolates. Tondella et al. (1994) further provided evidence that ribotyping was a powerful method to provide useful information about the clonal characteristics on study done on serogroup B epidemic isolates from Brazil.

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A problem with ribotyping, however, is that the similarity of ribosomal genes may potentially reduce the power of ribotyping in discriminating between some unrelated strains (Mazurek, 1993).

1.6.3.2 PCR-Restriction Fragment Length Polymorphism (RFLP)

In PCR-RFLP, specific genetic loci are amplified and digested with a restriction endonuclease. The resulting DNA restriction fragments are analyzed for polymorphisms, indicative of strain variation, by gel electrophoresis (Olive and Bean, 1999). This approach has been used successfully to type strains of both fungi and bacteria.

Ideally, a gene suitable for use in typing should show sufficient inter-strain variability but should be conserved among identical strains. Several genes have been used in epidemiological investigations and for characterizing invasive *N. meningitidis* isolates. These include PCR-RFLP of the *porA* gene (Kertesz et al., 1993; Malorny et al., 1996; Peixuan et al., 1995; Speers et al., 1997), PCR-RFLP of the *pilA/pilB* gene (Giorgini and Taha, 1995; Guibourdenche et al., 1997; Musilek et al., 1998) and PCR-RFLP of the *dhps* gene (Kristiansen et al., 1995).

Restriction digests are rapid highly reproducible, simple to interpret, discriminating and sensitive. However, the discriminatory power of the approach varies substantially for different species, loci and restriction enzymes.

1.6.3.3 Random Amplified Polymorphic DNA (RAPD)

RAPD, also known as arbitrary primed PCR (AP-PCR), is based on the use of short random primer sequences (~10bp in length), which hybridize with sufficient affinity to chromosomal DNA sequences at low annealing temperature. If two RAPD primer sequences bind within a few kilobases of each other in the proper orientation, a PCR product with the corresponding molecular size results. Following separation of the amplification products by agarose gel electrophoresis, a pattern of bands which is

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characteristic of the particular strain, results (Olive and Bean, 1999). Woods et al. (1994) demonstrated that RAPD analysis was a fast, simple and sensitive method for distinguishing genetically different meningococcal strains and has great discriminatory power during a university outbreak investigation in the U.S.

Although RAPD is an efficient method to type strains of meningococci, problems associated with RAPD include the lack of reproducibility and standardization. Since the primers are not directed against a specific genetic locus, many of the priming events are the result of imperfect hybridization between the primer and target site. Thus the amplification process is extremely sensitive to slight changes in the annealing temperature which can lead to variability in the banding pattern (Olive and Bean., 1999).

1.6.3.4 Pulsed-field Gel Electrophoresis (PFGE)

PFGE is a variation of standard agarose gel electrophoresis in which the orientation of the electric field across the gel is changed periodically ("pulsed") rather than being kept constant. This critical modification allows large (>500Kb) DNA fragments to be effectively separated by size. Consequently, by digesting whole intact bacterial chromosomal DNA by restriction enzymes that have few recognition sites, and analyzing the fragments generated by PFGE, one can generate a chromosomal restriction profile typically composed of 8-20 distinct, well-resolved bands. PFGE patterns generated are highly specific for strains from a variety of species and detect polymorphic variation throughout the genome of the organism. The relative simplicity of the restriction profiles generated facilitates the analysis and comparison of multiple isolates. PFGE has proven to be a highly discriminatory and reproducible tool, comparable or superior to other available techniques, and is suitable for typing both highly clonal and less-clonal populations of *N. meningitidis*, (Olive and Bean, 1999; Kauffman, 1998). Its usefulness in discriminating between strains in several outbreaks and in general population genetics studies have been well described (Bygraves and Maiden, 1992; Nicolas et al., 1997; Popovic et al., 2001; Strathdee et al, 1993)

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1.6.3.5 Multi-Locus Sequence Typing (MLST)

Many bacterial populations undergo extremely high levels of genetic exchange. Such recombination is especially apparent in naturally transformable bacteria such as members of the genus *Neisseria,* which has implications for epidemiology (Maiden et al., 2003). Because of the random changing of segments of DNA within the population, it is not always possible to determine genetic relationships amongst isolates by looking at single genetic loci. For this reason, the indexing of neutral variation of housekeeping genes in a typing system is desirable (Maiden et al., 1998). The use of multiple genetic markers distributed around the genome provides both a discriminating and robust means of determining strain diversity. Such an approach is used in the technique known as multi-locus sequence typing (MLST).

First described by Maiden et al (1998), MLST has since become a popular methodology for typing strains of *N. meningitidis*. Rather than comparing the electrophoretic mobilities of enzymes (as in MLEE), MLST involves the sequencing of seven housekeeping genes. The comparison between sequences and alleles on the global MLST website allows the determination of the allele for each locus. The allele combination at the seven loci characterizes the sequence type (ST) of the strain. Since the results of MLST are unambiguous, reliable and easily transferable between laboratories, for epidemiological purposes, MLST has become the reference method for *N. meningitidis* typing (Nicolas et al., 2000), with its usefulness in outbreak investigations and population genetic analyses well documented (Feavers et al., 1999; Jolley et al., 2000).

1.7 Epidemiology of *Neisseria meningitidis*

1.7.1 General Introduction

Meningococcal disease is a significant cause of morbidity and mortality throughout the world. Although rates of endemic disease range from 0.8-1.1/100,000 in the United

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States (Rosenstein et al., 1999) and Europe (Connolly et al., 1999) to 10-25/100,000 in many parts of the developing world (Riedo et al., 1995), this disease is noteworthy for causing major, periodic epidemics with attack rates exceeding 500/100,000 (Tikhomirov et al., 1997). Strains of serogroups A, B and C cause 90% of cases of meningococcal disease worldwide (Peltola, 1983), and almost all of the remaining disease is accounted for by serogroups Y and W135 (Caugant et al., 1987).

Epidemics of meningococcal disease occur throughout the world, although nowhere have recurrent epidemics caused more morbidity than in the countries forming the "meningitis belt" of sub-Saharan Africa (Fig. 2). The meningitis belt encompasses Benin, Burkina Faso, Cameroon, Chad, Ethiopia, The Gambia, Ghana, Guinea, Mali, Niger, Nigeria, Senegal, Sudan and Togo (Greenwood et al., 1987; Lapeyssonnie, 1963). In this region epidemic rates have approached 1000/100,000 (Riedo et al., 1995).

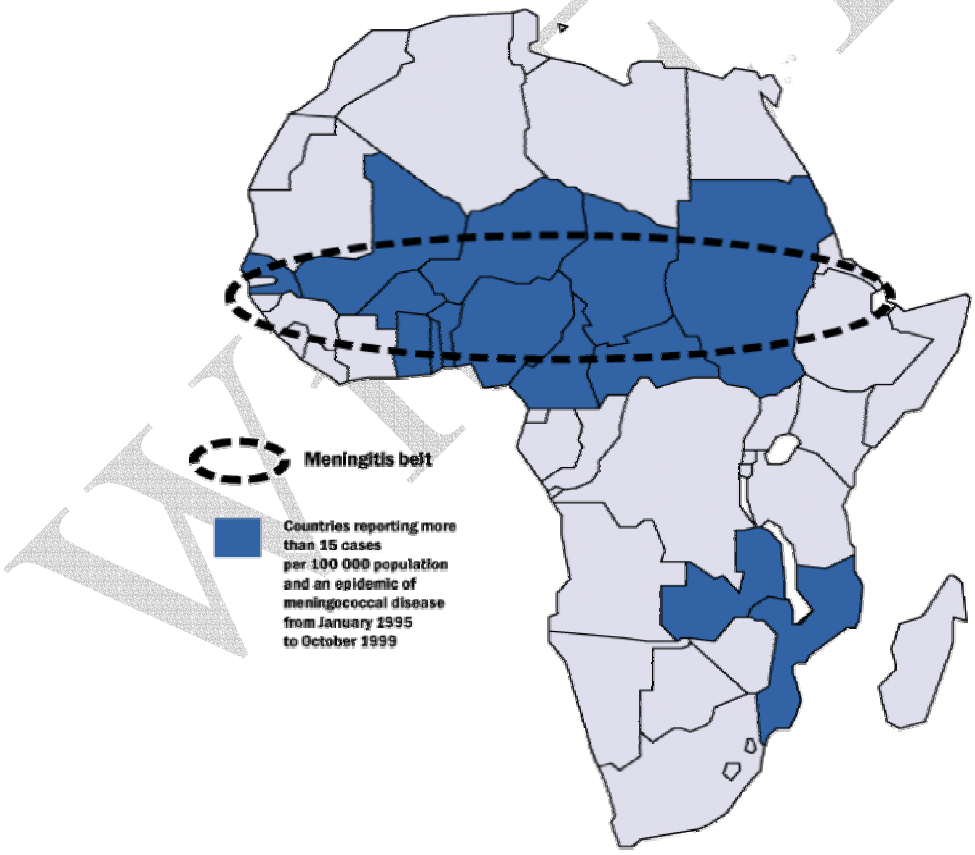


Figure 2. Diagrammatic Representation of the Meningitis Belt of sub-Saharan Africa. (http://www.who.int/disease-outbreak-news/n2001/february/20february2001.html)

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Epidemics are dynamic processes composed of cases clustered in time and space (Schwartz et al., 1989). The epidemiology of meningococcal disease undergoes 3 characteristic changes in the transition from endemic to epidemic disease: (1) an increase in the overall number of cases; (2) a shift in the age distribution of cases from predominantly <5 years of age toward older age groups; and (3) the predominance of a single bacterial strain rather than the heterogeneous mix of strains typical of endemic disease (Diermayer et al., 1999; Jackson et al., 1995).

Epidemics are favored by multiple factors related to the microorganism, the host and the environment. Interactions between these factors may explain the periodicity and seasonal patterns of epidemics, as well as the unusual age distribution among individuals who contract meningitis during an epidemic (Moore, 1992). Unlike endemic disease, risk factors for an epidemic affect an entire population, not just scattered individuals (Moore and Broome, 1994). The reasons for recurrent epidemic disease in the meningitis belt are unclear. One variable that may affect the occurrence of epidemic disease is the introduction of a virulent organism (Riedo et al., 1995). The introduction, transmission and acquisition of new meningococcal strains of virulent clonal groups or clonal complexes into a previously unexposed population may precipitate a meningococcal outbreak (Tzeng and Stephens et al., 2000). The combination of increased carriage and transmission of invasive clonal groups can result in large outbreaks. However, the presence of a virulent strain alone is usually not sufficient to result in an epidemic (Schwartz et al., 1989).

Host factors such as humoral immunity against meningococci might change in a population over time. Humoral immunity is an essential factor in the prevention of meningococcal disease (Schwartz et al., 1989). The general level of immunity in a population against an organism is called herd immunity. Waning herd immunity to a particular strain in a population may be necessary for an outbreak to occur and could contribute partially to the cyclic patterns of meningitis in sub-Saharan Africa (Moore and Broome, 1994). Decline in antibody levels and herd immunity may be a product of the appearance of new, susceptible birth cohorts (Moore, 1992). Nevertheless, the loss of

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immunity does not entirely explain the intriguing patterns of disease in the meningitis belt.

The seasonality of disease indicates that environmental factors are also pivotal (Moore and Broome, 1994). Climate factors play an important role in the seasonal upsurge of meningococcal disease. Peak activity is generally in periods of low absolute humidity, such as the winter in temperate climate zones and the dry season in Africa. Drought and dust storms in sub-Saharan Africa can also help the spread of infection while the onset of the rainy season often leads to the end of the epidemic (Tikhomirov et al., 1997). Therefore, it appears as though a combination of high temperatures and low humidity make people more prone to disease once infected (Moore and Broome, 1994). The complete role of climatic factors in precipitating epidemics, however, is unclear. Although epidemics typically occur during the dry season, many dry seasons may pass without epidemic disease (Schwartz et al., 1989).

Other risk factors that may potentially precipitate an epidemic include socio-economic status (or a correlate, such as sanitation or crowding), and the spread of an infectious co-factor such as respiratory pathogens other than meningococci.

The risk of epidemic meningococcal disease differs between serogroups. Serogroups A, B and C account for most cases of meningococcal disease throughout the world, with serogroups B and C responsible for the majority of cases in Europe and the Americas, and serogroups A and C predominating throughout Asia and Africa (Schwartz et al., 1989; Connolly et al., 1999; WHO, 1995). Within serogroups A, B, and C, most disease is caused by a limited number of groups of genetically related bacteria which have been referred to variously as complexes (Wang et al., 1993), clusters, subgroups (Wang et al., 1992) and hyperinvasive lineages (Maiden et al., 1998).

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1.7.2 Serogroup A Meningococcal Disease

Serogroup A meningococci have historically been the main cause of epidemic meningococcal disease, and still predominate in Africa during both endemic and epidemic periods. Epidemic serogroup A meningococcal disease has been documented in various parts of the developing world, with outbreaks beginning during the dry season and ending with the onset of the rainy season. Attack rates generally run from 100-500/100,000, with periodic outbreaks occurring across sub-Saharan Africa at intervals of 8-12 years (Schwartz et al., 1989). The highest rates of sporadic meningococcal meningitis tend to occur among infants (Moore, 1992). During serogroup A epidemics, however, older groups are often infected, with the age-specific attack rates among 5- to 10- year olds being higher than the rates among younger groups (Peltola et al., 1982; Peltola, 1983).

Although outbreaks of serogroup A meningococcal disease were common in developed countries early in the 20th century, outbreaks as well as sporadic cases, have been infrequent in these industrialized countries since World War II (Rosenstein et al., 2001). Limited outbreaks of serogroup A meningococcal disease in developed countries have taken place in Finland (Salmi et al., 1976), New Zealand (Lennon et al., 1988; Lennon et al., 1989), and the U.S. Pacific Northwest (Counts et al., 1984). These outbreaks, as well as outbreaks that occurred in the United States before the 1940s, had relatively low incidence rates (<15 cases/100 000) and affected lower socio-economic groups predominantly.

In contrast, intense meningococcal epidemics have been reported in a number of developing countries, including Brazil (Peltola, 1983), Nepal (Cochi et al., 1987), China, and various sub-Saharan African countries, such as Rwanda, Kenya, Ethiopia, Uganda, Sudan and Burundi (WHO, 1995). Attack rates during these epidemics can approach 1% of the population. Epidemic waves of meningococcal disease, usually caused by serogroup A meningococci, occur every 8-12 years in the meningitis belt region of sub-Saharan Africa (Hart and Cuevas, 1997). This region is uniquely susceptible to intense

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serogroup A meningococcal epidemics. Although many countries are grouped together in the meningitis belt, each country does not necessarily experience an epidemic of the same magnitude at the same time. For example, Burkina Faso, Niger and Mali experienced large outbreaks (200-350/100,000) whereas the concurrent incidence in Nigeria and Sudan (20-40/100,000) was only marginally higher than baseline levels (Riedo et al., 1995).

Different strains of serogroup A *N. meningitidis* appear to have different potentials for causing epidemics. Five subgroups of group A strains, designated I-V have been described with 22 genetically distinct clones (Olyhoek et al., 1987). The last three epidemic waves in the African meningitis belt have been caused by clone I-I, IV-I and III-I, respectively (Moore, 1992, Olyhoek et al., 1987; Peltola, 1983).

1.7.3 Serogroup B Meningococcal Disease

Serogroup B is the most common cause of meningococcal disease in many developed countries. It is recognized as the major cause of sporadic meningococcal disease in North America, South America, Canada and Europe (Kertesz et al., 1999; Poolman et al., 1986; Swaminathan et al., 1996). Serogroup B meningococci cause about one-third of all invasive disease in the United States (Tondella et al., 2000) and about 60-75% of cases of meningococcal disease in the UK (Cartwright et al., 2002). Compared to other serogroups, serogroup B causes a disproportionately large number of cases among infants (Tondella et al., 2000).

Historically, serogroup B is generally associated with sporadic disease and localized outbreaks of disease, but may occasionally cause epidemics. Epidemics tend to occur in developed nations, as was observed in Norway (mid-1970s), Cuba (1982-84), Chile (1986, 1993) and Brazil (1989), with attack rates of 50-100/100,000 (Apicella, 2000). Epidemics have also been observed in Belgium, Great Britain, the Faroe Islands (Denmark) and Iceland (Caugant et al., 1986; Peltola, 1983) as well as South Africa (Caugant et al., 1986).

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The epidemiology of serogroup B strains differ from serogroup A and C strains in terms of their epidemic processes. In contrast to serogroup A or C epidemics, which are usually characterized by a high incidence and tend to resolve in 1 to 3 years, serogroup B outbreaks begin slowly, with only a moderate rise in incidence per year and may persist for 5 to 10 years or longer, as seen in Norway (Bjune et al., 1991), and areas of Chile (Boslego et al., 1995) and New Zealand (Martin et al., 1998). A higher proportion of serogroup B disease occurs in children younger than 5 years of age (Wenger, 1999).

In most industrialized countries, serogroup B strains have prevailed over the last 30 years. Most of these strains belong to a few clonal complexes, identified as ET-5, lineage III, cluster A4 and ET-37 (Caugant, 1998).

1.7.4 Serogroup C Meningococcal Disease

Serogroup C disease occurs in both developed and developing nations where it has been implicated in large outbreaks (Broome et al., 1983), small disease clusters (Kaiser et al., 1974), and sporadic infections with attack rates as high as 500/100,000 (Schwartz et al., 1989). In Europe, serogroup C disease accounted for 28% of strains between 1993 and 1996, during which period, there was an increase in proportion from 26%-32%, consistent with increases in Austria, Belgium, the Czech Republic, England and Wales, Republic of Ireland, and Spain (Connoly at al., 1999; Van Looveren et al., 2001). In the U.S. serogroup C causes approximately 35% of all cases of meningococcal disease (Rosenstein et al., 1999). Although predominantly a cause of sporadic disease in the U.S., serogroup C accounts for most outbreaks in the U.S. (Woods et al., 1998). Since the early 1990s, the frequency of serogroup C meningococcal outbreaks has increased (Jackson et al., 1995), with outbreaks being reported in institutions (community jails, schools and universities) and communities (Jackson et al., 1995; Krause et al., 2002; Tappero et al., 1996; Zangwill et al., 1997). A similar increase has also been reported in Canada (Jackson et al., 1995). In addition to outbreaks seen in the U.S. and Canada, serogroup C outbreaks have also been identified in Burkina Faso (Broome et al., 1983), Brazil (De Morais et al., 1974), Denmark (Ronne et al., 1993), Mali (WHO, 1995), the

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United Kingdom (Masterton et al., 1988), Vietnam (WHO, 1995) and Spain (Alcala et al., 2002; Arreaza et al., 2000).

Most cases of meningococcal disease in the U.S., Canada and Europe are caused by a limited number of identical or closely-related isolates circulating slowly in the population. Endemic disease and outbreaks of serogroup C meningococci belonging to the ET-37 complex have been reported during the 1990s from North America (Ashton et al., 1991), Europe (Kriz et al., 1994), Brazil (Sacchi et al., 1992) and the Sahel region of West Africa (Broome et al., 1983; Wang et al., 1993). In the United States, 84% of serogroup C isolates analyzed from 1988-1994 belonged to closely-related members of the ET-37 complex (Raymond et al., 1997). In Canada, most serogroup C meningococci involved in endemic or epidemic disease and outbreaks belong to ET-15, a new variant within the ET-37 complex (Tsang et al., 2003) and has been linked with several outbreaks in Canada since 1991 (Ashton et al., 1991; Tsang, 2004). From 1980 to 1999, in Denmark, the ET-15 complex was responsible for 83% of serogroup C-associated cases of meningococcal disease (Jensen et al., 2002).

