The molecular characterisation of *Neisseria meningitidis* in Scotland before, during and after the initial introduction of the meningococcal serogroup C conjugate (MenC) vaccines

Mathew. A. Diggle

Scottish Meningococcus and Pneumococcus Reference Laboratory,

Stobhill Hospital, Glasgow, UK.

Presented for the degree of Doctor of Philosophy (PhD) in the Faculty of Science University of Glasgow

Supervisors:

Dr. S. C. Clarke[1,2]

[1]Scottish Meningococcus and Pneumococcus Reference Laboratory, Glasgow, UK.
[2]Institute of Biomedical and Life Sciences, Division of Infection and Immunity, University of Glasgow, Glasgow, UK

Prof. T. Mitchell[1]

[1]Institute of Biomedical and Life Sciences, Division of Infection and Immunity, University of Glasgow, Glasgow, UK

SEPTEMBER 2004

Declaration

This thesis is the original work of the author Mathew Anthony Diggle

Quod erat

demonstrandum

Acknowledgements

There are many people I would like to thank for their support helping me navigate the sometimes difficult waters of the degree of doctor of philosophy, or as my partner would like to call it my "phud". I would like to thank my supervisors both Dr. Stuart Clarke and Prof. Tim Mitchell for their professional guidance and knowledge without which I would have most certainly been drowning in a sea of science. I would like to thank my assessor Dr. Robert Davies for his comments on the project throughout its duration. I would like to thank all the Staff at the Scottish Meningococcus and Pneumococcus Reference Laboratory (SMPRL) where all my work was conducted and where in times of desperation were able to keep me going and were willing to give me generous support for so long. I am also grateful for all the support given by MWG-Biotech both their management and technical staff. I would like to thank Rachel my brave partner through all of this. I am very grateful for her endless stamina and unselfish support and without her I would not have been able to lock myself in a darkened room and attempt to make some sense of it all. Finally I would like to thank all my family and friends who have come to realise that doing research on the surface can seem like fun but making sense of it all can make you go crazy.

Table of Contents

Figures and Tables

Research Publications and Articles

1. Clarke SC, Diggle MA and Edwards GFS. Surveillance of pneumococcal disease in Scotland. *SCIEH Weekly Report* 2001, 35: 2.

2. Diggle MA. News from Alumni: Work on laboratory confirmation of meningococcal disease. *Alumni News-letter of The London School of Hygiene & Tropical Medicine* 2001; 20: 4.

3. Clarke SC, Diggle MA, Reid JA, Thom L and Edwards GFS. Introduction of an automated service for the laboratory confirmation of meningococcal disease in Scotland. *Journal of Clinical Pathology* 2001; 54: 556-557.

4. Clarke SC, Diggle MA and Edwards GFS. Semi-automation of multi-locus sequence typing (MLST) for the characterisation of clinical isolates of *Neisseria meningitidis*. *Journal of Clinical Microbiology* 2001; 39(9): 3066-3071.

5. Diggle MA, Edwards GFS and Clarke SC. Automated fluorescence-based PCR for confirmation of meningococcal disease. *Journal of Clinical Microbiology* 2001; 39(12): 4518-4519.

6. Diggle MA, Edwards GFS and Clarke SC. Developments in the diagnosis of meningococcal disease and the characterisation of *Neisseria meningitidis*. *Reviews in Medical Microbiology* 2001; 12(4): 211-217.

7. Clarke SC, Diggle MA and Edwards GFS. Automated non-culture based sequence typing of meningococci from body fluids. *British Journal of Biomedical Science*: 2001; 58: 230-234.

8. The United Kingdom Meningococcal Carriage Group. Reduced carriage of serogroup C meningococci in teenagers one year after the introduction of meningococcal C conjugate polysaccharide vaccine in the United Kingdom. *The Lancet* 2002; 359: 1829-1830.

9. Diggle MA and Clarke SC. What a load of old sequence!!! *Journal of Clinical Microbiology* 2002; 40: 2707.

10. Clarke SC and Diggle MA. Automated PCR and sequence product purification using commercial filtration plates. *Molecular Biotechnology* 2002; July; 21(3): 221-4.

11. Sandvang D, Diggle M and Platt, DJ. Translocation of integron-associated resistance in a natural system: acquisition of resistance determinants by Inc P and Inc W plasmids from *Salmonella enterica Typhimurium* DT104. *Microbial Drug Resistance* 2002; Fall; 8(3): 151-60.

12. Diggle MA and Clarke SC. Semi-automation of the polymerase chain reaction for the laboratory confirmation of meningococcal disease. *British Journal of Biomedical Science* 2002; 59(3): 137-40.

13. Clarke SC, Diggle MA and Edwards GFS. Multi-locus sequence typing and porA gene sequencing differentiates strains of *Neisseria meningitidis* during case clusters. *British Journal of Biomedical Science*, 2002; 59(3): 160-2.

14. Diggle MA and Clarke SC. Rapid assignment of nucleotide sequence data to allele types for multi-locus sequence analysis (MLSA) of bacteria using a novel database and modified alignment program. *Journal of Molecular Microbiology and Biotechnology* 2002; 4: 515-517.

15. Diggle MA and Clarke SC. Detection and genotyping of meningococci using a nested PCR approach. *Journal of Medical Microbiology* 2003; Jan; 52(1): 51-7.

16. Diggle MA, Smith K, Govan K, and Clarke SC. Evaluation of a fluorescence-based PCR method for identification of serogroup A meningococci. *Journal of Clinical Microbiology* 2003: 41(4); 1766-1768.

17 Diggle MA, Bell C and Clarke SC. Nucleotide sequence-based typing of meningococci directly from clinical samples. *Journal of Medical Microbiology* 2003: 52; 505-508.

18 Clarke SC, Diggle MA, Molling P, Unemo M and Olcen P. Analysis of porA variable region 3 in meningococci: implications for vaccine policy? *Vaccine*, 2003: (21); 2468-2473.

19. Lewis C, Diggle MA and Clarke SC. Nucleotide sequence analysis of the *sialytransferase* genes of meningococcal serogroups B, C, Y and W135. *Journal of Molecular Microbiology and Biotechnology*, 2003: (5); 82-86.

20. Diggle MA and Clarke SC. A novel method for the preparation of single-stranded DNA for Pyrosequencing™. *Molecular Biotechnology*, 2003: (24); 221-224.

21. Jefferies J, Clarke SC, Diggle MA, Smith A, Dowson C and Mitchell TJ. Automated pneumococcal MLST using liquid-handling robotics and a capillary DNA sequencer. *Molecular Biotechnology*, 2003: (24); 303-307.

22. Smith K, Diggle MA and Clarke SC. Comparison of commercial DNA extraction kits for extraction of bacterial genomic DNA from whole-blood samples. *Journal of Clinical Microbiology*, 2003: (41); 2440-2443.

23. Scott K, Diggle MA and Clarke SC. TypA is a virulence regulator and is present in many pathogenic bacteria. *British Journal of Biomedical Science*, 2003; (3) 168-169.

24. Smith K, Diggle MA and Clarke SC. Automation of a fluorescence-based multiplex PCR for the simultaneous detection of *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae*. *Journal of Medical Microbiology*, 2004; (53) 115-117.

25. Lowe, CA, Diggle MA and Clarke SC. A single nucleotide polymorphism identification assay for the genotypic characterisation of *Neisseria meningitidis* using MALDI-TOF mass spectrometry. *British Journal of Biomedical Science,* 2004; (61) 8-10.

26. Diggle, MA and Clarke, SC. Pyrosequencing – sequence typing at the speed of light. *Molecular Biotechnology,* 2004; (28) 129-138.

27. Diggle MA and Clarke SC. Genotypic characterisation of *Neisseria meningitidis* using Pyrosequencing™. *Molecular Biotechnology*, 2004; (28) 139-145.

28. Clarke, SC, Lawrie D and Diggle MA. Sequence typing identifies-resistant Streptococcus pneumoniae during case clusters. *Journal of Medical Microbiology*, 2004; (53) 1097-1099.

29 Muhamed-Kheir Taha, Jean-Michel Alonso, Stuart C Clarke, Dominique A. A.Caugant, Mathew A Diggle, Andrew Fox, Matthias Frosch, Stephen J. Gray, Malcolm Guiver, Sigrid Heuberger, Mary Cafferkey, Jitka Kalmusova, Anne-Marie Klem, Paula Kriz, Konstantinos Kesanopoulos, John Marsh, Paula.Mölling, Karen Murphy, Per Olcén, Oumar Sanou,Georgina Tzanakakil, Ulrich Vogel Interlaboratory comparison of PCR-based identification and genogrouping of Neisseria meningitidis. *Journal of Clinical Microbiology*, 2005; (43) 144-149

30. Sullivan, CB, Diggle MA and Clarke SC. MLST within Clinical Microbiology and Public Health. *Molecular Biotechnology*, ***in press.***

31 Diggle MA and Clarke SC. Increased diversity in the meningococcal population after the introduction of the UK conjugate vaccine. *Journal of Clinical Microbiology*, ***in press.***

32. Diggle MA and Clarke SC. A truncated *xanthine phosphoribosyltransferase* (*xpt*) gene present in invasive *Streptococcus pneumoniae* isolates. *Journal of Medical Microbiology*, ***submitted.***

33. Diggle MA and Clarke SC. Culture and non-culture techniques for the laboratory confirmation of meningococcal disease and its impact on public health management. *Journal of Clinical Microbiological Reviews*, ***submitted***.

Abbreviations

abcZ	House-keeping gene encoding putative ABC transporter
adk	House-keeping gene encoding adenylate kinase
AFLP	Amplified fragment length polymorphism
AO	Acridine orange
aroE	House-keeping gene encoding shinimate dehydrogenase
BBB	Blood-brain barrier
bp	Base pairs
CCD	Charged coupled device
CDC	Centers for Disease Control and Prevention
CDSC	Communicable Disease Surveillance Centre
CSF	Cerebrospinal fluid
ctrA	Gene encoding capsule polysaccharide export outer membrane protein CtrA
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assays
ET	Electrophoretic-type
FAFLP	Fluorescent amplified fragment length polymorphism
fumC	House-keeping gene encoding fumarate hydratase class 2 protein
gdh	House-keeping gene encoding glucose-6-phosphate dehydrogenase
HPA	Health Protection Agency
IL-1β	Interleukin-1β
LAT	Latex agglutination test
LOS	Lipo-oligosaccharide
MALDI-TOF	Matrix-assisted desorption/ionization time of flight
MenC	Meningococcal Serogroup C Conjugate
MD	Meningococcal disease
MLEE	Multi-locus enzyme electrophoresis
MLSA	Multi locus sequence analysis
MLST	Multi-locus sequence typing
MRU	Meningococcal Reference Unit

OMPs	Outer membrane proteins
OMV	Outer membrane vaccine
ONS	Office for National Statistics
ISD	Scottish Information and Statistics Division
ORF	Open reading frame
pa	Per annum
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pdhC	House-keeping gene encoding pyruvate dehydrogenase subunit
PFGE	Pulsed-field gel electrophoresis
pgm	House-keeping gene encoding phosphoglucomutase
porA	Gene encoding PorA protein
PorA porin,	Class 1 outer membrane protein
porB	Gene encoding PorB protein
PorB porin,	Major outer membrane protein (class 2 or 3)
PPi	Pyrophosphate
RFLP	Restriction fragment length polymorphism
RIVM	Institute for Public Health and the Environment
SCIEH	Scottish Centre for Infection and Environmental Health
siaD	Gene encoding polysialic acid capsule biosynthesis protein SiaD
SMPRL	Scottish Meningococcus and Pneumococcus Reference Laboratory
SNP	Single-nucleotide polymorphism
SQA	Sequence analysis
SSCP	Single-stranded conformational polymorphism
ST	Sequence-type
TNF-∝	Tumour necrosis factor ∝
USELAT	Ultrasound-enhanced latex immunoagglutination
VR	Variable region

Summary

The aim of this project was to develop and introduce an MLST system for all meningococci causing invasive disease in Scotland starting in 1999, and subsequently throughout 2000, 2001 and 2002. MLST was introduced because strains within the electrophoretic type 37 (ET-37) complex, particularly those of ST11, are often indistinguishable by traditional methods.

Nucleotide sequencing was performed on seven housekeeping genes and one outer membrane protein gene, *porA*. Data was analysed using databases and software available through the MLST website (www.mlst.net) and the *porA* websites (neisseria.org/nm/typing/porA/ and http://www.show.scot.nhs.uk/smprl/).

The introduction of MLST in Scotland was used as a routine method for the characterization of *Neisseria meningitidis* isolates in 1999. Coincidentally this is just prior to the introduction of the MenC vaccines in November 1999.

All *N. meningitidis* isolates from invasive disease were analysed. This has given valuable data highlighting the effect the MenC has on Serogroup C disease and its decline over the length of the vaccination campaign. At the same time, the overall numbers of cases were monitored including the effect on serogroup B between 1999 and 2002. This has been linked with historical data such as the typical cyclical pattern seen with serogroups B and C over the past decade. The incidences of ET-37 strains in serogroup C disease were assessed along with ET-37 strains of other serogroups. The

number of different ST's isolated were monitored from the introduction of the MenC vaccine and this has been linked with their corresponding serogroups and subtypes.

A decrease in serogroup C disease was observed, however this has been accompanied by an increase in serogroup B disease. The occurrence of capsule switch has been assessed and applied to the whole data set for subsequent conclusion. This thesis clearly shows that MLST is extremely important for the surveillance of meningococcal disease over a period of years and, and that this study, has been effective not only in monitoring the impact of the MenC vaccines, but also providing a detailed genotypic representation of strains now commonly associated with disease.

In addition to enhanced sequence based characterisation, development of a fluorescent -based PCR protocol using *N. meningitidis* specific probes has been developed to markedly improved sensitivity and specificity of meningococcal DNA detection. Using an automated platform employing Taqman chemistry, a detection system employs fluorescence-based chemistry that eliminates post PCR processing and has provided accurate real-time quantitative PCR. This development has identified previously undetectable quantities of meningococcal DNA, which have been subsequently used in a modified MLST system to produce genotypic results, which would not normally be created.

Chapter 1

Introduction

1.1 Developments in Molecular Biology

When a patient is suspected of having a life-threatening infectious disease it is important to obtain as much microbiological information as possible. The pace with which developments have occurred in molecular diagnostic and characterisation tools makes it seem increasingly likely that molecular techniques will play an even larger role in the diagnosis of diseases such as meningitis and septicaemia. It is important to utilise molecular methods and realise their tremendous value not only in the investigation of basic scientific questions, but also in application to a wide variety of problems and limitations affecting all agents that can affect the human condition.

Such stimuli have helped this area progress and develop so that investigators can adequately address the genetic basis of biological function. This had slowly emerged over the latter half of the twentieth century and has continually gathered pace over the last decade (Tait 1999; Boxer 2000). The development of genetics, specifically molecular genetics, has incorporated newly-evolving techniques and resulted in the start of the genetic revolution, which has led to a greater understanding of basic mechanisms of disease-causing pathogens (Boxer 2000; Pitt 2000). The primary motivation behind this unprecedented growth of molecular genetics was initially to benefit the understanding of human genetics. This has ultimately benefited other areas of molecular science including bacterial genetics (Pitt 2000). Virtually all major scientific disciplines will find molecular techniques increasingly necessary in areas

such as research, diagnosis, prognosis, and clinical management. This introduction will provide an up-to-date review on the molecular methods used in the laboratory confirmation of meningococcal disease (MD). It attempts to highlight the constant evolution and continual need for diagnostic procedures and molecular typing methods. Techniques required for the laboratory confirmation and characterisation of meningococci and the future prospects for the role of new technologies in such environments are described. In addition, it provides a background for the description of the research project undertaken for this thesis.

1.2 Historical Perspectives of Meningococcal Disease

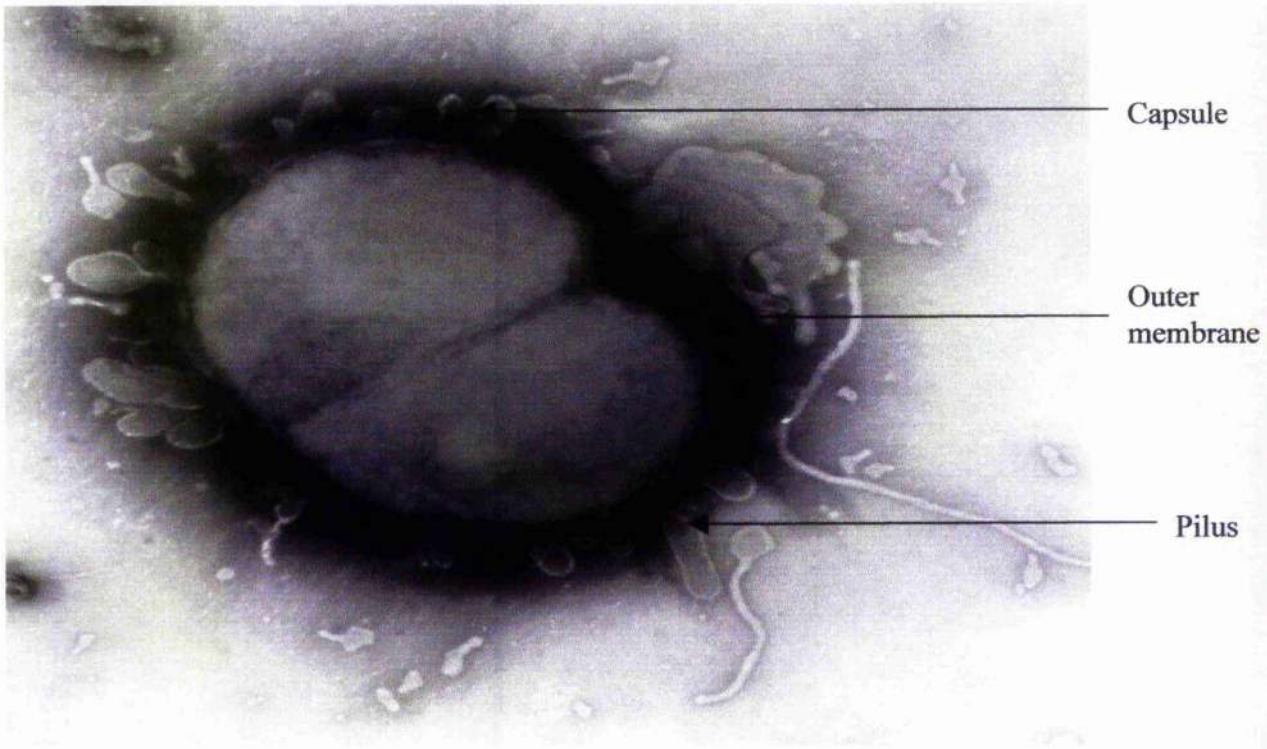
In 1919, long before the introduction of antibiotics, Herrick famously described MD with the words "no other infection so quickly slays". After more than 80 years of major developments in detection, characterisation and treatment, it still holds true in recent times. (Knapp 1988a; Ellis-Pegler 2003).

Neisseria meningitidis (the meningococcus) is a causative agent of septicaemia and meningitis. Meningitis is classically described as inflammation of the meninges (membranes covering the brain and spinal cord). The symptoms typically include a severe throbbing headache, photophobia, accompanied by a stiff neck, fever and can lead to delirium and coma. Meningococcal meningitis may be rapidly fatal (Ferguson et al. 2002). It is notable for its rapid progression and sometimes sporadic or epidemic presentation in both industrialised and developing countries. The unexpected appearance in previously healthy children and young adults has resulted in a great deal of public as well as medical attention. This has lead to a large amount of research

concerning diagnosis, characterisation, therapy and vaccine design (van der Ende et al. 1995; Molling et al. 2000; Richmond et al. 2001; Pollard and Moxon 2002; Riordan et al. 2002; Tunkel and Scheld 2002; Welch and Nadel 2002; Wildes and Tunkel 2002). The symptoms mentioned above have been associated with disease as far back as 400 BC, and subsequently recognised as possible meningitis (Fornara 1968). The cause of these conditions remained unknown until the late 19th century (Vieusseux 1806; Bovre 1984). The development of bacteriology and science in general over the late 19th century culminated in microbiologists making repeated attempts to isolate organisms from patients who had died from suspected meningitis (Knapp 1988b). In the 1870s the Italian physician, pathologist and neurologist Ettore Marchiafava in collaboration with Angelo Celi, identified and described micrococci within the cytoplasm of leukocytes, but they failed to culture any identifiable organisms (Marchiafava 1884). In Vienna, seven years later, Anton Weichselbaum made a breakthrough with the isolation of a coccoid bacterium from meningeal exudates. There was initial caution however, given the previous isolation of pneumococci from other cases of meningitis (Weichselbaum 1887; Kiefer 1896). Nevertheless this coccoid bacterium was later termed *Diplococcus intracellularis meningitides* and then *Neisseria meningitidis* (Figure 1.1).

Further investigations of small epidemics of meningitis were reported, with the same intracellular bacterium being isolated resembling the coccoid bacterium described by Weichselbaum. These investigations yielded a gram-positive chain of cocci similar to streptococci (Weichselbaum 1887). Because of low discrimination techniques between cultures at that time there was confusion over what was isolated and this continued for some time. The association between the meningococcus and meningitis

Figure 1.1 A pair of meningococci (*Neisseria meningitidis*) showing their pili and expressed capsules (With kind permission from Dr. Muhamed-Kheir Taha, National Reference Centre for Meningococci Unite Neisseria, Institute Pasteur).



was eventually defined by a series of publications from Weichselbaum's findings. It was only eight years after these initial findings that meningococci were isolated from lumbar punctures for the first time from patients clinically confirmed with meningitis (Knapp 1988b). Isolation of *N. meningitidis* and the introduction of techniques to obtain suitable clinical samples, such as the lumbar puncture, became routine clinical procedures and led the way for intraspinal immunotherapy in the early 20th Century. Importantly, at about the same time it was hypothesized that patients suffering from meningococcal meningitis could also carry the organism in the oropharynx (Knapp 1988b).

1.3 Meningococcal Disease

Since the isolation of meningococci over 115 years ago, studies have slowly highlighted the interrelationships between meningococci and the host (Peltola 1983). Meningococci employ complex mechanisms involving molecular mimicry and immune evasion and subsequent circumventation of immune responses to elicit disease (van Deuren and Meis 2001). The three principal stages involved in MD will be discussed, namely mucosal colonization, adhesion and penetration causing systemic disease and inflammation of the meninges (meningitis).

1.3.1 Mucosal Colonization

MD starts invariably with the colonization of the upper respiratory tract, mainly the mucosa of the nasopharynx and the tonsils. Meningococci are transmitted via droplets from a carrier of *N. meningitidis*. It is unknown as to the exact parameters pertaining to the successful transmission of meningococci from one person to another (Townsend

et al. 2002). Concentrated numbers of people can facilitate transmission, and this is one factor considered to contribute to successful acquisition of *N. meningitidis* (Imrey et al. 1995; Cardenosa et al. 2001; Round et al. 2001; Simmons et al. 2001; Taha et al. 2002). Invasive disease has been observed quickly after the pathogenic clone has been transmitted to a new host. This can be in the form of upper respiratory symptoms days prior to the development of generalized disease however most individuals are asymptomatic during colonization of the nasopharynx (Faoagali 1995; Townsend et al. 2002). There are many risk factors associated with the onset of disease. These include general social and environmental factors (Krause et al. 2001; Nelson et al. 2001), smoking (Fischer et al. 1997; Dobson 2000), passive smoking (Iles et al. 2001), socioeconomic deprivation (Kriz et al. 2000; Stuart et al. 2002), viral infections (Harrison et al. 1991) and even snoring and speech problems (Robinson et al. 2001) although contradictory reports debate correlations between respiratory infections and its association with the onset on MD (Yusuf et al. 1999; Robinson et al. 2001)

In the absence of globally effective vaccination programmes, meningococcal disease remains a major public-health problem worldwide. In Europe, North America, and Australasia most cases occur in young children and adolescents and are caused by group B or C meningococcal bacteria. The UK was the first country to introduce vaccination against meningococcal group C disease in November 1999 with a take-up assessed a year later at around 80-85% (Anon. 1999b; Miller et al. 2001; Ramsay et al. 2001; Balmer et al. 2002; Finn and Lakshman 2002).

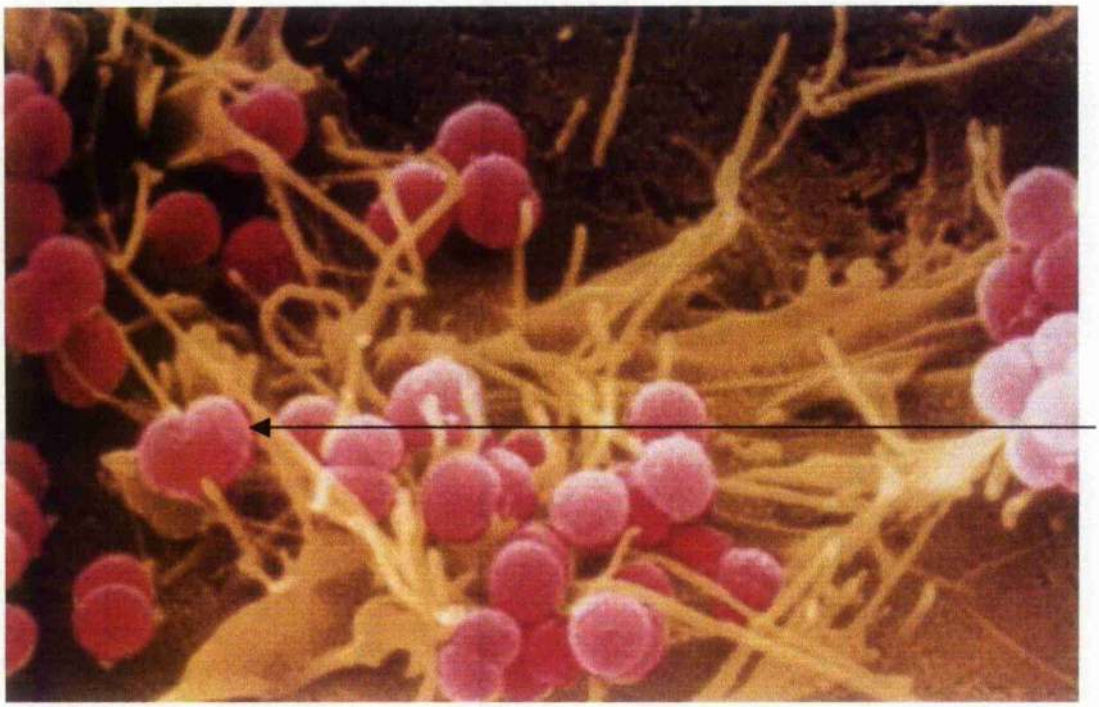
As a consequence of this vaccination campaign a multi-centre collaborative study involving centres from around the UK compared the carriage of meningococcal

bacteria from around 14,000 students aged 15-17 years at the start of vaccination in 1999, with around 16,500 students of the same age surveyed one year later (Maiden and Stuart 2002). The investigators report a two-thirds reduction in the carriage of group C meningococci after vaccination. This carriage study in combination with the implementation of the meningococcal C conjugate vaccines have highlighted its effect on meningococcal carriage and potentially protecting unvaccinated members of the population (Maiden and Stuart 2002).

1.3.2 Mucosal Adherence

Studies on human nasopharyngeal tissue cultures found that encapsulated, piliated meningococci adhere selectively to the microvilli on non-ciliated epithelial cells (Rayner et al. 1995) (Figure 1.2). To facilitate attachment to the epithelial cell layer, pathogenic meningococci possess class I and class II pili. Non-capsulated stains have a greater ability to adhere to these cell lines than capsulated strains (Rayner et al. 1995; Virji 1996). They also down-regulate the ciliary activity and one reason explaining this is that local inflammatory response mediators such as tumor necrosis factor ∝ (TNF-∝) and/or interleukin-1β (IL-1β) could be responsible for some or all the effects caused by colonization (Virji et al. 1996). It has also been noted that the class 3 outer membrane proteins termed opacity proteins (Opc) may facilitate adherence to endothelial cells *in vitro* (Virji et al. 1995; McNeil and Virji 1997). In general the major attributes of pathogenic neisseriae for interaction with nonphagocytic cells are:

Figure 1.2 Neisseria meningitidis interaction with human epithelial cells. (With kind permission of Prof. Mumtaz Virji, School of Medical Sciences, University of Bristol).



- Pilus mediated adhesion factors
- Pilin antigenic variation and modulation of adhesiveness through the formation of bundles of pili for cell-binding specificity including pilC proteins pilC1 which are likely candidates as adhesions
- Opacity proteins including opa/class 5 proteins and opc, which are involved in adhesion and invasion. These are known to be very efficient in endothelial cells
- Capsule
- Lipo-oligosaccharide (LOS).

1.3.3 Mucosal Penetration

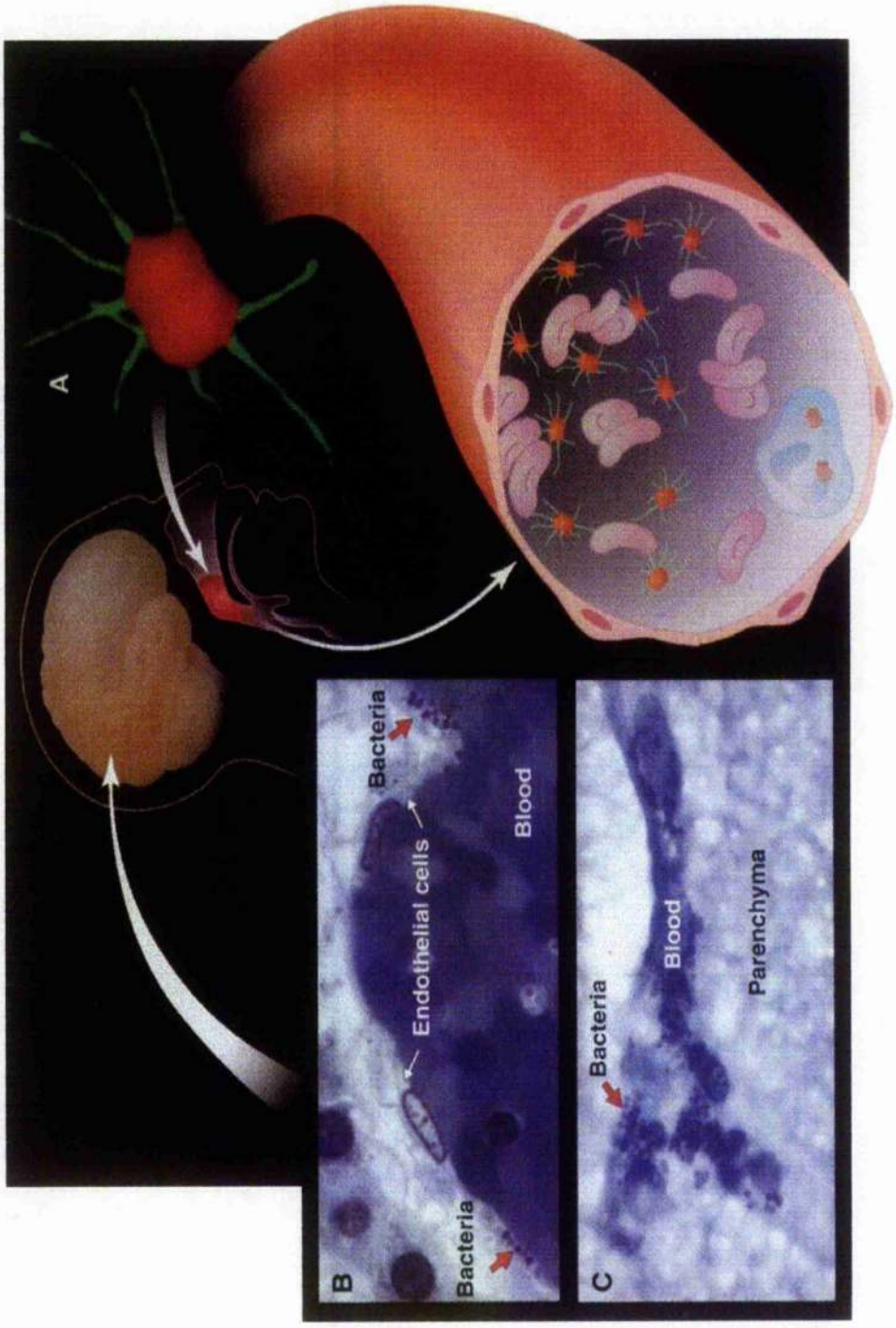
After the initial interaction of the meningococcus with the mucosal layer, there is a period of localised adaption and proliferation. Interestingly, there are a number of routes by which meningococci can penetrate the mucosal layer (Pujol et al. 1997; van Deuren et al. 2000; Tinsley and Nassif 2001). Non-capsulated strains can enter by endocytosis through non-ciliated epithelial cell lines without the use of membrane-bound phagocytic vacuoles (Nassif 1999). Alternatively, capsulated meningococci are transported via phagocytic vacuoles through the mucosal layer only 24 hours after initial colonization (Eugene et al. 2002). Although adhesion to endothelial cells is facilitated by pili, once the meningococci migrate from the upper mucosal layer these are down-regulated (Nassif 1999). Previous studies have highlighted a third route of entry through M cells present in the nasopharynx at the tonsillar sites. These could be used as a "mode of transport" as they continually endocytose material from the lumen and deliver it to a pocket formed by the basal plasma membrane consisting of lymphocytes and macrophages (Nassif 1999).

These stages can be summarized in several parts, the meningococcal subpopulation that expresses high levels of adhesive pilin and pilC1 would have an advantage and therefore have the capacity to colonise the host. Following the production of high levels of Opc/Opa the organism is likely to access the epithelial barrier and cause disease. These processes require the down regulation of the capsule so that the outer membrane proteins (OMPs) are available for interaction. The expression of either pilin or opacity proteins could explain why a small population can harbor colonizing meningococci without causing disease. The high regulation of pilin can encourage colonization of the mucosal surface, but expression of Opc or an appropriate class 5 variant is required for the meningococcus to cross the epithelial barrier (Figure 1.3) (Nassif 1999). Thus, production of adhesions without invasions could result in colonization, and the host would become one of approximately 10-20% of the population that carry *N. meningitidis* as a commensal organism on the nasopharynx (Verdu et al. 2001).

Once in the bloodstream the meningococcus can multiply and cause systemic infection, which clinically displays as a rash on the skin where the blood vessels have ruptured causing hemorrhaging. Patients who develop systemic meningococcal disease have a high risk of tissue damage, usually around the extremities, including the hands, fingers, feet and toes. Severe cases can result in amputations where lack of oxygen from damage to blood vessels has caused gangrene (Mele et al. 1997; Nolan and Sinclair 2001; Oostenbrink et al. 2002; Wheeler et al. 2003).

Interaction of meningococci with the blood-brain barrier (BBB) can be achieved through the initial interaction with the cerebrospinal fluid (CSF) via the bloodstream.

Figure 1.3 ***Neisseria meningitidis*** **interaction with the host** (Adaptation from the WHO website: http://www.who.org/).

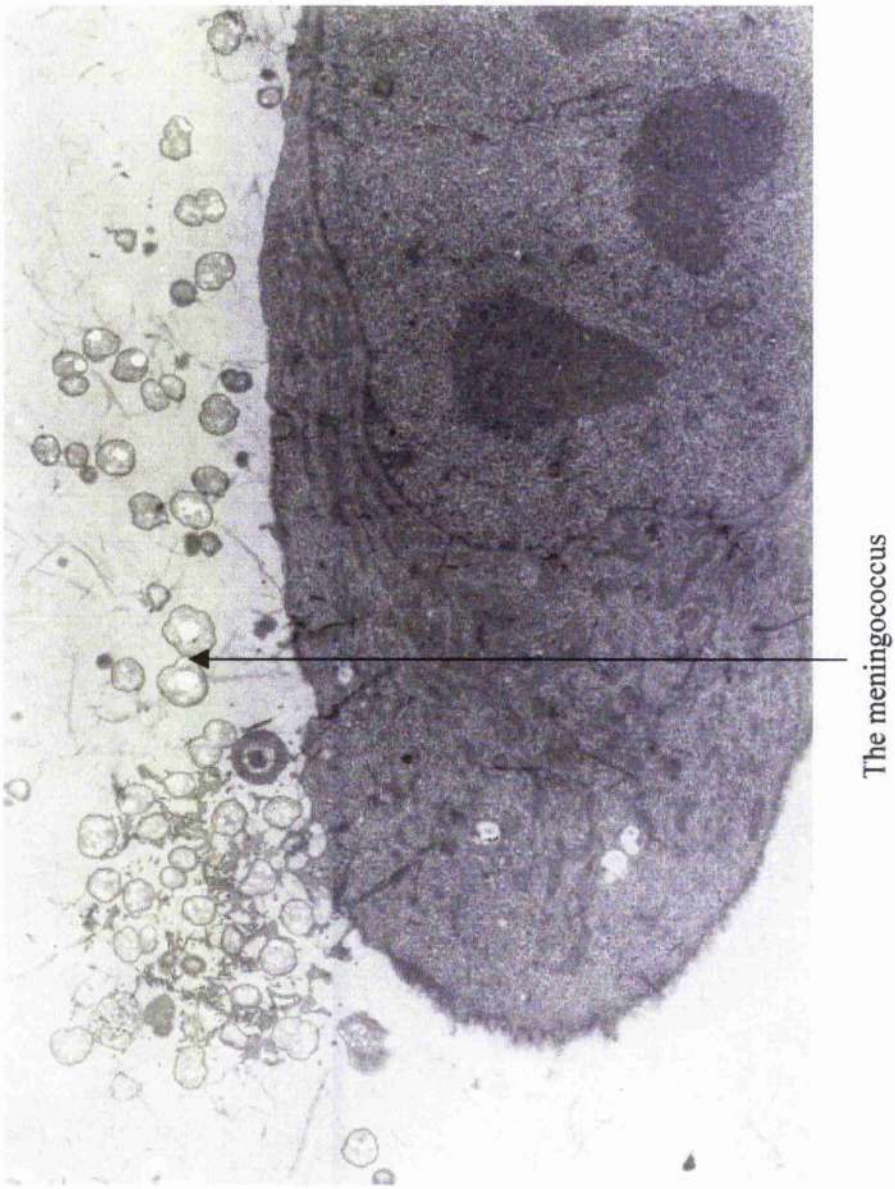


This has been demonstrated with recovery of *N. meningitidis* from CSF after inoculation into the blood stream of animal models. It is also well established that after clinical presentation of classic meningitis, *N. meningitidis* can often be cultured from blood samples (Nassif et al. 2002). From the blood, the meningococcus can gain access to the meninges by crossing the capillaries of the meninges (Figure 1.4), either by transcytosis or by passage after disruption of tight junctions between cells (Hardy 2000). After entry, the meningococcus can cause inflammation of the local site that clinically causes a whole range of symptoms including, as mentioned before, headaches, photophobia, stiff neck and nausea (van Deuren et al. 2000).

1.4 Historical Trends in Meningococcal Disease

During the early 20th Century there were large epidemics of MD described throughout the world, in New York between 1904-1905 (Counts and Petersdorf 1980), East Germany between 1905-1907 (Ballada 1990), Brazil between 1971-1972 (Barata Rde 1988) and India in 1985 (Sarkar et al. 1987). Over thirty major epidemics of meningococcal meningitis also occurred throughout the world between 1971 and 1997 (WHO 1998). Because of these large epidemics, European microbiologists developed agglutinating antibodies by immunising rabbits and horses with meningococci. Consequently, this led to the first human trials of anti-serum for the treatment of meningococcal meningitis. These initial human trials involved primary subcutaneous injections followed by further doses administered by intra-spinal injections. Preliminary reports showed a 67% survival rate compared with 70-80% mortality rate at the time (Rockowitz and Tunkel 1995). By 1908, several groups working in both the USA and Europe reported over 400 cases, which had been treated with

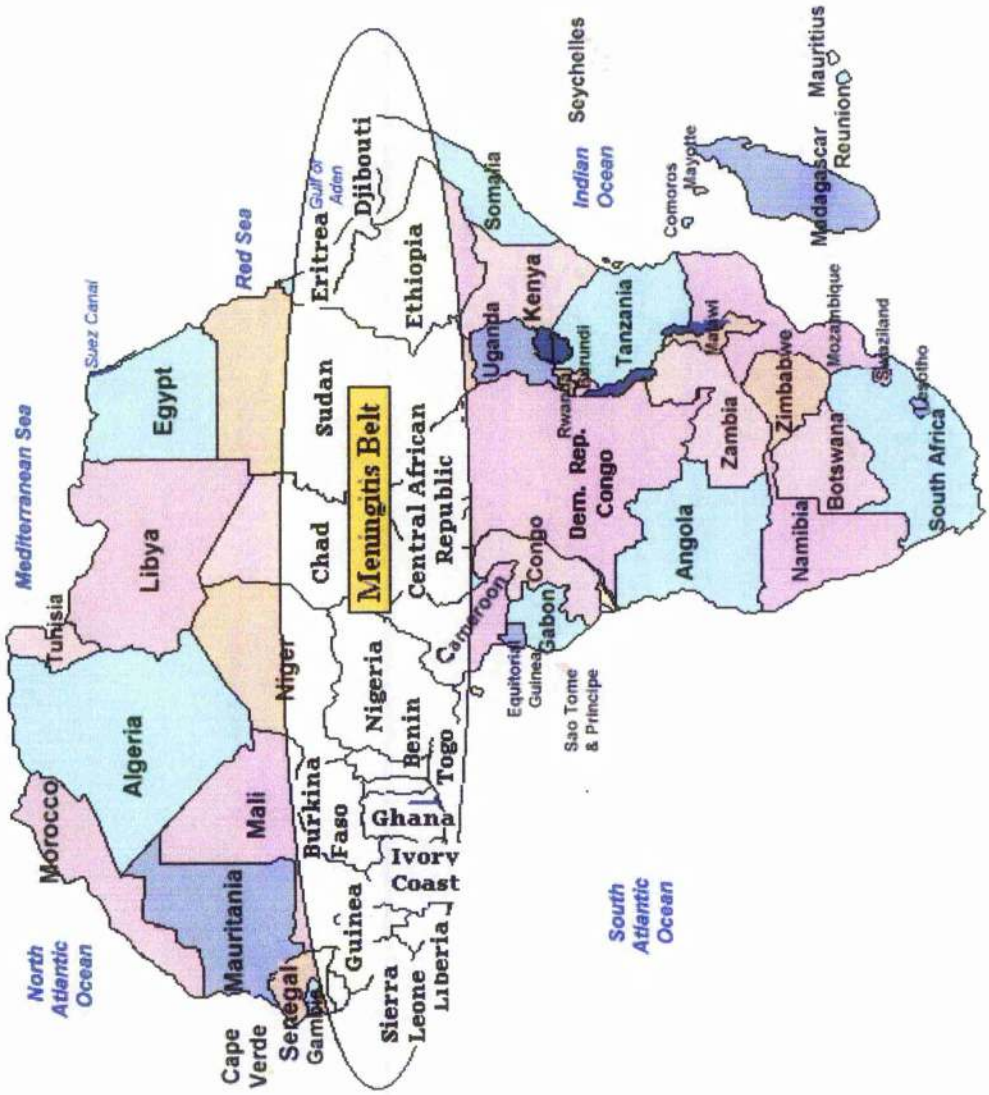
Figure 1.4 ***Neisseria meningitidis*** **crossing the capillaries of the meninges** (With kind permission from Dr. Xavier Nassif, Faculte' Necker Enfants Malades, Paris, France).



meningococcal anti-serum and the mortality rate was reduced to approximately 25%. Although there were many side effects to this treatment and its mode of application, such as arthritis and skin eruptions, this therapy remained in use until the 1930s (Schwartz et al. 1989).

Within the African Continent, epidemic meningitis has been known to occur for as long as within other areas of the world. It was reported from the West Coast of Africa by G. William in 1909 and has been present consistently since that time (Greenwood et al. 1984; Greenwood et al. 1985; Molesworth et al. 2002). From the early 1940s meningococcal disease has constituted a permanent public health problem in many countries throughout the world however, by the 1960s it was no longer considered a serious problem in most European and North American countries. Unfortunately, this was not a trend mirrored in some tropical countries, particularly those located in the African meningitis belt (Figure 1.5). During the 1970s epidemics were again commonplace throughout the world and meningococcal disease increased in a number of countries of the Americas, Asia and Europe. This pattern was characterised by recurrent epidemics and persistent sporadic disease. Significant increases in disease were observed in Mongolia and in the former USSR between 1973-1974 (Hjetland et al. 1990; Tsend et al. 1992), Argentina in 1974 (Anon. 1998a), Vietnam and Rwanda between 1977-1978 (Oberti et al. 1981; Vimont-Vicary and Rogerie 1983), Algeria and Chile in 1979 (Anon. 1998a), and in Brazil since 1971 (Anon. 1998a). From the early 1980s, epidemics of meningococcal disease have occurred in Chile in 1986, Faroe Islands between 1980-1981, but mainly throughout Africa, from Ethiopia in 1989 in the East, to the Côte d' Ivoire in 1983 and 1985 in the West, including Sudan in 1988. This culminated in more than 30,000 reported cases during 1988 in Sudan

Figure 1.5 The "meningitis belt" of Central Africa.



and over 40,000 reported cases in Ethiopia in 1989 (Anon. 1998a). In the 1990s the epidemic spread in West Africa, with more than 25,000 reported cases in Niger during 1995 and more than 16,000 cases the following year. In total an estimated 800,000 cases have been observed during a 20-year period between 1970 and 1992 and over a further 200,000 cases were observed during 1995 and 1997 (Anon. 1998a). This level has had significant effects on the spread of the disease outside its normal boundaries, and if this is a true reflection, it could be due to increased mobility of the population, or the introduction of a new meningococcal strain into susceptible populations.

1.5 Global Trends in Meningococcal Disease

Meningococcal disease was described as far back as 1805 with an outbreak in Geneva, Switzerland. The causative agent was not identified for a further 82 years. The documentation of meningococcal disease has highlighted major global outbreaks at the time of both World Wars One and Two. Epidemics have been periodically reported on the African continent since the turn of the 20th century (Norheim et al. 2000; Anon 2001a; Lewis et al. 2001; Pollard and Maiden 2003). In Asia, major epidemics have been documented during the past 30 years in China, Vietnam and Mongolia (Skalova 1984; Achtman et al. 2001). There have also been epidemics in Europe and in both North and South America during the past 30 years, but these have not reached the high incidence levels of epidemics documented in sub-Saharan Africa (Pollard and Maiden 2003). The characterisation of meningococci from different continents shows trends that are closely associated with these regions of the world. The major epidemics in the meningitis belt are commonly associated with serogroup A disease, whereas in recent years the most common cause of meningococcal disease

in the United States of America has been serogroup Y (Lingappa et al. 2001; Rosenstein et al. 2001b). In Europe, a fluctuation between two serogroups, namely serogroup B and serogroup C meningococci, has been well documented (Cartwright et al. 2001).

1.6 European Trends in Meningococcal Disease

In many European countries, serogroup B predominates, while in others such as the Czech Republic, United Kingdom and the Slovak Republic, serogroup C has been more common (Noah 2001). Serogroup C has been known to be responsible for case clusters and outbreaks, with a high morbidity and mortality rate, especially among certain age groups such as 16-24 years of age (Round et al. 2001). This, combined with its ever-increasing responsibility in causing MD prompted the development and implementation of a UK national vaccine campaign. This campaign involved the administration of a meningococcal serogroup C conjugate vaccine (MenC) in November 1999 initially to high-risk groups (under 17 year old) and then to all ages less than 24 years of age (Miller et al. 2001). Serogroup W135 has recently emerged as a potential source of outbreaks, and vaccination against this serogroup is now mandatory for all persons traveling from European countries to high-risk regions, such as Saudi Arabia for the annual Hajj pilgrimage (Hahne et al. 2002; Wilder-Smith et al. 2003).

Meningococcal disease incidence rates in Europe can be grouped into three main categories, category 1 containing incidence of 3.0 cases/ 100,000, category 2 containing incidence of 1.0-2.9 cases/100,000 and category 3 containing incidence of

<1.0 cases/100,000 (Cartwright et al. 2001) (Table 1). Due to the variation in individual specimen culturing and disease reporting practices, the amount of data that can be compared and interpreted is limited. However there are efforts in place to construct a pan-European infrastructure for the investigation and surveillance of meningococcal disease (EuMenNet), which will be built up for the analysis of the meningococcal population structure and the dynamics of the spread of the causative organism, *N. meningitidis*, this now includes the European Meningococcal MLST Centre (EMMC) part of the European Meningococcal Epidemiology Centre (EMEC). In addition, the European Union Invasive Bacterial Infections Surveillance (EU-IBIS) project has established surveillance networks within the European Union (EU) for invasive *H. influenzae* and *N. meningitidis* disease in 2000. The overall aims of this project are to improve epidemiological information and laboratory capacity to characterise isolates of these two invasive bacterial infections within the EU. These networks were established with the assistance of existing networks within Europe. Specialist microbiologists and epidemiologists from each of the 15 EU countries, and the Czech Republic, Iceland, Israel, Malta, Norway, and Australia are collaborating to achieve the surveillance network aims, including improvements to the laboratory capacity to characterise accurately the isolates of *H. influenzae* and *N. meningitidis* using standardised methods and to evaluate the impact of vaccination with conjugate vaccines on the epidemiology of *H. influenzae* and *N. meningitidis*. From recent literature the epidemiology of meningococcal disease during 1998/1999 was chosen as a suitable overview. The incidence of disease was highest in the Republic of Ireland (7.9 cases/100,000) with Malta and Scotland above 6.0 cases/100,000. In contrast, Poland and Romania had the lowest incidence (0.1 cases/100,000). The degree of variation could be due to underreporting, and countries that are classified as category

Table 1.1 Incidence of meningococcal disease in Europe, during 1998-1999 (adapted from Cartwright *et al*, 2001).

Incidence Category		
Category 1	Category 2	Category 3
Republic of Ireland	Switzerland	France
Malta	Latvia	Estonia
Scotland	Norway	Germany
England and Wales	Denmark	Israel
Northern Ireland	Russia (Moscow)	Slovenia
Iceland	Slovak Republic	Croatia
The Netherlands	Spain	Portugal
Greece	Finland	Italy
Belgium	Austria	Sweden
	Czech Republic	Poland

Table 1.1 Category 1 (≥3.0 cases/ 100,000), Category 2 (1.0-2.9 cases/100,000) and Category 3 (<1.0 cases/100,000).

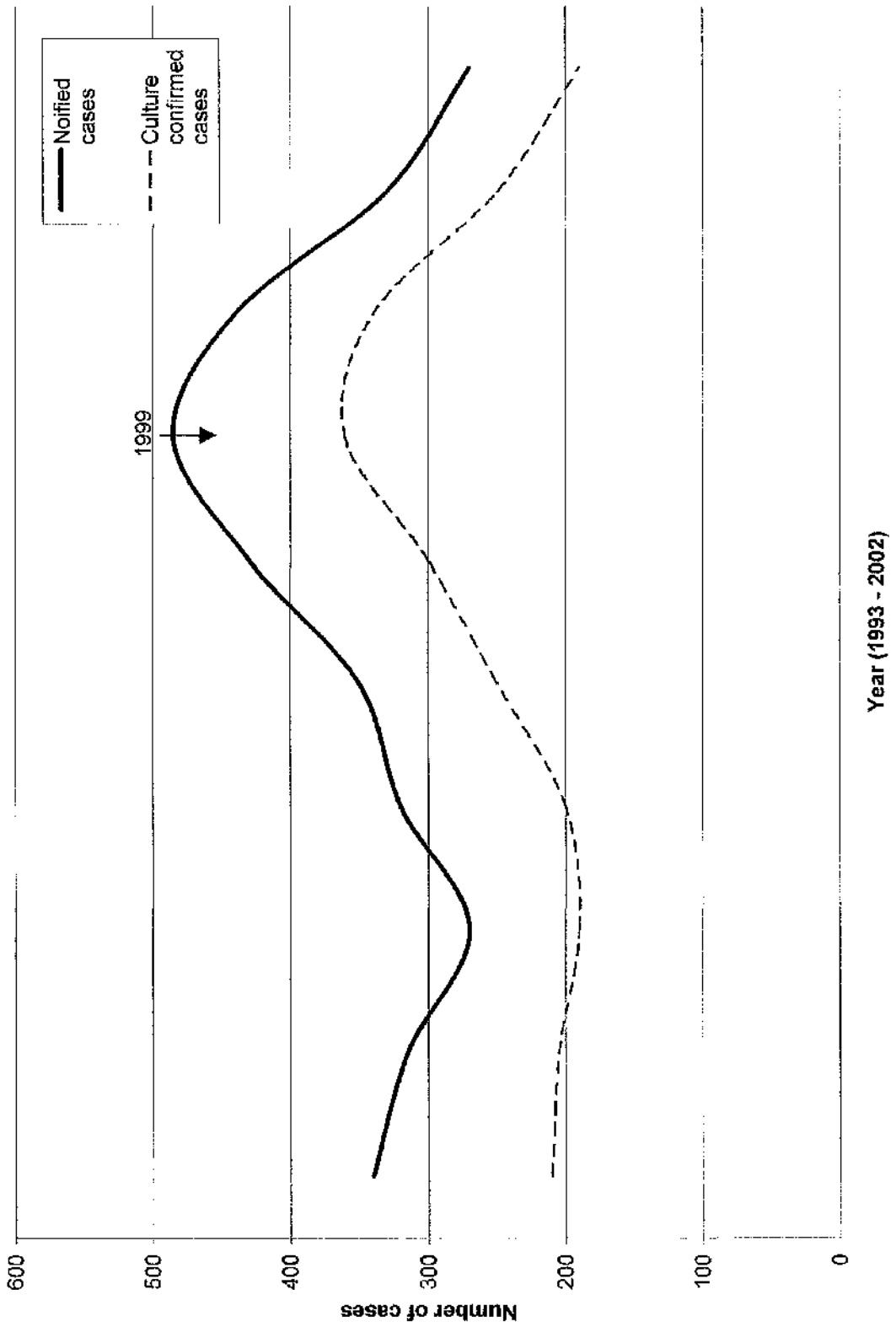
3, could have a high underreporting rate compared with countries within category 2 and 1 (Kremastinou et al. 1999a; Achtman et al. 2001; Cartwright et al. 2001; Alcala et al. 2002).

