Molecular characterisation of *Neisseria meningitidis* **serogroup B isolates in South Africa, 2002 – 2006**

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Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg in fulfillment of the requirements for the degree of Master of Science in Medicine.

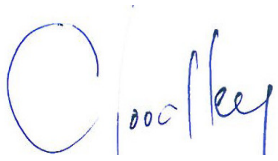
Johannesburg, November 2010

‘I was taught that the way of progress is neither swift nor easy.’

Marie Curie

DECLARATION

I, Chivonne Moodley, declare that this dissertation is my own work. Experiments described were conducted under the supervision of Dr. Mignon du Plessis and Dr. Anne von Gottberg at the Respiratory and Meningeal Pathogens Research Unit, National Institute for Communicable Diseases, National Health Laboratory Service, Johannesburg. It is being submitted for the degree of Master of Science in Medicine to the Faculty of Health Sciences at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination to this or any other university.



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For Julien

PRESENTATIONS

Oral – Wits Research Day 2010

Moodley C., du Plessis M., von Gottberg A., Mothibeli K., de Gouveia L., and Klugman K.P. Molecular characterisation of *Neisseria meningitidis* serogroup B isolates causing invasive disease in South Africa, 2002-2006. *Wits Research Day*, Faculty of Health Sciences, Witwatersrand University, South Africa, 22 September 2010.

Poster - International Pathogenic Neisseria Conference 2010

Moodley C., du Plessis M., Mothibeli K., de Gouveia L., Klugman K.P., von Gottberg A. Global clones and capsule switching identified among invasive *Neisseria meningitidis* isolates, South Africa, 2005. *17th International Pathogenic Neisseria Conference*, Banff, Canada, 11-16 September 2010.

Poster - FIDSSA Congress 2009

Moodley C., du Plessis M., von Gottberg A., Mothibeli K., de Gouveia L., and Klugman K.P. Molecular characterisation of *Neisseria meningitidis* serogroup B isolates causing invasive disease in South Africa, 2002-2006. *3rd Congress of the Federation of Infectious Diseases Societies of Southern Africa,*. Sun City, South Africa, 20-23 August 2009.

Poster - NICD Academic Day 2008

Moodley C., du Plessis M., von Gottberg A., de Gouveia L., and Klugman K.P. Genetic characterisation of serogroup B meningococci in South Africa, 2000-2003. *National Institute for Communicable Diseases Academic Day 2008*, James Gear Auditorium, PRF Centre, Sandringham, Johannesburg, South Africa, 11 November 2008.

Oral - NICD Research Forum/Scientific Talk 2008

Moodley C. Serogroup B meningococci in South Africa - implications for vaccine development. *National Institute for Communicable Diseases bimonthly scientific presentation/discussion,* James Gear Auditorium, NICD, Sandringham, Johannesburg, South Africa, 27 August 2008.

ABSTRACT

Despite being a fulminant pathogen, *Neisseria meningitidis* (meningococcus) is part of the commensal flora of the human nasopharynx. Globally, five meningococcal serogroups (A, B, C, Y and W135) cause the majority of invasive disease. Most serogroup B cases occur sporadically but may be endemic or epidemic within a geographic region. In South Africa, there are limited data on invasive serogroup B clones and the antigenic diversity of certain meningococcal outer membrane proteins. This study examined the molecular epidemiology of serogroup B meningococci in South Africa from 2002 through 2006.

Invasive meningococcal isolates were submitted to a national laboratory-based surveillance system. For this study, serogroup B isolates were characterised by pulsed-field gel electrophoresis (PFGE), PorA, FetA and multilocus sequence (MLST) typing. PorA, FetA and multilocus sequence (MLST) typing were performed on all 2005 isolates (n=58) and randomly selected isolates from other years (n=25).

A total of 2144 invasive cases were reported over the study period. Of these, 76% (1627/2144) had viable isolates available for serogrouping and 307 (19%) were serogroup B. Serogroup B cases were reported from across the country however

the majority were from the Western Cape province. The highest incidence of serogroup B was in children less than 5 years of age.

Isolates displayed a high level of diversity by PFGE. Despite this diversity the majority of serogroup B meningococci collected over the 5-year period could be grouped into several clonal clusters representative of global invasive MLST clonal complexes. Overall, the most predominant MLST clones in South Africa were ST-32/ET-5 and ST-41/44/lineage 3. In addition, at least 19 PorA types and 16 FetA types were determined among selected isolates.

Globally invasive serogroup B disease is caused by heterogeneous strains however, prolonged outbreaks in several countries have been due to strains of the ST-32/ET-5 and ST-41/44/lineage 3 clonal complexes. At present, serogroup B disease in South Africa is not dominated by an epidemic clone, however, global clonal complexes ST-32/ET-5 and ST-41/44/lineage 3 are circulating in Western Cape and Gauteng, respectively.

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To God – thank you for bringing me to this unit, for blessing me with good opportunities and granting me strength and perseverance.

GERMS-SA

The surveillance network that made this project possible.

Data contribution by colleagues

A component of this study, namely characterisation of serogroup B isolates from 2005 by MLST, PorA and FetA typing, was carried out by scientists Ms. Kedibone Mothibeli and Dr. Mignon du Plessis. This component was funded by Pfizer (formally Wyeth) Vaccines Research.

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NOMENCLATURE

~ - approximately

eBURST – based upon related sequence types

CA - California

CO_2 – carbon dioxide

conc. - concentrated

°C – degrees Celsius

DVL – double locus variant

EDTA – disodium ethylenediaminetetraacetic acid

g – gram

h - hour

HCl – hydrochloric acid

L – liter

µl – microliter

mg - milligram

ml – milliliter

min - minute

M – molar

NaOH – sodium hydroxide

n - number of isolates

OMP - outer membrane protein

PCR – polymerase chain reaction

% - percentage

rpm - revolutions per minute

s - second

SDS – sodium dodecyl sulphate

SVL – single locus variant

TBE – Tris-Boric-EDTA

TE – Tris-HCL-EDTA

Tris – tris[hydroxymethyl]aminomethane

U – unit

USA – United States of America

V – volt

CONTENTS

1 LITERATURE REVIEW

Meningoccocal disease causes substantial morbidity and mortality, and despite treatment with antibiotics approximately 10% of cases are fatal. Among those who survive, 11% to 19% suffer long-term sequelae such as hearing loss, neurological disabilities, loss of a limb or paralysis [1;2]. Although the disease affects all age groups, children less than 5 years of age are most at risk [3]. Despite being a fulminant pathogen, *Neisseria meningitidis* (meningococcus) is part of the normal commensal flora of the human nasopharynx and, outside of epidemics, is carried by ~10% of the human population [4].

1.1 The bacterium - *Neisseria meningitidis*

Although the disease was first described in Geneva, Switzerland in 1805, it was decades later in 1887 that the causative organism was discovered in Vienna by Anton Weichselbaum, an Austrian pathologist. Weichselbaum was able to isolate the organism from meningeal exudates of six cases of cerebrospinal fever. He called the bacterium *Diplococcus intracellular meningitidis,* which was later changed to *Neisseria meningitidis* after the German scientist Albert Neisser [2;5].

1.1.1 Microbiologic characteristics

N. meningitidis belongs to the genus *Neisseria* [6]. Other members of the genus include the pathogenic *N. gonorrhoeae* as well as the common commensal *N. lactamica*. The bacterium is a Gram-negative, aerobic diplococcus. Cocci occur in pairs and have a characteristic bean shape **(Figure 1.1)**. Meningococci require enriched media for growth. Blood-enriched media incubated at 35°C to 37°C in a moist atmosphere with 5% CO_2 supports the fastidious growth of this organism. Colonies are convex and appear a translucent grey.

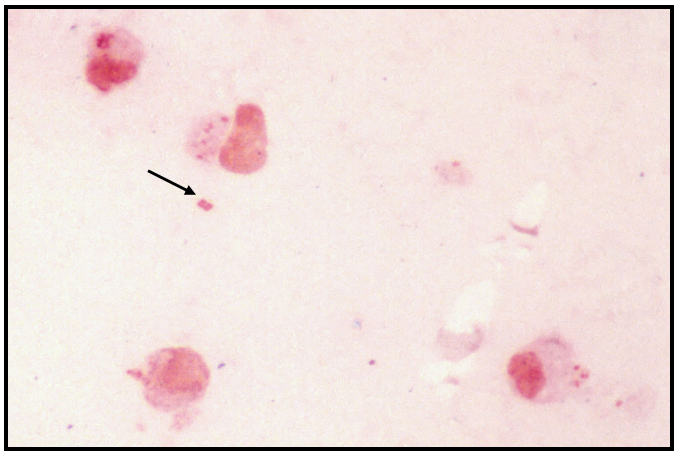


Figure 1.1 Gram stain of cerebrospinal fluid (CSF) with *Neisseria meningitidis*. (Magnification x100, oil immersion objective)

1.1.2 The polysaccharide capsule

Meningococci may be encapsulated or unencapsulated **(Figure 1.2)**. The cell produces a polysaccharide capsule that envelops the cell and serves as a major virulence factor [7]. It protects the underlying outer membrane, membrane proteins and enzymes from host defense mechanisms during transmission and

invasion. The capsule and certain outer membrane components have been studied extensively for vaccine development and are the basis for a classification system.

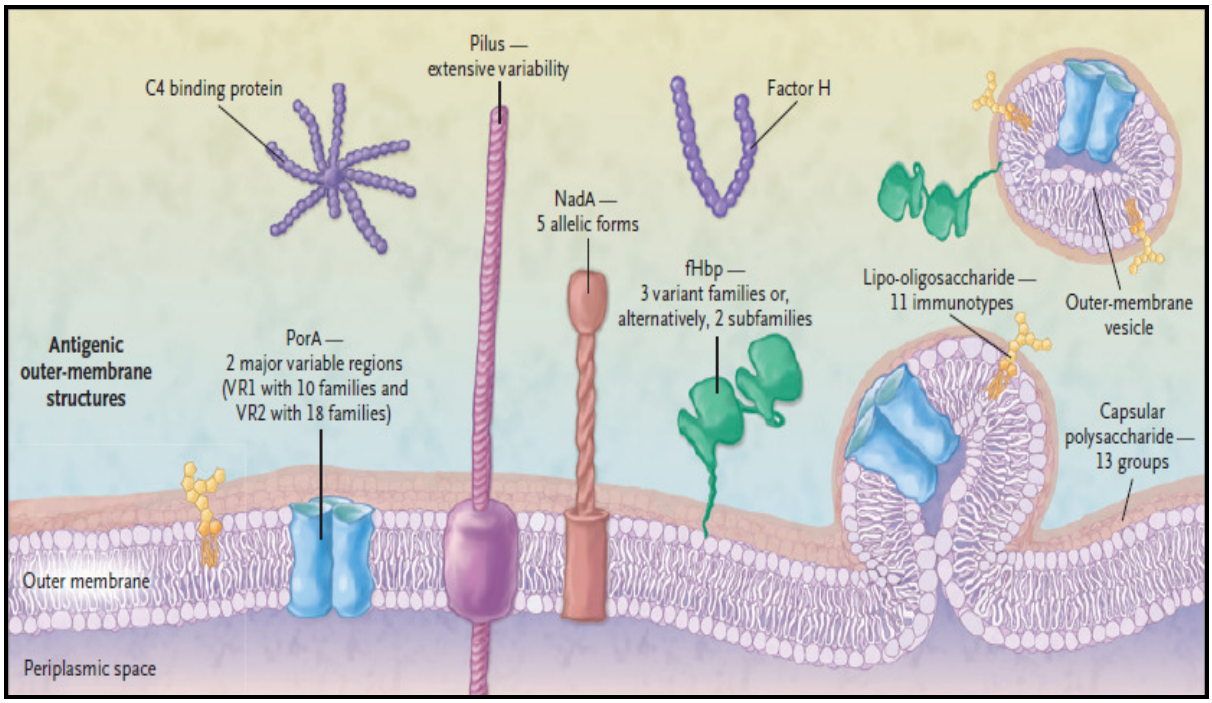


Figure 1.2 Schematic of outer membrane components of encapsulated meningococci [8].

1.1.3 Classification

Meningococcal isolates may be classified into serogroups, serotypes and serosubtypes [9]. Initial classification was done using serological methods and was based on reactivity with immunological reagents (monoclonal antibodies).

Serogrouping is based on structural differences of the polysaccharide capsule [10]. Thirteen different serogroups have been described, ie. A, B, C, D, H, I, K, L,

W135, X, Y, Z and 29E, however, serogroups A, B, C, Y, W135 and X are responsible for virtually all cases of invasive disease. Further classification of meningococci into serotypes and serosubtypes are based on the antigenic differences of essential outer membrane proteins (OMPs) [11]. PorB (a class 2 or 3 OMP) and PorA (a class 1 OMP), which function as porins, determine serotype and serosubtype, respectively. More recently FetA, an iron-regulated OMP has been included in routine classification [12]. Another classification system is immunotyping. This system classifies strains into lipooligosaccharide (LOS, also known as endotoxin) types L1 to L12 based on variations in the oligosaccharide side-chain [5;13]. Immunotypes L1 through L8 are associated with serogroups B and C.

Classification systems have greatly improved by the replacement of serological methods with molecular techniques – targeting the nucleotide sequence rather than the protein itself. However, for immediate results serogrouping kits with monoclonal or polyclonal antisera are still used. While each laboratory will choose a classification system that suits their purpose, classification targets and a standardised nomenclature has been proposed by Jolley *et al*. 2007 [12]. The current scheme is as follows :

	Serogroup	: PorA type	: FetA type	: Sequence type (clonal complex)
E.g.	B	: P1.19,15	: F5-1	: ST33 (ST-32/ET-5)

1.2 Carriage

The human nasopharnyx is the only known reservoir for the meningococcus and ~10% of a population may be carriers at any given time [14;15]. The carriage state is asymptomatic and influenced by various host-environmental factors.

Meningococci are transmitted via airborne droplets of salivary and mucosal secretions **(Figure 1.3).** Close contact is important for transmission as the bacterium is susceptible to drying [4;16]. Once acquired, the bacteria colonises the nasopharyngeal mucosa leading to carriage. The duration of carriage varies with individuals, it may be brief, days or weeks or last several months. Shortly after carriage the bacteria may cross the epithelial barrier and invade the bloodstream resulting in invasive disease. Since disease incidence is rare relative to carriage rates it is suggested that transmission and carriage, rather than invasive disease, is important for the life cycle of the meningococcus.

Several characteristics and risk factors have been associated with the frequency of carriage [17;18]. Carriage rates are low in children under 5 years of age, peak in adolescents and young adults and decline with increasing age. There are more male than female carriers. Close contact at schools, universities, social gatherings, as well as over-crowded conditions are major factors for the increase of transmission and carriage. Social behavior such as smoking, kissing and attendance of pubs and clubs also increase the risk of carriage. Carried

meningococci are far more genetically diverse than disease-causing isolates [17]. In addition, almost 50% of carried isolates may lack a capsule which may facilitate colonisation. Although high rates of carriage have not been directly linked to invasive disease, asymptomatic carriers are the source of transmission and spread of the bacterium.

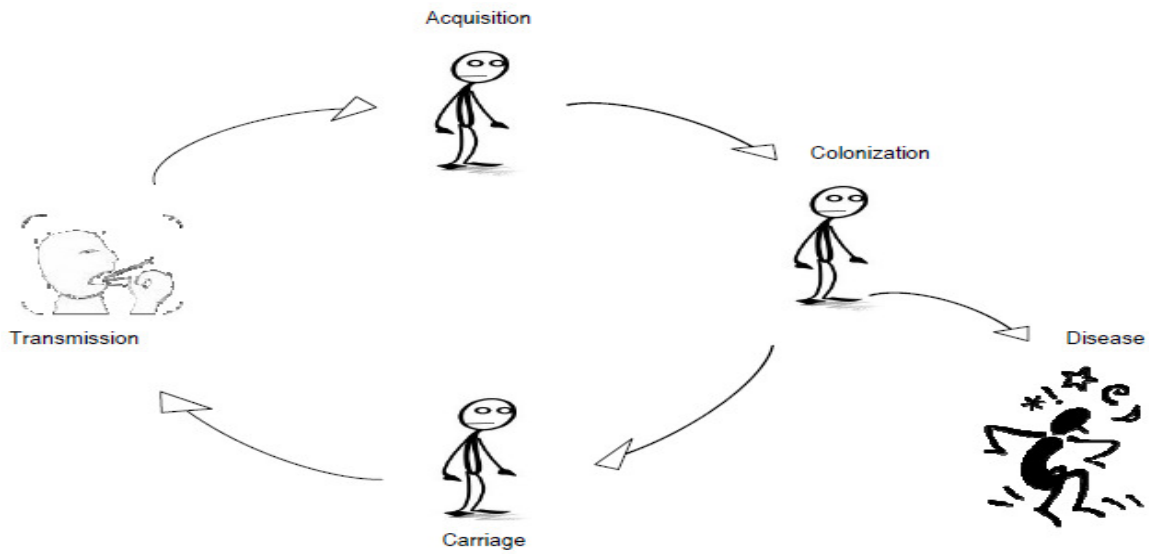


Figure 1.3 The meningococcal transmission cycle.

1.3 Disease epidemiology

Although meningococcal disease occurs all over the world, there are geographical differences in incidence and distribution of the serogroups that cause disease [19;20]. Globally, serogroups A, B and C are the most common causes of meningococcal disease **(Figure 1.4).**

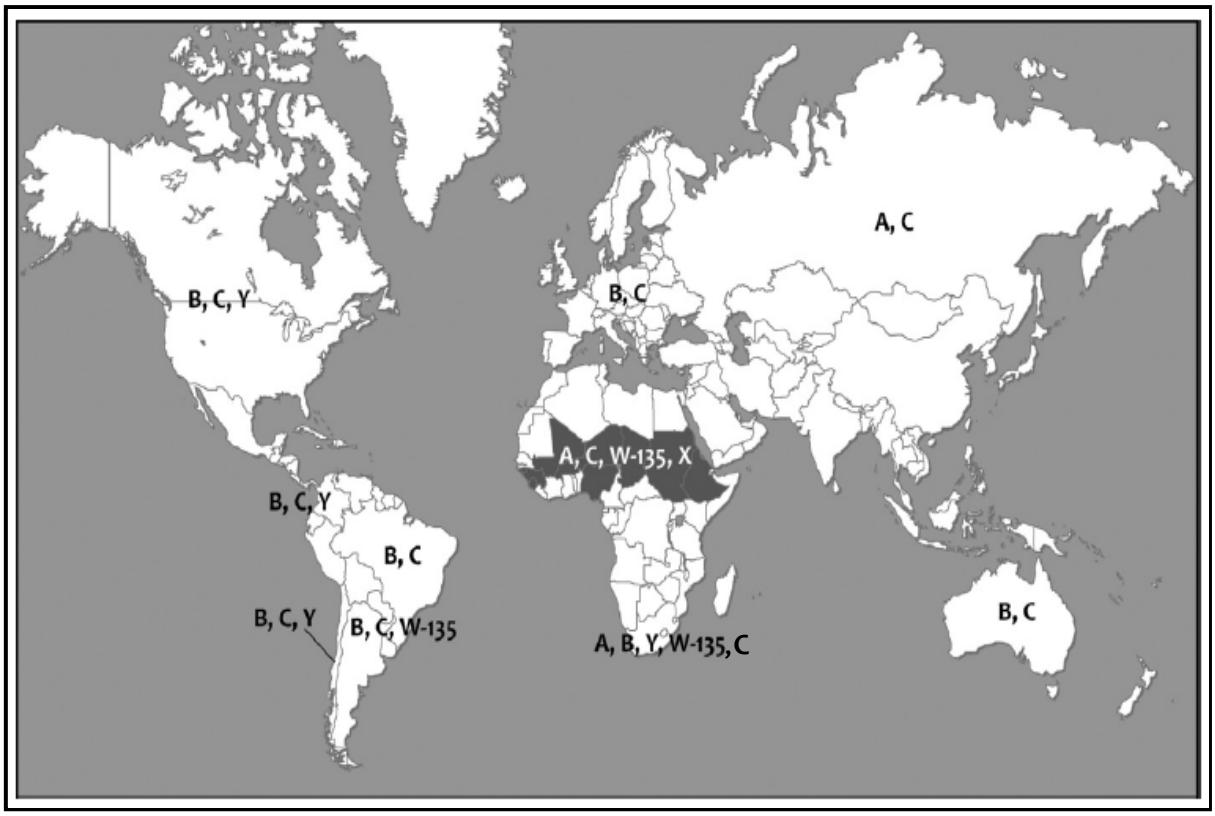


Figure 1.4 Global distribution of meningococcal serogroups causing invasive disease (Adapted from [21]).

1.3.1 The meningitis belt

Globally, the highest incidence of meningococcal meningitis occurs in the meningitis belt **(Figure 1.5)**, a region of savannah within sub-Saharan Africa that extends from Senegal in the west to Ethiopia in the east. Since its estimated arrival on the African continent in the mid 19th century, the meningococcus has caused over 100 years of epidemic meningococcal disease within the meningitis belt [22].

Large epidemic waves occur every 5 to 12 years and last for several years [23;24]. Annual rates of disease follow a seasonal cycle, peaking in the dry season (December–May) and disappearing with the onset of the rainy season. Endemic conditions in sub-Saharan Africa are often considered epidemic elsewhere in the world.

From 1970 through 1992 there was an estimated 800 000 meningococcal cases [3]. During a severe epidemic from 1996 to 1997, which affected Burkino Faso, Mali, Niger and Nigeria, approximately 190 000 cases were reported from January through October 1996. During this epidemic almost 20 000 cases were fatal. The cause of these mass epidemics was serogroup A. While serogroups A, C, W135 and X are also responsible for endemic and epidemic cases within the meningitis belt [22;25], serogroup A predominates.

1.3.2 Serogroup distribution

Serogroup A

Serogroup A is known to cause large epidemics [26]. Global spread of invasive serogroup A clones occurred over a 20 year period from the 1960s to 1980s, resulting in outbreaks in a number of countries, including South Africa [27]. Serogroup A disease is endemic in developing countries of Africa and Asia [10;28]. Although serogroup A has not been common in developed countries there has been a recent increase in invasive cases in countries such as the United Kingdom, Greece and Canada [29-31].

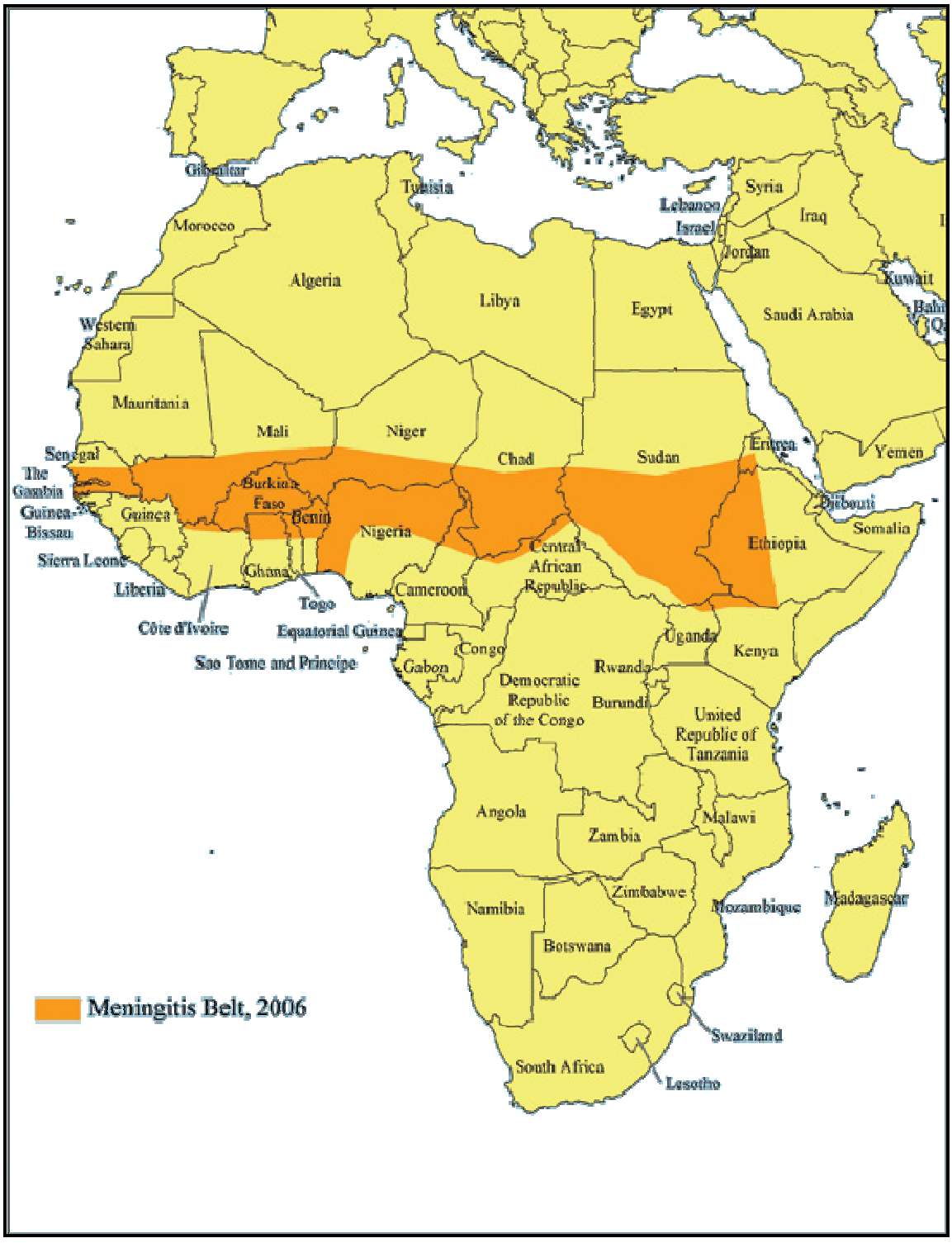


Figure 1.5 The sub-Saharan African meningitis belt [32].

Serogroup C

Serogroup C is associated with sporadic cases and is mostly endemic in developed countries [33-35], however small-scale outbreaks have occurred world wide [36]. The introduction of meningococcal serogroup C vaccines in parts of Europe, Canada and North and South America America has lead to significant decreases in serogroup C disease [21;37].

Serogroup W135

Endemic cases of serogroup W135 have been documented in the meningitis belt (section 1.3.1) and in countries such as Brazil [38] and Sweden [39]. The largest outbreaks occurred in Saudi Arabia during the Hajj pilgrimage in 2000 [40] and in Burkino Faso in 2001 [41]. As Hajj pilgrims travelled home to different continents, the outbreak clones spread globally [42].

Serogroup Y

Serogroup Y prevalence is low and scattered across the globe. In Europe, serogroup Y is responsible for a small percentage of cases [43]. A case report from London of unusual clinical presentations associated with serogroup Y disease, suggested that the organism may be capsule switching from serogroup C to Y to evade vaccine induced immunity against serogroup C [44]. The USA and Canada have experienced an increase in serogroup Y cases since the early 1990s [45-47].

Serogroup X

While the majority of invasive cases worldwide are caused by serogroups A, B, C, Y and W135, serogroup X has emerged as an important invasive serogroup in recent years [48]. Serogroup X has been identified in Europe, North America and Australia, while outbreaks have occurred in Africa [49-51].

Serogroup B

Serogroup B is the main cause of meningococcal disease in industrialised and developing countries contributing to at least 30-80% of disease in the United States [52], the Netherlands [53], Europe [37], and Australia [54]. In contrast, serogroup B does not cause disease in the meningitis belt [3;23], the reason for this is still unknown.

The United States conducts meningococcal disease surveillance through an active, population- and laboratory-based surveillance system [52;55]. Serogroup B accounted for approximately 32% of endemic disease (http://www.cdc.gov/abcs/survreports/meningo08.pdf) in 2008. When comparing the surveillance report from 2007 and 2008, an increase in serogroup B incidence from 2007 to 2008 was noted. In Europe, two networks, EU-MenNet and EU-IBIS, have contributed to meningococcal surveillance from 1999 to 2006 [21;37;56]. Since 2007, surveillance has been conducted by the European Centre for Disease Prevention and Control. The majority of cases were caused by serogroup B and C.

Meningococcal disease prevalence in Brazil has been reported from individual states [38;57;58]. Sao Paulo, a large metropolitan state, documented an increase in serogroup B disease during the 1980s. The southern most state of Brazil, Rio Grande do Sul, reported an overall decrease in serogroup B disease between 2003 and 2005. Approximately 60% of available meningococcal isolates were nevertheless serogroup B.

Worldwide, serogroup B disease occurs mostly as sporadic cases but is endemic to many regions [2;38;59-61]. Prolonged outbreaks have occurred in Brazil [57], Chile [62], Cuba [63] and Oregon [64]. Serogroup B epidemics can span several years, as in the case of New Zealand [65], Norway [66] and the Netherlands [53;59].

1.3.3 South Africa

"Epidemic cerebrospinal meningitis, cerebrospinal fever or 'spotted fever', is a specific disease due to the meningococcus, and should not be confused with meningitis of other bacteriological etiology."

Dr. David Ordmen, SAMJ, 1932 [67]

Meningitis was made a notifiable condition in South Africa in 1920 but the condition, was documented as early as 1905 in the Johannesburg Municipality [67]. The South African mining sector contributed a great deal to the description and documentation of disease characteristics [68-70]. Meningococcal outbreaks

were common in mines due to the crowded living and working conditions. The constant influx of new labourers meant the introduction of susceptible individuals as well as new, possibly invasive strains, into the mining community. Epidemiological descriptions of age, race and geographical distribution were based only on notifications. According to Ordmen [67], the meningococcus was the etiological agent responsible for the majority of meningitis cases amongst the miners.

In 1979, serogroups A, B, C and D were reported from the Western Cape Province [71;72]. At that time serogrouping was not routinely done. The city of Cape Town was experiencing a meningococcal outbreak and therefore isolates from the Western Cape from 1977 through August 1979 (n=76), were further characterised and serogroup B accounted for 57% (43/76).

By the mid 1980s serogroup B had established itself as the dominant serogroup in the country [73]. A 2-year retrospective study of bacterial meningitis in Johannesburg, from May 1980 through May 1982 described serogroups A and B as the cause of the majority of meningococcal cases [74]. The South African Department of Health continued to monitor and report on meningococcal disease, and although serogroup was not routinely reported [75;76], when determined, serogroup B was predominant in the Western Cape [77;78]. A meningococcal outbreak occurred in Johannesburg in 1996 [27] and the isolates were serogrouped (n=111). Although serogroup A (n=77) caused the outbreak,

serogroups B (n=18), C (n=1), Y (n=11) and W135 (n=3) were also responsible for some cases.

Serogroup B caused 41% (251/615) of meningococcal cases in the country during the period 1999 to 2002 [79]. The predominance of serogroup B in the Western Cape and serogroup A in Gauteng was also noted for this period. Since 1999, routine surveillance of meningococcal disease has been conducted through an active, national, laboratory-based surveillance system (http://www.nicd.ac.za /units/germs). South Africa is unique because, unlike many other countries where only one or two serogroups dominate, five serogroups (A, B, C, Y and W135) are responsible for causing disease [79-82]. In 2003, a shift in the dominant serogroup from A to W135 in Gauteng was noted. Currently, serogroups B and W135 cause the majority of cases in South Africa [83]. Serogroup B still dominates in the Western Cape and serogroup W153 in Gauteng.

1.4 Pathogenesis

Following acquisition, meningococci may colonise the nasopharnyx which can result in asymptomatic carriage [2;84]. Invasive disease occurs when the bacterium crosses the nasopharyngeal mucosa and enters the systemic circulation **(Figure 1.6)**. The disease progresses quickly as the bacterium is able to proliferate rapidly in the bloodstream and disseminate through the body, producing various symptoms as it reaches different organs. From the

bloodstream the meningococcus is able to cross the blood-brain barrier into the cerebrospinal fluid (CSF). Bacteria multiply in the CSF, which fills the subarachnoid space around the brain and spinal cord, causing inflammation of the leptomeninges [84;85]. Meningitis, septicaemia and meningitis with septicaemia are the most common presentations of meningococcal disease [1;10]. Other clinical signs and symptoms occur in varying combinations which may be similar to more common but less serious illnesses thus making diagnosis of meningococcal disease difficult. These symptoms include headache, fever, neck stiffness, altered mental status, non-blanching petechial or purpuric rash [1;2;10;86;87], vomiting, abdominal pain, lethargy, myalgia [1;84], pneumonia [2;84] and haemorrhagic skin lesions [88].

Diagnosis

Laboratory diagnosis requires specimens from normally sterile (CSF, blood, pleural fluid) or other infected sites [2;10;89]. Gram staining of sediment from blood and CSF as well as commercial latex agglutination kits (CSF and blood specimens only) can be used for rapid identification and serogroup results. Specimens are cultured but may or may not yield growth of meningococci due to antibiotic treatment prior to specimen taking. PCR has become an important diagnostic tool especially in the case of culture-negative specimens.