1.7.5 Serogroup W135 Meningococcal Disease

After its description in 1968, *N. meningitidis* W135 was considered a minor serogroup of little clinical importance. Only in the 1980s was this organism described as a fully pathogenic strain, as an important new cause of disease in Europe and the United States and as an emerging cause of meningococcal disease in Africa (Brandstetter et al., 1981; Denis et al., 1982). The presence of W135 has been confirmed in Africa for some time. In Burkina Faso in 1980, 1.3% of the meningococcal strains isolated from rhinopharyngeal carriers belonged to serogroup W135. In 1981-82, 4% and 3% of strains in parts of Senegal and Niger, respectively, and 7% of strains in Gambia in 1984-85, belonged to serogroup W135 (Denis et al., 1982).

During the 1990s, *N. meningitidis* W135 represented up to 4% of all meningococci in the UK, U.S and France (Aguilera et al., 2002; Rosenstein et al., 2001). Until recently, *N.*

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meningitidis serogroup W135 was associated with 1%-8% of all cases of sporadic meningococcal disease worldwide (Jackson et al., 1995; Krause et al., 2002; Mayer et al., 2002; Rosenstein et al., 1999). Its ability to cause severe epidemics was first fully realized in 2000 in Saudi Arabia during the annual Hajj pilgrimage, where more than 400 cases of serogroup W135 meningococcal disease had been reported in Hajj pilgrims and their close contacts from 16 countries throughout the world including the United Kingdom, Belgium, the United States, France, Morocco, Kuwait, Saudi Arabia, Oman, Malaysia, Indonesia, Singapore, Denmark, Finland, Sweden, Norway, Germany, and The Netherlands (CDC, 2000; Aguilera et al., 2002; Issack and Ragavoodoo, 2002; Mayer et al., 2002; Popovic et al., 2000). All isolates associated with the outbreak were members of a single clone of the virulent ET-37 complex, which has caused hyperendemic disease and outbreaks worldwide (Mayer et al., 2002; Taha et al., 2000; Wang et al., 1993). Prior to the outbreak, ET-37 strains associated with the 2000 Hajj outbreak of W135-related meningococcal disease had been seen in Indonesia and Canada (1997) (Popovic et al., 2000) and were common among *N. meningitidis* strains isolated in Sweden during 1978-2000 (Molling et al., 2001).

In addition to the major serogroup W135 epidemic that occurred in Saudi Arabia in 2000, W135 was also implicated as the cause of major epidemics during Hajj 2001 and 2002, and Burkina Faso in 2002 (Tiendrebeogo et al., 2004).

Currently, meningococcal epidemics occur regularly in the African meningitis belt. Although serogroup A is predominantly involved in these epidemics, it is believed that large serogroup A epidemics may be masking small, low-intensity W135 epidemics (Mayer et al., 2002; Lingappa et al., 2003). Taha et al (2002) reported on the involvement of both serogroups A and W135 in meningitis cases in Burkina Faso and Niger at the end of the 2001 epidemic, and revealed that serogroup W135 accounted for 38% of the cases.

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1.7.6 Serogroup Y Meningococcal Disease

Serogroup Y has historically been a serogroup of minor significance. Together with serogroup W135, these two serogroups account for less than 10% of cases worldwide. In the United States, the proportion of meningococcal disease during 1989 to 1991, associated with serogroup Y, was approximately 2% of cases (Racoosin et al., 1998). From 1992-1996, however, the number of cases involving serogroup Y had increased from 10.6% in 1992 to 32.6% in 1996. This was best characterized by increases in the proportion of serogroup Y disease seen in Illinois, Connecticut, Maryland and Chicago in the U.S. (CDC, 1996; McEllistrem et al., 2004; Racoosin et al., 1998). In the U.S., serogroup Y disease was predominantly associated with elderly patients (Rosenstein et al., 1999), with patients displaying underlying illnesses twice as much as those with non-serogroup Y meningococcal disease. The most common underlying conditions were diabetes mellitus, chronic lung disease, lupus, premature birth and therapy with immunosuppressive agents (Racoosin et al., 1998). Presently, serogroup Y now accounts for approximately a third of all invasive meningococcal disease in the U.S. (Rosenstein et al., 2001; Winstead et al., 2000).

This relatively high incidence of serogroup Y disease in the United States is of particular interest, since in Europe serogroup Y disease is almost exclusively found in patients with terminal compliment deficiencies (Fijen et al., 1994). Sullivan et al., (2004) reported invasive meningococcal disease caused by serogroup Y in Scotland from 1978 to 2004, was associated with mostly with the young and old. Other than the U.S., Israel and Sweden are the only other countries, to our knowledge, to have reported an increase in serogroup Y disease (Connoly et al., 1999; Rosenstein et al., 2001). In parts of Canada, an increase in serogroup Y-associated meningococcal disease has been reported. Although the relative frequency of serogroup Y in Canada, in general, was 11.1% from January 1999-June 2001 (Tsang et al., 2002), almost half of those cases were from Ontario, a province in Canada that reported serogroup Y as accounting for 30% of laboratory confirmations (PHAC, 2001).

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Although primarily a source of sporadic disease, serogroup Y has also been responsible for outbreaks of meningococcal disease. Smilack et al., (1974) reported on a military outbreak that included 12 cases of serogroup Y meningococcal disease among members of an U.S. army combat training unit; and Koppes et al., (1977) reported a series of cases associated with serogroup Y meningococci in a group of U.S. Air Force recruits.

1.7.7 Meningococcal Epidemiology in South Africa

Meningococcal disease is endemic in South Africa, with only modest variations in the incidence of meningococcal disease observed from year to year, although outbreaks in communities can still occur (Ferrinho et al., 1993). Meningococcal infection has shown a cyclical occurrence pattern in South Africa since 1971, with major outbreaks every eight to ten years and minor outbreaks every two to three years, following the cyclical pattern described for *N. meningitidis* worldwide (Broome, 1986). The <5 age-group has the highest incidence rate in areas where the disease is endemic, whereas the epidemic situation is characterized by peak incidence between adolescents and teenagers (Donald et al., 1981; Ferrinho et al., 1993). Although meningococcal meningitis does occur sporadically in South Africa throughout the year, there is a peak in the number of cases between July and October, corresponding with the onset of winter (Balfour et al., 1999; Donald et al., 1981; Sonnenburg et al., 2000).

From 1986 to 1997, 8143 cases of meningococcal meningitis were reported in South Africa with an annual average of 683 cases (DOH, 1998). The incidence rate of meningococcal disease decreased from 2.35/100,000 in 1991 to 1.00/100,000 in 1997, with the average for the 12 year period being 1.75/100,000. The average CFR over this period was 9.5%, with the highest CFR in the African population (DOH, 1998). Three provinces had the majority of cases and deaths (Fig. 3): Western Cape, Gauteng and Eastern Cape. These provinces together had 76.7% of the total South African cases. Localized outbreaks have occurred in the Western Cape (1988, 1989 and 1990) and Gauteng (1996) (DOH, 1998). By 2003, the number of cases of meningococcal disease reported in South Africa had decreased to 368, with the resulting incidence rate of

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0.8/100,000 von Gottberg et al., 2004). Seventy-percent of the cases came from Gauteng and the Western Cape.



	Eastern Cape	Free State	Gauteng	KwaZulu-Natal	Limpopo	Mpumalanga	North West	Northern Cape	Western Cape	South Africa
2000	6,412,662	2,688,265	8,464,866	9,176,315	5,185,397	3,043,156	3,590,864	827,301	4,383,355	43,772,181
2001	6,441,776	2,702,878	8,762,888	9,378,800	5,253,145	3,107,506	3,654,136	824,029	4,497,091	44,622,249
2002	6,472,262	2,720,073	9,085,119	9,571,480	5,331,578	3,176,334	3,722,254	821,259	4,617,870	45,518,229
Average	**6,442,233**	**2,703,739**	**8,770,958**	**9,375,532**	**5,256,707**	**3,108,999**	**3,655,751**	**824,196**	**4,499,439**	**44,637,553**

Figure 3. Diagrammatic Representation of South Africa showing the Provinces and their Respected Population Densities (www.aboutsouthafrica.com)

Just as epidemics of meningococcal meningitis have shown a cyclical pattern, so has the predominant serogroup of meningococcal disease in South Africa as a whole appeared to be cyclical. Up to 1973, serogroup A dominated as the most prevalent serogroup seen in South Africa, though this may have been due to the massive epidemics of serogroup A disease on the mines in Gauteng (DOH, 1998). After 1973, serogroup B emerged as the dominant serogroup in the country as a whole, except for Gauteng province where serogroup A remained the primary cause of meningococcal disease (DOH, 1998). In 2002, serogroup A once again replaced serogroup B as the predominant serogroup, causing the highest proportion of meningococcal disease in South Africa (Coulson et al., 2003). Although serogroup A caused the highest number of cases in South Africa, the trend of most disease in the Western Cape being caused by serogroup B was still evident (Donald et al., 1981; Ryder et al; 1987; von Gottberg et al., 2004). At present, approximately equal numbers of cases of meningococcal disease due to serogroups A (predominantly in Gauteng) and B (predominantly in Western Cape) are seen (von Gottberg et al., 2004).

Although meningococcal disease in South Africa follows a predominantly endemic course, outbreaks do occasionally occur. Outbreaks of serogroup A disease, amongst adults in the community and on the mines in Gauteng (Balfour et al., 1999; Liebowitz et al., 1984; Sonnenberg et al., 2000), and outbreaks of serogroup B disease in the Western Cape have been reported (Donald et al., 1981; Ryder et al., 1987). Recently, a community-based outbreak of serogroup C was reported in Gauteng (Coulson et al., 2003). The outbreaks are often of short duration, and occur in densely populated settings where people live communally (e.g. army barracks, schools, college residences, prisons and worker hostels). Outbreaks occur in these settings because of the influx of new susceptible individuals into the community and proximity of contact, which enhance the rapid spread of a virulent strain.

At present, vaccination is not routinely employed for the control of meningococcal disease in South Africa, and is usually prescribed only for high-risk individuals (e.g. health care workers, mine workers).

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1.8 Study Objectives

Meningococcal disease, caused by the organism *Neisseria meningitidis*, is a serious health problem in developed and developing countries alike, responsible for approximately 500,000 cases and 50,000 deaths per annum (WHO, 1998). Most cases of meningococcal disease associated with epidemics and outbreaks are generally caused by strains of a limited number of genetically defined clonal groups, in contrast to meningococci causing sporadic disease, which are more variable (Caugant et al., 1986; Achtman, 1995). In addition to the finding that different serogroups have different potentials for causing epidemics, it has also been observed that different strains (clones) within a given serogroup, similarly, have different potentials for causing an epidemic (Moore, 1992).

To date, no comprehensive molecular epidemiological studies of *Neisseria meningitidis* in South Africa have been carried out. It was, therefore, our aim in this study to develop a basic understanding of the population genetics of *N. meningitidis* in South Africa through a detailed molecular epidemiological approach. The primary focus of this study was to identify, by different genotypic methods, which clonal groups from the major serogroups prevailing in South Africa (serogroups A, B, C, W135 and Y) were present and circulating in the population, with particular reference to their geographical distribution (amongst the various provinces) and their temporal variation (throughout the duration of the study).

The importance of conducting a molecular epidemiological study of *N. meningitidis* in South Africa can not be understated. Monitoring the changing epidemiology in such a manner allows us to identify and distinguish outbreaks from clusters of temporally and geographically proximate cases, and therefore, has direct implications for public health decisions, particularly with regards to the development of effective control and intervention strategies.

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CHAPTER 2: MATERIALS AND METHODS

2.1 Bacterial Strains

National surveillance for invasive disease caused by *Neisseria meningitidis* is part of ongoing active laboratory-based surveillance at the Respiratory and Meningeal Pathogens Research Unit (RMPRU), National Institute for Communicable Diseases (NICD), South Africa. National isolates for this study were selected from a 3-year period, August 1999 to July 2002. In total, 854 cases of meningococcal disease were reported countrywide from participating hospitals to the RMPRU for this period. Cases were defined as patients with *Neisseria meningitidis* isolated from a normally sterile body fluid (blood or cerebrospinal fluid, or both).

2.2 Culture

Disease-associated isolates were cultured directly from clinical specimens sent by the participating hospitals to the RMPRU. Bacteria were cultured on 5% blood agar (Diagnostic Media Products, Johannesburg, South Africa) for 18-24 hours at 37°C in an atmosphere of 5% CO_2. Bacteria identified as *Neisseria meningitidis* were then harvested using a cotton wool swab and resuspended in a 10% skim-milk suspension (Difco, Michigan, USA) before being stored frozen at -80°C.

2.3 Identification and Serogrouping

Cultured isolates were confirmed as *N. meningitidis* using standard biochemical tests (Gram stain, oxidase test and carbohydrate fermentation tests). A bacterial isolate was considered positive for *N. meningitidis* if it was a Gram-negative diplococcus that tested positive for both the oxidase test and the utilization of maltose and glucose.

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Confirmed *N. meningitidis* isolates were then serogrouped using slide agglutination testing with monoclonal antiserum (Murex Biotech Limited, Dartford, England) to meningococcal capsular polysaccharides A, B, C X, Y, Z, and W135.

Only those isolates collected from August 1999 - July 2002 that were positive for serogroup A, B, C, W135 or Y, were used in this study. Of the total number of 854 cases reported to the RMPRU, 604 viable isolates belonging to one of the five serogroups were available for genotypic characterization. The complete breakdown of the dataset for these available isolates, indicating serogroup proportions per province per year, is shown in Table 1.

2.4 Pulsed-Field Gel Electrophoresis (PFGE)

2.4.1 Plug Preparation and Electrophoresis

PFGE was performed on all viable *N. meningitidis* isolates of serogroup A, B, C, Y and W135 using a method adapted from Popovic *et al* (2001). Isolates were cultured on a 5% blood agar plate and incubated overnight at 37°C. Bacteria were harvested and resuspended in 2ml Cell Suspension Buffer (100mM Tris, 100mM EDTA pH 8.0). Concentration of the suspension was adjusted to a reading 0.48-0.52 using a Microscan Turbidity Meter (Dade, California, USA). Plugs were prepared with 400µl of the cell suspension, 20µl of proteinase K (20mg/ml stock solution), and an equal volume of 1% Seakem Gold® (Cambrex Bio Science, Rockland, USA)- 1% sodium dodecyl sulphate (SDS) agarose and dispensed into reusable plug moulds. Once the plugs had solidified (~15mins), they were then incubated at 54°C overnight in Cell Lysis Buffer (50mM Tris, 50mM EDTA pH 8.0 and 1% sarcosyl) and proteinase K.

Following overnight lysis, plugs were washed with preheated (54°C) sterile water twice, and then subsequently washed four times with preheated (54°C) sterile TE buffer (10mM Tris, 1mM EDTA pH 8.0). After the last wash with preheated TE buffer, sterile TE buffer at room temperature was added and the plugs stored at 4°C till ready for use.

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In preparation for electrophoresis, thin (2-3mm) slices of the agarose plugs were cut and incubated in restriction buffer (Restriction Enzyme Buffer M, Roche Diagnostics GmbH, Mannheim, Germany) for 10mins at 37°C. The buffer was then removed and the plugs subsequently incubated for 4 hours at 37°C in restriction buffer/restriction enzyme mix (20U *NheI*/plug slice) (Roche Diagnostics GmbH, Mannheim, Germany). The restriction buffer/enzyme mix was then replaced with 0.5X TBE and incubated at room temperature for 5mins. Plug slices were then placed onto the bottom of the comb teeth of a 15-tooth comb and allowed to air dry for 5mins. Plugs were then submerged in 1% Seakem Gold® agarose and allowed to solidify for 30mins.

Once the gel had set, PFGE was performed using a contour-clamped homogenous electric field apparatus (CHEF DR III, Bio-Rad Laboratories, California, USA) containing 0.5X TBE (Tris-borate EDTA) buffer cooled to 14°C and run at the desired electrophoresis conditions as follows: Block 1 had an initial switch time of 1sec and a final switch time of 30secs for a duration of 16hours; Block 2 had an initial switch time of 0.1secs and a final switch time of 6secs for a duration of 4hours. Both blocks were run at a current gradient of 6V/cm using a linear ramping factor and an angle of 120°.

2.4.2 Visualization, Capture and Data Analysis

After completion of the electrophoresis, the gel was removed and stained with ethydium bromide (1µg/ml) for 30mins and then destained with sterile water (4x15mins). The gel was then visualized using a ultraviolet transilluminator apparatus (UVP Ltd., California, USA) and an uncompressed TIFF image of the gel captured using Grab-IT v2.59 Annotating Grabber software (Synoptics, California, USA). For data analysis, PFGE restriction profiles were then analyzed and compared using the GelCompar™ v4.1 software (Applied Maths, Kortrijk, Belgium). Dendrograms and clusterings of patterns were created using the unweighted pair group method with arithmetic averages (UPGMA). Analysis of the banding patterns was performed with the Dice-coefficient, and a position tolerance of 1.5% for the band migration distance. A PFGE-based clonal

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group was defined as isolates sharing >80% similarity on dendrogram (Popovic et al., 2001).

2.5 Serogroup-Specific PCR (SS-PCR)

2.5.1 Whole-cell Preparations (Crude Extracts)

Based on the results of the PFGE analysis on the meningococcal isolates, clonal groups (complexes) of interest from each serogroup were observed. Representative isolates from each of these groups were chosen for further MLST analysis. Serogroup-specific PCR was conducted on these strains to confirm their serogroup as predicted by latex slide agglutination.

Isolates were cultured on a 5% blood agar plate and incubated in 5% CO_2 overnight at 37°C. Crude DNA extracts were obtained from theses isolates by harvesting bacteria from the blood plate and suspending them in TE buffer. The suspensions were then boiled for 10mins to in order to lyse the bacterial cell walls. Following boiling, the suspensions were briefly centrifuged (12,000rpm for 1min) to pellet the solid bacterial matter. Suspensions were then stored at -20°C.

2.5.2 Polymerase Chain Reaction (PCR)

Serogroups, as determined by latex agglutination, were confirmed by a serogroup-specific PCR, using a method adapted from Taha (2000). For serogroup prediction (serogroups A, B, C, Y and W135), a multiplex PCR was performed using primers specific for each of the serogroups (Table 2).

In each assay, the final 50µl reaction mixture consisted of 2µl crude DNA extract, 1X Buffer, 5mM $MgCl_2$, 0.4mM of each oligonucleotide, 1mM each deoxynucleoside triphosphate (dNTP) (Invitrogen, California, USA) and 2.5U of *Taq* polymerase (ABgene, New York, USA).

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The PCR assays were performed in a DNA thermal cycler (Hybaid Omnigene, Middlesex, UK) with the following parameters: a first cycle of denaturation at 94°C for 5mins, annealing at 55°C for 30secs and polymerization at 72°C for 20secs. The subsequent 35 cycles were as follows: 95°C for 1min, annealing at 55°C for 30secs, and polymerization at 72°C for 20secs. A final cycle of polymerization at 72°C for 1min was then performed.