1.7 United Kingdom Trends in Meningococcal Disease

Meningococcal disease trends within Scotland are similar to those experienced by England and Wales (Fallon et al. 1984; Fallon 1988; Smart and O'Brien 1997; Clarke 2002b; Kyaw et al. 2002c). However, Scotland has a different laboratory infrastructure compared with England and Wales and surveillance has been conducted in Scotland on an *ad hoc* basis from the early 1970s. Since 1993 funding for a Scottish Meningococcus Reference Laboratory has been made available to provide a national service relating to the laboratory confirmation of meningococcal disease and characterization of meningococci. Between 1993 and 2002, there have been a total of 2552 notified cases of meningococcal disease in Scotland (Figure 1.6) compared to over 1446 clinically notified cases in just five regional health authorities in England in 1998 (Clarke 2002b; Clarke et al. 2002; Davidson 2002).

From 1999 to 2002 there has been a decline in the number of meningococcal septicaemia notified cases in England and Wales. In 1999 there were 1822 cases compared to 842 in 2002 a decrease of over 50% over four years. Within Scotland, culture based methods have provided a laboratory confirmation of 1051 cases. Since 1993 there has been a steady increase in notified cases; this peaked in 1999 with 349 cases, and subsequently deceased to fewer than 100 by 2002. Within England and Wales the number of notified cases can be observed from as far back as 1912 when

Figure 1.6. Meningococcal infections in Scotland from 1993 - 2002 (adapted from Smart *et al*, 1995,1996,1998, Clarke *et al* 1999, 2001, 2002).

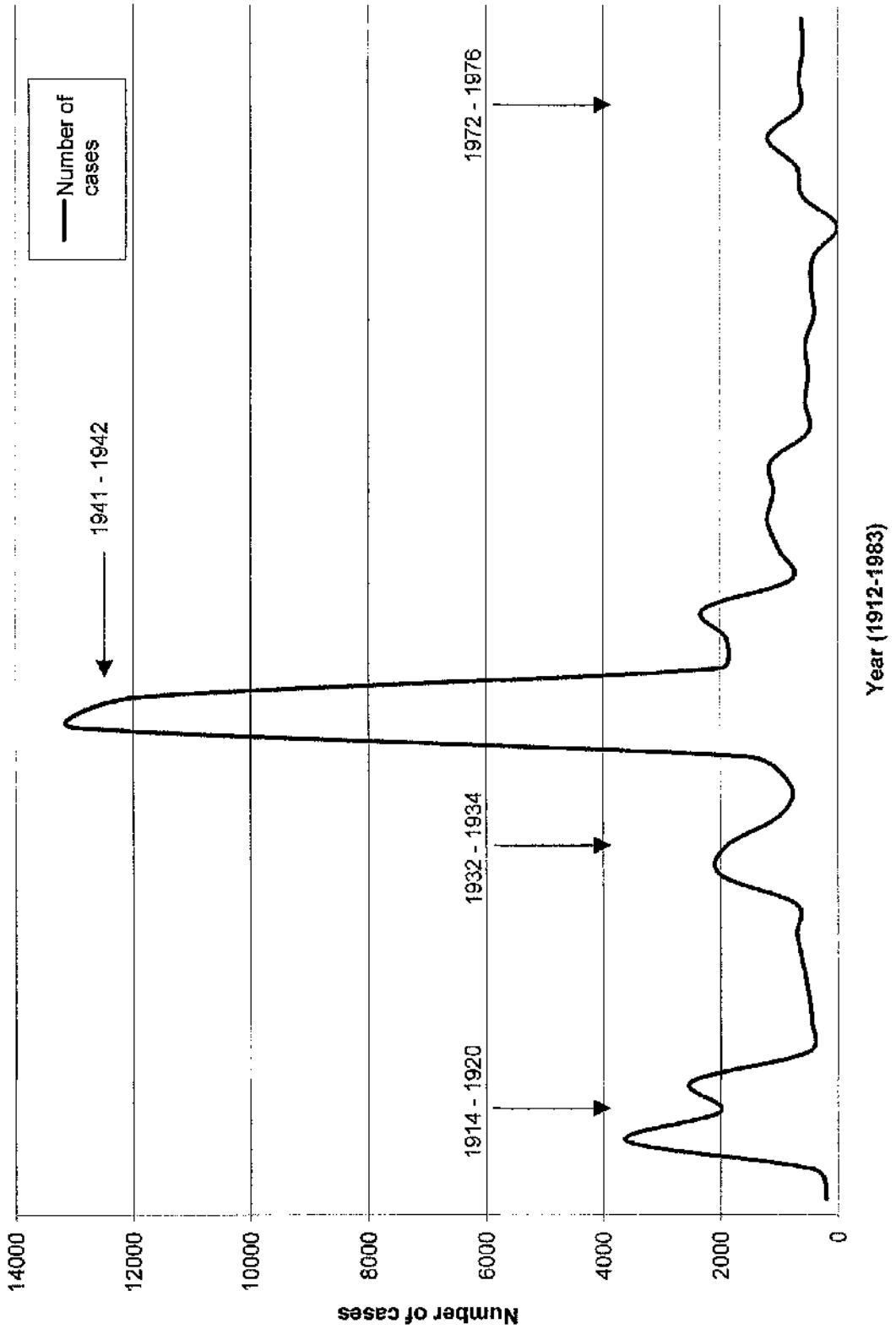


cerebrospinal fever was made statutorily notifiable. Coincidently, this monitoring system was in place to observe the great outbreaks during World War One when there were 3496 notified cases of MD in England and Wales alone. This level of notified cases remained high for the duration of both World Wars One and Two. Throughout World Wars One and Two many countries experienced large outbreaks of MD including Australia, Canada, Germany, New Zealand and the United Kingdom. The outbreaks were confined mainly in military troops whose over-crowded living conditions were ideal for the spread of MD (Gordon 1915; Abbott et al. 1985). After World War Two the number of notified cases decreased from over 12,000 cases in 1940 to over 300 cases in 1966, during this time there was a slight increase between 1950 and 1952. This level remained constant until 1967 when the number of cases increased dramatically with over 1200 in 1974. With enhanced surveillance systems in place present day levels are over 11,000 clinically diagnosed cases within England, Wales and Northern Ireland. This correlates to 8.0-9.2 per 100,000 people (Shigematsu et al. 2002) (Figure 1.7).

Over the past 30 years, the UK has observed a cyclical pattern of dominance in the meningococcal disease population between serogroup B and serogroup C meningococci. Throughout the 1990s there was a significant increase in serogroup C disease. This was more commonly observed in older teenagers with a much higher case fatality rate than with other age groups. This continuing increase in serogroup C disease prompted the development and introduction of a serogroup C conjugate vaccine (MenC). This was successfully introduced to the UK population in November 1999. Subsequent effects of the MenC have shown a decrease in serogroup C disease

Figure 1.7 Number of notified cases in England and Wales from 1912 – 1983

(adaption from Abbott *et al* 1985).



with the meningococcal disease population in the UK in 2003 predominantly serogroup B meningococci.

1.8 Periodicity and Seasonality of Meningococcal Disease

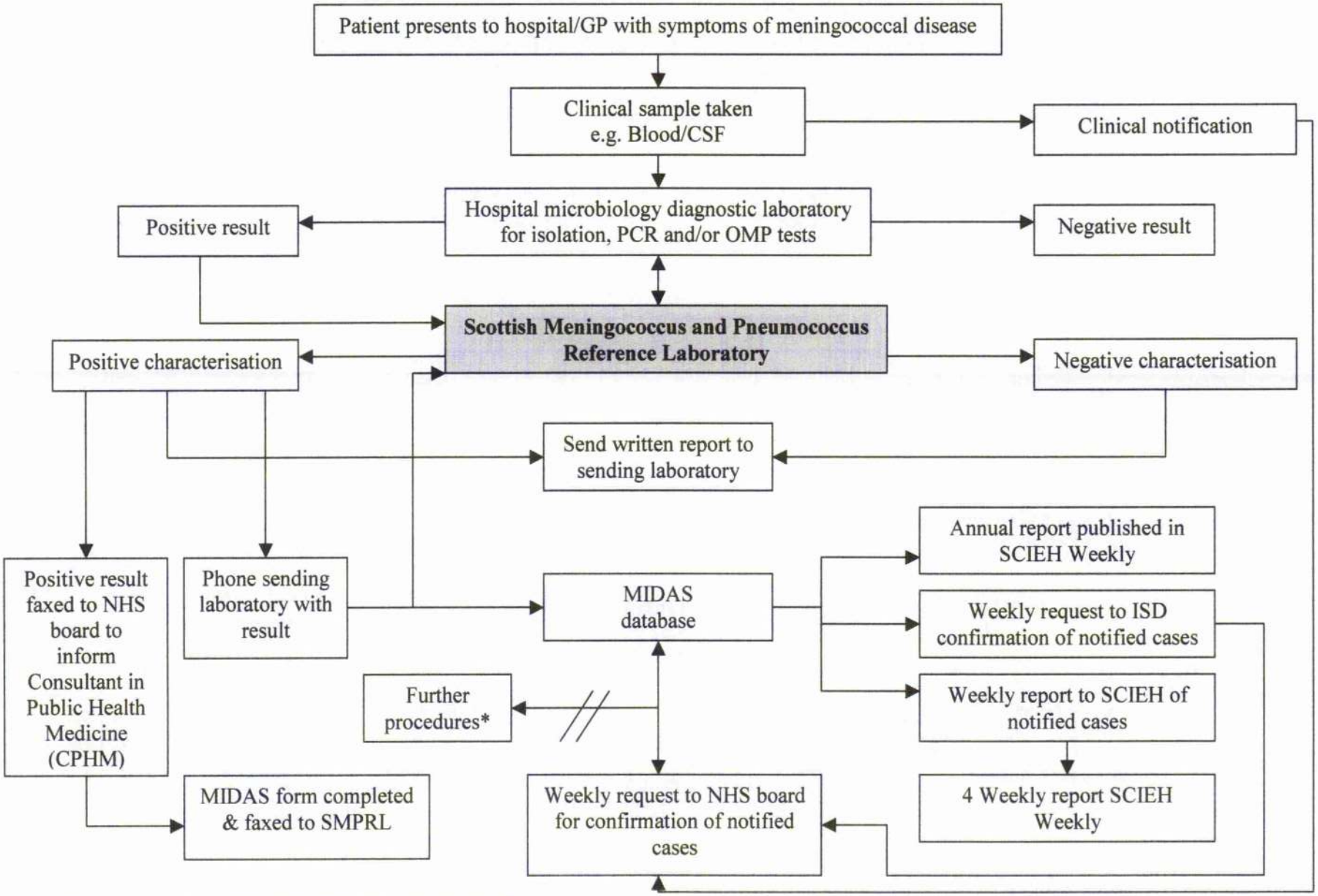
The temporal distribution of meningococci has been shown in the increased number of cases observed over the winter period in developed countries such as the UK (Wylie et al. 1997; Clarke et al. 2002). Climatic factors seem to play an important role in the occurrence of meningococcal disease. In tropical and temperate regions such as South America and central regions of Africa the transition from rainy to dry seasons are thought to be influential (1996; Weiss et al. 2001). This has been observed with an increase in incidence in the early dry season, when the climate is hot, dry and dusty. The incidence of MD peaks at the end of the dry season and virtually disappears at the start of the rainy season (Lapcyssonnic 1963; Peltola 1983; Achtman 1995; Riedo et al. 1995). In Africa this central region relating to high incidences of disease is called the "meningitis belt" (Figure 1.5) (Molesworth et al. 2002). Within this area, epidemics can last for as long as two to three dry seasons each lasting several months, separated by the intermittent rainy seasons. However, in small towns and villages, these epidemics can recede within a few weeks of initial onset (Hart and Cuevas 1997; Greenwood 1999; Norheim et al. 2000; 2001b; 2001a).

Within England, Scotland and Wales, doctors and medically trained staff have been required by statute since 1969 to notify clinically diagnosed cases of meningococcal meningitis and since 1989, cases of meningococcal septicaemia, to the proper officer of the local authority. In England and Wales this is the Consultant in Communicable

Disease Control (CCDC) and, in Scotland the Consultant in Public Health Medicine (CPHM). Initial detection is performed at the hospital laboratories. Subsequent characterisation including serogrouping, typing, sub-typing and antibiotic resistance profiles are conducted at the national reference laboratories, such as the Meningococcal Reference Unit for England and Wales (MRU) or the Scottish Meningococcus and Pneumococcus Reference Laboratory (SMPRL). Data on such cases are sent each week to the Office for National Statistics in England and Wales or the Scottish Information and Statistics Division (ISD) in Scotland and the Communicable Disease Surveillance Centre (CDSC) in England and Wales or the Scottish Centre for Infection and Environmental Health (SCIEH) in Scotland. This data is published in the *Communicable Disease Report Weekly* (CDR Weekly), from the Health Protection Agency (HPA) in England and Wales and *Scottish Centre for Infection and Environmental Health (SCIEH) weekly Report* from SCIEH in Scotland.

This infrastructure provides an excellent representation of meningococcal disease and its distribution throughout the year (Figure 1.8). With coordination between laboratories and communicable disease surveillance centres, the confirmation of disease along with the identity and characterisation can be correlated with detailed patient data. Trend analysis can then be undertaken and meningococcal disease distribution data throughout the country can be produced. From these surveillance systems, England and Wales, Scotland and Europe (through the European Surveillance Network (EU-IBIS)) display repeated patterns of meningococcal disease throughout each year, and within a single year a significant increase in incidence during the cooler, dryer months. This pattern is similar in other countries such as Australia, Canada, Italy and New Zealand (Fallon et al. 1984; Ballada et al. 1991;

Figure 1.8 Management of meningococcal disease notifications in Scotland.



If patient is notified but discharged from hospital. Public health are contacted for a convalescent serum. This is then sent to the MRU for analysis.

Jackson and Wenger 1993; Squires et al. 2000; Cartwright et al. 2001; Pollard and Scheifele 2001; Kyaw et al. 2002b; Lindsay et al. 2002; Moura et al. 2003).

1.9 Meningococcal Vaccines

N. meningitidis (the meningococcus) remains an important cause of meningitis and septicaemia worldwide and, in many countries, serogroup B meningococci account for a large percentage of disease for which there is no effective vaccine (Rosenstein et al. 2001a; Vermont and Van Den Dobbelsteen 2003). In the 1960s, vaccines consisting of purified polysaccharide antigens were developed against four of the five pathogenic serogroups (A, C, Y and W135) (Gotschlich 1985). These vaccines were highly effective in adults but are not efficacious in infants and young children. Although plain polysaccharide or conjugate vaccines are now available against serogroups A, C, Y and W135 (Rappuoli 2001a) there is an urgent need for a conjugate vaccine against the serogroup B meningococcus (Vermont and Van Den Dobbelsteen 2003; Zagursky et al. 2003; Capecchi et al. 2004). However, there are problems with the design of a capsule-based vaccine because the meningococcal polysaccharide is identical to a human carbohydrate ($\alpha(2\rightarrow8)$ *N*-acetyl neuraminic acid or polysialic acid and is therefore poorly immunogenic and induces only a transient antibody response (Finne et al. 1987; Hayrinen et al. 1995). The development of a serogroup B vaccine therefore requires a different approach, perhaps based on an essential virulence gene from data gained through the genome sequencing projects (Rappuoli 2000; Rappuoli 2001b; Zagursky et al. 2003; Capecchi et al. 2004; Newcombe et al. 2004). During such development, there is the option to develop vaccines based on other antigens, the most studied of which are those based on outer membrane proteins (OMP) (Ala'

Aldeen et al. 1996; Rosenstein et al. 2001a). Although OMP-based vaccines are not the final solution for combating serogroup B meningococcal infection, they may provide a short-term solution and have shown great promise for the control of serogroup B meniningococcal infection (Rappuoli 2001a). OMP's of classes 1 to 5 have been studied as potential vaccine candidates (Tsai et al. 1981). The class I PorA and class 2 PorB proteins have been studied most because they are used as phenotypic markers in the characterisation of meningococci, for the serosubtype and serotype, respectively. These proteins are variable because they are antigens exposed on the meningococcal cell surface; some regions of the proteins are hypervariable.

A two-dimensional structural model has been predicted for the meningococcal PorA protein. It consists of eight exposed surface loops (I to VIII) (van der Ley et al. 1991). Most variability is thought to occur in variable regions 1 and 2 (VR's 1 and 2) which correspond to loops I and IV, respectively (Maiden et al. 1991; van der Ley et al. 1991; McGuinness et al. 1993). Serosubtype-specific monoclonal antibodies react with peptide epitopes located in these loops and therefore serosubtyping is used to identify these PorA VR epitopes. A widely-recognised nomenclature is used which has been recently updated to take into account new data now available through DNA sequence typing. A novel subtyping nomenclature has also been proposed (Sacchi et al. 1998a; Maiden et al. 1999).

Experimental vaccines based on OMV's have demonstrated that bactericidal polyclonal antibodies are mainly directed against PorA. Over the last decade, single and mutli-valent vaccines have therefore been developed (Wedege and Michaelsen 1987; Rosenqvist et al. 1995; Peeters et al. 1996; van der Voort et al. 1996). These

have been based on the most prevalent serosubtypes circulating in distinct geographical areas (Van Der Ley and Poolman 1992; Peeters et al. 1996; Perkins et al. 1998; Cartwright et al. 1999; Tappero et al. 1999; de Kleijn et al. 2000a) because such proteins are antigenically highly variable and an OMP vaccine cannot protect against all types and subtypes of *N. meningitidis*. For example, the recombinant hexavalent PorA OMV vaccine developed by the Institute for Public Health and the Environment (RIVM) in the Netherlands is comprised of P1.7, 16; P1.19, 15; P1.5, 2; P1.5c, 10; P1.12, 13 and P1.7, 4 (Rouppe van der Voort et al. 2000). This composition accounted for over 80% of UK meningococcal case isolates in 1996 (Cartwright et al. 1999). The vaccine has shown promising results in phase I and phase II clinical trials (Peeters et al. 1996; Cartwright et al. 1999). Therefore, selected OMV's must be used based on the prevalent types and subtypes in a given country.

To develop such vaccines, high quality data must be gained to determine such prevalence. Although it is thought that VR1 and VR2 are the most variable regions within PorA, there is little data to support this. A number of new VR3 serosubtypes have been described (Arhin et al. 1998; Molling et al. 2000; Riesbeck et al. 2000; Molling et al. 2001; Clarke et al. 2003) which suggest that more data is required to determine the importance of this variable region for both disease surveillance and vaccine development. As most laboratories use monoclonal antibodies for the characterisation of meningococci to serotype and serosubtype level, new types and subtypes are missed unless nucleotide sequencing is performed.

1.10 Identification of Meningococci – The Gold Standards

Historically, culture has been used for the characterisation of meningococci. Once successfully isolated and cultured, the meningococcus can be examined by microscopy after Gram-staining as well as for oxidase production and sugar degradation profiles. Meningococcal characterisation can be obtained with subsequent sero-grouping, sero-typing and sub-typing of the capsule and outer membrane proteins respectively (Anon 1998d; Anon 1999a). This is accompanied by other tests such as antibiotic susceptibility profiles (Wall 2001). These clinical samples for culture are usually taken from either a non-invasive site such as the nasopharynx or invasive sites such as CSF and blood.

Culture has long been the "gold standard" of diagnosis and confirmation for MD, but with the advent of antibiotics and rapid and effective treatments, it is not always possible to obtain a culture for characterisation. Cultures are often negative due to the rapid administration of antibiotics before hospital administration. There are many reasons why isolation of meningococci could fail, including the transport media used, the isolation media used and the techniques used for culture isolation. These are a few reasons why non-culture detection and characterisation is being recognised as an increasingly important tool for reference laboratories to maximize the chances of confirmation followed by characterisation (Diggle et al. 2001a; Diggle 2003).

1.11 Laboratory Confirmation of Meningococcal Disease

Molecular biology by its very nature embraces modern molecular techniques. These include detection, isolation, identification and characterisation of pathogens such as *N. meningitidis* (van Deuren et al. 2000). Only five of the thirteen recognised serogroups, A, B, C, W135 and Y are at present considered clinically important worldwide (Horby et al. 2001; Clarke et al. 2002). Control of all these serogroups by methods such as immunisation has yet to be accomplished (2000b; 2000a; Pizza et al. 2000; Morley and Pollard 2001) consequently, modern laboratories utilize a wide range of techniques to provide information relating to the diagnosis and monitoring of infections caused by meningococci (Ragunathan et al. 2000; Clarke et al. 2001a; Diggle et al. 2001b; Clarke et al. 2002).

Typing of microorganisms has traditionally involved the subdivision of a single species utilising a standard set of characteristics. Historically these have included culture, microscopy, biochemical characterisation, antibiotic susceptibility and antigen characterisation (Pitt 2000). These typing methods are usually based on phenotypic markers, biological and biochemical pathways and some can be species specific. This can be a limitation because environmental pressures may adversely affect these fundamental criteria and compromise the reliability and reproducibility of the different methods (Williams 1999). A good example of this is the effect on the expression of outer membrane genes, which can be used for the characterization of meningococci (Urwin et al. 1998a; Urwin et al. 1998b; Vogel et al. 2000; Arreaza et al. 2001; Meyers et al. 2003). The necessity for molecular procedures based around DNA analysis can offer a more stable and universal approach to typing *N. meningitidis*.

These new approaches are used increasingly in microbiology laboratories, and can complement the historical phenotypic methods (Yakubu et al. 1999; Diggle et al. 2001b). In the not too distant future, genotypic typing methods will not only supplement but may also replace existing typing methods (Olive 1999; Diggle et al. 2001b). The basis of DNA typing methods is the differentiation in nucleotide variations. The only drawback from providing greater discrimination is the vast amount of information DNA typing methods inevitably provide.

1.12 Phenotypic Analysis of *Neisseria meningitidis*

The laboratory confirmation of MD is very important as it provides valuable information for therapy, short-term management of case clusters and contacts, and long-term epidemiological data. This can be complemented by the use of enhanced surveillance schemes and are particularly useful for monitoring important situations such as epidemics or national vaccine programmes (Clarke 2002d). The laboratory confirmation of MD is usually achieved using antigen detection, culture and serological methods. Traditionally, the characterisation of *N. meningitidis* has involved the analysis of phenotypic markers such as the capsule and outer membrane proteins using latex agglutination, co-agglutination and enzyme-linked immunosorbent assays (ELISA) methods (Clarke et al. 2002). However, these methods are not universally applicable to all circumstances when attempting to characterise meningococci. Recent developments in DNA analysis, together with the natural limitations of phenotypic methods, have resulted in a natural evolution towards genotypic procedures (Struelens 1996; Ragunathan et al. 2000; Clarke 2002a).

1.12.1 Specimens

In patients with suspected meningococcal disease, specimens from various sources such as blood, cerebrospinal fluid (CSF), eye and throat can be obtained (Demina and Orlova 1981). Skin scrapings and post mortem samples can also be obtained where lesions are present but this is not commonly practiced (Barker et al. 1999; Fischbacher et al. 2001; Anon 2002b). Isolation of different strains from different areas of the host is uncommon. It is therefore unlikely that a nasopharyngeal isolate from a suspected case of meningococcal disease will be attributed to their normal nasopharyngeal flora and is more likely to be attributed to causing meningococcal disease, if all invasive samples are negative. Usually a number of different sites are used to isolate meningococci and this is considered sufficient to limit attributing commensal strains as disease causing within the same patient (Olcen 2001). National guidelines are available in most countries explaining the protocols required for successful isolation of *N. meningitidis* (Anon 1998d; Anon 1999a; Anon 2002b).

1.12.2 Bacterial Culture

N. meningitidis are less nutritionally fastidious than other *Neisseria* species such as *N. gonorrhoeae* but are still likely to be present only in comparatively smaller quantities in clinical samples (Hackett et al. 2002). It is common for isolates of *N. meningitidis* or *Neisseria* species. required for serological characterisation to be transported on heated blood (chocolate) agar slopes or transport swabs. Subcultures are taken from organisms on these transported slopes or swabs to obtain fresh colonies for testing. *Neisseria* species are usually sub-cultured onto different types of agar plates, such as Columbia agar with 5% horse blood and heated Columbia blood agar (Isenberg 1992; Anon 1998d; Anon 1999a; Murray 1999). Inoculated plates are

commonly incubated at 37°C for greater than 18 hours air mixed with a final concentration of 5-10% carbon dioxide. Isolates taken from areas such as the nasopharynx can be inoculated onto selective media, which inhibit the growth of a large number of unrelated residing commensal species (Olcen 1979; Anon 1998b). After incubation colonies are characteristically pale grey and approximately 1mm in diameter (Olcen 2001). Furthermore all isolates can also be stored for future analysis in self-contained storage systems at –70°C (Anon 1998d; Anon 1999a).

1.12.3 Antimicrobial Susceptibility

The search for antibiotics began in the late 1800s, with the growing acceptance of the germ theory of disease, a theory which linked bacteria and other microbes to the causation of a variety of ailments. As a result, scientists began to devote time to searching for drugs that would kill these disease-causing bacteria (Wainwright 1990).

Penicillin along with the discovery of sulphonamides in 1932 both were widely used for bacterial infections (Braude 1953). This success led scientists to intensify the development of new antibiotics that could treat other bacterial diseases. Sulphonamides, first used in epidemics in World War II, were given as a two-day course of treatment which was very effective in eliminating carriage strains (Scheld and Mandell 1984). Eventually, resistance to sulphonamides became commonplace (Abbott et al. 1985) and to combat antibiotic resistance, chemical modifications and derivatives were produced to prevent widespread resistance (Wainwright 1990). By early 1945, in an interview with *The New York Times*, the British scientist and the discoverer of penicillin, Alexander Fleming warned of the misuse of antibiotics such as penicillin that could lead to selection of resistant forms of bacteria (Anon 1945).

Antimicrobial susceptibility testing is also important so that effective therapies can be used for patients and their contacts. The sulphonamides introduced in the late 1930s prompted a decline in the meningococcal mortality rate (Scheld and Mandell 1984) until significant resistance was noticed in the 1950s and 1960s. This increase in antibiotic resistance necessitated the switch to new antibiotics such as penicillin derivatives and chloramphenicol. Nevertheless, in recent years increased resistance has been reported in both penicillin and chloramphenicol (Van Esso 1987; Sutcliffe 1988; Jackson 1994; Galimand 1998; Kyaw et al. 2002a). In the United Kingdom in the early 1990s over 36% of serogroup B and 42% of serogroup C strains were resistant to sulphonamides (Oppenheim 1997). Therefore, unless an antibiotic susceptibility profile has been carried out on the causative organism, sulfonamide is not considered the first choice antibiotic treatment (Oppenheim 1997). Several different laboratory methods can be used for meningococcal antibiotic susceptibility testing (Hughes et al. 1993; Gomez-Herruz et al. 1995). These methods are based on the evaluation of minimal inhibition concentrations (MIC) by methods like the E-test (Hughes et al. 1993; Marshall et al. 1997), agar dilution, and disc diffusion (Il'ina 1992). In addition, slight decreases in penicillin and rifampicin susceptibility have also been observed in other countries throughout Europe and North America. (Bellete et al. 1994; Ringuette et al. 1995; Enting et al. 1996; Richter et al. 2001; Kyaw et al. 2002a). For cases involving penicillin-resistant strains, broad-spectrum cephalosporins such as ceftriaxone are recommended because of the resistance reported in both penicillin and chloramphenicol (Van Esso 1987; Sutcliffe 1988; Jackson 1994; Galimand 1998; Kyaw et al. 2002a). As a prophylatic in contacts with meningococcal disease (Richter et al. 2001) rifampicin is the most widely used chemoprophylactic agent at present, as many studies throughout the world have shown

little resistance to date. A standard course will eradicate carriage in over 70% of contacts (Begg 1995; Simmons et al. 2000).

In recent years, the primary treatment for meningococcal disease has been penicillin G or ampicillin in invasive cases (Anon 2003). Chloramphenicol is an alternative in developed countries because it is inexpensive and stable. The most frequently recommended antibiotics used where contact has been noted between persons and an invasive case(s) are ciprofloxacin or rifampicin (Green and Tillotson 1997; WHO 1998; Wall 2001). There has been a long-standing debate as to whether bactericidal antibiotics may increase the levels of LPS thus up-regulate inflammatory reactions (Shenep 1986). Treatment of meningococcal meningitis is similar to that of patients presenting with meningococcal septicemia (Anon 2003).

It is not uncommon to give broad-spectrum treatment for all meningeal pathogens including *N. meningitidis* before identification and characterisation is known. The most widely used combination of antibiotics remains penicillin and chloramphenicol. Over the past few years, third generation cephalosporins such as cefotaxine and ceftriaxone are considered on cases as first-line treatments due to the increased resistance of *H. influenzae* strains to chloramphenicol and the increased resistance of meningococci to penicillin (Oppenheim 1997; Wilcox and Modi 2000). The advantage with third generation cephalosporins is the high activity towards all common meningeal pathogens except *Listeria monocytogenes*, good penetration into the cerebro-spinal fluid (CSF) and ease of administration either once, twice or three times a day depending on the particular cephalosporins. They can also be used with pregnant women and young children (Tunkel and Scheld 2002). *N. meningitidis* was

previously considered extremely susceptible to penicillin, with most isolates showing minimal inhibitory concentrations of 0.06μg/mL. However, meningococcal isolates with decreased susceptibility to penicillin were reported from carriers over 30 years ago (Martin 1964). Since then, clinical meningococcal isolates with decreased susceptibility to penicillin have been widely described in many countries (Sutcliffe 1988; Saez Nieto et al. 1990; Tzanakaki et al. 1992; Woods et al. 1994; Blondeau et al. 1995). If a meningococcus is isolated and characterised as penicillin resistant, treatment can be easy tailored to suit the susceptibility profile of the organism and other antibiotics such as cephalosporins can be administered.

Seven days is generally considered adequate parenteral therapy of both meningococcal meningitis and septicemia, although this is dependent on several factors including the severity of the disease and patient response to treatment (Anon 2002b; Singhi et al. 2002). Therapy can therefore be shorter in the case of a good patient response and mild disease or of longer duration in cases where complications are evident including brain abscesses, prolonged fever or subdural empyaema (Anon 2002b). One drawback of third generation cephalosporins is the expense. They are still more expensive to administer than the older generation penicillin and chloramphenicol (Wall 2001; Singhi et al. 2002). This has ramifications on developing countries and thus in poorer countries penicillin remains the common treatment for meningococcal meningitis and septicemia (Molesworth et al. 2002). Chloramphenicol is not as common even though it is considered the choice antibiotic when clinical presentation without the characteristic rash is observed (Tondella et al. 2001). Chloramphenicol has other benefits including its flexibility of administration, either intravenously or intramuscularly, and in addition the oral route may be considered in improving cases

(Tondella et al. 2001). Several antibiotics can be used for close contact and persons connected with meningococcal disease for successful eradication of nasopharyngeal carriage as briefly stated earlier.

1.12.4 Biochemical Identification

In combination with biochemical techniques, basic techniques are still used in diagnostic laboratories. These make use of the specific biochemical composition of microbial structures, for example Gram staining using Gram and AO (acridine orange) stains according to defined protocols (Anon 1998d; Anon 1999a). Further to this simple microscopy can identify meningococci on the basis of their characteristic biochemical Gram stain, which highlights the pink-stained gram-negative diplococci arranged in pairs (Isenberg 1992; Murray 1999).

N. meningitidis utilizes glucose and maltose which is used as a standard biochemical identification of the bacterium using commercial or non-commercial reagents. These are common and simple carbohydrate utilization tests for identification based on the biochemical characteristics of acid production by the meningococcus. Simultaneously, use of other sugars such as lactose and sucrose can identify other *Neisseria* species like *N. lactamica* and *N. gonorrhoeae*. Spurious results can occur and commercially available tests such as API NH, which incorporate several specific biochemical reactions can be used to speciate and sub-speciate, identifying to genus and species level.

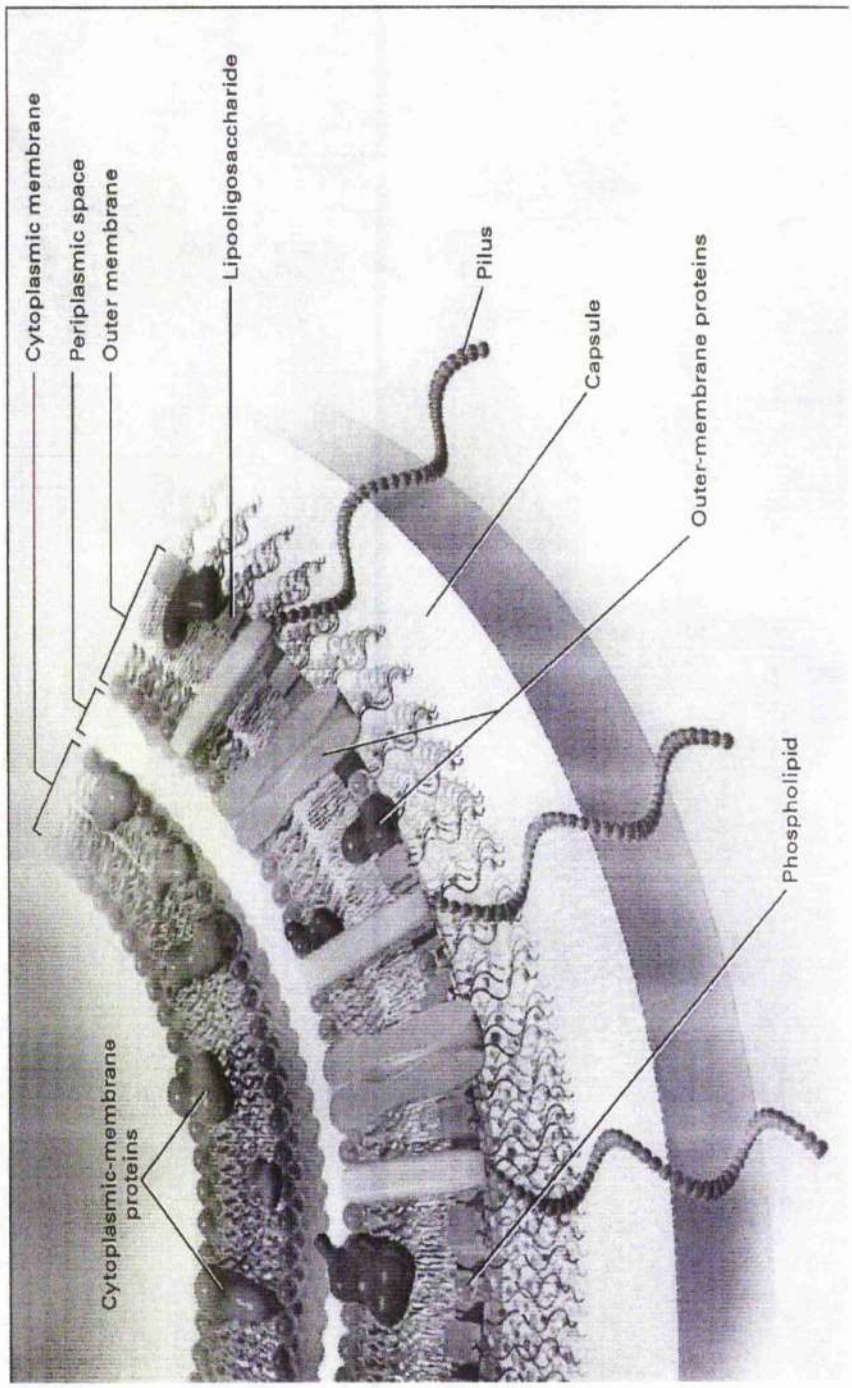
1.12.5 Capsular Grouping

Rapid tools for the non-culture diagnosis and serogrouping determination in cases of

meningococcal disease are crucial for effective patient treatment, contact management and epidemiological surveillance. Serogrouping of meningococci is important and often relies on accurate and sensitive methods for the characterisation of all or part of the polysaccharide capsule (Figure 1.9). The main serogroups of interest within Europe (B, C, Y and W135) boast a polysaccharide capsule which is composed of polysialic acid and are biochemically considered similar (Lewis et al. 2003). For example the capsule chemical composition of serogroups B and C are α2-8 linked sialic acid and α2-9 linked sialic acid, respectively, and serogroups Y and W135 are *N*-acetylneuraminic acid and D-glucose, and *N*-acetylneuraminic acid and D-galactose, respectively. All these serogroups with the exception of B can be *O*-acetylated or de-*O*-acetylated.

The gene cassettes of all the capsules contain four open reading frames (ORFs), (*siaA*, *siaB*, *siaC* and *siaD*) which code for different *sialytransferase* genes. From extensive studies the *siaD* sequence of serogroups B and C are individually distinct but are considered genetically related. The nucleotide sequence differs significantly from serogroups Y and W135 which are highly homogenous with each other even though they encode a similar protein. The capsular nucleotide sequence of serogroup A differs from the other disease-associated serogroups B, C, Y and W135. The capsular polysaccharide of serogroup A isolate is composed of repeating units of (α1→6)-linked-*N*-acetyl-D-mannosamine-1-phosphate (Swartley et al. 1998). This is in contrast to the common capsule types containing sialic acid (Claus et al. 1997). The capsular polysaccharide of *N. meningitidis* serogroup A is encoded by an operon containing four genes (*mynA*, *mynB*, *mynC*, and *mynD*) (formerly known as open reading frames 1 to 4) (Swartley et al. 1998) (Figure 1.10). Confusingly, these have

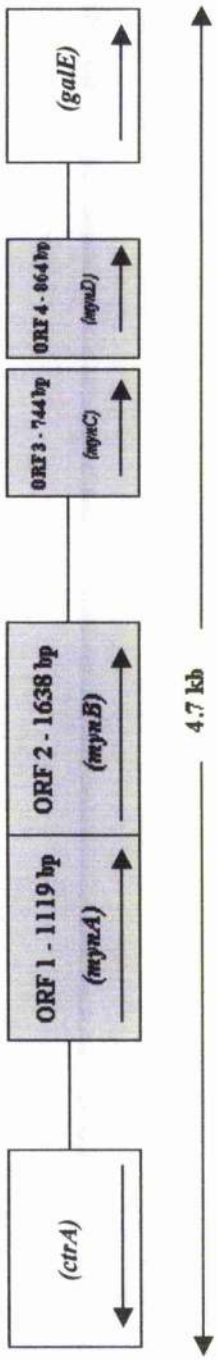
Figure 1.9 Schematic representation of the *Neisseria meningitidis* capsule (adapted from Rosenstein *et al* 2001).



also been referred to as *sacA, sacB, sacC and sacD*. However, this operon appears to be unique to members of *N. meningitidis* serogroup A. The *mynA* gene product is responsible for the first biosynthetic step in the production of the serogroup A capsule and is probably therefore the most conserved gene in the operon (Diggle et al. 2003b).

The usual methods for serogrouping are slide latex agglutination and co-agglutination, using serogroup specific rabbit antiserum. Agglutination denotes a positive reaction provided that it occurs with only one of the antisera. Groups A, C, Y, W135 and B/*E. coli* are tested and further co-agglutination with specific A, C, Y, and W135 groups can show specific serogroups (Eldridge 1978). *E. coli* K1 antisera is used for serogroup B detection due to the similarity between both *N. meningitidis* serogroup B and *E. coli* K1 antigens. More recently, ultrasound-enhanced latex immunoagglutination (USELAT) has been developed, which can provide enhanced sensitivity for the detection of capsular polysaccharide antigens from serogroups A, B, C, Y, and W135 in clinical samples without nonspecific reactivity (Gray et al. 1999; Sobanski et al. 2002; Porritt et al. 2003). Such phenotypic methods have proved less effective on clinical specimens such as CSF and blood samples due to the small amount of antigen or DNA present. Several methods can be used for the genotypic serogrouping of phenotypically non-groupable culture strains and clinical samples (Taha 2000; Tsang et al. 2001; Probert et al. 2002; Diggle et al. 2003b). These methods have demonstrated both the value of obtaining characteristics when phenotypic methods have failed and the importance of obtaining definitive serogroups especially in potential clusters and possible outbreaks.

Figure 1.10 The gene cluster containing four genes *myn*A, B, C and D.

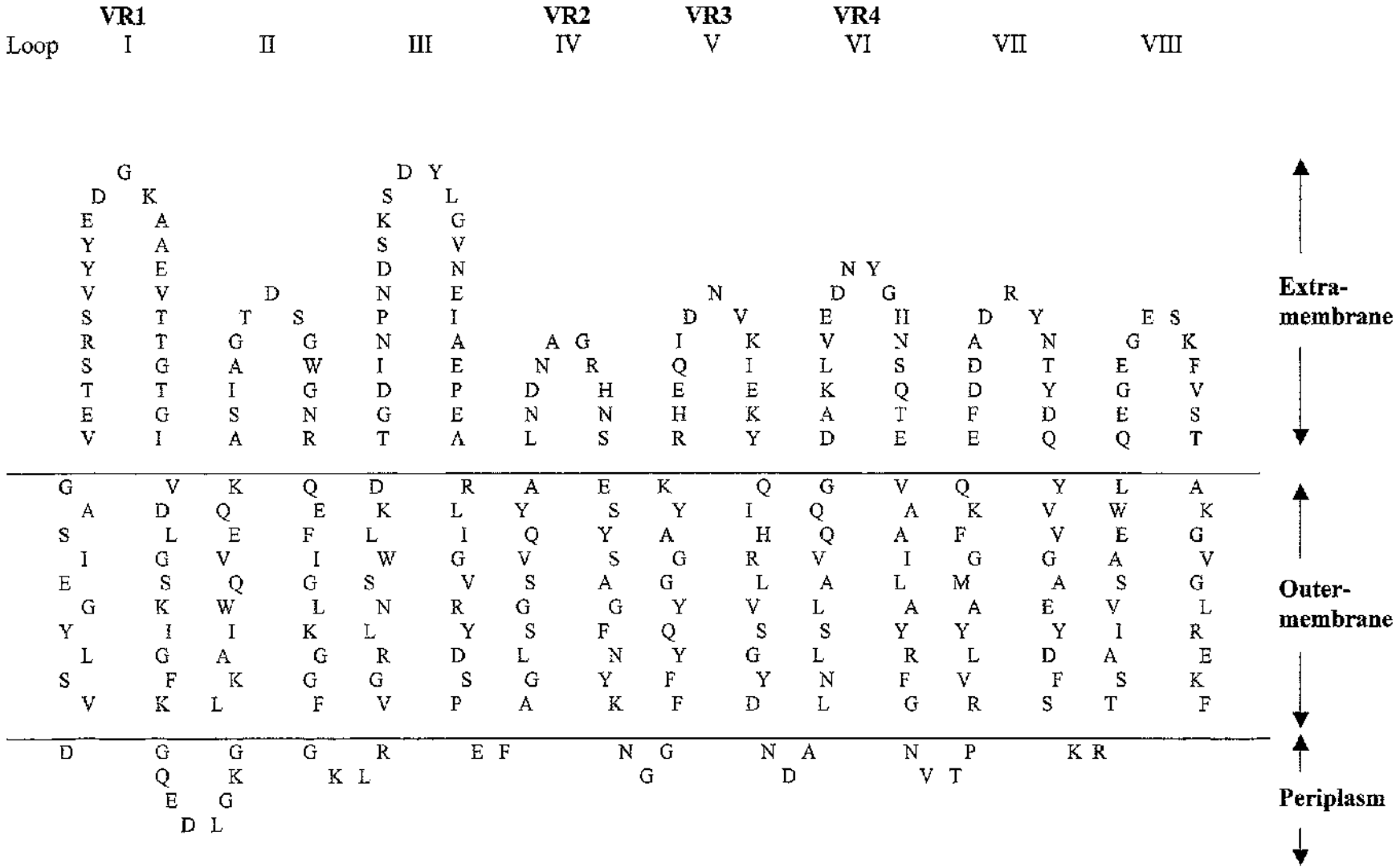


1.12.6 Porin Typing and Sub-Typing

OMPs that are expressed at high levels have been recognised for over 15 years (Frasch 1985). There are two OMPs which have been functionally characterised as porins. These are two different classes of protein from a collection of five different classes (1-5), isolated based on differences in molecular weight. Initial experiments revealed that the meningococcal class 1 porin protein (*porA*) and class 2 and 3 porin proteins (*porB*) perform as selective channels, which permit the passage of cations and anions across the cell membrane. Recently the advent of sequencing has confirmed the porin nature f these proteins with homology with well-characterised *E. coli* porins.

1.12.6.1 Class 2 and 3 Porin Proteins B (PorB). Meningococcal strains have the ability to possess either PorB2 (class 2) or PorB3 (class 3) OMPs that are predominant proteins of the outer membrane, mutually exclusively expressed by alternative alleles (*porB2* and *porB3*) at the *porB* locus (Hitchcock et al. 1986; Derrick et al. 1999). PorB topology models have been constructed on the basis of nucleotide sequence data (Maiden et al. 1991; van der Ley et al. 1991) and, more recently, the structural similarity between previously described *E. coli* porins OmpF and PhoE, and *Neisseria* porins, have generated three dimensional homology models for these Neisserial porins. From these predictions eight surface exposed loops combined with conserved outer membrane-spanning sequences have been postulated (Figure 1.11). As would be expected, the antigenically variable epitopes targeted in the host immune response were found to reside in the most exposed loops (McGuinness et al. 1990; Maiden et al. 1991).

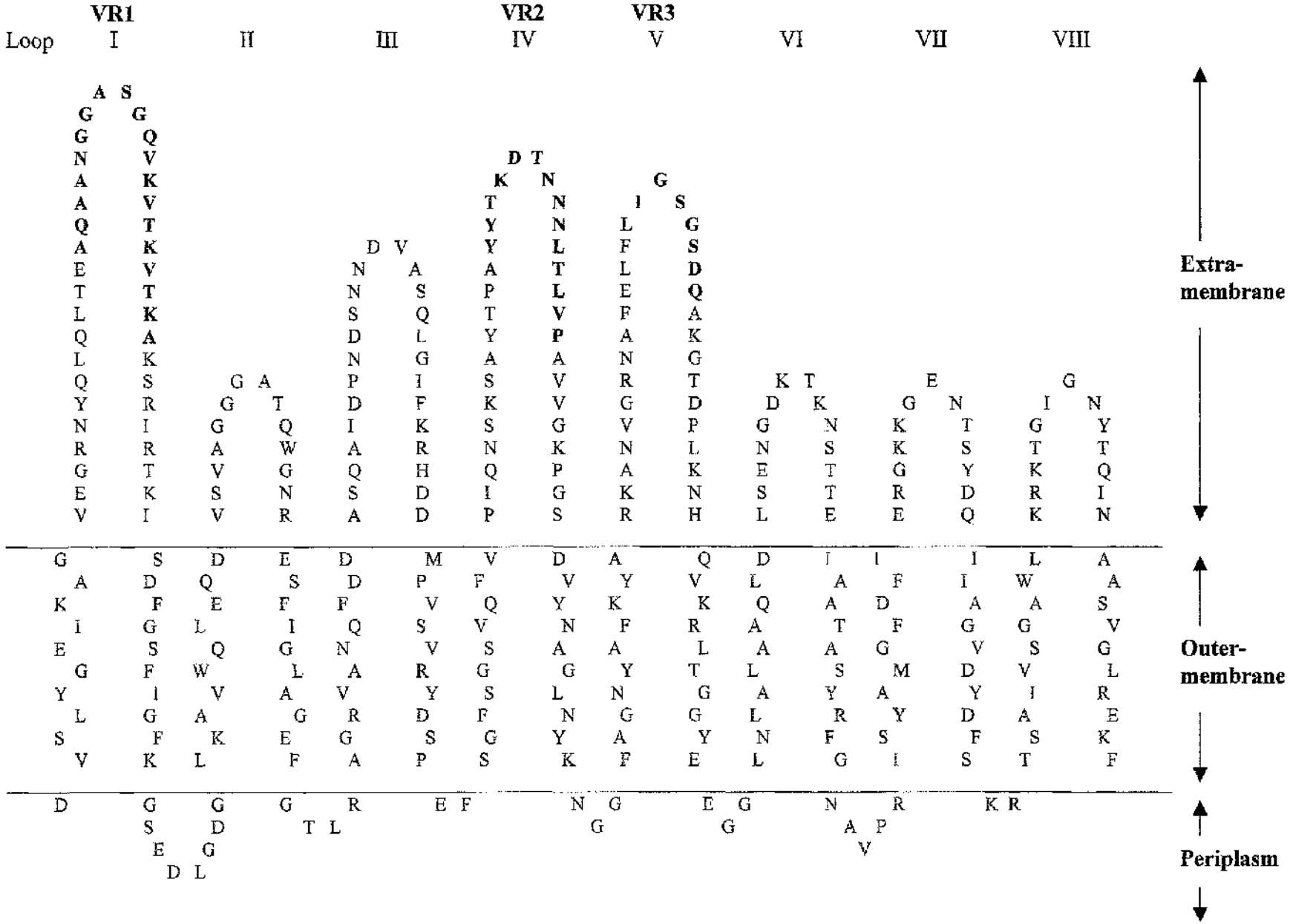
Figure 1.11 The amino acid topology of the class 2/3 porin protein B (PorB) variable regions (VR).


VR1
VR2
VR3
VR4
Loop
I
II
III
IV
V
VI
VII
VIII
Extra-membrane
Outer-membrane
Periplasm

1.12.6.2 Class 1 Porin Protein A (PorA). Virtually all meningococci possess the class 1 OMP (PorA) (Suker et al. 1994; van der Ende et al. 2003). The secondary structure of the class 1 OMP has been formulated and subsequent construction of topology models was possible. These displayed highly conserved trans-membrane regions with eight strain-variable exposed loops, which play a crucial role in the host immune response. These surface exposed loops are the most prominent regions within the protein and thus most exposed to immune responses. There are three regions considered important in sero-subtype characterisation; variable regions at loops 1, 4 and 5 correspond with variable regions I, II and III (VR1, VR2 and VR3) (Figure 1.12) (Molling et al. 2000; Tondella et al. 2000; Molling et al. 2002; Clarke et al. 2003).

Detection and characterisation of the outer membrane protein (OMP) genes *porB* and *porA* can be achieved by several methods (Frasch 1985; Diggle and Clarke 2003a). Typing may be performed on the basis of SDS-PAGE, specific monoclonal antibodies and whole cell ELISA typing methods (Hoehn 1990; Lehmann et al. 1999; Tondella et al. 2000; Wang et al. 2000). More recently, molecular characterisation of the meningococcal porins has included nucleotide gene sequencing of previously described variable regions or the whole gene (Urwin et al. 1998a; Clarke et al. 2003). These porins have not only been used for sero-typing and sero-subtyping but also for epidemiological analysis of meningococcal carriage and disease (Poolman et al. 1986), although the variability of these proteins means they are not entirely accurate as epidemiological markers (Urwin et al. 1998b).

Figure 1.12 The amino acid topology of the class 1 porin protein A (PorA) variable regions (VR).


VR1
VR2
VR3
Loop
I
II
III
IV
V
VI
VII
VIII
Extra-membrane
Outer-membrane
Periplasm

The class 4 OMP are antigenically stable (Munkley et al. 1991), and appear to be the most highly conserved between meningococcal strains. Although its cellular function is unknown, it shares sequence homology with *E. coli* Omp A (Klugman et al. 1989). Antibodies directed against this protein are non-bactericidal and have the additional ability to block the lytic effect of antibodies directed against other outer surface antigens (Munkley et al. 1991). In contrast to the other major OMP the heat modifiable class 5 opacity proteins, Opa and Opc are hypervariable (Stephens and McGee 1983; Tinsley and Heckels 1986) both in qualitative and quantitative expression (Achtman et al. 1991; Aho et al. 1991) but may induce bactericidal antibodies (Danelli et al. 1995). This group of proteins also elicits strong but strain-specific antibody responses and confers important interstrain antigenic differences which may be detected by MAbs (Zollinger et al. 1984).

1.13 Other Diagnostic Methods

1.13.1 Polymerase Chain Reaction (PCR)

The PCR method has been available for over a decade (Mullis et al. 1986) and has been applied in many different ways for the detection of *N. meningitidis*. During the past decade, the PCR has provided an invaluable tool and is considered a key advance in molecular biology (Wright 1990; Markman 1993). There are a number of different methods available for the detection or amplification of specific DNA sequences (Southern 1975; Viscidi 1987; Livak 1995; Tyagi 1996). The fundamental PCR method has found a home in many areas which utilise molecular techniques in research and non-research environments including microbiology, animal and human genetics and clinical diagnostics. Traditionally, PCR products are amplified in a

commercial thermocycler followed by visualisation of PCR products on a gel-based system (Sambrook 2000).

Some of these have disadvantages including low sensitivity, lack of specificity, high cost or their laborious methodology (Wright 1990). However, various technologies are now available to exploit the PCR method beyond its basic concept and this will be discussed in further sections.

Within routine microbiology laboratories it is still common to use gel-based PCR detection systems. The specific gene targets can vary in different laboratories, but the principle remains the same. Several genes are commonly used for the detection and characterisation of meningococci. The detection of the insertion element IS*1106* can be used to confirm the presence of *N. meningitidis* in suspected cases (Ni 1992; Newcombe et al. 1996). However, insertion sequences are genetically mobile with the ability to spread between species and even genera. The IS*1106* PCR has been evaluated and used for many years although recent studies evaluating the specificity have found a number of false positives caused by organisms other than *N. meningitidis* (Borrow 1998). As an alternative, the *ctrA* gene can be used for detection in clinical samples (Frosch et al. 1992). Although only present within the meningococcal genome as a single copy gene and compared to multiple copy genes such as the IS*1106*, lacks the sensitivity that IS*1106* offers, it is at present thought to be specific to *N. meningitidis*. IS*1106* was initially reported as a PCR target designed for the specific and sensitive detection of *N. meningitidis* DNA within clinical samples (Newcombe et al. 1996). There have been numerous ways attempted to produce a highly sensitive and highly specific PCR assay, using more than one gene,

and using species-specific genes. At present, it is true that PCR assays should not replace culture characterisation, but complement it as a valuable detection and surveillance system for meningococcal disease when culture fails to yield positive results.

1.13.2 Enzyme Linked Immunosorbent Assay (ELISA)

The ELISA method can be used to detect antibodies against meningococcal outer membrane proteins in serum from patients with suspected MD, or for the detection of specific meningococcal polysaccharide antibodies present within clinical samples taken from confirmed cases of MD (Rosenqvist et al. 1990; Scholten et al. 1994; Salih et al. 1995; Kremastinou et al. 1999b). This is based on the detection of immunoglobulin M (IgM) and immunoglobulin G (IgG) against meningococcal outer membrane proteins possessed by common serogroups such as B and C as a result of meningococcal infection. This type of technique can confirm MD when PCR or culture results are negative.