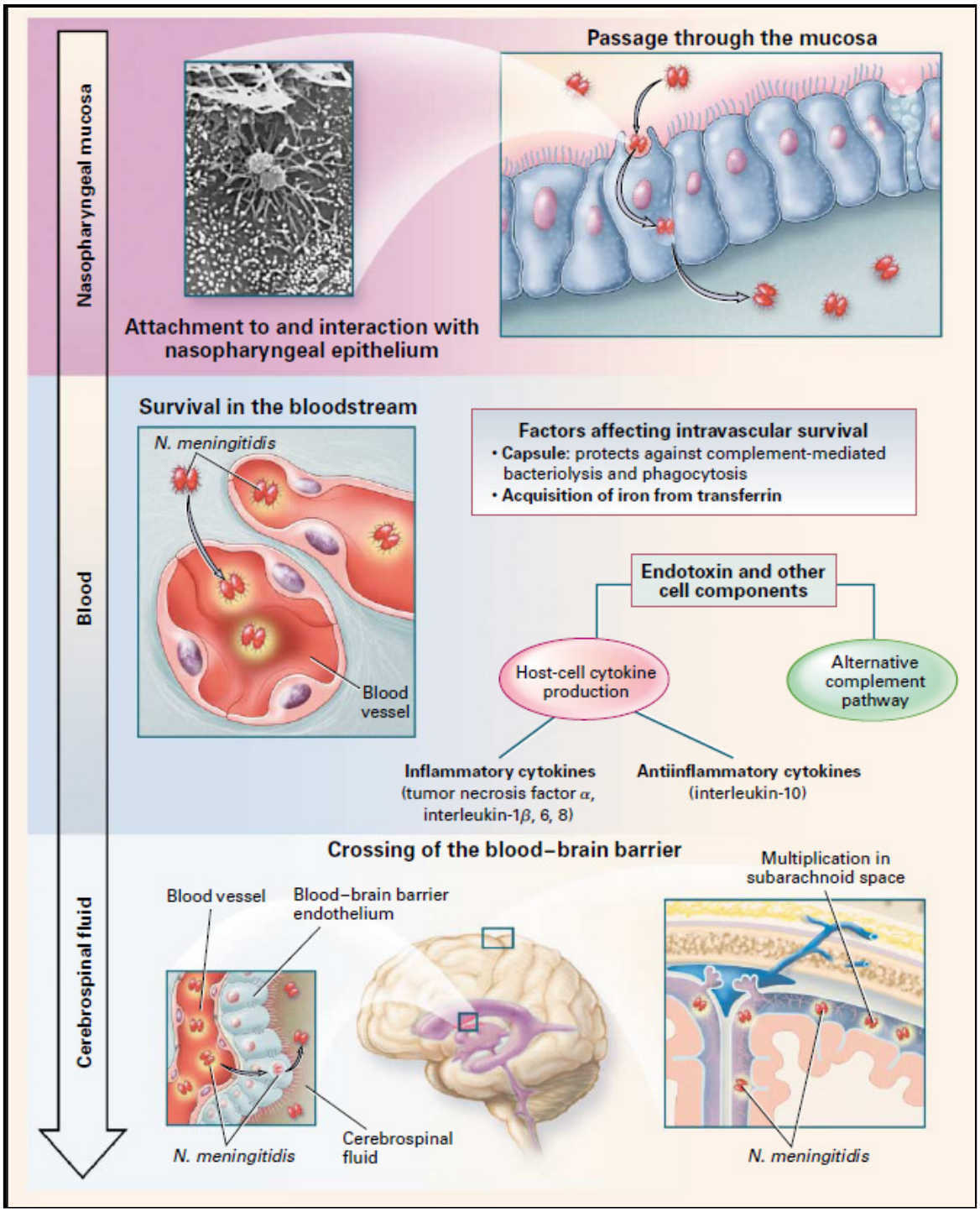


Figure 1.6 Colonisation of the nasopharyngeal mucosa by *N. meningitidis* and passage into the blood stream and cerebrospinal fluid [2].

1.5 Treatment and Prevention

Recovery from invasive disease requires the prompt administration of antibiotics which stop the proliferation of meningococci [2;10;89]. Since laboratory confirmation of meningococcal disease may take hours or days, antibiotic treatment is commenced following clinical suspicion of the disease. Penicillin, third-generation cephalosporins (ceftriaxone and cefotaxime) and chloramphenicol are effective in treatment of invasive disease. The South African National Department of Health Guidelines for the management, prevention and control of meningococcal disease in South Africa recommends ceftriaxone or cefotaxime as drugs of choice in suspected cases of bacterial meningitis [90].

Chemoprophylaxis for close contacts

In order to prevent secondary cases antibiotic prophylaxis must be given to close contacts at least 24 hours after the case has been identified [91]. Hence the importance of a prompt and accurate laboratory diagnosis. However careful consideration must be given as to who falls into the category of close contact [91] as mass chemoprophylaxis is not recommended. Rifampicin, ceftriaxone, azithromycin and ciprofloxacin are effective against eradicating nasopharyngeal carriage, therefore preventing potential invasion or ongoing transmission [2;10;20].

Antibiotic resistance

Decreased susceptibility to the above recommended antibiotics has been reported [92]. Reports include rifampicin resistance in Israel [93] and the United Kingdom [94], intermediate resistance to penicillin in South Africa [80] and Portugal [95], and ciprofloxacin resistance in India [96] and the United States [97]. Despite these reports, antibiotic resistance in meningococci is not widespread. However prevention by vaccine immunisation may be the best control strategy.

1.5.1 Vaccines

Vaccines against the major serogroups (A, C, Y, and W135) causing meningococcal disease have been available since the 1970s [20;98]. The polysaccharide capsule serves as the antigen for these vaccines. Individual formulations against serogroups A and C were developed first, followed by a tetravalent formulation containing antigens against serogroups A, C, Y and W135. The tetravalent polysaccharide vaccine has been safe and immunogenic but has limitations. Firstly, polysaccharides are T-cell-independent antigens that do not need T-cell help to induce an immune response which results in poor immunologic memory. Secondly, polysaccharide vaccines lack immunogenicity in children less than 2 years of age [89;98]. These limitations encouraged the development of meningococcal polysaccharide-conjugate vaccines in which the polysaccharide is conjugated to a protein [99]. Thus the advantages of the

meningococcal conjugate vaccines include a T-cell-dependent immune response resulting in lasting immunity and efficacy in children less than 2 years of age.

The success of meningocoocal serogroup C conjugate vaccine (MCC) has been demonstrated in the UK, which was the first country to implement the vaccine in November 1999 [100-102]. Other European countries soon followed. There was a reduction in invasive serogroup C disease as well as serogroup C carriage. The successes of MCC lead to the development of a serogroup A conjugate vaccine (MenAfriVac™) for Africa through the Meningitis Vaccine Project [103]. A tetravalent conjugate vaccine is also available, targeting serogroups A, C, Y and W135. This tetravalent formulation was implemented into the USA immunisation schedule in 2005 [104] while in Canada it has been approved for use in high risk groups [105].

1.5.2 Serogroup B

The serogroup B capsule

The serogroup B capsule is a homopolymer of α-2,8 linked sialic acid (N-acetylneuraminic acid) [106]. In comparison to capsules of serogroups A, C, Y and W135, serogroup B is unique in that it is structurally similar to human brain tissue [107], a characteristic it shares with the neonatal pathogen *Escherichia coli* serotype K1. Fetal brain tissue contains significant amounts of glycopeptides with N-acetylneuraminic acid residues bound by α-2,8 linkages. These glycopeptides are present in smaller amounts in adult brain tissue.

Thus the serogroup B capsule is poorly immunogenic in humans and cannot be used as a vaccine antigen. Efforts to increase immunogenicity by manipulating the polysaccharide structure, conjugation of polysaccharide to a protein carrier (e.g. tetanus toxoid) and selective use of adjuvants have given good results [107;108]. However there are concerns of inducing autoimmunity by using artificial components which may break down the body's natural tolerance to sialic acid. As a result vaccine development against serogroup B meningococci have sought non-capsular antigens most especially, outer membrane proteins (OMP).

OMP vaccines

Several OMPs have been used as vaccine targets including PorA, PorB and FetA [109-111]. These vaccines have had good safety, immunogenicity and efficacy profiles. One of the first vaccines to be developed and licensed (1989) against serogroup B was VA-MENGOC-BC® (Finlay Institute) [110;112]. The vaccine was developed to control a Cuban epidemic and was based on OMPs of the epidemic strain. Serogroup C capsular polysaccharide was also incorporated into the vaccine. The vaccine successfully controlled the Cuban epidemic and was also used in Brazil (the state of Sao Paulo), Uruguay and Colombia. This led to the development of other tailor-made OMP vaccines to potentially control epidemics in Norway and New Zealand [109;111;113].

However a vaccine with much broader coverage was required. The high level of diversity among OMPs (since they are exposed to the bacterial environment and are therefore under pressure for antigenic change) impeded the development of a broad vaccine. Other more conserved sub-capsular vaccine candidates were then sought [114]. The advent of reverse vaccinology vastly improved the identification of potential serogroup B vaccine candidates [115]. These genome-derived neisserial antigens (GNA) were obtained by whole–genome sequencing of a serogroup B reference strain followed by *in silico* analysis and selection of potential vaccine candidates.

To date the most promising GNA vaccine candidate is a surface–exposed lipoprotein, present in all *N. meningitidis* isolates tested so far, called factor H binding protein (fHbp) [116;117]. The human protein, factor H, is a component of the alternative complement pathway and is responsible for the down-regulation of the alternative pathway. Meningococci bind human factor H to the bacterial surface and since human factor H retains its activity the alternative pathway is down-regulated. The fHbp antibodies act by binding meningococcal fHbp. Currently, there are 2 recombinant protein vaccines that are in phase 2 ad 3 clinical trials. One targets fHbp only while the other is a 5–component vaccine that targets GNA1994,GNA2132, GNA2091,GNA1030 and fHbp [117-119].

1.6 Molecular characterisation

Why characterise?

Characterisation of meningococci is needed to distinguish between strains that 'exhibit minimal biochemical and biological differences' [120]. Characterisation data can be used to describe local disease epidemiology which is essential for control and management of cases and outbreaks [121]. Comparisons can be made to global findings in order to identify spread of hyperinvasive strains [122]. Serogroup B characterisation is especially important because of ongoing vaccine development.

Molecular techniques

DNA-based methods and technologies have largely replaced serological methods of characterisation [123]. The development of DNA methods and associated technologies have improved meningococcal disease diagnosis and epidemiology. Early antibiotic treatment is essential in cases of meningococcal disease [2;109] which compromises culture of the organism. Viable isolates are needed for serological serotyping and serosubtyping. Molecular techniques are quicker and can be applied to diagnose and characterise culture-negative cases. The advent of nucleotide sequencing has made molecular techniques the method of choice for diagnostics and epidemiology.

1.6.1 PFGE

Pulsed-field gel electrophoresis is used to obtain whole genome fingerprint patterns. Patterns are compared in order to determine genetic relatedness between isolates. Bacterial cells embedded in agarose plugs are lysed to release DNA **(Figure 1.7)** [124]. The chromosomal DNA is digested, by an infrequently cutting ('rare-site') restriction endonuclease enzyme, into a small number of large fragments [125]. The fragments are resolved on an agarose gel by pulsed-field electrophoresis which is specific for the separation of large fragments of DNA. The electric field in the electrophoresis unit changes direction **(Figure 1.7)** at regular intervals throughout the gel run, this is referred to as switch time. This pulsing action assists the DNA fragments in moving through the pores of the gel. A molecular weight marker is included on every gel in at least two positions **(Figure 1.8)**.

Using specialised software, the gel is captured digitally and the image normalised to reduce background noise and visual artifacts, ultimately improving the visual quality of the fingerprint. Bands may be manually or automatically assigned to each DNA fragment on a fingerprint and a dendrogram is generated. The dendrogram shows the genetic relatedness amongst a group of strains [126]. Band differences between fingerprints represent the occurrence of genetic events [127]. Guidelines for the interpretation of PFGE patterns from small collections of outbreak isolates have

been suggested [127], however these cannot be directly applied to large collections of isolates collected over a long period of time (several years).

Analysis of PFGE patterns from such collections are therefore subject to interpretation which may differ from person to person. In addition, interlaboratory comparisons are challenging, if not impossible. A pattern-relatedness breakpoint (i.e. greater than or equal to a certain percentage) is applied to the dendrogram to group isolates into clusters. The breakpoint is based on findings in the literature as well as the diversity of the organism. Clusters are also defined by a minimum number of isolates. Isolates within each cluster are considered clonal, deemed to have arisen from a common ancestor. Isolates that do not meet the cluster definition are referred to as outliers.

The DNA patterns generated are specific for each strain and therefore PFGE can be applied to outbreak investigations and to studies of evolution and structure of bacterial populations [128-130]. The use of specialised software resolves variation between gels and ultimately improves the accuracy and reproducibility of fingerprints [131].

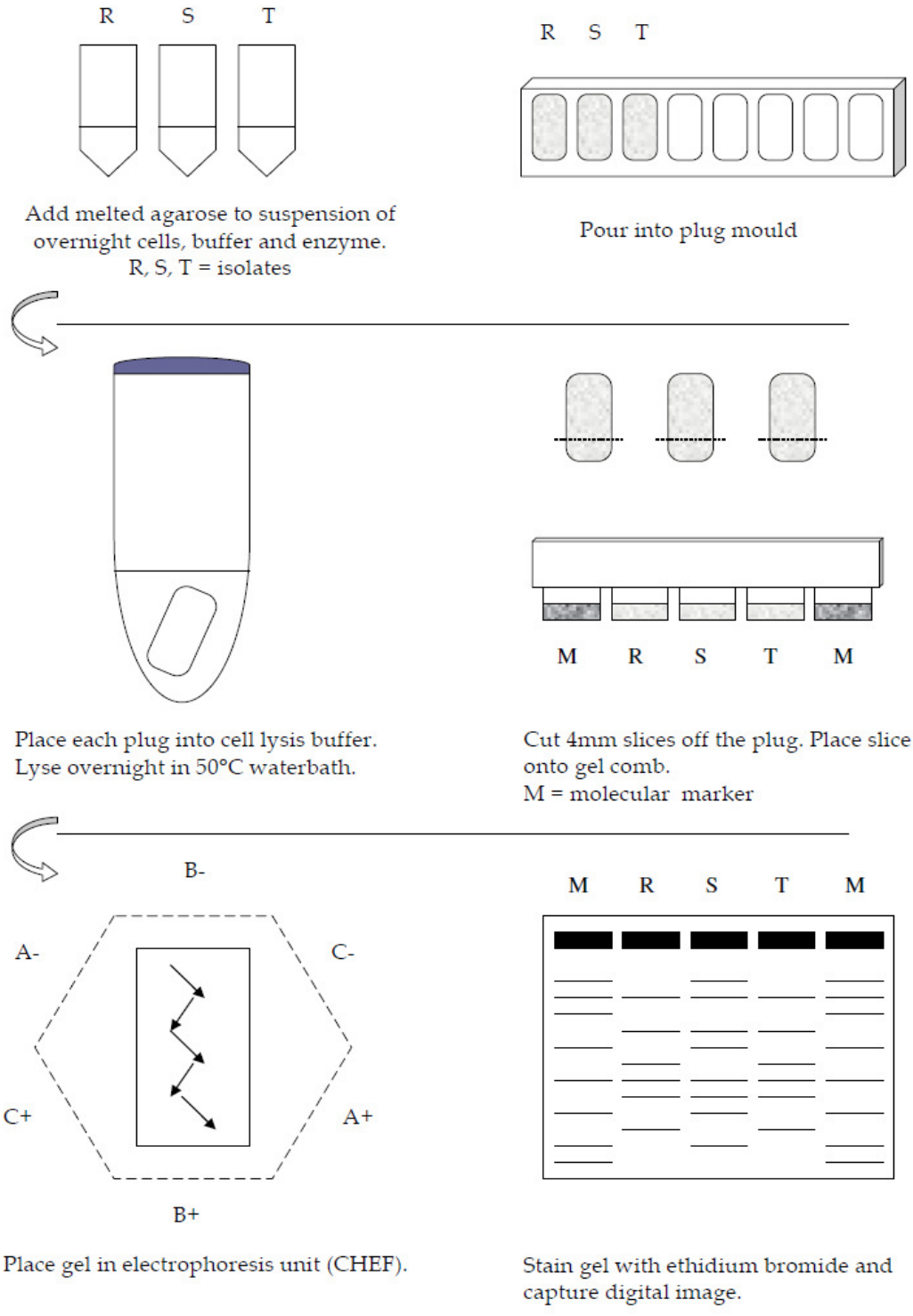


Figure 1.7 Pulsed-field gel electrophoresis.

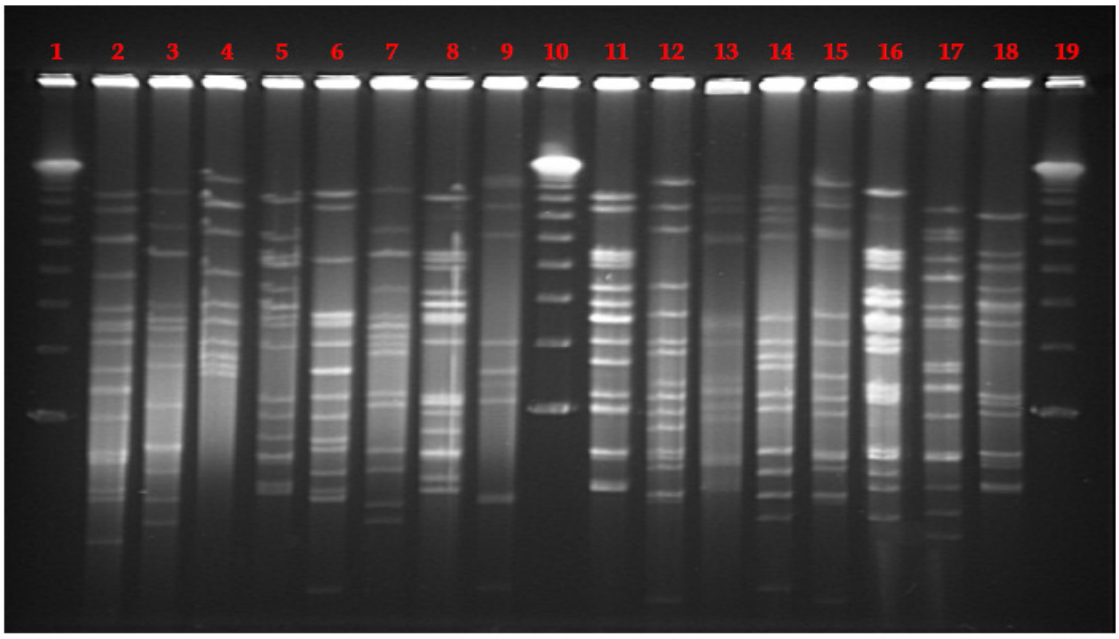


Figure 1.8 Representative PFGE fingerprint patterns of *N. meningitidis* serogroup B isolates. Lanes 1, 10 and 19 contain the molecular weight marker.

Still problems with reproducibility and standardisation between laboratories persist [132]. PFGE results are also subject to good quality chromosomal DNA, specific and consistent agarose plug, gel and loading conditions. Thus comparison of patterns between gels and between laboratories is challenging. Furthermore PFGE can only be applied to culture-positive cases. But despite these drawbacks the value of PFGE lies in its high discriminatory power, especially in outbreak situations, enabling distinction between isolates that may have been classified as similar or identical using other characterisation techniques, such as multilocus sequence typing.

1.6.2 Multilocus sequence typing (MLST)

MLST **(Figure 1.9)** is a nucleotide-based typing scheme that targets several house-keeping genes at various locations within the bacterial chromosome. The method was first proposed in 1998 by Maiden and colleagues [133]. The authors based MLST on the concept of a previous method, multilocus enzyme electrophoresis (MLEE) [134], and used *N. meningitidis* as the test organism. MLEE distinguishes isolates according to the electrophoretic mobility of several essential enzymes [135], however it is a laborious procedure and, as is the case with PFGE, difficult to compare between laboratories. MLST amplifies and sequences the genes that code for essential enzymes. These house-keeping genes or enzymes are under selection for conservation of function and therefore undergo very slow genetic variation [136]. A slow evolutionary rate makes house-keeping genes a reliable marker to determine genetic relationships between bacteria over long periods of time.

To improve discriminatory power several loci are selected [136]. A total of 107 *N. meningitidis* isolates representative of global strains from invasive cases and healthy carriers were chosen for the initial MLST analyses [133]. Initially six of eleven genes were chosen based on correlation with MLEE data, *fumC* was later added to the set of *N. meningitidis* MLST genes. The genes, with their encoded enzyme shown in parentheses include: *abcZ* (putative ABC transporter), *adk* (adenylate kinase), *aroE* (shikimate dehydrogenase), *fumC* (fumarate hydratase),

gdh (glucose-6-phosphate dehydrogenase), *pdhC* (pyruvate dehydrogenase subunit), *pgm* (phosphoglucomutase).

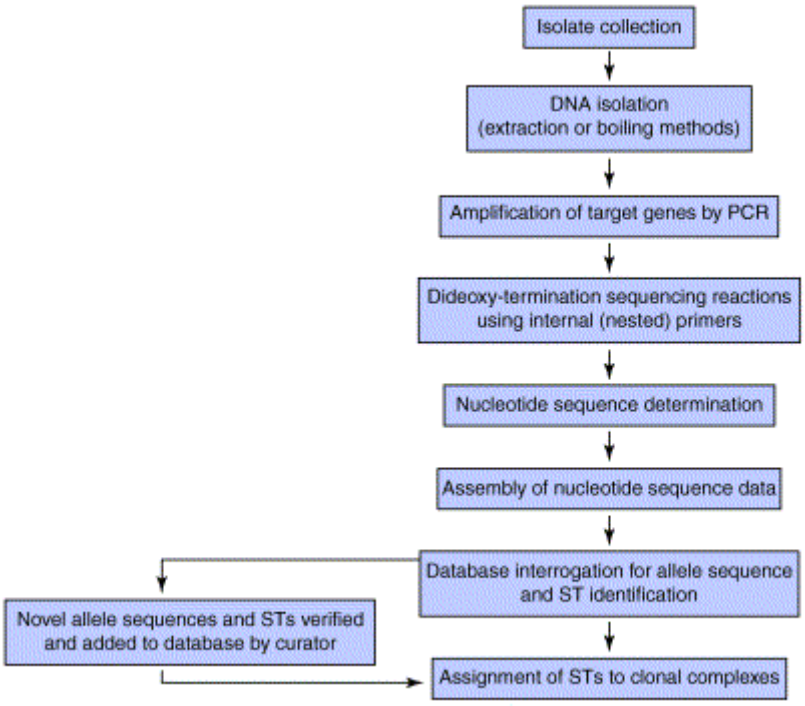


Figure 1.9 The MLST procedure [136].

Forward and reverse sequences of each of the seven loci are assembled using any DNA sequence alignment software. The resulting nucleotide sequences are submitted to the central internet MLST database and assigned an allele number, making up the allele profile for that isolate. The isolate is then assigned a sequence type (ST) based on the combination of alleles (allelic profile). Isolates that are closely related have identical STs or STs that differ at a few loci (usually three or less). Unrelated isolates have unrelated STs. Similarly, MLEE designated electrophoretic types (ET) to isolates based on a combination of

enzyme variants (electromorphs) [134]. Related sequence types can be grouped into clusters called clonal complexes [137].

A clonal complex is based on an ancestral ST (founder) and all related STs (isolates that share 4 or more alleles with the founder) that are grouped into the clonal complex (http://pubmlst.org/neisseria/mlst-info/nmeningitidis/complexes) [136]. Isolates that do not share the required number of alleles with existing founders are not assigned to a clonal complex on the MLST database.

An eBURST algorithm (http://eburst.mlst.net) is used to depict relatedness within a population based on the ST of each isolate. eBURST divides an MLST data set (STs and their allelic profiles) into groups based on a defined group definition. A stringent definition where members in a group share ≥6 of 7 alleles is recommended. In this case isolates within the group, defined by eBURST, will be considered to belong to a clonal complex. Groups can be displayed as an eBURST diagram **(Figure 1.10)**.

The primary founder of the group is the ST with the most number of single locus variants (SLVs). Double locus variants (DLVs) of the founder are only linked if the intermediate SLV is present. For instance, DLV ST11 **(Figure 1.10)** has to contain the same variation as ST10 and an additional variation. Thus eBURST provides a hypothesis for descent within a group of isolates. Subgroup founders are STs that have a number of SLVs of their own. eBURST uses a

bootstrapping procedure to indicate levels of confidence in the assignment of founders. An entire MLST dataset can be grouped as a single group by setting the group definition to 0 of 7 alleles. A single eBURST diagram can then be displayed. This is referred to as a population snapshot.

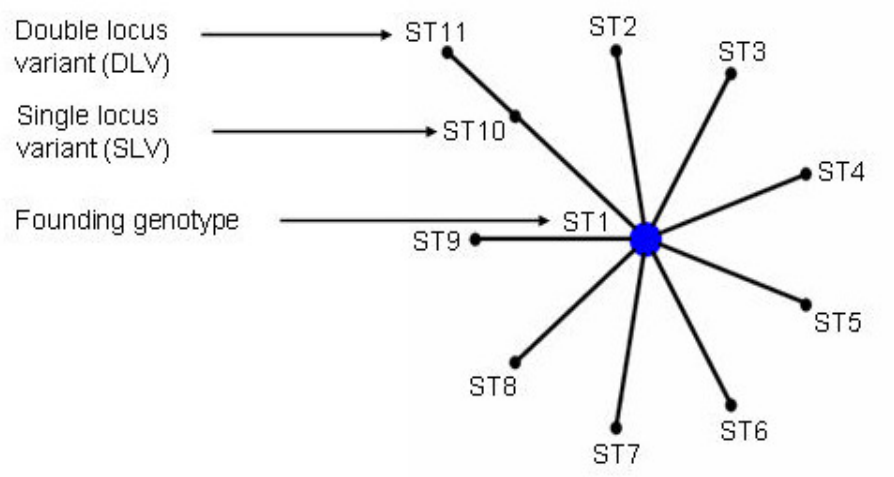


Figure 1.10 eBURST diagram. Primary founders are colored blue. The area of the circle is proportional to the prevalence of that ST in the input data. STs (SLVs and DLVs) are linked by solid black lines [138].

MLST data have been added to the expanding internet database since its inception in 1998. In 2004, new software was introduced that improved data collection and accessibility [139]. The MLST database has specific submission procedures and is monitored by curators, ensuring the quality and credibility of new sequence data. MLST is a generic nucleotide–based technology and as a result generates reproducible and unambiguous data. An advantage that MLST has over other bacterial typing schemes is that it is truly portable, available to laboratories via the World Wide Web. Most importantly, MLST can be applied

to culture-negative specimens [140;141], a common occurrence in meningococcal disease due to early antibiotic administration.

Correlation between PFGE and MLST

Isolates that cluster together on a PFGE dendrogram are considered clonal (genetically related) and have therefore arisen from a common ancestor [127;142]. If resources are limited, PFGE can be used to screen large numbers of isolates in order to get a sense of the overall clonality or diversity of a population, following which, random isolates may be selected for further characterisation by MLST. PFGE has been shown to be more discriminatory than MLST [143]. Therefore, although isolates within a PFGE cluster may have differences in their fingerprint patterns, they are still considered to be related and should, in most cases, belong to the same clonal complex [45;122]. Thus MLST of a few randomly selected isolates within a cluster may be used to infer clonal complex for the majority (if not all) isolates within the cluster.

1.6.3 PorA

PorA is a major porin of the meningococcal outer membrane. This surface exposed protein is abundant and acts as a pore, allowing nutrients to enter the cell [144]. The PorA structure crosses the cell membrane and contains 8 surface exposed loops **(Figure 1.11)**. The apex of loops I and IV have been identified as areas that undergo frequent antigenic change. These variable regions, termed

VR1 and VR2 respectively, are targets for characterisation and immunisation strategies [145-148].

OMPs are in direct contact with the bacterial environment and therefore are under strong selection for antigenic change. Since new types are constantly generated, serological characterisation using monoclonal antibody panels do not always cover all types [11;123]. Molecular serosubtyping is based on amplification and sequencing of the variable regions [146;149].

PorA nomenclature has been standardised [12;146] as follows: P1.VR1,VR2. The PorA type is designated by the prefix P1 and variable regions are separated by a comma. In addition, each variable region contains families, designated by a number, and within each family variants may arise i.e. variants of the original family member. Variants are also designated by numbers but separated from the original member by a hyphen. Thus the PorA type may be as follows: P1.7-2,4. PorA nucleotide sequences are submitted to an internet database (http://neisseria.org/nm/typing/pora), where the sequence is compared to existing sequences on the database for a match. New sequence data are submitted to curators of the database for PorA designation.

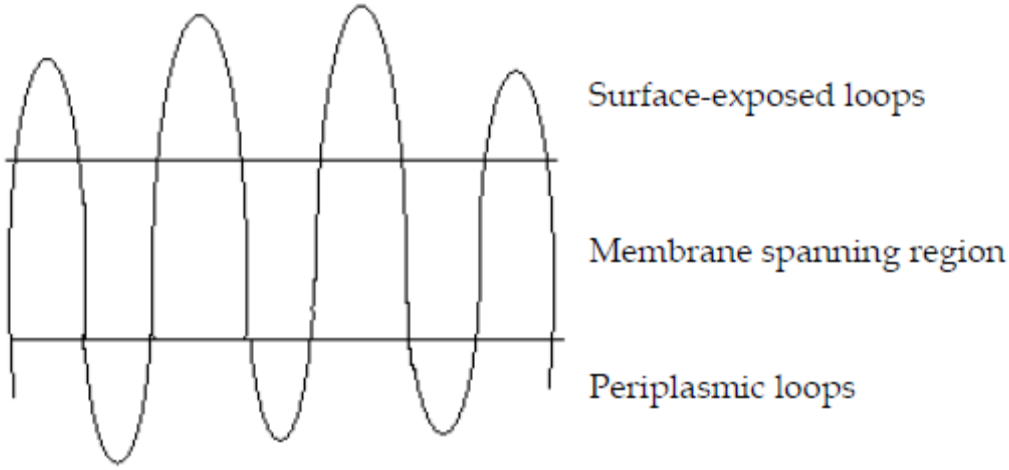


Figure 1.11 Schematic of the amphipathic beta sheet structure of the outer membrane proteins PorA and FetA [144;150].

1.6.4 FetA

FetA is a meningococcal surface-exposed iron receptor that is expressed under conditions of iron limitation. Free iron concentrations in the human body are low because iron is bound to storage and transport proteins such as hemoglobin, ferritin, transferrin and lactoferrin. Since iron is essential for bacterial growth [151], bacteria have developed iron-accumulating strategies. Some bacteria produce and secrete siderophores, organic chelating agents that solubilise iron and transport it back into the cell. The meningococcus acquires iron directly from lactoferrin [152] (at mucosal surfaces) and transferrin [153] (in serum).

Since the condition of iron limitation is met in the human body, the expression of FetA, previously known as FrpB [150;154], is ensured. This 70kDa protein is

employed in classification systems [12] and as a vaccine target. While iron limitation is required for expression, only a few isolates lack the genes encoding FetA [155]. Deletion of the *feta* gene seems to be a sporadic event that is not maintained.

A topology model was proposed in 1995 [150]. The FetA protein structure has 13 surface-exposed loops **(Figure 1.11)**. The largest of these, Loop 7, is a region of variability and is targeted for characterisation. The variable region (VR) is amplified and sequenced. FetA sequence data are submitted to a FetA internet database (http://neisseria.org/nm/fetA) for type designation. FetA types are divided into families and within each family there are variants. A family is designated by a number and preceded by an F (e.g. F1) and the variant follows after a hypen, e.g. F1-5. Like PorA typing, FetA typing is a generic sequence-based method and as a result provides portable and unambiguous characterisation data.

1.7 Molecular epidemiology

Portable DNA-based methods have vastly improved global meningococcal surveillance [156]. Invasive clones can be rapidly and unambiguously monitored as they spread between countries and across continents. Molecular surveillance is also important to track changes in population structure and evolution of *N. meningitidis*.

1.7.1 Genetic diversity in meningococcal populations

A naturally transformable bacterium

Bacterial cells reproduce asexually by binary fission resulting in daughter cells that are identical to the parent cell [157]. During cell division mutations occur giving rise to genetic variation. Mutations are maintained through successive generations but as the population grows selection processes favour isolates with certain mutations while removing others. The result is a relatively clonal population.

However bacterial cells are also able to exchange genetic material 'horizontally' [158;159]. Horizontal gene transfer is the exchange of small fragments of genetic material between cells that do not share the same parent and is referred to as 'localised sex'. If this process occurs at a high frequency, the clonal structure of the population is disrupted. Transformation (uptake of DNA fragments from the environment by competent cells and integration of the fragment into host DNA) is one of the ways by which horizontal gene transfer occurs. *N. meningitidis* is a naturally competent bacterium, able to take up homologous DNA from the environment [157;158;160], and incorporate it into the chromosome by recombination.

Phase variation

Phase variation brings about genetic diversity through mutation [161]. The meningococcal genome contains repetitive sequences of DNA that control the

expression of genes through a process called phase variation. The repeat sequences may be located anywhere in the genome and are used by the bacteria to adapt quickly to their current environment. Mutations are passed on to successive generations and selection forces choose variants with increased fitness.

Thus, a high degree of genetic diversity is brought on by increased rates of recombination and phase variation, in the meningococcus [123;162]. Despite this, clonal complexes within meningococcal populations persist over time and geographical distance [163]. So while *N. meningitidis* serogroup B isolates are extremely diverse, there are natural selective forces (as yet undetermined) that work to maintain a clonal population structure [164].

1.7.2 Invasive clonal complexes

When describing molecular disease epidemiology, isolates are often referred to by the clonal complex to which they belong (designated as ST-clonal complex number) [165]. Where available the corresponding ET lineage is included in the nomenclature (e.g ST-32/ET-5 complex). Carriage isolates are more genetically diverse and so belong to a greater number of clonal complexes [17;121;166], many of which are not associated with invasive isolates. Globally, the majority of global invasive disease is caused by strains that belong to a few clonal complexes refered to as hyperinvasive lineages [21;156;167]. Furthermore, clonal complexes may have a dominant serogroup, PorA and FetA types **(Table**

1.1). With regard to serogroup B invasive disease there are two important invasive lineages, ST-32/ET-5 and ST-41/44/lineage 3 [21]. Clones of these lineages have caused meningococcal outbreaks since the early 1970s **(Table 1.2)**.

1.7.3 South Africa

From 1973 to 1978 circulating serogroup B clones included ET26, ET27 and ET10 from clonal complexes ST-11/ET-37 and ST-8/Cluster A4 [168] with no evidence of the ST-32/ET-5 complex [169;170] **(Figure 1.12)**. From 1977 through 1979 South Africa experienced an increase in incidence of meningococcal disease. Isolates from cases during the outbreak revealed ET10 as the dominant clone. MLEE of a selection of serogroup B isolates (n=21) identified two strains of the ST-32/ET-5 complex (B:NT:P1.15) in South Africa in 1980 [169-171].