Amplicons were analyzed by electrophoresis on a standard 2% agarose gel stained with ethydium bromide. The size of the expected amplicons from this multiplex PCR are 400bp (serogroup A), 450bp (serogroup B), 250bp (serogroup C) and 120bp (serogroups Y and W135). As the amplicon size for serogroups Y and W135 are the same, a second PCR using only the serogroup Y and W135-specific primers in separate reactions was performed to differentiate between the two serogroups.

2.6 Multi-Locus Sequence Typing (MLST)

2.6.1 Chromosomal DNA Extraction

Chromosomal DNA was extracted by a modified method described by Ausubel et al. (1989). Briefly, meningococcal strains were cultured overnight at 37°C on 5% blood agar plates. Cells were harvested and resuspended in TE buffer (10mM Tris-HCL, 1mM disodium EDTA, pH 8.0) with sodium dodecyl sulphate (SDS) and proteinase K, and incubated at 37°C for 1 hour. This was followed by the addition of 5M NaCl and 10% cetyltrimethylammonium bromide (CTAB) and incubation at 65°C for 10 mins. DNA was then extracted with chloroform and precipitated with 70 % ethanol. DNA was resuspended in TE buffer and the final concentration estimated by means of agarose gel electrophoresis and ethydium bromide staining.

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2.6.2 MLST Polymerase Chain Reaction (PCR)

MLST was performed using the seven housekeeping genes: *abcZ* (putative ATP-binding cassette reporter), *adk* (adenylate kinase), *aroE* (shikimate dehydrogenase), *fumC* (fumarate hydratase), *gdh* (glucose-6-phosphate dehydrogenase), *pdhC* (pyruvate dehydrogenase subunit), and *pgm* (phosphoglucomutase) as previously described in Maiden et al. (1998).

Basically, each 100µl PCR reaction consisted of 1X buffer, 1.5mM $MgCl_2$, 1µM each oligonucleotide (Table 3), 0.25mM deoxynucleoside triphosphate (dNTPs), 2.5U *Taq* polymerase and 2µl of DNA from chromosomal DNA extraction. Cycling conditions for the PCR reaction included an initial denaturation step of 94°C for 2 mins, followed by 30 cycles of denaturation at 94°C for 1min, annealing at 60°C for 1min and extension at 72°C for 2 mins. A final extension step of 72°C for 7mins was then performed. The PCR reactions were then visualized on a standard 1% agarose gel to analyze the purity of the reaction (i.e. presence of spurious/non-specific bands).

2.6.3 Geneclean® II Gel Extraction (Short Protocol)

In order to remove any non-specific PCR products from the PCR reactions, the PCR reaction were cleaned using the Geneclean® II kit according to the manufacturer's instructions (BIO 101 Inc., La Jolla, CA). In essence, 40µl of the PCR product was run on a standard 1% agarose gel. The desired band, corresponding to the amplified gene, was then excised and added to 3 volumes 6M NaI stock solution, and incubated at 52°C until the agarose had completely dissolved. Five microliters of GLASSMILK® (Silica dioxide) was then added to the suspension and placed on ice following vigorous vortex. The GLASSMILK®/DNA complex was then pelleted at 12,000rpm for 15secs, and the resulting supernatant discarded. The pellet was then washed three times in NEW Wash buffer (0.1M NaCl, 10mM Tris pH 8.0, ethanol, water) and dried at 52°C. Purified DNA was then eluted in 20µl of dH_20 at 52°C for 5mins. Purity of the DNA was assessed by electrophoresis on a standard 1% agarose gel stained with ethydium bromide.

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2.6.4 Cycle Sequencing and Sequencing Analysis

Sequencing reactions, for both the forward and reverse orientations of each of the housekeeping genes, were performed by cycle sequencing using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, California, USA) as per manufacturer's protocols. The cycle sequencing reactions were: one cycle of 94°C for 1min; and 25 cycles of 95°C for 30secs, 50°C for 20secs, and 60°C for 4mins. Sequencing primers (Inqaba Biotech) are shown in Table 3. Sequencing reactions were cleaned using the DyeEx 2.0 Spin Kit (Qiagen GmbH, Hilden, Germany) as per manufacturer's instructions and sequencing was performed using an ABI 310 automated DNA sequencer (Applied Biosystems, California, USA). Sequence analysis and determination was through the use of the CHROMAS v1.43 (http://trishul.sci.gu.edu.au/~conor/chromas.html)) sequence analysis software.

2.6.5 MLST Website – Allele and ST Enquiries

Sequences obtained for the seven housekeeping genes for each isolate were submitted to the MLST website (http://neisseria.org/nm/typing/mlst). For each of the housekeeping genes, the derived sequence was assigned a particular allele according to its sequence. For each isolate, the allelic profile (combination of alleles at each of the seven loci) was used to define the sequence type (ST). This ST may also be assigned, by the MLST database, to an ST-complex. An ST-complex consists of STs that are closely-related to one another, sharing similarity at five or more of the loci. In addition to being assigned to an ST complex, isolates of certain STs may be linked to a specific ET-complex (or subgroup). Before MLST was uncovered, the population genetics of *N. meningitidis* was studied using MLEE. By this method, isolates were assigned ETs (electropherotypes), and closely-related isolates (ETs) were grouped into ET-complexes. This wealth of data, obtained by MLEE, has been incorporated into the MLST database. Consequently, isolates of certain STs may be assigned to a ST-complex and linked to the corresponding ET-complex.

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Table 1. Provincial Distribution of Meningococcal Isolates per Serogroup per Year of Study

YEAR	SEROGROUP	PROVINCE (n)									TOTAL
		EC	FS	GA	KZ	MP	NC	NP	NW	WC	**N** (%)
1	A	-	-	9	1	-	-	-	1	4	**15** (8)
Aug '99 – Jul '00	B	6	-	11	4	3	-	-	-	80	**104** (56)
	C	2	2	2	-	-	-	-	-	14	**20** (11)
	W135	-	-	3	3	-	-	-	-	4	**10** (5)
	Y	2	6	11	7	-	-	-	-	12	**38** (20)
	Total	**10**	**8**	**36**	**15**	**3**	**-**	**-**	**1**	**114**	**187**
2	A	-	-	34	2	5	-	-	3	1	**45** (24)
Aug '00 – Jul '01	B	6	3	12	3	1	-	-	1	56	**82** (43)
	C	3	-	3	-	1	-	-	1	4	**12** (6)
	W135	-	-	8	-	-	-	-	-	1	**9** (5)
	Y	2	14	13	1	3	1	2	1	4	**41** (22)
	Total	**11**	**17**	**70**	**6**	**10**	**1**	**2**	**6**	**66**	**189**
3	A	-	-	77	1	1	-	-	2	1	**82** (36)
Aug '01 – Jul '02	B	3	4	15	-	-	1	-	1	38	**62** (27)
	C	1	-	6	-	-	1	-	-	10	**18** (8)
	W135	1	-	9	-	-	-	-	1	2	**13** (6)
	Y	3	14	21	4	1	2	-	1	7	**53** (23)
	Total	**8**	**18**	**128**	**5**	**2**	**4**	**-**	**5**	**58**	**228**

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Table 2. Primer Sequences for Serogroup-Specific PCR

Serogroup	Primer	Sequence 5' – 3'	Product Size
A	orf-2 (F)	CGC AAT AGG TGT ATA TAT TCT TCC	400bp
	orf-2 (R)	CGT AAT AGT TTC GTA TGC CTT CTT	
B	siaD-F (B)	GGA TCA TTT CAG TGT TTT CCA CCA	450bp
	siaD-R (B)	GCA TGC TGG AGG AAT AAG CAT TAA	
C	siaD-F (C)	TCA AAT GAG TTT GCG AAT AGA AGG T	250bp
	siaD-R (C)	CAA TCA CGA TTT GCC CAA TTG AC	
Y	siaD-F (Y)	CTC AAA GCG AAG GCT TTG GTT A	120bp
	siaD-R (Y)	CTG AAG CGT TTT CAT TAT AAT TGC TAA	
W135	siaD-F (W135)	CAG AAA GTG AGG GAT TTC CAT A	120bp
	siaD-R (W135)	CAC AAC CAT TTT CAT TAT AGT TAC TGT	

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Table 3: MLST PCR Primer Sequences

Gene	Primer	Primer Sequence
abcZ	abcZ-P1C	TGT TCC GCT TCG ACT GCC AAC
	abcZ-P2C	TCC CCG TCG TAA AAA ACA ATC
adk	adk-P1B	CCA AGC CGT GTA GAA TCG TAA ACC
	adk-P2B	TGC CCA ATG CGC CCA ATA C
aroE	aroE-P1B	TTT GAA ACA GGC GGT TGC GG
	aroE-P2B	CAG CGG TAA TCC AGT GCG AC
fumC	fumC-P1B	TCC CCG CCG TAA AAG CCC TG
	fumC-P2B	GCC CGT CAG CAA GCC CAA C
gdh	gdh-P1B	CTG CCC CCG GGG TTT TCA TCT
	gdh-P2B	TGT TGC GCG TTA TTT CAA AGA AGG
pdhC	pdhC-P1B	CCG GCC GTA CGA CGC TGA AC
	pdhC-P2B	GAT GTC GGA ATG GGG CAA ACA
pgm	pgm-P1	CTT CAA AGC CTA CGA CAT CCG
	pgm-P2	CGG ATT GCT TTC GAT GAC GGC

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Table 4: MLST Sequencing Primer Sequences

Gene	Primer	Primer Sequence 5'-3'
abcZ	abcZ-S1A	AAT CGT TTA TGT ACC GCA GR
	abcZ-S2	GAG AAC GAG CCG GGA TAG GA
adk	adk-S1A	AGG CWG GCA CGC CCT TGG
	adk-S2	CAA TAC TTC GGC TTT CAC GG
aroE	aroE-S1A	GCG GTC AAY ACG CTG RTK
	aroE-S2	ATG ATG TTG CCG TAC ACA TA
fumC	fumC-S1	TCC GGC TTG CCG TTT GTC AG
	fumC-S2	TTG TAG GCG GTT TTG GCG AC
gdh	gdh-S3	CCT TGG CAA AGA AAG CCT GC
	gdh-S4C	RCG CAC GGA TTC ATR YGG
pdhC	pdhC-S1	TCT ACT ACA TCA CCC TGA TG
	pdhC-S2	ATC GGC TTT GAT GCC GTA TTT
pgm	pgm-S1	CGG CGA TGC CGA CCG CTT GG
	pgm-S2A	GGT GAT GAT TTC GGT YGC RCC

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CHAPTER 3: RESULTS

3.1 Genotypic Characterization of Serogroup A in South Africa

During the period August 1999-July 2002, a total number of 142 serogroup A *N. meningitidis* strains were collected through the national surveillance program. Of these isolates, PFGE analysis was performed on 136 of the isolates. The remaining six isolates were not available for genotypic comparison, either as a result of severe contamination, or the isolates had become non-viable during the freezing process.

Cluster A-1

PFGE analysis of the 136 serogroup A meningococci (Fig. 4) available for genotypic characterization revealed a total of 36 different PFGE patterns (pulsotypes). Of these 36 pulsotypes, a large clonal complex composed of 22 pulsotypes was observed. This complex (Cluster A-1) consisted of approximately 88% (120/136 isolates) of the total number of isolates characterized by PFGE, and was composed of closely-related isolates sharing at least 79% similarity to one another, with the vast majority (84%) of isolates sharing >88% similarity. During the first year of the study (Aug '99–Jul '00), isolates from this particular clonal complex were responsible for 47% (7/15) of disease caused by serogroup A countrywide. This percentage then increased to 91% (40/44) and 96% (74/77) for the following two years (Aug'00–Jul'01 and Aug'01-Jul'02), respectively (Table 7). Of the 120 isolates making up this clonal complex, 103 (86%) isolates were from Gauteng (Table 8). In Gauteng, the proportion of disease caused by Cluster A-1 increased from 63% in the first year, to 100% in the second year, and decreased marginally to 96% in the third year.

Nine representative isolates from among different PFGE groups within Cluster A-1 were selected for MLST characterization (Table 5). Isolates spanning collection dates throughout all three years of the study were chosen. These nine isolates were subjected to serogroup-specific PCR (SS-PCR) to confirm their serogroup status as was originally determined by standard latex slide agglutination. SS-PCR confirmed that all nine isolates

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selected from Cluster A-1 for further MLST analysis were positive for the meningococcal serogroup A capsule (Table 5).

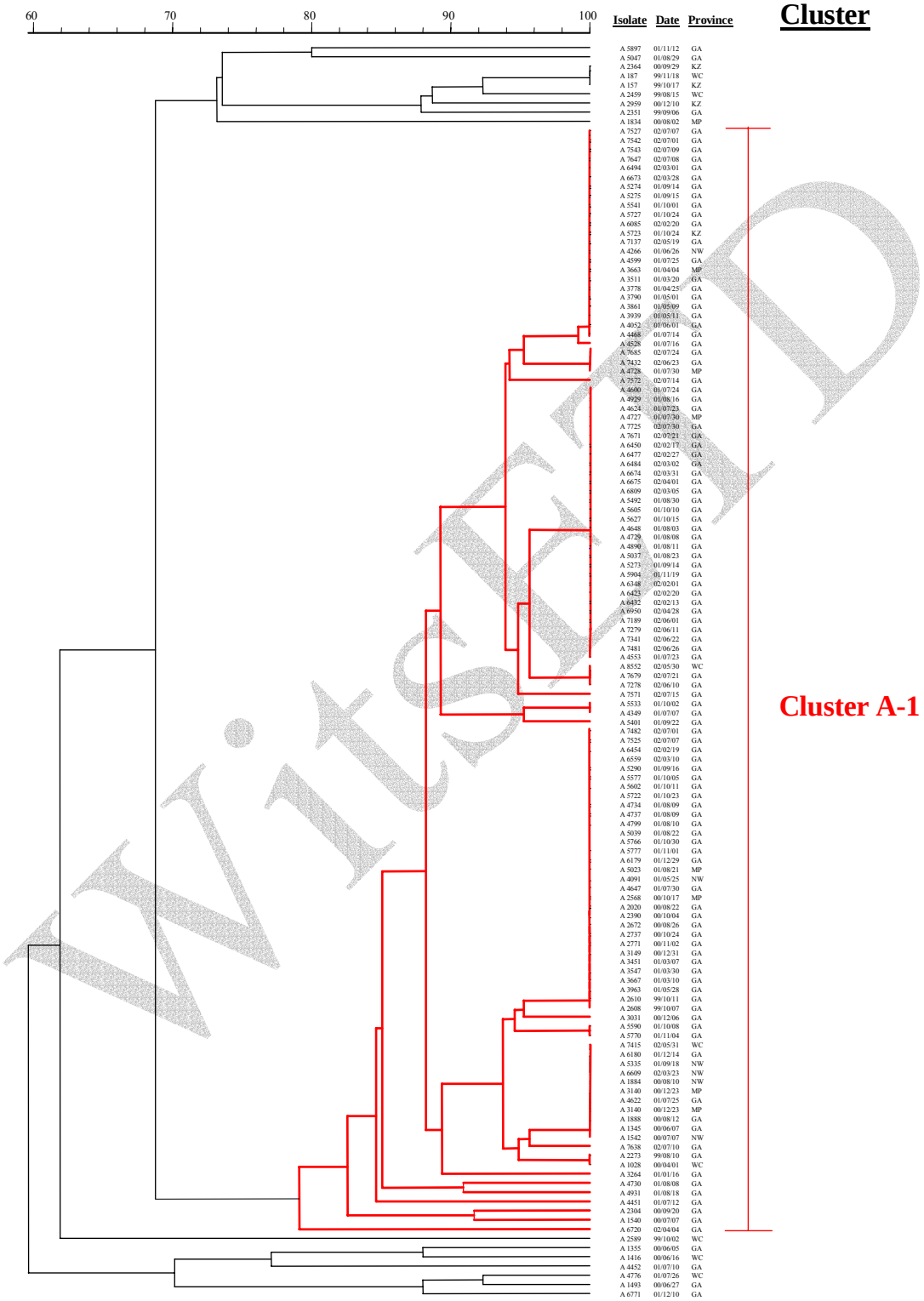
MLST analysis of these nine isolates from Cluster A-1 revealed that all the isolates selected from this complex had identical allelic profiles (Table 6). Isolates from this complex were all sequence type-1 (ST-1), the prototype ST for the broader ST-1 (Subgroup I/II) complex (Table 6, Fig. 5). An ST complex is a complex composed of closely-related STs that share homology in at least five of the sequencing loci (housekeeping genes). Therefore, it is probable that all isolates of Cluster A-1 belong to the ST-1 complex, meaning that the 88% of all serogroup A *Neisseria meningitidis* isolates collected in South Africa from August 1999 to July 2002 belong to the ST-1 (Subgroup I/II) complex. This clonal complex alone was responsible for 96% of the cases of meningococcal disease associated with serogroup A in the final year of the study.

Outliers

Sixteen isolates (12% of the total) in total did not fall into Cluster A-1. These isolates were distributed among 14 different pulsotypes, with only one pulsotype having multiple isolates (Fig. 4) and were not further characterized.

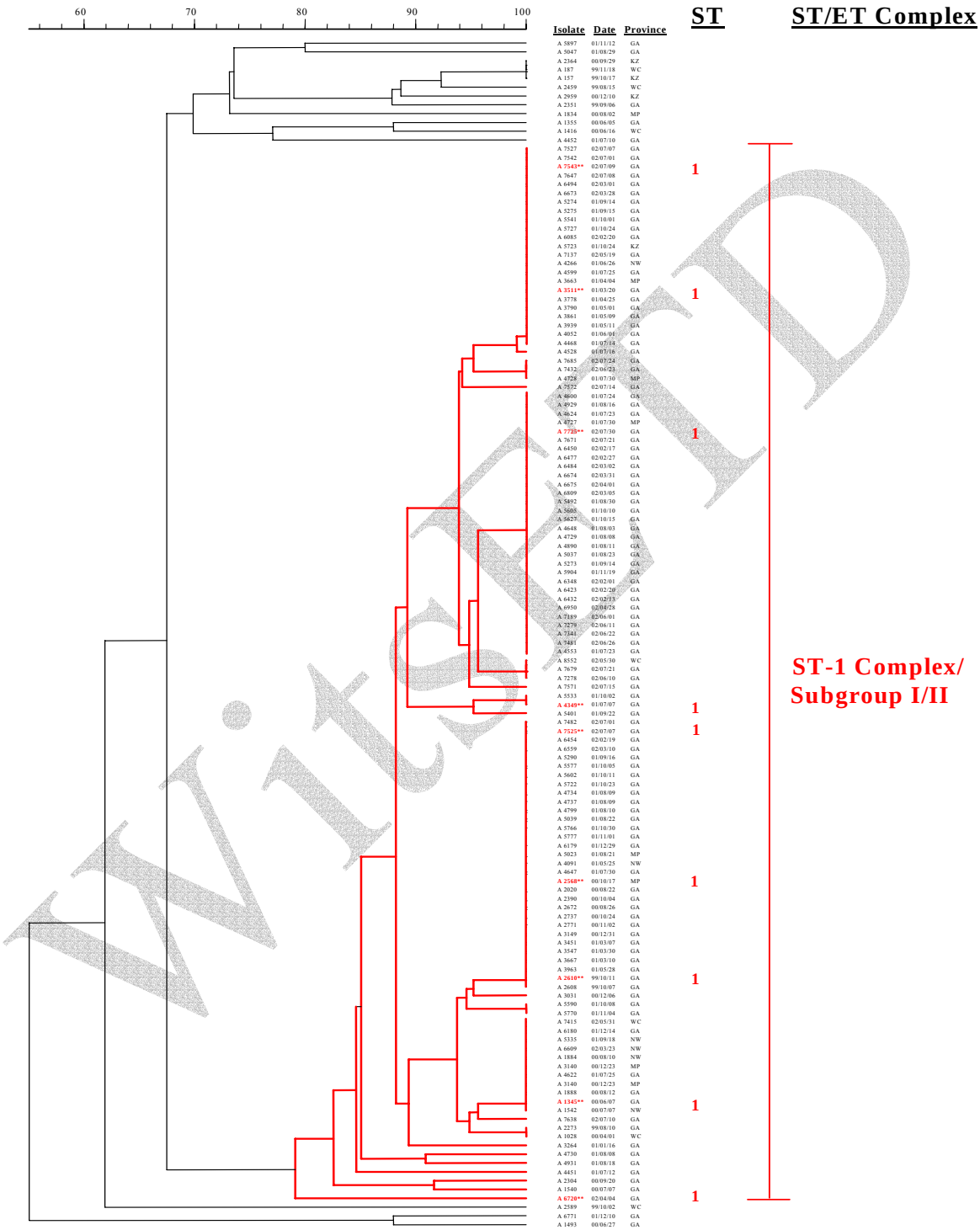
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Figure 4. PFGE Dendrogram Showing the Genetic Relationship Among Serogroup A Meningococci in South Africa August 1999 – July 2002



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Figure 5. PFGE Dendrogram of Serogroup A Meningococci Showing MLST Associations



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3.2 Genotypic Characterization of Serogroup B in South Africa

During the period August 1999-July 2002, a total number of 248 serogroup B *N. meningitidis* strains were collected through the national surveillance program. Of these isolates, PFGE analysis was performed on 245 of the isolates. The remaining 3 isolates were not available for genotypic comparison as they could not be recovered from the frozen stocks either as a result of severe contamination or the isolates had become non-viable during the freezing process.