1.13.3 Latex Agglutination

Latex agglutination tests (LAT's) for the detection of meningococcal antigens can offer a rapid diagnostic tool (Aguiar-Nogueira et al. 1989). This technique is more commonplace due to its relative low cost and low complexity (Coovadia et al. 1989), although recently PCR has emerged as the first-line investigation to confirm the presence of specific meningococcal antigens within clinical samples. LAT's are now usually performed in conjunction with PCR with attention focused on rapid, inexpensive easy to perform tests, which can be used during or after antibiotic treatment. The primary drawback of LAT's is the lack of sensitivity (Tarafdar et al.

2001) although recent developments have been made to improve and enhance the sensitivity of meningococcal antigen detection using modified LAT's with ultrasound technology (Sobanski et al. 2002).

1.14 Molecular Typing of *N. meningitidis*

Shortcomings in some phenotypic techniques for characterizing and typing meningococci have become more commonplace, an example being the increase in non-groupable, non-typeable and non-subtypeable strains. There are a number of reasons including the introduction of antibiotics, selective pressures such as climate, sociological factors and immunological factors, which have altered different biochemical structures, and processes hindering phenotypic methods such as, availability of reagents and availability of new technologies. Consequently, this has had a constructive effect on the development of new techniques and the development of molecular typing methods for enhanced characterisation and surveillance of microorganisms.

1.14.1 Selective Fragment Amplification Typing Methods.

In recent years, a range of PCR-based approaches have been developed and used with varying degrees of success for detecting meningococci (Frosch 1992; Ni 1992; Newcombe et al. 1996; Taha 2000). These molecular methods tend to be relatively quick and can be sensitive and specific. A typical test can take only a few hours before a definitive result is known. Because of the nature of PCR detection within clinical samples, inhibitors can sometimes be present such as immunoglobulin G in human plasma, hemoglobin and lactoferrin in erythrocytes and leukocytes,

respectively (Hackett et al. 2002). They have been identified as major inhibitors of diagnostic PCRs, which can decrease their efficacy (Al-soud 2000; Al-soud 2001). PCR-based detection and characterisation is especially useful when treatment has been given and culture has proven negative. The success of positive non-culture detection and characterisation ranges from 31% to 40% of suspected cases of meningococcal disease (Newcombe et al. 1996; Taha 2000; Clarke 2002b; Clarke et al. 2002).

A limited number of methods for non-culture diagnosis, that detect and type meningococci, have been described. These are based on the amplification of the *siaD* gene, *porB*, *porA* and 16S rRNA genes (Saunders 1993; Greisen 1994; Borrow et al. 1997; Sacchi et al. 1998b). With the exception of a few techniques, the majority of reported methods only identify the presence of *N. meningitidis* without serogroup or serotype prediction, and less can predict serogroups, serotypes and sero-subtypes.

There are initial trends towards the development of comprehensive non-culture characterisation methods based on PCR, which can identify group, type and sub-type, and in some cases further analysis of the appropriate genes. A good example of the limited techniques available is the lack of a test to detect serogroup A meningococci which, although not prominent in the majority of countries outside of the meningitis belt, is the serogroup encountered in the major epidemics in Africa (WHO 1998; Diggle et al. 2003b).

1.14.1.1 Developments in Selective Fragment Amplification Typing Methods. The initial growth in molecular techniques started with the first licensed kit using nucleic-acid based technology (Roche Diagnostics, Indianapolis, USA) and has continued

with yet another important growth in molecular typing techniques including real-time detection of PCR products (Guiver et al. 2000), and the move towards automation (Clarke 2002a). New chemicals, such as Taqman® and molecular beacons, have been developed commercially, to provide real-time PCR that is also more sensitive than the equivalent gel-based system because it is fluorescence-based (Livak 1995; Tyagi 1996). These chemistries have also allowed the further expansion of the applications of PCR into areas such as single nucleotide polymorphism (SNP) analysis (Morin 1999) whilst standard PCR has been developed into providing amplicons for microarray analysis (Shalon 1996). Automation has also recently become more affordable and therefore more accessible to standard laboratories. It is now used heavily in the pharmaceutical industry and more recently in academic research and clinical diagnostics (Clewley 1999; Seward and Towner 2000).

The three probe chemistries currently used for real-time detection of PCR products are;

- Hybridization probes which use a process of hybridization of small oligonucleotide probes to specific target DNA,
- Hydrolysis probes which rely on the 5'-3' exonuclease activity of Taq polymerase, which degrades a hybridized non-extendible DNA probe during the extension step of the PCR
- Hairpin probes which contain a duplex region adjacent to a single-stranded target capture region.

The basis of these three chemistries allows the elimination of post-PCR processing while allowing real-time analysis of PCR products produced during amplification.

Hairpin probe chemistry includes molecular beacons, which are oligonucleotide probes that can report the presence of specific nucleic acid sequences (Tyagi 1996). Molecular beacons are molecules with an internally quenched fluorophore whose fluorescence is restored when they bind to a target DNA sequence. They are designed in a way that allows the loop portion of the molecule to be the probe sequence for the target DNA.

The loop is attached to a stem formed by the annealing of complementary sequences of the probe sequence. On one arm of this stem is a fluorescent moiety and a quenching moiety is attached to the end of the other arm. The function of the stem is to maintain the close proximity of both the fluorescent and quencher moieties. The quencher prevents fluorescence by converting the absorbing fluorescent energy into heat. Only when the target probe encounters a complementary target molecule will it form a hybrid that is long and stable enough to maintain a conformational reorganization that forces the stem apart. This causes the two moieties to separate, leading to the re-establishment of fluorescence, which can be detected. This technology is very limited within the meningococcal field (Guiver et al. 2000; Diggle et al. 2001a; Diggle et al. 2003b), and given the value of this technology combined with the potential value of automation this should be further explored.

1.14.2 Gel-Based Typing Methods.

Systems which utilise various types of gels are commonly used for characterisation and population genetics, consequently isolates of meningococci can be resolved into limited numbers of genetically related types or clones each thought to contain strains with a common ancestry. A number of alternative techniques for the determination of

the clonal types present in bacterial populations have been investigated and these include but are not limited to, pulse-field gel electrophoresis (PFGE) (Popovic et al. 2001), restriction fragment length polymorphism (RFLP) (Fox et al. 1991; Kertesz et al. 1993), multi-locus enzyme electrophoresis (MLEE) (Caugant et al. 1986; Selander et al. 1986; Olyhoek et al. 1987; Selander et al. 1987; Tondella et al. 1999), amplified fragment length polymorphism (AFLP) and fluorescent amplified fragment length polymorphism (FAFLP) (Goulding et al. 2000; Goulding et al. 2003).

1.14.2.1 Pulsed-Field Gel Electrophoresis (PFGE). Pulsed-field gel electrophoresis (PFGE) uses chromosomal DNA isolated from strains of target organisms, in this case *N. meningitidis*, and is digested with restriction endonucleases that cut the chromosome into 10-40 fragments which are resolved into fingerprint patterns by the process of PFGE (Yakubu and Pennington 1995; Popovic et al. 2001).

PFGE involves embedding *N. meningitidis* in agarose, lysing it and digesting the DNA with restriction endonucleases that cleave occasionally. The resulting DNA fragments are reloaded into agarose wells and the various fragments are resolved into a specific pattern within the gel by an apparatus which switches the direction of the current in a predetermined pattern. Consequently, it can be difficult when comparing similar strains from different laboratories. Patterns produced are interpreted relatively simply and quickly, and are stable for at least eight passages of the strain *in vitro*. PFGE is used for comparison of these fingerprints, enabling distinction and identification of clonal subgroups of meningococcal isolates and allows evaluation of genetical relationships between strains belonging to the same clonal subgroup (Bygraves and Maiden 1992).

1.14.2.2 Restriction Fragment Length Polymorphism (RFLP). Restriction Fragment Length Polymorphism (RFLP) is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. If two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme. The similarity of the patterns generated can be used to possibly differentiate strains from one another.

An RFLP typing method was developed for *N. meningitidis* in which a cloned *Eco*R1 fragment from a serogroup B strain was used to probe Southern blots of total chromosomal DNA restriction fragments (enzyme *Ava*I). A group of 75 apparently unrelated organisms gave rise to twenty-six different RFLPs and two different groups of epidemiologically related strains had RFLP patterns that were distinct for each group. The technique was highly reproducible and discriminatory. The RFLP data were compared with the results of serotyping and subtyping and isoenzyme electrophoretotyping. This data was consistent with those from alternative typing methods (Swaminathan et al. 1996). The use of RFLP typing technology has proven to be of considerable epidemiological value (Speers and Jelfs 1997; Picard 2000; Stefanelli et al. 2001).

1.14.2.3 Multi-Locus Enzyme Electrophoresis (MLEE). Multi-locus enzyme electrophoresis (MLEE) has long been considered the gold standard for the molecular characterisation of *N. meningitidis* (Caugant et al. 1986). MLEE has been used for the genotyping of meningococci, to identify specific clones, and to study the genetic diversity of the meningococcus. Although the correlation between the electrophoretic

migration of individual enzymes and the genotype may be disrupted by horizontal gene transfer (Feil et al. 1995), the use of multiple enzymes make MLEE a moderately robust typing method for meningococci (Bart et al. 1998).

MLEE was first described in 1966 as a molecular approach to the study of genetic variation in eukaryotic systems, and microbiologists have as described been able to incorporate MLEE as a highly useful molecular typing tool. MLEE is fundamentally based around the different electrophoretic mobilities of constitutive enzymes from different amino acid changes. These enzymes were chosen on the basis of their powers to discriminate and type all strains and therefore allow enhanced population and evolutionary studies. Although only a small number of variants are detected at each locus, analyzing twenty or more loci can obtain a high level of resolution (Caugant et al. 1986). MLEE does have many attractive features for global epidemiology and epidemic-associated strain characterisation. Nowadays this procedure is considered labour intensive, and it can be subjective, relying on uncharacterized genomic differences between isolates while producing results which are difficult to compare between different laboratories. Given the emphasis on global epidemiology, due to the ability of meningococci to spread over whole countries and continents, tools are now being developed which not only maintain the same level of discrimination but also are easy to compare between far reaching laboratories.

1.14.2.4 Amplified Fragment Length Polymorphism (AFLP). AFLP is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. Fingerprints are produced without prior sequence knowledge using a limited set of generic primers. This technique can be considered as a combination of the

robustness and reliability of RFLP and the relative power of the PCR technique (Goulding et al. 2003). The genomic fingerprinting process can be separated into three appropriate steps. Firstly, the restriction enzyme digestion of the DNA and ligation of oligonucleotide adapters. Secondly, selective amplification of sets of restriction fragments and, thirdly, gel analysis of the amplified fragments using semi-automated scoring software of AFLP images (Myburg et al. 2001). Additionally, the PCR primers can be labeled fluorescently, and the dye-labeled fragments can be separated by capillary electorphoresis, which is currently considered one of the most suitable methods of fragment analysis. Using the capillary electrophoresis system with appropriate size standards enables fluorescent-AFLP (FAFLP) with a usual fragment accuracy of +/- 1bp. FAFLP can be used as a genotyping tool with a high resolution and accurate size determination (Goulding et al. 2000).

1.14.3 Sequence-Based Typing Methods.

Given the disadvantages of relying on uncharacterised genomic differences between isolates, sequence-based approaches have become the alternative to overcome this (Sun et al. 2000; Tinsley and Nassif 2001). DNA sequencing has many advantages over other methods that are used for national and global epidemiology as well as to distinguish apparently identical strains identified by other methods.

1.14.3.1 Multi-Locus Sequence Typing (MLST). MLST was envisaged as the "next-generation" typing tool developed from MLEE. As a consequence of utilising sequence technology MLST can successfully differentiate strains that appear identical by standard phenotypic typing methods (Maiden et al. 1998; Enright and Spratt 1999). The method can be used during outbreaks of MD (Feavers et al. 1999) and at a time

when sequencing has become a valuable tool, already a number of laboratories have started using multi-locus sequence typing (MLST) as a method for characterising isolates of *N. meningitidis* (Jolley et al. 2000; Clarke et al. 2001b; Van Looveren et al. 2001). MLST produces nucleotide sequence data of approximately 500 base-pair segments from seven house-keeping genes providing results that are digital and therefore highly portable between laboratories.

MLST was first validated on meningococci by Maiden and colleagues (Maiden et al. 1998). This method was originally evaluated on *N. meningitidis* because it provides a good example in which genetic recombination events are considered common. Originally, MLST for meningococci centred on ten loci from isolates that had been previously characterised. The meningococcal MLST house-keeping genes were subsequently reduced to use seven loci on the basis of its discriminatory power. This unique method was applied to a collection of 107 *N. meningitidis* isolates from invasive disease and healthy carriers that had been previously characterised by MLEE. MLST has now been validated for a number of important bacteria and fungi including *Bordetella pertussis* (Van Loo et al. 2002), *Campylobacter jejuni* (Dingle et al. 2001; Dingle et al. 2002), *Candida albicans* (Bougnoux et al. 2002), *Enterococcus faecalis* (Nallapareddy et al. 2002), *Enterococcus faecium* (Homan et al. 2002), *Escherichia coli* (Noller et al. 2003), *Haemophilus influenzae* (Meats et al. 2003), *Listeria monocytogenes* (Salcedo et al. 2003), salmonella (Kotetishvili et al. 2002), *Staphylococcus aureus* (Enright et al. 2000a), *Streptococcus pneumoniae* (Enright and Spratt 1998), *Streptococcus pyogenes* (Enright et al. 2001), and *Streptococcus suis* (King et al. 2002). The method is based on the well-tested principle of MLEE which

as mentioned, assigns a gel-based profile indirectly from the electrophoretic mobilities of gene products.

This can be incorporated with similar enhanced systems in most laboratories as a routine method and consequently can provide data which is useful for the public health management of clusters or outbreaks in institutions such as schools, and also for general disease surveillance on a national basis, thereby providing information to determine national vaccine policy. This, however, is not yet commonplace in all laboratories responsible for detection and characterisation of *N. meningitidis*.

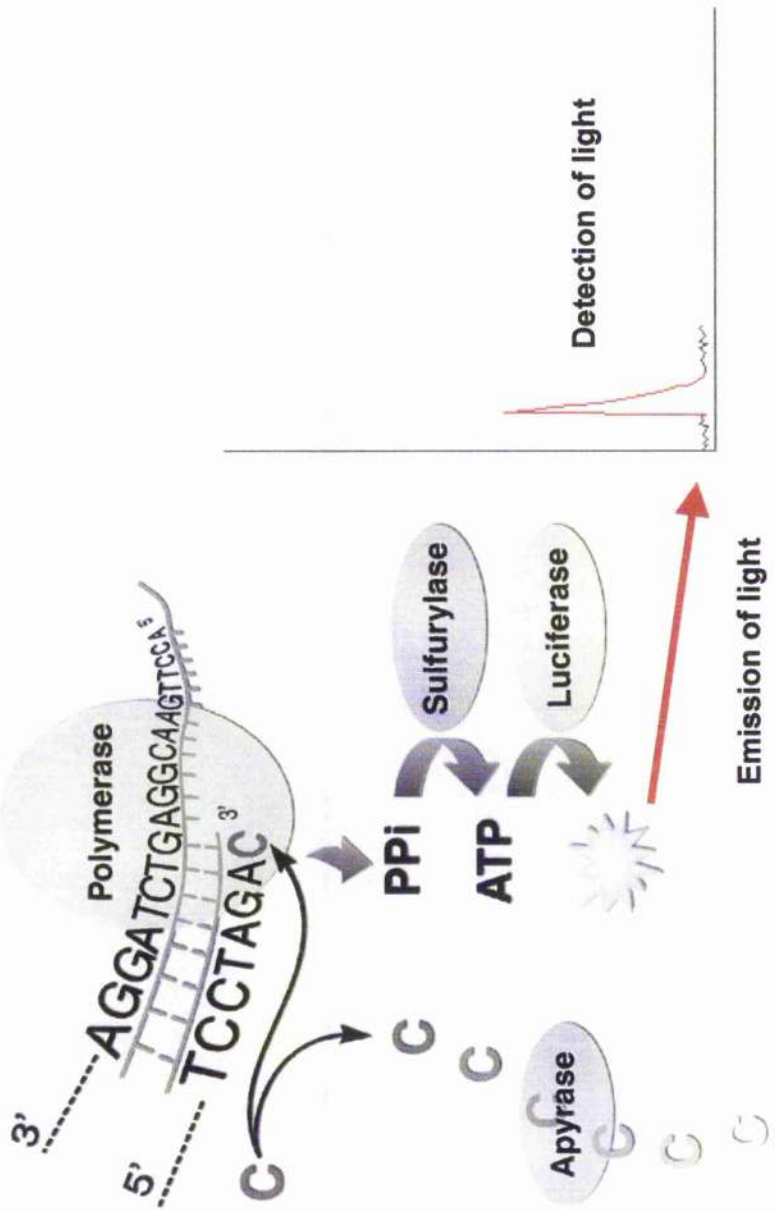
1.14.3.2 Single-Stranded Conformational Polymorphism (SSCP) Analysis. Single-stranded conformational polymorphism (SSCP) was developed in order to obtain the sub-specific typing information on the meningococci present in clinical samples, such as blood and CSF. Initially the gene used for SSCP was variable region 1 (VR1) of *porA* (Newcombe et al. 1997). This is a target used for many serotyping antibodies and, a vast amount of information is known on the genetic variation at this locus (Poolman 1995). SSCP analysis of post PCR products allows detection of single nucleotide point mutations within target DNA. This has been used to demonstrate the identities and non-identities of meningococci between clinical specimens. This sort of method is not unlike a number of single-nucleotide polymorphism (SNP) methods available for identifying single-base variations in a DNA sequence, usually represented by two or three different bases at a single position and can occur in a population at greater than 1% allele frequency. SNP's can be considered highly abundant within bacterial genomes and along with the tools available to determine the

exact variant present in a DNA target sequence, these are useful molecular typing tools.

1.14.3.3 Matrix-Assisted Desorption/Ionization Time of Flight (MALDI-TOF) Analysis. MALDI-TOF mass spectrometry has become, in recent years, a tool of choice for large-molecule analysis (Beavis 1996). Essentially MALDI-TOF is a technique for measuring large molecular masses precisely and is accurate enough to detect the differences between single nucleotides. The principle, using bacterially identified reference strains as a marker, could be used to detect differences in specific DNA sequences, but techniques such as this have not been widely reported for the discovery of different DNA sequences with *N. meningitidis* (Lowe 2004).

1.14.3.4 Pyrosequencing. Fundamentally, Pyrosequencing ™ involves the synthesis of a number of different chemicals, which enables a fast and accurate analysis of DNA sequences. After DNA amplification and subsequent single stranded DNA formation, a sequencing primer is hybridised to its complementary sequence on the template prior to the sequence of interest. Incubated enzymes are added with substrates to catalyse the incorporation of deoxynucleotide triphosphates into the DNA strand if it is complementary. Each incorporation event is accompanied by release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide, a complex cascade reaction follows that results in the generation of visible light in amounts proportional to the amount of dNTP originally incorporated. This is detected by a charged coupled device (CCD) camera and finally represented as a peak in a pyrogram displayed on a computer screen (Ronaghi 2001). As the process continues, the complementary DNA strand is built up and the nucleotide sequence is determined

Figure 1.13 The chemical cascade system of Pyrosequencing™.



from the collection of signal peaks in the Pyrogram. The reaction is simple and robust and the technology can be adapted to create dedicated tools for specific applications (Diggle and Clarke 2004b) (figure 1.13).

Pyrosequencing™ could be used to develop typing assays for the characterisation of *N. meningitidis*. This could involve developing methods for the nucleotide sequence analysis of important variable regions contained on capsule and outer membrane proteins responsible for grouping, typing and sub-typing *N. meningitidis*. These, as described previously, include *siaD*, *porB* and *porA* genes, respectively. To achieve this, one would have to successfully perform amplification of all three genes, *siaD, porB,* and *porA,* from the four main serogroups B, C, Y and W135. To improve reliability and reproducibility the method could also be automated. Recent studies using over 700 strains isolated in Scotland during 1999, 2000 and 2001 have shown that Pyrosequencing can be used for the single nucleotide polymorphic (SNP) and sequence analysis (SQA) characterisation of meningococci (Diggle and Clarke 2003b).

1.14.3.5 Microarrays. Microarray expression analysis has become one of the most widely used functional genomics tools (Hegde 2000). Microarrays are DNA segments representing a collection of genes or the various combinations of a single gene to be assayed. These are amplified by PCR and mechanically spotted at high density on glass microscope slides using simple x-y-z stage robotic systems. This results in a microarray containing thousands of elements. These microarrays can easily be constructed within a relatively short period of time. Using fluorescence-based technology, hybridisation of complementary DNA segments upon the microarray with

target DNA can be detected and immediately identified. This has already been reported for the discrimination of six gene sequences, which are representatives of different bacterial identification assays for *E. coli gyrA*, *Salmonella gyrA*, *Campylobacter gyrA*, *E. coli parC*, *Staphlococcus mecA*, and *Chlamydia* cryptic plasmid (Westin 2001). It was also reported to have the ability of discriminating strains carrying antibiotic resistance single-nucleotide polymorphism mutations. The evidence provided by several reports highlights microarrays as simple characterisation processes, allowing minimal processing of samples and required practical experience after system set-up (Bavykin 2001). The limitations with such new technology include cost and the practicality within laboratories with limited resources and technical support. The challenge with such technology is to find ways in which this can be used to study different aspects of the bacterial population. For example, the comparison of selected genes expressed by a pathogenic strain compared with that expressed by a non- pathogenic stain by hybridization to an array made from the genome of the pathogenic strain (Clewley 2000). In this way, this method could be used to develop DNA arrays that are suitable for both diagnosis and typing of bacterial species such as *N. meningitidis* (Borrow 2001).

Ultimately, the identification of meningococcal genes involved in meningococcal pathogenesis will provide an insight into the pathogenic mechanisms that underlie infection. These genotypic studies could eventually lead to the routine genome-wide analysis of *N. meningitidis* for complete genotypic characterisation, and thus provide all the attributes of *N. meningitidis* required for disseminated infection and may lead to new interventions to prevent and treat meningococcal infection.

1.15 Aims of The Project

The aim of this project was to develop and introduce an MLST system for all meningococci causing invasive disease in Scotland during years 1999 - 2002. MLST will be introduced because strains within the electrophoretic type 37 (ET-37) complex, particularly those of ST11, are often indistinguishable by traditional methods.

Nucleotide sequencing will be performed on seven housekeeping genes and one outer membrane protein gene, *porA*. Data will be analysed using databases and software available through the MLST website and the *porA* websites.

The introduction of MLST in Scotland will be used as a routine method for the characterization of *Neisseria meningitidis* isolates in 1999. Coincidentally, this is just prior to the introduction of the MenC vaccines in November 1999.

All *N. meningitidis* isolates from invasive disease will be analysed. This will give valuable data highlighting the effect the MenC has on Serogroup C disease and its potential decline over the length of the vaccination campaign. At the same time, the overall number of cases will be monitored including the effect on serogroup B between 1999 and 2002. This will then be linked with historical data such as the typical cyclical pattern seen with serogroups B and C over the past decade. The incidence of ET-37 strains in serogroup C disease will be assessed along with ET-37 strains of other serogroups. The number of different ST's isolated will also be monitored from the introduction of the MenC vaccine and this will be linked with

their corresponding serogroups and subtypes.

Hopefully a decrease in serogroup C disease will be observed, but it remains to be seen if this is accompanied by an increase in serogroup B disease. Any occurrence of Capsule switch will be clarified and applied to the whole data set for subsequent conclusion. This will hopefully show that MLST is extremely important for the surveillance of meningococcal disease over a period of years and, and that this study, will have been effective not only in monitoring the impact of the MenC vaccines, but also providing a detailed genotypic representation of strains now commonly associated with disease.

In addition to enhanced sequence based characterisation, development of a fluorescent based PCR protocol using *N. meningitidis* specific probes will be developed to markedly improved sensitivity and specificity of meningococcal DNA detection. Using an automated platform employing Taqman chemistry, a detection system will employ fluorescence-based chemistry that eliminates post PCR processing and will provide accurate real-time quantitative PCR. This development will identify previously undetectable quantities of meningococcal DNA, which can subsequently be used in a modified MLST system and ultimately produce genotypic results, which would not normally be created.

Chapter 2

Materials and Methods

2.1 Handling of Hazardous Chemicals

All chemicals were handled with care and the necessary precautions taken according to health and safety guidelines such as guidance notes for the control of substances hazardous to health (COSHH), Clinical Pathology Accreditation (UK) Ltd. (CPA) and the Advisory Committee on Dangerous Pathogens (ACDP). Health and Safety guidance and protection is based on the recommendations of the "Code of practice for the prevention of infection in clinical laboratories and post-mortem rooms" – 1978, H.M.S.O. (working party chairman Sir James Howie). This was drawn up to fulfil the department's obligations under the Health and Safety at Work, Act (1974) and associated health and safety legislation. Subsequent updated documentation has been based on these findings. Appropriate safety wear and extractor hoods were used when necessary. The guidelines require that no activity, (teaching or non-teaching), which is liable to expose employees, students or any other person to any substance that is hazardous to health may be carried out unless a suitable and sufficient risk assessment has been completed. In addition each risk assessment must be reviewed and, where necessary, changed where there is reason to suspect that it is no longer valid or there has been a significant change in the activity to which it relates (Anon 2002a). Chemical databases are available on-line and a material safety data sheet can be provided for most substances upon request. Sites include: http://hazard.com/msds/ and http://www.msdssearch.com/.

2.2 Patients and Specimens

All invasive *N. meningitidis* isolates received by the SMPRL during the years 1999, 2000, 2001 and 2002 were included. These were chosen as (i) notified cases with isolates and (ii) all isolates that had caused invasive meningococcal disease in Scotland during that time period. These isolates originated from blood, eye, CSF and throat, from patients with confirmed meningococcal disease. All isolates were initially isolated from diagnostic microbiology departments throughout Scotland.

2.3 Routine Culture of Bacterial Strains

All isolates were grown on horse blood agar (Oxoid, Basingstoke, UK) at 37°C in an atmosphere of 5% CO_2. Serogrouping of *N. meningitidis* was performed by latex agglutination, co-agglutination, *mynA* and *siaD* PCR as previously described (Borrow et al. 1997; Borrow et al. 1998; Clarke et al. 2002; Diggle et al. 2003b). Serotyping, sero-subtyping and antibiotic sensitivity profiles were performed as previously described (Clarke et al. 2001b; Clarke et al. 2002).

2.4 Genomic DNA Extraction

One fresh colony was inoculated into 0.5ml of 18MΩ distilled water and boiled for 1 min. The suspension was centrifuged at 15 000 g for 2 min and the supernatant was used as a source for the detection of meningococcal genomic DNA.

2.5 Storage of Bacterial Strains

A sweep of single colonies was taken using a sterile cotton bud. This was immersed into a vial containing protect beads in solution. The cotton bud was aggregated and bacterial colonies were displaced into the solution. The solution was mixed with the beads and then removed leaving behind the bacteria bound to the beads. The vial was marked with the appropriate strain designation and stored at –70°C until required. Bacterial strains were recovered by taking a sterile loop containing a single bead, plating it onto a horse blood agar plate and incubated as described in section 2.3.

2.6 Sterilisation of Buffers, Media, Solutions and Equipment

All buffers, media, solutions and equipment requiring sterilisation before use were either autoclaved at 121°C, 15psi for 15 minutes or washed with 70% alcohol and 1% Virkon solution (Antec International Limited, Sudbury, UK).

2.7 Media

Various different media were required for the isolation and characterisation of *N. meningitidis* that were not used in this study. These included chocolate blood agar, DST agar with 5% horse blood, horse blood agar, Mueller Hinton agar, Mueller Hinton agar with 5% lysed horse blood, lysed horse blood, Flynn Watkins sugars, Mueller Hinton broth, *o*-nitrophenyl-ß-D-galacopyranoside (ONPG) medium, ONPG stock solution, 0.2% phenol red, 10% saponin and 10% sugar solutions.

2.8 Miscellaneous Reagents

Various other reagents were required for the isolation and characterisation of *N. meningitidis*, that were not used in this study. These included 0.2M acetic acid, blocking buffer for outer membrane protein (OMP) ELISA, dilution buffer for OMP ELISA, dilution buffer for monoclonal antibodies, dilution buffer for protein A conjugate, 0.2M Di-sodium hydrogen orthophosphate, ferric nitrate (0.5%), 1% glutamine, sodium deoxycholate buffer, TMB 3,3,5,5 tetramethylbenzidine solution and TMB 3,3,5,5 tetramethylbenzidine substrate.

2.8.1 Agarose Gel Loading Buffer.

To make 20ml of DNA electrophoresis loading buffer, 2ml of 50x electrophoresis buffer and 4ml of 20% glycerol was added to 14ml of sterile distilled water. Bromothymol blue was added to give a final concentration of approximately 0.001%.

2.8.2 X10 Annealing Buffer.

The measurement of 24.2g of Tris and 10.7g of magnesium acetate-tetrahydrate was dissolved into 900ml of 18MΩ water. The pH was adjusted to 7.6 (at room temperature) with the addition of 4M acetic acid. Finally, 18MΩ water was added to make 1 litre.

2.8.3 X1 Annealing Buffer.

The measurement of 100ml of X10 Annealing buffer was mixed with 900ml 18MΩ water. There was no pH adjustment required.

2.8.4 Production of 5% Carbon Dioxide Atmosphere.

Approximately 10ml of 15% hydrochloric acid was dispensed into a universal container. The universal container with the acid was placed in the plastic holder inside the jar. One sodium bicarbonate tablet was added to the acid in the container, and the lid was quickly place on the jar. The cultures were incubated within this environment overnight at 37°C.

2.8.5 X50 ELFO Buffer.

The measurement of 242g of Tris and 18.61g EDTA were added to 900ml of distilled water. The pH was adjusted to 7.7 with approximately 50ml of glacial acetic acid and then the final volume made to 1000ml with distilled water.

2.8.6 Ethidium Bromide.

To make a 10mg/ml solution of ethidium bromide, 1g of ethidium bromide powder was added to 100ml of distilled water and mixed to ensure that the powder had completely dissolved. The container was wrapped with aluminium foil and stored at 4°C.

2.8.7 20% and 50% Glycerol.

20% glycerol was made by the addition of 20ml of glycerol to 80ml of distilled water. The solution was then mixed thoroughly and autoclaved. 50% glycerol was made by the addition of 50ml of glycerol to 50ml of distilled water.

2.8.8 15% Hydrochloric Acid.

The measurement of 1,190ml of de-ionised water was added into a 2 litre flask. Concentrated hydrochloric acid (810ml) was measured in a 1 litre measuring cylinder. The acid was slowly added to the water, mixing gently and allowed to cool. This was transferred to a 5 litre plastic container.

2.8.9 Orange G.

The measurement of 1g ficol was dissolved in 1ml distilled water. A 0.1% solution of orange G in distilled water was prepared. A 1:10 dilution of 50x electrophoresis buffer was made.

2.8.10 Phenol Chloroform.

Equal amounts of phenol and DNA-grade chloroform were mixed and equilibrated by extracting with 0.1M Tris HCl pH7.6. The equilibrated mixture was stored under a layer of 0.01M Tris HCl pH 7.6 at 4°C.

2.8.11 Phosphate Buffered Saline.

One phosphate buffered saline tablet pH 7.2 was dissolved in 100ml de-ionised water in a glass bottle and autoclave at 115°C for 10 minutes.

2.8.12 Sterile Saline.

Approximately 8.5g – 8.7g of sodium chloride was dissolved in 1 litre of de-ionised water. It was distributed in 2ml amounts in bijoux bottles and sterilised by autoclaving at 121°C for 15 minutes.

2.8.13 0.2M Sodium Acetate.

The measurement of 8.2g sodium acetate was weighed out and mixed in 500ml of distilled water. Distributed in 500ml glass bottle. The solution was then autoclaved.

2.8.14 3M Sodium Acetate.

The measurement of 408g of sodium acetate· $3H_2O$ was added to 800ml of distilled water and mixed well. The pH was adjusted to 5.2 using glacial acetic acid and the volume made to 1000ml with distilled water. The solution was then autoclaved.

2.8.15 5M Sodium Acetate.

The measurement of 116.9g of sodium acetate was dissolved in 300ml of distilled water. Once dissolved the final volume was made to 400ml with distilled water. The solution was then autoclaved.

2.8.16 10% Sodium Dodecyl Sulphate (SDS).

The measurement of 100g of SDS was added to 900ml of distilled water, mixed well and heated to 60°C to aid the dissolving process and adjusted to 1 litre. The pH was then adjusted to 7.2 using concentrated HCl and autoclaved.

2.8.17 1M Sodium Hydroxide.

The measurement of 4g of sodium hydroxide was weighed and added to 90ml of distilled water. Mixed well and transferred into a 100ml glass bottle and made upto 100ml with distilled water.

2.8.18 Substrate Buffer.

The measurement of 25.7ml of 0.2M di-sodium hydrogen orthophosphate was mixed with 24.3ml of 0.1M citric acid, then added to 50ml of de-ionised water and dispensed into a sterile 100ml glass bottle.

2.8.19 2M Sulphuric Acid.

The measurement of 340ml of de-ionised water was measured and transferred into a litre flask and placed in a fume cupboard. 20ml of concentrated sulphuric acid was measured and transferred into a 50ml measuring cylinder. The acid was slowly added to the water, mixing gently throughout the procedure. It was allowed to cool and transferred into 500ml bottles.

2.8.20 0.1M Tris-HCl Buffer.

The measurement of 50ml of 0.2M Tris was mixed with 12ml of 0.2M HCl and made up to 200ml with the addition of distilled water.

2.8.21 X10 TBE Buffer.

The measurement of 9.3g of EDTA was mixed with 27.5g of boric acid and 162g of Trizma base and added to 1 litre of 18MΩ water in a 2 litre conical flask. This was placed on a heated plate with a magnetic stirrer at 56°C and mixed until dissolved. The pH at 56°C was maintained between 7.2 and 7.4. The buffer was transferred into two sterile 500ml Duran bottles and stored at 4°C.

2.8.22 2% Virkon.

The measurement of 2g of virkon powder was weighed out and added to 100ml of distilled water in a glass bottle. The powder was mixed until dissolved.

2.8.23 Wash Buffer.

The measurement of 2.5ml of Tween 80 was added to 10 litres of water. This was mixed well and stored.

2.9 Phenotypic Confirmation and Characterisation.

2.9.1 Biochemical Confirmation and Characterisation.

A number of biochemical methods were required for the characterisation of *N. meningitidis*, which were used by the SMPRL to perform tests outside of this study. These included acid production, antimicrobial susceptibility testing (E-Test), serogrouping by latex agglutination, serogrouping by co-agglutination and whole cell enzyme-linked immunosorbent assay (ELISA) for typing and in cases where identification was not conclusive confirmation, by API NH was carried out. (BioMérieux UK Ltd. Basingstoke).

2.10 Genotypic Confirmation and Characterisation.

A number of biochemical methods were required for the confirmation or characterisation of *N. meningitidis*, which were used by the SMPRL to perform tests outside of this study. These included, the *IS1106* PCR (Knight et al. 1992; Ni et al. 1992).

2.10.1 Primer Design

Primers were designed using the GeneFisher computer program (http://bibserv.techfak.uni-bielefeld.de/genefisher/). Primers specifically for *N. meningitidis* genes were confirmed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

2.10.2 Primers

Specifically designed PCR amplification and DNA sequencing primers were used. Primer annealing temperatures were confirmed using the OligoCalc program (http://www.pitt.edu/~rsup/OligoCalc.html).

Table 2.1 PCR amplification and sequencing primers used for MLST

Associated Gene	Primer Sequence	Reference
Amplification Primers		
porA-F	5'- ATGCGAAAAAAACTTACCGCCCTC – 3'	Suker *et al* 1994
porA-R	5'- AATGAAGGCAAGCCGTCAAAAACA – 3'	Maiden *et al* 1992
abcZ-F	5'- AATCGTTTATGTACCGCAGG – 3'	Maiden *et al* 1998
abcZ-R	5'- GTTGATTTCTGCCTGTTCGG – 3'	Maiden *et al* 1998
adk-F	5'- ATGGCAGTTTGTGCAGTTGG – 3'	Maiden *et al* 1998
adk-R	5'- GATTTAAACAGCGATTGCCC – 3'	Maiden *et al* 1998
aroE-F	5'- ACGCATTTGCGCCGACATC – 3'	Maiden *et al* 1998
aroE-R	5'- ATCAGGGCTTTTTTCAGGTT – 3'	Maiden *et al* 1998
fumC-F	5'- CACCGAACACGACACGATGG – 3'	Feavers *et al* 1999
fumC-R	5'- ACGACCAGTTCGTCAAACTC – 3'	Feavers *et al* 1999

gdh-F	5'- ATCAATACCGATGTGGCGCGT – 3'	Maiden *et al* 1998
gdh-R	5'- GGTTTTCATCTGCGTATAGAG – 3'	Maiden *et al* 1998
pdhC-F	5'- GGTTTCCAACGTATCGGCGAC – 3'	Maiden *et al* 1998
pdhC-R	5'- ATCGGCTTTGATGCCGTATTT – 3'	Maiden *et al* 1998
pgm-F	5'- CTTCAAAGCCTACGACATCCG – 3'	Maiden *et al* 1998
pgm-R	5'- CGGATTGCTTTCGATGACGGC – 3'	Maiden *et al* 1998
abcZ (OF)	5'- TTCTTTTGCCATCGGCCACGT –3'	Diggle *et al* 2003a
abcZ (OR)	5'- GGCATTGCCGTTCTGTTTGGT –3'	Diggle *et al* 2003a
adk (OF)	5'- CCAAAAAGTTGCCGGCTTGTG –3'	Diggle *et al* 2003a
adk (OR)	5'- CTCAAGCCTCCGACAAACTGT –3'	Diggle *et al* 2003a
aroE (OF)	5'- TTCAGACGGCATCGTTCCCAT –3'	Diggle *et al* 2003a
aroE (OR)	5'- GCCTGATGGCGTTTTGAATGC –3'	Diggle *et al* 2003a
fumC (OF)	5'- CCGTCTGAACGCTTTTCAGAC –3'	Diggle *et al* 2003a
fumC (OR)	5'- GAGGGAAAGATTAGCGCGGAT –3'	Diggle *et al* 2003a
gdh (OF)	5'- TCACATATTCCCACGCGGCTT –3'	Diggle *et al* 2003a
gdh (OR)	5'- AATTCTTCGCGCAAGCCTGTG –3'	Diggle *et al* 2003a
pdhC (OF)	5'- AGCTACGCCAACAGCAACTTC –3'	Diggle *et al* 2003a
pdhC (OR)	5'- TCGGGCATTCGGTTTTCAGAC –3'	Diggle *et al* 2003a
pgm (OF)	5'- CGGACGCGGAGATTTTTAAGC –3'	Diggle *et al* 2003a
pgm (OR)	5'- GTGCTATGCCGTCTGAAAACC - 3'	Diggle *et al* 2003a
IS1106-1	5'- ATTATTCAGACCGCCGGCAG –3'	Newcombe *et al* 1996
IS1006-8	5'- TGCCGTCCTGCAACTGATGT –3'	Newcombe *et al* 1996
NMPD1F	5'- CGTAAGCAGATTGGCAGTCAGAATTGC –3'	Diggle *et al* 2003
NMPD1R	5'- GAAGACATATCGGGTGTTTGCCCGAT –3'	Diggle *et al* 2003

ctrAF	5'- TTGTGTGGAAGTTTAATTGTAGGATGC –3'	Guiver *et al* 2000
ctrAR	5'- TCAGATTGTTGCCCTAAAGAGACA –3'	Guiver *et al* 2000
Sequencing Primers		
porA-F	5'- AACGGATACGTCTTGCTC – 3'	Suker *et al* 1994
porA-R	5'- TCCGTACGCTACGATTCTCC – 3'	Maiden *et al* 1992
abcZ-F	5'- GAGAACGAGCCGGGATAGGA – 3'	Maiden *et al* 1998
abcZ-R	5'- GAGAACGAGCCGGGATAGGA – 3'	Maiden *et al* 1998
adk-F	5'- AGGCTGGCACGCCCTTGG – 3'	Maiden *et al* 1998
adk-R	5'- CAATACTTCGGCTTTCACGG – 3'	Maiden *et al* 1998
aroE-F	5'- GCGGTCAACYTACGCTGATT – 3'	Maiden *et al* 1998
aroE-R	5'- ATGATGTTGCCGTACACATA – 3'	Maiden *et al* 1998
fumC-F	5'- TCGGCACGGGTTTGAACAGC – 3	Feavers *et al* 1999
fumC-R	5'- CAACGGCGGTTTCGCGCAAC – 3'	Feavers *et al* 1999
gdh-F	5'- CCTTGGCAAAGAAAGCCTGC – 3'	Maiden *et al* 1998
gdh-R	5'- GCGCACGGATTCATATGG – 3'	Maiden *et al* 1998
pdhC-F	5'- TCTACTACATCACCCTGATG – 3'	Maiden *et al* 1998
pdhC-R	5'- ATCGGCTTTGATGCCGTATTT – 3'	Maiden *et al* 1998
pgm-F	5'- CGGCGATGCCGACCGCTTGG – 3'	Maiden *et al* 1998
pgm-R	5'- GGTGATGATTTCGGTTGCGCC 3'	Maiden *et al* 1998

2.10.3 Preparation of Agarose Gel

The appropriate amount of powder was added to 100ml ELFO buffer to obtain the required percentage agarose. The usual concentrations used were 1% and 2% and therefore 1g and 2g of agarose powder were added to 100ml ELFO buffer respectively. The solution was heated with care until the agarose was fully dissolved.

For each 100ml solution 5ul of ethidium bromide (10mg/ml) was added. The agarose solution was then stored at 55°C until required.

2.10.4 Agarose Gel Electrophoresis

Appropriate amounts of amplified DNA were transferred to the appropriate wells of an agarose gel in an electrophoresis tank; this ranged from between 5 and 10µl. Each sample of amplified DNA already contained a dye and precipitant from the master mix, this facilitated gel loading and visualisation under UV light.

2.10.5 *CtrA* Dual End-Point Fluorescence (DEF)-PCR.

This was based on a procedure described by Diggle *et al* (Diggle et al. 2001a). DNA was extracted using the previously described method. After DNA extraction, samples were transferred into 1.5ml NCC tubes and placed in the sample rack of the Roboseq 4204 SE robotic liquid handling system, possessing an integrated thermocycler and fluorescence reader (MWG Biotech, Milton Keynes, United Kingdom). All components of the system were in a 96-well microtiter plate format, allowing standardization and high-throughput methodology. Programming of the liquid handling robot was performed according to the manufacturer's instructions. All PCR reagents were maintained at 4°C on the robotic platform. Each reaction was performed in a final volume of 25µl consisting of 20µl of 1.1x Reddymix PCR Master Mix (ABgene) containing 1.25 U of *Taq* DNA polymerase; 75 mM Tris-HCl (pH 8.8 at 25°C); 20 mM $(NH_4)_2SO_4$; 1.5 mM $MgCl_2$; 0.01% (v/v) Tween 20; a 0.2mM concentration (each) of dATP, dCTP, dGTP, and DTTP; 1µl of each primer (1 pmol) (MWG Biotech); 1µl of dual-labelled probe (0.5 pmol) (MWG Biotech); and 2µl of extracted DNA. Each reaction was set up automatically by the robot within a

refrigerated 96-well microtitre plate using disposable tips. Cross-contamination was avoided by the use of these tips, which were discarded automatically into a waste container. After PCR setup, optically clear disposable strips (ABgene) were manually placed over the wells to seal the contents. The microtitre plate was then automatically placed into the integrated thermocycler for the period of thermocycling. After amplification, the microtitre plate was automatically removed from the thermocycler into the integrated Bio-Tek FL600 fluorescence plate reader. Selected wavelengths of 485 to 420 nm and 530 to 525 nm for excitation and emissions respectively were used to detect the fluorescence emissions caused by carboxyfluorescein. A total of 100 endpoint readings were taken from each well, and the average was calculated using the KC4 software (MWG Biotech). The KC4 software was programmed to calculate a cutoff value based on the subtraction of the average of the three controls from the positive control. Serogrouping of *ctrA*-positive samples was performed using the *siaD* gene, as described.

2.10.6 PCR for Serogroups B and C

2.10.6.1. *SiaD* Gene Amplification. PCR grouping was performed on all phenotypically non-groupable meningococci and on all clinical samples which gave a positive result for the *ctrA* meningococcal PCR (Borrow et al. 1997). The PCR mix used 300µl of 1.1x Reddymix PCR Master Mix (ABgene) containing 1.25 U of *Taq* DNA polymerase; 75mM Tris-HCl (pH 8.8 at 25°C); 20mM $(NH4)_2SO_4$; 3.5mM $MgCl_2$; 0.01% (v/v) Tween 20; a 0.2mM concentration (each) of dATP, dCTP, dGTP, and DTTP, to each of six eppendorf tubes. 4µl of positive sense primer at 50pmol concentration and 4µl of negative sense primer at 50pmol concentration was added to

each eppendorf (these primers are designed specifically for the amplification of serogroups B and C). This was mixed well and store at 4^0C. A single tube of PCR B and C grouping PCR master mix and a single tube of both group B and group C DNA extracted controls was removed from storage. These were mixed well and an aliquot of 40µl of PCR master mix was transferred into the appropriate number of PCR tubes (one tube per test). 10µl of purified water was added to one tube (negative control) and 10µl of control DNA to a further two tubes (positive B and C controls). 10µl of test DNA was transferred into the remaining tubes. This was mixed well and the caps secured. Placed into the thermocycler and the specific program started. The PCR conditions were 95°C for 5 min, 40 cycles of 94°C for 25 sec, 53°C for 40 sec (temperature gradient of 2.0°C/sec), 72°C for 1min (temperature gradient of 2.0°C/sec) and finally 72°C for 5 min.

2.10.6.2. RFLP of the *siaD* Gene in Serogroups B and C. For each test 11µl of purified water, 3µl 10x restriction buffer and 1µl of Taq 1 was added to a PCR tube. 15µl of the amplified *siaD* gene product was added from the previous stage. This was mixed and placed into the thermocycler. The digest conditions were 65°C for 90 min. After digestion they were removed from the thermocycler and the products generated from the first stage and the second stage were loaded on a 2% agarose gel. A 100bp DNA molecular weight ladder was loaded alongside the samples. The electrophoresis conditions were 50 volts for 15-20 min. The gel was removed from the electrophoresis tank and place under UV light to examine the products. The products sizes were as follows, initial PCR product of 460 bp and digested products of 460 bp for serogroup B and 207 bp, 200 bp and 53 bp for serogroup C.

2.10.7 PCR for Serogroups Y and W135

2.10.7.1. *SiaD* Gene Amplification. PCR grouping was performed on all phenotypically non-groupable meningococci and on all clinical samples that gave a positive result for the *ctrA* meningococcal PCR (Borrow et al. 1998). To create the PCR mix, 300µl of 1.1x Reddymix PCR Master Mix (ABgene) containing 1.25 U of *Taq* DNA polymerase; 75mM Tris-HCl (pH 8.8 at 25°C); 20mM $(NH_4)_2SO_4$; 3.5mM $MgCl_2$; 0.01% (v/v) Tween 20; a 0.2mM concentration (each) of dATP, dCTP, dGTP, and dTTP, was added to each of 6 eppendorf tubes. Seven microtitres of positive sense primer at 50pmol concentration and 7µl of negative sense primer at 50 pmol concentration was added to each eppendorf (these primers are designed specifically for the amplification of serogroups Y and W135). These were mixed well and store at 4^0C. A single tube of PCR Y and W135 grouping PCR master mix and 1 tube of both group Y and group W135 DNA extracted controls were removed from storage. These were mixed well and 40µl of PCR master mix was transferred into selected PCR tubes (one tube per test). 10µl of purified water was added to one tube (negative control) and 10µl of control DNA to a further two tubes (positive Y and W135 controls). 10µl of test DNA was transferred into the remaining tubes. These were mixed well and the caps secured. The samples were placed in the thermocycler and the specific program started. The PCR conditions were 95°C for 5 min, 40 cycles of 94°C for 25 sec, 53°C for 40 sec (temperature gradient of 2.0°C/sec), 72°C for 1min (temperature gradient of 2.0°C/sec) and finally 72°C for 5 min.

2.10.7.2. RFLP of the *siaD* Gene in Serogroups Y and W135. For each test 11µl of purified water, 3µl 10x restriction buffer and 1µl of *XbaI* was added to a PCR tube.

15µl of the amplified *siaD* gene product from the previous stage was added. This was mixed and place into the thermocycler. The digest conditions were 37°C for 90 min. After digestion they were removed from thermocycler and the products generated from the first stage and the second stage were loaded on a 2% agarose gel. A 100bp DNA molecular weight ladder was run alongside the samples. The electrophoresis conditions were 50 volts for 15-20 min. The gel was removed from the electrophoresis tank and placed under UV light to examine the products. The products sizes were as follows, initial PCR product of 460bp and digested products of 460bp for serogroup Y and smaller fragments for serogroup W135.

2.10.8 MLST DNA Amplification.

This was based on a procedure described by Clarke *et al.* (Clarke et al. 2001b). Programming of the RoboAmp-4200 ® liquid handling system was performed according to the manufacturer's instructions (figure 2.1). All PCR reagents were maintained at 4°C on the platform. Each PCR reaction was performed in a final volume of 25µl using 1.1 x Reddymix™ PCR Master Mix, containing 1.25U of *Taq* DNA polymerase (Abgene ®, Surrey, UK), 75 mM Tris-HCl (pH 8.8 at 25°C), 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.01% (v/v) Tween 20, 0.2 mM each of dATP, dCTP, dGTP and dTTP and red dye for gel electrophoresis. For a 25µl reaction, 20µl of PCR master mix and 1µl of each MLST or *por*A primer pair were added to produce a master mix volume of 22µl. These pre-prepared master mixes were placed on the RoboAmp-4200® refrigerated reagent rack and the DNA preparation samples were placed on the sample area. Within a refrigerated non-cross-contamination (NCC) 96-well plate, 22µl of master mix was automatically added to appropriate wells using a washable tip along with 3µl of DNA preparation, making a final 25µl reaction mixture.

After each stage of the set-up, the washable tip was automatically washed with 2ml of 18MΩ distilled water. The NCC 96-well plate was automatically placed into the integrated MWG-Biotech Primus 96 thermocycler. The PCR conditions were altered from Maiden and colleagues to a step-down PCR method to ensure complete and reproducible amplification of all eight genes. The step-down PCR conditions were 94°C for 2 min; 3 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 2 min; 3 cycles at 94°C for 1 min, 58°C for 1 min and 72°C for 2 min; 3 cycles at 94°C for 1 min, 56°C for 1 min and 72°C for 2 min; 20 cycles at 94°C for 1 min, 54°C for 1 min and 72°C for 2 min; and finally 72°C for 10 min. After PCR the NCC plate was automatically removed from the thermocycler to a refrigerated block.

2.10.9 Liquid Phase PCR Product Purification

A 5µl aliquot of each PCR product was subsequently transferred into another NCC 96-well plate. 0.5U of Shrimp Alkaline Phosphatase (SAP) (USB Corporation, Ohio, USA) and 0.1U of Exonuclease I (USB Corporation, Ohio, USA) were added to each PCR product and automatically placed in the integrated thermocycler. The cycle conditions were 37°C for 15 min followed by 80°C for 15 min. This process removed unused primers and dNTP's that could interfere with sequencing.

2.10.10 Solid Phase PCR Product Purification

This was based on a procedure described by Clarke *et al.* (Clarke and Diggle 2002). A Millipore MultiScreen™ 96 or 384-PCR plate (Millipore, Watford, UK) was placed on a vacuum manifold; this was done automatically onto the integrated vacuum

RoboManager NT

GLP Report

ID No.	Creation Date	Script Name
1	00/00/0000	C: \RoboManagerNT\Scripts\Routine\MenMLST

Report

User Name: Mathew
Computer Name: SMPRL
Software: C:\RoboManagerNT\RoboManager.exe
Software Version: 4. 0. 0 Build 798
Database Engine: SDE 5. 14. 03 (p4) 32 bit, Apollo 5. 0. 2. 0, 12/2/1999
Robot Type: Rosys
Robot SN:

```
SCRPIT LISTING:

Include C: \RoboManagerNT\Config\Config2397.rss
0001    Initialise System
0002    Flush Tips
0003    Flush Tips
0004    Flush Tips
0005    Flush Tips
0006    Flush Tips
>Platform equipment
0008    Put Empty Plate "Men MLST Amplification plate 1-12" on P1
0009    Put Empty Plate "DNA extracts 1-12" on P3
0010    Put Empty Tube " AMP Mix" on Positions A1,B1,C1,D1,E1,F1,G1,H1 on Reagent Rack
0011    Put Empty Tube " SEQ F Mix" on Positions A2,B2,C2,D2,E2,F2,G2,H2 on Reagent Rack
0012    Put Empty Tube " SEQ R Mix" on Positions A3,B3,C3,D3,E3,F3,G3,H3 on Reagent Rack
0013    Put Empty Tube "PCR re-elution 1-6" on position 1 on Reagent Rack
0014    Put Empty Tube "PCR re-elution 7-12 on position 2 on Reagent Rack
0015    Put Empty Tube "SEQ re-elution 1-6" on positions 3,4 on Reagent Rack
0016    Put Empty Tube "SEQ re-elution 7-12" on positions 5,6 on Reagent Rack
0017    Put Empty Plate "Plate sealer 1-6" on position P2
0018    Put Empty Plate "Plate sealer 1-6" on position P4
0019    Put Empty Plate "SEQ plate 1-6" on position A1 on refrigeration stacker
0020    Put Empty Plate "SEQ plate 1-12" on position A2 on refrigeration stacker
0021    Put Empty Plate "MEGA plate 1-6" on position B1 on refrigeration stacker
0022    Put Empty Plate "MEGA plate 7-12" on position B2 on refrigeration stacker
0023    Put Empty Plate "PCR Millipore" on position A5 on refrigeration stacker
0024    Put Empty Plate "SEQ Millipore" on position B5 on refrigeration stacker
0025    Put Tip Rack Starlab on Tip Rack Slot 1
0026    Put Tip Rack Starlab on Tip Rack Slot 2
0027    Put Tip Rack Starlab on Tip Rack Slot 3
0028...Put Tip Rack Starlab on Tip Rack Slot 4
0029    Put Tip Rack Starlab on Tip Rack Slot 5
0030    Put Tip Rack Starlab on Tip Rack Slot 6
```

```
SCRIPT RUN PROTOCOL:

09:00:00:    0001:    Initialise System
                      Current Tip Tool Type: Disposibe
09:00:30:    0002:    FlushTips
09:00:46:    0003:    FlushTips
09:01:02:    0004:    FlushTips
09:01:18:    0005:    FlushTips
09:01:34:    0006:    FlushTips
09:01:48:    0007:    >Platform equipment
09:01:48:    0008:    Put Empty Plate "Men MLST Amplification plate 1-12" on P1
                      Plate "Men MLST Amplification plate 1-12" was put on P1
```

manifold using the Roboseq liquid handling robot. The entire volume (range 25µl - 50µl) of each post-PCR reaction was added, up to a maximum volume of 100µl, to the appropriate well of the Multiscreen 384 well plate. The PCR product was purified by applying a vacuum at 350 millibars pressure for 15 min or until the wells were dry. To ensure full purification, the vacuum was applied for a further 1 min after the last well was dried. The PCR products were re-suspended by adding 20µl of 18MΩ distilled water and repeated pipetting a volume of 15µl 100 times slowly at 50% pipetting speed on the liquid handling robot. The purified PCR products for 24 MLST samples were redistributed into four 96-well microtitre plates.