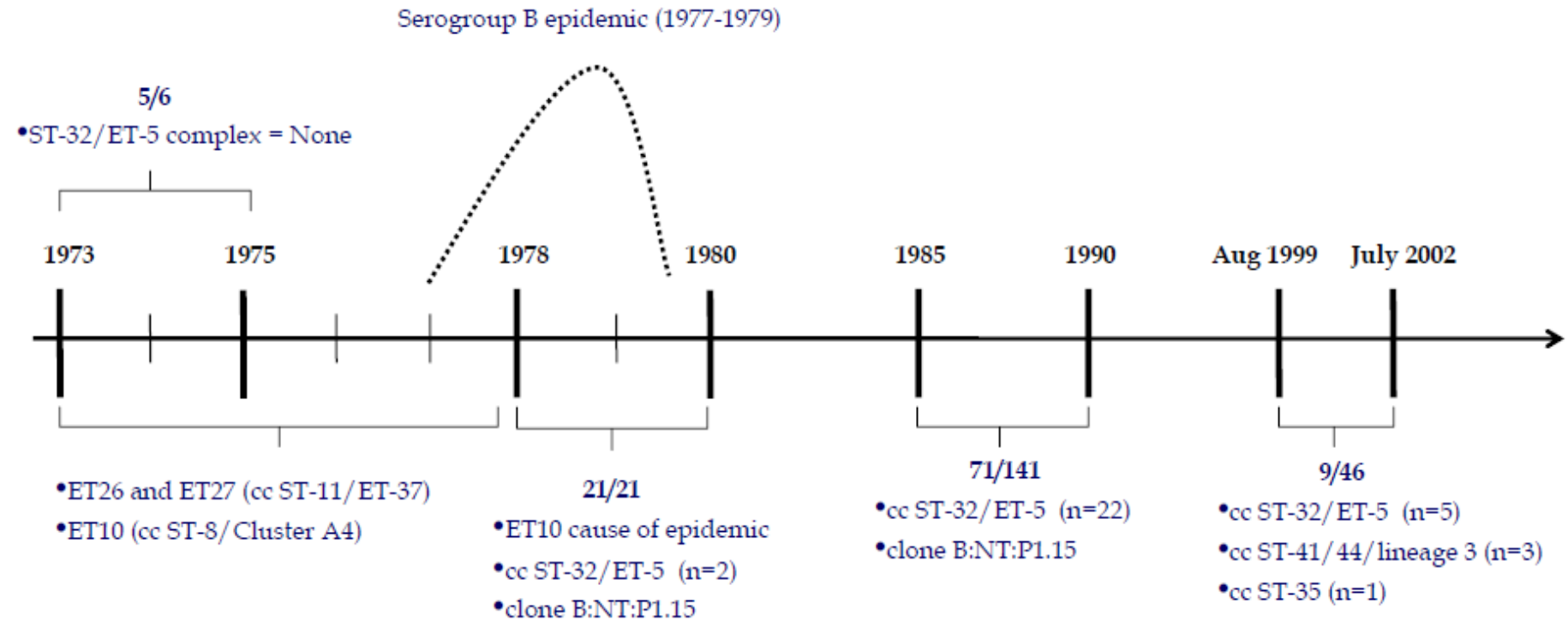
Orren et al. [172] analysed 124 routinely collected invasive isolates from the Western Cape from 1985 to 1990. A total of 22 isolates belonged to clonal complex ST-32/ET-5. The most common strain among routine isolates was B:4:P1.15. Then a local molecular epidemiology study by Coulsen et al. [79] on meningococcal isolates collected through our national laboratory-based surveillance system from August 1999 through July 2002, showed, once again, the circulation of the ST-32/ET-5 clone in the country. The Coulson study also identified the presence of ST-41/44/lineage 3 strains in South Africa.

Table 1.1 Important *N. meningitidis* clonal complexes compiled from the MLST database (February 6, 2009) [165].

Clonal complex	MLEE designation	No. of isolates	No. of STs	Dominant serogroups (%)	Dominant PorA	Dominant FetA
ST-1 complex	Subgroup I/II	204	49	A(97)	5-2,10	F3-5
ST-5 complex	Subgroup III	627	33	A(99)	20,9	F3-1
ST-8 complex	Cluster A4	283	107	B(51),C(35)	5-1,2-2	F3-6
ST-11 complex	ET-37 complex	1142	239	C(57),W135(24),B(12)	5,2	F3-6
ST-18 complex	Cluster J1	208	175	B(85)	22,14	F3-6
ST-22 complex		363	243	W135(52),NG(25)	18-1,3	F4-1
ST-23 complex	Cluster A3	385	154	Y(62),NG(18)	5-1,2-2	F4-1
ST-32 complex	**ET-5 complex**	**1028**	**350**	**B(85)**	**19-15**	**F5-1**
ST-35 complex		329	214	B(59),NG(25)	22-1,14	F4-1
ST-41/44 complex	**Lineage 3**	**1796**	**1274**	**B(70)**	**7-2,4**	**F1-5**
ST-53 complex		272	93	NG(76)	7-2,30	F1-7
ST-60 complex		225	148	B(30),29E(22),NG(19)	5,2	F1-7
ST-103 complex		127	84	B(26),NG(22),C(16)	18-1,3	F3-9
ST-162 complex		140	63	B(74),NG(13)	22,14	F5-9
ST-167complex		201	144	Y(47),NG(36)	5-1,10-4	F3-4
ST-198 complex		166	76	NG(76)	18,25-15	F5-5
ST-213 complex		187	165	B(74),NG(16)	22-14	F5-5
ST-254 complex		148	107	NG(35),B(24),29E(12)	5-1,16	F1-7,F3-6
ST-269 complex		415	312	B(73)	22,9	F5-1
ST-334 complex		106	64	C(58),B(33)	5-1,2-2	F1-5

Table 1.2 Genotypes of *N. meningitidis* serogroup B epidemic strains [21].

Location	Time period	Antigenic profile
ST-32/ET-5 complex		
Norway	1960s and 1970s	B:15:P1.7,16
Spain	1970s	B:4:P1.19,15
Cuba, Brazil	1980s	B:4:P1.19,15
Chile	1980s	B:15:P1.3
Oregon, U.S.	1990-2000s	B:15:P1.7,16
ST-41/44/lineage 3 complex		
Netherlands	1980s	B:4:P1.4
New Zealand	1990s-present	B:4:P1.7b,4



* ET- electrophoretic type. ST-sequence type. cc-clonal complex. n/N- number of serogroup B isolates/ total number of *N. meningitidis* isolates examined from South Africa.

Figure 1.12 Timeline depicting clones identified in South Africa.

1.8 Aim

To update serogroup B molecular epidemiology data by investigating the molecular diversity of circulating *Neisseria meningitidis* serogroup B isolates in South Africa from January 2002 through December 2006.

1.9 Objectives

1. To characterise all *Neisseria meningitidis* serogroup B isolates (n=307) available from cases reported to the national laboratory-based surveillance system from January 2002 through December 2006, using PFGE.
2. To fully characterise all serogroup B isolates (n=58) from one year (2005) using MLST, *porA* and *fetA* typing.
3. To characterise randomly selected serogroup B isolates from other collection years using MLST, *porA* and *fetA* typing.
4. To compare the above results to existing PFGE and MLST data for serogroup B isolates collected from August 1999 through July 2002.

2 METHODS

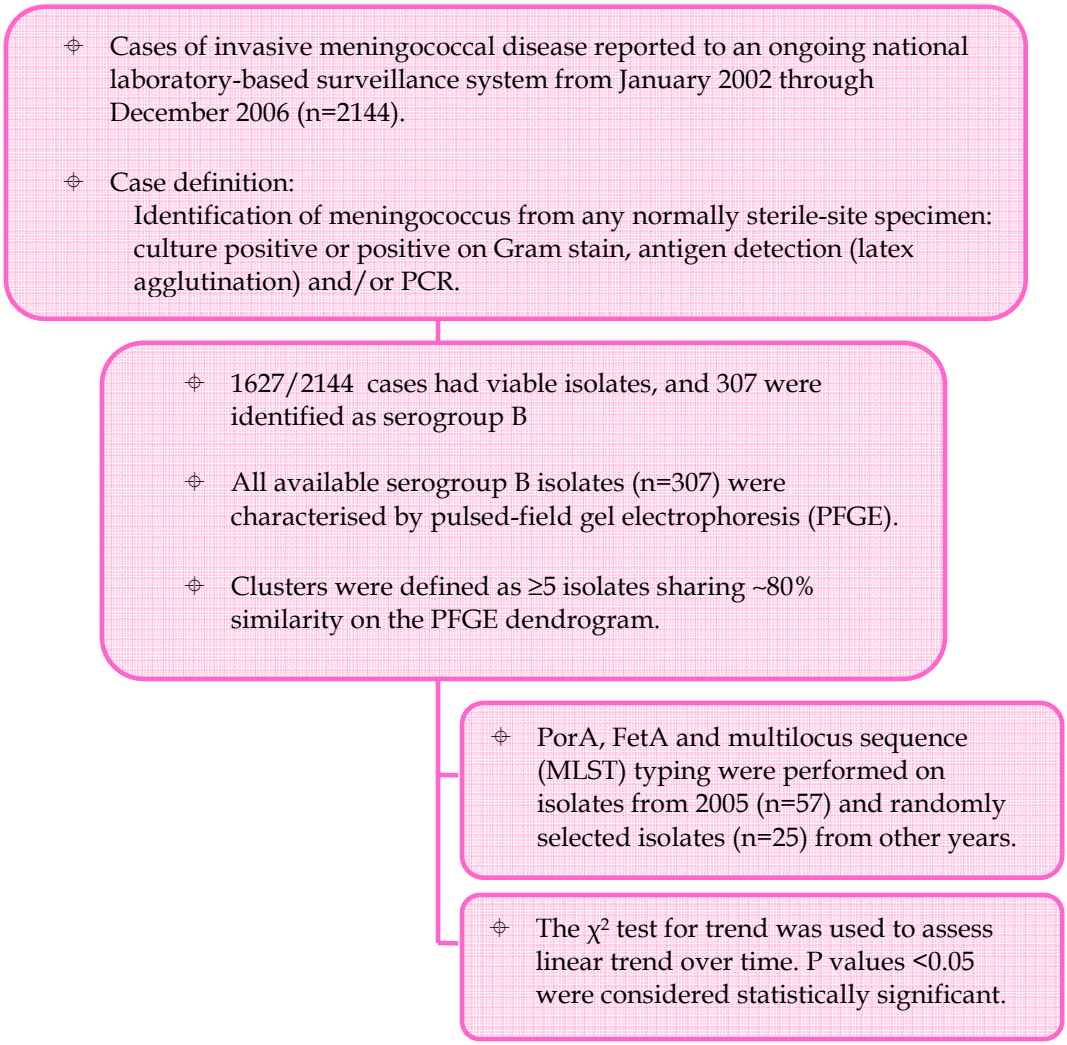


Figure 2.1 A brief overview of the methodology used in this study.

2.1 Bacterial isolates

N. meningitidis serogroup B isolates were obtained from the Respiratory and Meningeal Pathogens Research Unit (RMPRU) at the National Institute for Communicable Diseases (NICD), of the National Health Laboratory Service (NHLS), Johannesburg, South Africa. The unit conducts ongoing national laboratory-based surveillance for invasive disease caused by bacterial respiratory

pathogens including *N. meningitidis.* Viable isolates, together with patient and specimen details, are routinely submitted to the unit from approximately 180 private, public, military and mining hospital laboratories country wide *(Appendix A3)*. In 2003, surveillance was enhanced at 26 sentinel sites *(Appendix A1)* and cases from these sites were accompanied by additional patient data such as final outcome, discharge diagnosis, severity of illness, HIV status, history of tuberculosis treatment, and antibiotic use prior to and during admission. Data were collected from cases by on-site surveillance officers according to a standardised case report form *(Appendix A2).* Surveillance officers gathered information by interviewing patients or patient contacts, and/or from available medical records.

Once received, isolates were cultured from Dorset egg transport media [173] (Diagnostic Media Products, a division of the NHLS, Johannesburg, South Africa) onto Columbia agar (Oxoid Ltd, Basingtoke, United Kingdom) supplemented with 5% horse blood (South African Vaccine Producers, a division of the NHLS, Johannesburg, South Africa) and incubated at 37°C in 5% CO_2. Isolates were serogrouped by slide agglutination by first using polyclonal antisera to capsular polysaccharides A, B, C and D and, X, Y, Z and W135 (Remel Europe Ltd, Dartford, England). Then a reaction to determine the precise serogroup was carried out using monoclonal anitisera (Remel Europe Ltd, Dartford, England). Pure cultures were prepared for long term storage by suspension in 1 ml of 10% skim milk (Oxoid Ltd, Basingtoke, United Kingdom) and stored at -70°C.

2.2 Case definition

Cases were defined as patients with *N. meningitidis* identified from normally sterile body fluids (e.g. blood, cerebrospinal fluid, joint fluid and pleural fluid).

2.3 Pulsed-field gel electrophoresis

PFGE was carried out as previously described by Gautom [124]. A total of 307 serogroup B isolates causing invasive meningococcal disease in South Africa from January 2002 through December 2006 were available for PFGE.

2.3.1 Plug preparation

Each isolate was sub-cultured from -70°C onto 5% horse blood agar and incubated overnight at 37°C in 5% CO_2. Cultures were then directly suspended with cotton swabs into 1.5 ml of TE buffer *(Appendix B3)* and adjusted to a cell concentration of 0.5-1.0 using a Dade Microscan Turbidity Meter (Dade, California, USA). 200 µl of cell suspension was transferred to a 1.5 ml microcentrifuge tube, to which 20 µl of 10 mg/ml lysozyme (Sigma Aldrich, Steinheim, Germany) *(Appendix B4)* and 10 µl of 20 mg/ml proteinase-K (Roche, Mannheim, Germany) *(Appendix B5)* were added for cell lysis. Cell suspensions were incubated at 37°C for 15 min. Seakem Gold agarose (Lonza, Rockland, USA) solution was made up to a 1.2% concentration *(Appendix B8)* and maintained in a 55°C waterbath. Following incubation, 20 µl of 10% SDS *(Appendix B9)* was added to each cell suspension. Finally, 260 µl of the melted 1.2% Seakem Gold agarose was mixed into each

bacterial suspension. The bacterium-agarose mixture was immediately pipetted into a plug mould and allowed to solidify for 20 to 30 min at room temperature. The solidified plug was transferred into 2 ml of Cell Lysis Buffer *(Appendix B10)* and incubated in a shaking water bath at 50°C for a minimum of 2 h. After incubation, cell lysis buffer was poured off and plugs were washed in 8 to 10 ml of preheated (50°C) sterile water for 15 min at 50°C. The plugs were then washed 3 times (15 min each) with 8 to 10 ml preheated (50°C) TE buffer. All washes were done in an oscillating water bath at 50°C. Plugs were subsequently transferred to 2 ml of TE buffer (room temperature) and stored at 4°C for further use.

2.3.2 Restriction endonuclease digestion

For restriction endonuclease digestion, 4 mm thick slices of the plug were incubated in 100 µl of 1x restriction buffer *(Appendix B11)* at 37°C for 15 min. Restriction buffer was removed and DNA within the agarose plug was digested with 100 µl *Nhe*I restriction enzyme (Roche Diagnostics, Mannheim, Germany) cocktail *(Appendix B12)*.

2.3.3 Electrophoresis

Digested slices were then carefully loaded onto a comb, placed onto a gel tray and allowed to air dry for 5 to 8 min. A commercially available DNA lambda ladder (Bio-Rad, Hercules, CA) was used as a molecular marker and was placed in the first and last lanes on the gel. A 160 ml 1.1% Seakem Gold agarose solution was prepared. The solution was placed on a stirrer-hot plate and equilibrated to 54°C to

56°C. The agarose solution was poured into the gel tray and allowed to set. Approximately 3 L of 0.5x TBE buffer *(Appendix B7)* was equilibrated to 14°C in the PFGE chamber. Once solidified the gel was placed into the electrophoresis chamber. Electrophoresis was performed using the following conditions: 6 V/cm, 120° angle and switch times of 1-30 s for 16 h followed by 0.1-6 s for 4 h.

2.3.4 Visualisation and gel capture

Upon completion of electrophoresis, gels were stained in 300 ml of ethidium bromide solution *(Appendix B13)* for 15 min. The gel was washed 3 times (for 10 min each) in ~300 ml of distilled water and viewed on an ultraviolet transilluminator for fingerprint patterns. The gel was photographed using a polaroid camera and digitally captured using a gel documentation system (Vacutec, Johannesburg, South Africa).

2.3.5 PFGE image analysis

Digital images were analysed using GelCompar™ v6.1 software (Applied Maths, Kortrijk, Belgium). Fingerprints were added to an existing *N. meningitidis* PFGE database. This database contained the following fields: isolate number, serogroup, collection year, collection date, province, sequence type, clonal complex, PorA type, and FetA type. Genetic relatedness was determined by creating dendrograms using the unweighted pair group method with arithmetic averages (UPGMA) and Dice coefficient using an optimisation of 1.5% and 1.5% position tolerance. A cluster was defined as five or more isolates that shared ~80% similarity on the

dendrogram [79;126;127]. Isolates that not meet the cluster definition were referred to as outliers.

2.4 Multilocus sequence typing (MLST)

The MLST procedure was based on the method described on the MLST website: (http://pubmlst.org/neisseria/). Approximately 10% of the total number of isolates available for PFGE (n=307), were randomly selected for MLST. A web-based random number generator (http://www.random.org/sequences/) was used to generate a selection of 30 isolates. An additional four isolates from 2002 (n=2) and 2003 (n=2) had MLST data only. MLST was also performed on all serogroup B isolates from 2005 (n=58).

2.4.1 DNA extraction

Crude DNA extracts were prepared by suspending a loopful of overnight culture in 100 µl sterile distilled water. Cell suspensions were boiled at 95°C for 10 min to lyse the cells, followed by centrifugation at 12 000 rpm for 15 s. The supernatant contained the DNA.

2.4.2 PCR of house-keeping genes

PCR was used to amplify internal fragments of the following seven house-keeping genes: *abcZ* (putative ABC transporter), *adk* (adenylate kinase), *aroE* (shikimate

dehydrogenase), *fumC* (fumarate hydratase), *gdh* (glucose-6-phosphate dehydrogenase), *pdhC* (pyruvate dehydrogenase subunit), *pgm* (phosphoglucomutase). The primer pairs used for PCR amplification are depicted in **Table 2.1**.

Table 2.1 Meningococcal MLST PCR primers.

Gene	Primer
abcZ	abcZ-P1C 5-TGTTCCGCTTCGACTGCCAAC-3
	abcZ-P2C 5-TCCCCGTCGTAAAAAACAATC-3
adk	adk-P1B 5-CCAAGCCGTGTAGAATCGTAAACC-3
	adk-P2B 5-TGCCCAATGCGCCCAATAC-3
aroE	aroE-P1B 5-TTTGAAACAGGCGGTTGCGG-3
	aroE-P2B 5-CAGCGGTAATCCAGTGCGAC-3
fumC	fumC-P1B 5-TCCCCGCCGTAAAAGCCCTG-3
	fumC-P2B 5-GCCCGTCAGCAAGCCCAAC-3
gdh	gdh-P1B 5-CTGCCCCCGGGGTTTTCATCT-3
	gdh-P2B 5-TGTTGCGCGTTATTTCAAAGAAGG-3
pdhC	pdhC-P1B 5-CCGGCCGTACGACGCTGAAC-3
	pdhC-P2B 5-GATGTCGGAATGGGGCAAACA-3
pgm	pgm-P1 5-CTTCAAAGCCTACGACATCCG-3
	pgm-P2 5-CGGATTGCTTTCGATGACGGC-3

PCR cycling conditions were as follows:

1 cycle : 94°C for 2 min

25 cycles : 94°C - 1 min, 56-62°C - 1 min, 72°C - 1 min

1 cycle : 72°C for 5 min

Annealing temperatures vary for the different primer sets : *abcZ*, *adk*, *pdhC* and *gdh* anneal at 58°C, while *aroE*, *fumC* and *pgm* anneal at 56°C, 60°C, and 62°C respectively.

2.4.3 Cleanup of PCR products

PCR products were purified using the MSB® Spin PCRapace purification kit (Invitek, Berlin, Germany). Clean up was performed as per the manufacturer's instructions. Briefly, the PCR sample was mixed thoroughly with binding buffer. The solution was then transferred onto a spin filter and centrifuged for 3 min at 12 000 rpm. Elution buffer was added directly onto the center of the spin filter in order to elute the PCR fragments. The spin filter with elution buffer was incubated at room temperature for 5 min before centrifuging for 1 min at 10 000 rpm. The eluate, containing the purified PCR fragments, were stored at 4°C.

2.4.4 Cycle sequencing

Following amplification, sequencing of both forward and reverse strands was carried out using the Big Dye v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI 3130 Genetic Analyser (Applied Biosystems). Sequencing reactions for each strand (forward and reverse) were set up and prepared to a final volume of 10 µl, using the following components: 1 µl Terminator Ready Reaction Mix, 1.5 µl 5x Sequencing Buffer,

0.5 µl purified DNA, 0.5 µl of a 0.5 µM forward or reverse primer and 6.5 µl sterile deionised water. Reaction conditions were as follows :

1 cycle : 94°C for 1 min

25 cycles : 95°C - 30 s, 50°C - 20 s and 60°C - 4 min

Sequencing primers are depicted in **Table 2.2**.

Table 2.2 Meningococcal MLST sequencing primers.

Gene	**Primer**
abcZ	abcZ-S1A 5-AATCGTTTATGTACCGCAGR-3
	abcZ-S2 5-GAGAACGAGCCGGGATAGGA-3
adk	adk-S1A 5-AGGCWGGCACGCCCTTGG-3
	adk-S2 5- CAATACTTCGGCTTTCACGG-3
aroE	aroE-S1A 5- GCGGTCAAYACGCTGRTK-3
	aroE-S2 5-ATGATGTTGCCGTACACATA-3
fumC	fumC-S1 5-TCCGGCTTGCCGTTTGTCAG-3
	fumC-S2 5-TTGTAGGCGGTTTTGGCGAC-3
gdh	gdh-S3 5-CCTTGGCAAAGAAAGCCTGC-3
	gdh-S4C 5-RCGCACGGATTCATRYGG-3
pdhC	pdhC-S1 5-TCTACTACATCACCCTGATG-3
	pdhC-S2 5-ATCGGCTTTGATGCCGTATTT-3
pgm	pgm-S1 5-CGGCGATGCCGACCGCTTGG-3
	pgm-S2 5-GGTGATGATTTCGGTYGCRCC-3

In order to remove unincorporated dye terminators from the sequencing reaction, cycle sequencing products were cleaned using one of two kits : DyeEx 2.0 Spin Kit (Qiagen Inc., CA, USA) or NucleoSEQ (Macherey-Nagel, Düren, Germany). Spin columns with a hydrated gel matrix were centrifuged at 2700 rpm for 3 min. Cycle sequencing samples were then added drop-wise to the center of the dehydrated gel matrix and centrifuged at 3000 rpm for 5 min to recover purified sequences. Samples were dried in a vacuum centrifuge and resuspended in 12 µl of Hi-Di Formamide (Applied Biosystems). 10 µl of each sample was loaded into a 96-well reaction plate for capillary injection electrophoresis.

2.4.5 Analysis of sequences

Sequence data were analysed using DNAStar Lasergene v7 software (Madison, Wisconsin, USA). Sequences were trimmed to the appropriate length using a reference sequence (for each gene of interest) obtained from the MLST website. Sequences were submitted to the MLST database (http://pubmlst.org/nesseria/allelicprofile/STdatabase) for allele and sequence type assignment. eBURST diagrams depicting genetic relatedness by sequence type were generated using software at http://eburst.mlst.net. New allele profiles, resulting from the identification of a new allele or new allelic combination, were submitted to the MLST database for designation of a new ST.

2.5 PorA typing

PorA typing was done as described by Sacchi and collegues [174]. All randomly selected serogroup B isolates (n=30), isolates with existing MLST data (n=4) and those from 2005 (n=58), were selected for analysis by PorA typing. The variable regions of PorA (VR1 and VR2) were amplified by PCR using primers listed in **Table 2.3**.

Table 2.3 PorA PCR and sequencing primers.

	Primer
PCR	P14 5-GGGTGTTTGCCCGATGTTTTT
	P22 5-TTAGAATTTGTGGCGCAAACCGAC-3
SequencingVR1	U86 5-GCCCTCGTATTGTCCGCACTG-3
	435 5-TTGCTGTCCCAAGGATTAATGGC-3
SequencingVR2	435 5-GCCATTAATCCTTGGGACAGCAA-3
	773 5-GGCATAGTTCCCGGCAAAACCGCCAT-3

PCR cycling conditions were as follows:

1 cycle : 94°C for 1 min

30 cycles : 94°C - 1 min, 60°C - 30 s, 72°C - 2 min

1 cycle : 72°C for 5 min

Nucleotide sequences were determined as described in section 2.4.4. Sequence data was analysed using DNAStar Lasergene v7 software. Sequences were

submitted to http://neisseria.org/nm/porA to identify PorA VR1 and VR2 regions.

2.6 FetA typing

FetA typing was performed as described on the Neisseria.org website (http://neisseria.org/nm/typing/feta/information). All randomly selected serogroup B isolates (n=30), those with existing MLST data (n=4) and those from 2005 (n=58), were selected for FetA typing. The variable region of FetA was amplified by PCR using primers as depicted in **Table 2.4**.

PCR cycling conditions were as follows:

1 cycle : 94°C for 1 min

30 cycles : 94°C - 1 min, 55°C - 1 min, 72°C - 90 s

1 cycle : 72°C for 1 min

Nucleotide sequences were determined as described in section 2.4.4. Sequence data were analysed using DNAStar Lasergene v7 software. Sequences were submitted to http://neisseria.org/nm/fetA to identify FetA types.

Table 2.4 FetA PCR and sequencing primers.

	Primer
PCR	S1 5-CGGCGCAAGCGTATTCGG-3
	S8 5-CGCGCCCAATTCGTAACCGTG-3
Sequencing	S12 5-TTCAACTTCGACAGCCGCCTT-3
	S15 5-TTGCAGCGCGTCTACAGGCG-3

2.7 Data analysis

Age-specific incidence rates were calculated for the last year (2006) of the study period. The total number of laboratory-confirmed cases reported to RMPRU in 2006 were divided by mid-year total population estimates provided by Statistics South Africa (http://www.statssa.gov.za/publications/ P0302/P03022006).

The following demographic data were captured on a Microsoft Excel spreadsheet: isolate collection date, serogroup, province, PFGE cluster, sequence type, clonal complex, PorA type and FetA type, patient gender, race, age category, outcome (died vs recovered), and specimen type (from which the organism was isolated). Comparisons by year, geographic area, PFGE cluster and sequence data were made. Differences in proportion were calculated using the χ^2-test (Mantel-Haentzel or Fisher's exact test), while trend over time was evaluated using the χ^2-test for trend. P values of less than 0.05 were considered significant. Statistical tests were performed using *Epi Info*, version 6.04d (Centers for Disease Control and Prevention, GA, USA).

2.8 Ethics

Ethics approval for national surveillance (PRC Reference no. 000105) and ethics clearance for this project (Protocol No. M080701) *(Appendix C)*, were obtained from the Human Research Ethics Committee (Medical) at the University of the Witwatersrand, Johannesburg.

3 RESULTS

3.1 Epidemiology of meningococcal disease in South Africa, 2002-2006

For the study period, January 2002 through December 2006, 2144 cases **(Figure 3.1)** of laboratory-confirmed meningococcal disease were reported to RMPRU. Gauteng (1234/2144, 58%) and the Western Cape (354/2144, 17%) recorded the highest number of cases among the 9 provinces **(Figure 3.2).** Of 2144 cases, 1627 (76%) had viable isolates and were therefore serogrouped **(Table 3.1).** Serogroup B (307/1627, 19%) and serogroup W135 (702/1627, 43%) were collectively responsible for 62% of cases in South Africa. Serogroup B prevalence decreased from 24% in 2002 to 14% in 2006 (p=<0.001). Serogroup B was most common in the Western Cape and W135 in Gauteng. Serogroup B cases were reported in all provinces **(Figure 3.3).**

Age was known for 94% (2005/2144) of all cases and 97% (299/307) of serogroup B cases. Children <5 years of age were at highest risk for meningococcal disease **(Figure 3.4).** With regard to gender, males seemed to be more affected by the disease than females but this was not significant (all disease, p=0.256 and serogroup B disease, p=0.685) **(Table 3.2).** Overall, 1418/2144 (66%) of all meningococcal disease cases were diagnosed by identification of the organism on CSF specimens alone, 249 (12%) from CSF and blood specimens, and 469 (22%) from blood specimens (without CSF). A minority of cases were identified from specimens that were not CSF or blood (2 cases from pleural fluid, 6 cases from synovial fluid or joint tissue). Serogroup B

isolates were cultured from predominantly CSF specimens (194/307, 63%) and CSF and blood specimens (43/307, 14%), while 70/307 (23%) were cultured from blood specimens alone. The proportion of cases of serogroup B identified from CSF specimens was not different to the proportion of non-serogroup B isolates from the same specimen (237/307, 77% vs 1021/1320, 77%, p=0.955). Of 307 serogroup B isolates received, only 100 were from cases at enhanced surveillance sites and had additional clinical details. Of these, 99 had documented clinical diagnosis: 90 (91%) with meningitis, and 9 with signs of generalized sepsis without meningitis. The final outcome of serogroup B disease was known for 97/100, and the case-fatality ratio was 9/97 (9%).

Figure 3.1 Number of cases of meningococcal disease in South Africa as reported to RMPRU, 2002-2006 (n=2144).

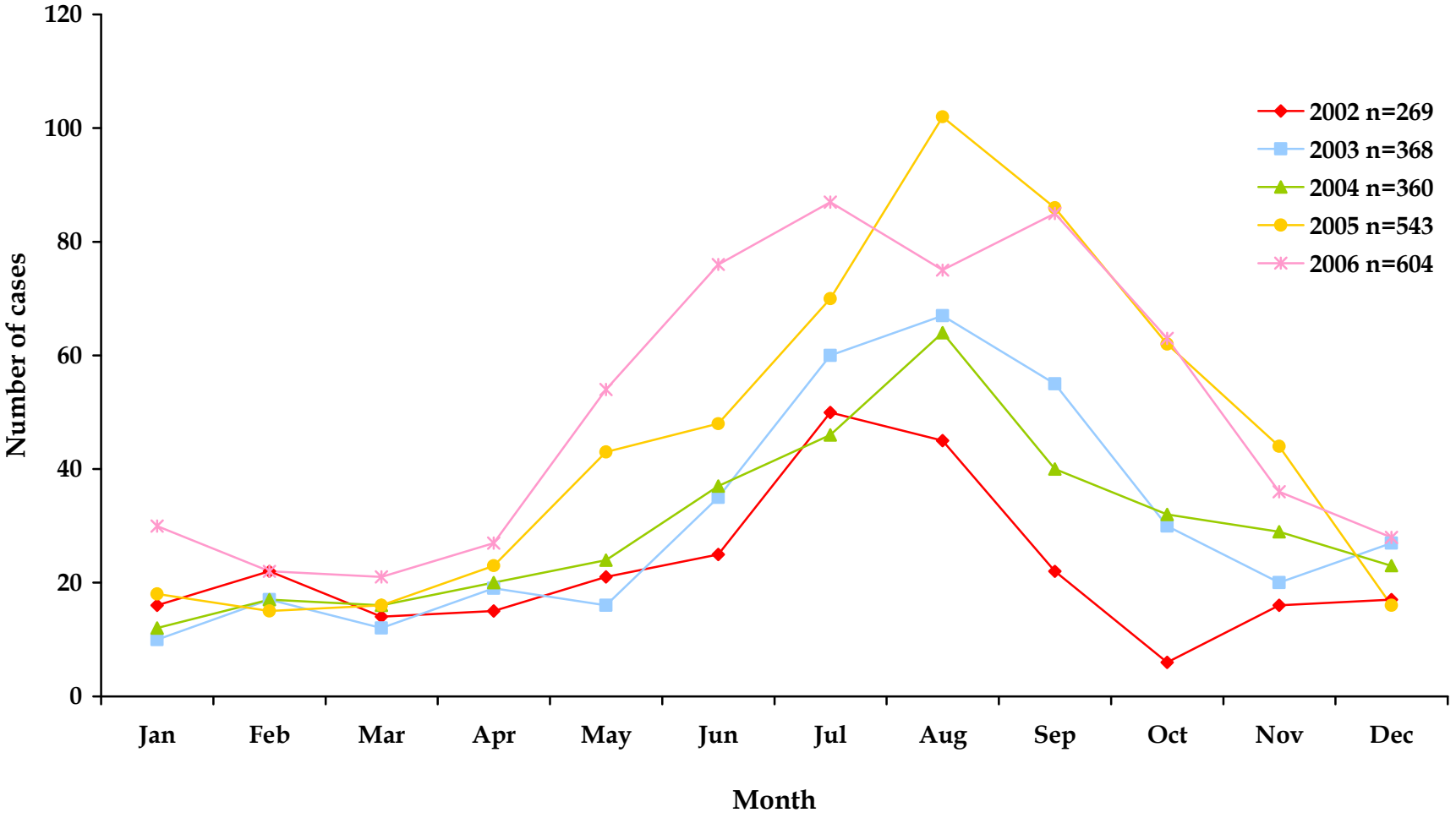
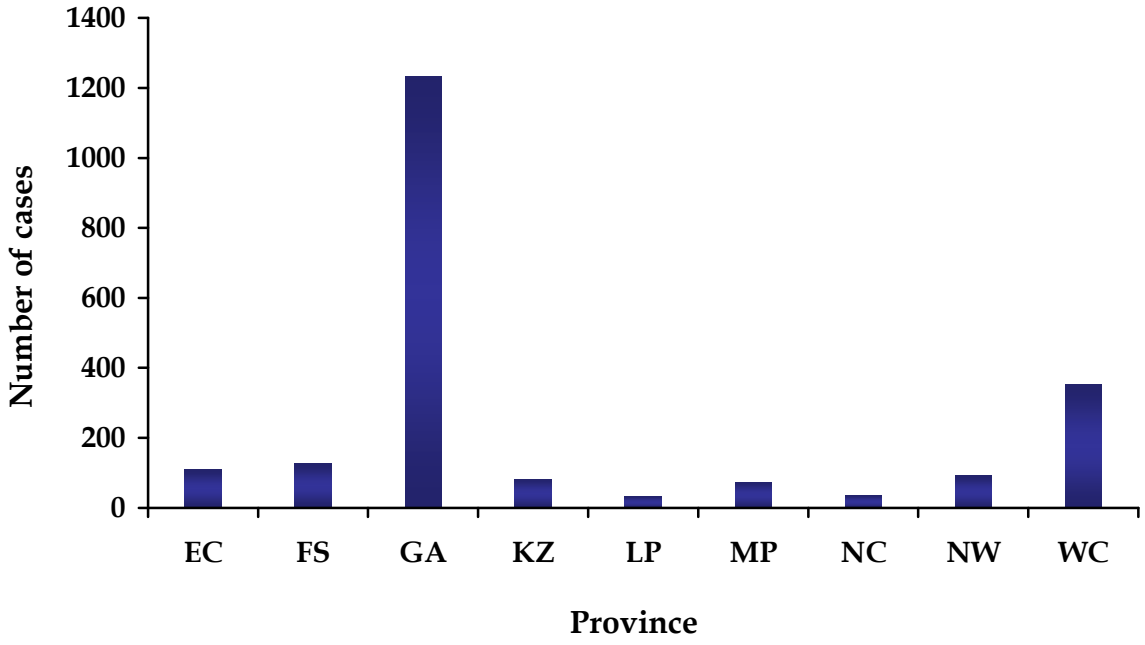


Figure 3.2 Number of cases of meningococcal disease by province, South Africa, 2002-2006 (n=2144).