Cluster B-1

Of the 245 isolates of serogroup B *N. meningitidis* analyzed by PFGE, a total number of 162 PFGE patterns were observed. From the PFGE dendrogram (Fig. 6), three predominant complexes (Cluster B-1, Cluster B-2 and Cluster B-3) could be seen. The largest of these three complexes (Cluster B-1) contained 37% (90/245) of all the isolates, and consisted of 31 different pulsotypes (19% of all pulsotypes). The isolates in this complex shared >88% similarity with one another. Cluster B-1 remained stable for the first two years of the study (Aug '99-Jul '01) where it made up 35% (37/107 and 27/77, respectively) of all the serogroup B isolates for both years (Table 7). In the third year (Aug '01-Jul '02), the proportion of cases associated with Cluster B-1 serogroup B meningococci increased to 43% (25/58). This increase appeared to be predominantly as a result of an increase in Cluster B-1 cases in the Western Cape, where it increased from 37% (31/83) in the first year, to 42% (22/53) and 58% (21/36) in the second and third years, respectively. In total, 82% of all the isolates from Cluster B-1 over the 3-year period were from the Western Cape (Table 8), where this cluster was individually responsible for 41% (72/175) of the total number of cases of invasive meningococcal disease during the 3-year period in this province. Nine percent of all the isolates from Cluster B-1 were from Gauteng where it caused 22% (8/36) of the cases during the 3-year period in this province (Table 8).

Five representative isolates from this predominant complex (Cluster B-1) were selected for further genotypic characterization by MLST. Isolates spanning collection dates

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throughout all three years of the study were chosen. These five isolates were subjected to serogroup-specific PCR to confirm their serogroup status as was determined by standard latex slide agglutination. SS-PCR confirmed that all five isolates selected from Cluster B-1 for further MLST analysis were positive for the meningococcal serogroup B capsule (Table 5).

MLST analysis of the five representative isolates from Cluster B-1 revealed that they all shared near-identical allelic profiles (Table 6). Four of the five isolates had identical profiles correlating with ST-33, whereas the fifth isolate (isolate 342) had a different allele (allele-40 as opposed to allele-4) at the *fumC* locus. This particular allelic profile had never been reported before to the global MLST database (http://pubmlst.org/neisseria/) and, consequently, a new ST (ST-4239) was assigned to this isolate. All five of these isolates shared closely-related STs which belonged to the ST-32 complex/ET-5 complex (Table 6, Fig. 7).

Cluster B-2

The second largest complex (Cluster B-2) consisted of 22 isolates (9% of the total number of isolates) which displayed 12 distinct pulsotypes among them (Fig. 6). The isolates of this complex shared >83% similarity to one another. Cluster B-2 was predominantly found in the Western Cape (10/19) and Gauteng (8/19), with the remaining isolates from Eastern Cape (2/19), Kwazulu Natal (1/19) and Free State (1/19) (Table 8). Cluster B-2 was identified in 8% (9/107) of the isolates in the first year (Aug '99- Jul '00). This proportion subsequently decreased to 5% (4/77) in the following year, and then increased to 14% (9/58) of the total number of serogroup B meningococcal isolates in the third year (Aug '01-Jul '02) (Table 7). This decrease and increase seem to correlate with a corresponding decrease and increase in the proportion of Cluster B-2 isolates in Gauteng. In Gauteng, the proportion of disease caused by isolates of this cluster decreased from 27% (3/11) in the first year, to 0% (0/9) in the second year, and finally increased to 36% (5/15) in the third year.

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Three representative isolates of Cluster B-2 were selected for further MLST characterization. SS-PCR on these isolates confirmed that they were all positive for the meningococcal serogroup B capsule (Table 5). MLST analysis on the three isolates from Cluster B-2 revealed that all three isolates shared near-identical allelic profiles (Table 6). Two of the three isolates (isolates 1140 and 2255) had identical allelic profiles corresponding to ST-154, whereas the third isolate (isolate 5580) had a novel allele at the *abcZ* locus. The novel allele, subsequently assigned as allele-212, differed from allele-3 seen in the other two isolates by only single G>A mutation at position 59 in the *abcZ* locus. The resulting new ST for this isolate was assigned as ST-4242. All three of these isolates shared closely-related STs that belonged to the ST-41/44/Lineage 3 complex (Figure 7, Table 6).

Cluster B-3

The third complex (Cluster B-3) consisted of 15 isolates (6% of the total number of isolates) from which 12 different pulsotypes could be defined (Fig. 6). This complex was composed of isolates sharing >86% similarity to one another. Seven percent (7/107) of the total number of serogroup B isolates in the first year were associated with this complex. This figure decreased to 3% (2/77) in the second year and then increased to 10% (6/58) in the third year (Table 7). This complex showed no primary concentration in any given province, but rather was dispersed amongst several different provinces (Table 8).

A single isolate from Cluster B-3 was selected for MLST analysis. This isolate was subjected to serogroup-specific PCR to confirm its serogroup status as was determined by standard latex slide agglutination originally. SS-PCR confirmed that the isolate selected was positive for the meningococcal serogroup B capsule (Table 5). MLST analysis on this single isolate from Cluster B-3 revealed that the isolate had an allelic profile consistent with ST-35 meningococci (Table 6). ST-35 isolates, grouped together with a several other closely-related STs, form part of the broader ST-35 complex.

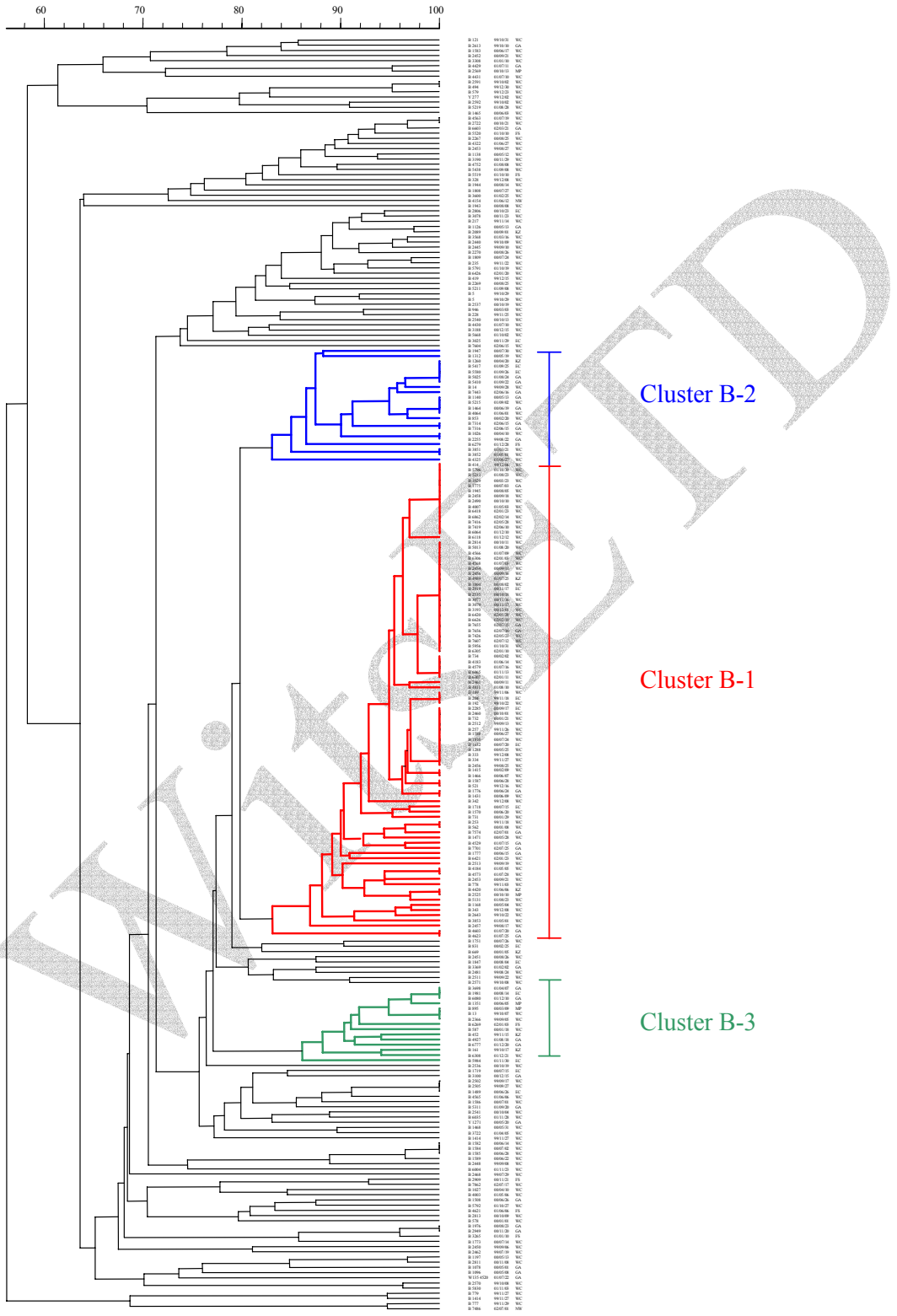
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Outliers

The remaining 118 (48% of all the isolates) isolates not falling into any of the three main clusters mentioned above were distributed among 109 pulsotypes (Fig. 6). Only six of the pulsotypes had more than one isolate associated with them, but never more than three. No further genotypic characterization by MLST of these strains was conducted.

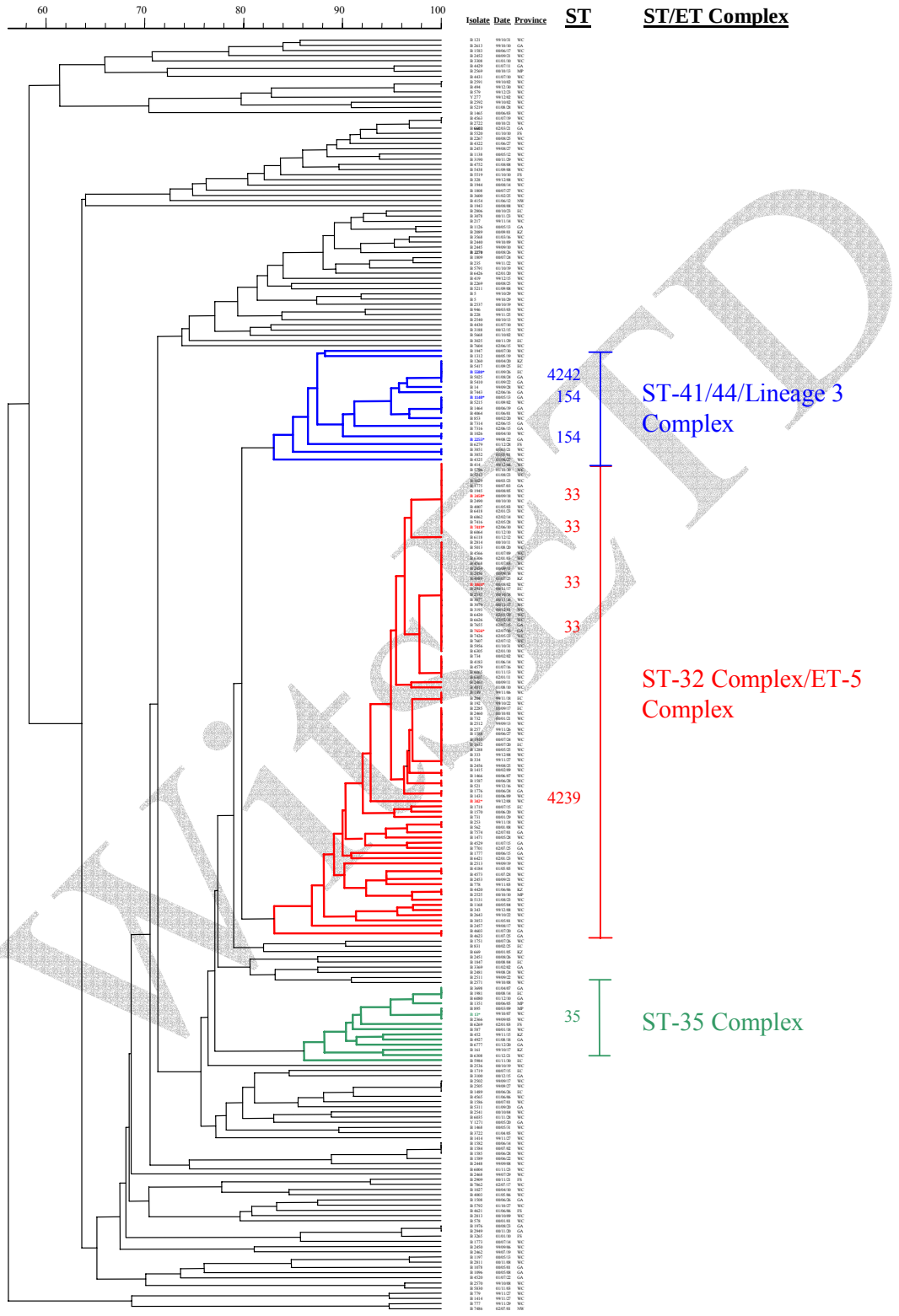
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Figure 6. PFGE Dendrogram Showing the Genetic Relationship Among Serogroup B Meningococci in South Africa August 1999 – July 2002



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Figure 7. PFGE Dendrogram of Serogroup B Meningococci Showing MLST Associations



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3.3 Genotypic Characterization of Serogroup C in South Africa

During the period August 1999-July 2002, a total number of 50 serogroup C *N. meningitidis* strains were collected through the national surveillance program. PFGE analysis was performed on 49 of the isolates. The remaining isolate was not available for genotypic comparison as it could not be recovered from the frozen stocks either as a result of severe contamination or the isolate had become non-viable during the freezing process.

Analysis of the 49 serogroup C meningococcal isolates by PFGE revealed a total of 37 different PFGE patterns (Fig. 8). Three main complexes (Cluster C-1, Cluster C-2 and Cluster C-3) could be clearly defined from the phylogenetic tree generated from the PFGE patterns.

Cluster C-1

The largest cluster of the three, Cluster C-1, was composed of 13 isolates (27% of the total number of isolates) distributed amongst a total of six different pulsotypes (Fig. 8). Isolates within this cluster shared > 81% similarity with one another, with the majority (11/13) sharing > 92% similarity. In the first year, strains of this cluster caused 25% (5/20) of serogroup C disease. This proportion increased to 42% (5/12) in the second year, and in the third year, this proportion subsided to 18% (3/17) (Table 7). Strains from this complex were distributed amongst several provinces and showed no particular concentration in a given province (Table 8).

Two representative isolates were selected from Cluster C-1 for further MLST characterization. Serogroup-specific PCR was performed on these two isolates to confirm their serogroup as originally determined by standard latex slide agglutination. SS-PCR results confirmed that both of the isolates were positive for serogroup C meningococcal capsule (Table 5). MLST analysis on the two isolates from Cluster C-1 revealed that both had identical allelic profiles consistent with ST-11 isolates (Table 6). ST-11 and a number of other closely-related STs form part of the broad ST-11/ET-37

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complex (Fig. 9, Table 6). Thus, 27% of all serogroup C isolates collected from August 1999 to July 2002 belonged to the ST-11/ET-37 complex.

Cluster C-2

The second major cluster observed, Cluster C-2, consisted of 9 isolates (18% of the total number of isolates) displaying a total of 6 different pulsotypes sharing >82% similarity with one another (Fig. 8). Isolates in this cluster were isolated throughout the duration of the study. The proportion of serogroup C cases caused by strains of this cluster demonstrated an increasing trend throughout the three years, with 15% (3/20) of serogroup C cases in the first year associated with this complex, and 17% (2/12) and 24% (4/17) in the following two years, respectively (Table 7). Isolates from this cluster came exclusively from the Western Cape (Table 8).

Two isolates from Cluster C-2 were chosen to be further characterized by MLST. SS-PCR on these isolates indicated that were both positive for serogroup C meningococcal capsule as was originally determined my latex slide agglutination (Table 5). MLST on the two representative isolates from Cluster C-2 indicated that both these isolates had identical allelic profiles belonging to ST-865 (Table 6), suggesting that 18% of our serogroup C isolates belonged to this particular ST type (Fig. 9, Table 6). This particular ST is not associated with any broader ST complex.

Cluster C-3

From the phylogenetic tree generated from the PFGE patterns of all the serogroup C isolates investigated, a third complex (Cluster C-3) was also evident (Fig. 8). This complex consisted of 8 isolates (16% of the total number of serogroup C isolates), sharing >86% similarity to one another, that were distributed amongst five different pulsotypes. All the isolates from this cluster came from the Western Cape exclusively, and were collected throughout the duration of the 3-year study (Table 8). Twenty-percent (4/20) of serogroup C isolates in the first year of the study came from this cluster. This proportion dropped to 8% (1/12) in the second year, and then increased to 18% (3/17) in the third year (Table 7).

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Two representative isolates from this cluster were selected for MLST analysis. SS-PCR conducted on both of these isolates confirmed that they were serogroup C meningococci as was originally predicted by latex slide agglutination (Table 5). MLST analysis of the two isolates from Cluster C-3 showed that both of these isolates had identical allelic profiles (Table 6). These two isolates were of the sequence type, ST-33. This particular ST, and a number of closely-related STs, is associated with a large clonal complex known as the ST-32/ET-5 complex (Fig. 9, Table 6).