2.10.11 Licor 4200 PCR Sequence-Labelling

This was based on a procedure described by Clarke *et al.* (Clarke et al. 2001b). A 3µl aliquot of each purified PCR product was automatically transferred using the washable tip into an open 96-well microtitre plate. From a refrigerated block on the platform, a pre-dilution was performed by adding 24µl of 18MΩ distilled water and 1.5µl of both positive and negative sense sequencing primer specific for each PCR product. All positive sense sequencing primers were 700nm infra red dye (IRD) tagged and all negative sense sequencing primers were 800nm IRD tagged. 4µl of each pre-diluted sequence mix was distributed into appropriate wells of another open 96 well plate, containing 2µl A, C, G and T from a Thermo Sequenase fluorescent labelled primer cycle sequencing kit (Amersham Pharmacia Biotech, Amersham, UK). Finally, 15µl of Chill-out 14 liquid wax (Genetic Research Instruments, USA) was added to each well. Appropriate washing of the washable tip occurred with 18MΩ distilled water throughout the procedure. The plate was automatically placed into the integrated thermocycler. The sequence cycle conditions were 95°C for 2 minutes, 30

cycles of 95°C for 15 seconds, 50°C for 30 seconds, 70°C for 30 seconds, and finally 72°C for 10 minutes. Afterwards the plate was automatically removed from the thermocycler and placed onto a refrigerated block. A 4µl aliquot of formamide loading dye / stop solution (Amersham Pharmacia Biotech, Amersham, UK) was automatically added to all 96 wells with the washable tip and subsequently placed into the integrated thermocycler at 65°C for 10 minutes. The process took approximately 8 hours for full automation from start to finish (figure 2.0).

2.10.12 MegaBACE 1000 Sequence-Labelling

Sequencing was performed using dideoxy dye-labeled terminators. At the start of the sequence set-up procedure, sequence master mixes for positive and negative sense nucleotide sequences of each of the eight genes of *N. meningitidis* were made. This consisted of fourteen serum tubes contained within a refrigerated reagent rack of the liquid handling robot. Each sequence master mix was made up from 4µl of sequence mix (DYEnamic™ ET Terminator sequence premix, Amersham Biosciences, Little Chalfont, UK) plus 1µl of positive or negative sense primer (5pmol/µL concentration) per 10µl of reaction mixture. 5µl of master mix was automatically transferred into the wells of a 96-well plate containing 5µl clean PCR product. Once the transfer was complete the plate was transferred into a thermocycler contained within the robotic platform.

2.10.13 Solid Phase Sequence Product Purification

This was based on a procedure described by Clarke and Diggle (2002) (Clarke and Diggle 2002). After performing the cycle sequence labelling reaction, according to previously described protocols, a Millipore MultiScreen™ 96 or 384-SEQ plate was

placed on a vacuum manifold; this was done automatically onto the integrated vacuum manifold using the Roboseq liquid handling robot. The volume was adjusted to 20µl with 0.3 mM EDTA (pH 8.0) prepared with 18MΩ distilled water. The entire volume of each post PCR reaction was transferred, usually ranging between 25µl -50µl depending on the gene being sequenced, up to a maximum volume of 100µl, onto the appropriate well of the Multiscreen plate. The product was purified applying a vacuum of 850 millibars for 5 min or until the wells were dry. The purified sequence labelled products were re-suspended by adding 20µl of 18MΩ distilled water and repeatedly pipetting a volume of 15µl 20 times, slowly at 50% pipetting speed using a liquid handling robot. The purified sequence-labelled products were redistributed into plates ready for DNA sequencing. When using the Multiscreen SEQ-384 plates for 24 MLST samples, the products were redistributed into four 96-well microtitre plates.

2.10.14 Single-Stranded DNA Sample Preparation for Sequencing.

This was based on a procedure described by Diggle and Clarke (2003c) (Diggle and Clarke 2003c). The liquid handling robot transfered a volume of 5 U/µl of lambda exonuclease (cloned) (Amersham Pharmacia Biotech) into 25ul of PCR product contained on the robot within a non-cross contamination plate with a washable tip. After each stage of the set-up, the washable tip was automatically washed with 2ml of 18MΩ distilled water. After liquid distribution, the microtitre plate was automatically placed into the integrated MWG-Biotech Primus 96 thermocycler. The thermocycler incubation was 37°C for 30 mins. To achieve optimum conditions for DNA concentration all parameters were assessed and evaluated including, enzyme concentration and length of incubation (Diggle and Clarke 2003c). After the cycle, the microtitre plate was automatically removed from the thermocycler onto a refrigerated

block. The single stranded DNA was then removed from each well and placed onto an open well plate and transferred onto another robot for the single stranded DNA clean-up procedure (Diggle and Clarke 2003c).

2.10.15 Single-Strand DNA Sample Clean-up for Sequencing.

This was based on a procedure described by Clarke and Diggle (2002) (Clarke and Diggle 2002). The liquid handling robot transferred each sample into a Millipore SEQ-384 clean-up plate (Millipore U.K. Ltd. Hertfordshire, UK). A vacuum was automatically applied and after 20 minutes the dry wells were re-eluted with 18mΩ distilled water (Clarke 2002c). A vacuum was reapplied to the wells and again after 20 minutes the dry wells were re-eluted with x1 annealing buffer (made to Pyrosequencing specifications).

2.10.16 Licor-4200 DNA Sequencing System

2.10.16.2 Preparation of Acrylamide Gel Mixture. 7.5ml of Rapid-Gel XL Solution was carefully measured out (40% concentrate) (USB, Ohio, USA) into a 100ml Duran bottle. 4ml of formamide, 21g of molecular grade urea, 5ml of X10 Tris buffer (162g Tris Base, 27.5g boric acid, 9.3g EDTA in 1 litre of 18MΩ distilled water) and 28mls of 18MΩ water was added. A magnetic pellet was placed into the bottle which was then sealed. This was placed onto a magnetic stirrer to dissolve the contents. Once dissolved, the pellet was removed and the bottle was placed into a sonicator bath for 3 minutes. This removed any air bubbles from the mixture. During sonication, 1.5ml of 18MΩ water was transferred into a specimen tube and a 150mg of ammonium persulphate was added and dissolved. After sonication, 350µl of the ammonium persulphate solution was added to the Duran bottle with 75µl of Temed

solution. This solution was quickly poured using a syringe into the prepared gel plate at a 45-degree angle, as described further in section 1.10.21.3. Once the ammonium persulphate and Temed solutions have been added the mixture has a limited self-life before solidification occurs.

2.10.16.3 Pouring of Acrylamide Gel. Using a pouring gel block the sequencing plates were placed into position. Using a 100ml syringe, approximately 40ml of acrylamide gel mixture was removed from the container and the injection into the gap between the two plates was performed, the syringe was moved from side to side between the two edges of the plate to get an even distribution of gel between the two plates. The gel mixture subsequently flowed down the inside of the plates combined with sporadic tapping ensuring no air bubble formation. Once the gel had reached the bottom of the plates, they were lifted and placed into a horizontal position. A Licor comb was inserted into the top of the plate creating a well at the top of the plate. This trough was later to be used in conjunction with the teeth from a comb to create individual wells for loading sequenced samples. Finally, an additional 350ml of ammonium persulphate solution and 75ml of temed solution were added into the remaining gel mixture and using the syringe, this was added to the top of the gel onto the comb to create an air-tight seal. The gel was left to set in a horizontal position for at least two hours at room temperature.

2.10.16.4 Preparation of Acrylamide Gel for Sequencing. Using white paper towels, excess gel matrix from around the Licor comb in place at the top of the gel plate was removed. Once the majority of the gel was removed, the comb was flooded with 18MΩ water. Once flooded, the comb was slowly removed from the gel plate

leaving a well in place at the top of the plate. Any excess gel matrix was removed using clean white paper towels. The back and front of the gel plates were cleaned with 70% alcohol and left to dry before placing into the Licor sequencer.

2.10.16.5 Loading Gel Plate. With the Licor 4200 in sleep mode the sequencing door was opened. The bottom buffer tank was placed into position on the bottom of the Licor sequencer. The prepared gel plate was loaded vertically into the sequencer and the side arms were slotted into the catches on the sequencer. The top buffer tank was placed into position at the top of the plate and tightened into position. 100ml of X10 Tris buffer was added into a 1 litre measuring cylinder and made up to 1 litre with 18MΩ water and mixed. The top buffer tank was filled with the X1 Tris buffer upto the maximum mark. The remaining X1 Tris buffer was carefully added to the lower tank. The lids on the top and bottom buffer tanks were placed on and the main sequencer door closed. The pre-run protocol was performed following the displayed commands.

2.10.16.6 Performing a Sequence Pre-run. After the gel plate was placed into the sequencer, the Licor sequencer software was opened and a sequence run file based on the present date, the initials of the user and the type of samples being sequenced was created. After the file was created the "begin pre-run" icon was selected and the sequencer began to focus the gel with respect to the position of the lasers before the samples were added.

2.10.16.7 Loading Sequenced Samples. After the pre-run protocol was complete, the "load samples" icon was selected on the computer screen. This ensured the high

voltage and laser light had automatically deactivated. The Licor sequencer door was then opened. The top buffer tank lid was removed and a white sheet of paper was placed behind the top of the plate between the plate and the heated sequence panel. This allowed the user to see samples being loaded into each well. The well was washed with the buffer solution within the top tank using a P5000 Gilson pipette. After well preparation, approximately 20µl of formamide loading dye was added along the length of the well to visualise the top of the gel. An appropriately sized comb was placed into the trough using the teeth to pierce the gel and create numerous well compartments. The loading dye was flushed out of the well compartment with a Gilson pipette and 1µl of each sample was added to each compartment on the comb until all samples were added. After the samples had been added, 1µl of formamide loading dye was added to either end of the comb. The top buffer tank lid was replaced and the white sheet from behind the plate was removed. The sequencer door was closed and the "start run" icon on the computer screen was selected.

2.10.16.8 Dismantling and Cleaning the Gel Plates. After sequencing was complete both the high voltage and the laser were automatically deactivated. The door was opened and the top and bottom buffer tank lids were removed and washed with warm water, dried and stored. Using the siphon tube the buffer solution from the top buffer tank was removed and discarded. The top buffer tank was removed and washed thoroughly with warm water, dried and stored. The gel plate was lifted out of the sequencer and placed horizontally on the gel-pouring block. The buffer from the bottom tank was removed and the tank was washed with warm water, dried and stored. The side screws holding the two plates together were loosened and removed leaving the two plates. With a separator, the two plates were carefully prised apart

exposing the used gel matrix. Using dry paper towels, the exposed gel was covered. After removing the paper towels removed the attached matrix was discarded in special waste containers. Once the matrix had been removed, the plates were washed with warm water and dried. The plates were stored in a special plate rack to ensure safe storage.

2.10.17 MegaBACE 1000 DNA Sequencing System

2.10.17.1 Preparation of Sequence Capillaries. Within the sequencing software the instrument control window was opened and the "prepare capillaries" icon was selected. The instructions on the instrument displays were followed. An empty water tank was loaded into the plate drawer and six water tubes were loaded into the tube drawer on the instrument. After re-filling the water tubes and several flush procedures the capillaries were adequately washed for either storage or matrix pre-fill.

2.10.17.2 Performing a MegaBACE 1000 Sequence Run. Within the sequencing software the instrument control window was opened and the "matrix pre-fill" icon was selected. The instructions were followed and six matrix tubes that have been prepared were loaded into the tube drawer. A prepared buffer plate containing TBE buffer was placed into the plate drawer. Equilibrium with the matrix was achieved and the instructions were followed samples injected and run protocols were followed. A plate containing clean sequence DNA was placed into the plate drawer and predetermined injection of DNA into the capillaries was performed. Once DNA injection was complete the plate was removed and stored at –20°C and the buffer tank was re-loaded for sequencing.

2.10.18 Sequence Interpretation for MLST Gene Fragments

Sequences were automatically read from the Licor L4200 sequencer with the integrated Licor base-calling software and from the MegaBACE 1000 sequencer with the integrated Cimarron v1.53 Slim Phredify base-calling software. The post sequence analysis software was based on a procedure described by Diggle and Clarke (2002a) (Diggle and Clarke 2002a). This was an adapted and modified alignment program which was held on a local department network and accessed through Windows operating software. The original DiscoverIR software was commercially available (Licor Biosciences Cambridge, UK). DiscoverIR10b software was installed using the manufacturer's instructions. Within the software there were eight main folders, two of which were of importance, namely (1) database and (2) sample. Within the database folder there were tdb. files which were used to hold the information for the database. New tdb. files were created as required; this would consist of 7 files each containing an MLST housekeeping gene with all the allele variations and number assignments currently available. The list of alleles was created by procuring annotation for the sequence (a descriptive tag for that sequence type) e.g. DATA;abc*01;. This annotation gave the type of information (data), the gene name (*abcZ*) and the sequence type (01). This annotation was followed with the actual sequence directly after the semi-colon. This was repeated for each allele starting on a new line for each one. For each new gene, a new tbd. file was created and the same process repeated. Once this was completed, a database of different files would exist containing alleles for MLST identification.

After MLST analysis program set-up, raw sequence data for each sequence was viewed as an electropherogram and this sequence was converted into FASTA (text)

format. This could be done using the sequencer programs. Each text file containing the sequence was downloaded into the previously described local database, containing the meningococcal MLST alleles. Using the MLST analysis software, the sample sequence was compared with all the other similar allele variants to produce a match and an allele number. This step could also be accomplished through the MLST website (www.mlst.net) or NCBI Blast server (www.ncbi.nih.gov/blast). Once all 7 genes for a given meningococcal isolate had been assigned allele numbers, this resulting 7-digit number was placed into the MLST web site (http://neisseria.org/nm/typing/mlst/). The sequence type (ST) could then be determined from a particular combination of alleles. When a new allele number or ST was detected, the trace files and isolate data were sent via a spreadsheet on the MLST web page to the MLST database curator for verification before its allocation and addition to the *N. meningitidis* MLST database (http://neisseria.org/nm/typing/mlst/). A new allele number would be given to the appropriate locus and a new ST type would be assigned to that combination of alleles.

2.10.19 Sequence Interpretation of *por*A Gene Fragments

After sequence confirmation and editing, the *porA* gene sequence was converted from its nucleotide sequence into an amino acid sequence using the Translate program (http://expasy.cbr.nrc.ca/tools/dna.html). The variable regions were identified and VR1 and VR2 downloaded onto the *por*A website (http://neisseria.org/nm/typing/pora/) where a variant number was assigned. New variants were sent to the Centre for the Epidemiology of Infectious Disease at the University of Oxford. The *por*A database was updated with all new variants only after detailed checks of all data collected and appropriate tests were repeated. The variant

number for VR3 was compared to data contained on the SMPRL website (http://www.show.scot.nhs.uk/smprl/)

2.11 Analysis of MLST Data

Analysis of initial nucleotide sequence data was conducted by a number of different computer analysis programs. This allowed detailed scrutiny of each sample that produces over eight thousand single nucleotide bases. There are a number of different programs available that compare the sequence generated with the meningococcal MLST database of all known allele sequences.

2.11.1 Basic Local Alignment Search Tool (BLAST).

This is a web-based tool that is used to compare sample nucleotide or protein sequence with that contained within the BLAST database of submitted nucleotide sequences worldwide. The nucleotide sequence data obtained from the sequencer is electronically pasted into the appropriate window within the BLAST website's selected nucleotide-nucleotide alignment icon (www.ncbi.nih.gov/blast). Depending on the demand this will take between seconds and minutes to conclude and display the results. This is used to check unknown sequence to either identify or confirm both the quality and/or origin of the sample nucleotide sequence.

2.11.2 Sequence Type Analysis and Recombinational Tests (START).

This is a web-based package of various tests which allow analysis of MLST data from *N. meningitidis* (Jolley 2001). This package can be separated into four main categories including summary of data, lineage assignment, recombinational tests and

tests for selection.

2.11.2.1 Data Summary – Allele Frequencies. Once the *N. meningitidis* allelic profiles had been entered, allele frequency analysis was performed. To run the analysis, the following icons were clicked "Analysis ... Data Summary ... Allele Frequencies". A table was displayed showing the frequencies of each allele at each locus. At the bottom of the table, the total number of different alleles present at each locus in the dataset was displayed.

2.11.2.2 Data Summary – Profile Frequencies. Once the allelic profiles were entered, profile frequency analysis was performed. To run the analysis, the following icons were clicked "Analysis ... Data Summary ... Profile Frequencies". A table was displayed showing the frequencies of each profile in the dataset in frequency order.

2.11.2.3 Data Summary – Polymorphism Frequencies. To determine polymorphism frequencies, both the allelic profiles and the allele sequences were loaded onto the software. To run the analysis, the following icons were clicked "Analysis ... Data Summary ... Polymorphism Frequencies". First a sequence map, showing the positions and identities of all polymorphic sites at each locus are displayed. Underneath this was a table that displayed the number of alleles in the dataset that have a particular nucleotide at each polymorphic site. If only one allele had a certain nucleotide, that allele was highlighted in bold. On the right-hand side of the table, data for strains in the dataset was handled in the same way.

2.11.2.4 Lineage Assignment – Based Upon Related Sequence Types (BURST). This was used to examine the relationships within clonal complexes while the relationships between different clonal complexes were ignored. BURST required allelic profile data only, and these also contained their ST numbers. To run the analysis, the following icons were clicked "Analysis ... Lineage Assignment ... BURST". Initially a dialog window, offering a number of options, was displayed. The first of these was the definition of a group. The number of loci that members of a group must share with at least one other member of the group was entered, which for this study was five. This means that for MLST data based on 7 loci, a cut-off point of 5 identical loci allows the inclusion of strains belonging to a single clonal complex, while excluding those that do not.

The next series of options concerned the display of a summary view. If an ancestral type was identified for a group, a summary view was displayed. This was displayed by a series of concentric circles surrounding the ancestral type. The middle circle contained STs that vary at a single locus (single-locus variants - SLVs) and the outer contained STs that vary at two loci (double-locus variants - DLVs). Outside of this circle were satellite STs that vary by more than two loci from the central type. STs were alos linked by lines showing whether they were SLVs or DLVs of other STs. The dialog window provided the option to select the colours and line styles of links, or whether or not to show SLV or DLV links. The program displayed a series of tables showing the members of each group and the number of SLVs, DLVs and satellites each of ST. If an ST had been identified as an ancestral type, its ST number would have an asterisk next to it and there would be a summary view, as described previous.

2.11.2.5 Lineage Assignment – UPGMA. The algorithm used a distance matrix constructed from allelic profile data only. Allele sequences were not used, so each allele number difference was treated identically. This is because in systems involving recombination, a single genetic event, i.e. recombination, may result in a large number of altered sites. This analysis required only the allelic profiles. To run the analysis, the following icons were clicked "Analysis ... Lineage Assignment ... UPGMA". Output displays were in the form of a tree. Allelic profiles were displayed along with the isolate numbers. The tree could be saved as a Windows Metafile (WMF) by right-clicking over it with the mouse and selecting "Save Picture As". This could then be imported into other packages for editing if required.

2.11.2.6 Tests for Recombination - Index of Association. The Index of Association (I_A) was calculated as follows: $I_A = V_O/V_E - 1$ if V_O was the observed variance of K and V_E was the expected variance of K, where K is the number of loci at which two individuals differ. If there was linkage equilibrium because of frequent recombination events, the expected value of I_A was zero. Clonal populations were identified by an I_A value that differed significantly from zero. This analysis required only the allelic profiles. To run the analysis, the following icons were clicked "Analysis ... Tests for Recombination ... Index of Association".

2.11.2.7 Tests for Selection - *d*S/*d*N Ratio. Nucleotide substitutions in genes coding for proteins can be either synonymous (no change of amino acid), alternatively called silent substitutions, or non-synonymous (change of amino acid). Usually, most non-synonymous changes would be expected to be eliminated by purifying selection, but under certain conditions Darwinian selection may lead to their retention. Investigating

the number of synonymous and non-synonymous substitutions may therefore provide information about the degree of selection operating on a system. This analysis required both allelic profiles and the allele sequences to be loaded. This analysis also required that the open reading frames were identified for each locus although the program would prompt for this information. If necessary, it would take you to the "Reading Frames" dialog where these could be auto-detected. To run the analysis, the following icons were clicked "Analysis ... Tests for Selection ... dS/dN ratio". A classical output result would include the mean number of synonymous and non-synonymous sites together with the number of coding sites analysed, number of pairwise comparisions made, the mean synonymous substitutions per synonymous sites, the standard deviation and confidence intervals, mean non-synonymous substitutions per non-synonymous sites with standard deviations and confidence intervals and finally the d_N/d_S value and the d_S/d_N value.

Chapter 3

Multi-Locus Sequence Typing (MLST)

3.0 Introduction

Using previously mentioned techniques this chapter concentrates on the characterisation of *N. meningitidis* causing disease in the Scottish population primarily using DNA sequencing technology. These meningococci were characterised and the molecular epidemiology over a number of recent years analysed in detail.

There are numerous groups within the meningococcal population that are commonly associated with disease, these include A, B, C, Y and W135. In the past ten years the most recent involved the domination of group C meningococci causing disease during the 1990s within the UK. Throughout the 1990s there was a significant increase of group C MD. This was seen more commonly in older teenagers and had a case fatality rate much higher than in other age groups. The continuing significant presence of group C MD coincided with the development and production of a group C meningococcal conjugate vaccine and subsequent implementation in a UK national vaccine campaign. This campaign required rapid implementation and thus mobilisation of health care professionals at both local and national level to achieve a successful administration of a MenC vaccine was undertaken in November 1999. This time scale was favoured owing to the need to prevent increasing numbers of outbreaks primarily effecting school pupils during the forthcoming winter season. The conjugate vaccine was therefore initially offered to high-risk groups (under 18 years old) and then

subsequently a follow-up immunisation campaign was implemented to all ages less than 24 years of age. This schedule was completed by the end of 2000 and in total, has immunised more than 15 million children and young people during a one-year period.

These recent changes resulting in group C dominance in the meningococcal population in Scotland add credence to the need for detailed surveillance of the meningococcal population. This study not only details the effect the MenC vaccine had on the meningococcal disease causing population but also highlight other serogoups and their sequence types which may fill the predicted void. The likelihood of a reduction in the serogroup C disease-causing population is very likely, and the serogroup B strain is also expected to fill the void. However this study will identify along with these predicted patterns the unknown sequence types prevailing after the introduction of the MenC vaccines. In addition the analysis of sequence data will highlight any interaction between different serogoups, possible evidence of a precursor event before capsule switch occurs due to selective pressures.

Ever since the isolation of meningococci over 115 years ago, studies have steadily highlighted the various inter-relationships between the meningococcus and the host (Peltola 1983). Meningococci have developed to employ various complex mechanisms involving molecular mimicry and subsequent immune evasion with resulting circumventation of immune responses to elicit disease (van Deuren and Meis 2001). Therefore, during this 4-year period between 1999 and 2002 it will be vital that enhanced surveillance is carried out to ascertain the effect of the MenC conjugate vaccine on the disease-causing meningococcal population.

Shortcomings in some phenotypic techniques for characterising and typing meningococci have become more evident, an example being the increasing presence of non-groupable, non-typeable and non-subtypeable strains. There are a number of reasons, including the introduction of antibiotics, selective pressures such as climate, sociological factors and immunological factors, which have altered different biochemical structures and processes hindering phenotypic methods and ultimately the lack of suitable reagents to identify these changes. Consequently, the development of new techniques and the development of molecular typing methods for enhanced characterisation and surveillance of microorganisms have had a constructive effect. MLST is an example whereby a collection of isolates from invasive disease and healthy carriers that had been previously characterised by multi-locus enzyme electrophoresis (MLEE) can be further characterised allowing the possibility of greater discrimination between previously identical strains (Maiden et al. 1998; Enright and Spratt 1999). This MLST tool has been successfully demonstrated during outbreaks of MD (Feavers et al. 1999). As a consequence of the production of nucleotide sequence data, results are recorded digitally and therefore, importantly, portable between laboratories. This can be useful for the public health management of clusters or outbreaks in institutions such as schools, and also for general disease surveillance on a national basis, thereby providing information to determine national vaccine policy.

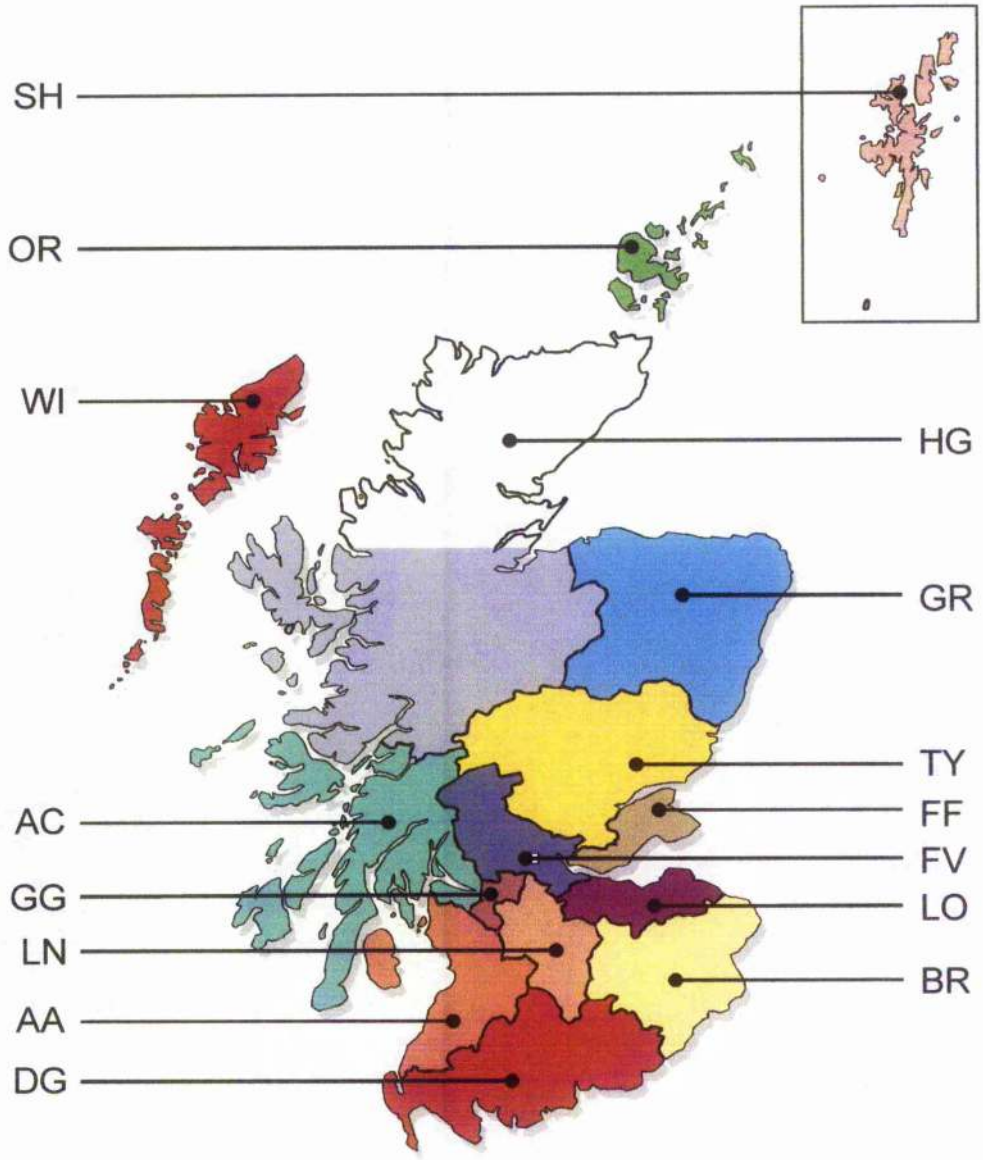
In order for the successful implementation of an MLST scheme within a clinical service, a semi-automated system was required. This enabled dependable results to be generated with the additional ability to perform sequencing on a large number of organisms with minimum complexity. Subsequent analysis was performed on an automated 96 capillary DNA sequencer, sequencing four 96-well plates during an

eight hour period. The four plates were equivalent to MLST of 24 bacterial isolates, each isolate consisting of positive and negative sense strand sequencing of seven housekeeping genes and one antigenic gene. To achieve the sequence preparation, a robotic liquid-handling system was programmed to perform the relevant tasks, including PCR amplification, PCR clean-up, sequence set-up and sequence clean-up. The semi-automated MLST system and subsequent sequence analysis was introduced in January 1999, coincidentally as a routine method for the characterization of *N. meningitidis* isolates prior to the introduction of meningococcal serogroup C conjugate (MenC) vaccines in November 1999 and has since been used prospectively to characterise all meningococci causing disease in Scotland.

This study will aim to collect and analyse data from all invasive meningococcal isolates received from all 15 NHS boards (figure 3.1) over a 4-year period between January 1999 and December 2002. The data will hopefully highlight MLST as an extremely important tool to identify and characterise the clonal population structure of meningococci causing disease over a sustained period, This study, will also have the potential to show the effectiveness not only in monitoring the impact of the MenC conjugate vaccines, but also providing a detailed genotypic representation of strains now commonly associated with disease to enable the construction of future vaccine policy.

MLST can be regarded as the detailed analysis of sequence data from a predetermined set of genes from bacterial, parasitic or fungal species, identified by their range of mutation and recombination enabling sufficient differentiation. In addition, genes such as antigenically variable genes, due to their relative high rate of mutation or

Figure 3.1 National Health Service (NHS) boards throughout Scotland



recombination may also be included to increase the discrimination power of MLST. The further expansion of traditional MLST incorporating antigenically variable genes provides a more powerful tool for short-term epidemiological studies and suspect outbreaks. The gene used with regards to *N. meningitidis* is the porA class 2 outer membrane porin protein. These *porA* sequence typing results and subsequent analysis will be discussed in chapter 4 – *porA sequence analysis*.

MLST can be a useful epidemiological tool and provide greater knowledge of the genetic variation that can occur within a species such as the *N. meningitidis* population. The sequence data obtained from MLST and subsequent analysis can determine population structures by analysing the extent of linkage disequilibrium between alleles and to identify the recombination events by the noncongruence of gene trees (Boyd et al. 1996) and by the presence of significant mosaic structures. For highly clonal species, the phylogenetic relationship between isolates can be inferred from the dendrogram derived from the pairwise differences between sequence types (STs), and independently from a consensus tree constructed from the gene sequence. In the case of weakly clonal species such as the meningococcus, MLST is valuable for the identification of currently circulating hyper-virulent lineages as these are recognised as clusters of isolates with identical or very similar sequence types (Maiden et al. 1998). Although nucleotide differences between alleles can go unscrutinised with just a simple allocation of an allele number and a ST, these nucleotide differences can determine the evolution of given alleles using nucleotide sequence analysis programs.

Most bacterial species have sufficient variation within housekeeping genes to provide many alleles per locus, allowing an almost infinite number of distinct allelic profiles to be distinguished using the chosen housekeeping loci. The analysis of data can be separated into four main categories:

- The summary of data.
- Lineage assignment.
- Recombination tests.
- Tests for selection.

Data summary can provide information regarding allele frequencies, profile frequencies, polymorphism frequencies, codon usage and GC content. Allele frequencies can be determined once allelic profiles have been ascertained. These results display the basic frequency of each allele at each locus. Profile frequencies are performed on complete allelic profiles. The profiles of a selected dataset will be displayed showing the frequency of each profile in the dataset in a basic frequency order. Polymorphism frequencies will highlight through a sequence map all the polymorphic sites at each locus. A summary table can be produced which displays the number of alleles in the dataset that have a particular nucleotide at each polymorphic site. Using the codon usage analysis requires both allelic profiles and allele sequences from the dataset. In addition, the open reading frames must be identified for each locus. This analysis will count the codons used across all loci and the dataset and the summary data is in the form of a table showing the number of occurrences of each codon. Finally, the GC content again requires both allelic profiles and allele sequences. The output data will highlight the %GC value for each strain at each locus

with the mean values for each strain and for each locus.

For lineage assignment, there are a number of packages available including BURST, which is a web-implemented clustering algorithm, designed for use on MLST data sets from bacterial pathogens. The approach specifically examines the relationships between very closely related genotypes within clonal complexes. The relationships between different clonal complexes (i.e. between more distantly related isolates) are ignored. This approach, therefore, bypasses the difficulties resulting from frequent recombination overwhelming the deep-rooted phylogenetic signal (Feil et al. 2001; Feil and Spratt 2001). There is also the Unweighted Pair Group Method with Arithmetic Mean method (UPGMA) (Sneath 1973) which is a straightforward method of tree construction. Its original purpose was to construct taxonomic phenograms, which are trees that reflect the phenotypic similarities between operational taxonomic units (OTUs).

Recombination may cause variation where phylogenetic analysis assumes that the evolutionary process is independent and identical at every region along a sequence alignment. These recombinational events can result in different regions of the same associated sequence having different phylogenetic histories, resulting in heterogeneous rates along sequences. Such recombination tests include Sawyer's Runs Tests, which looks for evidence of recombinational exchanges within a set of aligned sequences by determining if regions of sequence pairs have more consecutive identical polymorphic sites in common than would be expected by chance (Sawyer 1989; Drouin 1999). The maximum chi-squared test is used to identify potential recombination events between two sequences or between two sequences and a

putative derived sequence. The test compares the distribution of polymorphic sites along such sequences with those expected to occur by chance (Smith 1992). Index of Association (IA) measures the extent of linkage equilibrium within a population by quantifying the amount of recombination among a set of sequences and detecting association between alleles at different loci (Smith et al. 1993).

Selection is a process whereby the implementation of conditions allows the discrimination of specific isolates displaying a required phenotype/genotype. In terms of MLST, a test for selection can be performed on the specific housekeeping genes used in the scheme. Nucleotide substitutions within these genes that encode for proteins can either be synonymous or non-synonymous. Investigating the number of synonymous and non-synonymous substitutions that may occur within these genes can provide information relating to the degree of selection operating on such a system. Tests for selection include dS/dN ratios, these nucleotide substitutions in genes coding for proteins can be either synonymous (do not change amino acid), or alternatively silent substitutions, or non-synonymous (changes amino acid). Usually, most non-synonymous changes would be expected to be eliminated by purifying selection, but under certain conditions Darwinian selection may lead to their retention. Investigating the number of synonymous and non-synonymous substitutions may therefore provide information about the degree of selection operating on a system.

These are only brief summaries of the various forms of data analysis that are available once the initial sequence data has been generated. Depending on the hypothesis further more detailed analysis tools could be used.

3.1 Results

In the two year period before the introduction of the vaccine campaign (1998 and 1999) the proportion of cases confirmed as group C infection reached a high, comparable with group B incidence. The rise in the numbers of group C also took place alongside a steep yearly increase in meningococcal disease cases overall which began in the mid 1990s after a long period of relative stability. The effect of menC vaccine on the genetic diversity of group C meningococci and meningococcal disease overall will be analysed using molecular characterisation methods during the period from January 1999 until December 2002. These results will be sectioned into four different years and within each year described in specific sections, namely, serogroup diversity, sequence types, lineages and inter-lineage variability.

3.2 Serogroup Diversity

Over the past 30 years the UK has observed a cyclic pattern of dominance in the meningococcal disease population between serogroup B and serogroup C meningococci. Throughout the 1990s there was a significant increase in serogroup C disease. This continuing increase in serogroup C disease prompted the development and introduction of a MenC vaccine. A programme was initiated into the UK population in November 1999. Subsequent effects of the MenC vaccine have shown a decrease in serogroup C disease with the ratio of B:C increasing since the introduction of the MenC such that serogroup B menigococci now predominate.

3.2.1 Serogroup Diversity in 1999.

There were 349 cases of meningococcal disease in Scotland in 1999, representing to an incidence rate of approximately 6.4 cases per 100,000. The incidence of disease increased in all age groups except those aged 5-9 years and 10-15 years. The biggest increase occurred in those aged over 25 years. Serogroup C disease declined for the first time in recent years. Within the meningococcal population serogroup B and C isolates, comprising B:4:P1.4 and C:2a complex strains continued to predominate.

In total, 65 of the 130 isolates were serogroup B (50%), 58 were serogroup C (45%), 3 were serogroup X (2%) and both serogroups Y and W135 were represented by 2 isolates (1.5%) (Figure 3.2). Serogroup B isolates were further differentiated into 31 different STs contained within 5 recognised complex assignments. These included the ST-44 complex (34%), ST-269 complex (18%), ST-18 complex (8%), ST-35 complex (5%), ST-32 complex (3%) and the remaining 31% had no complex association. Serogroup C isolates were differentiated into 5 different STs contained within 2 recognised complex assignments. These included the ST-11 (93%), ST-269 complex (2%) and the remaining 5% had no complex association. Serogroup X isolates were differentiated into 2 different STs, neither ST being associated with a recognised complex. Serogroup Y isolates were differentiated into 2 different STs contained within 1 recognised complex. This included, ST-23, and a single isolate with no complex association. Serogroup W135 isolates were differentiated into a single ST type and a single sequence complex. These were ST-22 and ST-22 complex, respectively (Table 3.1).

Figure 3.2 Phylogenetic relationship of meningococci isolated in 1999 (year 1) and the association with ST complexes and serogroups. The UPGMA tree was constructed using the START program and ST complexes were assigned using BURST. A total of 130 isolates were characterised with each isolate represented by an individual number starting 99-. Each isolate has the sequence type and the allelic profile pertaining to this in brackets next to the isolate number. Each coloured region has the total number of strains represented as a total equal to N.

Serogroup B is shown in red	
Serogroup C in blue	
Serogroup Y in green	
Serogroup W135 in yellow	
Serogroup X in brown	

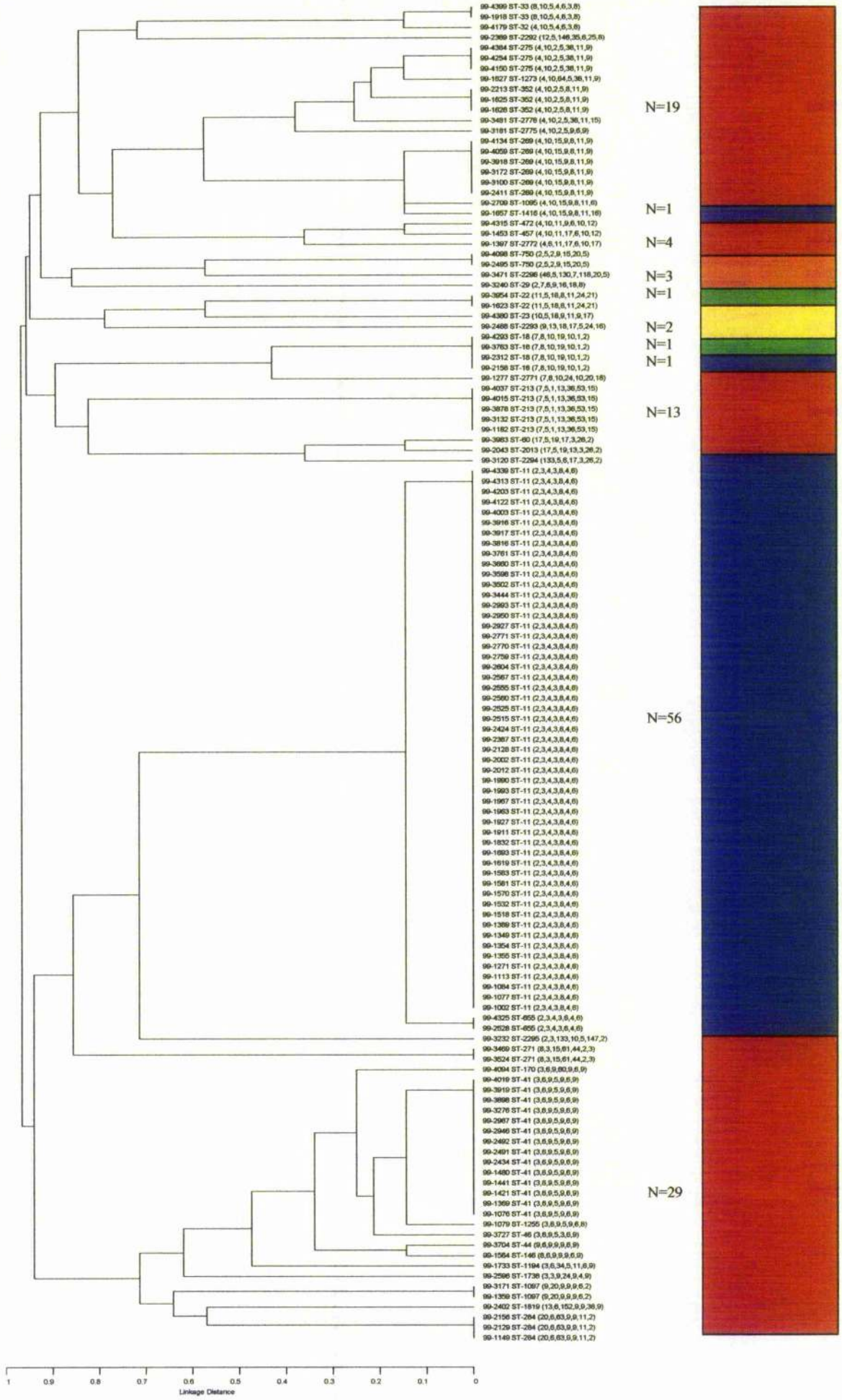

99-4399 ST-33 (8,10,5,4,6,3,8)
99-1918 ST-33 (8,10,5,4,6,3,8)
99-4179 ST-32 (4,10,5,4,6,3,8)
99-2369 ST-2292 (12,5,146,35,6,25,6)
99-4384 ST-275 (4,10,2,5,38,11,9)
99-4254 ST-275 (4,10,2,5,38,11,9)
99-4150 ST-275 (4,10,2,5,38,11,9)
99-1627 ST-1273 (4,10,84,5,38,11,9)
99-2213 ST-352 (4,10,2,5,8,11,9)
99-1625 ST-352 (4,10,2,5,8,11,9)
99-1626 ST-352 (4,10,2,5,8,11,9)
99-3481 ST-2776 (4,10,2,5,38,11,15)
99-3181 ST-2775 (4,10,2,5,9,6,9)
99-4134 ST-269 (4,10,15,9,8,11,9)
99-4059 ST-269 (4,10,15,9,8,11,9)
99-3918 ST-269 (4,10,15,9,8,11,9)
99-3172 ST-269 (4,10,15,9,8,11,9)
99-3100 ST-269 (4,10,15,9,8,11,9)
99-2411 ST-269 (4,10,15,9,8,11,9)
99-2709 ST-1095 (4,10,15,9,8,11,6)
99-1657 ST-1416 (4,10,15,9,8,11,16)
99-4315 ST-472 (4,10,11,9,6,10,12)
99-1453 ST-457 (4,10,11,17,6,10,12)
99-1397 ST-2772 (4,6,11,17,6,10,17)
99-4098 ST-750 (2,5,2,9,15,20,5)
99-2495 ST-750 (2,5,2,9,15,20,5)
99-3471 ST-2296 (46,5,130,7,118,20,5)
99-3240 ST-29 (2,7,6,9,16,18,8)
99-3954 ST-22 (11,5,18,8,11,24,21)
99-1623 ST-22 (11,5,18,6,11,24,21)
99-4380 ST-23 (10,5,18,9,11,9,17)
99-2488 ST-2293 (9,13,18,17,5,24,16)
99-4293 ST-18 (7,8,10,19,10,1,2)
99-3763 ST-18 (7,8,10,19,10,1,2)
99-2312 ST-18 (7,8,10,19,10,1,2)
99-2156 ST-18 (7,8,10,19,10,1,2)
99-1277 ST-2771 (7,8,10,24,10,20,18)
99-4037 ST-213 (7,5,1,13,36,53,15)
99-4015 ST-213 (7,5,1,13,36,53,15)
99-3878 ST-213 (7,5,1,13,36,53,15)
99-3132 ST-213 (7,5,1,13,36,53,15)
99-1182 ST-213 (7,5,1,13,36,53,15)
99-3983 ST-60 (17,5,19,17,3,26,2)
99-2043 ST-2013 (17,5,19,13,3,26,2)
99-3120 ST-2294 (133,5,6,17,3,26,2)
99-4339 ST-11 (2,3,4,3,8,4,6)
99-4313 ST-11 (2,3,4,3,8,4,6)
99-4203 ST-11 (2,3,4,3,8,4,6)
99-4122 ST-11 (2,3,4,3,8,4,6)
99-4003 ST-11 (2,3,4,3,8,4,6)
99-3916 ST-11 (2,3,4,3,8,4,6)
99-3917 ST-11 (2,3,4,3,8,4,6)
99-3816 ST-11 (2,3,4,3,8,4,6)
99-3761 ST-11 (2,3,4,3,8,4,6)
99-3680 ST-11 (2,3,4,3,8,4,6)
99-3598 ST-11 (2,3,4,3,8,4,6)
99-3502 ST-11 (2,3,4,3,8,4,6)
99-3444 ST-11 (2,3,4,3,8,4,6)
99-2993 ST-11 (2,3,4,3,8,4,6)
99-2950 ST-11 (2,3,4,3,8,4,6)
99-2927 ST-11 (2,3,4,3,8,4,6)
99-2771 ST-11 (2,3,4,3,8,4,6)
99-2770 ST-11 (2,3,4,3,8,4,6)
99-2759 ST-11 (2,3,4,3,8,4,6)
99-2604 ST-11 (2,3,4,3,8,4,6)
99-2567 ST-11 (2,3,4,3,8,4,6)
99-2555 ST-11 (2,3,4,3,8,4,6)
99-2560 ST-11 (2,3,4,3,8,4,6)
99-2525 ST-11 (2,3,4,3,8,4,6)
99-2515 ST-11 (2,3,4,3,8,4,6)
99-2424 ST-11 (2,3,4,3,8,4,6)
99-2387 ST-11 (2,3,4,3,8,4,6)
99-2128 ST-11 (2,3,4,3,8,4,6)
99-2002 ST-11 (2,3,4,3,8,4,6)
99-2012 ST-11 (2,3,4,3,8,4,6)
99-1990 ST-11 (2,3,4,3,8,4,6)
99-1993 ST-11 (2,3,4,3,8,4,6)
99-1957 ST-11 (2,3,4,3,8,4,6)
99-1963 ST-11 (2,3,4,3,8,4,6)
99-1927 ST-11 (2,3,4,3,8,4,6)
99-1911 ST-11 (2,3,4,3,8,4,6)
99-1832 ST-11 (2,3,4,3,8,4,6)
99-1693 ST-11 (2,3,4,3,8,4,6)
99-1619 ST-11 (2,3,4,3,8,4,6)
99-1583 ST-11 (2,3,4,3,8,4,6)
99-1581 ST-11 (2,3,4,3,8,4,6)
99-1570 ST-11 (2,3,4,3,8,4,6)
99-1532 ST-11 (2,3,4,3,8,4,6)
99-1518 ST-11 (2,3,4,3,8,4,6)
99-1389 ST-11 (2,3,4,3,8,4,6)
99-1349 ST-11 (2,3,4,3,8,4,6)
99-1354 ST-11 (2,3,4,3,8,4,6)
99-1355 ST-11 (2,3,4,3,8,4,6)
99-1271 ST-11 (2,3,4,3,8,4,6)
99-1113 ST-11 (2,3,4,3,8,4,6)
99-1084 ST-11 (2,3,4,3,8,4,6)
99-1077 ST-11 (2,3,4,3,8,4,6)
99-1002 ST-11 (2,3,4,3,8,4,6)
99-4325 ST-855 (2,3,4,3,8,4,6)
99-2528 ST-855 (2,3,4,3,8,4,6)
99-3232 ST-2295 (2,3,133,10,5,147,2)
99-3469 ST-271 (8,3,15,61,44,2,3)
99-3524 ST-271 (8,3,15,61,44,2,3)
99-4094 ST-170 (3,6,9,80,9,6,9)
99-4019 ST-41 (3,6,9,5,9,6,9)
99-3919 ST-41 (3,6,9,5,9,6,9)
99-3898 ST-41 (3,6,9,5,9,6,9)
99-3276 ST-41 (3,6,9,5,9,6,9)
99-2987 ST-41 (3,6,9,5,9,6,9)
99-2946 ST-41 (3,6,9,5,9,6,9)
99-2492 ST-41 (3,6,9,5,9,6,9)
99-2491 ST-41 (3,6,9,5,9,6,9)
99-2434 ST-41 (3,6,9,5,9,6,9)
99-1480 ST-41 (3,6,9,5,9,6,9)
99-1441 ST-41 (3,6,9,5,9,6,9)
99-1421 ST-41 (3,6,9,5,9,6,9)
99-1369 ST-41 (3,6,9,5,9,6,9)
99-1076 ST-41 (3,6,9,5,9,6,9)
99-1079 ST-1255 (3,6,9,5,9,6,8)
99-3727 ST-46 (3,6,9,5,3,6,9)
99-3704 ST-44 (9,6,9,9,9,6,9)
99-1584 ST-146 (6,6,9,9,9,6,9)
99-1733 ST-1194 (3,6,34,5,11,6,9)
99-2596 ST-1736 (3,3,9,24,9,4,9)
99-3171 ST-1097 (9,20,9,9,9,6,2)
99-1359 ST-1097 (9,20,9,9,9,6,2)
99-2402 ST-1819 (13,6,152,9,9,36,9)
99-2156 ST-284 (20,6,63,9,9,11,2)
99-2129 ST-284 (20,6,63,9,9,11,2)
99-1149 ST-284 (20,6,63,9,9,11,2)
N=19
N=1
N=4
N=3
N=1
N=2
N=1
N=1
N=13
N=56
N=29
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Linkage Distance

Table 3.1 Summary of meningococcal allelic profile frequencies from meningococci isolated in 1999. This represents a summary of all the different sequence types (ST) characterised in 1999. The table shows the ST followed by the allelic profile pertaining to that ST. Each allelic number represents each gene in order as stated in the table. The frequency number is the total number of meningococci represented by that ST followed by a percentage in relation to the total number of meningococci isolated.

ST	Allelic Profile (*abcZ, adk, aroE, fumC, gdh, pdhC, pgm*)	Frequency	% of dataset
11	2, 3, 4, 3, 8, 4, 6	53	40.77
41	3, 6, 9, 5, 9, 6, 9	14	10.77
269	4, 10, 15, 9, 8, 11, 9	6	4.62
213	7, 5, 1, 13, 36, 53, 15	5	3.85
18	7, 8, 10, 19, 10, 1, 2	4	3.08
284	20, 6, 63, 9, 9, 11, 2	3	2.31
352	4, 10, 2, 5, 8, 11, 9	3	2.31
275	4, 10, 2, 5, 38, 11, 9	3	2.31
1097	9, 20, 9, 9, 9, 6, 2	2	1.54
22	11, 5, 18, 8, 11, 24, 21	2	1.54
33	8, 10, 5, 4, 6, 3, 8	2	1.54
750	2, 5, 2, 9, 15, 20, 5	2	1.54
655	2, 3, 4, 3, 6, 4, 6	2	1.54
271	8, 3, 15, 61, 44, 2, 3	2	1.54
23	10, 5, 18, 9, 11, 9, 17	1	0.77
1255	3, 6, 9, 5, 9, 6, 8	1	0.77
2771	7, 8, 10, 24, 10, 20, 18	1	0.77
2772	4, 6, 11, 17, 6, 10, 17	1	0.77
457	4, 10, 11, 17, 6, 10, 12	1	0.77
146	8, 6, 9, 9, 9, 6, 9	1	0.77
1273	4, 10, 64, 5, 38, 11, 9	1	0.77
1416	4, 10, 15, 9, 8, 11, 16	1	0.77
1194	3, 6, 34, 5, 11, 6, 9	1	0.77
2013	17, 5, 19, 13, 3, 26, 2	1	0.77
2292	12, 5, 146, 35, 6, 25, 8	1	0.77
1819	13, 6, 152, 9, 9, 38, 9	1	0.77
2293	9, 13, 18, 17, 5, 24, 16	1	0.77
1738	3, 3, 9, 24, 9, 4, 9	1	0.77
1095	4, 10, 15, 9, 8, 11, 6	1	0.77
2294	133, 5, 6, 17, 3, 26, 2	1	0.77

2775	4, 10, 2, 5, 9, 6, 9	1	0.77
2295	2, 3, 133, 10, 5, 147, 2	1	0.77
29	2, 7, 6, 9, 16, 18, 8	1	0.77
2776	4, 10, 2, 5, 38, 11, 15	1	0.77
2296	46, 5, 130, 7, 118, 20, 5	1	0.77
44	9, 6, 9, 9, 9, 6, 9	1	0.77
46	3, 6, 9, 5, 3, 6, 9	1	0.77
60	17, 5, 19, 17, 3, 26, 2	1	0.77
170	3, 6, 9, 60, 9, 6, 9	1	0.77
32	4, 10, 5, 4, 6, 3, 8	1	0.77
472	4, 10, 11, 9, 6, 10, 12	1	0.77

The 41 different profile(s) in this dataset (displayed in descending order of frequency)

3.2.2 Serogroup Diversity in 2000.

There were 343 cases of meningococcal disease in Scotland in 2000 representing to an incidence rate of approximately 6.7 cases per 100,000 population. The incidence of disease decreased in years 9 or under but increased in ages 25 years and over. Serogroup C disease continued to decline. Within the meningococcal population serogroup B and C isolates, comprising B:4:P1.4, B:1:P1.14, B:NT:P1.9 and C:2a complex strains, continued to predominate.