NOTE: EC, Eastern Cape; FS, Free State; GA, Gauteng; KZ, KwaZulu-Natal; LP, Limpopo; MP, Mpumalanga; NC, Northern Cape; NW, North-West; WC, Western Cape.

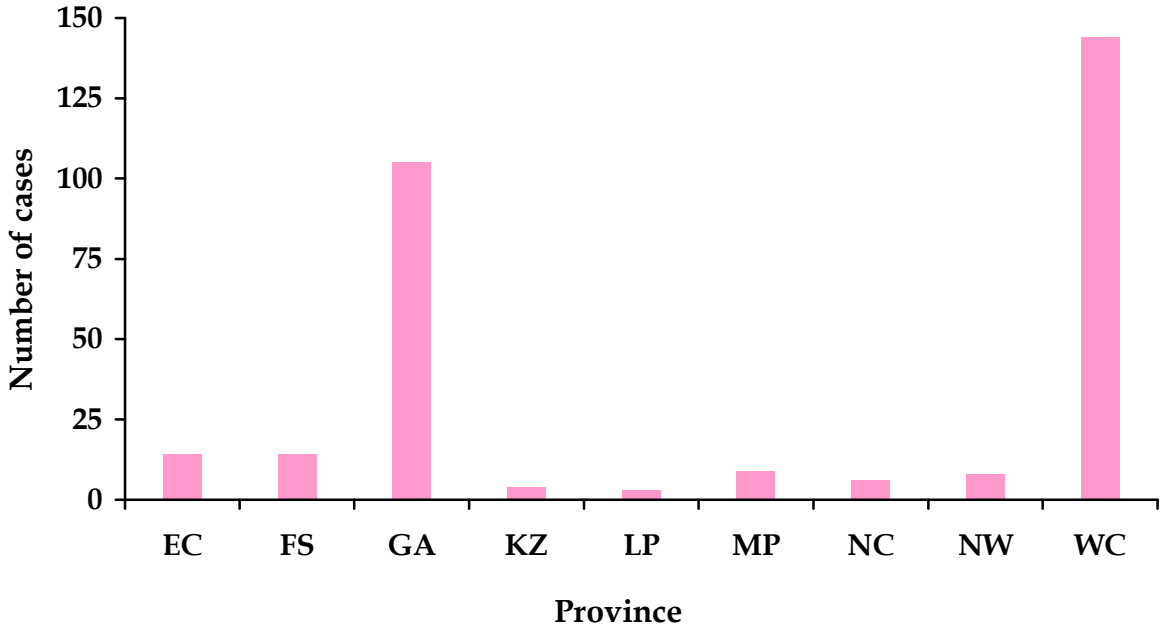
Table 3.1 Serogroups causing invasive meningococcal disease in South Africa, 2002-2006.

Year	Serogroup								No data[b]
	A	B	C	Y	W135	X	Z	NG	
2002	58	47	20	41	24	1	-	1	77
2003	87	76	31	43	26	1	2	-	102
2004	57	62	31	53	77	1	-	-	79
2005	24	58	21	52	257	2	-	-	129
2006	4	64	41	45	318	1	-	1	130
Total	230	307	144	234	702	6	2	2	517

NOTE:1627/2144 (76%) viable isolates were serogrouped. NG – non-groupable isolates.

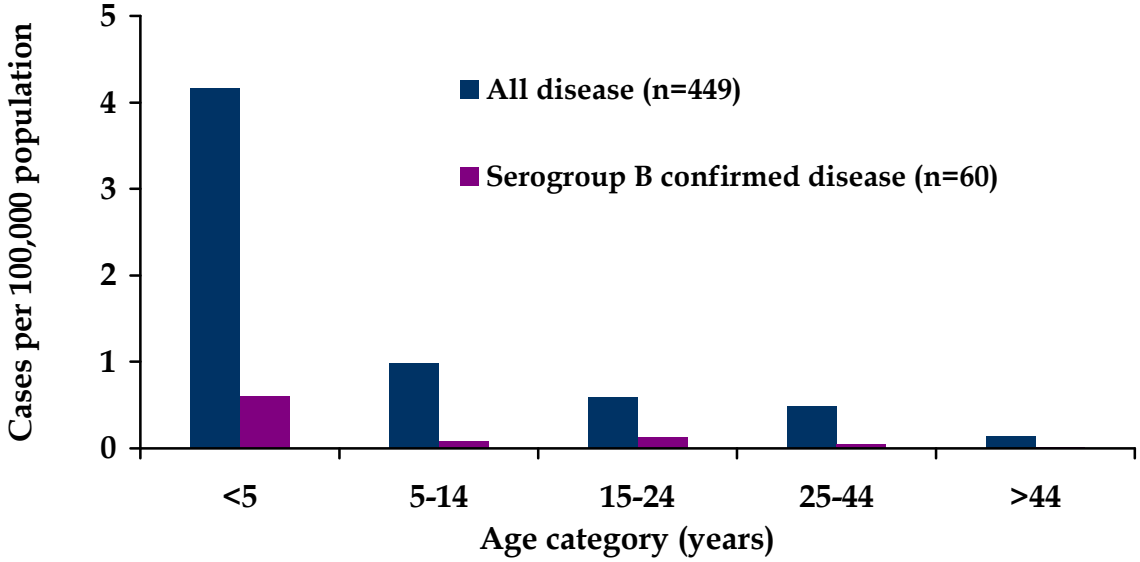
[b] Isolate not available for serogrouping

Figure 3.3 Number of cases of serogroup B meningococcal disease by province, South Africa, 2002-2006 (n=307).



NOTE: EC, Eastern Cape; FS, Free State; GA, Gauteng; KZ, KwaZulu-Natal; LP, Limpopo; MP, Mpumalanga; NC, Northern Cape; NW, North-West; WC, Western Cape.

Figure 3.4 Age-specific incidence rates for meningococcal disease, South Africa, 2006.



NOTE: 474/604 reported cases were serogrouped. 64/474 were serogroup B, age was unknown for 4 cases.Incidence rates were calculated based on mid-year population estimates provided by Statistics South Africa.

Table 3.2 Gender distribution of meningococcal disease, South Africa, 2002-2006.

Year	All disease				Serogroup B disease			
	Male (%)	Female (%)	Unknown (%)	Total	Male (%)	Female (%)	Unknown (%)	Total
2002	151 (56)	114 (42)	4 (1)	269	22 (46)	24 (51)	1 (2)	47
2003	221 (60)	138 (38)	9 (2)	368	43 (57)	32 (42)	1 (1)	76
2004	204 (57)	148 (41)	8 (2)	360	34 (55)	25 (40)	3 (5)	62
2005	306 (56)	223 (41)	14 (3)	543	35 (60)	21 (36)	2 (3)	58
2006	319 (53)	271 (45)	14 (2)	604	35 (55)	27 (42)	2 (3)	64
Total	1201 (56)	894 (42)	49 (2)	2144	169 (55)	129 (42)	9 (3)	307

3.2 Molecular characterisation of *N. meningitidis* serogroup B isolates, 2002-2006

From January 2002 through December 2006, 307 viable *N. meningitidis* serogroup B isolates from invasive cases were collected by the surveillance network. Of these, 305 (98%, 305/307) were available for characterization by pulsed-field gel electrophoresis (two isolates were no longer viable from their -70°C stocks). Of the 305 isolates characterized by PFGE, three isolates were excluded from the analysis due to poor fingerprint patterns.

On average, the restriction endonuclease enzyme *NheI*, digested DNA into 15-20 fragments **(Figure 1.8)**. A total of 260 fingerprint patterns were generated among the 302 isolates. Applying a ≥79% pattern relatedness breakpoint to the dendrogram, isolates were divided into 13 PFGE clusters (206/302, 68%), and 96 unrelated isolates (96/302, 32%). Clusters were designated B1 to B13 **(Figure 3.5)** *(Appendix D).*

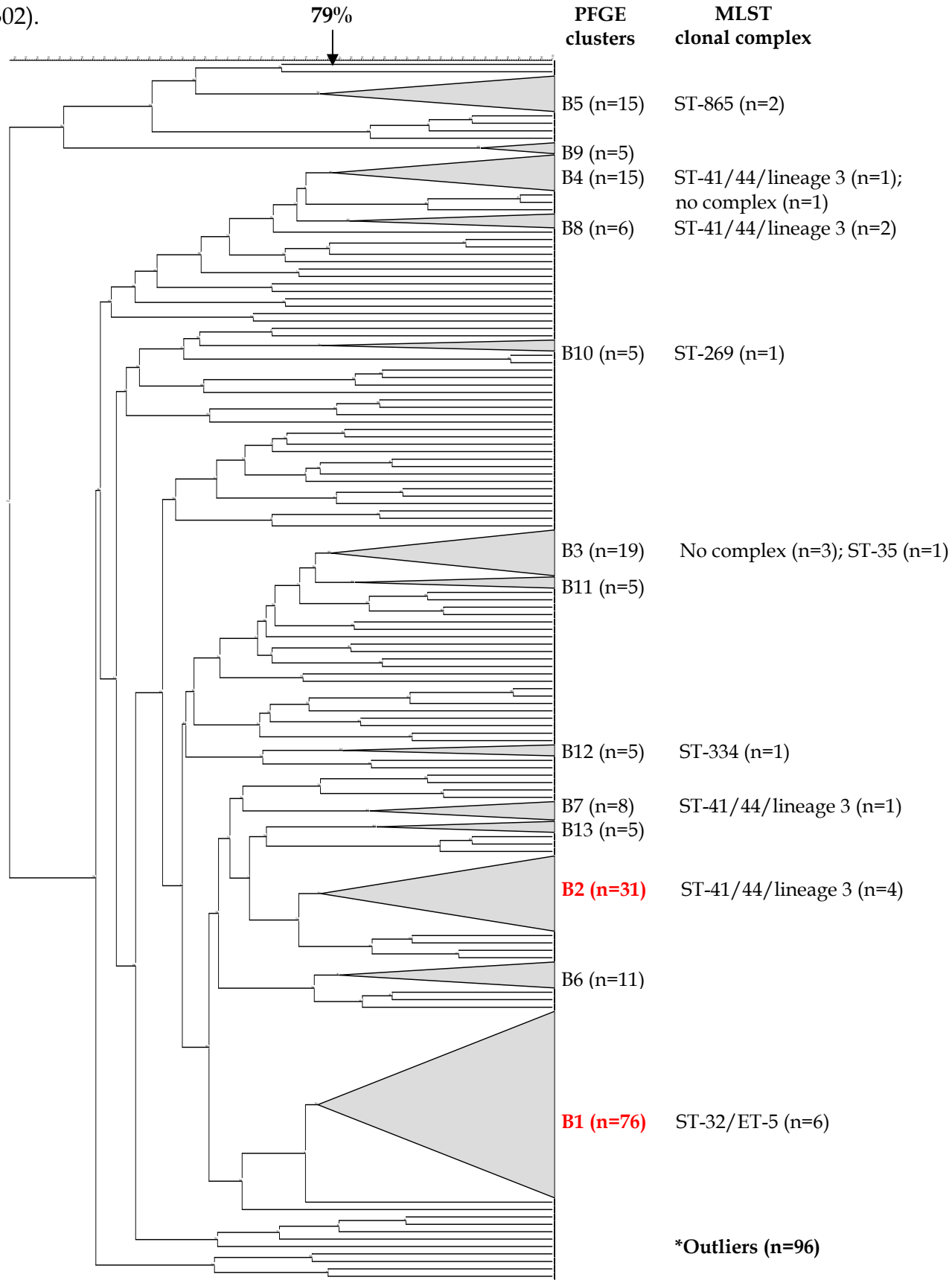
Approximately 10% of serogroup B isolates (n=302) were randomly selected for MLST, PorA and FetA characterisation, (2002, n=4; 2003, n=5; 2004, n=5; 2005, n=5; and 2006, n=11). Four serogroup B isolates from 2002 (n=2) and 2003 (n=2) had existing MLST data (only) from other studies and were also included in the analysis. These isolates were further characterised by PorA and FetA typing.

MLST results were obtained for 33/34 isolates. Four of seven genes could not be amplified for the one isolate. A total of 24 STs were identified. The most common clonal complexes were ST-41/44/lineage 3 (9/33) and ST-32/ET-5 (6/33) **(Table 3.3)**. Overall most isolates were not assigned to a clonal complex (12/33).

Table 3.3 Clonal complexes of *N. meningitidis* serogroup B isolates, 2002-2006.

Clonal complex	n (%)	STs (n)
No complex	12 (36)	ST3839, ST4240, ST4243, ST6688 (3), ST6990, ST6992 ST7222, ST7223, ST7392, ST7946
ST-41/44/lineage 3	9 (27)	ST41, ST43, ST154 (2), ST6590 (3), ST6698, ST7945
ST-32/ET-5	6 (18)	ST33 (5), ST6589
ST-865	2 (6)	ST6687, ST7195
ST-35	1 (3)	ST2437
ST-334	1 (3)	ST7978
ST-269	1 (3)	ST283
ST-1157	1 (3)	ST1157
Total	33	

Figure 3.5 PFGE dendrogram with MLST clonal complex information, representing the genetic relationship among serogroup B meningococci causing invasive disease in South Africa, 2002-2006 (n=302).



3.2.1 PFGE

Cluster B1

The largest cluster, B1, accounted for 25% (76/302) of isolates **(Figure 3.5).** There were 54 distinct PFGE fingerprint patterns within the cluster. B1 isolates were present throughout the study period and decreased over time, from 43% (20/47) in 2002 to 13% (8/62) in 2006 (p = 0.003) **(Table 3.4 and Figure 3.6).** Isolates were present in 4/9 provinces **(Table 3.5)**, but were most common to the Western Cape (58/76, 76%). MLST data were available for six isolates (6/76, 11%), 3 were randomly selected and 3 had available MLST data. All isolates belonged to cc ST-32/ET-5. Two STs, differing by a single allele were identified, namely ST33 (n=5) and ST6589 (n=1). Isolates expressed PorA types P1.19,15 (n=3) and P1.12-1,13-1 (n=3). FetA types included F5-1 (n=5) and F3-7 (n=1).

Cluster B2

Cluster B2 accounted for 10% (31/302) of isolates **(Figure 3.5)**. Present throughout the study period, B2 isolates showed no significant change over time [6% (3/47) in 2002 compared to 16% (10/62) in 2006 (p = 0.4)] **(Table 3.4 and Figures 3.6).** B2 isolates were present in 4/9 provinces **(Table 3.5)**, but were most common to Gauteng (18/31, 58%). Selected isolates from B2 (4/31, 13%) belonged to clonal complex ST-41/44/lineage 3. Three sequence types were identified, ST154 (n=2), ST6698 (n=1)

and ST41 (n=1). ST6698 and ST41 differed from ST154 by 1 allele. All 4 isolates expressed the same PorA, P1.7-2,4, and FetA, F1-5 type.

Table 3.4 Meningococcal serogroup B PFGE clusters, South Africa, 2002-2006.

Cluster		2002	2003	2004	2005	2006	p
	n	n	n	n	n	n	
B1	76	20	24	12	12	8	**0.003**
B2	31	3	7	5	6	10	0.487
B3	19	1	3	2	8	5	0.058
B4	15	1	4	5	5	0	0.128
B5	15	2	6	2	2	3	0.710
B6	11	0	2	2	4	3	0.391
B7	8	-	-	3	2	3	0.214
B8	6	-	1	-	-	5	**0.0004**
B9	5	1	1	3	-	-	0.191
B10	5	1	-	-	-	4	**0.017**
B11	5	-	1	4	-	-	**0.018**
B12	5	-	2	1	1	1	0.867
B13	5	1	2	1	1	-	0.812
Outliers	96	17	22	21	16	20	0.880
Total	302	47	75	61	57	62	

NOTE: Isolates within each cluster were compared to all other isolates (ie. those in clusters and outliers) over the 5-year period. Significant p values are in bold.

Cluster B3

Cluster B3 accounted for 6% (19/302) of isolates **(Figure 3.5)**. B3 isolates showed no significant change over time, 2% (1/47) in 2002 to 8% (5/62) in 2006 (p =0.058) **(Table 3.4 and Figure 3.6).** This cluster was consistently present from 2002 through 2006. B3 isolates were present in 5/9 provinces **(Table 3.5)**, but were most common to Gauteng (11/19, 58%). Selected isolates from B3 (4/19, 21%) were sequenced. Three isolates were ST6688 and were not assigned to a clonal complex on the MLST database. The remaining isolate (No. 7441) was ST2437 and belonged to clonal complex ST-35. ST6688 and ST2437 differed by 7 alleles. ST6688 isolates had the same PorA (P1.5,2) and FetA type (F5-8). ST2437 contained PorA type P1.22-1,14 while a FetA type could not be determined. A PCR product was obtained but sequencing did not yield results.

Cluster B4

Cluster B4 made up 5% (15/302) of all isolates **(Figure 3.5)**. B4 isolates was present from 2002 (1/47, 2%) to 2005 (5/57, 9%) with no reported isolates in 2006 **(Table 3.4 and Figure 3.6).** There was no significant increase or decrease (p=0.128) over time. The isolates were found in 5/9 provinces **(Table 3.5)** but were most common to Gauteng (7/15, 47%). Selected B4 isolates (2/15, 13%) were sequenced (1 randomly selected and 1 with existing MLST data). ST6590 belonged to clonal complex ST-41/44/lineage 3, while ST4243 was not assigned to a clonal complex on the MLST database. ST6590 and ST4243 differed by 3 alleles. Both isolates share the same PorA (P1.7,9) and FetA (F3-20) type.

Cluster B5

Cluster B5 also made up 5% (15/302) of all isolates **(Figure 3.5)**. The B5 cluster was consistently present throughout the 5-year period: 2002 (2/47, 4%) to 2006 (3/62, 5%) **(Table 3.4 and Figure 3.6)**, with no significant increase or decrease (p= 0.710) over time. B5 isolates were present in 4/9 provinces **(Table 3.5)**, but were most common to Western Cape (7/15, 47%). Isolates selected for sequencing (2/15, 13%) belonged to clonal complex ST-865. Two STs were identified, ST6687 and ST7195, and differed by 1 allele. ST7195 was a new ST. Both isolates share the same PorA (P1.7-1,1) and FetA (F1-6) type.

Cluster B6

Cluster B6 contained 4% of all isolates (11/302) **(Figure 3.5)**. The cluster appeared in 2003 (2/75, 3%) and was present till the end of the study period, 2006 (3/62, 5%), no significant increase or decrease was noted (p=0.391) **(Table 3.4 and Figure 3.6).** B6 isolates were found in 3/9 provinces **(Table 3.5)**, and were equally present in Gauteng (5/11, 45%) and the Western Cape (5/11, 45%).

Clusters B7-B13

Clusters B7 to B13 each contained <10 isolates **(Figure 3.5)**. Clusters B8, B10 and B11 increased from 2002 through 2006 **(Table 3.4)**. Isolates from these seven clusters were found in 2 to 4 of the 9 provinces **(Table 3.5)** but were mostly found in Gauteng and the Western Cape. Selected isolates from B7 (1/8, 13%) and B8 (2/6, 33%) analysed

and belonged to clonal complex ST-41/44/lineage 3. ST43 identified in cluster B7 contained PorA type P1.19,15-1 and FetA type F1-5. Isolates from cluster B8 were classified as ST6590 (n=2) and had the same PorA (P1.7,9) and FetA (F3-20) type. Selected isolates from clusters B10 (1/5, 20%) and B12 (1/5, 20%) belonged to ST-269 and ST-334 clonal complexes respectively. ST283 identified in cluster B10 contained PorA type P1.19-1,30-2 and FetA type F5-1. ST7978 (a new ST) identified in cluster B12 contained PorA type P1.12-1,16-8 and FetA type F5-2.

Figure 3.6 Meningococcal serogroup B PFGE clusters in South Africa, by year, 2002-2006 (n=302).

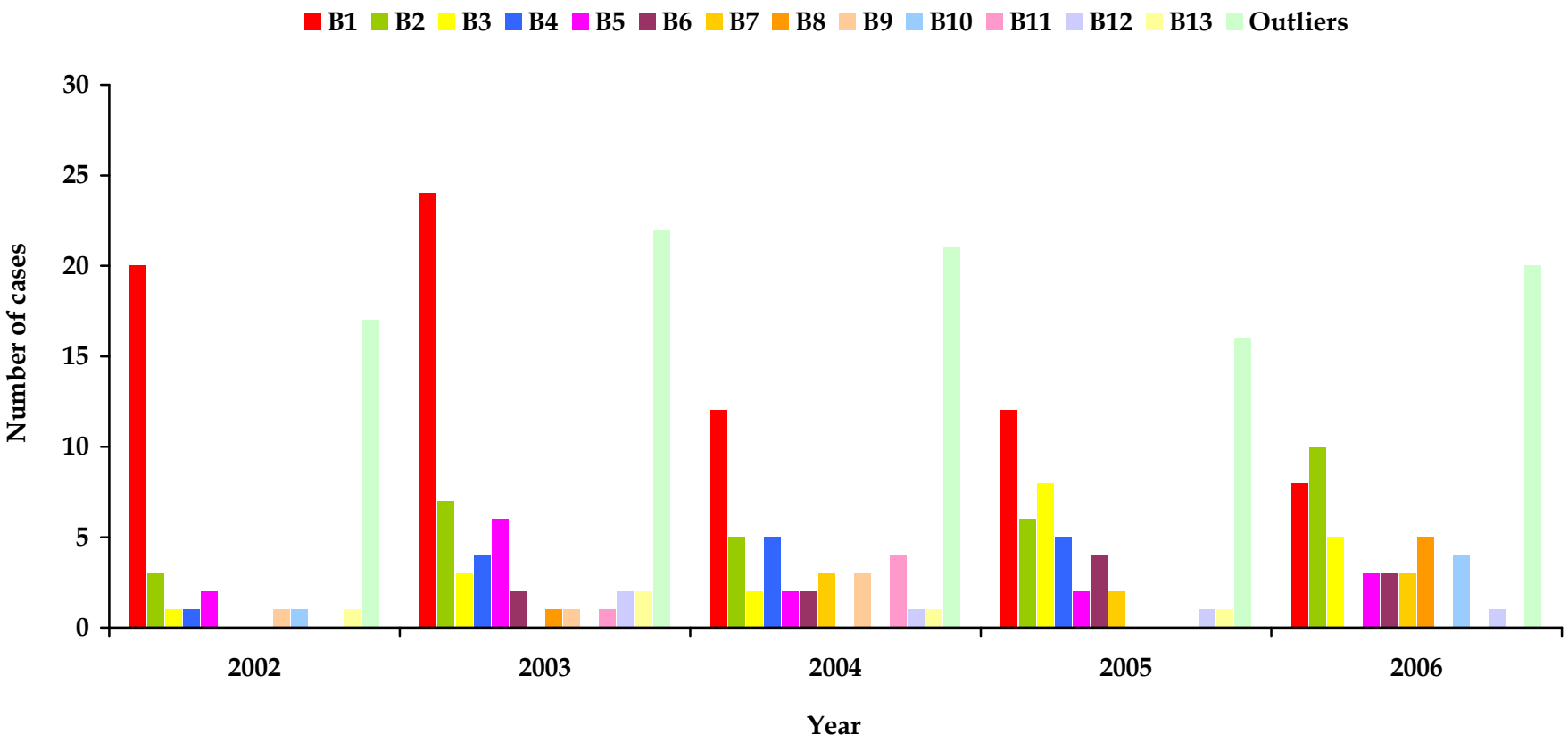


Table 3.5 Distribution of PFGE clusters causing invasive serogroup B meningococcal disease in South Africa, by province, 2002-2006 (n=302).

Province	PFGE clusters													Outliers	Total
	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13		n (%)
Western Cape	58	8	5	5	7	5	3	–	–	2	2	3	2	39	143 (47)
Gauteng	13	18	11	7	3	5	2	1	3	3	2	–	2	33	103 (34)
Eastern Cape	3	–	1	–	2	–	–	–	–	–	–	–	–	8	14 (5)
KwaZulu-Natal	–	–	–	–	–	–	–	–	–	–	–	1	–	3	4 (1)
Mpumalamga	–	3	1	–	–	–	2	–	–	–	–	–	–	2	8 (3)
North West	2	2	–	1	–	–	–	–	1	–	–	–	–	2	8 (3)
Free State	–	–	1	–	3	1	–	1	–	–	–	1	1	6	14 (5)
Limpopo	–	–	–	1	–	–	1	–	–	–	1	–	–	–	3 (1)
Northern Cape	–	–	–	1	–	–	–	–	1	–	–	–	–	3	5 (2)
Total	76	31	19	15	15	11	8	6	5	5	5	5	5	96	302

Outliers

Almost a third of the isolates (96/302, 32%) did not meet the cluster definition **(Figure 3.5)**. There were 96 distinct fingerprint patterns among outliers. These isolates were found across the country throughout the 5-year period **(Figure 3.6 and Table 3.5).** There was no significant change in outliers (p= 0.880), from 2002 (17/47, 36%) through 2006 (20/62, 32%) **(Table 3.4)**. Selected isolates (11/96, 11%) were analysed by MLST, PorA and FetA typing. Results were obtained for 10/11 (91%) isolates. A total of 10 STs were identified among these outliers**,** of which 8/10 (80%) were not assigned to a clonal complex on the MLST database **(Table 3.6)**. Five new STs were identified. Seven PorA types were identified: P1.21-7,16 (n=1), P1.17,16-4 (n=2), P1.19,15 (n=1), P1.7,9 (n=1),P1.5,2 (n=3), P1.5-1,2-2 (n=1) and P1.5-3,10-24 (n=1). Seven FetA types were identified: F3-16 (n=1), F3-20 (n=1), F3-27 (n=1), F4-1 (n=1), F5-34 (n=1), F5-5 (n=3), and F5-8 (n=2). One isolate (9127) could not be sequenced by MLST or FetA typing but had a PorA type (P1.22,23-1). This isolate did not yield a PCR product for 4/7 MLST genes. A very weak FetA PCR product was obtained which sequenced poorly.

Table 3.6 MLST clonal complexes and STs among meningococcal serogroup B PFGE outliers, South Africa, 2002-2006.

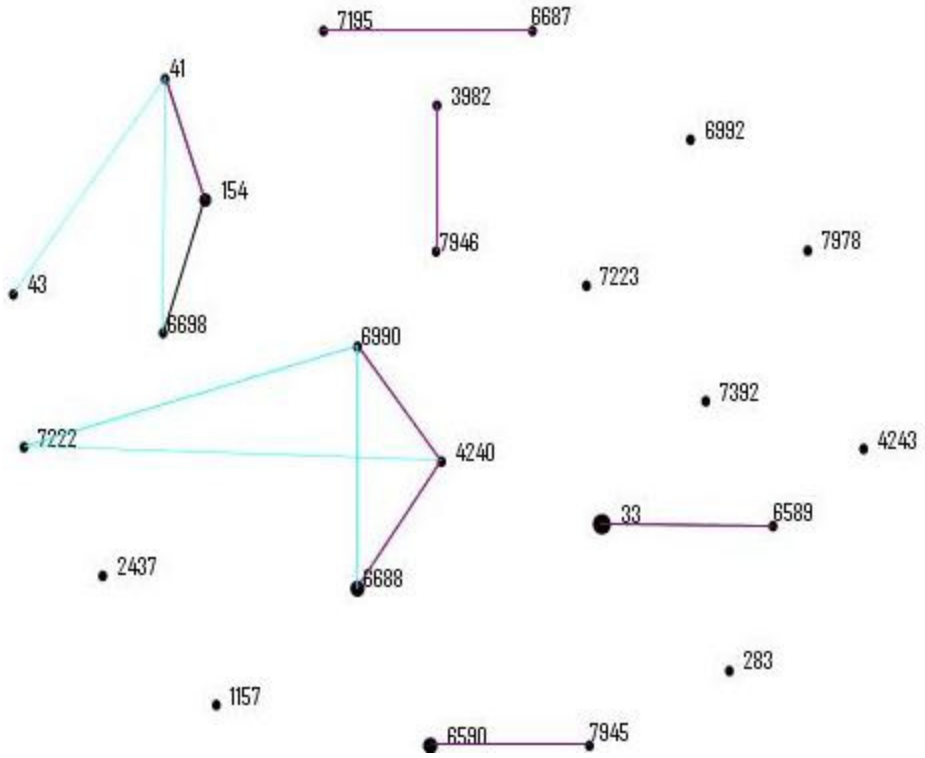
MLST complex	n	STs identified
no complex	8	**ST7392, ST7223**, ST3982, **ST7946, ST7222**, ST4240, ST6990, ST6992
ST-41/44/lineage 3	1	**ST7945**
ST-1157	1	ST1157
Total	10	

NOTE: New STs in bold.

3.2.2 eBURST analysis

The eBURST algorithm divided the isolates (based on STs) into 6 groups (n=23) and 10 singletons **(Table 3.7)**. The default setting of 6/7 identical loci for the group definition was used. A population snapshot was used for representation of all groups **(Figure 3.7)**. The diagram was edited to show possible relation of singletons.

Figure 3.7 A population snapshot of *N. meningitidis* serogroup B isolates, South Africa, 2002-2006 (n=33).



NOTE: Pink lines connect SLV. Blue lines connect DLV. Size of the circle is proportional to the number of isolates.

Table 3.7 eBURST analysis groups of meningococcal serogroup B isolates, South Africa, 2002-2006 (n=23).

Group	n	ST (n)	Alleles						
			abc	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*
1	4	154 (2)	3	**6**	9	5	**11**	6	9
		6698	3	**3**	9	5	11	6	9
		41	3	6	9	5	**9**	6	9
2	5	6688 (3)	15	5	**18**	**24**	8	19	62
		6690	15	5	18	**18**	8	19	62
		4240	15	5	**9**	24	8	19	62
3	6	33 (5)	8	10	5	**4**	6	3	8
		6589	8	10	5	**264**	6	3	8
4	4	6590 (3)	9	6	351	9	**9**	6	311
		7945	9	6	351	9	**26**	6	311
5	2	3982	46	11	79	129	**323**	9	3
		7946	46	11	79	129	**6**	9	3
6	2	7195	8	5	15	**53**	8	21	2
		6687	8	5	15	**8**	8	21	2

NOTE: ST, sequence type. Allele differences indicated in bold. The number of isolates belonging to each ST, if more than one, are indicated in parentheses.

3.2.3 PorA types

A PorA type was obtained for all isolates (n=34). There were 15 PorA types among the 34 isolates **(Figure 3.8)** *(Appendix E)*. Half of all isolates (19/34, 56%) were serosubtyped as one of four different PorA types **(Table 3.8)**. PorA type P1.5,2 (6/34, 18%) was exclusively associated with isolates that were not assigned to a clonal complex (6/6). Serosubtypes P1.19,15 (4/34, 12%) and P1.7,9 (5/34, 15%) were most associated with clonal complexes ST-32/ET-5 (3/4) and ST-41/44/lineage 3 (4/5).

Table 3.8 Most common PorA types and associated MLST complexes of *N. meningitidis* serogroup B isolates, South Africa, 2002-2006.

Clonal complex (n)	PorA types			
	P1.5,2	P1.19,15	P1.7,9	P1.7-2,4
ST-32/ET-5 (6)		3		
ST-41/44/lineage 3 (9)			4	4
no complex (12)	6	1	1	
Total	6	4	5	4

3.2.4 FetA types

FetA types were obtained for 94% (32/34) isolates *(Appendix E).* Isolates for which a result was not obtained included isolate 9127 (an outlier on PFGE with no MLST result) and 7441 (from PFGE cluster B3, belonging to the ST-35 complex). There were 12 FetA types among the 32 isolates **(Figure 3.9)**. The majority of isolates (21/32, 66%) contained one of four FetA **(Table 3.9)**. F5-1 (6/32, 19%) was most associated with ST-32/ET-5 (5/6). F1-5 (5/32, 16%) and F3-20 (5/32, 16%) were most associated with ST-41/44/lineage 3. F5-8 (5/32, 16%) was exclusively associated with isolates that were not assigned to a clonal complex. Overall, variants of FetA family 5 were most common.

Table 3.9 Most common FetA types and associated MLST complexes of *N. meningitidis* serogroup B isolates, South Africa, 2002-2006.

Clonal complex	FetA types			
	F5-1	F1-5	F5-8	F3-20
ST-32/ET-5 (6)	5			
ST-41/44/lineage 3 (9)		5		4
no complex (12)			5	1
ST-269 (1)	1			
Total	6	5	5	5

Figure 3.8 PorA types of *N. meningitidis* serogroup B isolates in South Africa, 2002-2006 (n=34).