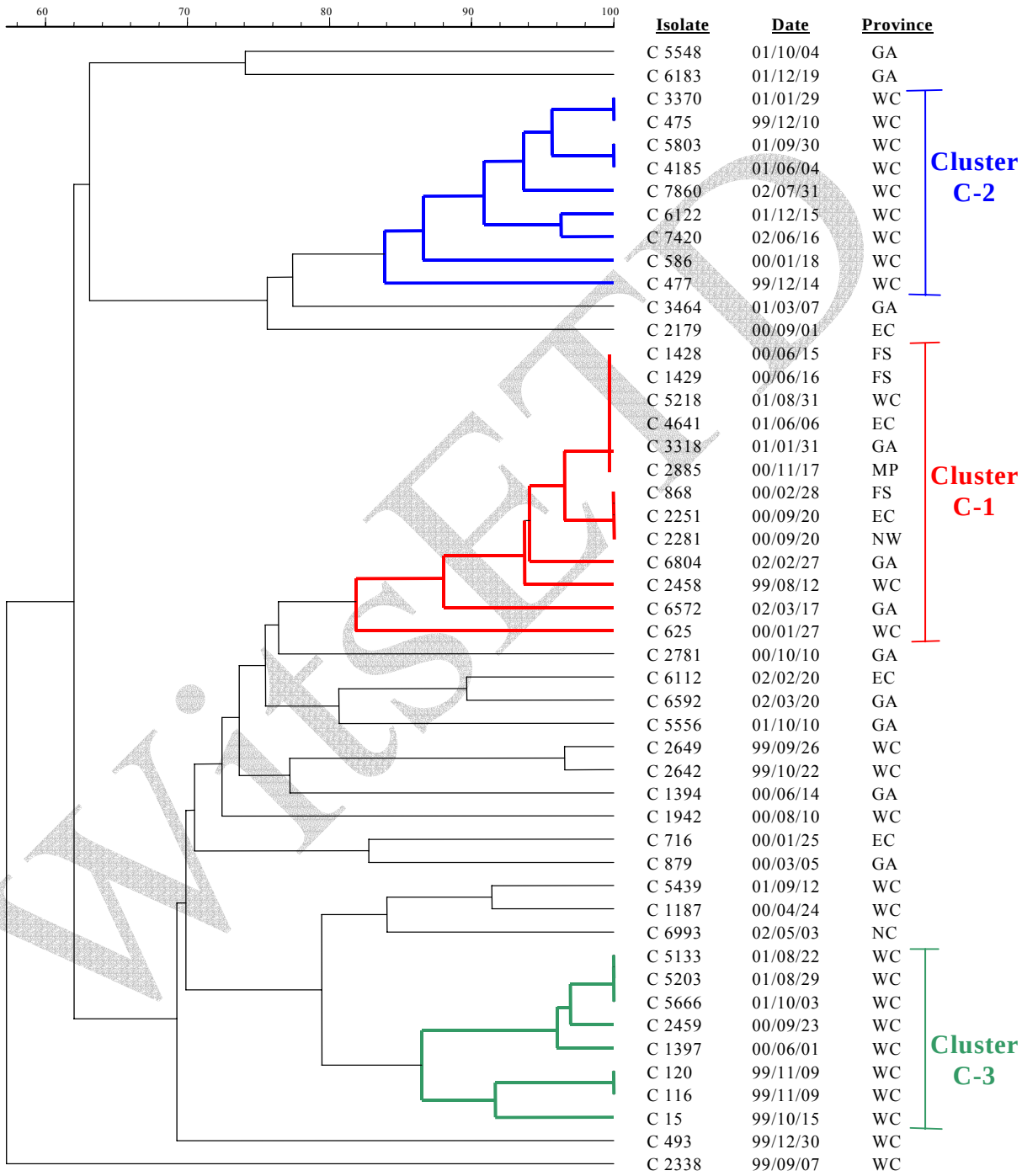
This ST-32/ET-5 complex is commonly associated with serogroup B meningococci, and evidence was found of the presence of serogroup B ST-32/ET-5 complex meningococci circulating in South Africa. To identify the genetic relationship, if any, between isolates of these two serogroups belonging to the ST-32/ET-5 complex, representative isolates from both serogroup B and C belonging to this complex were subjected to PFGE to analyze the similarity of their fingerprints (Figure 10). PFGE analysis revealed a close relationship between isolates of serogroup B and C belonging to the ST-32/ET-5 complex, with typically less than a three band difference between the isolates of both serogroups.

Outliers

The remaining 19 isolates (39% of all total number of serogroup C meningococci analyzed), not falling into any of the 3 major clusters, were distributed as single isolates belonging to 19 different pulsotypes (Fig. 8). No representative isolates were chosen from these outlier isolates for further characterization by MLST.

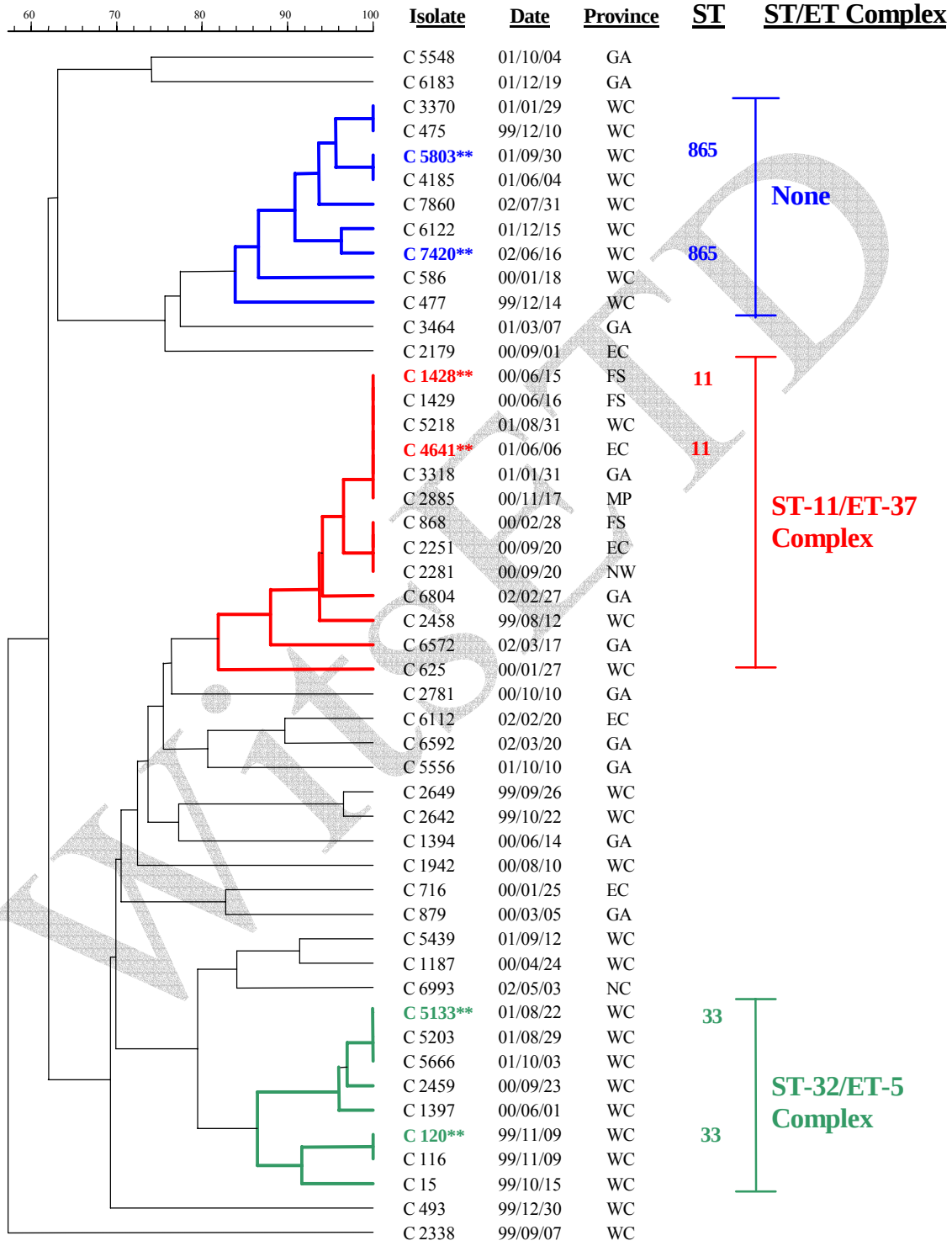
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Figure 8. PFGE Dendrogram Showing the Genetic Relationship Among Serogroup C Meningococci in South Africa August 1999 – July 2002



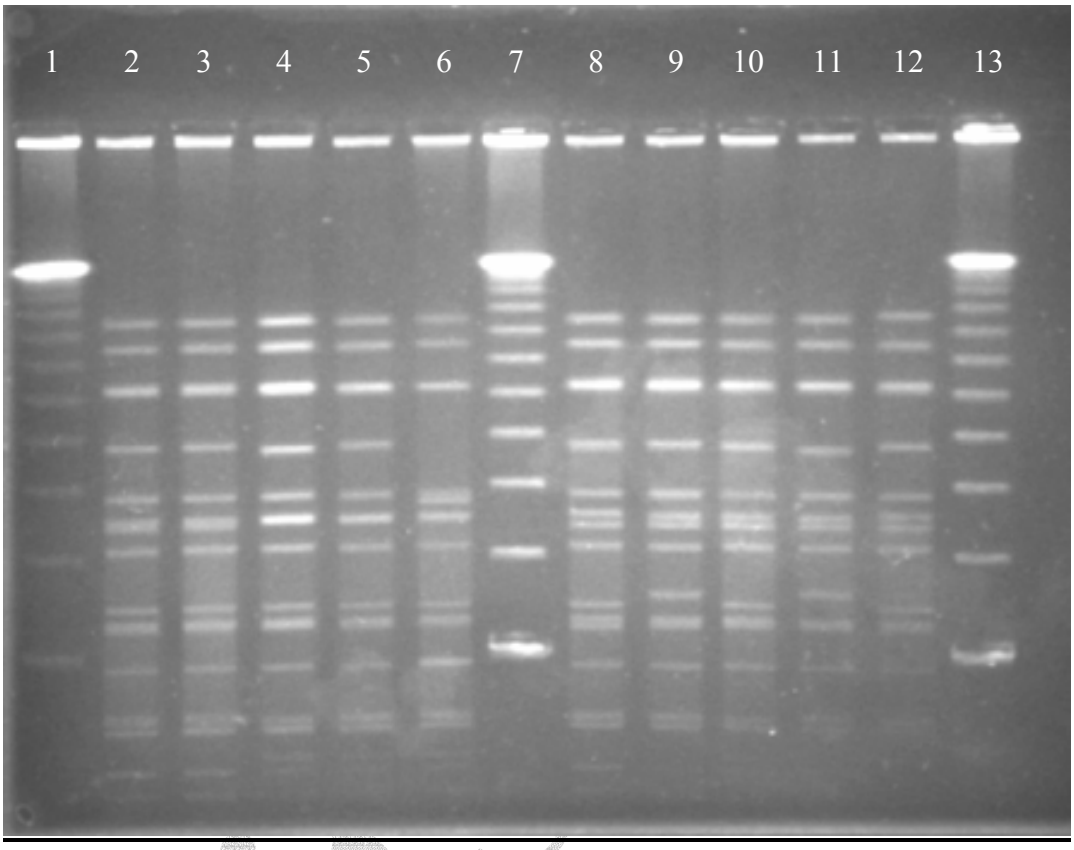
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Figure 9. PFGE Dendrogram of Serogroup C Meningococci Showing MLST Associations



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Figure 10. PFGE 1% Agarose Gel showing the Genetic Relationship between Serogroup B and Serogroup C Meningococci of the ST-32/ET-5 Complex



Lane 1, 7 and 13: Lambda DNA Molecular Weight Marker. **Lanes 2-6:** Serogroup C Isolates 5133, 5203, 120, 116 and 15. **Lanes 8-12:** Serogroup B Isolates 1029, 6418, 4566, 1431 and 1288.

3.4 Genotypic Characterization of Serogroup W135 in South Africa

During the period August 1999-July 2002, a total number of 32 serogroup W135 *N. meningitidis* strains were collected through the national surveillance program. PFGE analysis was performed on 30 of the isolates. The remaining isolate was not available for genotypic comparison as it could not be recovered from the frozen stocks either as a result of severe contamination or the isolate had become non-viable during the freezing process.

Cluster W-1

From the total number of 30 serogroup W135 meningococci isolates analyzed by PFGE, 14 different PFGE patterns (pulsotypes) were observed (Fig. 11). A distinct cluster of closely-related isolates (Cluster W-1), comprised 73% (22/30) of the total number of isolates. This cluster was represented by 7 pulsotypes sharing >83% similarity to one another. In the first year of the study, Cluster W-1 accounted for 56% (5/9) of the isolates analyzed. This increased to 88% (7/9) in the second year, and then decreased to 77% (10/13) in the final year of the study (Table 7).

Of the isolates collected countrywide, 77% (17/22) of the serogroup W135 meningococcal isolates from Cluster W-1 came from Gauteng alone (Table 8). In Gauteng, this cluster caused 85% (17/20) of the cases associated with serogroup W135 meningococcal disease, with some variation seen in the number of cases throughout the three-year period. In the first year, 67% (2/3) of isolates in Gauteng were associated with Cluster W-1. This proportion increased to 100% (8/8) in the following year, and then subsequently decreased to 78% (7/9) in the third year.

A total of five representative isolates from Cluster W-1 were selected for further MLST analysis (Table 5). These isolates were selected from different provinces and spanned collection dates throughout the three year period of the study. The five isolates were subjected to serogroup-specific PCR to confirm that they were serogroup W135 meningococci as was originally determined by standard latex slide agglutination. SS-

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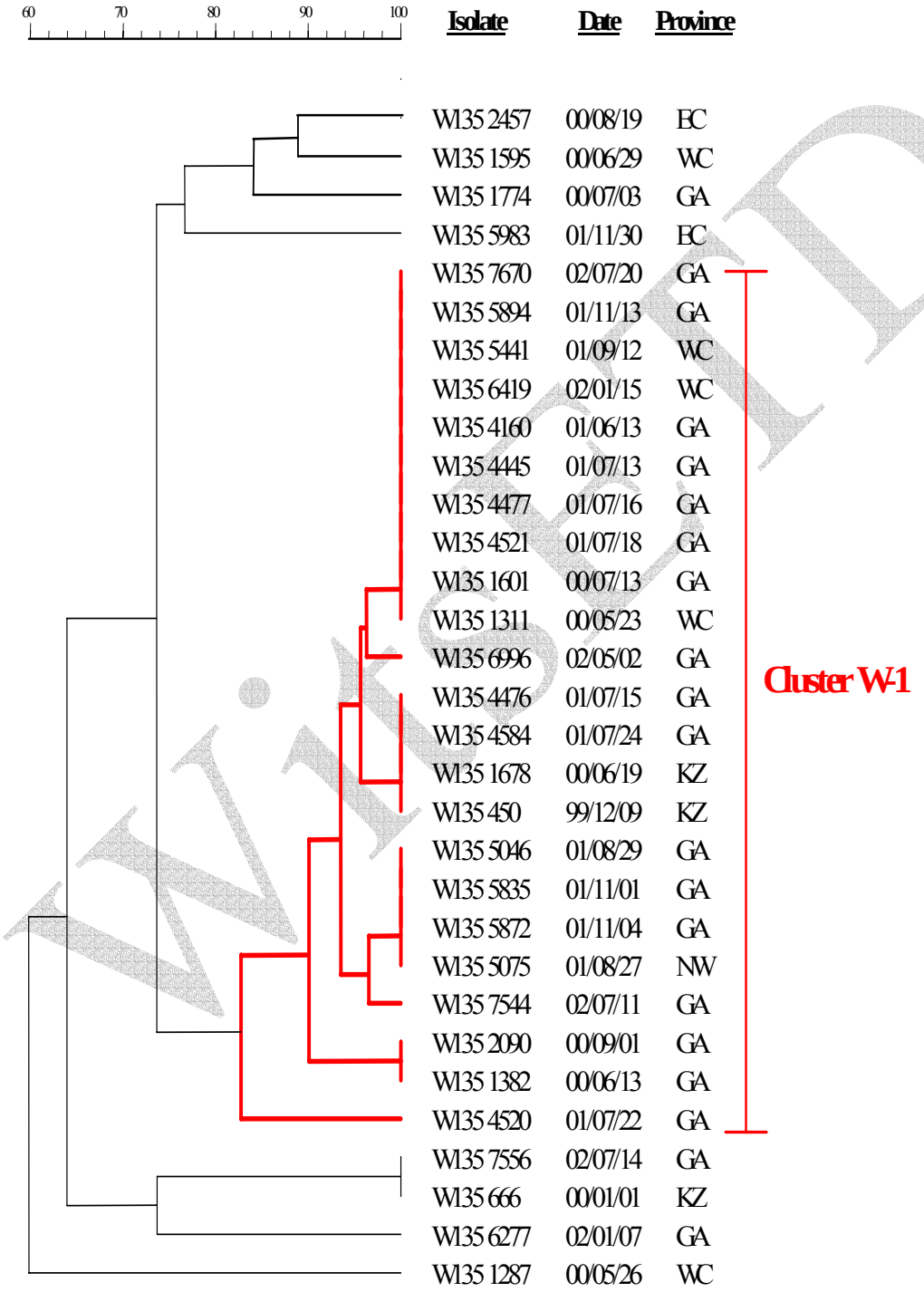
PCR results indicated that four of the five isolates were positive for the *N. meningitidis* serogroup W135 capsule (Table 5). MLST analysis on these four serogroup W135 isolates from Cluster-W1 showed that each of these isolates had an identical allelic profile which was consistent with ST-11 meningococci (Table 6). ST-11 meningococci, together with several other closely-related STs, belong to a large clonal complex known as the ST-11/ET-37 complex (Fig. 12, Table 6). The remaining isolate (isolate 4520) possessed a serogroup B meningococcal capsule. This isolate was subsequently removed from the serogroup W135 database and added to the serogroup B database.

Outliers

The remaining 8 isolates (27% of the total number of serogroup W135 meningococci analyzed by PFGE) were distributed among 7 different pulsotypes, with only one of the pulsotypes having >1 isolate (but not more than 2 isolates). No representative isolates from the outliers were selected for further MLST characterization.