In total, 74 of the 130 isolates were serogroup B (57%), an increase of 7% on the previous year, 46 were serogroup C (35%), a decrease of 10%, 6 were serogroup W135 (5%), an increase of 3.5%, and 4 isolates were serogroup Y (3%), an increase of 1.5% on the previous year (Figure 3.3). Serogroup B isolates were further differentiated into 40 different STs, an increase of 22.5% on the previous year, contained within 9 recognised complex assignments, an increase of 44%. These included the ST-44 complex (28%), ST-269 complex (19%), ST-32 complex (7%), ST-18 complex (5%), ST-11 complex (5%) and ST-35, 53, 92 and 254 complexes the latter group each accounted for 1% of the serogroup B population. The remaining 30% had no complex association. Serogroup C isolates were differentiated into 2 different STs contained within 2 recognised complex assignments. These included the ST-11 complex (96%), an increase of 3% within the serogroup C population, and ST-8 complex (4%), which had not been represented in previous years. Serogroup W135 isolates were differentiated into 4 different STs contained within 2 recognised complex associations. These included the ST-22 (67%) and the ST-11 complex was represented once and the remaining isolate with no complex association. Serogroup Y isolates were differentiated into 3 different STs, all with no recognised complex

assignment (Table 3.2).

Figure 3.3 Phylogenetic relationship of meningococci isolated in 2000 (year 2) and the association with ST complexes and serogroups. The UPGMA tree was constructed using the START program and ST complexes were assigned using BURST. A total of 130 isolates were characterised with each isolate represented by an individual number starting 00-. Each isolate has the sequence type and the allelic profile pertaining to this in brackets next to the isolate number. Each coloured region has the total number of strains represented as a total equal to N.

Serogroup B is shown in red	
Serogroup C in blue	
Serogroup Y in green	
Serogroup W135 in yellow	
Serogroup X in brown	

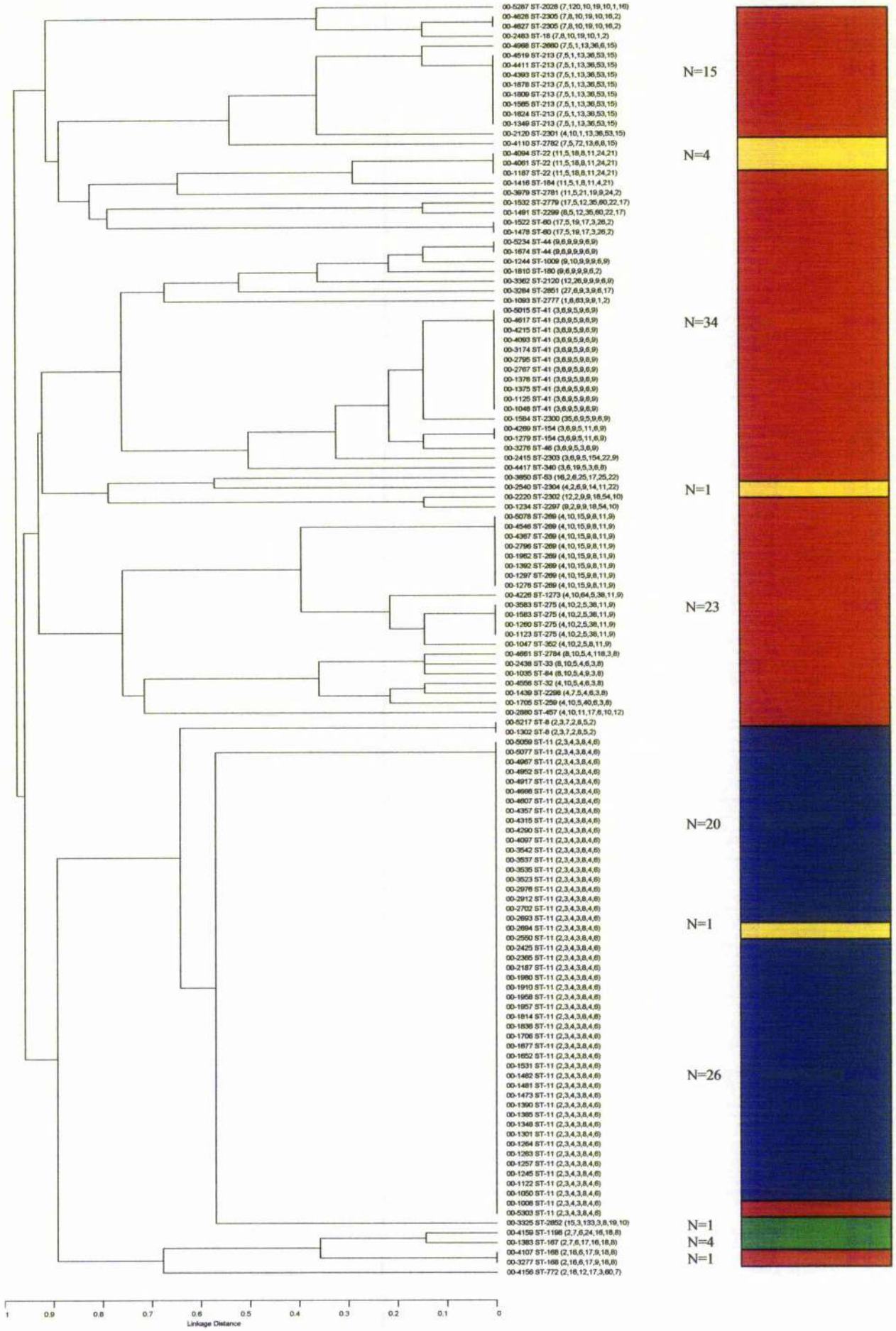

00-5287 ST-2028 (7,120,10,19,10,1,16)
00-4626 ST-2305 (7,8,10,19,10,16,2)
00-4627 ST-2305 (7,8,10,19,10,16,2)
00-2483 ST-18 (7,8,10,19,10,1,2)
00-4998 ST-2680 (7,5,1,13,36,6,15)
00-4519 ST-213 (7,5,1,13,36,53,15)
00-4411 ST-213 (7,5,1,13,36,53,15)
00-4393 ST-213 (7,5,1,13,36,53,15)
00-1878 ST-213 (7,5,1,13,36,53,15)
00-1809 ST-213 (7,5,1,13,36,53,15)
00-1585 ST-213 (7,5,1,13,36,53,15)
00-1624 ST-213 (7,5,1,13,36,53,15)
00-1349 ST-213 (7,5,1,13,36,53,15)
00-2120 ST-2301 (4,10,1,13,36,53,15)
00-4110 ST-2782 (7,5,72,13,6,8,15)
00-4094 ST-22 (11,5,18,8,11,24,21)
00-4081 ST-22 (11,5,18,8,11,24,21)
00-1187 ST-22 (11,5,18,8,11,24,21)
00-1416 ST-184 (11,5,1,8,11,4,21)
00-3679 ST-2781 (11,5,21,19,8,24,2)
00-1532 ST-2779 (17,5,12,35,60,22,17)
00-1491 ST-2299 (8,5,12,35,60,22,17)
00-1522 ST-60 (17,5,19,17,3,26,2)
00-1478 ST-60 (17,5,19,17,3,26,2)
00-5234 ST-44 (9,6,9,9,9,6,9)
00-1674 ST-44 (9,6,9,9,9,6,9)
00-1244 ST-1009 (9,10,9,9,9,6,9)
00-1810 ST-180 (9,6,9,9,9,6,2)
00-3362 ST-2120 (12,26,9,9,9,6,9)
00-3284 ST-2851 (27,6,9,3,9,6,17)
00-1093 ST-2777 (1,6,83,9,9,1,2)
00-5015 ST-41 (3,6,9,5,9,6,9)
00-4617 ST-41 (3,6,9,5,9,6,9)
00-4215 ST-41 (3,6,9,5,9,6,9)
00-4093 ST-41 (3,6,9,5,9,6,9)
00-3174 ST-41 (3,6,9,5,9,6,9)
00-2795 ST-41 (3,6,9,5,9,6,9)
00-2767 ST-41 (3,6,9,5,9,6,9)
00-1376 ST-41 (3,6,9,5,9,6,9)
00-1375 ST-41 (3,6,9,5,9,6,9)
00-1125 ST-41 (3,6,9,5,9,6,9)
00-1046 ST-41 (3,6,9,5,9,6,9)
00-1584 ST-2300 (35,6,9,5,9,6,9)
00-4269 ST-154 (3,6,9,5,11,6,9)
00-1279 ST-154 (3,6,9,5,11,6,9)
00-3276 ST-46 (3,6,9,5,3,6,9)
00-2415 ST-2303 (3,6,9,5,154,22,9)
00-4417 ST-340 (3,6,19,5,3,6,8)
00-3850 ST-53 (16,2,6,25,17,25,22)
00-2540 ST-2304 (4,2,6,9,14,11,22)
00-2220 ST-2302 (12,2,9,9,18,54,10)
00-1234 ST-2297 (9,2,9,9,18,54,10)
00-5078 ST-269 (4,10,15,9,8,11,9)
00-4546 ST-269 (4,10,15,9,8,11,9)
00-4367 ST-269 (4,10,15,9,8,11,9)
00-2796 ST-269 (4,10,15,9,8,11,9)
00-1982 ST-269 (4,10,15,9,8,11,9)
00-1392 ST-269 (4,10,15,9,8,11,9)
00-1297 ST-269 (4,10,15,9,8,11,9)
00-1276 ST-269 (4,10,15,9,8,11,9)
00-4226 ST-1273 (4,10,64,5,38,11,9)
00-3583 ST-275 (4,10,2,5,38,11,9)
00-1583 ST-275 (4,10,2,5,38,11,9)
00-1260 ST-275 (4,10,2,5,38,11,9)
00-1123 ST-275 (4,10,2,5,38,11,9)
00-1047 ST-352 (4,10,2,5,8,11,9)
00-4661 ST-2784 (8,10,5,4,118,3,8)
00-2438 ST-33 (8,10,5,4,6,3,8)
00-1035 ST-84 (8,10,5,4,9,3,8)
00-4556 ST-32 (4,10,5,4,6,3,8)
00-1439 ST-2298 (4,7,5,4,6,3,8)
00-1705 ST-259 (4,10,5,40,6,3,8)
00-2880 ST-457 (4,10,11,17,6,10,12)
00-5217 ST-8 (2,3,7,2,8,5,2)
00-1302 ST-8 (2,3,7,2,8,5,2)
00-5059 ST-11 (2,3,4,3,8,4,6)
00-5077 ST-11 (2,3,4,3,8,4,6)
00-4967 ST-11 (2,3,4,3,8,4,6)
00-4952 ST-11 (2,3,4,3,8,4,6)
00-4917 ST-11 (2,3,4,3,8,4,6)
00-4666 ST-11 (2,3,4,3,8,4,6)
00-4607 ST-11 (2,3,4,3,8,4,6)
00-4357 ST-11 (2,3,4,3,8,4,6)
00-4315 ST-11 (2,3,4,3,8,4,6)
00-4290 ST-11 (2,3,4,3,8,4,6)
00-4097 ST-11 (2,3,4,3,8,4,6)
00-3542 ST-11 (2,3,4,3,8,4,6)
00-3537 ST-11 (2,3,4,3,8,4,6)
00-3535 ST-11 (2,3,4,3,8,4,6)
00-3523 ST-11 (2,3,4,3,8,4,6)
00-2976 ST-11 (2,3,4,3,8,4,6)
00-2912 ST-11 (2,3,4,3,8,4,6)
00-2702 ST-11 (2,3,4,3,8,4,6)
00-2693 ST-11 (2,3,4,3,8,4,6)
00-2694 ST-11 (2,3,4,3,8,4,6)
00-2550 ST-11 (2,3,4,3,8,4,6)
00-2425 ST-11 (2,3,4,3,8,4,6)
00-2365 ST-11 (2,3,4,3,8,4,6)
00-2187 ST-11 (2,3,4,3,8,4,6)
00-1980 ST-11 (2,3,4,3,8,4,6)
00-1910 ST-11 (2,3,4,3,8,4,6)
00-1958 ST-11 (2,3,4,3,8,4,6)
00-1957 ST-11 (2,3,4,3,8,4,6)
00-1814 ST-11 (2,3,4,3,8,4,6)
00-1838 ST-11 (2,3,4,3,8,4,6)
00-1706 ST-11 (2,3,4,3,8,4,6)
00-1677 ST-11 (2,3,4,3,8,4,6)
00-1652 ST-11 (2,3,4,3,8,4,6)
00-1531 ST-11 (2,3,4,3,8,4,6)
00-1482 ST-11 (2,3,4,3,8,4,6)
00-1481 ST-11 (2,3,4,3,8,4,6)
00-1473 ST-11 (2,3,4,3,8,4,6)
00-1390 ST-11 (2,3,4,3,8,4,6)
00-1385 ST-11 (2,3,4,3,8,4,6)
00-1348 ST-11 (2,3,4,3,8,4,6)
00-1301 ST-11 (2,3,4,3,8,4,6)
00-1264 ST-11 (2,3,4,3,8,4,6)
00-1263 ST-11 (2,3,4,3,8,4,6)
00-1257 ST-11 (2,3,4,3,8,4,6)
00-1245 ST-11 (2,3,4,3,8,4,6)
00-1122 ST-11 (2,3,4,3,8,4,6)
00-1050 ST-11 (2,3,4,3,8,4,6)
00-1008 ST-11 (2,3,4,3,8,4,6)
00-5303 ST-11 (2,3,4,3,8,4,6)
00-3325 ST-2852 (15,3,133,3,8,19,10)
00-4159 ST-1198 (2,7,6,24,16,18,8)
00-1383 ST-167 (2,7,6,17,16,18,8)
00-4107 ST-168 (2,18,6,17,9,18,8)
00-3277 ST-168 (2,18,6,17,9,18,8)
00-4156 ST-772 (2,18,12,17,3,60,7)
N=15
N=4
N=34
N=1
N=23
N=20
N=1
N=26
N=1
N=4
N=1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Linkage Distance

Table 3.2 Summary of meningococcal allelic profile frequencies from meningococci isolated in 2000. This represents a summary of all the different sequence types (ST) characterised in 2000. The table shows the ST followed by the allelic profile pertaining to that ST. Each allelic number represents each gene in order as stated in the table. The frequency number is the total number of meningococci represented by that ST followed by a percentage in relation to the total number of meningococci isolated.

ST	Allelic Profile (*abcZ, adk, aroE, fumC, gdh, pdhC, pgm*)	Frequency	% of dataset
11	2, 3, 4, 3, 8, 4, 6	49	37.69
41	3, 6, 9, 5, 9, 6, 9	11	8.46
269	4, 10, 15, 9, 8, 11, 9	8	6.15
213	7, 5, 1, 13, 36, 53, 15	8	6.15
275	4, 10, 2, 5, 38, 11, 9	4	3.08
22	11, 5, 18, 8, 11, 24, 21	3	2.31
154	3, 6, 9, 5, 11, 6, 9	2	1.54
8	2, 3, 7, 2, 8, 5, 2	2	1.54
60	17, 5, 19, 17, 3, 26, 2	2	1.54
44	9, 6, 9, 9, 9, 6, 9	2	1.54
168	2, 16, 6, 17, 9, 18, 8	2	1.54
2305	7, 8, 10, 19, 10, 16, 2	2	1.54
352	4, 10, 2, 5, 8, 11, 9	1	0.77
84	8, 10, 5, 4, 9, 3, 8	1	0.77
2777	1, 6, 63, 9, 9, 1, 2	1	0.77
2297	9, 2, 9, 9, 18, 54, 10	1	0.77
1009	9, 10, 9, 9, 9, 6, 9	1	0.77
167	2, 7, 6, 17, 16, 18, 8	1	0.77
2298	4, 7, 5, 4, 6, 3, 8	1	0.77
184	11, 5, 1, 8, 11, 4, 21	1	0.77
2299	8, 5, 12, 35, 60, 22, 17	1	0.77
2779	17, 5, 12, 35, 60, 22, 17	1	0.77
2300	35, 6, 9, 5, 9, 6, 9	1	0.77
259	4, 10, 5, 40, 6, 3, 8	1	0.77
180	9, 6, 9, 9, 9, 6, 2	1	0.77
2301	4, 10, 1, 13, 36, 53, 15	1	0.77
2302	12, 2, 9, 9, 18, 54, 10	1	0.77
2303	3, 6, 9, 5, 154, 22, 9	1	0.77
33	8, 10, 5, 4, 6, 3, 8	1	0.77
2304	4, 2, 6, 9, 14, 11, 22	1	0.77

18	7, 8, 10, 19, 10, 1, 2	1	0.77
457	4, 10, 11, 17, 6, 10, 12	1	0.77
46	3, 6, 9, 5, 3, 6, 9	1	0.77
2851	27, 6, 9, 3, 9, 6, 17	1	0.77
2852	15, 3, 133, 3, 8, 19, 10	1	0.77
2120	12, 26, 9, 9, 9, 6, 9	1	0.77
53	16, 2, 6, 25, 17, 25, 22	1	0.77
2781	11, 5, 21, 19, 9, 24, 2	1	0.77
2782	7, 5, 72, 13, 6, 6, 15	1	0.77
772	2, 16, 12, 17, 3, 60, 7	1	0.77
1198	2, 7, 6, 24, 16, 18, 8	1	0.77
1273	4, 10, 64, 5, 38, 11, 9	1	0.77
32	4, 10, 5, 4, 6, 3, 8	1	0.77
340	3, 6, 19, 5, 3, 6, 8	1	0.77
2784	8, 10, 5, 4, 118, 3, 8	1	0.77
2660	7, 5, 1, 13, 36, 6, 15	1	0.77
2028	7, 120, 10, 19, 10, 1, 16	1	0.77

The 47 different profile(s) in dataset (displayed in descending order of frequency).

3.2.3 Serogroup Diversity in 2001.

There were 267 cases of meningococcal disease in Scotland in 2001, representing an incidence rate of approximately 5.2 cases per 100,000 population. There was an overall decrease in all groups aged 19 years or under, however the most significant decrease was in the 1-4 year age group. Serogroup C disease continued to decline, although, within the meningococcal population the C:2a complex strain together with serogroup B isolates, B:4:P1.4, B:1:P1.14, and B:NT:P1.9.complex strains continued to predominate.

In total, 51 of the 80 isolates were serogroup B (64%), an increase of 7% within the serogroup B population, 21 were serogroup C (26%), a decrease of a further 9%, 5 were serogroup W135 (6%), 2 isolates were serogroup X (2%), and 1 isolates was serogroup Y (1%) (Figure 3.4). Serogroup B isolates were further differentiated into 37 different STs, a decrease of 10%, contained within 6 recognised complex assignments, which was more representative of isolates in 1999. These included the ST-269 complex (27%), ST-44 complex (10%), ST-32 complex (8%), ST-18 complex (4%) and ST-11 and 364 complexes the latter group each accounted for 2% of the serogroup B population, the remaining 47% had no complex association, an increase of 17%. Serogroup C isolates were differentiated into 3 different STs contained within 2 recognised complex assignments. These included the ST-11 complex (90%), a decrease of 6% within the serogroup C population, and ST-8 complex (5%) the remaining isolate was not associated with any recognised sequence complex. Serogroup W135 isolates were differentiated into 3 different STs, contained within a single complex association, ST-22 complex. A single serogroup Y isolate had no recognised complex assignment (Table 3.3).

Figure 3.4 Phylogenetic relationship of meningococci isolated in 2001 (year 3) and the association with ST complexes and serogroups. The UPGMA tree was constructed using the START program and ST complexes were assigned using BURST. A total of 80 isolates were characterised with each isolate represented by an individual number starting 01-. Each isolate has the sequence type and the allelic profile pertaining to this in brackets next to the isolate number. Each coloured region has the total number of strains represented as a total equal to N.

Serogroup B is shown in red	
Serogroup C in blue	
Serogroup Y in green	
Serogroup W135 in yellow	
Serogroup X in brown	

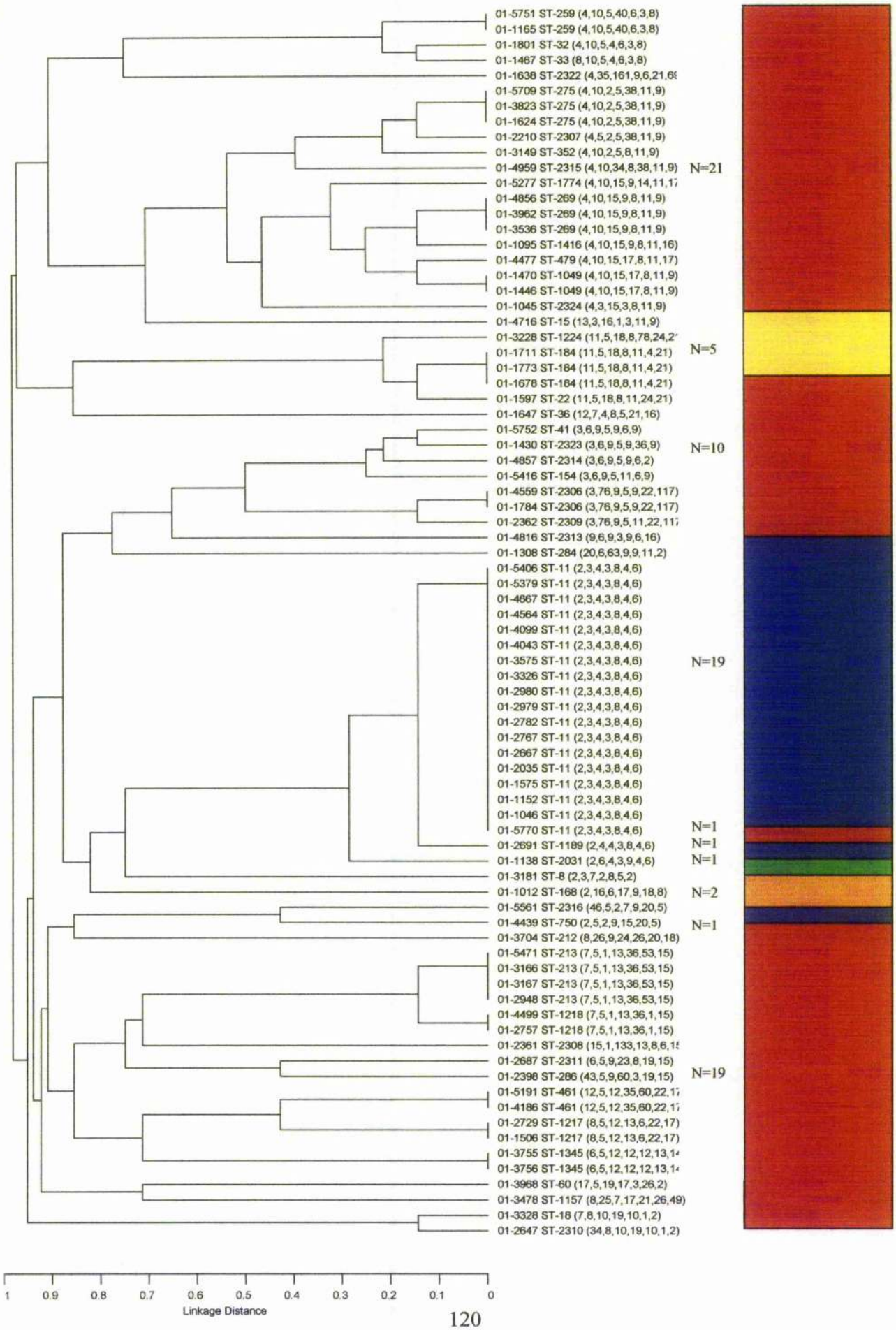

01-5751 ST-259 (4,10,5,40,6,3,8)
01-1165 ST-259 (4,10,5,40,6,3,8)
01-1801 ST-32 (4,10,5,4,6,3,8)
01-1467 ST-33 (8,10,5,4,6,3,8)
01-1638 ST-2322 (4,35,161,9,6,21,6
01-5709 ST-275 (4,10,2,5,38,11,9)
01-3823 ST-275 (4,10,2,5,38,11,9)
01-1624 ST-275 (4,10,2,5,38,11,9)
01-2210 ST-2307 (4,5,2,5,38,11,9)
01-3149 ST-352 (4,10,2,5,8,11,9)
01-4959 ST-2315 (4,10,34,8,38,11,9)
N=21
01-5277 ST-1774 (4,10,15,9,14,11,1
01-4856 ST-269 (4,10,15,9,8,11,9)
01-3962 ST-269 (4,10,15,9,8,11,9)
01-3536 ST-269 (4,10,15,9,8,11,9)
01-1095 ST-1416 (4,10,15,9,8,11,16)
01-4477 ST-479 (4,10,15,17,8,11,17)
01-1470 ST-1049 (4,10,15,17,8,11,9)
01-1446 ST-1049 (4,10,15,17,8,11,9)
01-1045 ST-2324 (4,3,15,3,8,11,9)
01-4716 ST-15 (13,3,16,1,3,11,9)
01-3228 ST-1224 (11,5,18,8,78,24,2
01-1711 ST-184 (11,5,18,8,11,4,21)
N=5
01-1773 ST-184 (11,5,18,8,11,4,21)
01-1678 ST-184 (11,5,18,8,11,4,21)
01-1597 ST-22 (11,5,18,8,11,24,21)
01-1647 ST-36 (12,7,4,8,5,21,16)
01-5752 ST-41 (3,6,9,5,9,6,9)
01-1430 ST-2323 (3,6,9,5,9,36,9)
N=10
01-4857 ST-2314 (3,6,9,5,9,6,2)
01-5416 ST-154 (3,6,9,5,11,6,9)
01-4559 ST-2306 (3,76,9,5,9,22,117)
01-1784 ST-2306 (3,76,9,5,9,22,117)
01-2362 ST-2309 (3,76,9,5,11,22,11
01-4816 ST-2313 (9,6,9,3,9,6,16)
01-1308 ST-284 (20,6,63,9,9,11,2)
01-5406 ST-11 (2,3,4,3,8,4,6)
01-5379 ST-11 (2,3,4,3,8,4,6)
01-4667 ST-11 (2,3,4,3,8,4,6)
01-4564 ST-11 (2,3,4,3,8,4,6)
01-4099 ST-11 (2,3,4,3,8,4,6)
01-4043 ST-11 (2,3,4,3,8,4,6)
01-3575 ST-11 (2,3,4,3,8,4,6)
N=19
01-3326 ST-11 (2,3,4,3,8,4,6)
01-2980 ST-11 (2,3,4,3,8,4,6)
01-2979 ST-11 (2,3,4,3,8,4,6)
01-2782 ST-11 (2,3,4,3,8,4,6)
01-2767 ST-11 (2,3,4,3,8,4,6)
01-2667 ST-11 (2,3,4,3,8,4,6)
01-2035 ST-11 (2,3,4,3,8,4,6)
01-1575 ST-11 (2,3,4,3,8,4,6)
01-1152 ST-11 (2,3,4,3,8,4,6)
01-1046 ST-11 (2,3,4,3,8,4,6)
01-5770 ST-11 (2,3,4,3,8,4,6)
N=1
01-2691 ST-1189 (2,4,4,3,8,4,6)
N=1
01-1138 ST-2031 (2,6,4,3,9,4,6)
N=1
01-3181 ST-8 (2,3,7,2,8,5,2)
01-1012 ST-168 (2,16,6,17,9,18,8)
N=2
01-5561 ST-2316 (46,5,2,7,9,20,5)
01-4439 ST-750 (2,5,2,9,15,20,5)
N=1
01-3704 ST-212 (8,26,9,24,26,20,18)
01-5471 ST-213 (7,5,1,13,36,53,15)
01-3166 ST-213 (7,5,1,13,36,53,15)
01-3167 ST-213 (7,5,1,13,36,53,15)
01-2948 ST-213 (7,5,1,13,36,53,15)
01-4499 ST-1218 (7,5,1,13,36,1,15)
01-2757 ST-1218 (7,5,1,13,36,1,15)
01-2361 ST-2308 (15,1,133,13,8,6,1
01-2687 ST-2311 (6,5,9,23,8,19,15)
01-2398 ST-286 (43,5,9,60,3,19,15)
N=19
01-5191 ST-461 (12,5,12,35,60,22,1
01-4186 ST-461 (12,5,12,35,60,22,1
01-2729 ST-1217 (8,5,12,13,6,22,17)
01-1506 ST-1217 (8,5,12,13,6,22,17)
01-3755 ST-1345 (6,5,12,12,12,13,1
01-3756 ST-1345 (6,5,12,12,12,13,1
01-3968 ST-60 (17,5,19,17,3,26,2)
01-3478 ST-1157 (8,25,7,17,21,26,49)
01-3328 ST-18 (7,8,10,19,10,1,2)
01-2647 ST-2310 (34,8,10,19,10,1,2)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Linkage Distance

Table 3.3 Summary of meningococcal allelic profile frequencies from meningococci isolated in 2001. This represents a summary of all the different sequence types (ST) characterised in 2001. The table shows the ST followed by the allelic profile pertaining to that ST. Each allelic number represents each gene in order as stated in the table. The frequency number is the total number of meningococci represented by that ST followed by a percentage in relation to the total number of meningococci isolated.

ST	Allelic Profile (*abcZ, adk, aroE, fumC, gdh, pdhC, pgm*)	Frequency	% of dataset
11	2, 3, 4, 3, 8, 4, 6	18	22.50
213	7, 5, 1, 13, 36, 53, 15	4	5.00
275	4, 10, 2, 5, 38, 11, 9	3	3.75
184	11, 5, 18, 8, 11, 4, 21	3	3.75
269	4, 10, 15, 9, 8, 11, 9	3	3.75
259	4, 10, 5, 40, 6, 3, 8	2	2.50
1049	4, 10, 15, 17, 8, 11, 9	2	2.50
1217	8, 5, 12, 13, 6, 22, 17	2	2.50
2306	3, 76, 9, 5, 9, 22, 117	2	2.50
1218	7, 5, 1, 13, 36, 1, 15	2	2.50
1345	6, 5, 12, 12, 12, 13, 14	2	2.50
461	12, 5, 12, 35, 60, 22, 17	2	2.50
168	2, 16, 6, 17, 9, 18, 8	1	1.25
2324	4, 3, 15, 3, 8, 11, 9	1	1.25
1416	4, 10, 15, 9, 8, 11, 16	1	1.25
2031	2, 6, 4, 3, 9, 4, 6	1	1.25
284	20, 6, 63, 9, 9, 11, 2	1	1.25
2323	3, 6, 9, 5, 9, 36, 9	1	1.25
33	8, 10, 5, 4, 6, 3, 8	1	1.25
22	11, 5, 18, 8, 11, 24, 21	1	1.25
2322	4, 35, 161, 9, 6, 21, 69	1	1.25
36	12, 7, 4, 8, 5, 21, 16	1	1.25
32	4, 10, 5, 4, 6, 3, 8	1	1.25
2307	4, 5, 2, 5, 38, 11, 9	1	1.25
2309	3, 76, 9, 5, 11, 22, 117	1	1.25
2308	15, 1, 133, 13, 8, 6, 15	1	1.25
286	43, 5, 9, 60, 3, 19, 15	1	1.25
2310	34, 8, 10, 19, 10, 1, 2	1	1.25
1189	2, 4, 4, 3, 8, 4, 6	1	1.25
2311	6, 5, 9, 23, 8, 19, 15	1	1.25

352	4, 10, 2, 5, 8, 11, 9	1	1.25
8	2, 3, 7, 2, 8, 5, 2	1	1.25
1224	11, 5, 18, 8, 78, 24, 21	1	1.25
18	7, 8, 10, 19, 10, 1, 2	1	1.25
1157	8, 25, 7, 17, 21, 26, 49	1	1.25
212	8, 26, 9, 24, 26, 20, 18	1	1.25
60	17, 5, 19, 17, 3, 26, 2	1	1.25
750	2, 5, 2, 9, 15, 20, 5	1	1.25
479	4, 10, 15, 17, 8, 11, 17	1	1.25
15	13, 3, 16, 1, 3, 11, 9	1	1.25
2313	9, 6, 9, 3, 9, 6, 16	1	1.25
2314	3, 6, 9, 5, 9, 6, 2	1	1.25
2315	4, 10, 34, 8, 38, 11, 9	1	1.25
1774	4, 10, 15, 9, 14, 11, 17	1	1.25
154	3, 6, 9, 5, 11, 6, 9	1	1.25
2316	46, 5, 2, 7, 9, 20, 5	1	1.25
41	3, 6, 9, 5, 9, 6, 9	1	1.25

The 47 different profile(s) in dataset (displayed in descending order of frequency).

3.2.4 Serogroup Diversity in 2002.

There were 178 cases of meningococcal disease in Scotland in 2001, representing an incidence rate of approximately 3.5 cases per 100,000 population. There was an overall decrease in notified cases for all age groups. Again the most significant decrease was in the 1-4 age group. Serogroup C disease continued to decline. Within the meningococcal population serogroup B isolates, comprsing B:4:P1.4, B:1:P1.14, B:15:P1.7;P1.16, B:NT:P1.14 and B:NT:P1.15 complex strains were predominatant.

In total, 69 of the 88 isolates were serogroup B (78%), an further increase of 14% within the serogroup B population, 10 were serogroup C (11%), a further decrease of 15%, 4 were serogroup W135 (5%), 3 isolates were serogroup Y (4%), and serogroups X and Z were represented once (1%) (Figure 3.5). Serogroup B were isolates further differentiated into 40 different STs, an increase of 8%, contained within 5 recognised complex assignments. These included the ST-269 complex (26%), ST-44 complex (19%), ST-32 complex (16%), ST-18 complex (4%) and ST-254 complexes, the latter group each accounted for 3% of the serogroup B population. The remaining 32% had no complex association, a decrease of 15%. Serogroup C isolates were differentiated into 2 different STs contained within 2 recognised complex assignments. These included the ST-11 complex (91%), and ST-269 complex (9%). Serogroup W135 isolates were differentiated into 2 different STs, contained within a single complex association the ST-22 complex. Serogroup Y isolates were differentiated into 3 different STs, with no recognised complex association. Serogroups X and Z had no recognised complex association (Table 3.4).

Figure 3.5 Phylogenetic relationship of meningococci isolated in 2002 (year 4) and the association with ST complexes and serogroups. The UPGMA tree was constructed using the START program and ST complexes were assigned using BURST. A total of 89 isolates were characterised with each isolate represented by an individual number starting 02-. Each isolate has the sequence type and the allelic profile pertaining to this in brackets next to the isolate number. Each coloured region has the total number of strains represented as a total equal to N.

Serogroup B is shown in red	
Serogroup C in blue	
Serogroup Y in green	
Serogroup W135 in yellow	
Serogroup X in brown	
Serogroup Z in purple	

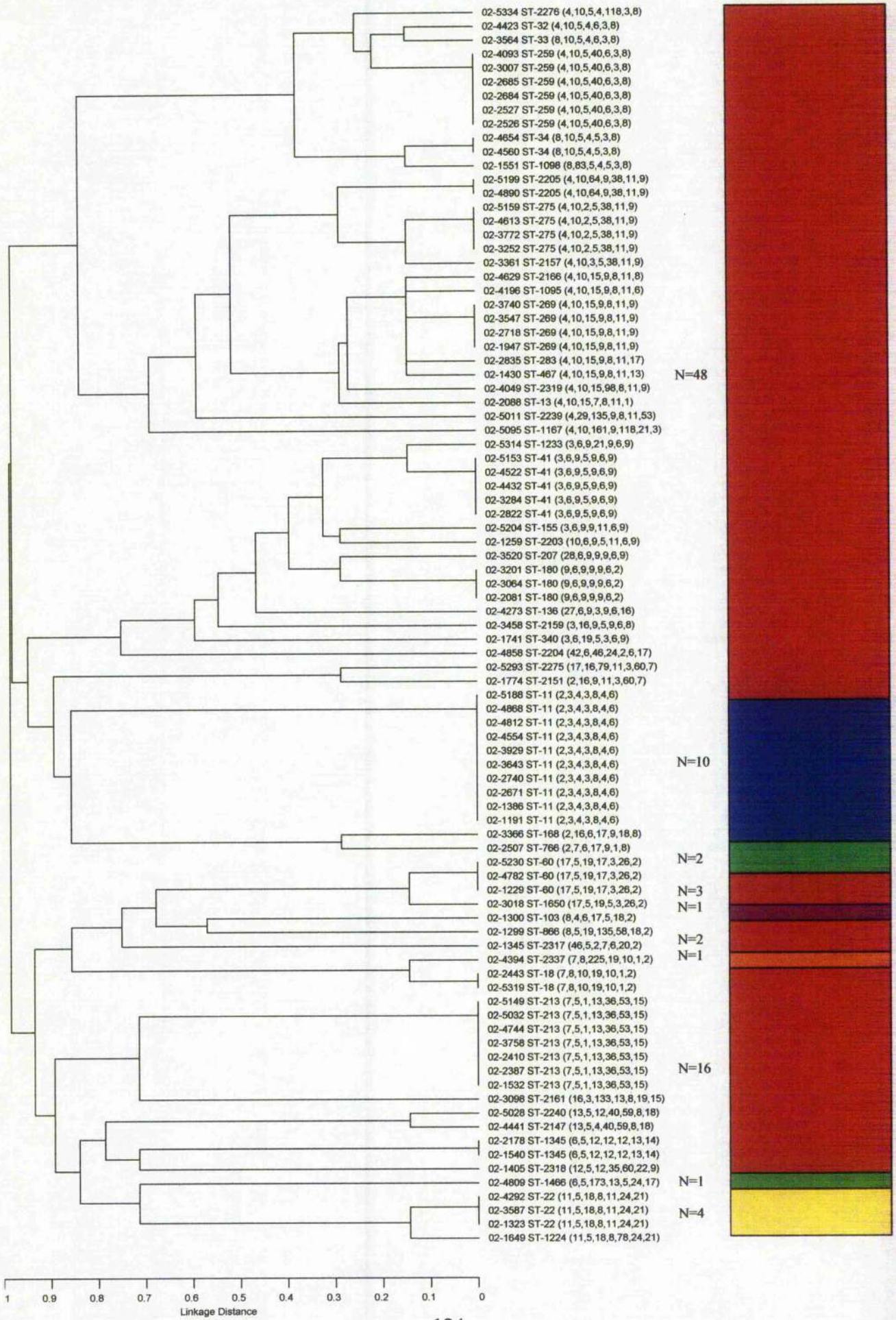

02-5334 ST-2276 (4,10,5,4,118,3,8)
02-4423 ST-32 (4,10,5,4,6,3,8)
02-3564 ST-33 (8,10,5,4,6,3,8)
02-4093 ST-259 (4,10,5,40,6,3,8)
02-3007 ST-259 (4,10,5,40,6,3,8)
02-2685 ST-259 (4,10,5,40,6,3,8)
02-2684 ST-259 (4,10,5,40,6,3,8)
02-2527 ST-259 (4,10,5,40,6,3,8)
02-2526 ST-259 (4,10,5,40,6,3,8)
02-4654 ST-34 (8,10,5,4,5,3,8)
02-4560 ST-34 (8,10,5,4,5,3,8)
02-1551 ST-1098 (8,83,5,4,5,3,8)
02-5199 ST-2205 (4,10,64,9,38,11,9)
02-4890 ST-2205 (4,10,64,9,38,11,9)
02-5159 ST-275 (4,10,2,5,38,11,9)
02-4613 ST-275 (4,10,2,5,38,11,9)
02-3772 ST-275 (4,10,2,5,38,11,9)
02-3252 ST-275 (4,10,2,5,38,11,9)
02-3361 ST-2157 (4,10,3,5,38,11,9)
02-4629 ST-2186 (4,10,15,9,8,11,8)
02-4196 ST-1095 (4,10,15,9,8,11,6)
02-3740 ST-269 (4,10,15,9,8,11,9)
02-3547 ST-269 (4,10,15,9,8,11,9)
02-2718 ST-269 (4,10,15,9,8,11,9)
02-1947 ST-269 (4,10,15,9,8,11,9)
02-2835 ST-283 (4,10,15,9,8,11,17)
02-1430 ST-467 (4,10,15,9,8,11,13)
N=48
02-4049 ST-2319 (4,10,15,98,8,11,9)
02-2088 ST-13 (4,10,15,7,8,11,1)
02-5011 ST-2239 (4,29,135,9,8,11,53)
02-5095 ST-1167 (4,10,161,9,118,21,3)
02-5314 ST-1233 (3,6,9,21,9,6,9)
02-5153 ST-41 (3,6,9,5,9,6,9)
02-4522 ST-41 (3,6,9,5,9,6,9)
02-4432 ST-41 (3,6,9,5,9,6,9)
02-3284 ST-41 (3,6,9,5,9,6,9)
02-2822 ST-41 (3,6,9,5,9,6,9)
02-5204 ST-155 (3,6,9,9,11,6,9)
02-1259 ST-2203 (10,6,9,5,11,6,9)
02-3520 ST-207 (28,6,9,9,9,6,9)
02-3201 ST-180 (9,6,9,9,9,6,2)
02-3064 ST-180 (9,6,9,9,9,6,2)
02-2081 ST-180 (9,6,9,9,9,6,2)
02-4273 ST-136 (27,6,9,3,9,6,16)
02-3458 ST-2159 (3,16,9,5,9,6,8)
02-1741 ST-340 (3,6,19,5,3,6,9)
02-4858 ST-2204 (42,6,46,24,2,6,17)
02-5293 ST-2275 (17,16,79,11,3,60,7)
02-1774 ST-2151 (2,16,9,11,3,60,7)
02-5188 ST-11 (2,3,4,3,8,4,6)
02-4868 ST-11 (2,3,4,3,8,4,6)
02-4812 ST-11 (2,3,4,3,8,4,6)
02-4554 ST-11 (2,3,4,3,8,4,6)
02-3929 ST-11 (2,3,4,3,8,4,6)
02-3643 ST-11 (2,3,4,3,8,4,6)
N=10
02-2740 ST-11 (2,3,4,3,8,4,6)
02-2671 ST-11 (2,3,4,3,8,4,6)
02-1386 ST-11 (2,3,4,3,8,4,6)
02-1191 ST-11 (2,3,4,3,8,4,6)
02-3366 ST-168 (2,16,6,17,9,18,8)
02-2507 ST-766 (2,7,6,17,9,1,8)
N=2
02-5230 ST-60 (17,5,19,17,3,26,2)
02-4782 ST-60 (17,5,19,17,3,26,2)
02-1229 ST-60 (17,5,19,17,3,26,2)
N=3
02-3018 ST-1650 (17,5,19,5,3,26,2)
N=1
02-1300 ST-103 (8,4,6,17,5,18,2)
02-1299 ST-866 (8,5,19,135,58,18,2)
N=2
02-1345 ST-2317 (46,5,2,7,6,20,2)
02-4394 ST-2337 (7,8,225,19,10,1,2)
N=1
02-2443 ST-18 (7,8,10,19,10,1,2)
02-5319 ST-18 (7,8,10,19,10,1,2)
02-5149 ST-213 (7,5,1,13,36,53,15)
02-5032 ST-213 (7,5,1,13,36,53,15)
02-4744 ST-213 (7,5,1,13,36,53,15)
02-3758 ST-213 (7,5,1,13,36,53,15)
02-2410 ST-213 (7,5,1,13,36,53,15)
02-2387 ST-213 (7,5,1,13,36,53,15)
N=16
02-1532 ST-213 (7,5,1,13,36,53,15)
02-3098 ST-2161 (16,3,133,13,8,19,15)
02-5028 ST-2240 (13,5,12,40,59,8,18)
02-4441 ST-2147 (13,5,4,40,59,8,18)
02-2178 ST-1345 (6,5,12,12,12,13,14)
02-1540 ST-1345 (6,5,12,12,12,13,14)
02-1405 ST-2318 (12,5,12,35,60,22,9)
02-4809 ST-1466 (6,5,173,13,5,24,17)
N=1
02-4292 ST-22 (11,5,18,8,11,24,21)
02-3587 ST-22 (11,5,18,8,11,24,21)
N=4
02-1323 ST-22 (11,5,18,8,11,24,21)
02-1649 ST-1224 (11,5,18,8,78,24,21)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Linkage Distance

Table 3.4 Summary of meningococcal allelic profile frequencies from meningococci isolated in 2002. This represents a summary of all the different sequence types (ST) characterised in 2002. The table shows the ST followed by the allelic profile pertaining to that ST. Each allelic number represents each gene in order as stated in the table. The frequency number is the total number of meningococci represented by that ST followed by a percentage in relation to the total number of meningococci isolated.

ST	Allelic Profile (*abcZ, adk, aroE, fumC, gdh, pdhC, pgm*)	Frequency	% of dataset
11	2, 3, 4, 3, 8, 4, 6	10	10.99
213	7, 5, 1, 13, 36, 53, 15	7	7.69
259	4, 10, 5, 40, 6, 3, 8	7	7.69
41	3, 6, 9, 5, 9, 6, 9	5	5.49
269	4, 10, 15, 9, 8, 11, 9	4	4.40
275	4, 10, 2, 5, 38, 11, 9	4	4.40
60	17, 5, 19, 17, 3, 26, 2	3	3.30
22	11, 5, 18, 8, 11, 24, 21	3	3.30
180	9, 6, 9, 9, 9, 6, 2	3	3.30
18	7, 8, 10, 19, 10, 1, 2	2	2.20
1345	6, 5, 12, 12, 12, 13, 14	2	2.20
1224	11, 5, 18, 8, 78, 24, 21	2	2.20
34	8, 10, 5, 4, 5, 3, 8	2	2.20
2205	4, 10, 64, 9, 38, 11, 9	2	2.20
2203	10, 6, 9, 5, 11, 6, 9	1	1.10
866	8, 5, 19, 135, 58, 18, 2	1	1.10
103	8, 4, 6, 17, 5, 18, 2	1	1.10
2317	46, 5, 2, 7, 6, 20, 2	1	1.10
2318	12, 5, 12, 35, 60, 22, 9	1	1.10
467	4, 10, 15, 9, 8, 11, 13	1	1.10
1098	8, 83, 5, 4, 5, 3, 8	1	1.10
340	3, 6, 19, 5, 3, 6, 9	1	1.10
2151	2, 16, 9, 11, 3, 60, 7	1	1.10
13	4, 10, 15, 7, 8, 11, 1	1	1.10
766	2, 7, 6, 17, 9, 1, 8	1	1.10
283	4, 10, 15, 9, 8, 11, 17	1	1.10
1650	17, 5, 19, 5, 3, 26, 2	1	1.10
2161	16, 3, 133, 13, 8, 19, 15	1	1.10
168	2, 16, 6, 17, 9, 18, 8	1	1.10
2157	4, 10, 3, 5, 38, 11, 9	1	1.10

2159	3, 16, 9, 5, 9, 6, 8	1	1.10
207	28, 6, 9, 9, 9, 6, 9	1	1.10
33	8, 10, 5, 4, 6, 3, 8	1	1.10
2319	4, 10, 15, 98, 8, 11, 9	1	1.10
1095	4, 10, 15, 9, 8, 11, 6	1	1.10
136	27, 6, 9, 3, 9, 6, 16	1	1.10
2337	7, 8, 225, 19, 10, 1, 2	1	1.10
32	4, 10, 5, 4, 6, 3, 8	1	1.10
2147	13, 5, 4, 40, 59, 8, 18	1	1.10
2166	4, 10, 15, 9, 8, 11, 8	1	1.10
1466	6, 5, 173, 13, 5, 24, 17	1	1.10
2204	42, 6, 46, 24, 2, 6, 17	1	1.10
2239	4, 29, 135, 9, 8, 11, 53	1	1.10
2240	13, 5, 12, 40, 59, 8, 18	1	1.10
1167	4, 10, 161, 9, 118, 21, 3	1	1.10
155	3, 6, 9, 9, 11, 6, 9	1	1.10
2275	17, 16, 79, 11, 3, 60, 7	1	1.10
1233	3, 6, 9, 21, 9, 6, 9	1	1.10
2276	4, 10, 5, 4, 118, 3, 8	1	1.10

The 49 different profile(s) in dataset (displayed in descending order of frequency).

3.3 Sequence Types, Lineages and Inter-Lineage Variability

The introduction of MLST in Scotland has provided a routine method for the characterisation of *N. meningitidis* isolates since January 1999. Coincidentally, this is just prior to the introduction of meningococcal MenC vaccines in November 1999.

All *N. meningitidis* isolates from invasive disease have been analysed. This has allowed the production of valuable data highlighting the effect the MenC vaccine has on serogroup C disease and its potential decline after the introduction of the immunisation campaign. At the same time, the overall number of cases was monitored including the effect on serogroup B between 1999 and 2002. This will then be linked with sequence typing data such as the typical complex patterns seen with serogroups B and C pre, during and post implementation of the initial MenC vaccination program. The incidence of ST-11/ET-37 strains in serogroup C disease will be assessed along with ST-11/ET-37 strains of other serogroups. The number of different STs isolated will also be monitored from the introduction of the MenC vaccine and this will be linked with their corresponding serogroups and subtypes.

A decrease in serogroup C disease will be expected, but it remains to be seen if this is accompanied by a relative increase in serogroup B disease. Any suspected capsule switch will be clarified and applied to the whole data set for subsequent conclusion. This will hopefully show that MLST is extremely important for the surveillance of meningococcal disease over a period of years and, and that this data, will have been effective not only in monitoring the impact of the MenC vaccines, but also providing a detailed genotypic representation of strains now commonly associated with disease.

3.3.1 Sequence Types, Lineages and Inter-Lineage Variability in 1999.

With reference to section 3.0 these isolates were categorised into 41 different STs (Figure 3.6). Using BURST analysis they were further differentiated into six distinct lineages, with 15 singleton types (11.5%). These lineages occurred between one (0.8%) and 55 (42%) times during 1999. The ST-11 complex accounted for the majority with 55 (42%) of all the isolates and these were divided into two STs; 53 were ST-11 (96%) and two were ST-655 (4%) a *gdh* locus variant of ST-11. The ST-44 complex accounted for 22 isolates (17%) and these were divided into eight different STs; 14 were ST-41 (64%), two were ST-1097 (9%) and ST-44, 46, 146, 170, 1194 and 1255 were each represented by a single isolate (4.5%). Within this lineage there were eight single locus variants combined with 20 double locus variants and 28 satellites. The ST-269 complex accounted for 17 isolates (13%), which were divided into eight different STs; ST-269 (35%) ST-275, and ST-352 (18%), and ST-1095 1273 1416 2775, and 2776 were represented by a single isolate (6%). Within this lineage there were eight single locus variants combined with nine double locus variants and 25 satellites. The ST-35 complex accounted for three isolates (2%), these were divided into three different STs, 457, 472 and 2772, and contained two single locus variants combined with two double locus variants and two satellites. The ST-32 complex accounted for three isolates; these were divided into two different STs, ST-33 (67%) and ST-32 (33%). There were two single locus variants. The remaining three isolates, ST-60, 2013 and 2294 were assigned to the final group but with no complex association. The singletons accounted for 27 isolates differentiated between 15 different STs; ST-213 (19%) ST-18 (15%) ST-284 (11%) ST-271, 750, and 22 (7%) and ST-23 29 1738 1819 2292 2293 2295, and 2771 were all represented once (4%) (Appendix 6.4) (Table 3.5). These represented STs that were not closely

associated with other STs within the population, although some are assigned to complex lineages.

Figure 3.6 Meningococcal analysis based upon related sequence types (BURST) from meningococci isolated in 1999 (year 1). The complete dataset of sequence types (ST) are grouped based upon the similarity of five or more allele numbers within an allelic profile. These are tabulated and presented as the ST combined with its frequency within the population and the genetic relatedness between other STs within the group. Each group is analysed based upon any single locus, double locus or satellite nucleotide changes between associated STs. If there are numerous STs associated to a single group a central ST is identified and is signified within the table with an asterisk (*). Furthermore this diagram highlighting the relatedness between different STs is displayed below the table. This diagram highlights the central ST within the centre of the circle and each ST located in the first outer circle will have a single locus variant (SLV) association with the central ST. Similarly, each ST within the second ring will have a double locus variant (DLV) association with the central ST. In addition, lines linking different STs will highlight either SLV or DLV associations with each other. Any STs that do not group are listed at the end and are termed "singletons". Because they have no other closely associated ST they are displayed with their ST and the frequency within the population.

Group: 1 (55 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
11	53	1	0	0
655	2	1	0	0

Group: 2 (22 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
1255	1	1	2	4
41*	14	3	3	1
1097	2	0	1	6
146	1	1	2	4
1194	1	0	2	5
44	1	1	3	3
46	1	1	3	3
170	1	1	4	2

Summary view:

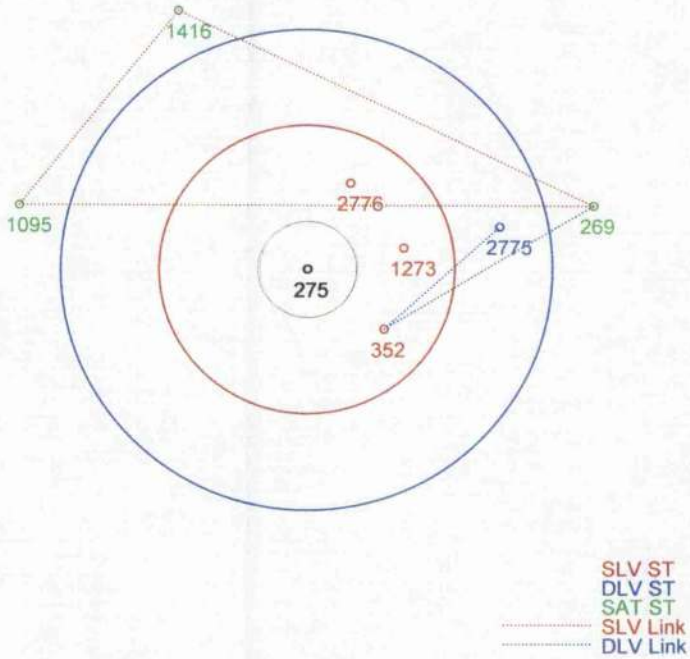
Group: 3 (3 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
2772	1	0	1	1
457	1	1	1	0
472	1	1	0	1

Group: 4 (17 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
352	3	1	4	2
1273	1	1	2	4
1416	1	2	0	5
269	6	2	1	4
1095	1	2	0	5
2775	1	0	2	5
2776	1	1	2	4
275*	3	3	1	3

Summary view:



Group: 5 (3 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
33	2	1	0	0
32	1	1	0	0

Group: 6 (3 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
2013	1	1	0	1
2294	1	0	1	1
60	1	1	1	0

Singleton sequence types (27 isolates)

ST	Frequency
23	1
284	3
213	5
2771	1
22	2
18	4
2292	1
1819	1
2293	1
750	2
1738	1
2295	1
29	1
271	2
2296	1

These isolates are grouped into 6 lineage(s), with 15 singleton sequence type(s).