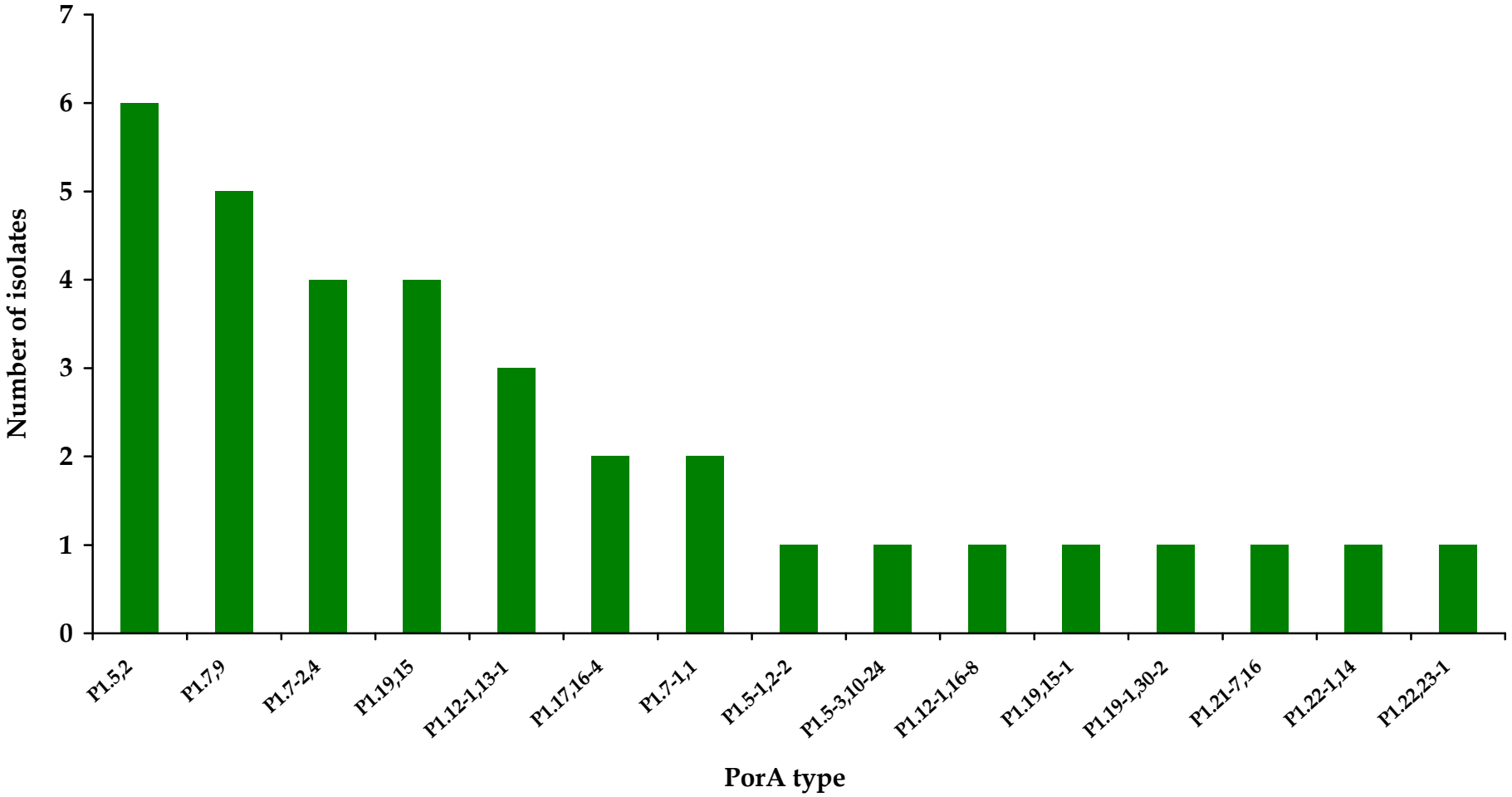
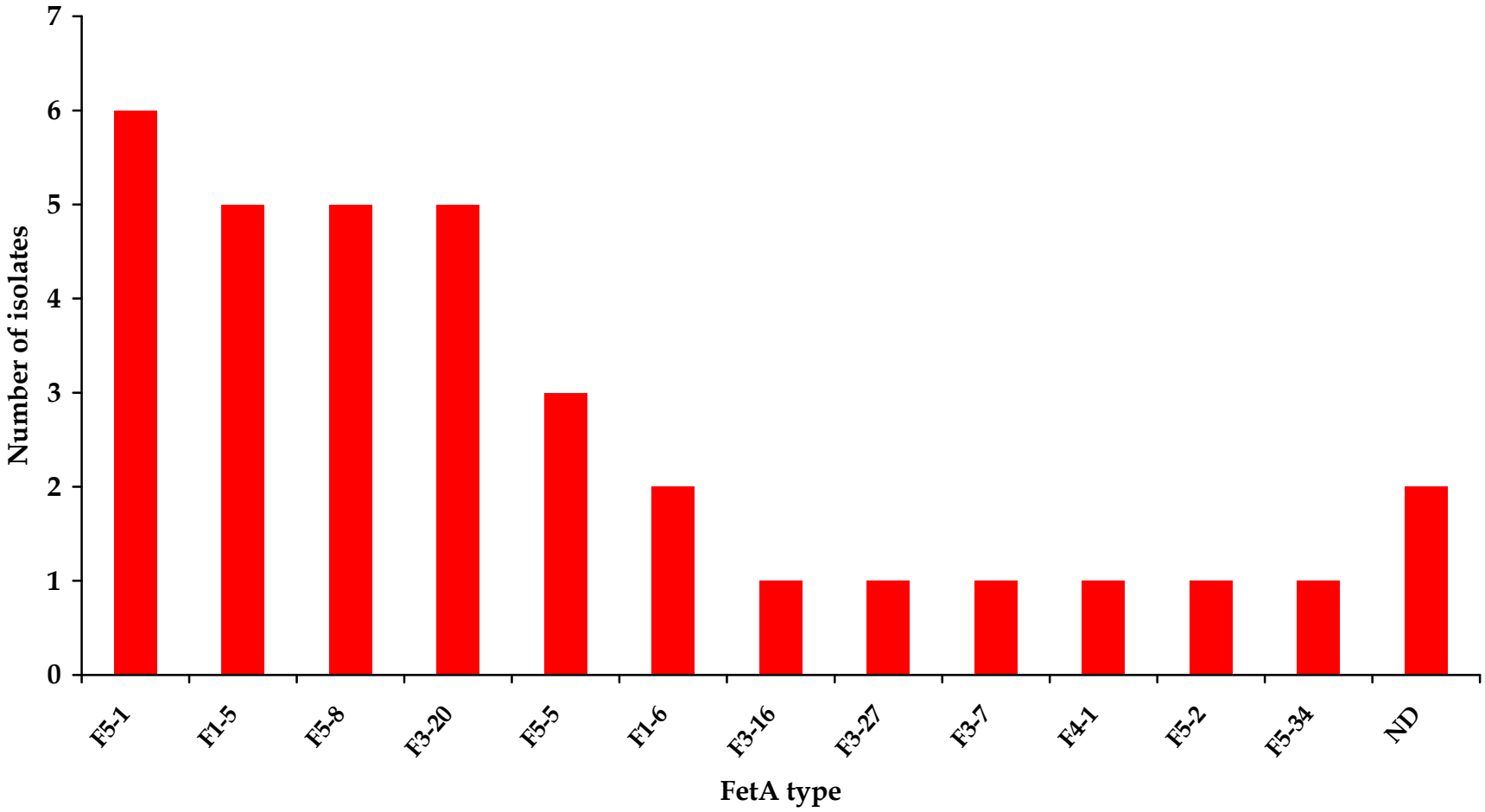


Figure 3.9 FetA types of *N. meningitidis* serogroup B isolates in South Africa, 2002-2006 (n=32).

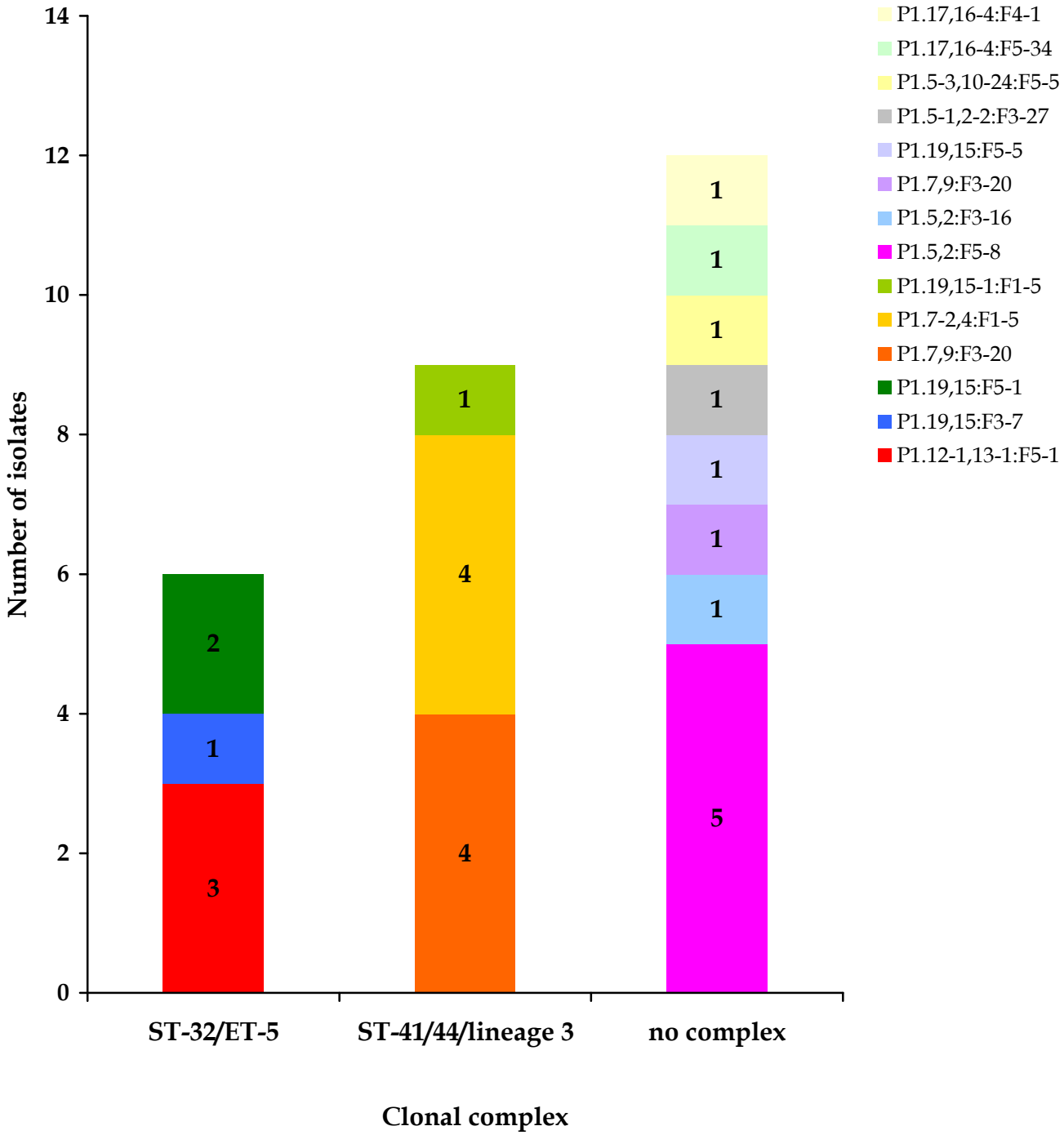


NOTE: ND, Not determined.

3.2.5 Genotypes

Isolates were assigned an MLST, PorA and FetA genotype. A genotype was determined for 32/34, (94%) **(Figure 3.10)**. The majority of isolates belonged to clonal complexes ST-32/ET-5 (6/34, 18%), ST-41/44/lineage 3 (9/34, 26%) and isolates not assigned to a clonal complex (12/34, 35%). A total of 14 genotypes were found, ST-32/ET-5 (3/14), ST-41/44/lineage 3 (3/14) and isolates not assigned to a clonal complex (8/14) **(Figure 3.10)**. The most common genotype overall was P1.5,2:F5-8:no complex (n=5). Common genotypes in the ST-32/ET-5 complex included P1.19,15:F5-1: ST-32/ET-5 (n=2) and P1.12-1,13-1:F5-1: ST-32/ET-5 (n=3). Genotypes P1.7,9:F3-20:ST-41/44/lineage 3 (n=4) and P1.7-2,4:F1-5: ST-41/44/lineage 3 (n=4) were equally common. The remaining genotypes were each represented by 1 isolate.

Figure 3.10 Genotypes of *N. meningitidis* serogroup B isolates, South Africa, 2002-2006.



3.3 Molecular characterisation of *N. meningitidis* serogroup B isolates, 2005

A total of 58 *N. meningitidis* isolates were identified as serogroup B in 2005. Of these, 57 were fully characterised by PFGE, MLST, PorA and FetA typing. One isolate was non-viable. Isolates were processed by MLST, PorA and FetA typing as part of a separate study.

3.3.1 PFGE

Using the cluster definition 49% (28/57) of isolates were divided into 3 clusters, B05-1, B05-2 and B05-3 while 51% (29/57) were outliers **(Figure 3.11).** A total of 54 fingerprint patterns were generated. Overall 93% (53/57) of isolates shared a >60% similarity by PFGE.

Cluster B05-1

The largest cluster, B05-1, contained 21% (12/57) of all isolates **(Figure 3.11)**. B05-1 isolates were distributed among 3 provinces **(Figure 3.12)**, but was most common to the Western Cape Province (9/12, 75%). All isolates belonged to clonal complex ST-32/ET-5 **(Table 3.11)**. Seven STs were identified, ST33, ST2400, ST639, ST484, ST6386, including 2 new STs ST6589 and ST6700. The most common ST was ST33 (5/12). Four serosubtypes and 3 FetA type were found in this cluster, P1.19.15 (5/12) and F5-1 (9/12) were the most common. ST-32/ET-5:P1.19.15:F5-1 was the most common genotype among the six genotypes identified **(Table 3.10)**.

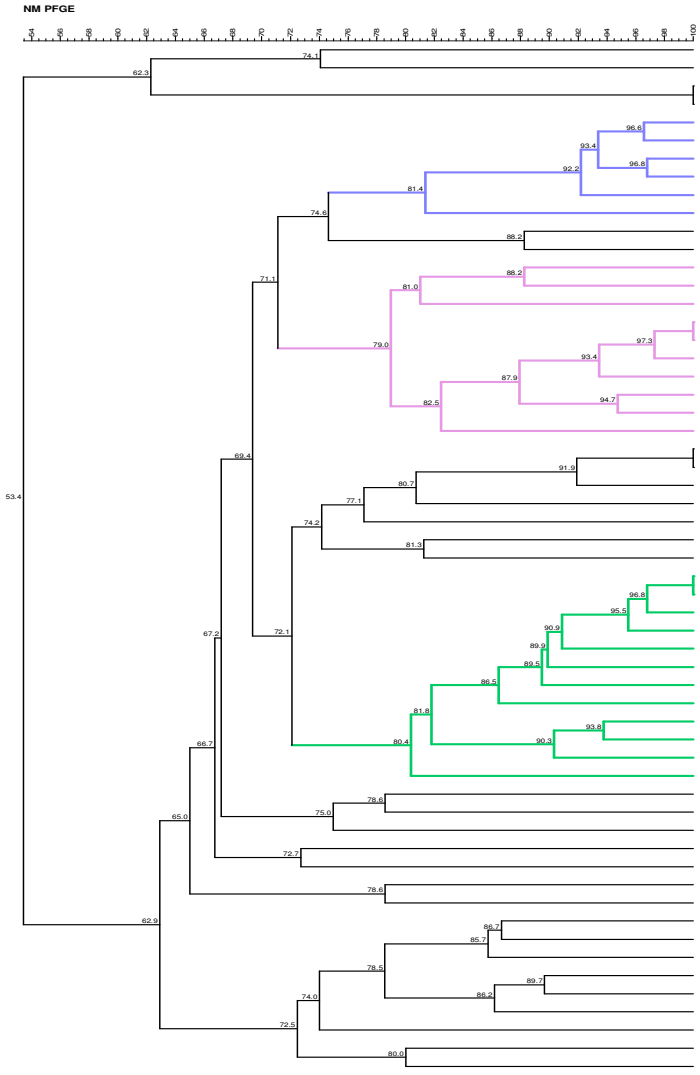
Cluster B05-2

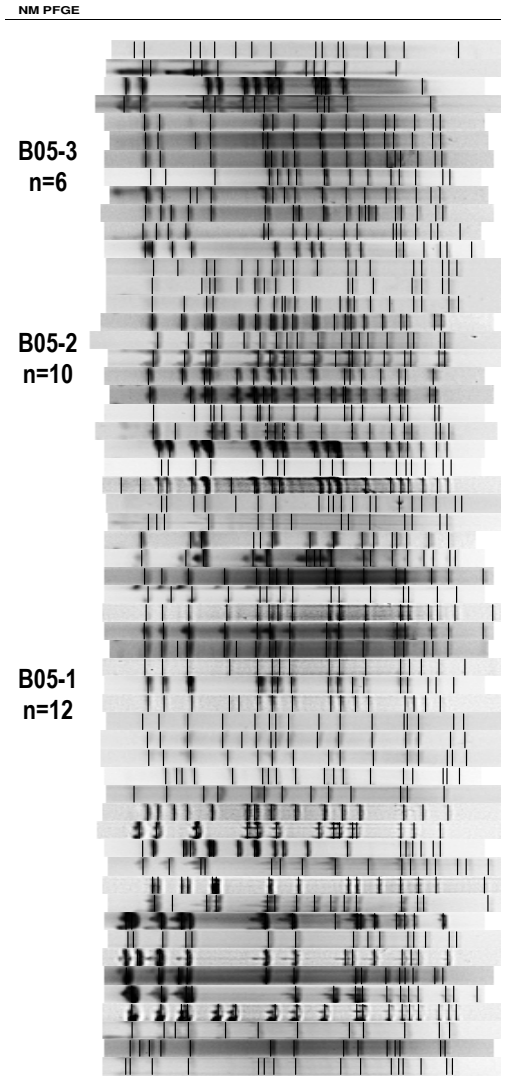
Cluster B05-1 contained 18% (10/57) of all isolates **(Figure 3.11)**. Isolates were distributed among 4 provinces **(Figure 3.12)**, but was most common to Gauteng Province (7/10, 70%). None of the isolates were assigned to a clonal complex **(Table 3.11)**. Five STs were identified, ST4240 and new STs ST6690, ST6692, ST6699 and ST6688, the most common being ST6688 (5/10). The cluster was dominated by one serosubtype and one FetA type, P1.5,2 and F5-8 respectively. Therefore clusterB05-2 contained one genotype, no complex:P1.5,2:F5-8 **(Table 3.10)**.

Cluster B05-3

Cluster B05-3 made up 11% (6/57) of all isolates **(Figure 3.11)**. Isolates were distributed between 2 provinces **(Figure 3.12)**, but was most common to Gauteng Province (5/6, 83%). All isolates belonged to clonal complex ST-41/44/lineage 3 **(Table 3.11)**. Five STs were identified, ST154 and ST1960 and new STs, ST6694, ST6697 and ST6698. The most common ST was ST154 (2/6). Two serosubtypes, P1.7-2,4 (5/6) and P1.7-2,13-1 (1/6) and 1 FetA type, F1-5, were found in this cluster. ST-41/44/lineage 3:P1.7-2,4:F1-5 was the most common genotype **(Table 3.10)**.

Figure 3.11 PFGE dendrogram with MLST, PorA and FetA type representing the genetic relationship among serogroup B meningococci causing invasive disease in South Africa, 2005 (n=57).





	Isolate no.	*	Year	ST	cc	PorA	FetA
	B 94	WC	2005	393	ST-269 complex	P1.5-1,10-1	F1-7
	B 1937	WC	2005	1878	ST-103 complex	P1.18-1,3	F3-9
	B 36	FS	2005	6687	ST-865 complex	P1.7-1,1	F1-6
	B 2826FR	WC	2005	865	ST-865 complex	P1.7-1,1	F1-6
◆	B 2379	GA	2005	6697	ST-41/44/lineage 3 comp.	P1.7-2,4	F1-5
◆	B 2640	GA	2005	6698	ST-41/44/lineage 3 comp.	P1.7-2,4	F1-5
◆	B 3288	WC	2005	154	ST-41/44/lineage 3 comp.	P1.7-2,4	F1-5
◆	B 1557	GA	2005	6694	ST-41/44/lineage 3 comp.	P1.7-2,4	F1-5
◆	B 2539	GA	2005	154	ST-41/44/lineage 3 comp.	P1.7-2,4	F1-5
◆	B 3813	GA	2005	1960	ST-41/44/lineage 3 comp.	P1.7-2,13-1	F1-5
	B 2351	GA	2005	180	ST-41/44/lineage 3 comp.	P1.19,15	F1-5
	B 1841	WC	2005	6696	ST-41/44/lineage 3 comp.	P1.18-1,34/34-2	F5-2
➲	B 652	GA	2005	6690	no complex	P1.5,2	F5-8
➲	B 1228	NC	2005	4240	no complex	P1.5,2	F5-8
➲	B 1027	GA	2005	6692	no complex	P1.5,2	F5-8
➲	B 3897	GA	2005	6688	no complex	P1.5,2	F5-8
➲	B 302FR	GA	2005	6688	no complex	P1.5,2	F5-8
➲	B 1966FR	GA	2005	6688	no complex	P1.5,2	F5-8
➲	B # 2439	MP	2005	6688	no complex	P1.5,2	F5-8
➲	B # 2825	GA	2005	6688	no complex	P1.5,2	F5-8
➲	B 2953	GA	2005	6699	no complex	P1.5,2	F5-8
➲	B 1766FR	WC	2005	4240	no complex	P1.5,2	F5-8
	B 2391	GA	2005	35	ST-35 complex	P1.22-1,14	ND
	B 372	WC	2005	35	ST-35 complex	P1.22-1,14	F3-7
	B 4660	WC	2005	6703	ST-35 complex	P1.22-1,14	ND
	B 1310	GA	2005	6693	ST-35 complex	P1.22-1,14	ND
	B 371FR	WC	2005	35	ST-35 complex	P1.22,13-37	F4-1
	B 2093	GA	2005	6709	no complex	P1.17,16-4	F5-5
	B # 4332	GA	2005	6992	no complex	P1.17,16-4	F4-1
✧	B 3422	WC	2005	33	ST-32/ET5 complex	P1.19,15	F5-1
✧	B 2136	WC	2005	2400	ST-32/ET5 complex	P1.19,15	F5-1
✧	B 4577	NW	2005	33	ST-32/ET5 complex	P1.12-1,13-1	F5-1
✧	B 3395	WC	2005	33	ST-32/ET5 complex	P1.19,15	F5-1
✧	B 2718	WC	2005	6589	ST-32/ET5 complex	P1.12-1,13	F5-1
✧	B 4661	WC	2005	639	ST-32/ET5 complex	P1.5,2-1	F5-18
✧	B 2516	GA	2005	33	ST-32/ET5 complex	P1.12-1,13	F3-7
✧	B 3954	WC	2005	6700	ST-32/ET5 complex	P1.19,15	F3-7
✧	B 995	WC	2005	639	ST-32/ET5 complex	P1.12-1,13-1	F5-1
✧	B 1835	WC	2005	33	ST-32/ET5 complex	P1.12-1,13-1	F5-1
✧	B 1834	WC	2005	484	ST-32/ET5 complex	P1.19,15	F5-1
✧	B 6	GA	2005	6368	ST-32/ET5 complex	P1.19,15	F5-1
	B # 1936	WC	2005	1157	ST-1157 complex	P1.21-7,16	F5-5
	B 4322	FS	2005	6702	ST-41/44/lineage 3 comp.	P1.18-1,3	F1-99
	B 3952	WC	2005	6991	no complex	P1.19,15	F3-16
	B # 2676	GA	2005	6990	no complex	P1.5,2	F3-16
	B 4035FR	EC	2005	6711	no complex	P1.7,9	F1-20
	B 5102	KZ	2005	6704	ST-334 complex	P1.5-2,10-1	F1-5
	B 3950FR	WC	2005	334	ST-334	P1.17,16-4	F4-23
	B 3482	GA	2005	6590	ST-41/44/lineage 3 comp.	P1.7,9	F3-20
	B 430FR	WC	2005	6689	ST-41/44/lineage 3 comp.	P1.7,9	F5-1
	B 4234	WC	2005	6701	ST-41/44/lineage 3 comp.	P1.19,15	F3-20
	B 2827	MP	2005	6710	ST-41/44/lineage 3 comp.	P1.7,9	F3-20
	B 2271	GA	2005	6590	ST-41/44/lineage 3 comp.	P1.7,9	F3-20
	B 4662	WC	2005	6993	no complex	P1.22,26	F3-20
	B 1767FR	WC	2005	6695	ST-41/44/lineage 3 comp.	P1.7,9	F3-20
	B 2909	GA	2005	1834	ST-41/44/lineage 3 comp.	P1.18,25/25-7	F3-9
	B 616FR	GA	2005	6989	ST-41/44/lineage 3 comp.	P1.7,9	F3-20

NOTE : * Province - EC, Eastern Cape; FS, Free State; GA, Gauteng; KZ, KwaZulu-Natal; LP, Limpopo; MP, Mpumalanga; NC, Northern Cape; NW, North West; WC, Western Cape. ST, sequence type. cc, clonal complex. ND, Not determined. # denotes isolates that were part of the random selection.

Outliers

Half of the 2005 serogroup B isolates were outliers (29/57, 51%) **(Figure 3.11)**. They were distributed among 6 provinces **(Figure 3.12)** but were most common to the Western Cape (14/29, 48%) and Gauteng (10/29, 34%) provinces. The isolates fell into different clonal complexes **(Table 3.11),** the majority (11/29) belonged to ST-41/44/lineage 3. A total of 26 STs were identified, all of which were represented by a single isolate except ST35 (n=3) and ST6590 (n=2). Most of the STs were new (18/26). There were 14 PorA types sand 14 FetA types among outliers and 21 PorA:FetA combinations.

Figure 3.12 Provincial distribution of *N. meningitidis* serogroup B PFGE clusters, South Africa, 2005 (n=57).

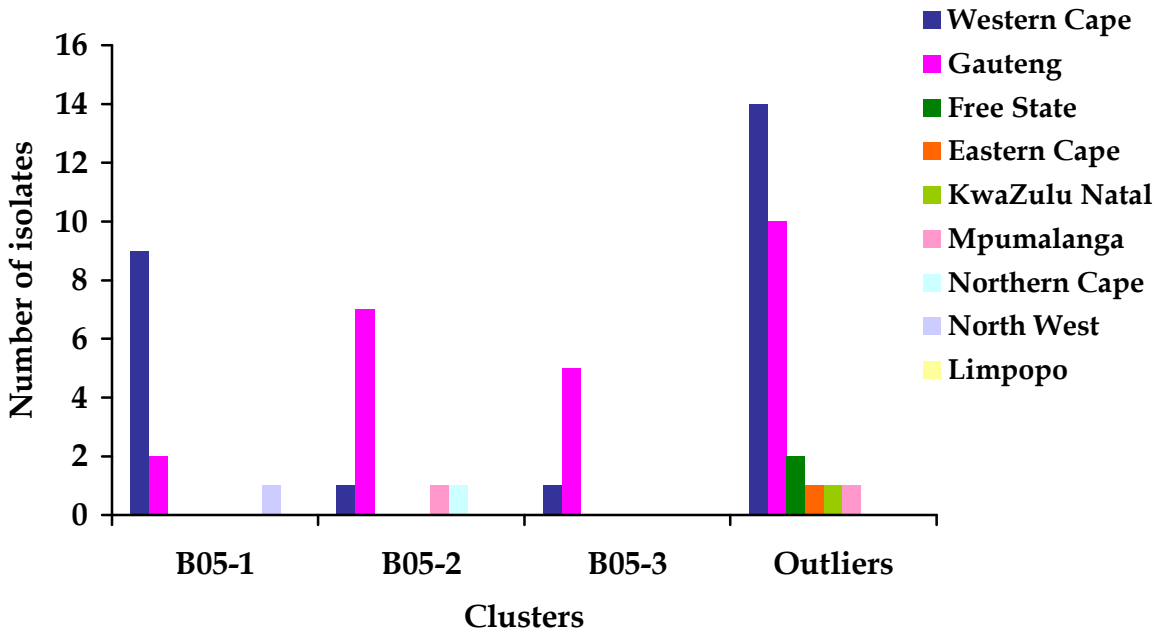


Table 3.10 Genotypes of *N. meningitidis* serogroup B isolates by PFGE clusters (n=28), South Africa, 2005.

Cluster		Genotypes			
	n	Clonal complex	PorA	FetA	n
B05-1	12	ST-32/ET-5	P1.19,15	F5-1	5
			P1.12-1,13-1	F5-1	3
			P1.12-1,13	F5-1	1
			P1.5,2-1	F5-18	1
			P1.12-1,13	F3-7	1
			P1.19,15	F3-7	1
B05-2	10	no complex	P1.5,2	F5-8	10
B05-3	6	ST-41/44/lineage 3	P1.7-2,4	F1-5	5
			P1.7-2,13-1	F1-5	1

Table 3.11 MLST clonal complexes of *N. meningitidis* serogroup B isolates, South Africa, 2005 (n=57).

Clonal complex	B05-1	B05-2	B05-3	Outliers
ST-32/ET-5	12			
no complex		10		6
ST-41/44/lineage 3			6	11
ST-35 complex				5
ST-334 complex				2
ST-865 complex				2
ST-103 complex				1
ST-1157 complex				1
ST-269 complex				1
Total	12	10	6	29

3.3.2 MLST

Most of the 2005 serogroup B isolates belonged to clonal complexes ST-32/ET-5 (12/57, 21%) and ST-41/44/lineage 3 (17/57, 30%) **(Table 3.11)** *(Appendix E)*. Almost a third were not assigned to a clonal complex. A total of 43 STs were identified. The most common STs were ST33 (n=5), ST6688 (n=5) and ST35 (n=3). ST639, ST154 and ST4240 were each represented by 2 isolates while the remaining STs (24/43, 56%) were each represented by a single isolate. A total of 44 isolates could be assigned a group by eBURST **(Table 3.12)**. A population snapshot **(Figure 3.13)** using eBURST grouped STs from the most important clonal complexes, ST-32/ET-5 and ST-41/44/lineage 3, as well as isolates not assigned to a complex. Predicted primary founders included ST33, ST154, ST6590 and ST6688.

Singletons

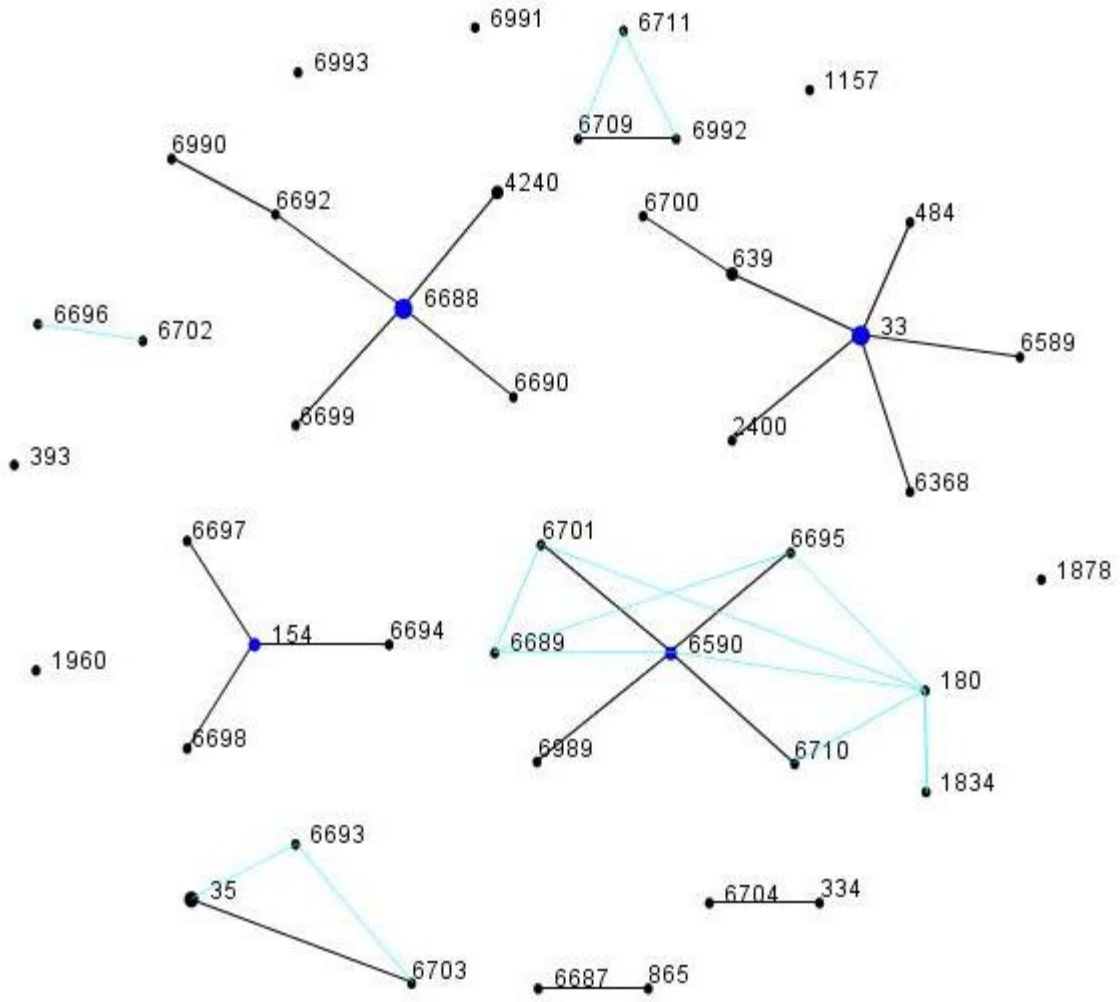
There were 13 singletons **(Figure 3.13)**. The diagram was manually edited to show the relationship of singletons to other STs. Seven singletons were shown to be DLVs of other STs. ST1157, ST393 and ST1878 belonged to clonal complexes ST-1157, ST-269 and ST-103 respectively and were each represented by a single isolate. ST6993 and ST6991 were not assigned to a clonal complex. ST6993 is a TLV of primary founder ST6950 but ST6991 was not a variant of any ST within the population. ST1960 belongs to ST41/44/lineage 3 but is a triple locus variant (TLV) of primary founder ST154.