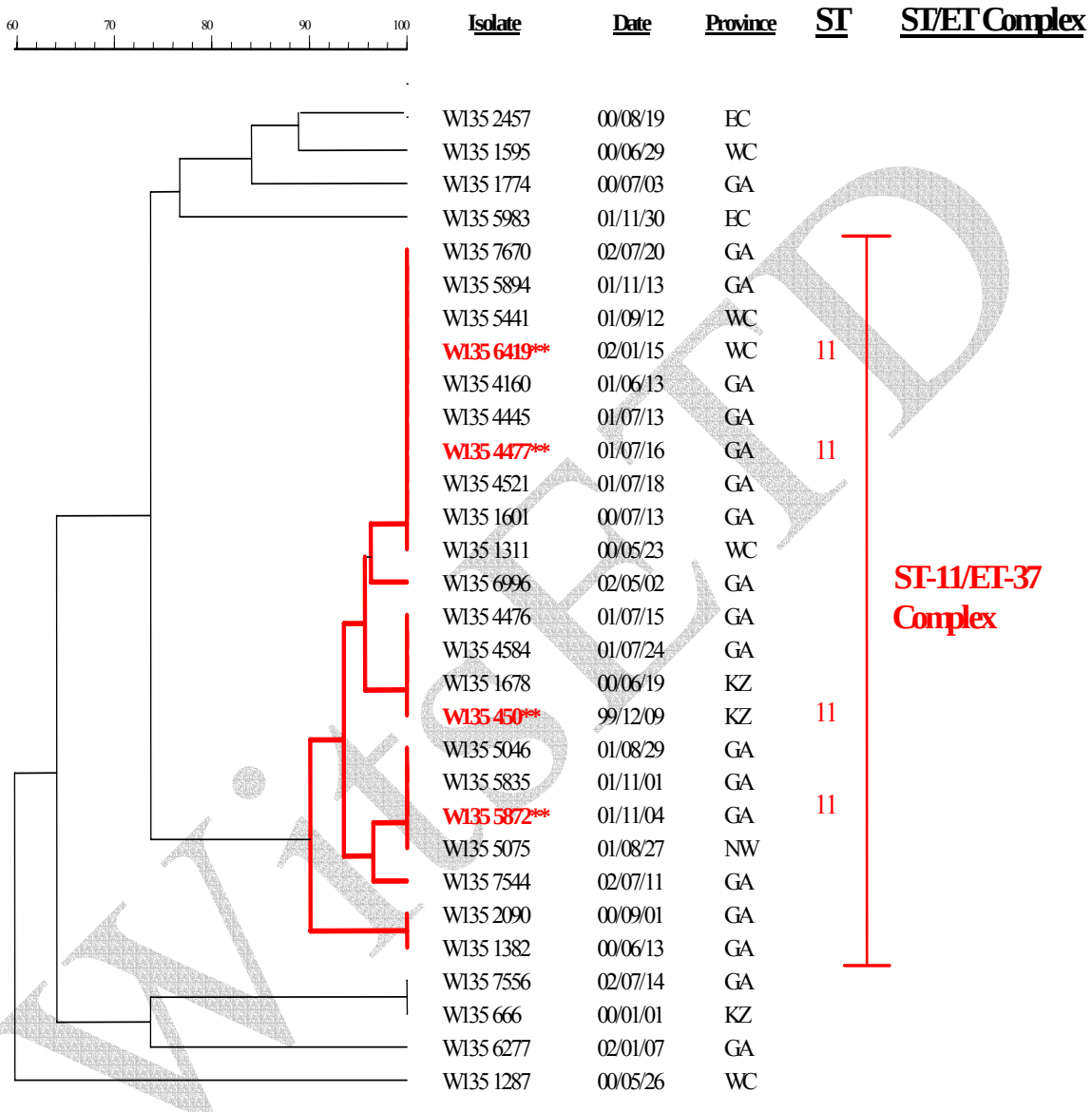
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Figure 11. PFGE Dendrogram Showing the Genetic Relationship Among Serogroup W135 Meningococci in South Africa August 1999 – July 2002



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Figure 12. PFGE Dendrogram of Serogroup W135 Meningococci Showing MLST Associations



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3.5 Genotypic Characterization of Serogroup Y in South Africa

During the period August 1999-July 2002, a total number of 130 serogroup Y *N. meningitidis* strains were collected through the national surveillance program. PFGE analysis was performed on 127 of the 130 isolates. The remaining three isolates were not available for genotypic analysis as they could not be recovered from the frozen stocks, either as a result of severe contamination or the isolates had become non-viable during the freezing process.

Analysis of the 127 serogroup Y meningococcal isolates by PFGE revealed a total of 49 distinct PFGE patterns (pulsotypes) (Fig. 13). The vast majority of these pulsotypes were distributed among two distinct clonal complexes (Cluster Y-1 and Cluster Y-2).

Cluster Y-1

The larger of the two complexes, Cluster Y-1, consisted of 68% (86/127) of the total number of isolates. These isolates were distributed amongst a total of 18 different pulsotypes that shared >82% similarity to one another. Serogroup Y meningococcal disease associated with this cluster decreased from 74% (28/38) in the first year, to 56% (22/39) in the second year, and then increased to 72% (36/50) in the final year.

Thirty-percent (26/86) of the strains belonging to this cluster were found in the Free State, where it caused 81% (26/32) of serogroup Y meningococcal disease in the region. In the Free State, the proportion of cases attributed to Cluster Y-1 was 67% (4/6) in the first year, and 83% (10/12) and 85% (11/13) in the second and third years respectively.

Twenty-eight percent (24/86) of the serogroup Y isolates belonging to Cluster Y-1 were found in Gauteng, where it was responsible for 56% (24/43) of disease associated with this serogroup. In Gauteng, the proportion of cases attributed to Cluster Y-1 was 80% (8/10) in the first year, and 40% (5/13) and 55% (11/20) in the second and third years respectively.

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A total of seven representative isolates were selected from Cluster Y-1 for further MLST analysis. These seven isolates were selected on the basis that they spanned most the provinces and collection dates from the entire time period of the study. The seven isolates were subjected to serogroup-specific PCR to confirm their serogroup as was originally predicted by standard latex slide agglutination. SS-PCR confirmed that each of the seven isolates were positive for the *Neisseria meningitidis* serogroup Y capsule (Table 5). MLST on these seven isolates from Cluster Y-1 revealed that each of the seven isolates all shared identical allelic profiles that were consistent with the sequence type, ST-175 (Table 6). This particular ST type does not belong to any particular ST complex. This MLST data suggests that the isolates in Cluster Y-1 probably all have ST-175 in common (Fig 14).

Cluster Y-2

A second, smaller clonal complex (Cluster Y-2) was also observed from PFGE analysis (Fig. 13). Cluster Y-2 consisted of a total of 15% (19/127) of the isolates collected during the three-year study. These 19 isolates were distributed amongst 15 different pulsotypes that shared >81% similarity to one another. The proportion of cases caused by this disease increased from 13% (5/38) in the first year, to 20% (8/39) in the second year, and then subsequently declined back to original levels of 12% (6/50) in the third year (Table 7). Although found in most provinces, 53% (10/19) of the serogroup Y isolates of this complex were found in Gauteng province (Table 8), where it was responsible for 23% (10/43) of the total cases associated with serogroup Y disease. Twenty-one percent (4/19) of isolates were from Free State (Table 8) where it caused 13% (4/32) of serogroup Y disease.

Three representative isolates from different provinces within Cluster Y-2 were selected for further MLST characterization. These three isolates were confirmed as serogroup Y meningococci by SS-PCR (Table 5). MLST analysis on these three isolates revealed that all three isolates had near-identical allelic profiles (Table 6). Two of the isolates (1160 and 1897) had identical STs that were identified as ST-23. The third (isolate 7471) differed slightly in its allelic profile in that it possessed allele-46 at the *abcZ* locus as

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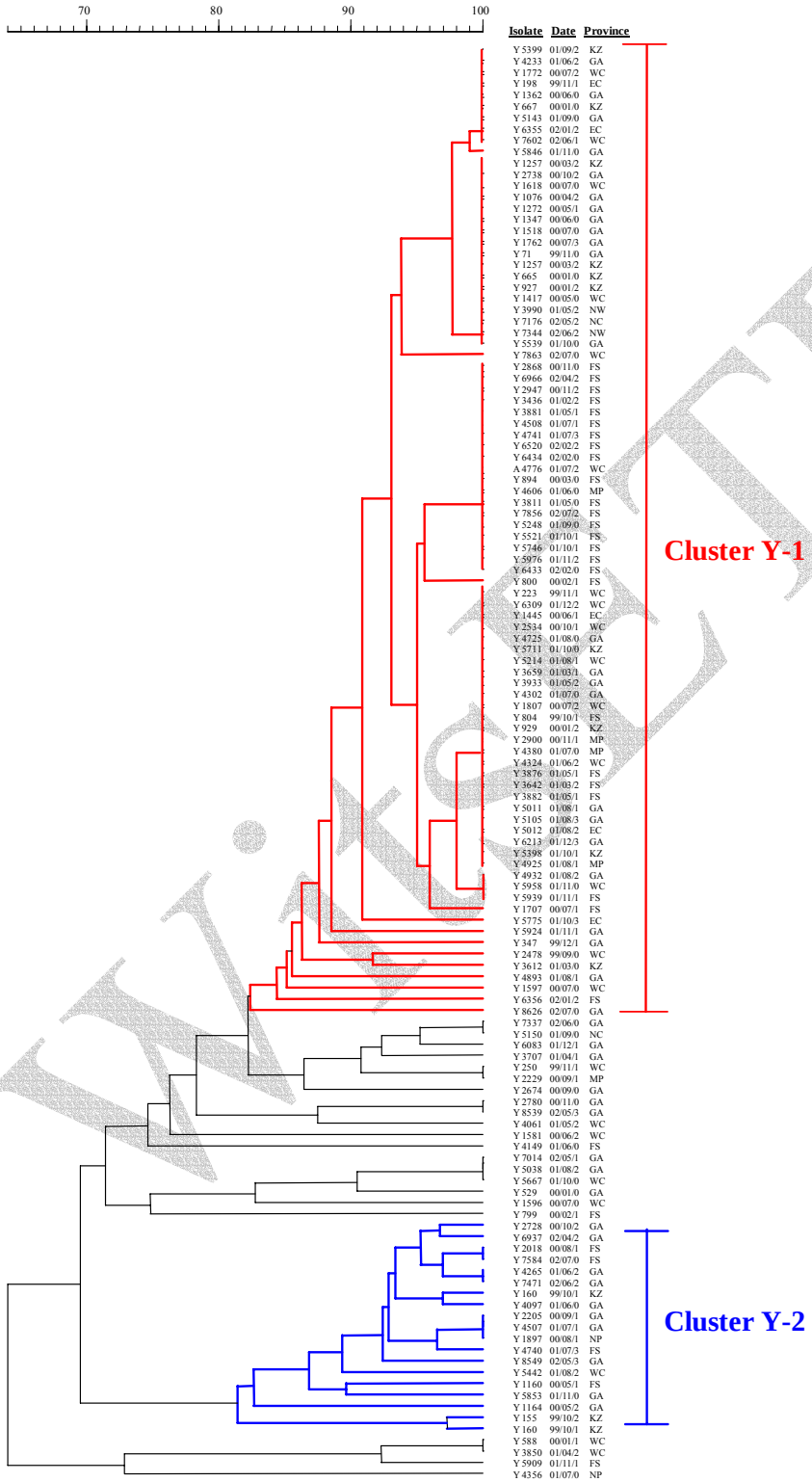
compared to allele-10 for the other two isolates. These two alleles differ from one another by 28 base pairs. This particular allelic profile, as seen in isolate 7471, was novel, and thus a new ST (ST-4245) was subsequently assigned to the isolate (Table 6). These two STs, ST-23 and ST-4245, group together with several other closely-related STs forming part of a broad clonal complex referred to as the ST-23 complex (Fig. 14, Table 6). Fifteen percent of serogroup Y meningococcal isolates from the period of this study therefore belonged to the ST-23 complex.

Outliers

The remaining 22 (17% of all the serogroup Y meningococci analyzed by PFGE) isolates that did not fall into either Cluster Y-1 or Y-2 were distributed among 25 different pulsotypes, with one pulsotype possessing three isolates, seven pulsotypes possessing two isolates, and the remaining pulsotypes occurring singly. Further MLST analysis was not conducted on any of these outlier isolates.

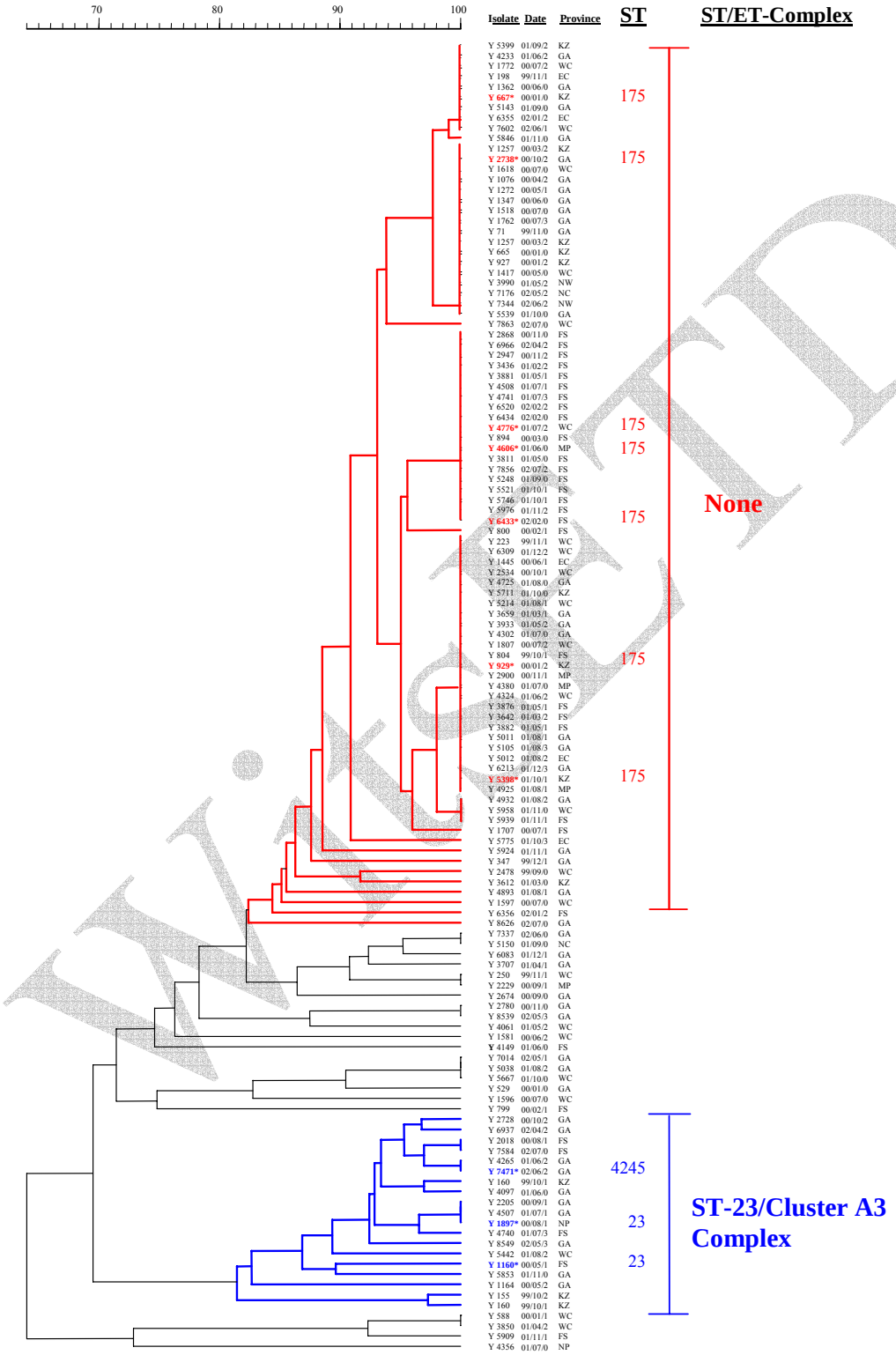
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Figure 13. PFGE Dendrogram Showing the Genetic Relationship Among Serogroup Y Meningococci in South Africa August 1999 – July 2002



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Figure 14. PFGE Dendrogram of Serogroup Y Meningococci Showing MLST Associations



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Table 5. Representative Isolates for MLST – Slide Agglutination and SS-PCR Results

SEROGROUP	CLUSTER	ISOLATE	SLIDE	SS-PCR	ST-Complex
A	Cluster A-1	1345	A	A	ST-1 Complex
		2568	A	A	ST-1 Complex
		2610	A	A	ST-1 Complex
		3511	A	A	ST-1 Complex
		4349	A	A	ST-1 Complex
		6720	A	A	ST-1 Complex
		7525	A	A	ST-1 Complex
		7543	A	A	ST-1 Complex
		7725	A	A	ST-1 Complex
B	Cluster B-1	342	B	B	ST-32 Complex
		1804	B	B	ST-32 Complex
		2458	B	B	ST-32 Complex
		7419	B	B	ST-32 Complex
		7565	B	B	ST-32 Complex
	Cluster B-2	1140	B	B	ST 41/44 Complex
		2255	B	B	ST 41/44 Complex
		2255	B	B	ST 41/44 Complex
	Cluster B-3	13	B	B	ST-35 Complex
C	Cluster C-1	1428	C	C	ST-11 Complex
		4641	C	C	ST-11 Complex
	Cluster C-2	5803	C	C	No Complex (ST-865)
		7420	C	C	No Complex (ST-865)
	Cluster C-3	120	C	C	ST-32 Complex
		5133	C	C	ST-32 Complex
W135	Cluster W-1	450	W135	W135	ST-11 Complex
		4477	W135	W135	ST-11 Complex
		5872	W135	W135	ST-11 Complex
		6419	W135	W135	ST-11 Complex
Y	Cluster Y-1	667	Y	Y	No Complex (ST-175)
		929	Y	Y	No Complex (ST-175)
		2738	Y	Y	No Complex (ST-175)
		4606	Y	Y	No Complex (ST-175)
		5398	Y	Y	No Complex (ST-175)
		6433	Y	Y	No Complex (ST-175)
	Cluster Y-2	1160	Y	Y	ST-23 Complex
		1897	Y	Y	ST-23 Complex
		7471	Y	Y	ST-23 Complex

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Table 6. MLST Allelic Profiles and Sequence Types (STs)

Serogroup	Cluster	Isolate	Alleles							ST	ST Complex
			abcZ	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*		
A	Cluster A-1	1345	1	3	1	1	1	1	3	**1**	ST-1/Subgroup I Complex
		2568	1	3	1	1	1	1	3	**1**	ST-1/Subgroup I Complex
		2610	1	3	1	1	1	1	3	**1**	ST-1/Subgroup I Complex
		3511	1	3	1	1	1	1	3	**1**	ST-1/Subgroup I Complex
		4349	1	3	1	1	1	1	3	**1**	ST-1/Subgroup I Complex
		6720	1	3	1	1	1	1	3	**1**	ST-1/Subgroup I Complex
		7525	1	3	1	1	1	1	3	**1**	ST-1/Subgroup I Complex
		7543	1	3	1	1	1	1	3	**1**	ST-1/Subgroup I Complex
		7725	1	3	1	1	1	1	3	**1**	ST-1/Subgroup I Complex
B	Cluster B-1	342	8	10	5	40	6	3	8	**4239***	ST-32/ET-5 Complex
		1804	8	10	5	4	6	3	8	**33**	ST-32/ET-5 Complex
		2458	8	10	5	4	6	3	8	**33**	ST-32/ET-5 Complex
		7419	8	10	5	4	6	3	8	**33**	ST-32/ET-5 Complex
		7656	8	10	5	4	6	3	8	**33**	ST-32/ET-5 Complex
	Cluster B-2	1140	3	6	9	5	11	6	9	**154**	ST-41/44/lineage III Complex
		2255	3	6	9	5	11	6	9	**154**	ST-41/44/lineage III Complex
		5580	212*	6	9	3	11	6	9	**4242***	ST-41/44/lineage III Complex
	Cluster B-3	13	4	10	11	18	6	10	12	**35**	ST-35 Complex

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Table 6. MLST Allelic Profiles and Sequence Types (STs) (Contd.)