Founder sequence types are marked with an asterisk (*).

Table 3.5 Distribution of meningococcal housekeeping genes represented by allele number from meningococci isolated in 1999 (year 1). A complete list of allele types directly correlated with the total number identified within each specific housekeeping gene from meningococci isolated during 1999.

Allele	*abcZ*	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*
1			5			4	
2	59		10			2	13
3	19	59		55	4	3	2
4	21		55	3		56	
5		15	3	26	2		3
6		25	2		9	23	56
7	10	1		1			
8	5	5		2	64		6
9	4		22	21	26	1	35
10	1	22	5	1	5	3	
11	2		3		4	19	
12	1						2
13	1	1		6			
15			10		2		6
16					1		2
17	2			5			2
18			4			1	1
19			2	4			
20	3	2				4	
21							2
24				2		3	
25						1	
26						3	
34			1				
35				1			
36					5		
38					5	1	
44					2		
46	1						
53						5	
60				1			

61				**2**			
63			**3**				
64			**1**				
118					**1**		
130			**1**				
133	**1**		**1**				
146			**1**				
147						**1**	
152			**1**				
Total	**14**	**8**	**18**	**14**	**13**	**16**	**12**

3.3.2 Sequence Types, Lineages and Inter-Lineage Variability in 2000.

With reference to section 3.0, these isolates were categorised into 47 different STs, an increase of 13% on the previous year (Figure 3.7). Using BURST analysis they were further differentiated into nine distinct lineages, an increase of 33% on the previous year, with a 27% decrease in singleton representation. These lineages occurred between one (0.8%) and 49 (38%) times during 2000. This was statistically similar to the previous year with a variation in range of less than 5%. Sequence type complex 11 continued to account for the majority with 49 (38%) isolates these were not associated with any other STs and represented all the STs within the ST-11 complex. This large proportion of isolates accounted for 80% of all singletons during 2000. The ST-44 complex accounted for 22 isolates (17%), which were divided into 10 different STs, an increase within the complex of 20%. There was a decrease of 14% in ST-41 isolates and the presence of only two further STs that were present in 1999, both ST-44 (9%) and ST-46 (4.5%). The remaining STs had not been isolated in the previous year. These were ST-154 (9%) and ST-180 340 1009 2120 2300, and 2303 (4.5%). Within this lineage there were 12 single locus variants, an increase of 33%, combined with 22 double locus variants, an increase of 10%, and 56 satellites, an increase of 100%. The ST-269 complex accounted for 14 isolates (11%), a decrease of 18%. These were divided into four groups, a decrease of 50% on the previous year, ST-269 (57%), an increase of 25%, ST-275 (29%), an increase of 25%, ST-352 and 1273 were represented once (7%). Inter-lineage variation accounted for four single locus variants, a decrease of 50%, four double locus variants, a decrease of 55%, and four satellites, a decrease of 84%. A further 11 (8%) isolates were grouped without any association to a ST complex. These were divided with eight ST-213 isolates (73%), an increase of 37.5% and three isolates, ST-2301 2660, and 2782 all represented once

(9%). These isolates were not represented in the previous year. This highlights an expansion of isolates closely associated with ST-213 by 27%. Inter-lineage variation accounted for two single locus variants combined with four double locus variants and six satellites. The ST-32 complex accounted for 6 isolates, an increase of 100%, these were represented by six individual STs, ST-32 33 84 259 2298, and 2784 (16.7%). Inter-lineage variation accounted for 12 single locus variants with 10 double locus variants and eight satellites. The ST-18 complex accounted for four isolates (3%), with two ST-2305 (50%) and one ST-18 and 2028 (25%). This complex highlights an expansion of a singleton ST-18 from the previous year however a 75% decrease in ST-18 isolates was observed. Inter-lineage variation accounted for two single and double locus variants combined with two satellites. The ST-22 complex had four isolates represented by three ST-22 strians (75%) and a single ST-184, with two double locus variants. This again was an expansion of a singleton ST-22 from the previous year with a 33% increase from the previous year. The three remaining ST groups had no complex association. The first group contained four isolates (3%) represented by two ST-168 (50%) isolates and a single ST-167 and 1198 (25%). Inter-lineage variation accounted for two single and double locus variants combined with two satellites. The second group contained two isolates (2%), ST-2779 and 2299, with inter-lineage variation accounting for two single locus variants. The final group contained two isolates (2%), ST-2297 and 2302, with inter-lineage variation accounting for two single locus variants. All three groups containing these STs had not been represented in the previous year. The singletons accounted for 61 isolates, an increase of 111% on the previous year. These were differentiated between 11 different STs, ST-11 accounting for the majority of isolates (80%), ST-8 and ST-60 (3%) with ST-53 457 772 2304 2777 2781 2851, and 2852 all represented once

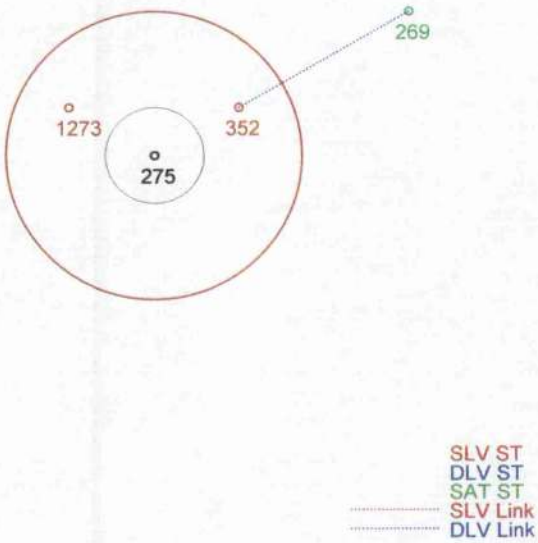
(2%) (Appendix 6.5) (Table3.6) with the exclusion of ST-11 isolates the singletons would account for 9% of the total number of isolates, a decrease of 11% on the previous year.

Figure 3.7 Meningococcal analysis based upon related sequence types (BURST) from meningococci isolated in 2000 (year 2). The complete dataset of sequence types (ST) are grouped based upon the similarity of five or more allele numbers within an allelic profile. These are tabulated and presented as the ST combined with its frequency within the population and the genetic relatedness between other STs within the group. Each group is analysed based upon any single locus, double locus or satellite nucleotide changes between associated STs. If there are numerous STs associated to a single group a central ST is identified and is signified within the table with an asterisk (*). Furthermore, this diagram highlighting the relatedness between different STs is displayed below the table. This diagram highlights the central ST within the centre of the circle and each ST located in the first outer circle will have a single locus variant (SLV) association with the central ST. Similarly, each ST within the second ring will have a double locus variant (DLV) association with the central ST. In addition, lines linking different STs will highlight either SLV or DLV associations with each other. Any STs that do not group are listed at the end and are termed "singletons". Because they have no other closely associated ST they are displayed with their ST and the frequency within the population.

Group: 1 (14 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
352	1	1	2	0
275*	4	2	0	1
269	8	0	1	2
1273	1	1	1	1

Summary view:



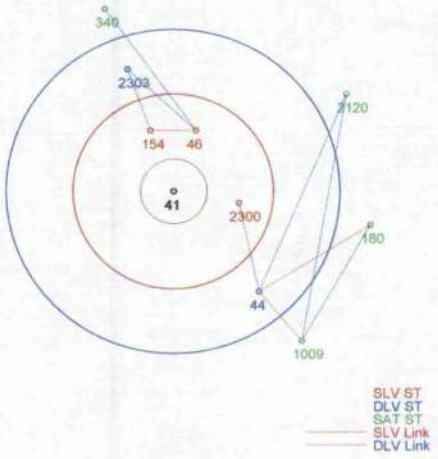
Group: 2 (6 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
84	1	2	1	2
2298	1	1	2	2
259	1	1	2	2
33	1	3	2	0
32	1	3	2	0
2784	1	2	1	2

Group: 3 (22 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
41*	11	3	2	4
1009	1	1	2	6
154	2	2	2	5
2300	1	1	3	5
44	2	2	3	4
180	1	1	1	7
2303	1	0	3	6
46	1	2	3	4
2120	1	0	2	7
340	1	0	1	8

Summary view:



Group: 4 (4 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
22	3	0	1	0
184	1	0	1	0

Group: 5 (2 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
2297	1	1	0	0
2302	1	1	0	0

Group: 6 (11 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
213	8	1	1	1
2301	1	0	1	2
2782	1	0	1	2
2660	1	1	1	1

Group: 7 (4 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
167	1	1	1	0
168	2	0	1	1
1198	1	1	0	1

Group: 8 (2 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
2299	1	1	0	0
2779	1	1	0	0

Group: 9 (4 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
18	1	1	1	0
2305	2	1	0	1
2028	1	0	1	1

Singleton sequence types (61 isolates)

ST	Frequency
11	49
2777	1
8	2
60	2
2304	1
457	1
2851	1
2852	1
53	1
2781	1
772	1

The isolates are grouped into 9 lineage(s), with 11 singleton sequence type(s).

Founder sequence types are marked with an asterisk (*).

Table 3.6 Distribution of meningococcal housekeeping genes represented by allele number from meningococci isolated in 2000 (year 2). A complete list of allele types directly correlated with the total number identified within each specific housekeeping gene from meningococci isolated during 2000.

Allele	*abcZ*	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*
1	1		11			3	
2	56	4	5	2			10
3	16	52		51	5	6	
4	20		49	5		50	
5		19	6	23		2	
6		22	6		6	24	49
7	14	3	2				1
8	4	3		4	61		11
9	5		24	17	23		34
10		22	4		4	1	3
11	5		1		6	15	
12	2		3				1
13				11			
14					1		
15	1		8				11
16	1	3			2	2	1
17	3			7	1		3
18			3		2	4	
19			3	5		1	
21			1				4
22						3	2
24				1		4	
25				1		1	
26		1				2	
27	1						
35	1			2			
36					10		
38					5		
40				1			
53						9	
54						2	

60					**2**	**1**	
63			**1**				
64			**1**				
72			**1**				
118					**1**		
120		**1**					
133			**1**				
154					**1**		
Total	**14**	**10**	**18**	**13**	**15**	**17**	**12**

3.3.3 Sequence Types, Lineages and Inter-Lineage Variability in 2001.

With reference to section 3.0, these isolates were categorised into 47 different STs, remaining consistent with the previous year (Figure 3.8). Using BURST analysis they were further differentiated into eight different lineages, a decrease of 11% from the previous year. These lineages occurred between one (1%) and 18 (23%) times during 2001. This range variation was significant smaller, with a 15% decrease in the upper percentage limit. This highlights a significant decrease in the representation of multiple isolates and the subsequent increase in single represented isolates. Sequence type complex 11 continued to account for the majority with 20 isolates (25%) containing 18 ST-11 isolates (90%) a 63% decrease on the previous year with both ST-1189 and 2031 represented once (5%). The highlighted an expansion in the ST-11 complex, neither closely associated ST-11 isolate had been represented in previous years. Inter-lineage variation accounted for two single locus variations combined with four double locus variations. The ST-269 complex accounted for 15 isolates (19%), an increase of 5% on the previous year. These were divided into 10 different STs, an increase of 150% on the previous year. ST-269 and 275 accounted for three isolates (20%), a decrease of 133% and a 25% increase on the previous year respectively. ST-352 was represented once (7%) and remained unchanged from the previous year. ST-1049 appeared twice (13%) and was not represented in previous years. The remaining STs, ST-479 1416 1774 2307 2315, and 2324 were all represented once (7%). Only ST-1416 was represented previously in 1999. Inter-lineage variation accounted for 10 single locus variations, a 120% increase combined with 24 double locus variations, a 500% increase and 56 satellites, an increase of 1300% increase. This highlighted statistically significant increases in sequence variation within this complex, which is a result of the expansion of closely associated STs within the complex. A further six

isolates (7.5%) were grouped without any association to an ST complex. This represented four ST-213 isolates (67%), a decrease of 50% on the previous year, and two ST-1218 isolates (33%). This highlighted a decrease in ST-213 and their closely associated isolates with inter-lineage variation accounted for only two single locus variations. The ST-22 complex had five isolates (6%), a 20% increase on the previous year with three ST-184 isolates (60%) a 67% increase, ST-22 and 1224 were represented once. Inter-lineage variation accounted for four single locus variants combined with two double locus variants. The ST-32 complex accounted for four isolates (5%) a 33% decrease. This complex was represented by two ST-259 isolates (50%) with ST-32 and 33 represented once. This complex shows significant contraction with a 50% decrease in the number of different STs represented within the complex. The remaining STs have all been represented previously and have consolidated their presence within the complex. As a consequence, the inter-lineage variation accounted for four single locus variants combined with two double locus variants. The ST-18/44 complex accounted for three isolates (4%), a 25% decrease on ST-18 complex isolates, with ST-18, 41, and 2310 all represented once. Again this complex has maintained its presence with closely associated isolates and although ST-18 and 41 are associated with different complexes, BURST analysis has grouped these together in the same group within this year. Inter-lineage variation accounted for two single locus variations, combined with one double locus variation and five satellites. The second ST-44 complex accounted for three isolates (4%), with a single representation of ST-154, 2314, and 2323, a significant decrease of 733% on the previous year. One remaining group had no complex association and accounted for three isolates (4%) represented by two ST-2306 strains and a single ST-2309. Inter-lineage variation accounted for two single locus variations. The singletons accounted

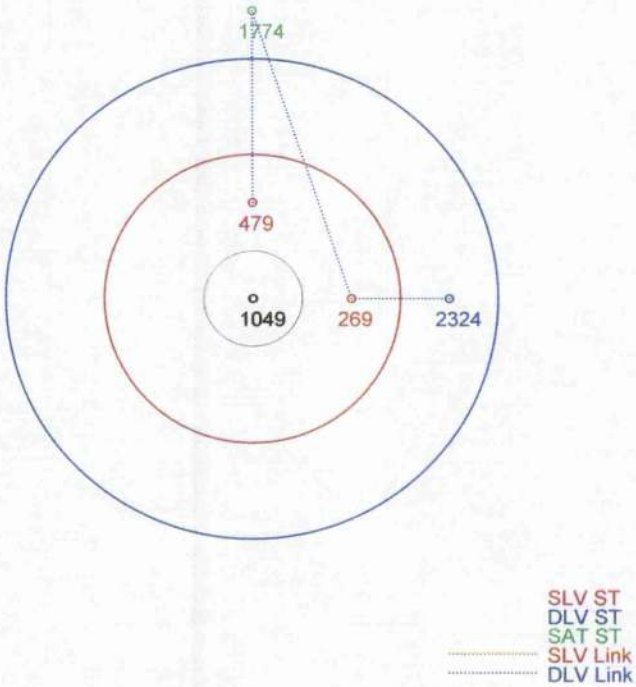
for 21 isolates (26%), with the exclusion of ST-11 isolates this represented a 43% increase in the previous year. These were differentiated into 18 different STs, ST-461, 1217 and 1345 were represented twice (10%), with ST-8, 15, 36, 60, 168, 212, 284, 286, 750, 1157, 2308, 2311, 2313, 2316, and 2322 represented once (Appendix 6.6) (Table 3.7).

Figure 3.8 Meningococcal analysis based upon related sequence types (BURST) from meningococci isolated in 2001 (year 3). The complete dataset of sequence types (ST) are grouped based upon the similarity of five or more allele numbers within an allelic profile. These are tabulated and presented as the ST combined with its frequency within the population and the genetic relatedness between other STs within the group. Each group is analysed based upon any single locus, double locus or satellite nucleotide changes between associated STs. If there are numerous STs associated to a single group a central ST is identified and is signified within the table with an asterisk (*). Furthermore, this diagram highlighting the relatedness between different STs is displayed below the table. This diagram highlights the central ST within the centre of the circle and each ST located in the first outer circle will have a single locus variant (SLV) association with the central ST. Similarly, each ST within the second ring will have a double locus variant (DLV) association with the central ST. In addition, lines linking different STs will highlight either SLV or DLV associations with each other. Any STs that do not group are listed at the end and are termed "singletons". Because they have no other closely associated ST they are displayed with their ST and the frequency within the population.

Group: 1 (8 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
2324	1	0	2	2
1049*	2	2	1	1
269	3	1	3	0
479	1	1	2	1
1774	1	0	2	2

Summary view:



Group: 2 (19 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
2031	1	0	2	0
11	17	1	1	0
1189	1	1	1	0

Group: 3 (4 isolates)

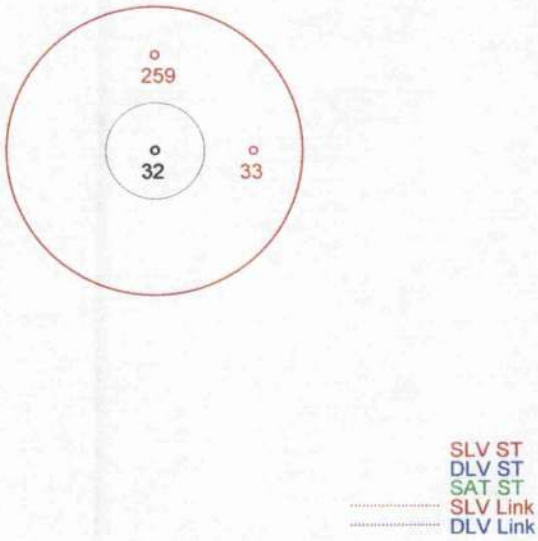
ST	Frequency	Single locus variants	Double locus variants	Satellites
2323	1	0	1	2
2314	1	1	1	1
154	1	1	1	1
41*	1	2	1	0

Summary view:

Group: 4 (3 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
33	1	1	1	0
32*	1	2	0	0
259	1	1	1	0

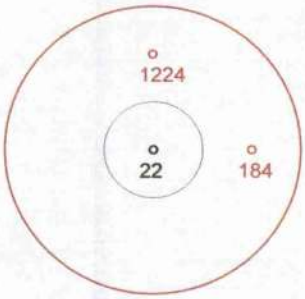
Summary view:



Group: 5 (5 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
22*	1	2	0	0
184	3	1	1	0
1224	1	1	1	0

Summary view:



Group: 6 (5 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
275	3	1	1	0
2307	1	1	0	1
2315	1	0	1	1

Group: 7 (3 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
2306	2	1	0	0
2309	1	1	0	0

Group: 8 (2 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
2310	1	1	0	0
18	1	1	0	0

Group: 9 (6 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
2321	1	0	2	0
213	4	1	1	0
1218	1	1	1	0

Singleton sequence types (19 isolates)

ST	Frequency
168	1
284	1
1217	2
2322	1
36	1
2308	1
286	1
8	1
1157	1
212	1
1345	2
60	1
461	2
15	1
2313	1
2316	1

The isolates are grouped into 9 lineage(s), with 16 singleton sequence type(s).

Founder sequence types are marked with an asterisk (*).

Table 3.7 Distribution of meningococcal housekeeping genes represented by allele number from meningococci isolated in 2001 (year 3). A complete list of allele types directly correlated with the total number identified within each specific housekeeping gene from meningococci isolated during 2001.

Allele	*abcZ*	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*
1		1	6	1		4	
2	23		7	1			6
3	7	21		22	3	4	
4	19	1	21	2		23	
5		23	4	12	1	1	2
6	3	7	1		7	5	20
7	7	1	2	1			
8	5	2		7	31		5
9	1		11	8	10		16
10		17	2		2		
11	5				6	17	
12	3		6	2	2		
13	1			9		2	
14					1		2
15	1		9		1		9
16		1	1				3
17	1			6			6
18			5			1	1
19			1	2		2	
20	1					3	
21					1	2	5
22						7	
23				1			
24				1		2	
25		1					
26		1			1	2	
34	1		1				
35		1		2			
36					6	1	
38					5		
40				2			

43	**1**						
46	**1**						
49							**1**
53						**4**	
60				**1**	**2**		
63			**1**				
69							**1**
76		**3**					
78					**1**		
117							**3**
133			**1**				
161			**1**				
Total	**16**	**13**	**17**	**17**	**16**	**16**	**14**

3.3.4 Sequence Types, Lineages and Inter-Lineage Variability in 2002.

With reference to section 3.0, these isolates were categorised into 49 different STs, a 4% increase on the previous year (Figure 3.9). Using BUSRT analysis they were further differentiated into nine different lineages, an increase of 11%on the previous year. These lineages occurred between one (1%) and 10 (11%) times during 2002. The range variation was again significantly smaller, with a 12% decrease in the upper limit. This was a further continuation towards a greater proliferation of different STs and thus a greater representation of single isolates. The ST-269 complex accounted for 17 isolates (19%), an increase of 12% on the previous year. These were divided into 10 different STs which remained consistent. ST-269 and 275 accounted for four isolates (24%), ST-2205 accounted for two isolates (12%), with ST-13, 283, 467, 1095, 2157, 2166, 2205 and 2319 represented once (6%). ST-269 and 275 both increased by 25% on the previous year and of the remaining STs none had been represented in previous years. Inter-lineage variation accounted for 24 single locus variations, a 140% increase, combined with 26 double locus variations, an 8% increase, with 40 satellites, a decrease of 29% on the previous year. The ST-44 complex accounted for 14 isolates (15%), an increase of 367% and more representative of the ST-44 complex in 2000. ST-41 accounted for five isolates (36%), with three ST-180 isolates (21%) and ST- 155, 207, 340, 1233, 2159 and 2203 were represented once (7%). Inter-lineage variation accounted for two single locus variations combined with 20 double locus variations and 34 satellites. This was more consistent with inter-lineage variation of this complex representation during 2000. ST-32 complex accounted for 13 isolates (14%) a 225% increase. This complex was represented by six different STs. ST- 259 accounted for seven isolates (54%), an increase of 250% with two ST-34 isolates (15%) and ST- 32, 33, 1098 and 2276

represented once (8%). This complex shows significant expansion with a 100% increase in the number of different STs represented within the complex. As a consequence the inter-lineage variation accounted for 10 single locus variations combined with 12 double locus variations and eight satellites. The ST-22 complex had five isolates (6%), with three ST-22 isolates (60%), and ST-1224 represented twice. This represented a consolidation of ST-22 and 1224 and the disappearance of ST- 184 from the previous year. Inter-lineage variation accounted for two single locus variations. The ST-18 complex accounted for three isolates (3%) with two ST-18 isolates and a single ST-2337. On this occasion ST-41 was not grouped within this complex and was considered more closely associated with alternative STs within 2002. Inter-lineage variation accounted for two single locus variations. The remaining four groups had no complex association. The first group contained four isolates (4%) with three ST-60 isolates and a single ST-1650, with an inter-lineage variation of two single loci. The non-complex associated group represents an expansion of ST-60 from a singleton sequence type in the previous year. The second group contained two isolates, ST-168 and 766 and again this was an expansion of a previously associated singleton sequence type in the previous year, with inter-lineage variation of two double loci. The third group contained two isolates, ST 2147 and 2240 with two single locus variations. The forth group contained two isolates, ST-2151 and 2275 with two double locus variations. Both previous groups had not been represented in the previous year. The singletons accounted for 29 isolates (32%), an increase of 28% on the previous year. These were differentiated into 13 different STs, ST-11 (35%), ST-213 (24%), ST-1345 (7%) and ST-103, 136, 866, 1167, 1466, 2161, 2204, 2239, 2317 and 2318 were represented once (Appendix 6.7) (Table 3.8). ST-11 decreased by a further 44% on the previous year. ST-213 increased by 43% on the

previous year although ST-213 was not closely associated with other STs during 2002. Existing singletons from 2002 had remained non-associated with other STs compared with the previous year.

Figure 3.9 Meningococcal analysis based upon related sequence types (BURST) from meningococci isolated in 2002 (year 4). The complete dataset of sequence types (ST) are grouped based upon the similarity of five or more allele numbers within an allelic profile. These are tabulated and presented as the ST combined with its frequency within the population and the genetic relatedness between other STs within the group. Each group is analysed based upon any single locus, double locus or satellite nucleotide changes between associated STs. If there are numerous STs associated to a single group a central ST is identified and is signified within the table with an asterisk (*). Furthermore, this diagram highlighting the relatedness between different STs is displayed below the table. This diagram highlights the central ST within the centre of the circle and each ST located in the first outer circle will have a single locus variant (SLV) association with the central ST. Similarly, each ST within the second ring will have a double locus variant (DLV) association with the central ST. In addition, lines linking different STs will highlight either SLV or DLV associations with each other. Any STs that do not group are listed at the end and are termed "singletons". Because they have no other closely associated ST they are displayed with their ST and the frequency within the population.

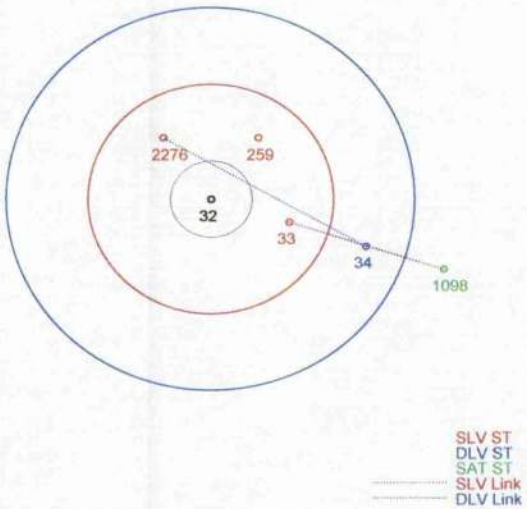
Group: 1 (14 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
2203	1	0	2	5
340	1	0	1	6
180	3	0	1	6
41	5	1	5	1
2159	1	0	1	6
207	1	0	4	3
155	1	0	4	3
1233	1	1	2	4

Group: 2 (12 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
1098	1	1	1	3
259	6	1	2	2
33	1	2	3	0
32*	1	3	1	1
34	2	2	2	1
2276	1	1	3	1

Summary view:



Group: 3 (3 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
1224	1	1	0	0
22	2	1	0	0

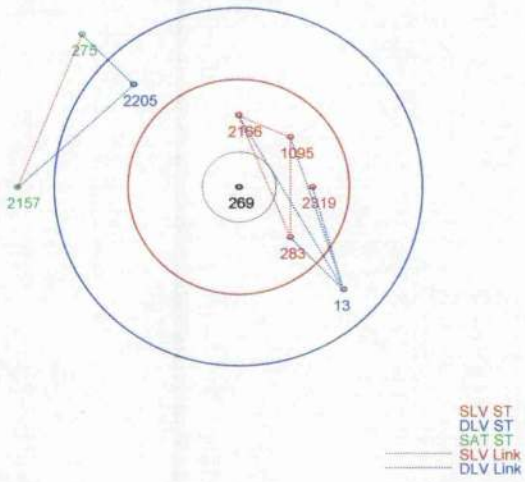
Group: 4 (2 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
2151	1	0	1	0
2275	1	0	1	0

Group: 5 (16 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
269*	4	4	2	2
13	1	0	5	3
283	1	3	2	3
275	4	1	1	6
2157	1	1	1	6
2319	1	1	4	3
1095	1	3	2	3
2166	1	3	2	3
2205	2	0	3	5

Summary view:



Group: 6 (3 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
18	2	1	0	0
2337	1	1	0	0

Group: 7 (2 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
766	1	1	0	0
168	1	1	0	0

Group: 8 (2 isolates)

ST	Frequency	Single locus variants	Double locus variants	Satellites
2147	1	1	0	0
2240	1	1	0	0

Singleton sequence types (30 isolates)

ST	Frequency
11	9
60	3
866	1
103	1
2317	1
2318	1
1345	2
213	6
2161	1
136	1
1466	1
2204	1
2239	1
1167	1

The isolates are grouped into 8 lineage(s), with 14 singleton sequence type(s).

Founder sequence types are marked with an asterisk (*).

Table 3.8 Distribution of meningococcal housekeeping genes represented by allele number from meningococci isolated in 2002 (year 4). A complete list of allele types directly correlated with the total number identified within each specific housekeeping gene from meningococci isolated during 2002.

Allele	*abcZ*	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*
1			7			4	1
2	13		5		1		13
3	9	11	1	11	7	13	1
4	28	1	11	6		10	
5		24	13	14	5		
6	3	15	3		10	16	11
7	10	1		2			2
8	6	3		5	22	2	17
9	3		15	17	14		23
10	1	30	2		3		
11	5			2	5	18	
12	1		4	2	2		
13	2			9		2	1
14							2
15			10				8
16	1	4					1
17	5			6			3
18			5			3	2
19			6	3		1	
20						1	
21				1		1	5
22						1	
24				1		6	
26						4	
27	1						
28	1						
29		1					
35				1			
36					7		
38					7		
40				9			

42	**1**						
46	**1**		**1**				
53						**7**	**1**
58					**1**		
59					**2**		
60					**1**	**2**	
64			**2**				
78					**2**		
79			**1**				
83		**1**					
98				**1**			
118					**2**		
133			**1**				
135			**1**	**1**			
161			**1**				
173			**1**				
225			**1**				
Total	**17**	**10**	**20**	**17**	**16**	**16**	**15**

3.4 Diversity of Housekeeping Genes and Sequence Types.

During this four-year study the distribution of housekeeping genes within a ST and the diversity of STs displayed specific patterns or lack of depending on the serogroup of the meningococci. Serogroup C isolates were extremely clonal and this was confirmed with an almost exclusive association with ST-11 and the ST-11 complex. The other major serogroup causing disease in Scotland displayed the opposite, with a wide range of different STs although these do conform to major ST complexes and these complexes have been consistently, and in some cases increasingly prominent, over the past four years.

The number of alleles present at each locus for each of the four years (1999, 2000, 2001 and 2002), ranged between 8 for *adk* in 1999 and 20 for *aroE* in 2002. The number of polymorphic sites present at each locus for each year ranged between 14 in 1999 (3% of sites for *adk*) and 128 in 2002 (26% of sites for *aroE*). Table 3.10 shows the comparison of this data for all four years. The number of alleles as a function of the number of isolates examined was calculated and there was a clear correlation between the increase in the alleles identified with an increase in the number of isolates examined (data not shown). This also correlated with previous studies where similar results were obtained (Holmes et al. 1999; Jolley et al. 2000).

It was found that the meningococcal population was highly diverse and an increasing trend of complex diversity was noted in individual years. The incidence of serogroup C meningococci changed significantly during the study, decreasing from 58 (45%) in year one to 10 (11%) in year four ($p = <0.0001$). The isolation of serogroup B

meningococci also changed resulting in a slight increase from 65 (50%) in year one to 69 (78%) in year four, although this was not significant (p = 0.06). Similarly, there was no significant change in the isolation of other serogroups (p = 0.6). In year one, serogroup C meningococci were assigned to only five different STs, 95% of which were contained within two complexes (ST-11 complex (93%) and ST-269 complex (2%)). Serogroup B meningococci were assigned to 31 different STs, 68% of which were contained within five complexes (ST-44 complex (34%), ST-269 complex (18%), ST-18 complex (8%), ST-35 complex (5%) and ST-32 complex (3%)). However, the serogroups associated with different STs changed between year one and four, such that, in year four, serogroup C meningococci were assigned to only two different STs, all of which were contained within two complexes (ST-11 complex (91%), and ST-269 complex (9%)). Serogroup B meningococci were assigned to 40 different STs, 68% of which were contained within five complexes (ST-269 complex (26%), ST-44 complex (19%), ST-32 complex (16%), ST-18 complex (4%) and ST-254 (3%)) (table 3.9). Importantly, over the study period, the serogroup C ET-37/ST-11 genetic lineage declined significantly (p = <0.0001) and there was no corresponding increase in serogroup B of the ET-37/ST-11 genetic lineage (p = 1). Moreover, there was no development of capsule switch as determined by serogroup and ST association. Analysis of allele frequency showed that the number of alleles present at each locus for each of the four years ranged between eight for *adk* (adenylate kinase) in year one and 20 for *aroE* (shikimate dehydrogenase) in year four. The number of polymorphic sites present at each locus for each year ranged between 14 in year one (3% of sites for *adk*) and 128 in year four (26% of sites for *aroE*). The number of alleles as a function of the number of isolates indicated a clear correlation between the increases in the alleles identified with an increase in the number of

isolates examined. This is further evidence of meningococcal genetic diversity.

Disregarding serogroup, in year one, meningococci were assigned to 41 different STs and six distinct lineages, with 15 singleton types (11.5%) (i.e. no complex association). These lineages occurred between one (0.8%) and 55 (42%) times during year one. Isolates of the ST- 11 complex accounted for 55 (42%) isolates and were assigned to two STs (ST-11 (n=53) and ST-655 (n=2)), the latter of which is a *gdh* (glucose-6-phosphate dehydrogenase) locus variant of ST-11. The ST-44 complex accounted for 22 isolates (17%) and these were divided into eight different STs, 14 were ST-41 (64%), two were ST-1097 (9%) and ST-44, 46, 146, 170, 1194 and 1255 were each represented by a single isolate (4.5%). In year two, meningococci were assigned to 47 different STs, an increase of 13% on the previous year. They were further differentiated into nine distinct lineages, an increase of 33% on the previous year, with a 27% decrease in singleton representation. These lineages occurred between one (0.8%) and 49 (38%) times during year two. This was statistically similar to the previous year ($p = 0.714$). Meningococci of the ST-11 complex continued to account for the majority (38%) of isolates. In year three, meningococci were assigned to 47 different STs and were differentiated into eight different lineages, a decrease of 11% from the previous year, demonstrating an increase in ST diversity. These lineages occurred between one (1%) and 18 (23%) times during year three. This range variation was significant smaller ($p = 0.0443$). This highlights a significant decrease in the representation of multiple isolates and the subsequent increase in single represented isolates. Meningococci of the ST-11 complex continued to account for the majority (25%) of isolates. In year four, meningococci were assigned 49 different STs, a 4% increase on the previous year and were differentiated into nine different lineages, an

increase of 11% on year three. These lineages occurred between one (1%) and ten (11%) times and were therefore again smaller, but not significantly so ($p = 0.2621$), resulting in a greater proliferation of different STs and thus a greater representation of single isolates. The ST-269 complex accounted for 17 isolates (19%), an increase of 12% on year three. These were divided into 10 different STs that remained consistent. ST-269 and 275 accounted for four isolates (24%), ST-2205 accounted for two isolates (12%), with ST-13, 283, 467, 1095, 2157, 2166, 2205 and 2319 represented once (6%). ST-269 and 275 both increased by 25% on year three and of the remaining STs none had been represented in previous years. Inter-lineage variation accounted for 24 single locus variations, a 140% increase, combined with 26 double locus variations, an 8% increase, with 40 satellites, a decrease of 29% on year three. The singletons accounted for 29 isolates (32%), an increase of 28% on the previous year. These were differentiated into 13 different STs, ST-11 (35%), ST-213 (24%), ST-1345 (7%) and ST-103, 136, 866, 1167, 1466, 2161, 2204, 2239, 2317 and 2318 were represented once each (3.5%). ST-11 decreased by a further 44% on year three. Mutation and/or recombination events were calculated by determining the number of substitutions per nonsynonymous (*d*N) and synonymous (*d*S) site within a particular nucleotide sequence, and by calculating the index of association (IA) (table 3.10). Firstly, *d*N /*d*S ratios were calculated for the 432 isolates in this study which showed that nonsynonymous nucleotide sequence changes were selected out of the population. This data was then compared to invasive meningococci contained within the global dataset (http://neisseria.mlst.net). The data was comparable, with only a few differences between the ratio values, except for *aroE*, suggesting that the proteins that they encode are well conserved. The *d*N /*d*S ratio value was >1 for *aroE* indicating that the proteins they encode are not so well conserved and nonsynonymous changes

were corrected faster than their occurrence by mutation (appendix 6.8). The index of association (IA) for the meningococcal population in years one to four was high (between 2.564 and 4.469) indicating a clonal population (table 3.10).

Table 3.9 Complex assignment, association, intra-complex variation and serogroup distribution from 1999 to 2002 (years 1 to 4). Sequence Type (ST) complexes displayed with their associated STs combined with both percentage nucleotide difference between represented STs within a complex (intra complex variation) and the serogroup distribution within each year.

ST complex assignment	ST associations	Intra complex variation (%)				Scrogroup distribution (%)			
		1999	2000	2001	2002	1999	2000	2001	2002
ST-11	11, 655, 1189, 2031.	(0 – 0.02)	(0 – 0.03)	(0 – 0.01)	(0)	C (99.98) B (0.02)	C (99.88) B (0.08) W135 (0.04)	C (99.98) B (0.11)	C (100)
ST-22	22, 184, 1224.	(0)	(0 – 0.05)	(0 – 0.04)	(0 – 0.05)	W135 (100) (100)	W135 (100)	W135 (100)	W135
ST-32	32, 33, 34, 259, 1098, 2276.	(0 – 0.08)	(0 – 0.08)	(0 – 0.08)	(0 – 0.08)	B (100)	B (100)	B (100)	B (100)
ST41/44	41, 44, 46, 146, 154, 155, 170, 180, 207, 340, 1009, 1097, 1194, 1233, 1255, 2109, 2120, 2203, 2300, 2303.	(0 – 0.1)	(0 – 0.07)	(0 – 0.08)	(0 – 0.09)	B (100)	B (100)	B (100)	B (100)
ST-213	213, 1218, 2301, 2660, 2782.	(0)	(0 – 0.06)	(0 – 0.006)	(0)	B (100)	B (100)	B (100)	B (100)
ST-269	13, 269,275, 283, 352, 467, 479, 1049, 1095, 1273, 1416, 1774, 2157, 2166, 2205, 2307, 2315, 2319, 2324, 2775, 2776.	(0 – 0.07)	(0 – 0.02)	(0 – 0.03)	(0 – 0.04)	B (99.93) C (0.07)	B (100)	B (100)	B (99.95) C (0.05)

3.5 Synonymous and Non-synonymous Nucleotide Substitutions

This represents the number of substitutions per synonymous (d_S) and non-synonymous (d_N) site within a particular sequence. This is calculated from the sequence of interest using the method of Nei and Gojobori and is implemented within the Sequence type analysis and recombinational tests (START) program located on the internet (http://outbreak.ceid.ox.ac.uk/software.htm). The START software will therefore determine the d_N/d_S ratio, which can be used as a marker of selection pressure. The individual d_S and d_N values were calculated and the ratio obtained. This data is compared with isolates pertaining to the same criteria as our population, namely the isolates were exclusively from invasive sites and patients with confirmed meningococcal disease. The data was comparable, with only a few differences between the ratio values, except for *aroE*. The d_N/d_S ration value was significantly higher then previously calculated for similar isolates (table 3.10).

Table 3.10 The synonymous (d_s), nonsynonymous (d_n) and index of association rates from 1999 to 2002 (years 1 to 4). The *dS/dN* ratio was obtained using the method developed by Nei *et al,* which estimates synonymous substitutions by means of an unweighted pathway method. The index of association (I_A) measures the extent of linkage equilibrium within a population. If there is frequent recombination events within the meningococci within that year the value will be close to zero. If clonal populations are identified the I_A will differ significantly from zero. These values are in relation to both a specific housekeeping gene and the year isolated.

	Year			
Housekeeping Gene	1999	2000	2001	2002
abcZ (putative ABC transporter)	0.0544	0.0570	0.0496	0.0647
adk (adenylate kinase)	0.0208	0.0137	0.0121	0.0166
aroE (shikimate dehydrogenase)	1.7749	1.6128	1.5805	1.6553
fumC (fumarate hydratase)	0.0115	0.0158	0.0178	0.0288
gdh (glucose-6-phosphate dehydrogenase)	0.0520	0.0494	0.0420	0.0557
pdhC (pyruvate dehydrogenase subunit)	0.0679	0.0688	0.0618	0.0646
pgm (phosphoglucomutase)	0.1275	0.1081	0.1162	0.1089
I_A (Index of association)	4.469	4.372	2.979	2.564

3.6 Discussion

A number of studies have been performed with collections of meningococci that have either caused disease or have been taken from healthy individuals who carry meningococci as a commensal residing at the back of the throat. This is represented by an ever-increasing number of ST types closely associated with meningococcal disease or carriage within the MLST database (Jolley et al. 2000; Clarke et al. 2001b; Nicolas et al. 2001; Van Looveren et al. 2001; Jacobsson et al. 2003). These collections have concentrated on sample populations and subsequent inference has been made on a limited number of strains in proportion to the perceived total population of both diseased patients and/or natural carriers (Clarke et al. 2001b; Maiden and Stuart 2002). This study had taken all available invasive isolates over a four-year period and characterised through sequencing, the genes responsible for the MLST and subtype profile of the organism, these include the seven housekeeping genes responsible for meningococcal MLST. The diversity of the different serogroups, types and subtypes within the meningococcal population from Scotland compared to data from the MLST website and annual reports released by various national reference facilities are not dissimilar to many other European countries therefore such unique data is extremely informative not only to Scotland. The diversity of housekeeping genes and STs over the four-year period remained stable, however, the continuous nature of the sampling technique and the number of isolates did give rise to a significant proportion of new combinations of alleles thus pertaining to new ST profiles. Interestingly, no novel gene sequences were obtained within the same population, except one novel *aroE* gene was identified. This would confirm the observations, which place *aroE* above all others with the highest percentage of polymorphic sites, with a range of between 25% and

26% of the total gene sequence.

This information combined with detailed patient data including immunisation status, age, location and sex (data not shown) can provide a detailed picture of the meningococci causing disease at a national level. As the immunisation of high-risk groups continues, the definitive effect of the vaccine has been analysed and primarily shows a significant decrease in serogroup C meningococcal disease since the MenC vaccine was introduced, however observing the national trend during the past 20 years does indicate a continual flux between serogroup B and C isolates predominating and this will require further observations.

Polysaccharide conjugate vaccines are providing essential and highly effective immunization against some of the major human pathogens (Rappuoli 2001a; Rosenstein et al. 2001a; Maiden and Stuart 2002; Ada and Isaacs 2003). To coincide with the introduction of the UK MenC immunisation programme, prospective enhanced surveillance of those meningococci associated with throat carriage and invasive disease was investigated (Anon 1998c; Clarke et al. 2001b; Maiden and Stuart 2002). The implementation of MenC vaccines has led to a reduced level of serogroup C meningococcal throat carriage and also a reduced level of invasive disease (Ramsay et al. 2001; Maiden and Stuart 2002; Ramsay et al. 2003). However, since the population dynamics of meningococcal disease is very fluid, with serogroup B and serogroup C historically undergoing a complementary cyclic pattern (Ramsay et al. 1997; Clarke et al. 2002), it is essential to understand the intra- and inter-lineage population dynamics of this disease. We used multi-locus sequence typing (MLST) to characterise a collection of 429 meningococci, isolated from cases of invasive

meningococcal disease between 1999 and 2002 (years one to four).

Four hundred and twenty nine isolates of *N. meningitidis* from invasive meningococcal disease in Scotland were analysed between January 1999 and December 2002 by MLST (Maiden et al. 1998; Clarke et al. 2001b). Genotypic characterisation through DNA sequencing followed by nucleotide sequence analysis and subsequent sequence type (STs) and complexes allocation was performed as previously described (Chan 2001; Clarke et al. 2001b; Clarke et al. 2002; Diggle and Clarke 2002a). Furthermore, intra- and inter-lineage comparisons were made using the START facility of programs, including previously described sequence type specialty programs such as BURST (http://neisseria.org/nm/typing/mlst/). The calculation of synonymous (*d*S) and nonsynonymous (*d*N) rates within specific sites within a particular sequence was calculated from the allele sequence using the method of Nei and Gojobori which is included in the START suite (http://outbreak.ceid.ox.ac.uk/software.htm). A summary of the single multi-locus measure of linkage disequilibrium was achieved by the index of association (IA) (Smith et al. 1993) using the START programs. In addition further statistical analysis highlighting the likelihood of these sample populations and subsequent hypothesis being significant was calculated using the standard t-test formula to achieve a two-tailed p value.

The clonality within the meningococcal population reduced year by year with a significant reduction observed between years two, three and four. This naturally coincides with the reduction in serogroup C ST-11 complex strains that are associated as a highly clonal complex. The large number of isolates represented on the

meningococcal MLST website is a reflection of the numerous studies performed with collections of meningococci that both cause disease and are present as carriage strains, and this is subsequently represented by a significant number of ST's closely associated with either meningococcal disease or carriage within the MLST database (Maiden et al. 1998; Feil et al. 1999; Jolley et al. 2000; Clarke et al. 2001b; Tzanakaki et al. 2001).

However, these collections have centred upon both carriage and disease and therefore, subsequent conclusions based on disease-causing isolates are limited. In addition, these studies have been performed during time periods where the selective pressures are normal (i.e. outwith vaccine programmes). Consequently, this study has taken all available invasive isolates over a 4-year MenC vaccine implementation period and determined the change in the genetic diversity of the meningococcal population.

The genetic diversity of meningococci has significantly increased after the introduction of MenC vaccines, initially significant changes in genetic diversity within the meningococcal population were not observed between years one and two, however a significant increase in diversity was observed between years two, three and four and this increase was directly reflected in a significant decrease in serogroup C ET-37/ST-11 meningococci. Interestingly, this was not accompanied by an increase in serogroup B meningococci of the same genetic lineage, as this may highlight a possible capsular switch between both serogroup C and B meningococci.

The diversity of the current meningococcal population has changed from 41 different profiles representing the meningococcal population in year one to 49 different profiles

in year four, an increase of over 16%. However, a significant increase of over 12% was observed between years one and two even though ST-11 maintained a significant presence in the population (37.7%). This increase in diversity was due, however, to the presence of new combinations of pre-existing alleles resulting from a process of recombination, resulting in new ST profiles, rather than novel allele sequences resulting from mutation. The increase in recombination between years one to four must therefore, be due to natural selection compounded by the lacking ability with which non ST-11 meningococci compensate for the artificial rapid decline in ST-11 complex meningococci with any subsequent increase in alternative non ST-11 meningococci.

Interestingly, this niche has been gradually filled by meningococci of numerous STs and from a number of clonal complexes including ST-41-44, ST213 and ST-269. Currently, therefore, there is no single ST complex or subsequent hyper-endemic clone present in the UK that has directly replaced the ST-11 clone with the same percentage of the population. However, some clones, such as the ST-41-44 and the ST-269 complex, must be monitored closely as they now represent a significant percentage of the meningococcal disease-causing population. These results will have an important impact on conjugate vaccine strategy for the control of meningococcal disease, as well as other bacterial pathogens, because the medium to long term effects of vaccines directed against a single serogroup clearly have an effect on the meningococcal population biology. Although introduction of MenC vaccines has not led directly to serogroup switch (to serogroup B), a definite increase in clones of serogroup B has been observed although much of this may be due to the typical cyclical pattern between serogroup B and serogroup C incidence previously described.

As a consequence continued analysis of the meningococcal population must be performed in order to gain further insights into the long-term effects of the MenC vaccine upon the whole meningococcal population, importantly this should include both invasive and non-invasive carriage isolates, as this combination will highlight the true emergence of important genetic lineages in the future.

Chapter 4

Genosubtyping and Analysis of PorinA (porA) Variable Regions

4.0 Introduction

The genus *Neisseria* comprises a number of species associated with mucosal carriage and disease in animals and humans (Morse 1992; Barrett and Sneath 1994), including *N. meningitidis*, a major cause of meningitis and septicemia worldwide (Cartwright 1995) and *Neisseria gonorrhoeae*, the etiological agent of gonorrhoea (Sherrard 1995). Studies of these two pathogenic species (Guibourdenche et al. 1986), are helpful in understanding both the pathogenesis of meningococcal and gonococcal infections and the evolutionary events that led to their emergence. Such studies are particularly valuable when they target cell components potentially involved in immunity or other host-pathogen interactions, such as OMPs (van der Ley et al. 1991; Ward et al. 1992; Suker et al. 1993).

Serogroup B meningococci significantly contribute to the cause of meningitis and septicaemia worldwide, mainly because there is no effective vaccine (Rosenstein et al. 2001a). As a consequence there is an urgent need for effective vaccines against the serogroup B meningococcus. However, there are difficulties with *N. meningitidis* serogroup B that are not encountered with plain polysaccharide or conjugated vaccines designed against serogroups A, C, Y and W135 (Rappuoli 2001a)

The meningococcus serogroup B capsular polysaccharide topology is identical to a human carbohydrate ((2 8) *N*-acetyl neuraminic acid or polysialic acid). As a

consequence a vaccine based on this construct configuration would be poorly immunogenic and would ultimately induce only a transient antibody response (Finne et al. 1987; Hayrinen et al. 1995). A vaccine based on the serogroup B meningococcus therefore requires the development of a vaccine based on an alternative target. This could be based on different genes that have been identified as possible candidates from complete genome sequence analysis termed reverse vaccinology (Rappuoli 2001a). An effective candidate could be virulence-based vaccine.

Using vast amounts of sequence-based data, numerous genes could be identified as potential candidates. The most studied of which are those based on OMPs (Al'Aldeen and Cartwright 1996; Rosenstein et al. 2001a). Although OMP-based vaccines are not the ideal solution for combating serogroup B meningococcal infection, they may provide a short-term solution. Previous research has shown great promise for the control of serogroup B meningococcal infection based on these vaccines (Rappuoli 2001a).

There are many OMPs possessed by the meningococcus and like other bacteria these are ordered into different classes, namely 1-5. Numerous OMPs from classes 1–5 have been studied as potential vaccine candidates (Tsai et al. 1981). Phenotypic markers class 2/3porB and class 1 porA protines are used for typing and subtyping meningococci respectively. These have been used for many years and as a consequence have been studied extensively as possible vaccine candidates. These are porin protines that have surface exposed regions subjuected to external forces and selective pressures. As a result these exposed porins contain variable and

hypervariable regions. From topological studies a two-dimensional structural model has been predicted for the meningococcal PorA protein that predicts eight surface exposed loops (I–VIII) (van der Ley et al. 1991). From this structural model the most exposed and therefore the most variability are thought to occur in variable regions 1 and 2 (VR1 and VR2) which correspond to loops I and IV, respectively (Maiden et al. 1991; van der Ley et al. 1991; McGuinness et al. 1993).

Phenotypic studies involving serosubtype-specific monoclonal antibodies react with peptide epitopes located in these loops and therefore serosubtyping is used to identify these PorA VR epitopes. A widely recognised nomenclature is used which has been recently updated to take into account new data now available through nucleotide sequence typing. A novel subtyping nomenclature has also been proposed (Sacchi et al. 1998a; Maiden et al. 1999) but this has not been adopted.

The major OMPs that are expressed at high levels have been recognised for over 15 years (Frasch 1985). There are two OMPs that have been functionally characterised as porins. These are two different classes of protein from a collection of five different classes (1-5), isolated based on differences in molecular weight. Initial experiments revealed that the meningococcal class 1 porin protein (*porA*) and class 2 and 3 porin proteins (*porB*) perform as selective channels, which permit the passage of cations and anions across the cell membrane. Recently the advent of sequencing has confirmed the porin nature of these proteins with homology with well-characterised *Escherichia coli* porins.

Interest in the antigenic variability of the *Neisseria* porins, from both typing and vaccination perspectives, has resulted in the generation of many sequences of their genes. Detailed interpretation of this database of sequences has been hindered by the lack of a molecular structure for the gene products, although a two-dimensional model has been proposed on the basis of sequence similarity (Maiden et al. 1991; Morse 1992). This model comprises a β-barrel porin structure, similar to that of the *Escherichia coli* porins (Cowan 1992), with regions that are relatively conserved among *Neisseria* sequences forming the β barrel. Other regions, which are less well conserved among and within species, form surface-exposed loops which protrude from the bacterial surface into the surrounding environment (Maiden et al. 1991; Morse 1992). While this model was useful, it was not three-dimensional, being proposed before the molecular structures of the *E. coli* porins were established; the assignment of residues to membrane-embedded or surface-exposed environments was somewhat uncertain.

These *Neisseria* porins, maintain a distinct class within the porin superfamily (Jeanteur 1991) and are major components of the outer membranes of all members of the genus *Neisseria* (Ward et al. 1992; Suker et al. 1993; Feavers and Maiden 1998). Most *Neisseria* species express only one, referred to as Por. The meningococcus exceptionally expressing two; PorA and PorB (Hitchcock 1989). The gonococcus is the only other *Neisseria* species known to have a *porA* gene, which is not expressed due to frameshift and promoter mutations (Feavers and Maiden 1998). The meningococcal and gonococcal porins are targets for serological typing schemes (Frasch et al. 1985; Gill 1991), candidates for inclusion in vaccines (Frasch et al. 1985), and have been implicated in pathogenesis (Rudel 1996).

Virtually all meningococci possess the class 1 OMP (PorA) (Suker et al. 1994; van der Ende et al. 2003). The secondary structure of the class 1 OMP has been formulated and subsequent construction of topology models were possible these displayed highly conserved trans-membrane regions with eight strain-variable exposed loops, which play a crucial role in the host immune response. These surface exposed loops are the most prominent regions within the protein and thus most exposed to immune responses. There are three regions considered important in sero-subtype characterisation; variable regions at loops 1, 4 and 5 correspond with variable regions I, II and III (VR1, VR2 and VR3) (Figure 1.12) (Molling et al. 2000; Tondella et al. 2000; Molling et al. 2002; Clarke et al. 2003).

Detection and characterisation of the outer membrane protein (OMP) gene *porA* can be achieved by several methods (Frasch 1985; Diggle and Clarke 2003a). Typing may be performed on the basis of SDS-PAGE, specific monoclonal antibodies and whole cell ELISA typing methods (Hoehn 1990; Lehmann et al. 1999; Tondella et al. 2000; Wang et al. 2000). More recently, molecular characterisation of the meningococcal porins have included nucleotide gene sequencing of previously described variable regions or the whole gene (Urwin et al. 1998a; Clarke et al. 2003). These porins have not only been used for sero-typing and sero-subtyping but also for epidemiological analysis of meningococcal carriage and disease (Poolman et al. 1986), although the variability of these proteins means they are not entirely accurate as epidemiological markers (Urwin et al. 1998b).

As previously noted, vaccines based on OMPs are being developed against the serogoup B meningococcus. These may contain a number of different common circulating porB and porA types and subtypes, respectively. Although the probability of a highly successful vaccine is dependent on circulating variants within the population. This is confounded by the limited amount of data available from all the different circulating types and subtypes throughout Europe and the world.