Table 3.12 eBURST analysis groups of meningococcal serogroup B isolates (n=44), South Africa, 2005

Group	n	ST (n)	Alleles						
			abc	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*
1	12	33 (5)	8	10	5	4	6	3	8
		639 (2)	8	10	5	**9**	6	3	8
		484	8	10	5	4	**9**	3	8
		6589	8	10	5	**264**	6	3	8
		6368	8	**35**	5	4	6	3	8
		2400	8	**5**	5	4	6	3	8
		6700	8	10	5	**9**	**9**	3	8
2	11	6688 (5)	15	5	18	24	8	19	62
		4240 (2)	15	5	**9**	24	8	19	62
		6692	15	5	18	**9**	8	19	62
		6690	15	5	18	**18**	8	19	62
		6990	15	5	**9**	**9**	8	19	62
		6699	**12**	5	18	24	8	19	62
3	6	6590 (2)	9	6	351	9	9	6	311
		6701	9	6	351	9	9	6	**3**
		6695	9	6	351	9	9	6	**17**
		6710	9	6	**483**	9	9	6	311
		6989	9	6	351	**24**	9	6	311
4	5	154 (2)	3	6	9	5	11	6	9
		6697	3	6	9	**24**	11	6	9
		6694	3	6	9	**4**	11	6	9
		6698	3	3	9	**5**	11	6	9
5	2	865	8	5	15	17	8	21	2
		6687	8	5	15	**8**	8	21	2
6	2	6992	47	3	482	**4**	21	59	49
		6709	47	3	482	**76**	21	59	49
7	2	334	14	5	6	9	3	8	18
		6704	14	5	6	**76**	3	8	18
8	4	35 (3)	4	10	11	18	6	10	12
		6703	**47**	10	11	18	6	10	12

NOTE: ST, sequence type. Allele differences indicated in bold. The number of isolates belonging to each ST, if more than one, are indicated in parentheses. Underlined STs are predicted primary founders.

Figure 3.13 A population snapshot of *N. meningitidis* serogroup B isolates, South Africa, 2005 (n=57).



NOTE: Size of circle is proportionate to the number of isolates included in the analysis. Blue circles depict the predicted primary founder. Pale blue lines join single-locus variants.

3.3.3 PorA

A serosubtype was obtained for all 57 isolates **(Figure 3.14)**. There were 19 PorA types. Almost half of all isolates (32/57, 56%) contained 1 of 3 PorA types **(Table 3.13)**, P1.5,2 (n=11), P1.19,15 (n=9) and P17,9 (n=7).

Table 3.13 Most common *N. meningitidis* sergroup B PorA types and associated MLST complexes, South Africa, 2005.

Clonal complex (n)	PorA types			
	P1.5,2	P1.19,15	P1.7,9	P1.7-2,4
no complex (16)	11	1	1	
ST-32/ET-5 (12)		6		
ST-41/44/lineage 3 (17)		2	6	5
Total	11	9	7	5

3.3.4 FetA

FetA types were obtained for 54/57 isolates processed **(Figure 3.15)**. The 3 isolates for which a FetA type could not be determined belonged to clonal complex ST-35 and were of the following STs, ST35, and new STs, ST6703 and ST6693. There were 16 FetA types among the 2005 serogroup B isolates. The majority of isolates (35/54, 65%) contained 1 of 4 FetA types **(Table 3.14)**.

Table 3.14 Most common *N. meningitidis* sergroup B FetA types and associated MLST complexes, South Africa, 2005.

Clonal complex (n)	FetA types			
	F5-1	F5-8	F1-5	F3-20
no complex (16)		10		1
ST-32/ET-5 (12)	9			
ST-41/44/lineage 3 (17)	1		1	6
ST-334 (2)			7	
Total	10	10	8	7

Figure 3.14 PorA types of *N. meningitidis* serogroup B isolates in South Africa, 2005 (n=57).

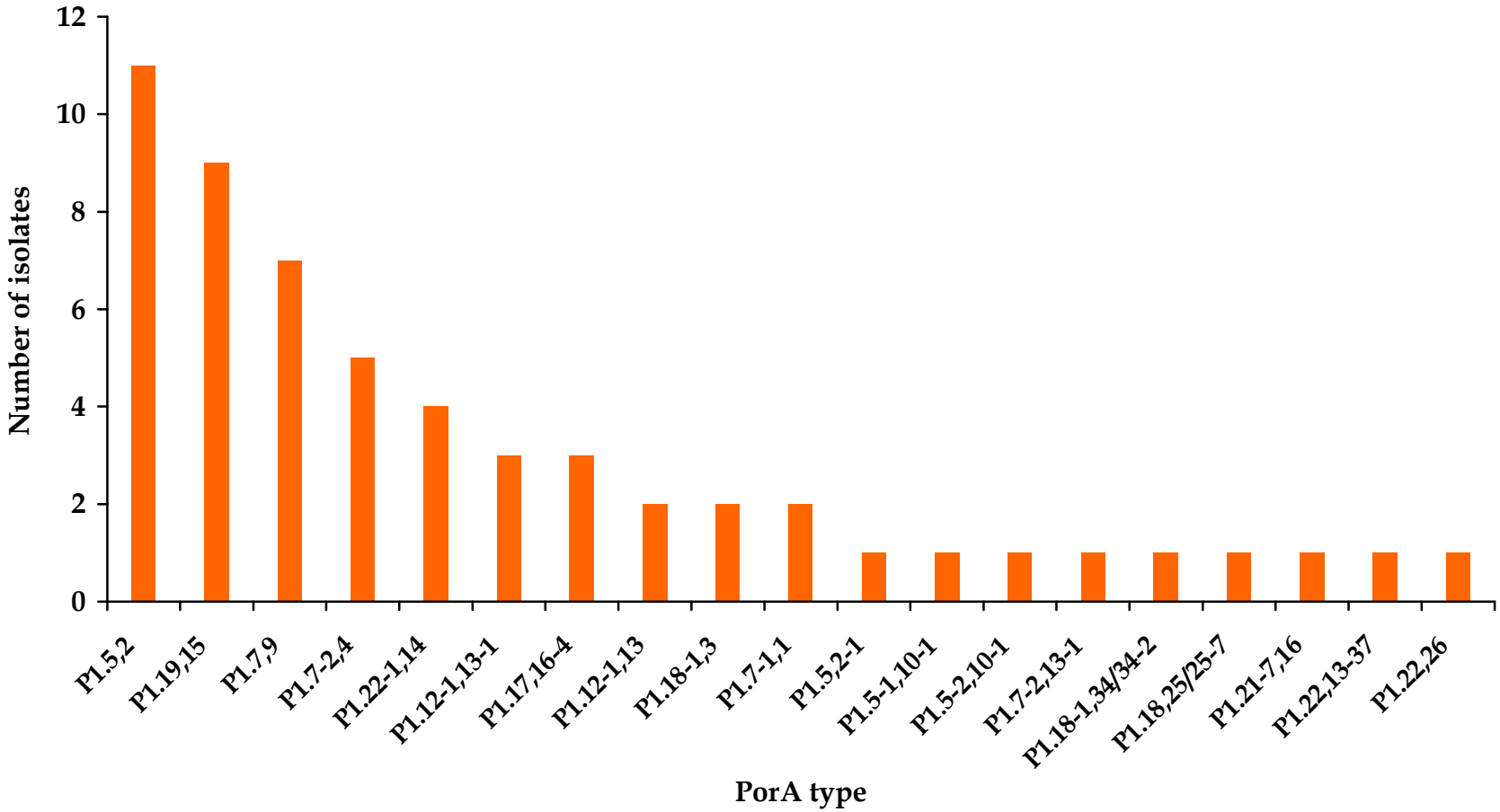
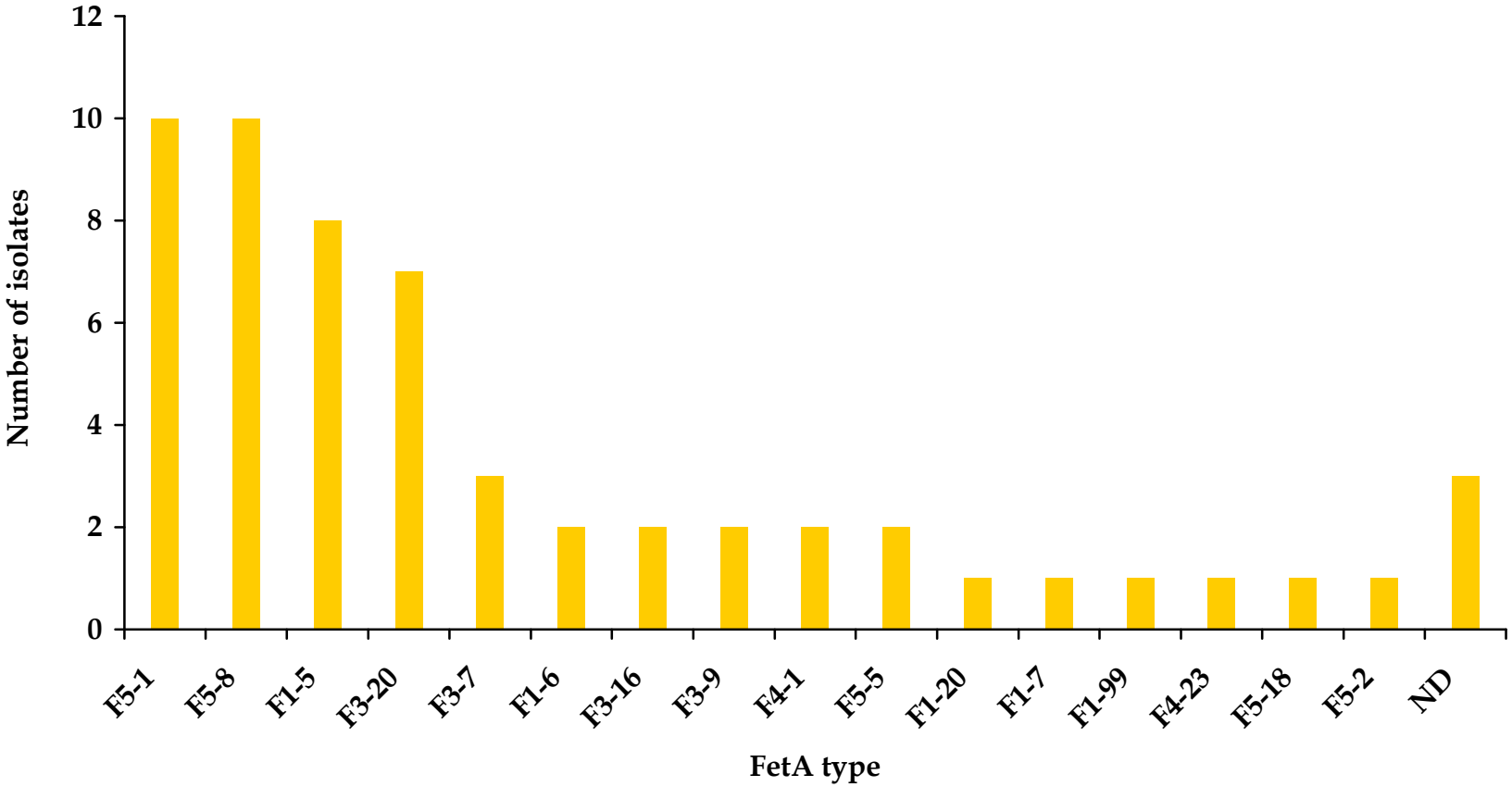


Figure 3.15 FetA types of *N. meningitidis* serogroup B isolates in South Africa, 2005 (n=57).



NOTE: ND, Not determined.

4 DISCUSSION

South Africa has documented meningococcal disease since the early 1900's [67] and is set apart from the rest of Africa in terms of serogroups responsible for invasive cases. Serogroup B causes 60-80% of meningococcal disease in countries in North and South America, parts of Europe, middle-east Asia, New Zealand and Australia [21;165]. However cases of serogroup B are rarely seen in most African countries.

Limited data are available regarding the molecular epidemiology of serogroup B meningococci in our country. Coulson et al. [79] described the epidemiology of invasive meningococcal disease in South Africa from August 1999 through July 2002 where serogroup B caused 41% of laboratory-confirmed cases during this period. Selected isolates from the three main serogroup B PFGE clusters were analysed by MLST, and hyperinvasive clonal complexes ST-32/ET5 and ST-41/44/lineage 3 were identified. The current study describes the genotypes of circulating serogroup B meningococci over a five-year period in South Africa, from 2002 to 2006.

Meningococcal disease epidemiology in South Africa, 2002-2006

From January 2002 through December 2006 sporadic cases of meningococcal disease occurred throughout each year with seasonal increases in the winter and spring months (May to October). The year round occurrence of meningococcal disease, including seasonal variation, has been previously described in South Africa [72;79]

and the United States [52;55]. Cases occurred across the country but were most commonly reported in the Western Cape and Gauteng provinces. Two serogroups, B and W135, were predominant, accounting for almost two thirds of cases. These were reported from all regions in the country, but were most common to the Western Cape and Gauteng provinces respectively. Improved reporting, through the national surveillance system, from these metropolitan areas may also have contributed to the higher numbers of confirmed cases. There was an overall decrease in serogroup B disease over the 5-year period.

Age-specific incidence rates were highest in children less than 5 years of age, for overall disease as well as serogroup B disease. Incidence rates were similar to those seen in the United States [52], Europe and Ireland [165;175]. Coulson et al. also reported the highest incidence in the less than 5-year age group, particularly in infants less than 1 year of age. With regard to gender, slightly more than half of invasive serogroup B cases (55%) occurred in males, however this was not statistically significant.

Among those cases wehee the final outcome was known the case-fatality ratio (CFR) was 9%. Several studies have reported similar and lower CFRs for serogroup B disease outside of epidemics [175-178]. In the United States, a significant association has been shown to exist between outbreaks (after adjusting for serogroups) and

increased CFR compared to sporadics cases [179]. Studies have reported higher CFRs for serogroups A, C and W135 [81;177;178] .

Serogroup B characterisation by PFGE and MLST

Serogroup B PFGE and MLST data from 2005 demonstrated the correlation between the two methods and therefore supported inferences made with the larger collection of isolates. As expected, PFGE showed substantial diversity among invasive isolates which is charactersistic of serogroup B globally [52;60], especially in countries where disease is mostly sporadic. Compared with serogroups A, C, Y and W135 which tend to be more clonal [80;126;128], serogroup B strains causing sporadic disease are generally more diverse. Serogroup B meningococci isolated in South Africa from a previous study also demonstrated high levels of diversity [79]. This diversity is attributed to high rates of recombination and phase variation within the naturally transformable meningococcal population [157;162]. Despite this diversity, the majority of isolates collected over the 5-year period could be grouped into several clonal clusters.

Overall, the most predominant clone in South Africa was clonal complex ST-32/ET-5. From August 1999 through July 2002, the ST-32/ET-5 clone was responsible for 37% (90/245)of serogroup B disease [79]. However, of the serogroup B isolates analysed for this study, ST-32/ET-5 caused approximately 25% of cases, indicating a decline in the prevalence of this clone. This clonal complex was found circulating mostly in the

Western Cape with a significant decrease over the five-year period. ST-32/ET-5 strains are considered hyperinvasive and have spread between continents since the 1970s causing outbreaks of varying magnitudes [169;170]. In this study, eBURST showed that ST33 was the primary founder of ST-32/ET-5 strains. ST33 is a SLV of ST32 and a subgroup founder within the ST-32/ET-5 complex [180]. A substantial amount of invasive meningococcal disease in Europe is due to ST-32/ET-5 [37;56;181] however proportions of cases due to this clone differ greatly between European countries [182].

Norway experienced an epidemic caused by a single ST-32/ET-5 clone [59;166] which spanned at least 20 years (1970s-1990s) [66]. In the United States the state of Oregon continues to experience an outbreak, which began in 1993 [52;64], caused by ST-32/ET-5 strains. Excluding isolates from Oregon, approximately one third of invasive serogroup B isolates collected in the USA through the ABC Surveillance system, from 2000 through 2005, belonged to clonal complex ST-32/ET-5 [55]. In 1983, Cuba experienced a peak in incidence of their meningococcal epidemic [63]. The predominant clone was ST-32/ET-5, the same clone that caused an increase in disease incidence in Sao Paulo, Brazil [38;57;58]. This clone also caused an outbreak in the city of Iquique, Chile in the 1980s [62;183]. In addition, Japan and Taiwan have also reported disease caused by ST-32/ET-5 strains [61;122].

The second most predominant clonal complex in South Africa was ST-41/44/lineage 3. From August 1999 through July 2002, the ST-41/44/lineage 3 clone was responsible for 9% (22/245)of serogroup B disease and was also the second most predominant complex [79].Unlike ST-32/ET-5, the ST-41/44/lineage 3 clonal complex was found mostly in Gauteng, where it was previously identified by Coulson et al. [79]. Strains of the ST-41/44/lineage 3 complex are highly diverse [182]. It is the only complex for which two central STs have been defined. ST41 is largely associated with invasive disease while ST44 tends to be associated with meningococcal carriage [184]. In this study, eBURST identified ST154 and ST6590 as primary founders of ST-41/44/lineage 3 strains. ST154 is a SLV of ST41 and a subgroup founder within the clonal complex [180]. ST110 is a SLV of ST44 and a subgroup founder within the ST-41/44/lineage 3 complex. ST6590 is a DLV of ST110. Strains of the ST-41/44/lineage 3 clonal complex have been shown to cause substantial disease in Europe [37;56], Japan [61] and Taiwan [122], more so than ST-32/ET-5.

ST-41/44/lineage 3 strains emerged in the 1980s in the Netherlands [167]. During this time the Netherlands experienced an increase in meningococcal disease incidence, mostly caused by this clone [53;59]. In New Zealand, ST-41/44/lineage 3 strains were responsible for a 10-year epidemic [65]. ST154 was responsible for a substantial amount of disease during this time. The epidemic began in mid-1991, reached peak incidence in 2001 (17.4 cases/100 000 population) and subsequently began to wane [185].

A substantial proportion of invasive isolates in this study belonged to STs that were not assigned to a known clonal complex. Some STs were related to each other. Among these STs eBURST predicated the primary founder to be ST6688. The majority of these ST's were new and appear to be unique to South Africa at this time. Unassigned STs have been reported in the USA [186], Europe [37;56] and elsewhere [58;61;110;122]. Other important clonal complexes that were identified included ST-269, ST-35, ST-334 and ST-103. Clonal complex ST-269 has expanded in Europe and is responsible for a substantial portion of invasive disease [37;56;187;188]. There have been reports of an increase in ST-269 following the introduction of the meningococcal C vaccine in Canada [189] and Scotland [188] with suggestions of possible capsule switching (between C and B) in Canada. ST-269 strains have also been identified in the USA [186], Cuba [110] and Europe [182].

ST-35 made up approximately 4% of serogroup B isolates. From August 1999 through July 2002 Coulson et al. [79] identified the ST-35 clone as the third most predominant complex (15/245, 6%) preceded only by clones ST-32/ET-5 and ST-41/44/lineage 3. ST-35 strains have been described in the USA and Europe [182;186]. Two ST-35 isolates (ST35 and ST2437) did not fall into the same PFGE cluster as other ST-35 strains. PFGE was repeated on these isolates to ensure that the fingerprint patterns were correct, and the same result was obtained. ST35 was an outlier while ST2437 (a SLV of the ancestral ST35) was closely related to strains that were not assigned to a

clonal complex. It is possible that recombination events led to substantial changes in the genome resulting in related STs having different PFGE fingerprint patterns.

ST-334 strains have been described in the USA [186]. Clonal complex ST-103 was represented by one isolate (ST1878, among outliers) identified as a SLV of the ancestral ST (ST103). ST-103 strains have been described in Cuba [110], the USA [186] and Brazil [58]. Identification of serogroup B isolates belonging to these clonal complexes indicates possible capsule switching from serogroup C to B since strains of ST-334 and ST-103 are mostly associated with serogroup C [186] according to the central MLST database.

ST-865 comprised approximately 5% of all serogroup B isolates in this study. Coulson et al. [79] identified serogroup C ST-865 strains (9/49, 18%) in South Africa. At the time ST-865 was the second most predominant complex among serogroup C isolates preceded by the ST-11/ET-37 clone (13/49, 27%) A recent study in our unit (unpublished data) showed that ST-865 has expanded, as the majority of serogroup C isolates from 2005 belonged to ST-865. Capsule switching among ST-865 strains was indicated by serogroup C isolates from 2005 having the same genotype, P1.7-1,1:F1-6:ST865, as a serogroup B strain isolated in the same year. It is not always possible to determine the direction of caspular switching, however, since ST-865 is usually associated with serogroup B (according to the central MLST database), it is possible that the direction of the switch was from serogroup B to C.

For other organisms eg. *Streptococcus pneumoniae*, capsular switching following routine vaccine implementation, has been demonstrated [190;191]. Recently, Harrison et al. [186] reported that capsular switching, especially amongst serogroup B, C and Y meningococcal isolates, was evident among USA invasive isolates prior to the introduction of the meningococcal C conjugate vaccine (MCV4). The study confirmed that capsular switching can occur as a natural occurrence in the absence of vaccine or other selective pressures. [186;192-194]. To date, capsular switching has not been identified in the United Kingdom where routine MCV4 vaccination has been implemented [195]. The SA strains exhibited capsular switching in the absence of any vaccine pressure. A potential switch between sergroup Y and B has been reported in Canada [196]. While ST-865 strains were not identified among serogroup B and C isolates in the USA [186], they were reported among serogroup B isolates in Taiwan [122].

PorA types

As expected, there was substantial PorA diversity among the isolates. The most common PorA types during the 5-year period included P1.5,2; P1.19,15; P1.7,9 and P1.7-2,4 These serosubtypes have been identified elsewhere [110;186;197-201]. P1.19,15 was associated with high disease incidence in Cuban, Brazil and Spain. Based on data from the central MLST database, P1.19,15 was the dominant PorA type of the ST-32/ET-5 clonal complex [165]. P1.7-2,4 is associated with the clone

responsible for the New Zealand epidemic. Devoy et al. [202] showed that this PorA type was relatively stable throughout the epidemic and therefore could be used in an OMP-based vaccine [202]. Based on data from the central MLST database P1.7-2,4 is the dominant PorA type of the ST-41/44/lineage 3 clonal complex [165]. Previous studies using meningococci from South African complement-sufficient and complement-deficient individuals determined PorA type, however monoclonal antibodies were used and determined either VR1 or VR2 [172;203]. Therefore it was difficult to compare findings. Multiple mechanisms of phase variation, which lead to a high degree of diversity, have been described for PorA [204]. Deletion of PorA has been reported to occur via recombination involving palindromic RS3 core sequences [205]. However, PorA-deficient isolates have been reported to cause invasive disease [206].

FetA types

Isolates were also quite diverse by FetA. The most common FetA types were F5-1, F5-8, F1-5 and F3-20. These FetA types have been identified globally [110;147;186;207]. In this study, F5-1 and F1-5 were the dominant types of the ST-32/ET-5 and ST-41/44/lineage 3 clonal complexes, respectively. The FetA type of four isolates could not be determined. Repeated attempts failed to produce PCR products fro three of the four isolates. The fourth isolate produced a PCR product that was slightly smaller than the expected band size. Very poor sequencing results were obtained for this product. No further work was done on these isolates. These isolates belonged to

different STs of the ST-35 clonal complex but had the same PorA type. FetA gene deletions have been reported in different clones but mostly among isolates of the ST-35 complex and have been found in both invasive and carriage isolates [155;208]. Marsh et al. [208] attributed the deletions to genetic recombination of RS3 repetitive elements, similar to PorA deletions, while Claus et al. [155] showed that, in addition to RS3 recombination, different mechanisms of intragenomic recombination and rearrangement were responsible for the *fetA* deletions. PorA in three of the isolates was the same type as those FetA deficient isolates reported from the USA [208] and Germany [155].

Genotypes

Common genotypes included P1.5,2:F5-8:no complex; P1.19,15:F5-1:ST-32/ET-5; P1.7-2,4:F1-5:ST-41/44/lineage 3 and P1.7,9:F3-20:ST-41/44/lineage 3. P1.5,2:F5-8:no complex has been reported in Germany but among serogroup C isolates [207]. P1.19,15:F5-1:ST-32/ET-5 was frequently identified in Cuba [110]. P1.7-2,4:F1-5:ST-41/44/lineage 3 was prevalent among German [207] and USA [186] isolates. Urwin et al. [147] used a global collection of isolates representative of hyperinvasive lineages to examine the antigenic diversity of PorA, FetA and PorB among meningococci. The study identified the P1.7-2,4:F1-5 combination as the most common among ST-41/44/lineage 3 strains [147]. The genotype causing the ongoing epidemic in Oregon, USA (P1.7,16:F3-3:ST-32/ET-5) [186] was not identified among the South African isolates.

5 CONCLUSIONS

Serogroup B molecular epidemiology in South Africa from 2002 through 2006 is similar to that described globally, for sporadic disease. The isolates displayed a high level of diversity. Over the study period South Africa had a predominance of the two most important hyperinvasive serogroup B clonal complexes, ST-32/ET-5 and ST-41/44/lineage 3. ST-32/ET-5 strains have decreased over time while ST-41/44/lineage 3 complex strains increased since they were first reported between 1999 and 2002 [79]. In addition, other clones, ST-269, ST-35, ST-334, ST-103 and ST865, were identified. ST-269 has recently expanded in parts of Europe [187] and in Canada [189]. Studying the characteristics and structure of meningococcal populations, is therefore important for monitoring shifts in meningococcal disease epidemiology.

PFGE and MLST are both valuable techniques for characterisation and should be used in conjunction depending on the epidemiological circumstances. PFGE, while technically subjective and laborious, has a higher discriminatory power where MLST may characterise isolates as the same ST [143]. A combination of DNA-based typing schemes is more useful. This would provide further resolution between isolates as well as unambiguous data. While capsule switching occurs naturally within the meningococcal population [186] the use of vaccines against serogroups A, C, Y and W135 may act as a selective pressure under which isolates may switch to other

serogroups. Capsule switching in this study was identified in the absence of any vaccine pressure.

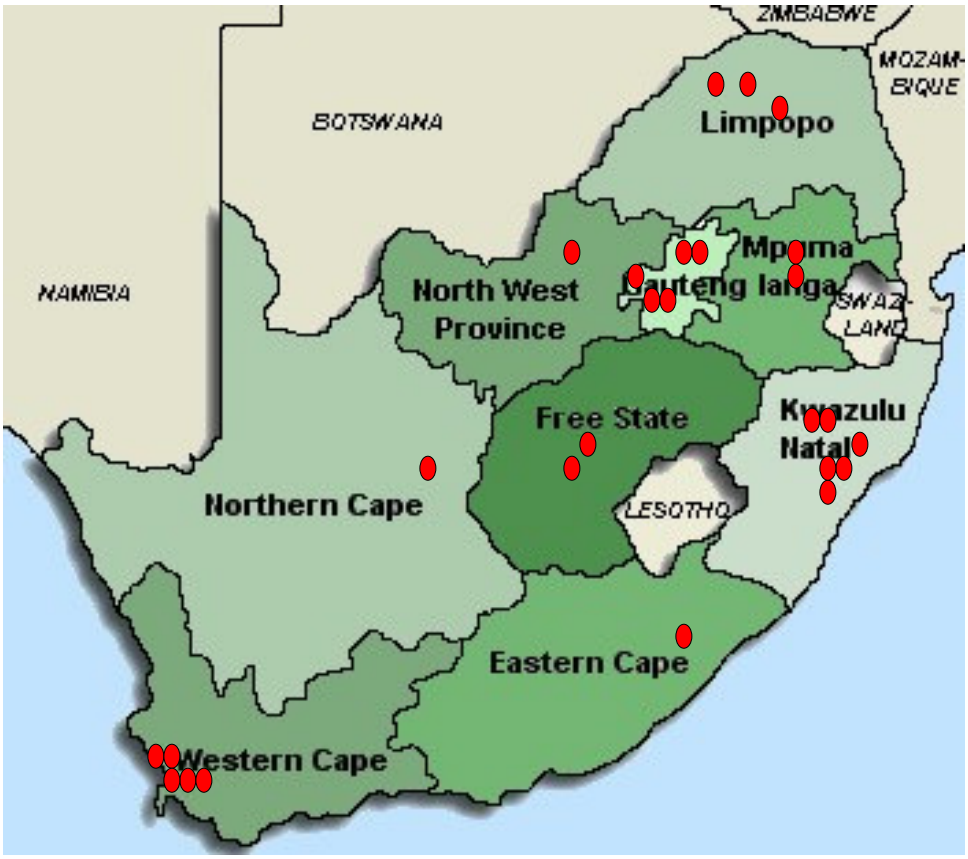
In the absence of an effective vaccine against serogroup B, widespread group B outbreaks may result in substantial morbidity and mortality. The use of strain-specific OMP vaccines based on PorA type have been successful in controlling outbreaks in Chile, Cuba, The Netherlands and New Zealand. However, since the vaccines target strains responsible for the outbreaks, they are therefore limited to geographic region. Isolates that cause endemic disease are more diverse. South African isolates displayed a level of diversity that would require a multivalent vaccine of at least four PorA types and four FetA types to prevent at least 50% of invasive disease. In addition, deletions of PorA and FetA gene sequences may limit the use of these OMPs as vaccine targets because isolates may benefit from these deletions under vaccine pressure. A more conserved protein, such as fHBP [209], may be more suitable as a vaccine target, affording more comprehensive control of meningococcal disease.

N. meningitidis clones have traversed the globe [170]. Continued molecular surveillance using multiple typing methods is therefore crucial for monitoring out-break-associated and emerging clones. Mapping the disease epidemiology within a region will assist in public health control strategies by providing data for vaccine development and implementation.

6 APPENDICES

Appendix A

1. Sentinel sites (dots) across the 9 provinces participate in an active enhanced surveillance program.



2. Case report form

GERMS-SA: National Laboratory-based Surveillance for Enteric, Respiratory and Meningeal Bacterial and Fungal Diseases in South Africa
Protocol Version 1.4 (January 2009)
Clinical Case Report Form
National Microbiology Surveillance Unit (NMSU)
TEL: 011 386 6234 OR 011 555 0353 FAX: 011 386 6077

Surveillance officer name: Signature: Date:

Sources of data: Patient/Guardian ☐ Clinician ☐ Medical records ☐ No record found ☐ Refused participation ☐

Lab Specimen No: Laboratory Name:

Hospital Name: Hospital Number: Ward: Adult Ward ☐ Paed Ward ☐

Gender: M ☐ F ☐ Unk ☐ Race: Asian ☐ Black ☐ Coloured ☐ White ☐ Unk ☐

Date of Birth: D D M M Y Y Y Y DOB Unk ☐ Age: Unit: Days ☐ Months ☐ Years ☐ Age Unk ☐

Patient Surname: Patient First Names:

Address: Town/City: Province:

Tel no: (H) (W) (C) (Neighbour)

Has patient stayed in SA for the last month: Yes ☐ No ☐ Unk ☐ If no, which country has patient come from:

ID No. Unk ☐ ARV No. Unk ☐

Was patient referred from a hospital or chronic-care facility: Yes ☐ No ☐ Unk ☐ If yes, specify:

Date of admission to acute hospital: D D M M Y Y Y Y Unk ☐

Was patient transferred to a step-down hospital: Yes ☐ No ☐ Unk ☐ Date of transfer: D D M M Y Y Y Y

If yes, name of step down hospital:

Final outcome of patient: Discharged ☐ Died ☐ RHT/ Absconded ☐ Unk ☐ Outcome date: D D M M Y Y Y Y

If discharged, patient discharged to: Home ☐ TB Hosp/Chronic care facility ☐ Other ☐ Specify: Unk ☐

Discharge diagnosis:

Meningitis ☐ LRTI ☐ Dysentery ☐ Diarrhoea ☐ Fungaemia/Bacteraemia without focus ☐ Other ☐ Specify:

Organism isolated: *Cryptococcus* sp. ☐ *Haemophilus* sp. ☐ *N. meningitidis* ☐ *Shigella* sp. ☐ *S. pneumoniae* ☐ *P. jirovecii* ☐ *Salmonella* sp. ☐

Date of specimen collection: D D M M Y Y Y Y

Site of specimen collection: CSF ☐ Blood ☐ Joint Fluid ☐ Other ☐ Specify:

Severity of illness (on the day the positive specimen was taken):

Temp: °C Unk ☐ BP: / Unk ☐ Mechanical Ventilation: Yes ☐ No ☐ Unk ☐ Cardiac Arrest: Yes ☐ No ☐ Unk ☐

GCS: /15 Unk ☐ Mental Status: Alert ☐ Disorientated ☐ Stuporous ☐ Comatosed ☐ Unk ☐

Previous admissions in the last 12 months: Yes ☐ No ☐ Unk ☐ Number of admissions:

Cotrimoxazole prophylaxis and TB treatment (from the last 3 months and current)

Cotrimoxazole prophylaxis: Yes ☐ No ☐ Unk ☐ Dosage: Date initiated: D D M M Y Y Y Y Compliant in last month: Yes ☐ No ☐ Unk ☐

TB Treatment: Drugs: Yes ☐ No ☐ Unk ☐ 1. 2. 3. 4. Date initiated: D D M M Y Y Y Y Date stopped: D D M M Y Y Y Y

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GERMS-SA: National Laboratory-based Surveillance for Enteric, Respiratory and Meningeal Bacterial and Fungal Diseases in South Africa
Protocol Version 1.4 (January 2009)
Clinical Case Report Form

National Microbiology Surveillance Unit (NMSU)
TEL: 011 386 6234 OR 011 555 0353 FAX: 011 386 6077

Laboratory Specimen Number: ☐☐☐☐☐☐☐☐☐☐

Immunocompromising conditions:

Alcohol dependency ☐	Chronic renal failure ☐	Heart failure ☐	Kwashiokor/ Marasmus ☐	Valvular heart disease ☐
Asthma ☐	Current smoker ☐	History of head injury/head surgery ☐	Nephrotic syndrome ☐	Malignancy ☐ Specify:_____
Burns ☐	Coronary Artery Disease ☐	Hydrocephalus with VP shunt ☐	Sickle cell anaemia ☐	Organ transplant ☐ Specify:_____
CVA/Stroke ☐	Diabetes mellitus ☐	Immunoglobulin deficiency ☐	Splenectomy/ asplenia ☐	Other ☐ Specify:_____
Cirrhosis/ liver failure ☐	Emphysema/COPD ☐	Immunosuppressive rx (steroid,chemo) ☐	Systemic Lupus Erythematosus (SLE) ☐	None ☐ Unknown ☐

HIV status prior to this admission: Pos ☐ Neg ☐ Unk ☐
HIV status at this admission: Pos ☐ Neg ☐ Unk ☐
For children <18 months: HIV PCR Done: Yes ☐ No ☐ Unk ☐
If HIV unknown, why was patient not tested: Patient died ☐ Patient not seen ☐ No guardian ☐ Patient confused/ comatose ☐ Pt referred for VCT elsewhere ☐ Refused consent ☐
HIV related counseling offered by SO: Yes ☐ No ☐
HIV test performed by SO: Yes ☐ No ☐
If HIV unknown, is there clinical suspicion of HIV: Yes ☐ No ☐ Unk ☐
Reason for refusal: _____ Unk ☐

Clinical markers of HIV: Diarrhoea >10days ☐ Oral candidiasis ☐ Suspected PCP ☐ None ☐
Kaposis sarcoma ☐ Tuberculosis ☐ HIV wasting ☐ Unk ☐

CD4 count closest to specimen collection date: Absolute: Unk ☐ Percentage: % Unk ☐ Date taken: D D M M Y Y Y Y

Viral load closest to specimen collection date: <400 ☐ 400-10,000 ☐ >10,000 ☐ Unk ☐ Date taken: D D M M Y Y Y Y

Any antiretroviral use: Yes ☐ No ☐ Unk ☐ If yes: Current ☐ Previous ☐ Perinatal ☐ Unk ☐

If HIV positive and no **current** ARV use, has the patient been referred to an ARV clinic: Yes ☐ No ☐ Died ☐ Unk ☐

PLEASE COMPLETE RELEVANT SECTIONS FOR SPECIFIED ORGANISMS

***Haemophilus* spp., *S. pneumoniae*, *N. meningitidis*, *Salmonella* spp., *Shigella* spp. ONLY**

Number of children, <18 years, living with patient: None ☐ Number ☐ Place of safety ☐ Unk ☐
Have any of these children been hospitalised in the last 3 months: Yes ☐ No ☐ Unk ☐

Antibiotic use prior to this specimen collection date:

ABX in 24hr before specimen: Yes ☐ No ☐ Unk ☐ Date initiated: D D M M Y Y Y Y
Name of antibiotic: 1. 2. 3. 4.