Serogroup	Cluster	Isolate	Allele							ST	ST Complex
			abcZ	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*		
C	Cluster C-1	1428	2	3	4	3	8	4	6	**11**	ST-11/ET-37 Complex
		4641	2	3	4	3	8	4	6	**11**	ST-11/ET-37 Complex
	Cluster C-2	5803	8	5	15	17	8	21	2	**865**	None
		7420	8	5	15	17	8	21	2	**865**	None
	Cluster C-3	120	8	10	5	4	6	3	8	**33**	ST-32/ET-5 Complex
		5133	8	10	5	4	6	3	8	**33**	ST-32/ET-5 Complex
W135	Cluster W-1	450	2	3	4	3	8	4	6	**11**	ST-11/ET-37 Complex
		4477	2	3	4	3	8	4	6	**11**	ST-11/ET-37 Complex
		5872	2	3	4	3	8	4	6	**11**	ST-11/ET-37 Complex
		6419	2	3	4	3	8	4	6	**11**	ST-11/ET-37 Complex
	Cluster W-2	2457	11	5	18	76	11	24	21	**4241***	ST-22 Complex
Y	Cluster Y-1	667	6	7	4	56	26	18	8	**175**	None
		929	6	7	4	56	26	18	8	**175**	None
		2738	6	7	4	56	26	18	8	**175**	None
		4606	6	7	4	56	26	18	8	**175**	None
		4776	6	7	4	56	26	18	8	**175**	None
		5398	6	7	4	56	26	18	8	**175**	None
		6433	6	7	4	56	26	18	8	**175**	None

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Table 6. MLST Allelic Profiles and Sequence Types (STs) (Contd.)

Serogroup	Cluster	Isolate	Allele							ST	ST Complex
			abcZ	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*		
Y	Cluster Y-2	1160	10	5	18	9	11	9	17	**23**	ST-23 Complex
		1897	10	5	18	9	11	9	17	**23**	ST-23 Complex
		7471	46	5	18	9	11	9	17	**4245**	ST-23 Complex

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Table 7. Temporal Variation of the Major Clonal Complexes for Each Serogroup

SEROGROUP	COMPLEX	YEAR			TOTAL
		1 % (n/N)	2 % (n/N)	3 % (n/N)	% (n/N)
A	ST-1 Complex	47 (7/15)	91 (40/44)	96 (74/77)	89 (120/136)
	Outliers	53 (8/15)	9 (4/44)	4 (3/77)	11 (15/136)
B	ST-32 Complex	35 (37/107)	35 (27/77)	43 (25/58)	37 (90/245)
	ST-41/44 Complex	8 (9/107)	5 (4/77)	14 (9/58)	9 (22/245)
	ST-35 Complex	7 (7/107)	3 (2/77)	10 (6/58)	6 (15/245)
	Outliers	50 (54/107)	57 (44/77)	31 (18/58)	48 (118/245)
C	ST-11 Complex	25 (5/20)	42 (5/12)	18 (3/17)	27 (13/49)
	No Complex(ST-865)	15 (3/20)	17 (2/12)	24 (4/17)	18 (9/49)
	ST-32 Complex	20 (4/20)	8 (1/12)	18 (3/17)	16 (8/49)
	Outliers	40 (8/20)	33 (4/12)	41 (7/17)	39 (19/49)
W135	ST-11 Complex	56 (5/9)	88 (7/8)	77 (10/13)	73 (22/30)
	Outliers	44 (4/9)	12 (1/8)	23 (3/13)	27 (8/30)
Y	No Complex(ST-175)	74 (28/38)	56 (22/39)	72 (36/50)	68 (86/127)
	ST-23 Complex	13 (5/38)	20 (8/39)	12 (6/50)	15 (19/127)
	Outliers	18 (5/38)	23 (9/39)	16 (8/50)	27 (22/127)

n = number of isolates of the serogroup belonging to that particular complex collected in that given year.

N = total number of isolates collected during that year for that serogroup.

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Table 8. Geographic Distribution of the Major Clonal Complexes for Each Serogroup

SEROGROUP	COMPLEX	PROVINCE (%)								
		EC	FS	GA	KZ	MP	NC	NP	NW	WC
A	ST-1 Complex	-	-	86	<1	6	-	-	5	3
B	ST-32 Complex	6	-	9	2	1	-	-	-	82
	ST-41/44 Complex	9	5	36	5	-	-	-	-	45
	ST-35 Complex	13	7	27	13	13	-	-	-	27
C	ST-11 Complex	15	23	23	-	8	-	-	8	23
	No Complex(ST-865)	-	-	-	-	-	-	-	-	100
	ST-32 Complex	-	-	-	-	-	-	-	-	100
W135	ST-11 Complex	-	-	74	9	-	-	-	5	14
Y	No Complex(ST-175)	2	29	28	12	5	1	-	2	17
	ST-23 Complex	-	21	53	16	-	-	5	-	5

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CHAPTER 4: DISCUSSION

4.1 Molecular Epidemiology of Serogroup A in South Africa

Genotypic characterization of serogroup A meningococcal isolates from South Africa during the period August 1999 to July 2002 revealed that serogroup A meningococci circulating in the South African population were of a highly clonal nature, with PFGE fingerprint patterns remaining relatively stable over time (Bygraves and Maiden, 1992). This finding of the highly clonal nature of serogroup A in South Africa is not surprising, however. It has been well recognized that the rate of genetic variation in serogroup A meningococci is relatively slow, and consequently, serogroup A meningococci tend to have clonal population structures, with clones persisting for long periods of time (Jacobsson et al., 2003). Compared to the other serogroups, serogroup A meningococci are genetically more homogenous (Achtman, 1995). It has been shown that only a relatively small number of serogroup A clones seem to be present concurrently in one area (Olyhoek et al., 1987; Peltola et al., 1983). This was evident in our study, where in Gauteng, the province where the majority of serogroup A disease is reported, 93% of all the strains belonged to the ST-1/Subgroup I complex.

ST-1/Subgroup I Complex

In South Africa, the majority of meningococcal disease caused by serogroup A, during the period of the study, was caused by a small number of closely-related clones belonging to the ST-1/Subgroup I complex. This complex appeared to follow an increasing trend as it established itself in the population, with the proportion of serogroup A meningococcal disease caused by this complex increasing from 47% to 96% during the three year period.

The presence of ST-1/Subgroup I strains, particularly in such high proportion, is significant. Strains of Subgroup I have caused both endemic and epidemic disease worldwide for more than four decades now. Strains of Subgroup I first caused epidemics in the African meningitis belt and the Mediterranean in the 1960s (Olyhoek et al., 1987), and by the mid-1970s had spread causing epidemics in North and West Africa, Canada,

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the American Northwest and Brazil (Achtman et al., 1992; Olyhoek et al., 1987; Wang et al., 1992). Later epidemics in the mid-1970s to early 1980s in Nigeria, Rwanda and Burkina Faso (Olyhoek et al., 1987), and outbreaks in the mid-1980s in New Zealand (Schwartz et al., 1989) and Australia (Patel et al., 1997) were also caused by this particular subgroup.

Subgroup I meningococci have also been isolated worldwide from non-epidemic areas such as Pakistan, the Philippines, West Germany, the United States, Australia from the mid-1960s to early 1980s (Olyhoek et al., 1987). Subgroup I strains (specifically identified as ST-1 strains), were also isolated in Algeria in 1992 (Nicolas et al., 2000).

In South Africa, strains of Subgroup I-1 had been isolated from cases of endemic disease from 1968-1983 (McGee et al., 1998; Olyhoek et al., 1987), and were the cause of an outbreak in neighboring Mozambique in 1991 (Wang et al., 1992). Although it had not been isolated for many years in the meningitis belt (Nicolas et al., 1997), in 1996, McGee et al (1998) showed that most meningococcal isolates from this period in South Africa belonged to Subgroup I. The continued isolation of meningococcal strains belonging to the serogroup A Subgroup I complex suggests the persistent circulation of this clone in South Africa. Furthermore, not only is there persistent circulation in the population, but the increase in proportion of disease associated with this particular clonal complex during the period of study suggests that it has firmly established itself, and clonal expansion may be taking place.

It is interesting to note that 85% of all serogroup A disease in South Africa was reported from Gauteng, and ~90% of serogroup A disease in Gauteng was caused by isolates of the ST-1/Subgroup I complex. Therefore, strains of serogroup A (particularly of the ST-1/Subgroup I complex) appeared to be almost exclusively concentrated in this province. The exact reason for this concentration, and the apparent increase, in Gauteng is not clear. Endemic disease caused by serogroup A Subgroup I strains was evident in South Africa since the late 1960s (Olyhoek et al, 1987). Since the 1970s till quite recently, the number of cases due to serogroup A countrywide decreased as serogroup B emerged as

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the dominant serogroup in the country (DOH, 1998). This decrease in serogroup A disease may have been a result of the continuous exposure of the general population to serogroup A disease. Through continued exposure, an increase in the population herd immunity to this serogroup would have been expected. Consequently, an increase in herd immunity may have lead to a decrease in the number of susceptible individuals, and therefore, the number of cases of disease associated with this serogroup. Although the prevalence of serogroup A decreased countrywide as a result, it is possible that the steady influx of travelers and immigrants, from serogroup A endemic countries in southern Africa, into the financial and mining hub of Gauteng, may have provided a source of repeated introduction of Subgroup I into this province. Alternatively, if these immigrants were from non-endemic regions, they may have provided a steady population of susceptible individuals. Either of these possibilities, or both, may have allowed serogroup A meningococci of the Subgroup I complex to firmly establish itself in the population in Gauteng.

Additionally, the possible roles that environmental or socioeconomic factors (e.g. crowding) in this particular geographic region may be playing can not be excluded (Moore, 1994; Tzeng and Stephens, 2000).

It is interesting to note that Subgroups III and IV-I, two resident subgroups in the African meningitis belt which have been continuously isolated from endemic meningitis since the early 1960s, as well as from epidemics in the early 1960s and 1980s (Olyhoek et al., 1987), were not found at all during the three year period of this study. Major epidemics due to Subgroup III in particular have occurred throughout Africa, including Burundi, Chad, Ethiopia, Guinea, Kenya, Niger, Nigeria, Nepal, Saudi Arabia, Senegal, Sudan, United Republic of Tanzania and Zambia (Haimanot et al., 1990; Luo et al., 1998; Nicolas et al., 2000; Norheim et al., 2004; Olyhoek et al., 1987; Pinner et al., 1992; Salih et al, 1990; WHO, 1995). McGee et al (1998) documented the first evidence of clone III-1 in South Africa in 1996, where it was found that 15% of serogroup A isolates during belonged to this subgroup.

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4.2 Molecular Epidemiology of Serogroup B in South Africa

Most serogroup B meningococcal populations are non-clonal, or consist of short-lived clones. Among endemic disease in the UK, it is rare to see a PFGE fingerprint represented more than once in a sample of such strains (Bygraves and Maiden, 1991; Yakubu and Pennington, 1995). For serogroup B, MLEE studies have shown that serogroup B lineages diversify during spread, resulting in complexes of genetically related, but non-identical, isolates (Olyhoek et al., 1987; Wang et al., 1992; Wang et al., 1993). Some serogroup B meningococci, however, do belong to well-defined clonal groupings which are repeatedly isolated from cases of disease (Achtman, 1995). A particular example of this is the ET-5 complex, which is a group of related meningococci that have caused hyperendemic disease in numerous countries since the 1970s (Caugant et al., 1987; Caugant et al., 1989). These organisms generally have conserved PFGE fingerprint patterns (Maiden, 1998).

In the present study of serogroup B meningococci in South Africa, we observed a genetic picture consistent with previous reports. Out of 254 serogroup B isolates, a total of 162 distinct pulsotypes were observed, indicating a fairly high degree of diversity in the serogroup B population. Greater than 50% of all the serogroup B meningococcal isolates analyzed by PFGE were non-clonal, non-identical isolates. The remaining isolates fell into one of three clonal complexes, the largest of which, the ST-32/ET-5 Complex, consisted of a large proportion of isolates sharing relatively conserved PFGE patterns.

ST-32/ET-5 Complex

In this study, three main complexes were observed. The largest of the three complexes, consisting of strains belonging to the ST-32/ET-5 complex, was responsible for 37% of all the cases of serogroup B meningococcal disease in South Africa during the period of the study. Of all the cases of disease associated with this complex, 82% were from the Western Cape alone (Table 8), where an increasing trend in proportion was noted over the three year period, as clones of this complex further established themselves in the population.

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The prevalence of the ST-32/ET-5 complex in South Africa is epidemiologically important. It has been well documented that the majority of strains causing serogroup B outbreaks and epidemics in industrialized countries are caused by strains of this complex. ET-5 strains were first identified in 1974 as the cause of a serogroup B epidemic in northern Norway (Caugant et al., 1986). Strains of this complex then spread to other parts of northern Europe in the late 1970s, and was identified as a prevalent clone of meningococcal outbreaks in several countries including Norway, Belgium and the UK (Caugant et al., 1986; Poolman et al., 1986). In the late 1970s to 1980s, it had spread to parts of Cuba, Chile, Brazil and Thailand (Cruz et al., 1990; Poolman et al., 1986; Sacchi et al., 1992). During the 1990s, ET-5 strains had also spread to North Africa, Israel and Australia (Caugant, 1998). In the 1990's an epidemic outbreak caused by the ET-5 clonal complex occurred in the U.S. Pacific Northwest, Oregon (CDC, 1995). By 1994, 14% of US endemic strains belonged to the ET-5 complex (Reeves et al., 1995), with an increasing presence in the Pacific Northwest.

Of the 119 STs that make up the serogroup B ST-32/ET-5 complex, the majority of our ET-5 complex strains were ST-33, which differs from the predominant ST in the ET-5 complex (ST-32) at only the *abcZ* locus, where it possesses allele-4 rather than allele-8. This particular ST, ST-33, was responsible for elevated disease in the UK (1988-1990) and Cuba (1992) (Bygraves et al., 1999)

In the late 1970s, strains of the ET-5 complex were discovered in isolates from South Africa (Caugant et al., 1986). To the best of our knowledge, this was the first report of ET-5 complex strains in South Africa. Although it was found that 25% of cases in the Cape Province, more complete data on the proportion of disease caused by this complex countrywide was not available from that study. Since its first identification in South Africa in the late 1970s, our data suggests that strains of this hypervirulent complex have established themselves throughout South Africa, with persistent circulation particularly in the Western Cape. At this particular juncture in time it is difficult to comment on the slight increase in the proportion of serogroup B disease associated with this complex from the second year of the study (35%) to the third year (43%) as one would normally

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expect some degree of variation in cases from one year to the next, as is the nature of endemic disease. Continued analysis of this complex in the years following our study would give a true indication of whether this is indeed an increasing phenomenon, or merely a reflection of annual variation.

ST-41/44/Lineage III Complex

In our study we report for the first time, to our knowledge, the presence of strains belonging to the hyperinvasive ST-41/44/lineage III complex in South Africa. This complex was responsible for 8% of serogroup B disease in South Africa during the three-year period of the study. Of particular interest is the fact that even though the greater majority of serogroup B disease occurs in the Western Cape, 42% of strains of the ST-41/41/lineage III complex were discovered in Gauteng (Table 8), a province almost exclusively associated with serogroup A meningococcal disease. While the absolute numbers of strains belonging to this complex are small, the persistent presence of this complex is concerning, as the ST-41/ST-44/lineage III complex has been associated with high virulence worldwide.

Strains of lineage III were first encountered in The Netherlands, and have caused disease there since the early 1980s (Caugant et al., 1990; Scholten et al., 1994). Subsequently, strains have spread to other European countries, including Finland, Norway, and Iceland, although only a few cases occurred in these countries (Caugant, 1998). In the early 1990s, New Zealand experienced a sharp rise in cases caused by lineage III strains (Martin et al., 1998), and in the second half of the 1990s, an increasing number of cases were observed in the United Kingdom, Belgium, Chile, Greece and Austria (Caugant, 1998; Van Looveren et al., 1998; Van Looveren et al, 2001). Recently, reports of the ST-41/44/lineage III complex causing disease in Japan and the Czech Republic have been published (Jolley et al, 2000; Takahashi et al., 2004).

Sequence type 154 (ST-154), the predominant ST seen in our South African isolates of the ST-41/44/lineage III complex, is one of approximately 712 unique STs belonging to the large ST-41/44/lineage III complex. Strains of invasive meningococcal disease

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specifically caused by ST-154 have been discovered previously in Canada, New Zealand, Australia and Taiwan (http://pubmlst.org/neisseria/; Maiden et al, 1998; Sacchi et al., 1998), and have been associated with epidemics.

ST-35 Complex

The third meningococcal serogroup B complex found in South Africa was the ST-35 complex. The ST-35 complex was found to cause 6% of serogroup B meningococcal disease in South Africa, and displayed a varied geography, with isolates of this complex coming from provinces throughout South Africa (Table 8). The ST-35 complex is composed of 135 unique STs, which are found amongst non-groupable isolates, as well as serogroup B and C meningococci. The majority of isolates of this complex, however, belong to serogroup B, of which ST-35 (the prototype for this complex) is the most common ST observed. Interestingly, isolates of this complex, as reported to the global MLST database, are more commonly associated with carriage than invasive disease (http://pubmlst.org/neisseria/). However, ST-35 strains causing invasive meningococcal disease have been discovered in Canada, Tunisia and Switzerland (Maiden et al., 1998; http://pubmlst.org/neisseria/), yet these were associated with sporadic/endemic disease, and none with epidemic disease. Since they have been primarily associated with carriage rather than invasive disease, it is possible that the patients with meningococcal disease associated with this particular complex were uniquely susceptible, possibly as a result of an immunocompromised status or exhibiting other host factors predisposing towards disease. Therefore, the presence of this complex does not appear to be of major epidemiological significance, as it is associated with only sporadic disease and not epidemic/outbreak disease.

It is interesting to note that strains of the ET-37 complex causing meningococcal disease were not evident in our study during this three year period. Strains belonging to the ET-37 complex, which can also express serogroup C, W135 and Y polysaccharide capsule are found worldwide (Caugant, 1998), and are commonly associated with epidemic disease (http://pubmlst.org/neisseria). In the 1960s ET-37 strains caused outbreaks among military personnel in the United States (Caugan., 1998). In the 1970s, strains

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expressing the serogroup B capsule ET-37 were isolated in China, and in the late 1970s serogroup B ET-37 strains were recovered from an epidemic in South Africa (Caugant, 1998; Coetzee et al., 1983).

4.3 Molecular Epidemiology of Serogroup C in South Africa

Isolates of epidemic serogroup C meningococci often belong to clonal groupings, but the overall population of serogroup C meningococci is thought to be non-clonal (Smith et al., 1993). Serogroup C organisms that cause sporadic, endemic disease are genetically diverse over time, and during intercontinental spread (Caugant et al., 1990). Sporadic serogroup C meningococcal disease is often caused by a limited number of related strains that appear to circulate slowly in the population (Raymond et al., 1997). This was evident in our study, where 61% of all the cases of serogroup C disease in South Africa during August 1999-July 2002 were caused by one of three complexes of closely-related strains.