This highlights the importance in the comprehensive analysis of OMP genes such as *porA*. As described previously this gene encodes a protein with eight surface exposed loops of which three main variable regions, VR1, VR2 as well as VR3, of the *por*A gene from *N. meningitidis* have been analysed. This analysis highlighted that with in 429 strains studied, there were a total of 86 different strains. No new VR1 or VR2 alleles were found but five new VR3 alleles are described (Appendix 6.9 – 6.13). This indicates the importance of analysing these variable regions of *porA* and also highlights, in general terms, the need for genosubtyping meningococci. Such analyses could have major implications for the design of new meningococcal OMP vaccines.

In this chapter, there have been a number of different techniques used, including phylogenetic analysis, individual variable region phenograms and sequence alignments enabling a more precise identification of the differences between individual isolates. Phylogenetic analyses of the porins from different meningococcal isolates were enhanced when the model was used to assist sequence alignment and to identify structurally conserved parts of the proteins, identifying features likely to be important in the emergence of novel sequences within a particular serogroup.

4.1 Results

Numerous vaccines containing single and multi-valent components have been developed and evaluated over the past decade (Wedege and Michaelsen 1987; Rosenqvist et al. 1995; Peeters et al. 1996; Rouppe van der Voort et al. 1997). These have been based on several factors, including most importantly geographical area and prevalent circulating variants (Van Der Ley and Poolman 1992; Peeters et al. 1996; Perkins et al. 1998; Cartwright et al. 1999; Tappero et al. 1999; de Kleijn et al. 2000b). However the numbers of different variants are ever increasing due to the very nature of the highly antigenic regions, therefore OMP vaccines will not protect against all variants including all the different types and subtypes of *N. meningitidis*. For example, the Institute for Public Health and the Environment (RIVM) based in the Netherlands has developed a recombinant hexavalent PorA outer membrane vesicle (OMV) vaccine comprised of P1.7, 16; P1.19, 15; P1.5, 2; P1.5c, 10; P1.12, 13 and P1.7h, 4 (Rouppe van der Voort et al. 2000). These subtypes correlate to genosubtype families 2, 5, 7, 10,12, 13, 15, 16 and 19. This vaccine has undergone trials and is being developed for the commercial market. This OMV vaccine was represented in over 80% of UK meningococcal case isolates in 1996 (Cartwright et al. 1999) and in phase I and phase II clinical trials has shown promising results (Peeters et al. 1996; Cartwright et al. 1999). As a result successful OMP vaccines must use high quality data pertaining to the prevalent types and subtypes in any given geographical area such as a country or collection of countries.

It has been widely documented that VR1 (loop I) and VR2 (loop IV) are highly variable regions (Figure 1.12). However genosubtyping involving PorA VR3 (loop V)

have also been described to a much lesser extent (Arhin et al. 1998; Molling et al. 2000; Riesbeck et al. 2000; Clarke et al. 2001b; Molling et al. 2001). As a consequence this suggest that more data is required to determine the significance of VR3 and any other variable regions which are subjected to an immune response and are subsequently important in both disease surveillance and vaccine development.

Monoclonal antibody testing is still performed in most laboratories as a method for serotyping and serosubtyping meningococci, however with new types and subtypes occurring these are going undetected unless alternative techniques such as nucleotide sequencing are performed. Here, this chapter describes the efforts of performing sequence-based genosubtyping of VR1, VR2 as well as VR3 on all invasive meningococci isolated throughout Scotland over a period of four-years and the subsequent effects of the MenC vaccine on this dynamic population.

The meningococcal subtype is determined by amino acid sequence variation in the cell surface exposed loops I, IV and V of the porin protein PorA (van der Ley et al 1991). These antigenically variable loops are encoded by variable regions 1, 2 and 3 (VR1, VR2 and VR3) of the *porA* gene respectively. This was first recognised with regards VR1 and VR2 by using murine monoclonal antibodies (Mabs) (Abdillahi and Poolman 1988).

This chapter uses meningococcal subtype nomenclature based primarily on the amino acid sequence deduced from the nucleotide sequence. This is to minimise any possible non-typable results and therefore can include novel sequences since *porA* is underimmune selection in humans. The results displayed will contain the

conventional genetic designation PorA followed by a number representative of the original amino acid sequence of each sample (Appendix 6.1, 6.2 and 6.3).

4.1.1 Genosubtyping of meningococcal isolates from 1999

4.1.1.1 Serogroup C isolates. The majority of serogroup C isolates from 1999 fell into a single group within variable region one (VR1) and two distinct groups within VR2 and VR3. The first variable region was represented by seven different variable types within six distinct families namely, 5, 7, 17, 18, 19 and 21 with over 87% characterised as variable regions 5 and 5-1. The second variable region was represented by nine different variable types within six distinct families namely, 2, 3, 4, 10, 15 and 16 with over 68% allocated to variable regions 10-1, 10-4 and 10-8. The third variable region was represented by five different variable types within three distinct families namely, 36, 37, and 38 with over 87% allocated to variable region 36-2. This followed a similar pattern to the MLST data whereby large clonal complexes were formed from large collections of serogroup C data, although a greater proliferation and subsequent differentiation was observed with the addition of *porA* sequence data. The variable region distribution chart for 1999 (Figure 4.1 – 4.7) highlights a pattern whereby in general within the serogroup C population a few VR1 types expand into a larger number of VR2 types before converging into a single VR3 type (Figure 4.1 - 4.7). This association between these different variable regions is highly conserved not only within specific variable regions but also within this serogroup. Therefore it is rare to find serogroup C isolates with alternative variable region profiles as highlighted with the statistical distribution described previously.

The first variable region was represented in the majority from the type 5-family or the P1.5 family (n=50). Within this family to date there are eleven different variants associated with the original representative amino acid sequence. The subsequent

variants range between one and three amino acids either by substitution, insertion or mutation from the original twelve amino acid sequence (Appendix 6.1). The two represented in Scotland were 5 (n=12) and 5-1 (n=38). The latter varies with the addition of an extra amino acid within the middle of the region. This correlates with the top of the VR2 loop displayed in within Chapter 1 (Figure 1.12).

The second variable region was represented in the majority from the type 10-family or the P1.10 family (n=39) and to a lesser extent the type 2-family or the P1.2 family (n=11). This variable region contains more variant types in comparison to the other two. Within the type 10-family there are to date forty different variants of the representative variant. This family has variants between one and seven amino acids either by substitution, insertion or mutation from the original fifteen amino acid sequence (Appendix 6.2). The three represented in Scotland were 10-1 (n=4), 10-4 (n=17) and 10-8 (n=18). The first type had a mutation in the latter region, whereas type 10-4 had the addition of a recognised "QNK" (Glutamine, Asparagine, Lysine) amino acid motif repeated along with the same mutation present in type 10-1. The third type had the addition of another recognised "QNQ" (Glutamine, Asparagine, Glutamine) amino acid motif repeated again along with the same mutation present in type 10-1. The variant type 10-1 in relation to variant 10 had a substitution of an arginine for a proline. This resulted in the exchange of a basic charged amino acid into a neutrally charged amino acid and as a consequence the overall molecular weight and hydrophobicicty were not significantly affected. The variant type 10-4 in relation to 10-1 had the substitution of glutamine for a lysine. This resulted in the exchange of a basic charged amino acid into a neutrally charged amino acid and as a consequence the overall molecular weight and hydrophobicity were not significantly affected.

Within the type 2-family there are to date thirty-one different variants of the representative variant. This family has variants between one and six amino acids either by substitution, insertion or mutation from the original fifteen amino acid sequence (appendix 6.1). The two represented in Scotland were 2 (n=10) and 2-2 (n=1). The first variant was the original whereby all others are compared. The second type varies by the mutation of a single amino acid within the centre of the amino acid sequence.

A single variant type represents the third variable region in the vast majority of serogroup C meningococci and this was from the type 36-family (n=52). This variable region has recently been added to the profile in the last few years to increase discrimination of more clonal types (Clarke et al 2003). As a consequence the number of different variants is smaller in comparison to the other two. The 36-family to date contains four variants and each representative type varies by only a single amino acid by mutation (appendix 6.3). The majority of serogroup C strains were represented by the type 36-2 (n=50) variant. This variant had a single mutation within the centre of the amino acid sequence when aligned with the original representative type.

4.1.1.2 Serogroup B Isolates. The distribution of isolates was much more extensive within the serogroup B population. The first variable region was represented by thirteen different variable types, this was over double the amount when compared to the serogroup C isolates. These were contained within eight distinct families namely, 5, 7, 12, 17, 18, 19, 21 and 22 with over 74% allocated to variable regions 7-2, 18-1, 19 and 22. The second variable region was represented by eighteen variable types, again this was over double the amount when compared to the serogroup C isolates.

These were contained within twelve families namely, 2, 3, 4, 9, 10, 13, 14, 15, 16, 23, 25 and 30 with over 69% allocated to variable regions 3, 4, 9, 14 and 15. The third variable region was represented by nine different variable types within five distinct families namely, 35, 36, 37, 38 and 40 with over 90% allocated to variable regions 35-1, 36, 36-2, 37 and 38.

The first variable region was represented by a number of distinct variable types from the 7, 18, 19 and 22 family or the P1.7, P1.18, P1.19 and P1.22 family. Within this collection the 22 variant (n=18) was represented by the original variant with the exception of a single isolate 22-1 which had three amino acid changes compared to the original (Appendix 6.1). The P1.22 family has to date 8 different variants with an eighteen amino acid chain length. The 7-variant family (n=18) has to date seventeen distinct variants with between 20-25 amino acids (Appendix 6.1). The represented types in Scotland were 7 (n=4) and 7-2 (n=14). The first type was the original representative type. The second type had the deletion of a recognised "VTK" (Valine, Threonine, Lysine) amino acid motif removed from the end of the region. This resulted in the removal of two neutrally charged amino acids and a single basic charged amino acid and as a consequence the overall length was decreased and the molecular weight within this region was reduced by 382.46. The 19-variant family (n=14) has to date eighteen distinct variants with between 14-19 amino acids (Appendix 6.2). The represented types in Scotland were 19 (n=11) and 19-1 (n=3). The first was the original representative type and the second had a single amino acid mutation in the centre of the region. The 18-variant family (n=7) has to date twelve distinct variants with a fourteen amino acid chain length (Appendix 6.1). The represented variant in Scotland was the 18-1, which had a single amino acid change at

the start of the region.

The type 3, 4, 9, 14 and 15 families or the P1.3, P1.4, P1.9, P1.14 and P1.15 families, represented the majority in the second variable region. The 4-variant family (n=15) has seven distinct variants with between 8-21 amino acids. The representative types were all from the original type 4-variant. The 14-variant family (n=11) has to date thirteen distinct variants, and has variants between one and two amino acid changes either by substitution or mutation from the original ten amino acid sequence (Appendix 6.2). The two represented in Scotland were 14 (n=10) and 14-6 (n=1). The first type was the original representative variant, whereas the second had three consecutive amino acids changes at the latter end of the variable region. The 15-variant family (n=11) has to date twenty-two distinct variants, and has variants between one and six amino acid changes either by substitution, insertion or mutation from the original thirteen amino acid sequence (Appendix 6.2). The two represented in Scotland were 15 (n=8) and 15-11 (n=3). The first type was the original representative variant, whereas the second had a single amino acid change within the centre of the region. Finally, the 9-variant family (n=8) has to date seven distinct variants, and has variants between one and two amino acid changes either by substitution or mutation from the original ten amino acid sequence (Appendix 6.2). The two represented in Scotland were 9 (n=7) and 9-4 (n=1). The first was the original representative variant, whereas the second had a single amino acid change within the centre of the region.

A considerable collection of different variants types represents the third variable region. These include 35-1 (n=11), 36 (n=20), 36-2 (n=6), 37 (n=16) and 38 (n=8).

The 36-family represents a significant proportion of these isolates. Unlike serogroup C isolates, the 36 variant rather than the 36-2 variant was mainly represented. This is the original representative variant in the family. The 36-2 variant is differentiated by a single amino acid change at the end of the region. The 37-family has two representative variants and these are significantly different to the 36-family with an amino acid length of nine (rather than eight). Again the original representative variant was predominant. The 35-family has three representative variants and the 35-1 was predominant. This eight amino acid chain has a single amino acid change at the start of the region. Finally, the 38-family represents a small proportion of serogroup B isolates. The family contains two variants and has an amino acid length of ten. All the represented isolates were from the 38-variant (Appendix 6.3).

4.1.1.3 Miscellaneous Serogroup Isolates. These represented less than 5% of the total population in 1999. These were serogroups W135 (n=2), Y (n=2) and X (n=3). At this stage these isolates could represent future dominant strain characteristics. Therefore, although they were statistically insignificant, they could be important markers to monitor and analyse.

Variable regions 18-1, 3 and 38, represented serogroup W135 isolates. These within 1999 have also been associated with serogroup B isolates. Variable regions 21, 16 and 37-1, represented two serogroup X isolates. These have been associated with both serogroup B and C isolates. Variable regions 7-1, 4 and 37-1, represented the remaining serogroup X isolate. This displays significant differences within the VR1 and VR2 and are closely associated with a clonal serogroup B collection, namely 7-2, 4, 37-1. The serogroup X VR1 had an additional five amino acids, a "GA" (Glycine,

Alanine) amino acid insertion and an additional "KVT" (Lysine, Valine, Threonine) amino acid repeat. This resulted in the addition of four neutrally charged amino acids and a single basic charged amino acid and as a consequence the insertions produced a slightly larger variable region with the additional molecular weight of 546.62. Variable regions 5-1, 10-2, 2-13 and 36-2, represented serogroup Y isolates. The VR1 and VR3 were consistent with a clonal serogroup C collection. The VR2 was different within each isolates and were unique to the meningococcal population in 1999. The 10-2 was more closely associated with the original representative variant 10 than the commonly found 10-4 and 10-8, although there are conserved regions at the start and at the end. The 2-13 was more closely associated with VR2 2-2, which had only a single amino acid change at the start of the region. Both VR2s were close relations to a clonal serogroup C collection in 1999.

Figure 4.1 Association between porin A variable region 1 (VR1), variable region 2 (VR2) and variable region 3 (VR3) from meningococci isolated in 1999 (year 1). Each identified porin A profile (VR1, VR2, VR3) is linked through a series of individually coloured and styled arrows. The total number represented within that variable type is indicated within the brackets. Following the single VR1 22-1 (green) the dotted line finishes at VR2 14 (purple) with 9 others VR1s. These are further distributed through a dotted line to VR3 36 (n=8), 36-2 (n=1) and 38 (n=1).

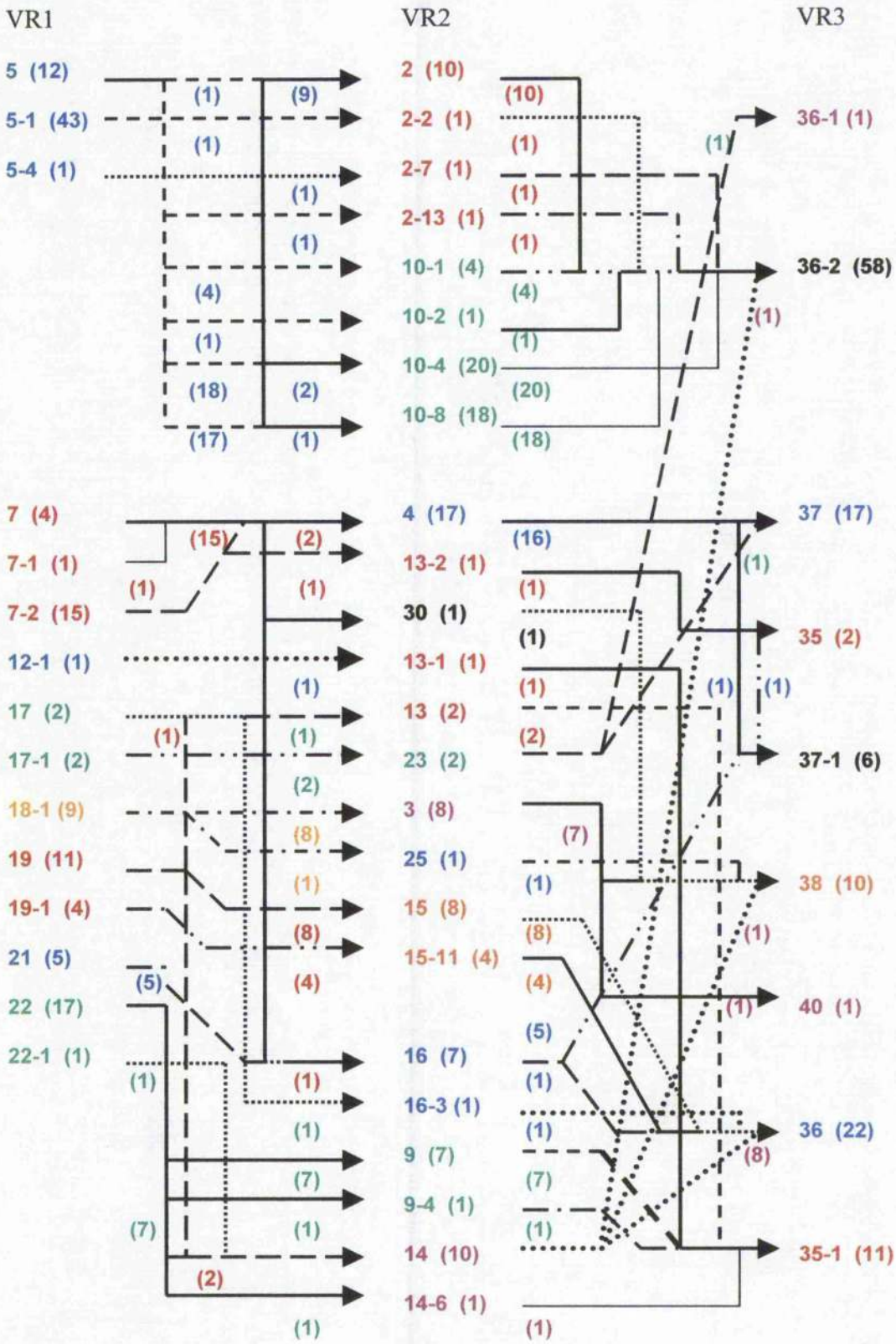

VR1
VR2
VR3
5 (12)
5-1 (43)
5-4 (1)
(1)
(1)
(1)
(1)
(4)
(1)
(18)
(17)
(9)
(1)
(2)
(1)
2 (10)
2-2 (1)
2-7 (1)
2-13 (1)
10-1 (4)
10-2 (1)
10-4 (20)
10-8 (18)
(10)
(1)
(1)
(1)
(4)
(1)
(20)
(18)
(1)
36-1 (1)
36-2 (58)
(1)
7 (4)
7-1 (1)
7-2 (15)
12-1 (1)
17 (2)
17-1 (2)
18-1 (9)
19 (11)
19-1 (4)
21 (5)
22 (17)
22-1 (1)
(15)
(1)
(1)
(1)
(1)
(2)
(8)
(1)
(8)
(5)
(4)
(1)
(1)
(1)
(7)
(7)
(1)
(2)
(1)
4 (17)
13-2 (1)
30 (1)
13-1 (1)
13 (2)
23 (2)
3 (8)
25 (1)
15 (8)
15-11 (4)
16 (7)
16-3 (1)
9 (7)
9-4 (1)
14 (10)
14-6 (1)
(16)
(1)
(1)
(1)
(1)
(2)
(7)
(1)
(8)
(4)
(5)
(1)
(1)
(7)
(1)
(1)
(1)
(1)
(1)
(1)
37 (17)
35 (2)
37-1 (6)
38 (10)
40 (1)
36 (22)
35-1 (11)

Figure 4.2 Distribution of porin A variable region 1 (VR1) from meningococci isolated in 1999 to 2002 (years 1 to 4).

The year 1999 in light blue	
The year 2000 in plum	
The year 2001 in light yellow	
The year 2002 in pale blue	

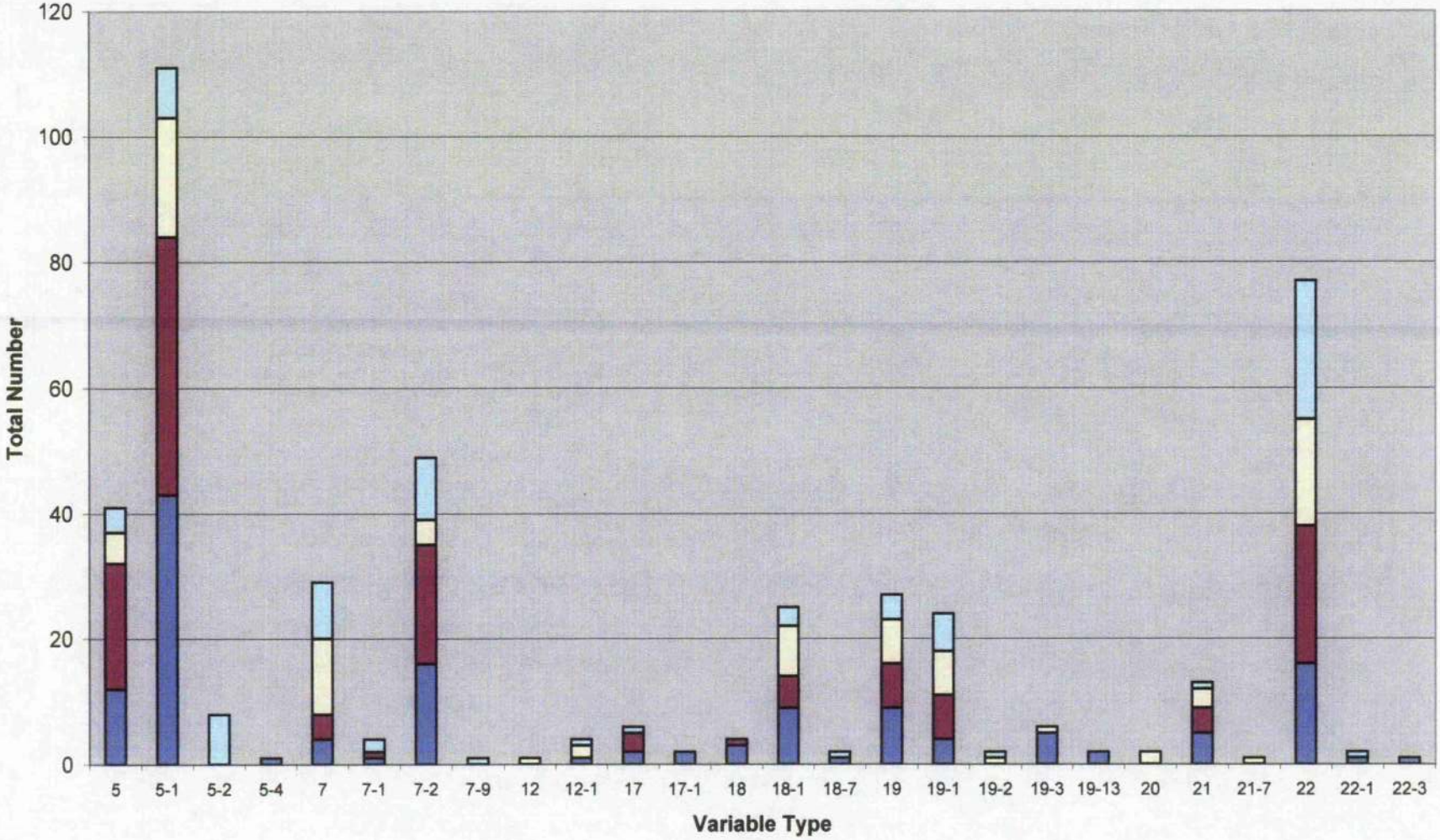

120
100
80
60
40
20
0
Total Number
5
5-1
5-2
5-4
7
7-1
7-2
7-9
12
12-1
17
17-1
18
18-1
18-7
19
19-1
19-2
19-3
19-13
20
21
21-7
22
22-1
22-3
Variable Type

Figure 4.3 Distribution of porin A variable region 2 (VR2) from meningococci isolated in 1999 to 2002 (years 1 to 4).

The year 1999 in light blue	
The year 2000 in plum	
The year 2001 in light yellow	
The year 2002 in pale blue	

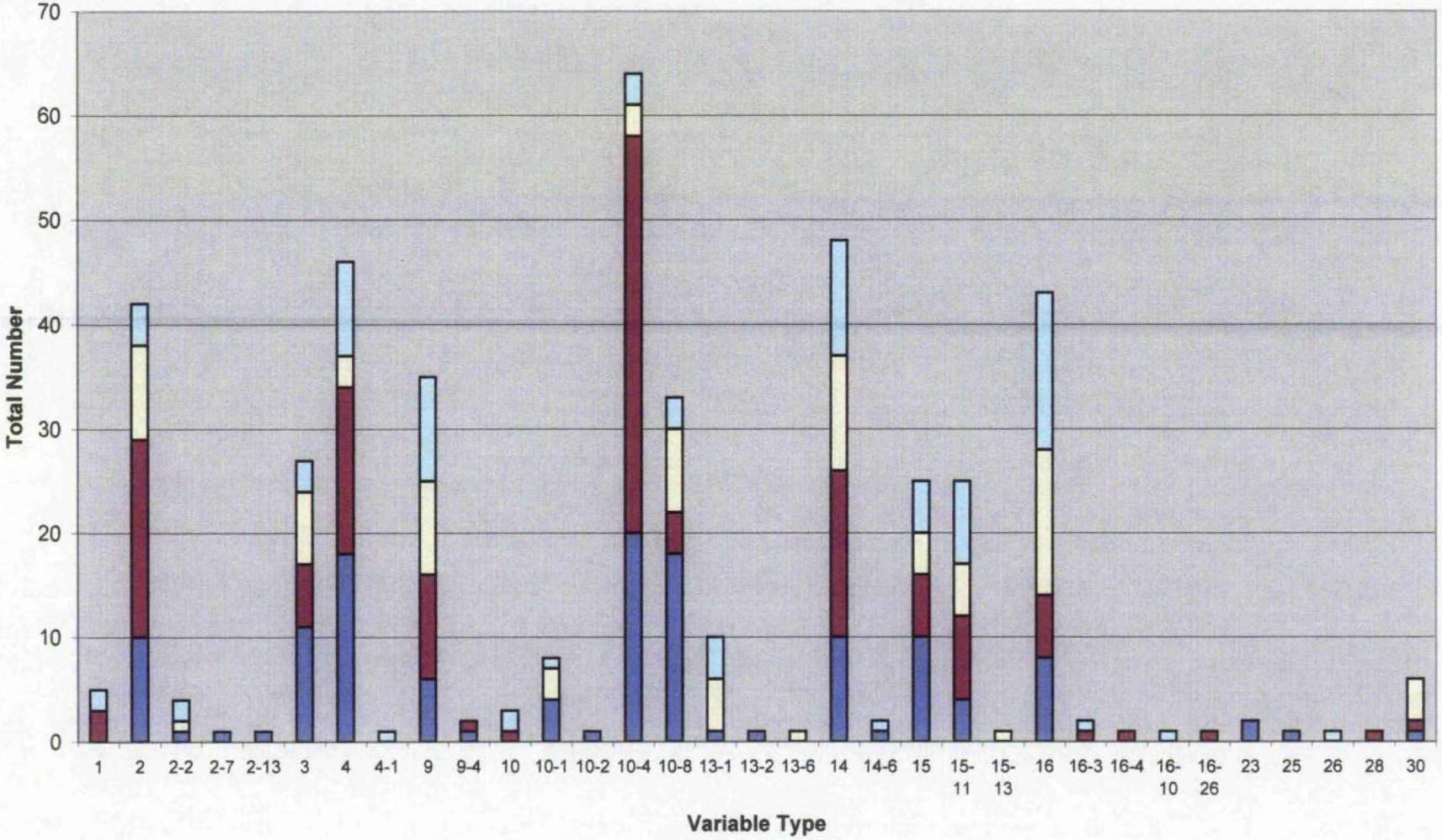

70
60
50
40
30
20
10
0
Total Number
1
2
2-2
2-7
2-13
3
4
4-1
9
9-4
10
10-1
10-2
10-4
10-8
13-1
13-2
13-6
14
14-6
15
15-11
15-13
16
16-3
16-4
16-10
16-26
23
25
26
28
30
Variable Type

Figure 4.4 Distribution of porin A variable region 3 (VR3) from meningococci isolated in 1999 to 2002 (years 1 to 4).

The year 1999 in light blue	
The year 2000 in plum	
The year 2001 in light yellow	
The year 2002 in pale blue	

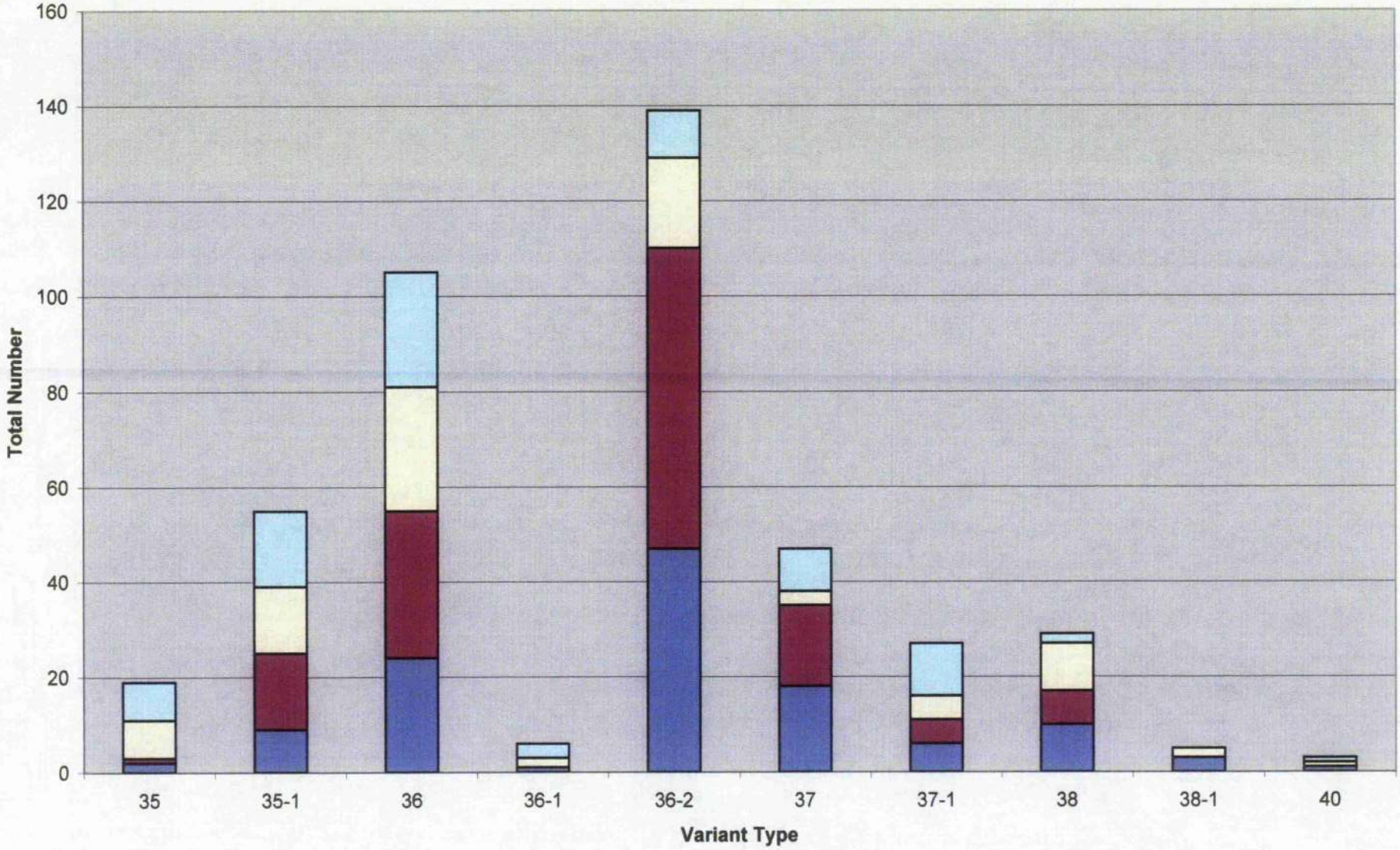

160
140
120
100
80
60
40
20
0
Total Number
35
35-1
36
36-1
36-2
37
37-1
38
38-1
40
Variant Type

Figure 4.5 Association between porin A variable region 1 (VR1) and related serogroups from meningococci isolated in 1999 to 2002 (years 1 to 4). Each variable type is represented by year with the proportion of each serogroup present within that variant type displayed by a colour-coded line.

The year 1999 in dark red	
The year 2000 in blue	
The year 2001 in light purple	
The year 2002 in yellow	
Serogroup B is shown in red	
Serogroup C in blue	
Serogroup Y in yellow	
Serogroup W135 in purple dash	
Serogroup X in green	
Serogroup Z in brown	
Serogroup NG in black	

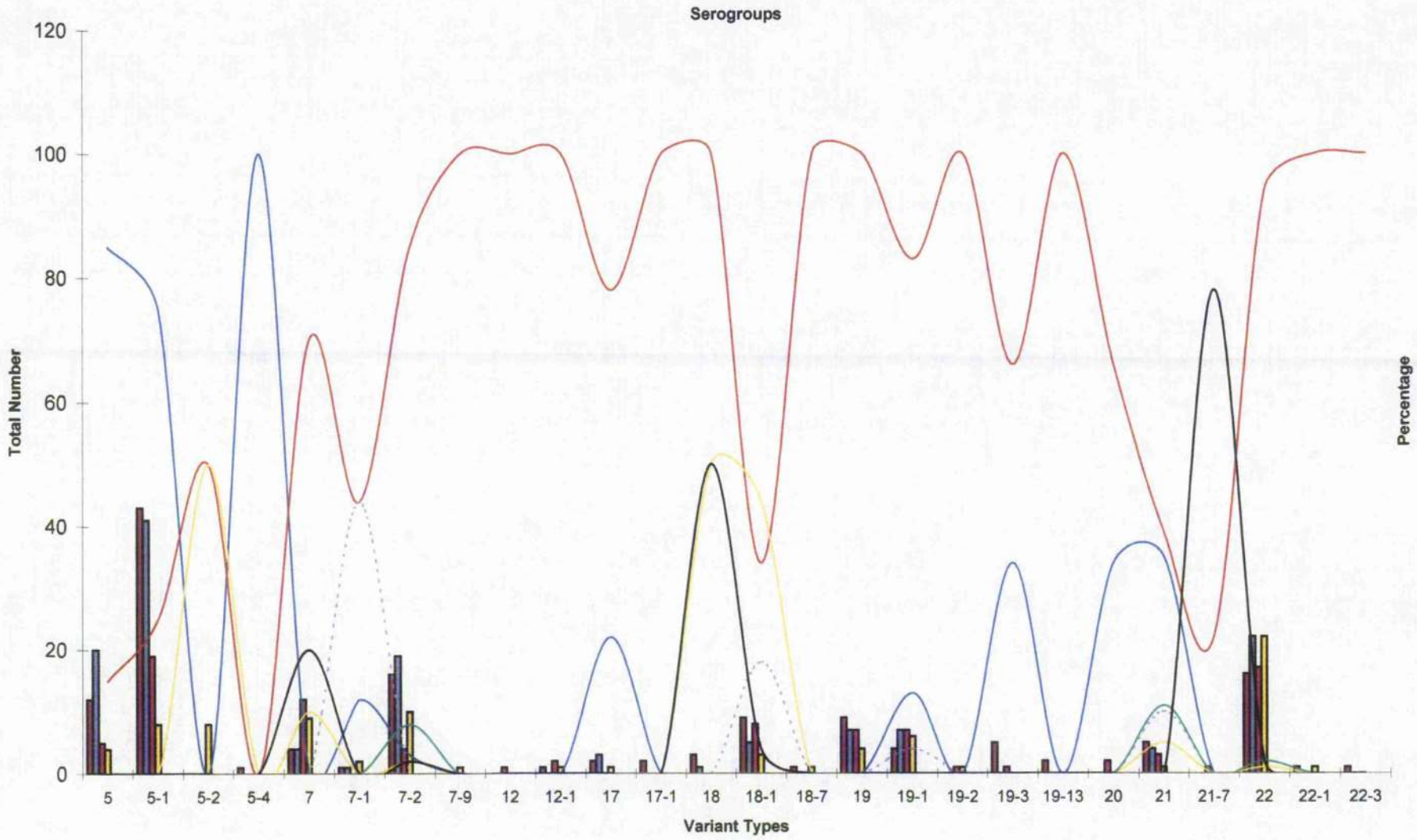

Serogroups
120
100
80
60
40
20
0
Total Number
Percentage
5
5-1
5-2
5-4
7
7-1
7-2
7-9
12
12-1
17
17-1
18
18-1
18-7
19
19-1
19-2
19-3
19-13
20
21
21-7
22
22-1
22-3
Variant Types

Figure 4.6 Association between porin A variable region 2 (VR2) and related serogroups from meningococci isolated in 1999 to 2002 (years 1 to 4). Each variable type is represented by year with the proportion of each serogroup present within that variant type displayed by a colour-coded line.

The year 1999 in dark red	
The year 2000 in blue	
The year 2001 in light purple	
The year 2002 in yellow	
Serogroup B is shown in red	
Serogroup C in blue	
Serogroup Y in yellow	
Serogroup W135 in purple dash	
Serogroup X in green	
Serogroup Z in brown	
Serogroup NG in black	

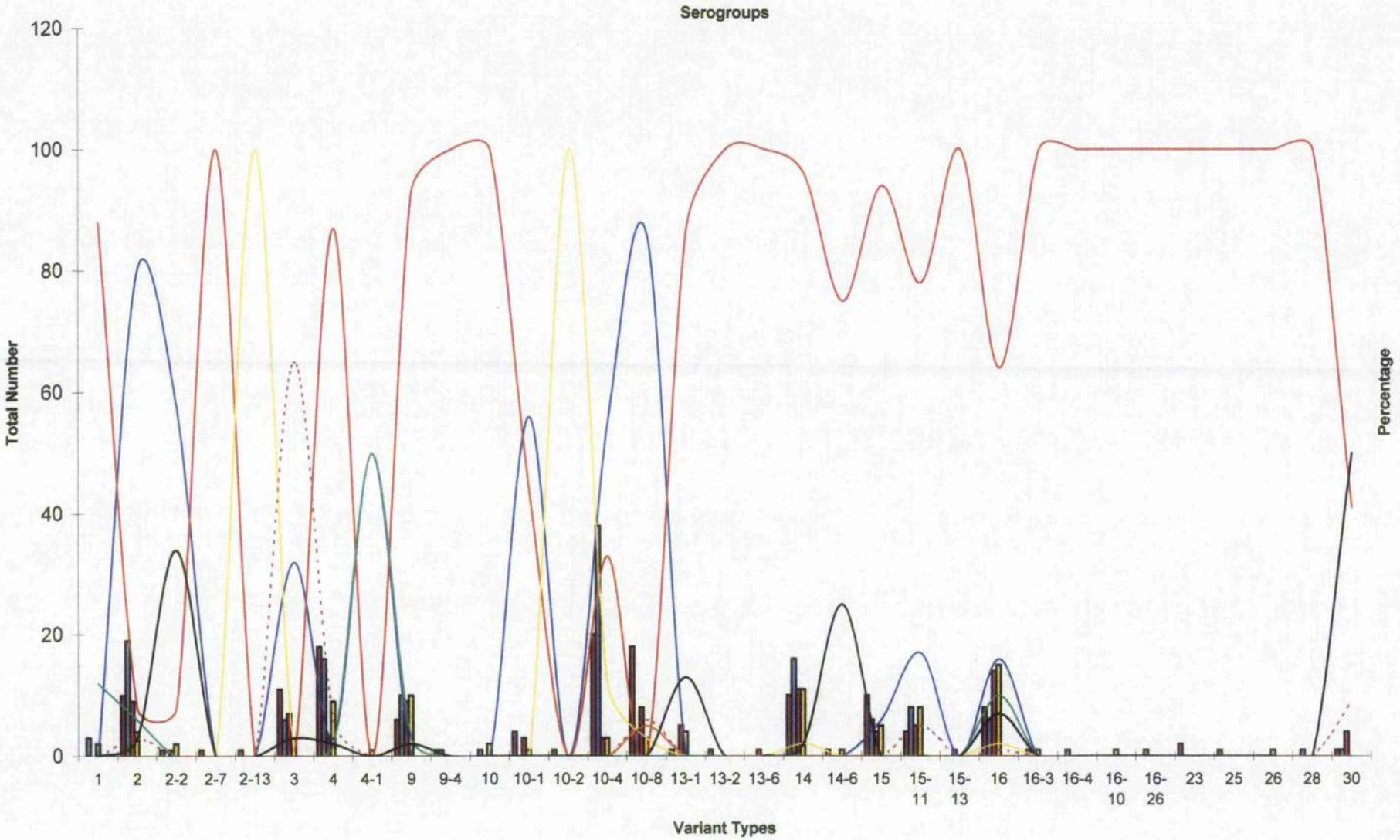
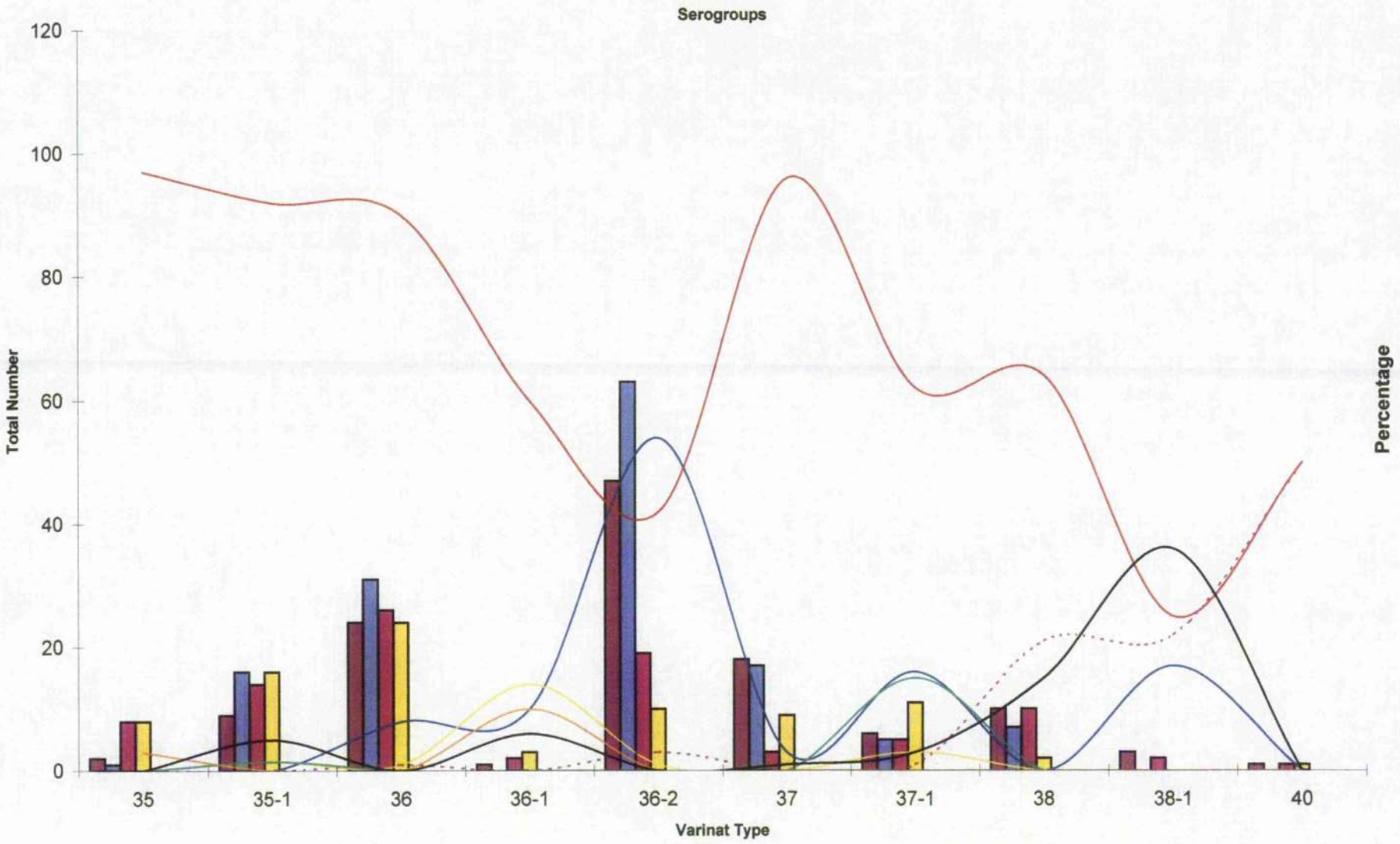

Serogroups
120
100
80
60
40
20
0
Total Number
Percentage
1
2
2-2
2-7
2-13
3
4
4-1
9
9-4
10
10-1
10-2
10-4
10-8
13-1
13-2
13-6
14
14-6
15
15-11
15-13
16
16-3
16-4
16-10
16-26
23
25
26
28
30
Variant Types

Figure 4.7 Association between porin A variable region 3 (VR3) and related serogroups from meningococci isolated in 1999 to 2002 (years 1 to 4). Each variable type is represented by year with the proportion of each serogroup present within that variant type displayed by a colour-coded line.

The year 1999 in dark red	
The year 2000 in blue	
The year 2001 in light purple	
The year 2002 in yellow	
Serogroup B is shown in red	
Serogroup C in blue	
Serogroup Y in yellow	
Serogroup W135 in purple dash	
Serogroup X in green	
Serogroup Z in brown	
Serogroup NG in black	


Serogroups
120
100
80
60
40
20
0
Total Number
Percentage
35
35-1
36
36-1
36-2
37
37-1
38
38-1
40
Varinat Type

4.1.2 Genosubtyping of Meningococcal Isolates in 2000.

4.1.2.1 Serogroup C Isolates. The majority of isolates fall into a single group within VR1, VR2 and VR3. There had been a consolidation within VR2 and VR3 into single groups rather than two groups previously described in section 4.1.1.1. The first variable region was represented by four different variable types within only two distinct families namely, 5 (n=45) and 21 (n=1) with over 97% allocated to variable regions 5 (n=10), 5-1 (n=34) and 5-4 (n=1). This represents a dramatic decrease in the distribution throughout different families from six into two. The second variable region was represented by four different variable types within three distinct families namely, 2 (n=11), 10 (n=34) and 16 (n=1) with over 98% allocated to variable regions 2, 10-4 (n=31) and 10-8 (n=3). The third variable region was represented by two different variable types within two distinct families namely, 36 (n=45) and 37 (n=1) with over 98% allocated to variable region 36-2. This followed a similar pattern to the previous year with VR1, VR2 and VR3 patterns consistently appearing with the same profile compared with 1999. There had been a dramatic consolidation of different variable types and consequently specific variable regions associated with large clonal complexes were predominant and more diverse variable types had disappeared in 2000. A single exception was the isolation and characterisation of a C, 2B, non-subtypable (NST) strain with the *porA* profile 21, 16, 37-1. This was unique within the Scottish meningococcal population. The *porA* profile was more commonly associated with serogroup B and X isolates, although the MLST profile (ST8) was consistent with serogroup C Scottish isolates from the 1980s.

The first variable region was represented in the majority from the type 5-family. The

three represented were 5 (n=10), 5-1 (n=34) and 5-4 (n=1). This follows a similar pattern to serogroup C isolates characterised in 1999. The total number of serogroup C isolates had decreased, however the major variant types still remained dominant.

The type 10-family (n=34) and the type 2-family (n=11) represent the second variable region. The type 10 variants represented were 10-4 (n=31) and 10-8 (n=3) and the type 2 (n=11) represents the 2-family. This displays a similar variant distribution compared with isolates from 1999.

The type 36-family (n=45) and the type 37-family (n=1) represent the third variable region. As with 1999 the majority of serogroup isolates (over 97%) were represented by the type 36-2 (n=45). However there was a single representation from the type 37-family from the 37-1 variant as discussed previously

4.1.2.2 Serogroup B Isolates. The first variable region was represented by thirteen different variable types. This again was significantly larger than the dispersion of VR1s between serogroup C isolates, although this represented the same number of variants compared with serogroup Bs in 1999. These were represented within seven distinct families namely, 5, 7, 17, 18, 19, 21 and 22 with over 91% allocated to variable region families 5, 7, 19 and 22. The second variable region was represented by fifteen variable types, a slight reduction in the total number of different VR2s compared with 1999. These were contained within nine families namely, 1, 2, 4, 9, 10, 14, 15, 16 and 30 with over 82% allocated to variable regions 4, 9, 14, 15 and 16. This highlighted a change in the dominant VR2s with variable type 3 diminishing and variable type 16 significantly emerging. The third variable region was dispersed

between seven different variable types within four distinct families namely, 35, 36, 37 and 38 with over 91% allocated to variable regions 35-1, 36, 36-2 and 37.

The first variable region was represented by a number of distinct variable types from the 5, 7, 17, 18, 19, 21 and 22. Within this collection the 7 variant (n=23) was represented by three variant types 7 (n=4), 7-1 (n=1) and 7-2 (n=18). Both 7 and 7-2 had been consistently prominent throughout 1999 and 2000, though a single 7-1 variant had emerged. This variant had been observed in a single serogroup X isolate from 1999 (section 4.1.1.3), however this had not been previously observed in serogroup B isolates. The 22-variant family (n=20) is represented by 22 (n=19) and 22-3 (n=1). The type 22 had been observed previously in 1999 whereas the 22-3 had not been identified before. This variant had a single amino acid change compared with the original type 22 variant. The 19-variant family (n=16) was represented by three different variant types namely, 19 (n=7), 19-1 (n=7) and 19-3 (n=2). Both 19 and 19-1 had been observed previously, whereas 19-3 had not, this variant type had a single amino acid change compared to the original type 19 variant. The 5-variant family (n=9) was represented by both 5 (n=6) and 5-1 (n=3). These were rarely associated with meningococcal serogroup B isolates in Scotland and more commonly found associated with serogroup C isolates, although a small number were observed associated with serogroup B isolates in 1999. The emergence of type 5 within the serogroup B population in 2000 had not been observed previously except in large clonal complexes within the serogroup C population.

The type 4, 9, 14, 15 and 16 represented the majority in the second variable region. All the 4-variant family (n=16) were represented by the type 4 variant and continued

to dominate within the type 4 sub-population. The 15-variant family (n=14) were represented by the 15-variant (n=6) and the 15-11-variant (n=8). These variants had been observed previously. In 2000 a notable shift between the 15-variant and 15-11-variant was noted. The 14-variant family (n=13) had maintained a significant presence in the population in 2000 and the single type 14-variant represented these. This represented a consolidation in this variant type within the population. The 9-variant family (n=10) followed a similar pattern to 1999 with a significant presence from the 9-variant (n=9) and a single isolate from the 9-4-variant. Finally, the 16-variant family (n=8) had increased and proliferated during 2000 with the presence of variant-16 (n=5) and the single representation from variant 16-3, 16-4 and 16-26, both 16-4 and 16-26 had not been observed before. The 16-4-variant had 3 amino acid changes compared to the original representative variant, although when compared with 16-3 only had a single amino change. The 16-26 was more closely associated with the original representative 16-variant with a deletion of the "KD" (Lysine, Aspartic acid) amino acid motif and the insertion of an "N" (Asparagine) amino acid. This resulted in the exchange of both an acidally charged amino acid and a basic charged amino acid for a single neutally charge amino acid and as a consequence slightly reduced both the length and molecular weight of this region by 147.21.

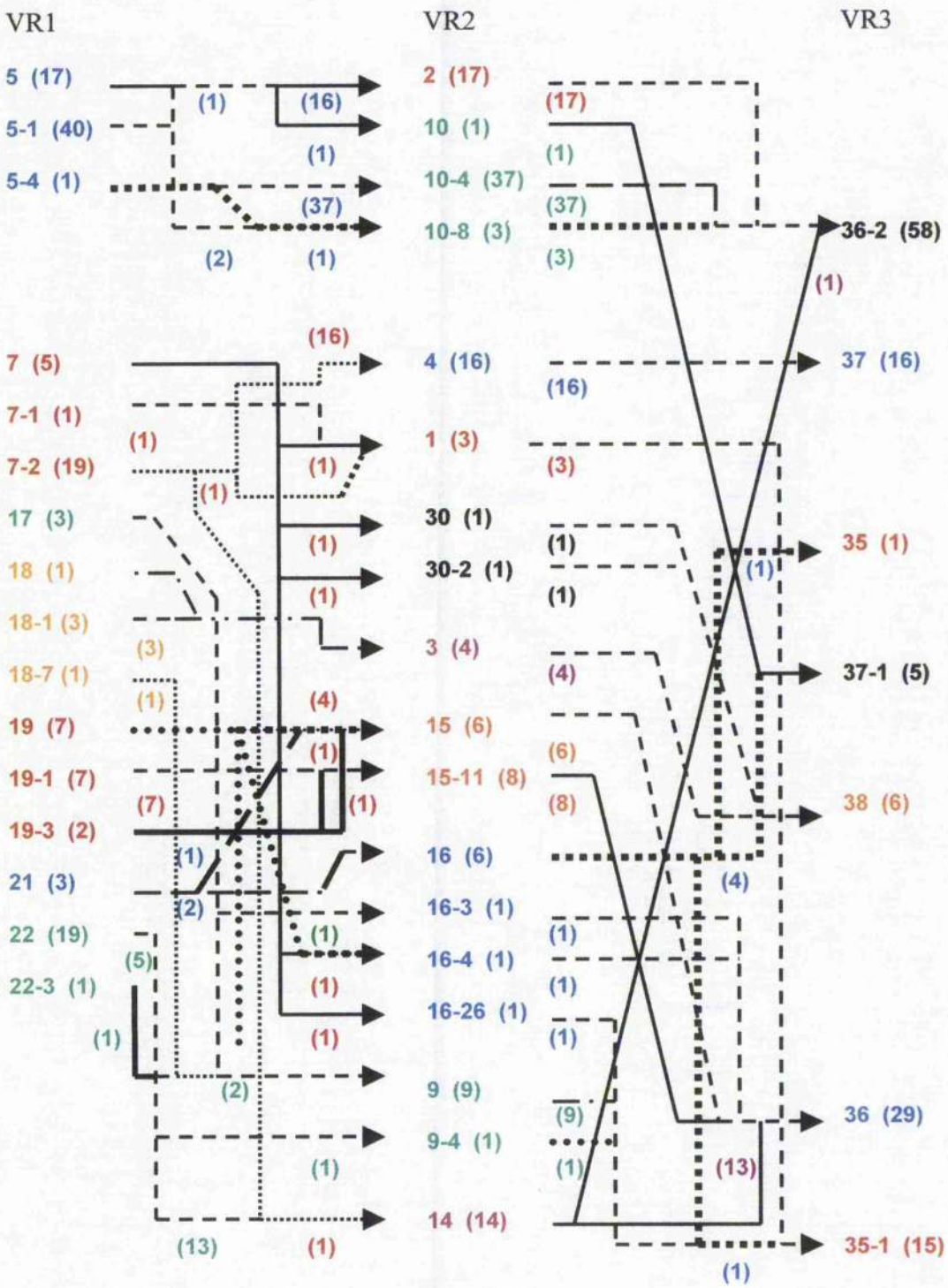
The third variable region was represented by 35 (n=1), 35-1 (n=15), 36 (n=28), 36-2 (n=9), 37 (n=16), 37-1 (n=4) and 38 (n=1). The distribution was similar to that displayed in 1999. The 36-family again represented a significant proportion of these isolates. These were mainly represented by the 36-variant. There had been an increase in the proliferation of other variants within families, such as the emergence of

variant 35, and variant 37-1, from a pre-existing population containing closely related variants, notably 35-1 and 37 respectively.