Other ABX in last 2 months: Yes ☐ No ☐ Unk ☐
Name of antibiotic: 1. 2.
In last 30 days: Yes ☐ No ☐ Unk ☐
In last 30 to 60 days: Yes ☐ No ☐ Unk ☐

Antibiotic use in hospital during this admission (excluding TB therapy)

Weight: ☐☐☐.☐ kg Unk ☐ Antimicrobial therapy unknown: ☐ Antimicrobial therapy not prescribed: ☐

Name of antimicrobial	Dose	Route	Date initiated	Total doses given/no. of days
1.			D D M M Y Y Y Y	
2.			D D M M Y Y Y Y	
3.			D D M M Y Y Y Y	
4.			D D M M Y Y Y Y	
5.			D D M M Y Y Y Y	

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National Microbiology Surveillance Unit (NMSU)
TEL: 011 386 6234 OR 011 555 0353 FAX: 011 386 6077

Laboratory Specimen Number: ☐☐☐☐☐☐☐☐☐☐☐☐☐

Haemophilus spp. and _S. pneumoniae_ ONLY

Vaccination status for ***Haemophilus influenzae***:
If <15 years old, did patient receive *Haemophilus influenzae* type b (Hib) vaccine? Yes ☐ No ☐ Unk ☐

Dose	Dose given?	Date given
6 weeks	Yes ☐ No ☐ Unk ☐	D D M M Y Y Y Y
10 weeks	Yes ☐ No ☐ Unk ☐	D D M M Y Y Y Y
14 weeks	Yes ☐ No ☐ Unk ☐	D D M M Y Y Y Y
18 month booster (Pentaxim)	Yes ☐ No ☐ Unk ☐	D D M M Y Y Y Y
Catch up/ Other	Yes ☐ No ☐ Unk ☐	D D M M Y Y Y Y
Catch up/ Other	Yes ☐ No ☐ Unk ☐	D D M M Y Y Y Y
Catch up/ Other	Yes ☐ No ☐ Unk ☐	D D M M Y Y Y Y

Vaccination status for ***S. pneumoniae***:
If <15 years old, did patient receive conjugate vaccine for *S. pneumoniae*? Yes ☐ No ☐ Unk ☐

Dose	Dose given?	Date given
6 weeks	Yes ☐ No ☐ Unk ☐	D D M M Y Y Y Y
10 weeks	Yes ☐ No ☐ Unk ☐	D D M M Y Y Y Y
14 weeks	Yes ☐ No ☐ Unk ☐	D D M M Y Y Y Y
Catch up/ Other	Yes ☐ No ☐ Unk ☐	D D M M Y Y Y Y
Catch up/ Other	Yes ☐ No ☐ Unk ☐	D D M M Y Y Y Y

Has the patient (all ages) received the 23 valent polysaccharide pneumococcal vaccine? Yes ☐ No ☐ Unk ☐

If yes give date most recently given: D D M M Y Y Y Y

Source of vaccine status information:

The Road to Health Card seen by S. officer ☐ Verbal report from caregiver ☐ Drs notes from RTHC ☐ Drs notes from verbal report ☐

Directly from clinic ☐ Other ☐ Specify:

Cryptococcus spp. ONLY

Antifungals prior to this admission:

Fluconazole Yes ☐ No ☐ Unk ☐ If yes, date initiated D D M M Y Y Y Y Dose Daily ☐ BD ☐

Amphotericin B Yes ☐ No ☐ Unk ☐ If yes, date initiated D D M M Y Y Y Y Dose

Is this the first episode of cryptococcosis? Yes ☐ No ☐ Unk ☐ Weight ☐☐☐.☐ kg Unk ☐

Management during this admission: Antifungal therapy unknown ☐ Antifungal therapy not prescribed ☐

	Dose	Frequency	Date initiated	Total number of doses/ number of days
Fluconazole		Daily ☐ BD ☐	D D M M Y Y Y Y	
Amphotericin B		Daily ☐ BD ☐	D D M M Y Y Y Y	

Rifampicin Yes ☐ No ☐ Unk ☐

Was opening intracranial pressure documented at time of first LP? Yes ☐ No ☐ Unk ☐

If yes, what was the recorded opening pressure: _____ cm H_20 Unk ☐

On discharge, was patient given fluconazole: Yes ☐ No ☐ Unk ☐ Died ☐

Pneumocystis jirovecii ONLY

PCP treatment during this admission: Weight ☐☐☐.☐ kg Unk ☐

	Dose	Route	Date initiated	Total number of doses/ number of days
Cotrimoxazole			D D M M Y Y Y Y	
Dapsone			D D M M Y Y Y Y	
Other			D D M M Y Y Y Y	
Prednisone			D D M M Y Y Y Y	
Hydrocortisone			D D M M Y Y Y Y	

PCP therapy unknown ☐ PCP therapy not prescribed ☐

On discharge was patient given cotrimoxazole: Yes ☐ No ☐ Unk ☐ Died ☐ Discharge dose/ number of days:

3. Laboratory sterile site isolate form

STERILE SITE ISOLATES

Regional Laboratory Data Form for Invasive
H. influenzae, S. pneumoniae, N. meningitidis, Salmonella spp.
Shigella spp. *and C. neoformans*
PLEASE ATTACH A COPY OF LABORATORY REPORT OR WORKING CARD

NATIONAL HEALTH LABORATORY SERVICE

RESPIRATORY AND MENINGEAL PATHOGENS RESEARCH UNIT (RMPRU)
TEL: (011) 555 0315/7
FAX: (011) 555 0437

MYCOLOGY REFERENCE UNIT (MRU)
TEL: (011) 555 0384
FAX: (011) 555 0435

ENTERIC DISEASES REFERENCE UNIT (EDRU)
TEL: (011) 555 0333/4
FAX: (011) 555 0433

REGIONAL LABORATORY SPECIMEN NUMBER: ☐☐☐☐☐☐☐☐☐☐

Hospital Name: _____ (Where patient is admitted) Laboratory Name: _____

Laboratory Contact Person: _____ Laboratory Tel: ☐☐☐☐☐☐☐☐☐☐

PATIENT DETAILS

Surname: First Name: _____

Sex: ☐ M ☐ F Date of Birth:(dd/mm/yyyy) ☐☐ ☐☐ ☐☐☐☐

WARD: Age/Units: ☐ Days ☐ Months ☐ Yrs

Hospital No.: ☐☐☐☐☐☐☐☐☐☐ Admission Date:(dd/mm/yyyy) ☐☐ ☐☐ ☐☐☐☐

Province: _____ Town: _____

DIAGNOSIS & OUTCOME

Diagnosis: ☐ Meningitis ☐ LRTI ☐ Bacteraemia/Fungaemia ☐ Dysentery ☐ Diarrhoea

☐ Other, specify _____ ☐ Unknown

Outcome at current date: ☐ Recovered ☐ Died ☐ Unknown

SPECIMEN DETAILS

Collection Date: (dd/mm/yyyy) ☐☐ ☐☐ ☐☐☐☐

Gram stain result: _____ India ink: ☐ Pos ☐ Neg ☐ Not done

Latex Test Results (if tested):

Cryptococcal ☐ Pos ☐ Neg ☐ Not Done

Bacterial ☐ Pos Specify: _____ ☐ Neg ☐ Not Done

Organism isolated? ☐ Yes ☐ No

Identification of organism:

☐ *S. pneumoniae* ☐ *H. influenzae* ☐ *N. meningitidis* ☐ *C. neoformans*

☐ *Salmonella typhi* ☐ Non-typhoidal *Salmonella* spp. ☐ *Shigella* spp. ☐ Other, specify: _____

SOURCE OF ISOLATE

☐ Blood Culture ☐ CSF ☐ Blood Culture and CSF ☐ Other, specify: _____

Did original specimen yield mixed culture? ☐ Yes ☐ No If yes, specify organisms: _____

PAST HISTORY

Did patient have any of the above isolates previously? ☐ Yes ☐ No If yes, specify organism: _____

Date of previous isolate: (dd/mm/yyyy) ☐☐ ☐☐ ☐☐☐☐

Regional laboratory number: ☐☐☐☐☐☐☐☐☐☐

Draft 6: January 2006

FORMSFIT 083 325 2389

Appendix B

1. 1 M Tris-HCL

- 121.1 g Tris (Sigma Aldrich, Steinheim, Germany)
- 800 ml distilled water

Adjust pH to 8.0 by adding conc. HCL. Adjust volume to 1 L and autoclave.

2. 0.5 M EDTA

- 186.1 g EDTA (Merck Chemicals, Gauteng, South Africa)
- 800 ml distilled water

Dissolve completely. Adjust pH to 8.0 with NaOH pellets.

Make up to 1 L and autoclave.

3. TE Buffer

- 5 ml 1M Tris-HCL (pH 8.0)
- 1 ml 0.5M EDTA (pH 8.0)
- 494 ml distilled water

Mix and autoclave at 121°C.

4. Lysozyme

- 10 mg lysozyme (Sigma Aldrich, Steinheim, Germany)
- 1 ml distilled water

1 ml stock solutions stored at -20°C.

5. Proteinase-K

- 20 mg proteinase-K (Roche, Mannheim, Germany)
- 1 ml distilled water

1 ml stock solutions stored at -20°C.

6. 5x TBE

- 54 g Trizma® (Sigma-Aldrich, Steinheim, Germany)
- 27.5 g Boric acid (Sigma-Aldrich, Steinheim, Germany)
- 4.65 g EDTA (Merck Chemicals, Gauteng, South Africa)

Make up to 1 L with deionised water.

7. 0.5x TBE

- 100 ml 5x TBE buffer
- 900 ml distilled water

Mix well.

8. 1.2% Seakem Gold Agarose

- 0.3 g Seakem Gold Agarose (Lonza, Rockland, USA)
- 25 ml 0.5x TBE buffer

Dissolve agarose completely by boiling.

9. 10 % SDS

- 100 g SDS (BDH Lab Supplies, Poole, England)
- 900 ml distilled water

Heat to 68°C to assist dissolution. Adjust pH to 7.2 by adding conc. HCL

Adjust to 1 L. Do not autoclave!

10. Cell Lysis Buffer (25 ml)

- 25 ml EDTA (0.5 M, pH 8.0)
- 0.25 g sarkosyl [final = 1 %] (Sigma-Aldrich, Steinheim, Germany)
- 2.5 mg Proteinase-K [final = 0.1mg/ml] (Roche Diagnostics, Mannheim, Germany)

Make up fresh.

11. 1x Restriction Buffer

- 10 µl 10x restriction buffer (Roche Diagnostics , Mannheim, Germany)
- 90 µl distilled water

Make up fresh.

12. *Nhe*I Restriction Enzyme Cocktail (100 µl)

- 0.5 µl of 40 U/µl *Nhe*I restriction enzyme (Roche Diagnostics, Mannheim, Germany)
- 10 µl 1x restriction buffer
- 90 µl distilled water

Make up fresh.

13. Ethidium Bromide Solution (300 ml)

- 300 ml 0.5x TBE buffer
- 30 µl of 10 mg/ml ethidium bromide [final = 1 mg/ml]

Use twice before discarding.

Appendix C

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Moodley

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M080701**
PROJECT	Molecular Characterization of Neisseria Meningitidis Serogroup B Isolates in South Africa 2002-2006
INVESTIGATORS	Miss C Moodley
DEPARTMENT	Pathology Department
DATE CONSIDERED	08.07.25
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 08.07.28 **CHAIRPERSON** .....
(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Dr M du Plessis

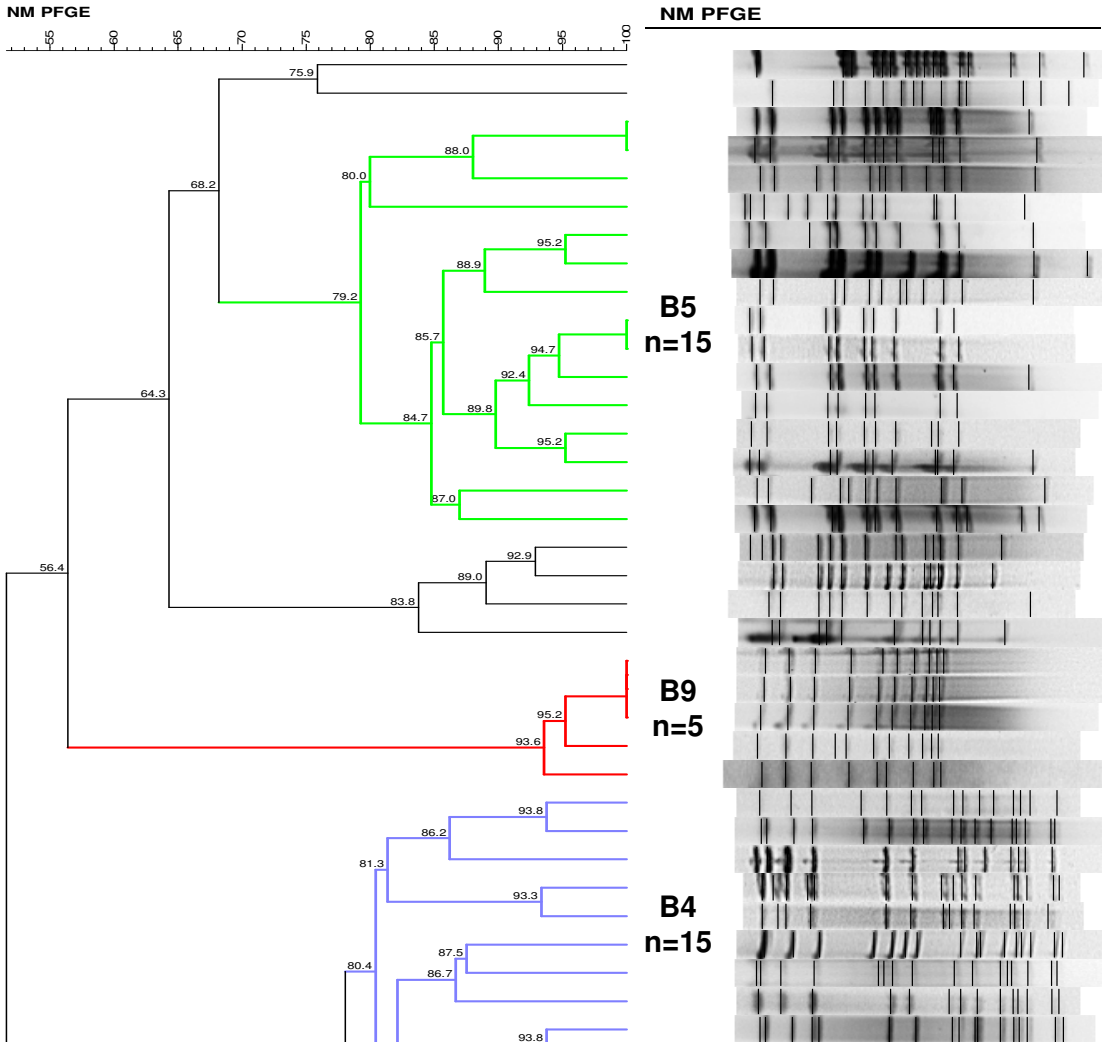
DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

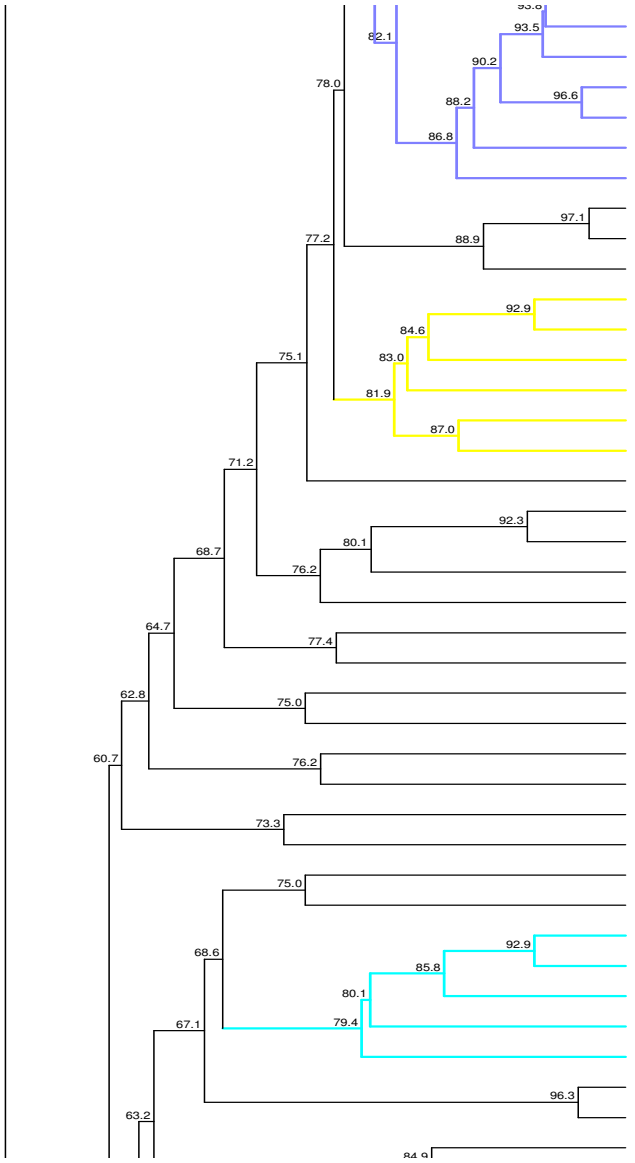
PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix D

PFGE dendrogram with MLST, PorA and FetA type representing the genetic relationship among serogroup B meningococci causing invasive disease in South Africa, 2002-2006 (n=302).



	Isolate no.	*	Year	ST	cc	PorA	FetA
	B 6067	GA	2006				
	B 6770	FS	2006				
●	B 36	FS	2005	6687	ST-865 complex	P1.7-1,1	F1-6
●	B 2826FR	WC	2005	865	ST-865 complex	P1.7-1,1	F1-6
●	B # 9203FR	GA	2003	6687	ST-865 complex	P1.7-1,1	F1-6
●	B 8018R	WC	2002				
●	B 8149	WC	2002				
●	B 5932	FS	2006				
●	B 17732	EC	2004				
●	B 11358	WC	2003				
●	B 14863	FS	2004				
●	B 8944	EC	2006				
●	B 10173	WC	2003				
●	B 10853	GA	2003				
●	B # 13072	WC	2003	7195	ST-865 complex	P1.7-1,1	F1-6
●	B 12039	WC	2003				
●	B 5856	GA	2006				
	B 14407	WC	2004				
	B 16058	GA	2004				
	B 13001	EC	2003				
	B 1937	WC	2005	1878	ST-103 complex	P1.18-1,3	F3-9
★	B 17323	GA	2004				
★	B 17670	NC	2004				
★	B 15197	GA	2004				
★	B 12658	GA	2003				
★	B 7486R	NW	2002				
◆	B 11779	GA	2003				
◆	B 14676	GA	2004				
◆	B 4234	WC	2005	6701	ST-41/44/lineage 3.	P1.19,15	F3-20
◆	B 15214	LP	2004				
◆	B 15585	GA	2004				
◆	B 17069	NC	2004				
◆	B 616FR	GA	2005	6989	ST-41/44/lineage 3.	P1.7,9	F3-20
◆	B 10725	NW	2003				
◆	B 13150	WC	2004				



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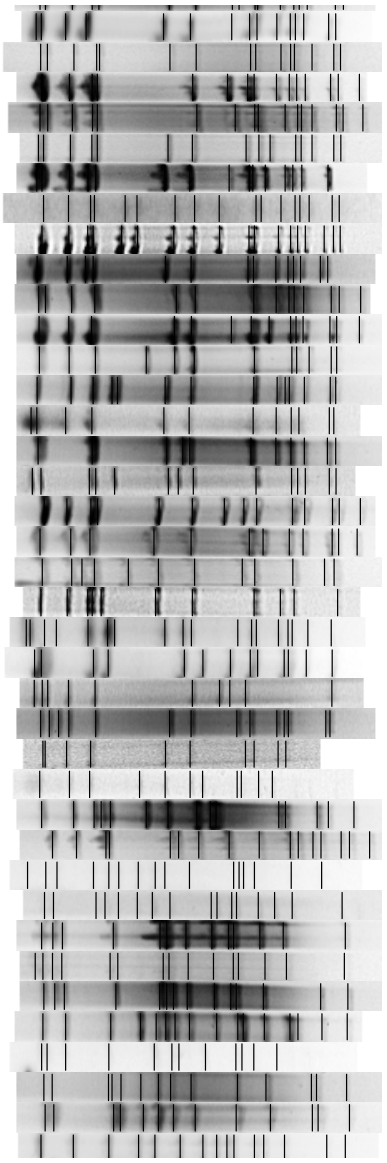
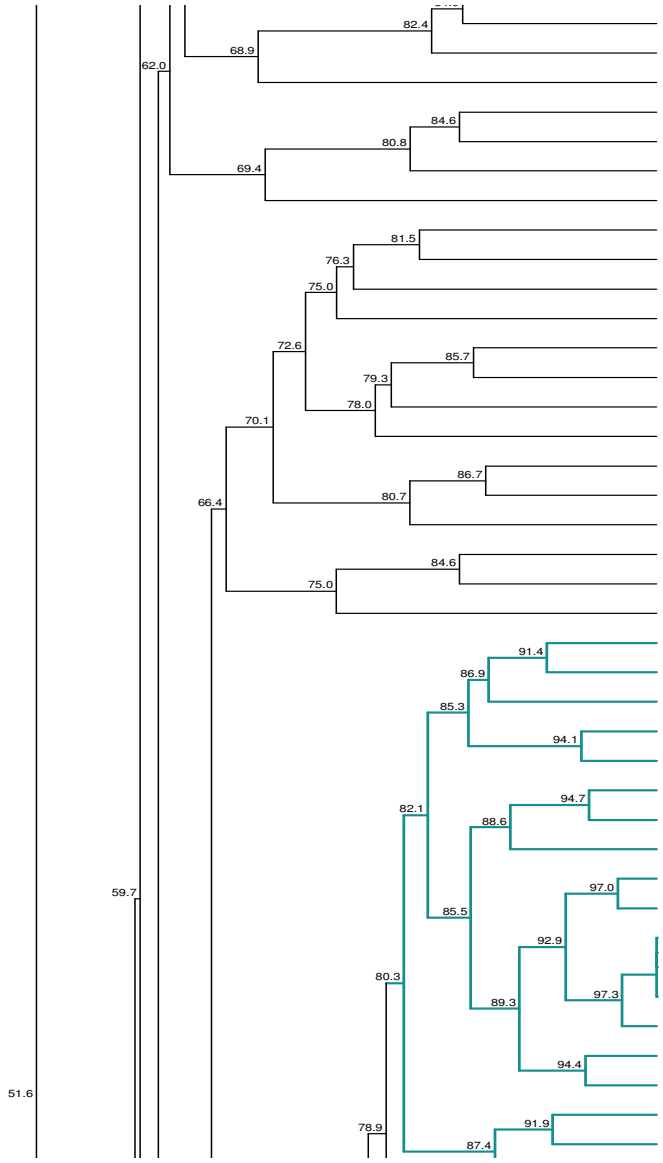
B10 n=5



◆	B	9158	WC	2003				
◆	B	# 6305FR	WC	2002	4243	no complex	P1.7,9	F3-20
◆	B	2271	GA	2005	6590	ST-41/44/lineage 3.	P1.7,9	F3-20
◆	B	9076FR	GA	2003	6590	ST-41/44/lineage 3.	P1.7,9	F3-20
◆	B	430FR	WC	2005	6689	ST-41/44/lineage 3.	P1.7,9	F5-1
◆	B	3482	GA	2005	6590	ST-41/44/lineage 3.	P1.7,9	F3-20
	B	7604R	WC	2002				
	B	4662	WC	2005	6993	no complex	P1.22,26	F3-20
	B	2827	MP	2005	6710	ST-41/44/lineage 3.	P1.7,9	F3-20
⊙	B	# 7277	WC	2006	6590	ST-41/44/lineage 3.	P1.7,9	F3-20
⊙	B	# 9800	FS	2006	6590	ST-41/44/lineage 3.	P1.7,9	F3-20
⊙	B	9825	WC	2006				
⊙	B	8927	WC	2006				
⊙	B	10629	GA	2003				
⊙	B	8013	WC	2006				
	B	13649	WC	2004				
	B	10552	FS	2006				
	B	5419	WC	2006				
	B	1767FR	WC	2005	6695	ST-41/44/lineage 3.	P1.7,9	F3-20
	B	# 15733	WC	2004	7945	ST-41/44/lineage 3.	P1.7,9	F3-20
	B	8019	WC	2002				
	B	9101	EC	2002				
	B	14507	GA	2004				
	B	2909	GA	2005	1834	ST-41/44/lineage 3.	P1.18,25/25.	F3-9
	B	6603R	GA	2002				
	B	10177	WC	2003				
	B	# 9127	WC	2006	ND	ND	P1.22,23-1	ND
	B	4035FR	EC	2005	6711	no complex	P1.7,9	F1-20
	B	7858R	GA	2002				
	B	94	WC	2005	393	ST-269 complex	P1.5-1,10-1	F1-7
■	B	9504	GA	2006				
■	B	# 8477FR	WC	2006	283	ST-269 complex	P1.19-1,30-2	F5-1
■	B	7469	WC	2006				
■	B	5433	GA	2006				
■	B	7823	GA	2002				
	B	7961	MP	2006				
	B	8371	GA	2006				
	B	10013	FS	2003				



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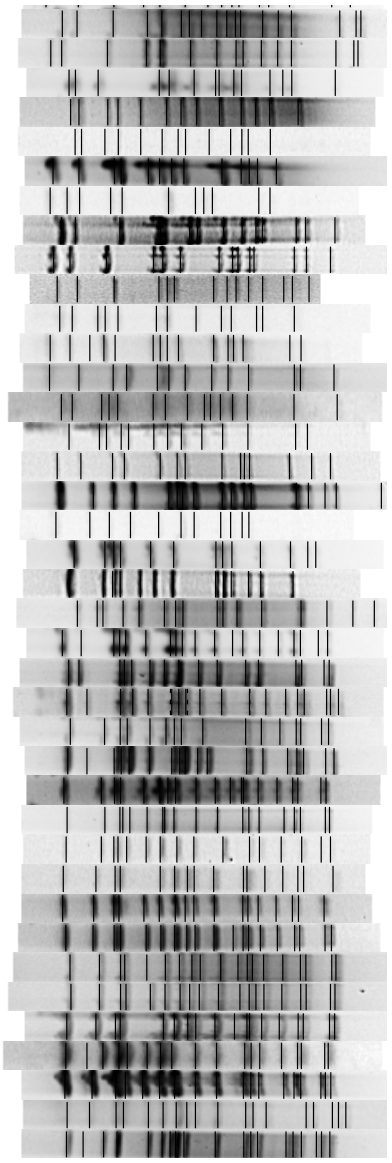
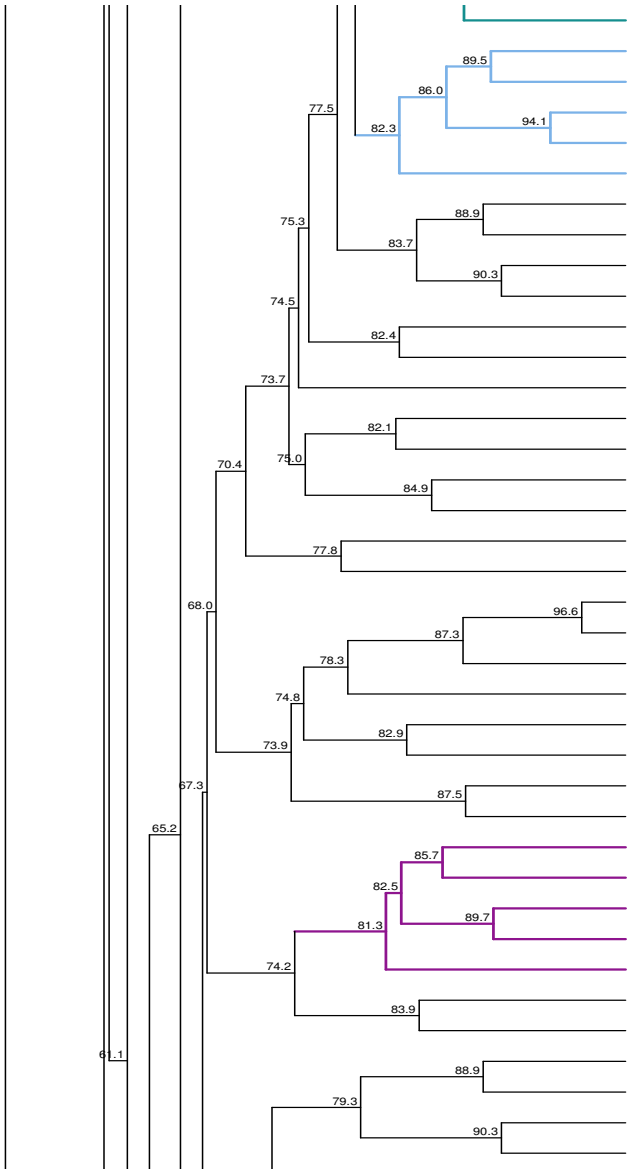


	B	10238	WC	2006				
	B	6184	WC	2006				
	B	9580	GA	2003				
	B	7862	WC	2002				
	B	13755-R	GA	2004				
	B	7819	WC	2006				
	B	11479	KZN	2003				
	B	15938	GA	2004				
	B	3952	WC	2005	6991	no complex	P1.19,15	F3-16
	B	6269R	FS	2002				
	B	12141	GA	2003				
	B	11532	GA	2003				
	B	# 1936	WC	2005	1157	ST-1157 complex	P1.21-7,16	F5-5
	B	# 6894R	NC	2002	7392	no complex	P1.5-1,2-2	F3-27
	B	11129	GA	2003				
	B	16362	WC	2004				
	B	9714	GA	2006				
	B	11232	GA	2003				
	B	9470	GA	2003				
	B	15696	EC	2004				
	B	15865	WC	2004				
⅄	B	10174	WC	2003				
⅄	B	14674	FS	2004				
⅄	B	1766FR	WC	2005	4240	no complex	P1.5,2	F5-8
⅄	B	15316	WC	2004				
⅄	B	7638	EC	2006				
⅄	B	# 2825	GA	2005	6688	no complex	P1.5,2	F5-8
⅄	B	2953	GA	2005	6699	no complex	P1.5,2	F5-8
⅄	B	10838	GA	2003				
⅄	B	12620	GA	2003				
⅄	B	# 2439	MP	2005	6688	no complex	P1.5,2	F5-8
⅄	B	3897	GA	2005	6688	no complex	P1.5,2	F5-8
⅄	B	# 10412.	GA	2006	6688	no complex	P1.5,2	F5-8
⅄	B	302FR	GA	2005	6688	no complex	P1.5,2	F5-8
⅄	B	1966FR	GA	2005	6688	no complex	P1.5,2	F5-8
⅄	B	7798	GA	2002				
⅄	B	6017	WC	2006				
⅄	B	652	GA	2005	6690	no complex	P1.5,2	F5-8
⅄	B	8450	WC	2006				