Caugant et al. (1987) studied 108 disease-causing serogroup C meningococci from worldwide sources, and noted a large genetic diversity. However, they noted that genotypes identified in one geographic region at a given time tend to be closely related to one another. These data and those of Raymond et al. (1997) are consistent with the concept of gradual evolution and slow spread of virulent serogroup C meningococcal clones in geographically defined human populations. We found evidence of such closely-related genotypes in one geographic region (Western Cape), where two clonal complexes of closely-related strains (ST-32/ET-5 complex and the ST-865 cluster) made up 63% of the isolates collected from this province during the three year study.

ST-11/ET-37 Complex

In this study, three main serogroup C complexes were observed. The largest of the three complexes, consisting of strains belonging to the ST-11/ET-37 complex, was responsible for 27% of all the cases of serogroup C disease in South Africa during the three year period of the study. The predominant presence of strains belonging to this complex in

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South Africa is epidemiologically important. Strains of the ET-37 complex are known to be hypervirulent meningococci commonly associated with epidemics and outbreaks, and cause severe disease particularly among adolescents and young adults (Vogel et al., 2000). ET-37 complex isolates accounted for the majority of endemic serogroup C isolates in the 1980s in the United States (Pinner et al., 1991) and Italy (Mastrontonio et al., 1991) and were the second most common isolates in Canada (Ashton et al., 1991) and Norway (Caugant et al., 1986). ET-37 complex strains also caused a considerable proportion of all meningococci from Mali, Burkina Faso and Ghana (Wang et al., 1993). In the 1990s, endemic disease and outbreaks caused by serogroup C meningococci of the ET-37 complex have been reported from North America (Ashton et al., 1991; Jackson et al., 1995; Pinner et al., 1991), Europe (Kritz et al., 1994), Brazil (Sacchi et al., 1992) and the Sahel region of West Africa (Broome et al., 1983; Wang et al., 1993). In a study conducted on sporadic serogroup C meningococcal isolates in Atlanta 1988-1994, Georgia USA., 85% of isolates belonged to the ET-37 complex (Raymond et al., 1997). Strains of the ET-37 complex have also caused outbreaks in in Brazil (De Morais et al., 1974), South Africa (Wang et al., 1993) and Toronto, Canada (Tsang et al., 2003).

ST-11 is the most common ST, out of 145 unique STs, associated with strains of the ET-37 complex (van Looveren et al., 2001; http://pubmlst.org/neisseria/). From 1992-1997, the increasing incidence of invasive meningococcal disease among persons 15-24 years of age in Maryland, USA was largely due to a clonal group of serogroup C strains belonging to the ST-11 complex (McEllistrem et al., 2004). A serogroup C university outbreak in UK was found to be associated with ST-11 (Feavers et al., 1999). In South Africa, a community-based outbreak caused by strains of the ST-11/ET-37 complex was observed in 2003 (Coulson et al., 2003), which displayed the self-limiting nature previously noted for many ET-37 serogroup C outbreaks (Jackson et al., 1995).

ST-865 (No ST Complex)

A second complex, composed of isolates belonging to the ST, ST-865, was responsible for 18% of the total number of isolates, and consisted of isolates from the Western Cape exclusively. The finding of serogroup C disease associated with this particular clone is

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unexpected as this specific ST, and closely-related STs (sharing homology at five or more MLST gene loci), is more frequently associated with carriage than invasive disease (http://pubmlst.org/neisseria/). It is also more commonly found in non-groupable isolates. Of the groupable isolates, it has been found in serogroup B isolates in the UK and Taiwan, serogroup X isolates in Spain and serogroup 29E isolates in the USA. However, no ST-865 isolates or closely-related STs have been reported for serogroup C strains before. Therefore, to our knowledge, we document the first serogroup C strains of ST-865. Interestingly, a closely-related ST (ST-1387) was identified previously by a Norwegian research group from a South African serogroup B isolate collected in 2001 (http://pubmlst.org/neisseria/). This ST differs at only one locus (*fumC*) from ST-865, where it possesses allele-9 in place of allele-17. Although this particular genotype (ST) associated with Serogroup C could have arisen out of a transformation/recombination, the absence of any closely-related STs associated with serogroup C may suggest otherwise. It is therefore possible that the ST-865 isolates associated with the serogroup C capsule, identified in our study, arose out of a capsule switching event. Presumably, as a result of co-colonization of the nasopharynx with a serogroup B, or other, strain of the ST-865 genotype and a serogroup C meningococcus, genetic exchange of the capsule biosynthesis genes by transformation could have occurred, thus resulting in genotypically identical strain, expressing a serogroup C capsule (Swartley et al., 1997).

It is difficult to comment on the apparent increase in proportion of serogroup C disease associated with this complex, as the numbers involved are very small (Table 7). Although the proportion of disease associated with this ST over the three years may suggest an increase, the absolute numbers suggest otherwise, with only minor variations from one year to the next, and never more than a change of two cases between adjacent years.

The reason that serogroup C meningococci associated with this particular ST come exclusively from the Western Cape is not certain. The composition and prevalence of different STs is known to vary with geographic location in populations of *N. meningitidis*. Such geographic differentiation may be the result of gene flow restricted by

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distance, or may be maintained by natural selection, perhaps in response to the genetic makeup of the host population (Wilson et al., 2004).

ST-32/ET-5 Complex

A third cluster of isolates, belonging to the ST-32/ET-5 complex, was also observed in South Africa during the three year period. Strains of this complex composed approximately 16% of the total number of serogroup C strains collected during the period of the study. All of the isolates belonging to this complex came exclusively from the Western Cape. The reason for this concentration in the Western Cape is not entirely clear, but may be due to similar reasons as those proposed for strains of the ST-865 clone. That is, the geographic isolation of these clones may be due to the particular genetic makeup of the resident population or a restricted gene flow out of the population (Wilson et al., 2004).

The presence of this particular complex in South Africa is epidemiologically significant. Strains of the ET-5 complex have caused outbreaks and epidemics in North America, South America, the UK, Europe and Africa (Caugant et al., 1986; Cruz et al., 1990; Poolman et al., 1986; Reeves et al., 1995; Sacchi et al., 1992). Although most of these outbreaks caused by strains of the ET-5 complex were associated with serogroup B (the predominant serogroup for the ET-5 complex), serogroup C meningococcal strains belonging to the ET-5 complex have also been known to cause disease. In a Pacific Northwest outbreak, 17% of ET-5 complex strains were serogroup C strains, and in Oregon 1994, 17% of serogroup C isolates were ET-5 complex strains (Swartley et al., 1997). Disease associated with serogroup C strains of the ET-5 complex have also been reported in Norway, Sweden, Cameroon, Brazil and Spain (Caugant et al., 1986; Maiden et al., 1998; Wang et al., 1993; http://pubmlst.org/neisseria/).

These serogroup C strains of the ET-5 complex possibly arose from a capsule switching from a serogroup B strain of the same complex. Strains from both serogroups of the ET-5 complex have been reported to share identical serotypes and ET types (Swartley et al., 1997). Closer comparison of our serogroup C strains of the ET-5 complex with the

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serogroup B strains of the same complex revealed that strains of both serogroups had similar PFGE patterns (Fig. 9) and identical MLST sequence types (Table 6), indicating that they shared a common lineage, and possibly diversified one from the other.

4.4 Molecular Epidemiology of Serogroup W135 in South Africa

In South Africa, from August 1999 to July 2002, the population genetics of serogroup W135, as determined by PFGE and MLST, appeared to suggest a fairly clonal population circulating in the population, with almost three-quarters of the isolates collected during this time falling into a single distinct clonal complex of closely-related isolates belonging to the ST-11/ET-37 complex.

ST-11/ET-37 Complex

Genotypic analysis of serogroup W135 strains from South Africa, collected during the period of the study, revealed that the majority (>75%) of serogroup W135 meningococcal isolates belong to the ST-11/ET-37 complex. Serogroup W135 strains of the ET-37 complex are very similar to serogroup C strains of the same complex and may have developed from serogroup C meningococci by a capsule-switching event (Swartley et al., 1997). The presence of this particular complex associated with serogroup W135 disease in such high proportion in the country is concerning, as this complex has been responsible for major epidemics and outbreaks worldwide in recent years. Most notably, serogroup W135 strains of the ST-11/ET-37 complex were responsible for a large outbreak in 2000 in Saudi Arabia during the annual Hajj pilgrimage. More than 400 cases of serogroup W135 meningococcal disease, at an attack rate of 9/100,000, had been reported in Hajj pilgrims and their close contacts from 16 countries throughout the world including the United Kingdom, Belgium, the United States, France, Morocco, Kuwait, Saudi Arabia, Oman, Malaysia, Indonesia, Singapore, Denmark, Finland, Sweden, Norway, Germany, and The Netherlands (Aguilera et al., 2002; CDC, 2000; Issack and Ragavoodoo, 2002; Mayer et al., 2002; Popovic et al., 2000). All outbreak associated isolates were members of the virulent ET-37 complex (specifically strains belonging to the ST-11 complex), which has caused hyperendemic disease and outbreaks worldwide

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(Mayer et al., 2002; Taha et al., 2000; Wang et al., 1993). Prior to the outbreak, ET-37 strains associated with the 2000 Hajj outbreak of W135-related meningococcal disease had been seen previously in Indonesia and Canada (Popovic et al., 2002) and were common among *N. meningitidis* strains isolated in Sweden over a 23-year period, from 1978-2000 (Molling et al., 2001).

Strains of serogroup W135 invasive meningococcal disease belonging to the ST-11/ET-37 complex have subsequently been discovered in the UK, France, US, The Netherlands, Spain, Finland, Singapore and a host of other countries in Europe (http://pubmlst.org/neisseria/). Since 2000, it has been discovered in several African countries including Niger, Cameroon, Madagascar, Senegal, Burkina Faso, Central African Republic and Chad (Guibourdenche et al., 1996; Popovic et al., 2000; Taha et al., 2000; Taha et al., 2002; http://pubmlst.org/neisseria/).

The reason for this particular complex (and serogroup W135 as a whole) appearing to be concentrated primarily in Gauteng, is not entirely clear, and may resemble a process similar to the introduction of serogroup A strains into Gauteng. It is possible that the influx of travelers or immigrants, from countries of the African meningitis belt where serogroup W135 is endemic or epidemic, into Gauteng may be providing a constant source of repeated introduction. Alternatively, if these travelers or immigrants come from countries outside of the meningitis belt, they may therefore be presenting a susceptible population to W135 disease in Gauteng, South Africa where the disease is known to be endemic. The role that particular environmental and/or socio-economic factors in Gauteng may be playing in propagating disease in this province can not also be excluded.

4.5 Molecular Epidemiology of Serogroup Y in South Africa

It has been reported previously that serogroup Y is fairly clonal, not as clonal as serogroup A but certainly more homogeneous than serogroups B and C (Caugant et al., 1987). Serogroup Y disease in South Africa appeared to follow a similar trend in that the

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majority of disease associated with this serogroup was caused by a limited number of strains. These strains belonged chiefly to two main clonal complexes related to the STs, ST-175 and ST-23.

ST-175/No Complex

The results from the PFGE and MLST characterization of the serogroup Y meningococcal isolates collected from August 1999-July 2002 in South Africa indicated that a predominant clonal complex, composed of isolates associated with the ST, ST-175, was prevalent. Strains of this complex caused 70% of meningococcal disease associated with serogroup Y in South Africa, and where isolated countrywide. The presence of this particular ST in South Africa, particularly the high proportion in which it was found, is significant. To our knowledge, strains of invasive serogroup Y meningococcal disease belonging to ST-175 have previously only been seen in isolates collected in 1988 in Gambia (http://pubmlst.org/neisseria/). Disease caused by ST-175 isolates have been reported for other serogroups, however. An ST-175 isolate belonging to serogroup W135 had been reported for Rwanda and Niger, and a closely-related ST (ST-2881) belonging to a non-groupable isolate had been reported from Japan (http://pubmlst.org/neisseria/).

ST-23/Cluster A3

A second, smaller clonal complex was also identified, consisting of isolates belonging to the ST-23/Cluster A3 complex. This particular complex was responsible for approximately 15% of serogroup Y meningococcal disease in South Africa. The ST-23 complex is composed of 99 unique STs, consisting predominantly of serogroup Y, but also contains non-groupable strains and strains belonging to serogroups B, C and W135. Forty-six unique STs belonging to serogroup Y alone make up part of the ST-23 complex. Strains of invasive serogroup Y meningococcal disease belonging to the ST-23 complex/Cluster A3 have previously been discovered in Norway, Canada, Germany, Israel, Japan, Taiwan and France and tend be associated more with sporadic/endemic disease rather than epidemic disease (Maiden et al., 1998; http://pubmlst.org/neisseria/). In a study from Scotland, 1978-2004, the most common serogroup Y complex observed was the ST-23/Cluster A3 complex (Sullivan et al., 2004). In parts of the United States

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serogroup Y strains belonging to ET-508 (an ET associated with the ST-23 complex), and related clones, emerged in the mid-1990s as an important cause of endemic case clusters. Currently, approximately one-third of the cases in certain areas of the United States are due to this serogroup Y strain (Dolan-Livengood et al., 2003; Kellerman et al., 2002; Racoosin et al., 1998; Stephens, 1999). During a study on serogroup Y isolates over an eight year period (1992-1999) in Maryland, U.S.A, it was determined that nearly all the isolates tested belonged to the ST-23 complex (McEllistrom et al., 2004).

Although no isolates of the ST-23/Cluster A3 complex have been reported to cause epidemics, their repeated isolation from endemic and sporadic cases of serogroup Y disease in South Africa, and worldwide, has significance for the continued monitoring of the circulation of this complex in South Africa.

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CHAPTER 5: CONCLUSIONS

Neisseria meningitidis is an important cause of morbidity and mortality worldwide. Because of the severe and potentially life-threatening nature of this disease, and its propensity to cause outbreaks and epidemics, a thorough understanding of the epidemiology of this organism is vital for efforts to control it. One of the primary components of this understanding is the complete elucidation of the population genetics of the meningococcus, allowing us to determine the role of specific clones or clonal complexes in causing invasive meningococcal disease.

The meningococcus has a complex population biology, which can in part be attributed to its natural competence to take up exogenous DNA. The rate of horizontal genetic exchange by transformation in this species is sufficiently high that the clonal population structure is disrupted, and different members of the species (i.e. serogroups) can exhibit different population structures (Bygraves et al., 1999). This was evident in our study where the population structure of the various serogroups analyzed differed markedly, with serogroups A and Y showing a distinct clonal nature as opposed to serogroup B which was non-clonal. As a result of frequent recombination in meningococci, hypervirulent lineages tend to emerge at intervals within the population and slowly diversify through these highly localized recombinational events (Maiden et al., 1998). Ultimately, these lineages may diversify to such an extent that they can no longer be distinguished from the background meningococcal population (Maiden et al., 1998). Thus, the shuffling of genetic markers among clonal lineages, as a result of recombination, makes following the spread of clones and, hence, epidemiology more difficult than with other strictly clonal species.

Although this may be the case, several effective molecular typing methods for *N. meningitidis* have been developed that have allowed us to unravel the complex population genetics of this organism. Research on the population structure of the meningococcus, using these molecular approaches, has revealed that though populations of meningococci typically consist of large, heterogeneous collections of isolates that rarely cause disease, a

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small number of groups of closely-related strains (clones or lineages) are particularly associated with outbreaks of disease, termed "hypervirulent lineages" (Maiden et al., 1998). In contrast to the genetic diversity of carrier populations of meningococci (Jolley et al., 2000), most isolates obtained from patients with meningococcal disease belong to a small number of clonal complexes (Caugant et al., 1987).

This study represented the first comprehensive study of the population genetics of *Neisseria meningitidis* in South Africa, with the goal of determining the prevalence and distribution of particular clones and/or clonal complexes causing the majority of endemic meningococcal disease in the country. For the purpose of our study, a combination of PFGE and MLST was used to determine the molecular epidemiology of this organism in South Africa.

Pulsed-field gel electrophoresis has been demonstrated as being a highly discriminatory method for strains of *N. meningitidis* collected over the short term, typically not more than a few years. As a result, its main application has been in identifying strains belonging to an outbreak and differentiating them from background strains collected in geographic and temporal proximity. In our study, PFGE allowed us to successfully identify the genetic relatedness of meningococcal strains within each serogroup, thus permitting us to resolve the clonal population structures for each serogroup. Having identified the major clones and clonal complexes of interest through the application of PFGE, MLST proved to be a valuable tool to determine the specific identity of these clones and clonal complexes by assigning them to an ST, and where applicable, to an ST-complex.

The results of this study indicated that for each of the serogroups investigated, a very limited number of clones or clonal complexes were responsible for the preponderance of sporadic meningococcal disease in South Africa during the period of the study. Most significant was the discovery that almost all of the predominant clones and clonal complexes identified, excluding the ST-865 clone identified in serogroup B meningococci and the ST-175 clone identified in serogroup Y meningococci, have been

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identified worldwide as being hypervirulent clones and/or complexes. Particularly strains of the ST-44 complex (lineage III), ST-32 complex (ET-5 complex) and ST-11 complex (ET-37 complex), which have been termed "hyperinvasive lineages" (Maiden et al., 1998), and strains of the ST-1 complex (Subgroup I/II) which have been responsible for major outbreaks and epidemics globally.

The continued isolation of strains of these "hyperinvasive lineages" over a period of years may suggest that there is prolonged, persistent carriage of these disease-causing lineages in the population, or slow transmission in normal populations, or both (Raymond et al., 1997). Importantly, carriage of the hyperinvasive lineage clones appears not to always result in disease. This was evident by the absence of outbreaks attributed to these complexes, or any other, in South Africa during this period. It has been found, though, that strains of the hyperinvasive lineages (particularly strains of the ST-32/ET-5 complex and ST 41/44/lineage III complex) do tend to circulate slowly through the population with a low transmissibility, but a high degree of virulence (Achtman 1995; Caugant, 1998; Peltola, 1983).

Although no epidemics or outbreaks were reported during the period of our study, a serogroup C outbreak in South Africa 2003 (Coulson et al., 2003), associated with the ST-11/ET-37 complex, served as a severe reminder of the ability of these "epidemic-prone" clones to cause outbreaks unexpectedly. The presence of these virulent clones and clonal complexes, particularly the high proportion in which they occur in, is concerning. Having said this, the relationship between epidemic outbreaks and sporadic disease in populations has not yet been established (Raymond et al., 1997). It has been recognized, though, that the presence of a virulent strain alone is usually not sufficient to result in an epidemic (Schwartz et al., 1989). Multiple factors related to the microorganism, the host and the environment are required for an epidemic to occur.

A more thorough analysis combining the data from such population genetics studies and the comprehensive epidemiological information available may potentially help determine risk factors associated with particular clones or clonal complexes, and allow us to define

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individuals or populations at risk. The availability of this information would have direct implications for public health with regards to preparedness, and the development of effective intervention and control strategies.

In conclusion, meningococcal disease continues to be a serious health concern in South Africa, with approximately 200-300 cases per annum. In this study we identified that the vast majority of meningococcal disease in South Africa could be attributed to just a few virulent clones or clonal complexes, most of which have been identified worldwide as important sources of epidemic disease. Due to their persistent presence and predominance in South Africa, continued long-term population genetics studies monitoring the spread of these strains in the population is essential. In association with the current epidemiological surveillance program, these studies can provide valuable information for the development of public health strategies to minimize or control the risk of outbreaks and epidemics.

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