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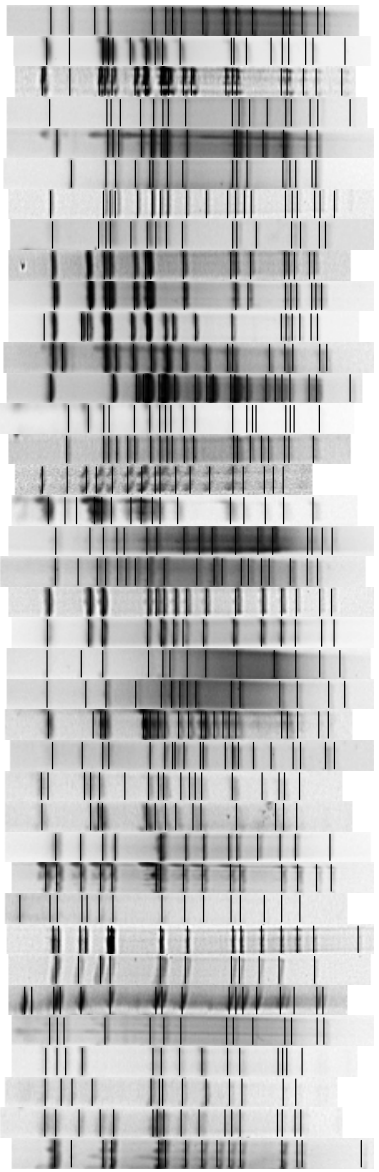

⮝	B	# 7441	GA	2006	2437	ST-35 complex	P1.22-1,14	ND
Ⅱ	B	10145	WC	2003				
Ⅱ	B	15720	LP	2004				
Ⅱ	B	13133	GA	2004				
Ⅱ	B	15825	GA	2004				
Ⅱ	B	15868	WC	2004				
	B	# 17653	GA	2004	7222	no complex	P1.5,2	F5-8
	B	1228	NC	2005	4240	no complex	P1.5,2	F5-8
	B	14010	WC	2004				
	B	6149	EC	2006				
	B	# 2676	GA	2005	6990	no complex	P1.5,2	F3-16
	B	10587	WC	2006				
	B	5890	EC	2006				
	B	8891	WC	2002				
	B	13829	NC	2004				
	B	6426R	WC	2002				
	B	12087	WC	2003				
	B	9809	GA	2006				
	B	# 7466FR	EC	2006	4240	no complex	P1.5,2	F5-8
	B	17731	WC	2004				
	B	10023	WC	2006				
	B	13651	WC	2004				
	B	14479	WC	2004				
	B	12989	KZN	2003				
	B	1027	GA	2005	6692	no complex	P1.5,2	F5-8
	B	10414	NW	2003				
	B	12823	WC	2003				
☼	B	6771	FS	2006				
☼	B	# 9542FR	WC	2003	6704	ST-334	P1.12-1,16-8	F5-2
☼	B	10493	WC	2003				
☼	B	5102	KZ	2005	6704	ST-334 complex	P1.5-2,10-1	F1-5
☼	B	16571	WC	2004				
	B	13398	WC	2004				
	B	371FR	WC	2005	35	ST-35 complex	P1.22,13-37	F4-1
	B	11847	WC	2003				
	B	10301	GA	2003				
	B	12998	EC	2003				
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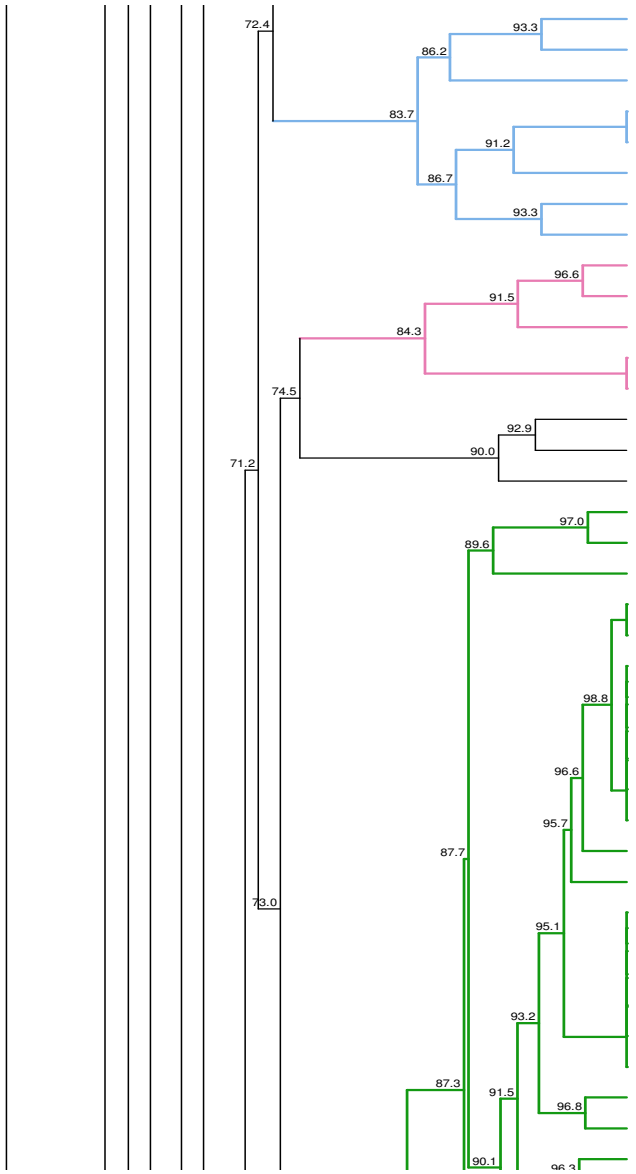

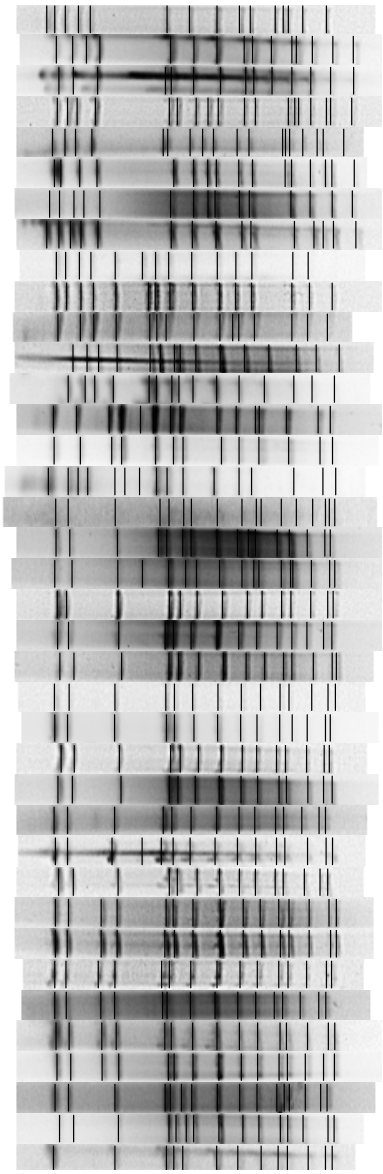

Ⅱ	B	16354	GA	2004				
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Ⅱ	B	16842	MP	2004				
Ⅱ	B	2351	GA	2005	180	ST-41/44/lineage 3.	P1.19,15	F1-5
Ⅱ	B	1841	WC	2005	6696	ST-41/44/lineage 3.	P1.18-1,34/.	F5-2
Ⅱ	B	15541	LP	2004				
Ⅱ	B	10450	WC	2006				
➔	B	12547	WC	2003				
➔	B	4322	FS	2005	6702	ST-41/44/lineage 3.	P1.18-1,3	F1-99
➔	B	13397	WC	2004				
➔	B	10570	GA	2003				
➔	B	8242	GA	2002				
	B	7829	GA	2006				
	B	8411	GA	2006				
	B	8797	GA	2002				
×	B	7093R	WC	2002				
×	B	7818	WC	2006				
×	B	# 5430	WC	2006	41	ST-41/44/lineage 3.	P1.7-2,4	F1-5
×	B	17305	GA	2004				
×	B	8150	NW	2006				
×	B	10429	NW	2003				
×	B	11159	MP	2003				
×	B	9394	GA	2003				
×	B	# 16186	GA	2004	154	ST-41/44/lineage 3.	P1.7-2,4	F1-5
×	B	14175	GA	2004				
×	B	2379	GA	2005	6697	ST-41/44/lineage 3.	P1.7-2,4	F1-5
×	B	10856	MP	2003				
×	B	16182	GA	2004				
×	B	10901	GA	2003				
×	B	11963	GA	2003				
×	B	15473	GA	2004				
×	B	2640	GA	2005	6698	ST-41/44/lineage 3.	P1.7-2,4	F1-5
×	B	# 10170	GA	2006	6698	ST-41/44/lineage 3.	P1.7-2,4	F1-5
×	B	9823	GA	2006				
×	B	3288	WC	2005	154	ST-41/44/lineage 3.	P1.7-2,4	F1-5
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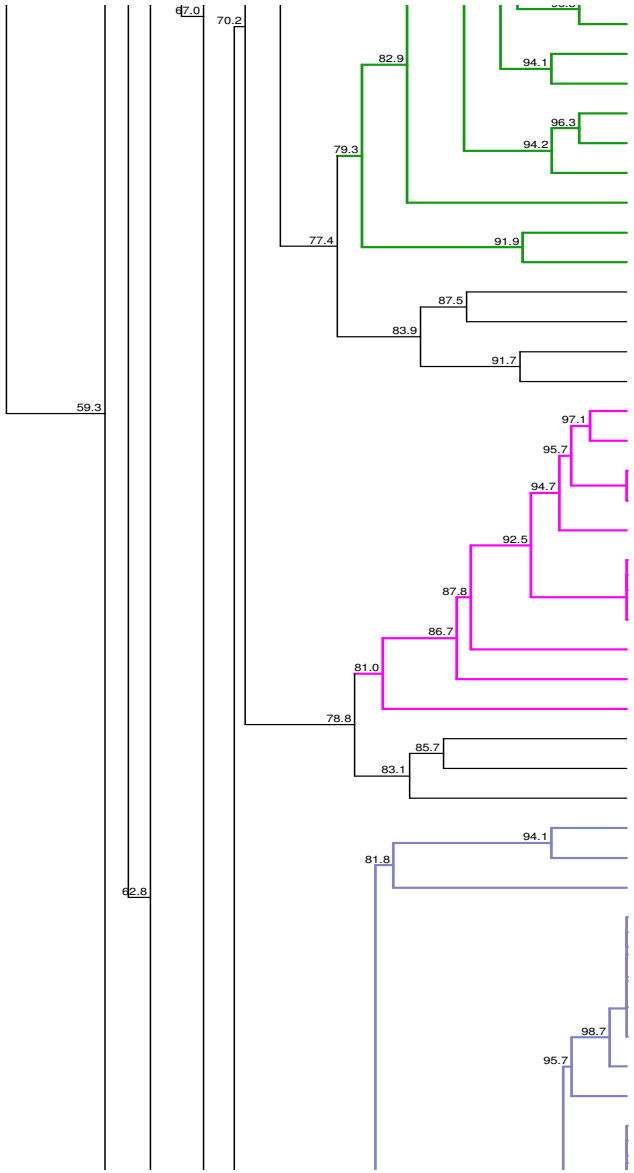

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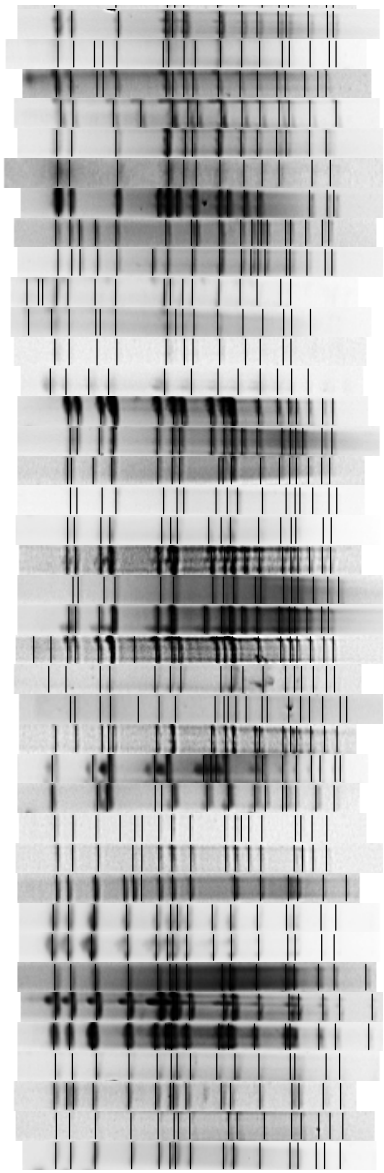

×	B	5628	WC	2006				
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×	B	6515	GA	2006				
×	B	9690	WC	2006				
×	B	7443R	GA	2002				
×	B	# 7637	GA	2006	154	ST-41/44/lineage 3.	P1.7-2,4	F1-5
×	B	3813	GA	2005	1960	ST-41/44/lineage 3.	P1.7-2,13-1	F1-5
×	B	9085	MP	2006				
	B	7846	GA	2002				
	B	8199	GA	2002				
	B	7314R	GA	2002				
	B	11626	GA	2003				
	B	2391	GA	2005	35	ST-35 complex	P1.22-1,14	ND
	B	5524	GA	2006				
	B	14698	WC	2004				
	B	372	WC	2005	35	ST-35 complex	P1.22-1,14	F3-7
	B	10184	GA	2003				
	B	16014	FS	2004				
	B	10413	WC	2006				
	B	9658	GA	2006				
	B	4660	WC	2005	6703	ST-35 complex	P1.22-1,14	ND
	B	12701	WC	2003				
	B	1310	GA	2005	6693	ST-35 complex	P1.22-1,14	ND
	B	15103	GA	2004				
	B	# 4332	GA	2005	6992	no complex	P1.17,16-4	F4-1
	B	2093	GA	2005	6709	no complex	P1.17,16-4	F5-5
◇	B	11040	GA	2003				
◇	B	3954	WC	2005	6700	ST-32/ET5 complex	P1.19,15	F3-7
◇	B	13807	GA	2004				
◇	B	10505	WC	2003				
◇	B	10504	WC	2003				
◇	B	3422	WC	2005	33	ST-32/ET5 complex	P1.19,15	F5-1
◇	B	7718	GA	2006				
◇	B	9327	EC	2006				
◇	B	5854	WC	2006				
◇	B	13221	WC	2003				
◇	B	10507	WC	2003				
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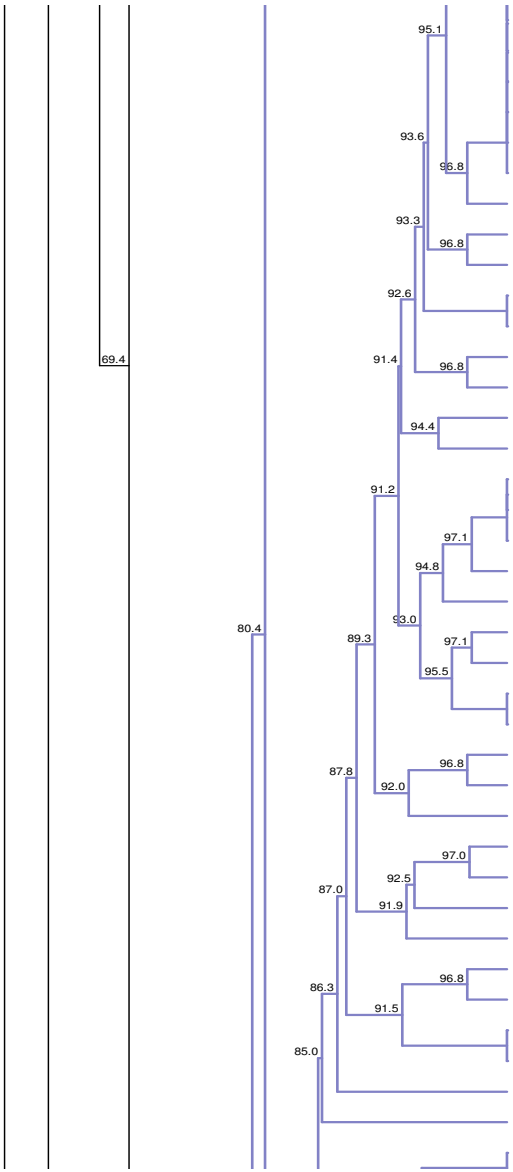

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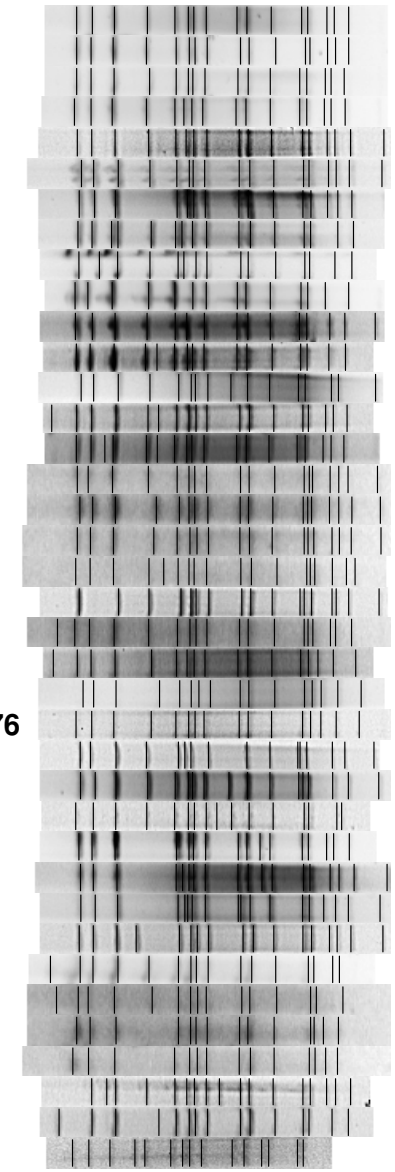

B	10176	WC	2003			
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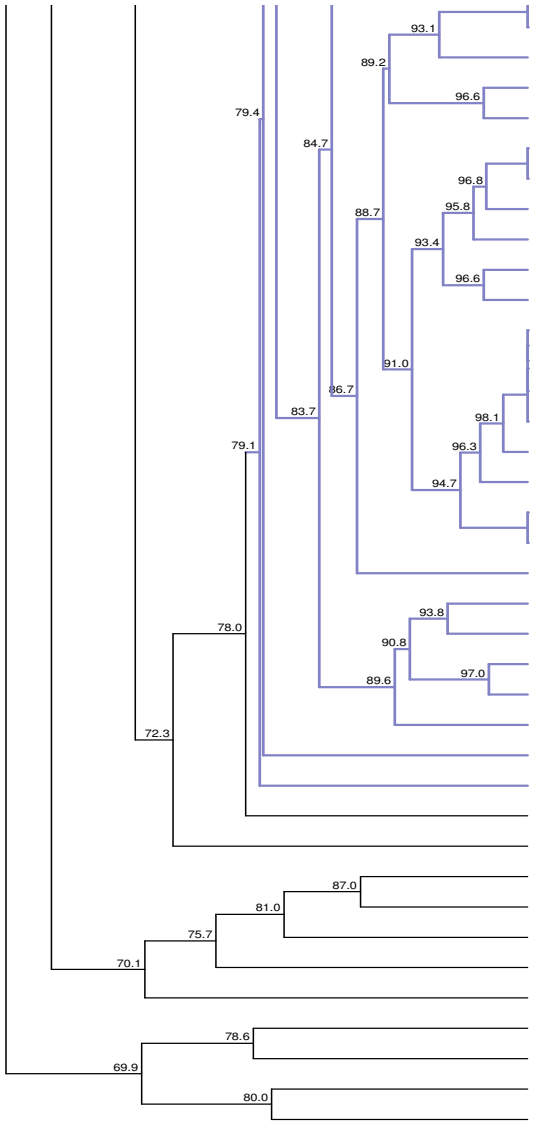
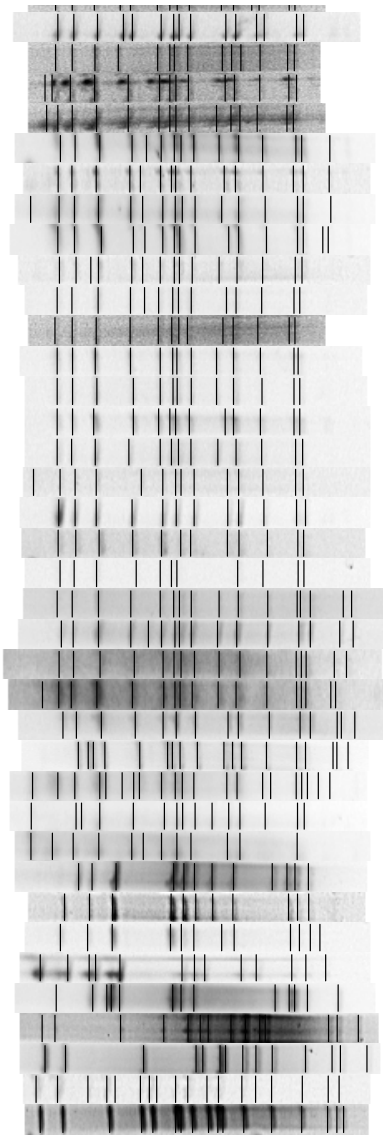
B	10175	WC	2003			
B	9954	GA	2003			
B	4577	NW	2005	33	ST-32/ET5 complex P1.12-1,13-1	F5-1
B	12780FR	WC	2003	6589	ST-32/ET5 complex P1.12-1,13-1	F5-1
B	8943	EC	2006			
B	10647	WC	2006			
B	2136	WC	2005	2400	ST-32/ET5 complex P1.19,15	F5-1
B	9219	WC	2003			
B	3395	WC	2005	33	ST-32/ET5 complex P1.19,15	F5-1
B	14517	WC	2004			
B	15946	WC	2004			
B	14516	WC	2004			
B	2718	WC	2005	6589	ST-32/ET5 complex P1.12-1,13	F5-1
B	7607R	WC	2002			
B	7655R	GA	2002			
B	7656R	GA	2002	33	ST-32/ET5 complex P1.12-1,13-1	F5-1
B	6626R	WC	2002			
B	16902	WC	2004			
B	7419R	WC	2002	33	ST-32/ET5 complex P1.19,15	F5-1
B	14418	WC	2004			
B	13394	GA	2004			
B	4661	WC	2005	639	ST-32/ET5 complex P1.5,2-1	F5-18
B	16402	WC	2004			
B	8452	WC	2006			
B	15098	WC	2004			
B	2516	GA	2005	33	ST-32/ET5 complex P1.12-1,13	F3-7
B	6494	WC	2006			
B	8215	GA	2006			
B	16999	GA	2004			
B	8398	WC	2002			
B	7426R	WC	2002			
B	# 7574R	GA	2002	33	ST-32/ET5 complex P1.19,15	F3-7
B	7701R	GA	2002			
B	15097	WC	2004			
B	14914	WC	2004			
B	6421R	WC	2002			





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B	6307R	WC	2002					
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B	6418R	WC	2002					
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B	10956	WC	2003					
B	8259	WC	2002					
B	8269	WC	2002					
B	11173	WC	2003					
B	12092	WC	2003					
B	6420R	WC	2002					
B	11323	WC	2003					
B	12098	WC	2003					
B	12094	WC	2003					
B	12090	WC	2003					
B	11352	WC	2003					
B	# 12691	EC	2003	33	ST-32/ET5 complex	P1.19,15	F5-1	
B	12548	WC	2003					
B	12093	WC	2003					
B	995	WC	2005	639	ST-32/ET5 complex	P1.12-1,13-1	F5-1	
B	1835	WC	2005	33	ST-32/ET5 complex	P1.12-1,13-1	F5-1	
B	6862R	WC	2002					
B	7416R	WC	2002					
B	1834	WC	2005	484	ST-32/ET5 complex	P1.19,15	F5-1	
B	6	GA	2005	6368	ST-32/ET5 complex	P1.19,15	F5-1	
B	8899	GA	2002					
B	8959	WC	2002					
B	8150	WC	2002					
B	7817	WC	2006					
B	15863	WC	2004					
B	12088	WC	2003					
B	12408	GA	2003					
B	8268	WC	2002					
B	# 10438	FS	2003	7223	no complex	P1.17,16-4	F5-34	
B	# 13172	GA	2004	3982	no complex	P1.19-15	F5-5	
B	11266	FS	2003					
B	# 14149	KZN	2004	7946	no complex	P1.5-3,10-24	F5-5	

NOTE: # denotes randomly selected isolates. ST, sequence type. cc, clonal complex. ND, not determined

Appendix E

***N. meningitidis* serogroup B isolates sequenced by MLST, PorA and FetA typing, 2002-2006 (n=85)**

Year	Lab no.	Alleles							ST	Clonal complex	PorA VR1,VR2	FetA
		abc	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*				
2002	7574	8	10	5	4	6	3	8	33	ST-32/ET-5	P1.19, 15	F3-7
	6305	9	6	350	9	8	21	311	4243	no complex	P1.7, 9	F3-20
	8257	8	10	5	4	6	3	8	33	ST-32/ET-5	P1.12-1,13-1	F5-1
	6894	47	3	512	31	8	87	13	7392	no complex	P1.5-1,2-2	F3-27
	7656	8	10	5	4	6	3	8	33	ST-32/ET-5	P1.12-1,13-1	F5-1
	7419	8	10	5	4	6	3	8	33	ST-32/ET-5	P1.19, 15	F5-1
2003	9542	14	6	6	76	3	8	18	6704	ST-334	P1.12-1, 16-8	F5-2
	9203	8	5	15	8	8	21	2	6687	ST-865	P1.7-1, 1	F1-6
	13072	8	5	15	53	8	21	2	7195	ST-865	P1.7-1, 1	F1-6
	12691	8	10	5	4	6	3	8	33	ST-32/ET-5	P1.19, 15	F5-1
	10438	12	2	15	455	58	11	3	7223	no complex	P1.17, 16-4	F5-34
	9076	9	6	351	9	9	6	311	6590	ST41/44/lineage 3	P1.7, 9	F3-20
	12780	8	10	5	264	6	3	8	6589	ST-32/ET-5	P1.12-1,13-1	F5-1
2004	14149	46	11	79	129	6	9	3	7946	no complex	P1.5-3, 10-24	F5-5
	16186	3	6	9	5	11	6	9	154	ST41/44/lineage 3	P1.7-2, 4	F1-5
	17653	15	5	9	8	28	19	62	7222	no complex	P1.5, 2	F5-8
	15733	9	6	351	9	26	6	311	7945	ST41/44/lineage 3	P1.7, 9	F3-20
	13172	46	11	79	129	323	9	3	3982	no complex	P1.19, 15	F5-5
2005	36	8	5	15	8	8	21	2	6687	ST-865 complex	P1.7-1,1	F1-6
	6	8	35	5	4	6	3	8	6368	ST-32/ET5	P1.19,15	F5-1
	94	4	35	15	9	8	11	9	393	ST-269	P1.5-1,10-1	F1-7
	302	15	5	18	24	8	19	62	6688	no complex	P1.5,2	F5-8

Year	Lab no.	Alleles							ST	Clonal complex	PorA VR1,VR2	FetA
		abc	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*				
2005	371	4	10	11	18	6	10	12	35	ST-35 complex	P1.22,13-37	F4-1
	372	4	10	11	18	6	10	12	35	ST-35	P1.22-1,14	F3-7
	430	9	6	351	9	9	3	9	6689	ST-41/44 /lineage 3	P1.7,9	F5-1
	652	15	5	18	18	8	19	62	6690	no complex	P1.5,2	F5-8
	616	9	6	351	24	9	6	311	6989	ST-41/44 /lineage 3	P1.7,9	F3-20
	995	8	10	5	9	6	3	8	639	ST-32/ET5	P1.12-1,13-1	F5-1
	1027	15	5	18	9	8	19	62	6692	no complex	P1.5,2	F5-8
	1228	15	5	9	24	8	19	62	4240	no complex	P1.5,2	F5-8
	1310	14	10	11	9	6	10	12	6693	ST-35	P1.22-1,14	ND
	1557	3	6	9	4	11	6	9	6694	ST-41/44 /lineage 3	P1.7-2,4	F1-5
	1835	8	10	5	4	6	3	8	33	ST-32/ET5	P1.12-1,13-1	F5-1
	1841	8	6	9	17	9	6	9	6696	ST-41/44 /lineage 3	P1.18-1,34/34-2	F5-2
	1766	15	5	9	24	8	19	62	4240	no complex	P1.5,2	F5-8
	1767	9	6	351	9	9	6	17	6695	ST-41/44 /lineage 3	P1.7,9	F3-20
	1834	8	10	5	4	9	3	8	484	ST-32 /ET5	P1.19,15	F5-1
	1936	8	25	7	17	21	26	49	1157	ST-1157	P1.21-7,16	F5-5
	1937	8	4	6	17	5	8	2	1878	ST-103	P1.18-1,3	F3-9
	1966	15	5	18	24	8	19	62	6688	no complex	P1.5,2	F5-8
	2093	47	3	482	76	21	59	49	6709	no complex	P1.17,16-4	F5-5
	2136	8	5	5	4	6	3	8	2400	ST-32 /ET5	P1.19,15	F5-1
	2271	9	6	351	9	9	6	311	6590	ST-41/44 /lineage 3	P1.7,9	F3-20
	2351	9	6	9	9	9	6	2	180	ST-41/44 /lineage 3	P1.19,15	F1-5
	2391	4	10	11	18	6	10	12	35	ST-35	P1.22-1,14	ND
	2439	15	5	18	24	8	19	62	6688	no complex	P1.5,2	F5-8
	2379	3	6	9	24	11	6	9	6697	ST-41/44 /lineage 3	P1.7-2,4	F1-5

Year	Lab no.	Alleles							ST	Clonal complex	PorA VR1,VR2	FetA
		abc	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*				
2005	2516	8	10	5	4	6	3	8	33	ST-32 /ET5	P1.12-1,13	F3-7
	2539	3	6	9	5	11	6	9	154	ST-41/44 /lineage 3	P1.7-2,4	F1-5
	2826	8	5	15	17	8	21	2	865	ST-865	P1.7-1,1	F1-6
	2640	3	3	9	5	11	6	9	6698	ST-41/44 /lineage 3	P1.7-2,4	F1-5
	2676	15	5	9	9	8	19	62	6990	no complex	P1.5,2	F3-16
	2718	8	10	5	264	6	3	8	6589	ST-32 /ET5	P1.12-1,13	F5-1
	2827	9	6	483	9	9	6	311	6710	ST-41/44 /lineage 3	P1.7,9	F3-20
	2825	15	5	18	24	8	19	62	6688	no complex	P1.5,2	F5-8
	2909	9	6	9	9	8	6	9	1834	ST-41/44 /lineage 3	P1.18,25/25-7	F3-9
	2953	12	5	18	24	8	19	62	6699	no complex	P1.5,2	F5-8
	3288	3	6	9	5	11	6	9	154	ST-41/44 /lineage 3	P1.7-2,4	F1-5
	3422	8	10	5	4	6	3	8	33	ST-32 /ET5	P1.19,15	F5-1
	3395	8	10	5	4	6	3	8	33	ST-32 /ET5	P1.19,15	F5-1
	3482	9	6	351	9	9	6	311	6590	ST-41/44 /lineage 3	P1.7,9	F3-20
	3954	8	10	5	9	9	3	8	6700	ST-32 /ET5	P1.19,15	F3-7
	3952	6	5	2	4	143	16	17	6991	no complex	P1.19,15	F3-16
	3813	3	116	9	5	9	22	9	1960	ST-41/44 /lineage 3	P1.7-2,13-1	F1-5
	4035	4	3	482	9	21	59	49	6711	no complex	P1.7,9	F1-20
	3950	14	5	6	9	3	8	18	334	ST-334	P1.17,16-4	F4-23
	3897	15	5	18	24	8	19	62	6688	no complex	P1.5,2	F5-8
	4234	9	6	351	9	9	6	3	6701	ST-41/44 /lineage 3	P1.19,15	F3-20
	4322	259	6	9	129	9	6	9	6702	ST-41/44 /lineage 3	P1.18-1,3	F1-99
	4332	47	3	482	4	21	59	49	6992	no complex	P1.17,16-4	F4-1
	4661	8	10	5	9	6	3	8	639	ST-32 /ET5	P1.5,2-1	F5-18
	4662	41	6	351	76	3	6	311	6993	no complex	P1.22,26	F3-20

Year	Lab no.	Alleles							ST	Clonal complex	PorA VR1,VR2	FetA
		abc	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*				
	4663	8	10	5	4	6	3	8	33	ST-32 /ET5	P1.12-1,13-1	F5-1
	4660	47	10	11	18	6	10	12	6703	ST-35	P1.22-1,14	ND
	5102	14	5	6	76	3	8	18	6704	ST-334	P1.5-2,10-1	F1-5
2006	7637	3	6	9	5	11	6	9	154	ST41/44/lineage 3	P1.7-2, 4	F1-5
	9127	ND	ND	ND	ND	ND	ND	ND	ND	ND	P1.22,23-1	ND
	7277	9	6	351	9	9	6	311	6590	ST41/44/lineage	P1.7, 9	F3-20
	10170	3	3	9	5	11	6	9	6698	ST41/44/lineage 3	P1.7-2, 4	F1-5
	7466	15	5	9	24	8	19	62	4240	no complex	P1.5,2	F5-8
	5430	3	6	9	5	9	6	9	41	ST41/44/lineage 3	P1.7-2, 4	F1-5
	8477	4	10	15	9	8	11	17	283	ST269	P1.19-1,30-2	F5-1
	9800	9	6	351	9	9	6	311	6590	ST41/44/lineage 3	P1.7, 9	F3-20
	7441	14	10	11	212	6	10	12	2437	ST35	P1.22-1,14	ND
	10412	15	5	18	24	8	19	62	6688	no complex	P1.5,2	F5-8
	7032	12	6	9	9	9	6	9	43	ST41/44/lineage 3	P1.19,15-1	F1-5

NOTE : ND, Not determined. ST, sequence type.

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