4.1.2.3 Miscellaneous Serogroup Isolates. These represented over 9% of the total population in 2000. These were serogroups W135 (n=6), Y (n=4) and two non-groupable isolates. Variable region one was represented by 18 (n=1), 18-1 (n=3), 5 (n=1) and 7 (n=1) within the W135 population. The W135 population had shown an increased proliferation of its variable region characteristics. Previously, W135 was exclusively associated with variant 18-1, and this was subsequently associated with serogroup B isolates. The proliferation in 2000 included variant 18, which was closely associated to 18-1. Although these variants had not been observed during 2000 in other major serogroups, the emergence of the 18-7-variant within the serogroup B population was noted. The variant 5 is strongly, associated with serogroups B and C and the variant 7, which, was apart from a single W135 exclusively associated with the serogroup B population. VR2 was represented by a smaller set of variants that were 2 (n=1), 3 (n=4) and 30-2 (n=1). Variant 2 was associated with VR1 type 5 and consequently had a VR3 36-2 type. This was a common profile within both the serogroup B and C populations. Variant 3 was associated with the VR1 type 18 family and consequently had a VR3 38 type. This was a common profile within the serogroup W135 population. Variant 30-2 was associated with VR1 type 7 and subsequent VR3 38 type. This *porA* profile had not been observed before although the VR2 variant 30-family had been represented within the serogroup B and non-groupable populations. The *porA* profiles of both non-groupable isolates were, 7-2, 4, 37 and 7, 30, 38. These had been observed before within the serogroup B population in both 1999 and 2000.

Variable regions 5-1 (n=3) and 7-2 (n=1) represented the VR1s within the serogroup Y population. Both these were associated with serogoups C and B respectively. Subsequent variable regions 10-4 (n=3) and 14 (n=1) represented VR2s and variable regions 36-2 (n=3) and 36 (n=1) represented VR3s with both close associated with the VR1s and consequently again represented common *porA* profiles with serogroups C and B respectively. Previously serogroup Y *porA* profiles were closely associated with the serogroup C population and this represented a slight shift towards common association between both serogroups B and C. Finally, two non-groupable isolates were identified during 2000 that had *porA* profiles 7-2, 4, 37 and 7, 30, 38 respectively. These had been observed before within the serogroup B population.

Figure 4.8 Association between porin A variable region 1 (VR1), variable region 2 (VR2) and variable region 3 (VR3) from meningococci isolated in 2000 (year 2). Each identified porin A profile (VR1, VR2, VR3) is linked through a series of individually coloured and styled arrows. The total number represented within that variable type is indicated within the brackets. Following the 18-1 VR1 variants (yellow) the dash/dot line finishes at VR2 3 (purple) (n=4). These are further distributed through a dashed line to VR3 38 (n=6).


VR1
VR2
VR3
5 (17)
5-1 (40)
5-4 (1)
(1)
(16)
(1)
(37)
(2)
(1)
7 (5)
7-1 (1)
7-2 (19)
17 (3)
18 (1)
18-1 (3)
18-7 (1)
19 (7)
19-1 (7)
19-3 (2)
21 (3)
22 (19)
22-3 (1)
(16)
(1)
(1)
(1)
(1)
(3)
(1)
(4)
(1)
(7)
(1)
(1)
(2)
(1)
(5)
(1)
(1)
(2)
(1)
(13)
(1)
2 (17)
10 (1)
10-4 (37)
10-8 (3)
4 (16)
1 (3)
30 (1)
30-2 (1)
3 (4)
15 (6)
15-11 (8)
16 (6)
16-3 (1)
16-4 (1)
16-26 (1)
9 (9)
9-4 (1)
14 (14)
(17)
(1)
(37)
(3)
(16)
(3)
(1)
(1)
(1)
(4)
(6)
(8)
(4)
(1)
(1)
(1)
(9)
(1)
(13)
(1)
36-2 (58)
37 (16)
35 (1)
37-1 (5)
38 (6)
36 (29)
35-1 (15)
(1)
(1)

4.1.3 Genosubtyping of Meningococcal Isolates in 2001.

4.1.3.1 Serogroup C Isolates. The total number of serogroup C isolates characterised in 2001 fell by over 50% compared with the previous year. The majority of isolates fell into a single VR1 family, two distinct VR2 families and a single VR3 family. This displayed a similar pattern to those described in 1999 rather than 2000 described in section 4.1.1.1. The first variable region was represented by four different variable types within only two distinct families namely, 5 (n=19) and 19 (n=2) with over 90% allocated to variable regions 5 (n=5) and 5-1 (n=14). This maintained the trend towards consolidation into a smaller sub-population within a single variant family. There had been a change in the representation of the remaining variants types within the serogroup C population, the shift from type 21 to type 19 was observed. This type was more commonly associated with serogroup B isolates, although the previous type was also commonly represented in the serogroup B population. The second variable region was represented by six different variable types within three distinct families namely, 2 (n=9), 10 (n=10) and 15 (n=2) with over 90% allocated to variable regions 2, 10-1 (n=2), 10-4 (n=1) and 10-8 (n=7). There had been a dramatic decrease in type 10-4 although other variant types within this family had been unaffected consequently with the emergence of previously unobserved variants such as 10-1. The third variable region was represented by three different variable types all contained within a single distinct family namely 36 (n=21). These were dispersed into variant type 36 (n=4), 36-1 (n=2) and 36-2 (n=15). There had been a greater proliferation into different variable types observed in 2001 compared with the previous year and this represented a similar pattern observed in 1999. Specific variable regions had maintained a presence within this serogroup although a change within specific

families was observed. Again two exceptions were the isolation and characterisation of a C, 2A, non-subtypable and a non-groupable that was later confirmed as a group C, 1, P1.15. The first was classified non-subtypable by traditional ELISA methods and subsequently sequenced. This was considered an unusual *porA* profile for a serogroup C isolate. The second was successfully sub-typed and sequenced and both correlated to produce another unusual result for a serogroup C isolate.

The first variable region was represented in the majority from the type 5-family. The two represented were 5 (n=5) and 5-1 (n=14). This followed a similar distribution to 1999 and 2001. The total number of serogroup C isolates had decreased by over 50% but the major variants types still remained dominant.

The type 10-family (n=10) and the type 2-family (n=9) represented the second variable region. The type 10 variants had reduced in number but had expanded to include 10-1 (n=2) as well as previously observed variants 10-4 (n=1) and 10-8 (n=7). This followed a similar family distribution to 1999 and 2000, although specific variants had changed in number and type.

The type 36-family (n=21) represented the third variable region. As with 1999 and 2000 the majority of serogroup isolates (over 71%) were represented by the type 36-2 (n=15). This highlighted a consolidation towards a single family type, although variation within this family to include type 36 (n=4) and type 36-1 (n=2) was observed.

4.1.3.2 Serogroup B Isolates. The first variable region was represented by thirteen different variable types. This remains significantly larger than the dispersion of VR1s between serogroup C isolates, although this remains consistent within the serogroup B populations from 1999 and 2000. These were contained within eight distinct families namely, 5, 7, 12, 18,19, 20, 21 and 22 with over 68% allocated to variable region families 7, 19 and 22. This displayed exactly the same pattern as 2000 with the exception of the absence of type 5 from the significant contributors. However, type 5 was not eliminated, rather than significantly reduced with variant type 5-1 (n=4) contributing less than 8% of the total serogroup B population. The second variable region was represented by fifteen variable types, the same number of types observed in 2000. These were contained within ten families namely, 2, 3, 4, 9, 10, 13, 14, 15, 16 and 30 with over 76% allocated to variable regions 9, 13, 14, 15 and 16. This highlighted another change in the dominant VR2s with variable type 4 diminishing and variable type 13 significantly emerging. The third variable region was represented by eight different variable types within four different families namely, 35, 36, 37 and 38 with over 74% allocated to variable regions 35, 35-1, 36 and 36-2.

The first variable region was represented by a number of distinct families from the 5, 7, 12, 18, 19, 20, 21 and 22. Within this collection all the 22 variant types (n=14) were represented by the original representative type-22. This variant continued to maintain a significant presence within the serogroup B population with a similar variant distribution to 1999 and 2000. The 19-varinat family (n=13) was represented by three different variant types namely, 19 (n=7), 19-1 (n=5) and 19-2 (n=1). Both 19 and 19-1 continued to dominate as previously described, whereas 19-2 had not, this variant type has a single amino acid change compared to the original type 19 variant.

Interestingly, this mutation was found in the same position as the mutation that produced the 19-3 the previous year. The 7-varinat family (n=8) was represented by two variant types namely, 7 (n=5) and 7-2 (n=3). Both 7 and 7-2 had been observed in significant numbers previously throughout 1999 and 2000. There had been a notable consolidation and significant reduction of isolates pertaining to the type 7 family.

The types 9, 13, 14, 15 and 16 represented the majority in the second variable region. With the demise of the type 4-variant family in 2001 the type 16-variant family (n=10) had continued to increase during this time, although consolidation into a single representative type had been observed and no other variants types had been characterised during this time period. The 9-variant family (n=8) follows a similar pattern to 1999 and 2000 with a significant presence from the single variant type 9. The 14-variant family (n=8) had maintained a significant presence and the single type 14-variant had represented these. This represented a continued consolidation with this particular type within the population. The 15-variant family (n=7) were represented by 15 (n=3), 15-11 (n=3) and 15-13 (n=1). With the exception of 15-13 these variants had been observed in previous years. The notable shift observed in 2000 between variant 15 and 15-11 had re-adjusted to similar levels. The variant 15-13 had not been characterised previously and displayed a close relationship to 15-11 with a repeat insertion of the "QNN" (Glutamine, Asparagine, Asparagine) amino acid motif within the central region. This resulted in the addition of three neutrally charged amino acids and as a consequence increased both the length and the molecular weight by 410.39. This insertion in relation to this relatively small type of variable region has significantly extended it by another 30%. Finally, the 13-variant family (n=6) had been introduced during 2001 with the presence of variant 13 (n=1), 13-1 (n=4) and 13-

6 (n=1). These types were not observed during 2000, however variant 13 and 13-1 were observed during 1999 and variant 13-6 had been observed for the first time. The 13-variant family has an 18 amino acid chain length and has 18 different variants ranging from between one and four amino acid changes either by substitution insertion or mutation when compared to the original representative type (Appendix 6.2). The newly emerged 13-6 had an additional "TTTT" (Threonine, Threonine, Threonine, Threonine) amino acid motif inserted within the existing "TTTT" (Threonine, Threonine, Threonine, Threonine) amino acid region when compared to the 13-1 variant and an additional "TTT" (Threonine, Threonine, Threonine) amino acid within the same region when compared to the original 13 variant. This resulted in the addtion of three or four threonine neutrally chaged amino acids and as a consequence increased both the length and the molecular weight by either 357.36 or 476.48.

The third variable region was represented by 35 (n=4), 35-1 (n=12), 36 (n=19), 36-2 (n=3), 37 (n=3), 37-1 (n=4), 38 (n=4) and 38-1 (n=2). The distribution had similarities to 1999 and 2000, although a significant reduction if type 37 was observed. The 35-family (n=16) and the 36-family (n=22) represented a significant proportion of the isolates. These were mainly 35-1 and 36 variants. There had been a continued trend towards greater proliferation within families with the emergence of the 38-1-variant, which has not been observed since 1999.

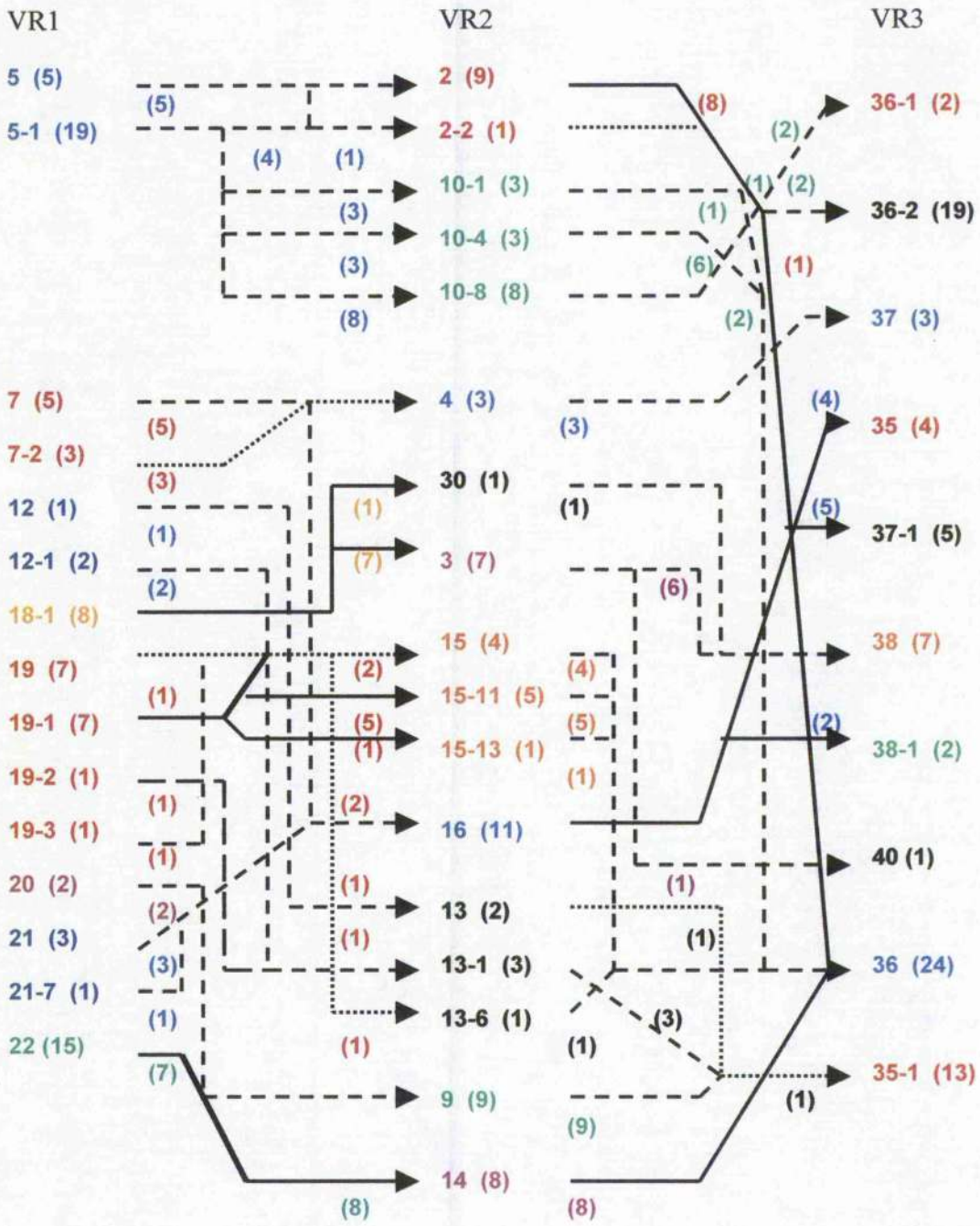
4.1.3.3 Miscellaneous Serogroup Isolates. These represented over 12% of the total population in 2001. These were serogroups W135 (n=5), X (n=2), Y (n=1) and two non-groupable isolates. Variable region one was represented by 18-1 (n=4) and 19-1 (n=1) within the W135 population. The W135 population in 2001 had a close

association to the W135 population of 1999 rather than 2000, where a greater number of different variants types were identified. The emergence of 19-1 had not been observed before and is more closely associated with serogroup B isolates, as is 18-1. VR2 was represented by variants 3 (n=4) and 15-11 (n=1). These were associated with 18-1 and 19-1 respectively. Interestingly, although these conform to pre-existing VR1 and VR2 profiles, the VR3 showed greater variation than expected. VR3 was represented by 36 (n=1), 38 (n=3) and 40 (n=1), of these *porA* profile 18-1, 3, 40 had not been identified previously within the W135 population although a single serogroup B isolate from 1999 did share the same *porA* profile.

The serogroup X profiles were 21, 16, 37-1 and 22, 9, 35-1. The first profile had been observed before, however the second profile had not been observed in serogroup X isolates and was commonly identified within common serogroup B complexes.

A single serogroup Y isolated was identified as 5-1, 10-8, 36-2. This profile had a slight variation in the VR2, which had been identified previously as a 10-2 or 10-4 and as a consequence had not been observed before within the serogroup Y population. This profile was commonly identified in serogroup C isolates.

Figure 4.9 Association between porin A variable region 1 (VR1), variable region 2 (VR2) and variable region 3 (VR3) from meningococci isolated in 2001 (year 3). Each identified porin A profile (VR1, VR2, VR3) is linked through a series of individually coloured and styled arrows. The total number represented within that variable type is indicated within the brackets. Following the 12-1 VR1 variants (blue) the dashed line finishes as VR2 13-1 (black) (n=3). These are further distributed through a dashed line to VR3 35-1 (n=13).


VR1
VR2
VR3
5 (5)
5-1 (19)
7 (5)
7-2 (3)
12 (1)
12-1 (2)
18-1 (8)
19 (7)
19-1 (7)
19-2 (1)
19-3 (1)
20 (2)
21 (3)
21-7 (1)
22 (15)
2 (9)
2-2 (1)
10-1 (3)
10-4 (3)
10-8 (8)
4 (3)
30 (1)
3 (7)
15 (4)
15-11 (5)
15-13 (1)
16 (11)
13 (2)
13-1 (3)
13-6 (1)
9 (9)
14 (8)
36-1 (2)
36-2 (19)
37 (3)
35 (4)
37-1 (5)
38 (7)
38-1 (2)
40 (1)
36 (24)
35-1 (13)

4.1.4 Genosubtyping of Meningococci Isolates in 2002.

4.1.4.1 Serogroup C Isolates. For the second year in a row the total number of serogroup C isolates fell by over 47% compared to the previous year. The majority of isolates fell into one single VR1 family, two distinct VR2 families and one single VR3 family. This pattern had been consistent throughout this four-year study. The first variable region was represented by five different variable types within four distinct families namely, 5 (n=8), 19-1 (n=1), 21 (n=1) and 22 (n=1) with over 70% allocated to variable regions 5 (n=5) and 5-1 (n=3). This highlighted a slight increase in dispersion throughout a number of different family variants, although the majority of isolates still remained in the same families and family variants compared to previous years. The second variable region was represented by five different variable types within four distinct families namely, 2 (n=5), 10 (n=4), 15(n=1) and 16 (n=1) with over 70% allocated to variable regions 2, 10-4 (n=1) and 10-8 (n=3). The level of type 10-4 had remained low since 2000 and type 10-8 remained dominant within the family. The third variable region was represented by four different variable types contained within two distinct families namely, 36 (n=10) and 37 (n=1) with over 90% allocated to variant types 36 (n=1), 36-1 (n=2) and 36-2 (n=7). The dispersion within this family remained consistent with patterns observed in the previous year. Overall a similar pattern was observed within the serogroup C population compared with the previous year, although there was the continued presence of variable types that had been commonly associated with serogroup B isolates.

The first variable region was represented in the majority from the type 5-family. The two represented are 5 (n=5) and 5-1 (n=3). This followed a similar distribution to

1999, 2000 and 2001. The total number of serogroup C isolates had decreased by over 47% but the major variant types still remained dominant. The existence of variants 19-1 (n=1), 21 (n=1) and 22 (n=1) were observed, these had been characterised within the serogroup C population previous but usually either the 19 or the 21 was observed, this was the first instance where both had been observed within the population within a single year. As previously described these were more commonly observed within the serogroup B population.

The type 2-family (n=5) and the type 10-family (n=4) represented a large proportion of the VR2 variants with the type 15-family and type 16-family represented by a single isolate respectively. With the exception of the single representatives this followed a similar family distribution to the previous years.

The type 36-family (n=10) and the type 37-family (n=1) represented the third variable region. As with all previous years the majority of serogroup C isolates (over 63%) were represented by the type 36-2 (n=7). From previous years this trend was shown to consolidate towards a single variant type, however there had been a decrease in the percentage identified as type 36-2 and an increase in the presence of other types either within the same family such as type 36 (n=1) and 36-1 (n=2) or from other family types namely, 37-1 (n=1).

4.1.4.2 Serogroup B Isolates. The first variable region was represented by fifteen different variable types and this highlighted an increase of over 10% on the previous year. These were contained within eight distinct families namely, 5, 7, 12, 17, 18, 19, 21 and 22. Consequently, an increase in variable types within the same number of

variant families highlights increased diversity within specific families. However type 20 was not observed in 2002 and type 17 was observed and this maintained stability in the total number of families observed within the serogroup B population. Within these eight distinct families over 79% were allocated to variable region families 7, 19 and 22. These dominant variant families remain the same compared with the previous year. The variant type 5 that was significantly reduced in previous years had not been observed in 2002 although variants 5-1 (n=3) and 5-2 (n=2) maintained a presence. The second variable region was represented by fifteen variable types, again the same number of types were observed in the previous year. These were contained within ten families namely, 1, 2, 4, 9, 10, 13, 14, 15, 16 and 26 with over 82% allocated to variable regions 4, 9, 14, 15 and 16. This highlighted another change within this group of dominant families with variable type 4 re-emerging and type 13 maintaining a presence albeit slightly reduced. The third variable region was represented by six different variable types within three different families namely 35, 36 and 37 with even distribution across each family with 35%, 41% and 24% respectively within variant types 35 (n=9), 35-1 (n=15), 36 (n=24), 36-2 (n=4), 37 (n=9) and 37-1 (n=8).

The first variable region was represented by a number of distinct families from the 5, 7, 12, 17, 18, 19, 21 and 22. The 22-variant family (n=22) was represented by two variant types namely, 22 (n=21) and 22-1 (n=1). This represented an expansion of the 22-variant family with the emergence of 22-1 although the type 22 had remained consistently dominant over a number of years within the serogroup B population. The 7-variant family (n=22) was represented by four variant types namely, 7 (n=9), 7-1 (n=2), 7-2 (n=10) and 7-9 (n=1). Both 7 and 7-2 had been observed in the previous year however 7-1 and 7-9 had not although 7-1 had been identified in 2000. There

was a notable consolidation and significant reduction of isolates pertaining to the type 7 family that in 2002 had been significantly reversed. The newly emerged 7-9 had a "GANGGAS" (Glutamine, Alanine, Asparagine, Glutamine, Glutamine, Alanine, Serine) amino acid motif inserted within the central region compared to the commonly identified 7-2. This resulted in the addtion of seven neutrally chaged amino acids and as a consequence of the size of this inserted motif it increased the size of the variable region by over 40% and increased the molecular weigth by 640.60. The 19-variant family (n=11) continued to maintain a presence within the population with representative types 19 (n=4), 19-1 (n=6) and 19-2 (n=1) following a similar pattern from the previous year.

The types 4, 9, 14, 15 and 16 represent the majority within the VR2 group. The type 16-variant family (n=16) had continued to increase during this time with proliferation into 16 (n=14), 16-3 (n=1) and 16-10 (n=1) whereas in previous years type 16 predominated as the single representative type. The 16-10 variant had not been observed previous and has a "TKD" (Threonine, Lysine, Aspartic acid) amino acid motif deleted compared to the type 16-varinat. This resulted in the deletion of a single neutrally, basic and acidicly charged amino acid with the combined molecular weight of 398.41. The motif was reduced in length by over 20%. The 14-varinat family (n=13) had maintained a significant presence with two variant types, namely 14 (n=12) and 14-6 (n=1). This represented an expansion within the type 14-family and mirrored a similar distribution pattern to 1999. The 15-variant family (n=10) were represented by 15 (n=5) and 15-11 (n=5). This highlighted a consolidation into two representative variant types more indicative of the type-15 population in 1999 and 2000 rather than 2001. The demise of the type 4-varinat (n=9) was short lived and its

re-emergence had been observed in 2002. The 9-variant family (n=9) followed a similar pattern to previous years with a significant impact from the single variant type 9.

The third variable region was represented by 35 (n=9), 35-1 (n=15), 36 (n=24), 36-2 (n=4), 37 (n=9) and 37-1 (n=8). This highlighted a consolidation into three main families with the inconsistent appearance and non-appearance of the 38-family over the past few years mainly associated with the serogroup W135 population. The 35-family (n=24) and 36-family (n=28) maintained a dominant presence within the population although the 37-family had significantly increased and displayed levels similar to 1999 and 2000.

4.1.4.3 Miscellaneous Serogroup Isolates. These represent over 10% of the total population in 2002. These were serogroups W135 (n=4), X (n=1), Y (n=3) and Z (n=1). The serogroup W135 population was represented by a single VR1 and VR2 and two distinct VR3s. The first variable region was represented by 18-1 (n=4) these represented a common VR1 associated with serogroup W135 isolates and displayed a similar pattern to previous years. Variable region two was represented by variant type 3 (n=4) and again this was a common VR2 associated with both the VR1 and the W135 population. Variable region three was represented by two types namely, 38 (n=3) and 40 (n=1). The first type was common with pre-existing W135 *porA* profiles however the second type was less common although had been identified previously.

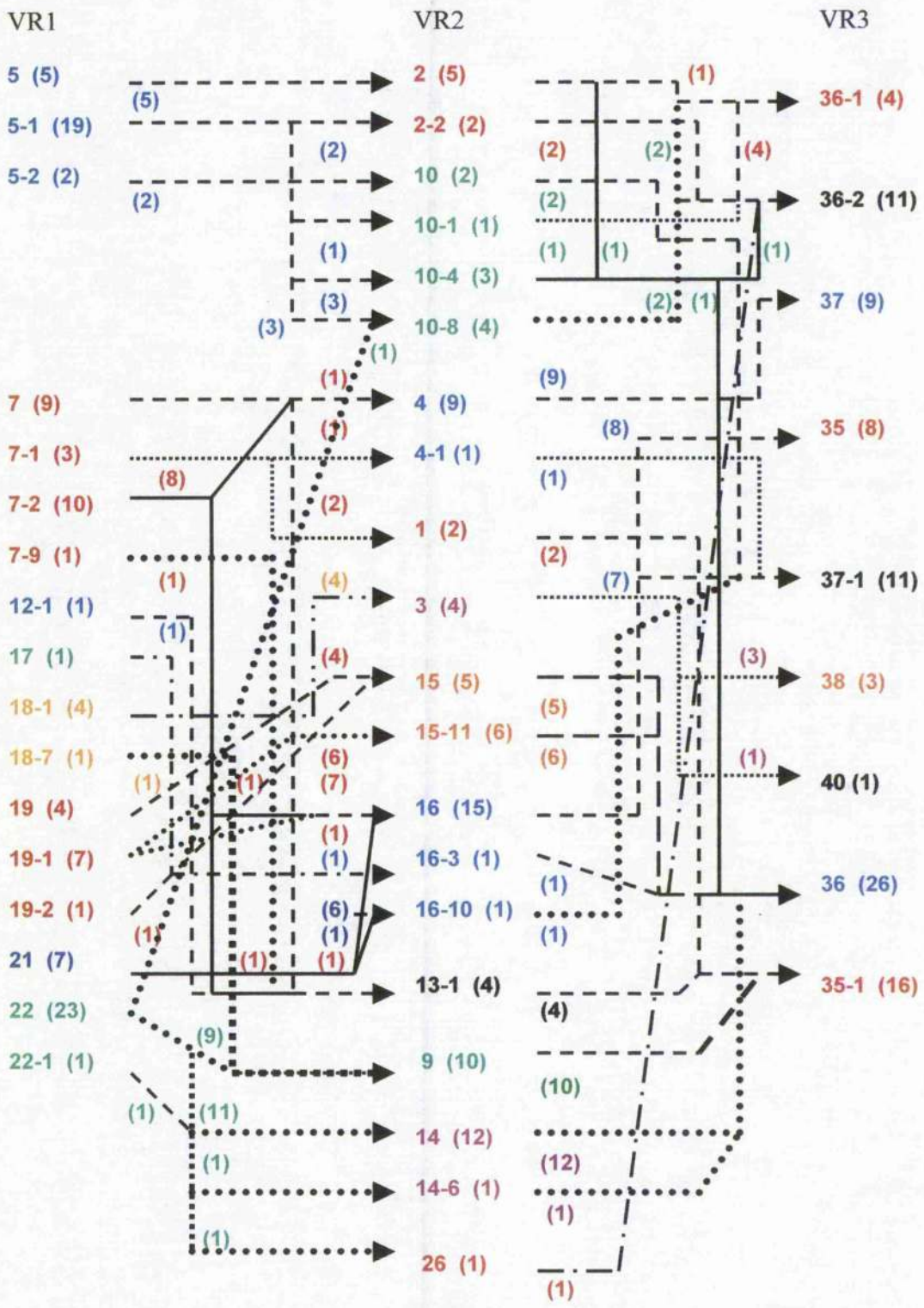
The serogroup Y (n=3) population was represented by two VR1s, VR2s and two VR3s. The first isolate had a 22, 9, 35-1 *porA* profile that was more commonly

associated with serogroup B isolates. The remaining two isolates had 5-1, 10-4 and two VR3s namely, 36 and 36-1. The variable region one and two were consistent with previous serogroup Y profiles however both VR3s were not expected within both this *porA* profile and the serogroup Y population. These VR3s were more commonly associated with serogroup B isolates whereas previous years had displayed variant type 36-2, which was more commonly associated with serogroup C isolates.

The single serogroup X profile was 7-1, 4-1 and 37-1. This profile has a slight variation on a previously identified serogroup X isolate from 1999. The second variable region has a single amino acid mutation and both these variant types are commonly identified within serogroup B isolates.

The single serogroup Z profile was 5-1, 10-8 and 36-1. This profile has a common association with serogroup C profiles. This serogroup is rare and this profile has not been identified within this serogroup previously consequently comparisons with isolates out with this population within the MLST database do confirm this profile is unique.

Figure 4.10 Association between porin A variable region 1 (VR1), variable region 2 (VR2) and variable region 3 (VR3) from meningococci isolated in 2002 (year 4). Each identified porin A profile (VR1, VR2, VR3) is linked through a series of individually coloured and styled arrows. The total number represented within that variable type is indicated within the brackets. Following the 5 VR1 variants (blue) the dashed line finishes at VR2 2 (red) (n=5). These are further distributed through a dashed line to VR3 36-1 (n=1) and 36-2 (n=4).


VR1
VR2
VR3
5 (5)
5-1 (19)
5-2 (2)
7 (9)
7-1 (3)
7-2 (10)
7-9 (1)
12-1 (1)
17 (1)
18-1 (4)
18-7 (1)
19 (4)
19-1 (7)
19-2 (1)
21 (7)
22 (23)
22-1 (1)
2 (5)
2-2 (2)
10 (2)
10-1 (1)
10-4 (3)
10-8 (4)
4 (9)
4-1 (1)
1 (2)
3 (4)
15 (5)
15-11 (6)
16 (15)
16-3 (1)
16-10 (1)
13-1 (4)
9 (10)
14 (12)
14-6 (1)
26 (1)
36-1 (4)
36-2 (11)
37 (9)
35 (8)
37-1 (11)
38 (3)
40 (1)
36 (26)
35-1 (16)

4.2 Discussion.

This study has highlighted that the VR3 region, along with VR1 and VR2, was useful for the analysis of meningococcal strains. Strains were isolated from a number of different NHS boards within Scotland. A total of 86 different strains were identified from 431 isolates. Five new VR3 variants were identified (Appendix 6.9 – 6.13) indicating the variability of this gene and this showed previous and on-going variation of the VR3 region of *porA*. Such variation, like that in VR1 and VR2, will have an impact on the development of *porA* vaccines, which may include VR3 in their composition. Although VR3 has not been extensively researched in other laboratories, the significance of the immune response to *porA* VR3 is poorly understood, however a number of VR3 components are contained within the RIVM OMV vaccine, though, those VR3 components designations have not been published yet. The availability of VR3 nucleotide sequence information and typing combined with the VR1 and VR2 profile data has been clearly demonstrated in this chapter and is therefore important. This sequence surveillance of bacterial infections is important and leads to a better understanding of diseases.

The porin A variable region distribution over the four years shows a significant shift from VRs closely associated with serogroup C, namely variant 2, 5 and 36-2 families towards a wider distribution of VRs closely associated with serogroup B meningococci, namely 3, 4, 7, 14, 15, 16, 18, 19, 22, 35, 36 and 37 families. The clonality with which serogroup C meningococci are expressed phenotypically and genotypically is reflected in the *porA* profiles displayed within this serogroup population. A significant reduction as described in section 4.1 was observed within

variant 2, 5, 10 and 36-2 families between years two, three and four. However there still remain a significant number of these variant families present in the population.

Interestingly, the number of different variant family types associated with serogroup B meningococci did not increase even though the proliferation of different MLST types has been documented. However there has been a greater variation between pre-existing *porA* profiles whereby new combinations of previously characterized profiles have been observed. This would highlight the continued ability with which the meningococcus can display a wide range of different OMP configurations.

These molecular methods have further improved our knowledge of the population structure of some bacteria and this has been exemplified by the use of MLST and genotyping (Enright and Spratt 1999; Yakubu et al. 1999; Clarke et al. 2001b; Tzanakaki et al. 2001). The use of sequence-based systems for bacterial typing have been highlighted previously (Maiden et al. 1992; Enright and Spratt 1999; Spratt 1999) and the availability of a national genotyping service for the characterisation of pathogens with public health significance, such as *N. meningitidis*, have been documented and clearly highlight their importance (Clarke et al. 2001b; Clarke et al. 2001c). Such systems can often provide rapid results when cultures are available and be useful for public health management (Clarke et al. 2001b) as well as invaluable data where culture negative clinical samples are used to obtain the genotype. As a consequence the provision of this information also provides long-term surveillance data which can then be used for informing vaccine policy. The development of vaccines relies on the availability of such data so that the design can be representative of strains circulating in the community.

With the successfully implementation of the serogroup C vaccines and the availability of serogroup A, Y and W135 vaccines, there is an urgent need for a serogroup B meningococcal vaccine as this serogroup is now very common, if not the most common, serogroup of infection in many countries (Connolly and Noah 1999). For example, it accounts for over 60% of disease in the UK (Connolly and Noah 1999). Although a serogroup B vaccine is now undergoing clinical trials, OMP vaccines may provide a short-term solution by providing protection in countries where predominant serosubtypes exist.

However, the sequencing of *porA* VR1, VR2 and VR3 provides a vast amount of information than is gained through phenotypic characterisation and this is useful for strain characterization and differentiation especially in case cluster scenarios. In this chapter some strains, which appeared identical by phenotypic analysis, were in fact different when the VR1 VR2 and VR3 profile information was examined.

In conclusion this chapter combines *porA* profiles this MLST profiles, and the cost of performing additional nucleotide sequencing on several small but highly discriminatory regions is minimal compared to the extra discrimination gained. However, this chapter illustrates that a universal nomenclature is required for the PorA VR regions which are being analysed to avoid duplication or misinterpretation. This would also be important for those working within microbiology, but not necessarily the meningococcal field, where the understanding and interpretation of meningococcal typing may currently be confusing. Further development of the meningococcal *porA* website (http://www.neisseria.org/nm/typing/pora) would therefore be required to incorporate this extra variable region and enable

standardisation of genotyping of meningococci. These results within this chapter include *por*A VR3 and this has displayed a greater level of discrimination than using just VR1 and VR2 alone. This ultimately enables more complete *por*A characterisation and data that can be useful for epidemiological purposes and vaccine design.

Chapter 5

General Discussion

5.0 Introduction

Neisseria meningitidis has been documented as a causative agent of meningitis and septicaemia for over 100 years and has been well documented during this time period as a disease of rapid onset with high mortality and morbidity if untreated. After more than 80 years of major developments in detection, characterization and treatment, it still holds true today (Knapp 1988a). However after recent advances in engineering, molecular techniques and analysis systems the answers to questions raised over decades of research are becoming achievable. This study was in the first instance conducted over a numbers of years and set out to develop an automated system that performed MLST from an extracted isolate and as a consequnce allow the genotypic anaysis on meningococci over a number of years spanning the introduction of the MenC Vaccine. This would ultimately highlight the effect the MenC vaccine had on the disease-causing meningococcal population. Combined with detailed genotyic analysis of carriage strains, this would ultimately give a realistic picture of the dynamics of meningococcal within the environment. This involved in the first instance detailed trials with automated liquid handling robots and as a consequence development and optimisation of novel chemical protocols to achieve MLST. Consequently, development of analysis software was required to characterise a relatively large number of nucleotide sequences. Finally detailed analysis of the data set allowed the generation of conclusions based on preformed hypotheses as described

in chapter 2.

5.1 Is Semi-Automated MLST and *porA* Sequencing Useful?

The identification and speciation of bacteria has a long history and is important because it enables the monitoring of the occurrence, differences and similarities between different groups of bacteria. Data provided from such analysis improves our understanding of bacterial species, genera, and populations, which supplies important information for epidemiological and public health interventions. Microbiological techniques have moved on since the early days of bacterial identification and new methods enable the availability of a much greater breadth of information. As time has progressed the cost of analysing bacteria to a DNA level has decreased such that routine analysis of genes from bacteria is possible and subsequently enable us to decipher the secrets within a whole genome. It is these latter methods that have become important in modern microbiology where the characterisation and differentiation of bacteria is increasingly important.

Ultimately semi-automation of this MLST scheme has been highly successful and subsequently essential where high numbers of isolates are analysed involving the amplification and sequencing of multiple loci within a reference-laboratory environment. As a consequence it has reduced cross-contamination, enabled continuity of performance, and reduced the cost per sample, all key goals in settings in which throughput is relatively high (Clarke 2002a). The semi-automated protocol described in this thesis fulfils these requirements.

The combination of *N. meningitidis* MLST housekeeping genes and the OMP *porA* within a semi-automated procedure enabled quick, reliable and reproducible production of results that aided public health management of meningococcal disease in Scotland. There have been many advantages highlighted from using a sequence-based system for typing organisms such as *N. meningitidis* (Maiden et al. 1998; Enright and Spratt 1999; Spratt 1999; Clarke et al. 2001b). The use of MLST and *porA* gene sequencing integrated within a semi-automated set-up gives a greater level of discrimination than performing MLST alone (Clarke 2002d). This has been highlighted repeatedly throughout this study from successful differentiation of large clonal complexes to small case clusters (Clarke et al. 2001b; Clarke 2002d; Clarke et al. 2003).

The importance of enhanced molecular methods and subsequent enhanced surveillance has been proven with this four-year study on the effect of the MenC vaccine on the disease-causing meningococci in Scotland pre, during and post vaccination campaign. At this stage it is important to emphasise the requirement for continual use of these systems to monitor the long-term effect as well as the short-term effect of this vaccine campaign. It has been well documented as to the predicted reduction of serogroup C disease, however it is far more difficult to predict possible replacement serogroups, and their genetic profiles. The continual use of these systems will enable the scientific community to monitor any possible capsule switch between common serogroups and the detailed genetic profile of future dominant strains such as serogroup B isolates. These profiles will also highlight any fundamental genetic switch that may occur between common and less common serogroups.

5.2 Is the Meningococcal Serogroup C Conjugate Vaccine Effective?

During the 1990s, the incidence of MD was higher in the UK than most other European countries. This was primarily due to an increase in serogroup C disease, particularly those within the ET-37/ST-11 genetic lineage. MenC vaccines were introduced in the UK at the end of 1999 with an uptake rate of approximately 70% by the end of 2000. However no meningococcal genetic population study has yet confirmed that serogroup C disease has decreased. This study clearly shows both an overall decrease in serogroup C disease over the first fours years since January 1999 and a significant increase in the diversity within the disease-causing population of meningococci after the introduction of the MenC vaccines, although there has been no genetic evidence for capsule switch.

This study found that the meningococcal population was highly diverse with an increasing trend of complex diversity coinciding with a decrease in serogroup C disease. A number of studies have been performed with collections of meningococci that cause disease within the MLST database (Maiden et al. 1998; Feil et al. 1999; Jolley et al. 2000; Clarke et al. 2001b; Tzanakaki et al. 2001). These collections have centred upon carriage and disease and therefore subsequent conclusions have been based on a limited number of disease-causing isolates. Moreover, these studies have been performed during time periods where the selective pressures are considered normal (i.e. out-with large-scale vaccine programmes). This unique study has taken into account all invasive isolates over a four-year period during which the MenC vaccine programme was executed. This study has shown that the genetic diversity of meningococci has significantly increased and in reflection the serogroup C ET-37/ST-

11 meningococcal population has significantly decreased. This increase in diversity has been represented in the majority by the serogroup B meningococci, although this niche has been filled by a number of different STs from a number of different clonal complexes. Therefore currently, there is no hyper-endemic clone present in Scotland that has directly replaced the void left by the ST-11 clone.

In summary the main conclusions drawn from this study include:

- In 1999, under half (43.5%) of all meningococcal disease was due to serogroup C meningococci
- In 2000, 2001 and 2002, this decreased to 35%, 26% and finally 12% of the total disease casuing population.
- The proportion of serogroup C's characterised as ST-11 remained high between 1999 and 2002 ranging from 93% to 100%.
- Less than 1% of the STs were only associated with a single year.
- In 1999, 2000, 2001 and 2002, 19%, 18.5%, 35% and 31% of STs had no complex association.
- The analysis of *N. meningitidis* MLST data from patients with MD pre, during and post MenC vaccine implementation and subsequent success of this campaign on the targeted meningococcal serogroup C disease causing population.
- The analysis of *N. meningitidis* MLST data ascertaining the subsequent sequence profile within the meningococcal population after the anticipated reduction in the serogroup C population.

- No clear conformity was observed between meningococcal serogroup and *porA* variable region profile.
- Genosubtyping of the *porA* gene increases the power of differentiation within clonal meningococcal populations.
- *porA* profiles associated with serogroup C have decreased significantly.
- *porA* profiles specifically associated with serogroup B have increased.
- The diversity of other serogrous within the population has notably increased.
- Non-groupable meningococci are specifically associated with *porA* profiles namely, VR1 variants 7 and 18, VR2 variants 2, 16 and 30 and VR3 variants 36 and 38.
- Specific porA variants such as VR1 5, 7, 18, 21, VR2 3, 4 and 16 and VR3 36-1, 37-1 and 38-1 had mixed serogroup populations which could be conducive to capsular switch under selection pressure.

5.3 Further Work

Multi-Locus Sequence Analysis (MLST) incorporating a combination of either housekeeping genes alone or in combination with more variable and discriminating genes can be incorporated with similar laboratory methods in most laboratories as a routine technique and consequently can provide data which is useful for the public health management of clusters or outbreaks in institutions such as schools, and also for general disease surveillance on a national basis, thereby providing information to determine national vaccine policy. This, however, is not yet commonplace in all laboratories responsible for detection and characterisation of *N. meningitidis*. However the development of a centralised facility within the European Union based in

Oxford will allow access to this technique for all European member states and EMGM members (EU-Men-Net), whereby specific facilities working in the meningococcal field within Europe can utilise the expertise and equipment enabling them to perform MLST on their selected sample populations. This undoubtedly will allow members access to highly technical techniques with which they have no ability to obtain within their own scientific communities and allow greater definition of meningococcal populations throughout Europe and with its success hopefully expansion throughout the world.

A large proportion of cases within the UK are not culture confirmed due to the effective administration of antibiotics on suspicion of MD, consequently there is a need for nucleotide sequence-based typing of meningococci directly from clinical samples. Non-culture characterisation has been mainly basic detection and confirmation of MD rather than detailed characterisation through serogrouping, typing and sub-typing as well as full sequence typing with MLST. As MLST is a PCR-based technique, it has the advantage that it can be applied directly to culture negative clinical specimens such as cerebrospinal fluid (CSF) (Enright et al. 2000b) and blood. The development of such techniques is paramount in highlighting the true picture of meningococcal disease within the population. Recent studies have described nested MLST approaches to the laboratory confirmation and characterisation of meningococci directly from clinical sample (Diggle et al. 2003a). Along with other non-culture methods for the laboratory confirmation of MD, non-culture MLST will in the future play an important part in the surveillance of the disease. It is important to note that treatment with antibiotics can successfully limit the onset or decrease the level of disease and is recommended treatment upon immediate suspicion of

meningitis (Cartwright et al. 1992). Although this therapy may lead to culture-negative samples, DNA usually remains in a clinical sample taken after onset of MD and can therefore be detected and characterised as demonstrated by non-culture MLST. Nucleotide sequencing, once an expensive and highly specialised technique, has become more widely available, mainly due to reduced consumable and equipment costs (Diggle and Clarke 2002b). The availability of MLST data is important as, along with serogroup (Diggle et al. 2003b) and sero-subtype (Diggle and Clarke 2003a) information, it can be used to monitor capsule switch in meningococci. Moreover, with *porA* nucleotide sequence data that can be gained by culture (Clarke 2002d) or non-culture methods (Diggle and Clarke 2003a), it can be used for public health management during case clusters (Feavers et al 1999). These highly sensitive detection and characterisation methods will provide an invaluable source of information to aid public health management of MD as more effective treatments continue to be used.

With the decrease in serogroup C disease and therefore the apparent success of the MenC vaccine campaign within the UK, serogroup B meningococci account for a large percentage of disease for which there is no effective vaccine (Rosenstein et al. 2001a). Although plain polysaccharide or conjugate vaccines are now available against serogroups A, C, Y and W135 (Rappuoli 2001a) there is an urgent need for a vaccine against the serogroup b meningococcus. With the well documented problems the development of a serogroup B vaccine therefore requires a different approach, perhaps based on an essential virulence gene gained from data gained through genome sequencing projects (Rappuoli 2001a). During such development, there is the option to develop vaccines based on other antigens the most studies of which are those based

on OMPs (Al'Aldeen and Cartwright 1996; Rosenstein et al. 2001a). Although OMP-based vaccines are not the optimal solution for combating serogroup B meningococcal infection, they may provide a short-term solution and have shown great promise for the control of serogroup B meningococcal infection (Rappuoli 2001a).

The publication of complete genomes of a number of different *N. meningitidis* isolates has provided a comprehensive profile of the genetic complement of this important organism. However, the genome sequences emphasise inadequacies in our understanding of meningococcal pathogenesis. For example, it is not known which of the 2,158 *N. meningitidis* genes are involved in the disease process. Therefore, with the increased accessibility of genome sequencing methods for high-throughput, analysis of gene functions are needed to exploit the vast amount of information from an ever-increasing pool of genome projects. This could lead to the development of potential vaccines targeted at problematic serogroups such as serogroup B. This technique, namely reverse vaccinology, has already been attempted with the *N. meningitidis* genome.

The evidence provided by several reports highlights microarrays as simple characterisation tools, allowing minimal processing of samples and required practical experience after system set-up (Bavykin 2001). The limitations with such new technology include cost and the practicality within laboratories with limited resources and technical support. The challenge with such technology is to find ways in which this can be used to study different aspects of the bacterial population. For example, the comparison of selected genes expressed by a pathogenic strain compared with that expressed by a nonpathogenic stain by hybridization to an array made from the

genome of the pathogenic strain (Clewley 2000). In this way, this method could be used to develop DNA arrays that are suitable for both diagnosis and typing of bacterial species such as *N. meningitidis* (Borrow R, personal communication).

The use of single nucleotide polymorphisms as a practical approach for identifying genetic diversity within a bacterial population has been available for a number of years (Emonts et al 2003), although with the advent of large scale nucleotide sequencing, SNP analysis has become much more accessible. There have been recent developments in SNP analysis which utilise such techniques as MLST to develop straightforward approaches to SNP-based bacterial typing, whereby computer analysis packages can identify highly informative set of SNPs within a database of gene sequence variants (Robertson et al 2004). These have the potential to rapidly identify and possibly differentiate specific sequence types within a population. This is one example where manipulation of sequence data can enable future development of SNP analysis tools

Variable Number Tandem Repeats (VNTRs) are represented as repeats, usually representing a single locus and show inter-individual length variability and are thought to occur due to slipped-strand mispairing. This type of typing scheme has been used on a number of different bacteria including, *H. influenzae*, *Staphylococcus aureus*, and *N. meningitidis* (van der Ende et al, 2000, van Belkum et al, 1998 and van Belkum et al 1997). The selected genes include opacity surface proteins (*opa*) and outer membrane proteins (*porA*). Within this study *porA* was used in combination with the *N. meningitidis* MLST scheme to provide a comprehensive profile of each organism.

These approaches have provided high-level discrimination between isolates from the same and different species and will no doubt provide the basis for future typing methods. These techniques described can be extended to any organism for which extensive nucleotide sequence is available. As eluted to previously, these digital procedures studying pathogenic related genomic regions may in the future be applied to those organisms that are either fastidious or nonculturable. Once whole genome sequences of bacterial organisms become available, both diagnostic information and data on the evolutionary status of the strains involved can be obtained and in some cases obtained with the use of a single PCR or multiplex PCR test.

Bibliography

Abbott, J. D., D. M. Jones, M. J. Painter and S. E. Young. 1985. The epidemiology of meningococcal infections in England and Wales, 1912-1983. J Infect. 11,(3), 241-257.

Abdillahi, H., and J. T. Poolman. 1988. Definition of meningococcal class 1 OMP subtyping antigens by monoclonal antibodies. FEMS Microbiol. Immunol. 1, 139-144.

Achtman, M. (1995). Global epidemiology of meningococcal disease. Meningococcal Disease. K. Cartwright. Chichester, UK, Wiley: 159-175.

Achtman, M., A. van der Ende, P. Zhu, I. S. Koroleva, B. Kusecek, G. Morelli, I. G. Schuurman, N. Brieske, K. Zurth, N. N. Kostyukova and A. E. Platonov. 2001. Molecular epidemiology of serogroup A meningitis in Moscow, 1969 to 1997. Emerg Infect Dis. 7,(3), 420-427.

Achtman, M., R. A. Wall, M. Bopp, B. Kusecek, G. Morelli, E. Saken and M. Hassan-King. 1991. Variation in class 5 protein expression by serogroup A meningococci during a meningitis epidemic. J Infect Dis. 164,(2), 375-382.

Ada, G. and D. Isaacs. 2003. Carbohydrate-protein conjugate vaccines. Clin Microbiol Infect. 9,(2), 79-85.

Aguiar-Nogueira, J., H. Lecour and M. Luz-Dias. 1989. Use of a latex agglutination test in rapid diagnosis of acute meningitis. Enferm Infecc Microbiol Clin. 7,(4), 186-188.

Aho, E. L., J. A. Dempsey, M. M. Hobbs, D. G. Klapper and J. G. Cannon. 1991. Characterization of the opa (class 5) gene family of *Neisseria meningitidis*. Mol Microbiol. 5,(6), 1429-1437.

Ala'Aldeen, D. A. and K. A. Cartwright. 1996. *Neisseria meningitidis*: vaccines and vaccine candidates. J Infect. 33,(3), 153-157.

Ala' Aldeen, D. A., A. H. Westphal, A. De Kok, V. Weston, M. S. Atta, T. J. Baldwin, J. Bartley and S. P. Borriello. 1996. Cloning, sequencing, characterisation and implications for vaccine design of the novel dihydrolipoyl acetyltransferase of *Neisseria meningitidis*. J Med Microbiol. 45,(6), 419-432.

Alcala, B., C. L., Salcedo, S. Arreaza, L. Berron, De La Fuente and J. A. Vazquez. 2002. The epidemic wave of meningococcal disease in Spain in 1996-1997: probably a consequence of strain displacement. J Med Microbiol. 51,(12), 1102-1106.

Al-soud, W. A., and P. Rädstrom. 2001. Purification and characterisation of PCR-inhibitroy components in blood cells. J Clin Microbiol. 39, 485-493.

Al-soud, W. A., L. J. Jonsson, and P. Rädstrom. 2000. Identification and characterisation of immunoglobulin G in blood as a major inhibitior of diagnostic PCR. J Clin Microbiol. 38, 345-350.

Andreasson, H., A. Asp, A. Alderborn, U. Gyllensten and M. Allen. 2002. Mitochondrial sequence analysis for forensic identification using pyrosequencing technology. Biotechniques. 32,(1), 124-126, 128, 130-133.

Anon 1945. Thanking discoverer of penicillin. The New York Times. New York: 11.

Anon. 1996. Meningococcal infection in Africa. Commun Dis Rep CDR Wkly. 6,(22), 189-192.

Anon 1998a. Control of epidemic meningococcal disease., World Health Organisation.

Anon. 1998b. Diagnosing meningococcal disease by throat swabs. Commun Dis Rep CDR Wkly. 8,(6), 49-52.

Anon. 1998c. Enhanced surveillance of meningococcal disease in five English regions: first quarter 1998. Commun Dis Rep CDR Wkly. 8,(21), 183-186.

Anon 1998d. Laboratory methods for the diagnosis of meningitis caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae.*, Centre for Disease Control and Prevention.

Anon 1999a. Laboratory methods for the diagnosis of meningitis caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae.*, World Health Organisation.

Anon. 1999b. Monitoring the impact of the serogroup C meningococcal conjugate vaccination programme. Commun Dis Rep CDR Wkly. 9,(49), 435- 438.

Anon. 2000a. Meningococcal disease prevention and control strategies for practice-based physicians (Addendum: recommendations for college students). Committee on Infectious Diseases. Pediatrics. 106,(6), 1500-1504.

Anon. 2000b. Prevention and control of meningococcal disease. Recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Morb Mortal Wkly Rep. 49,(RR-7), 1-10.

Anon. 2001a. Epidemics of meningococcal disease. African meningitis belt, 2001. Wkly Epidemiol Rec. 76,(37), 282-288.

Anon. 2001b. Meningococcal disease, African meningitis belt. Wkly Epidemiol Rec. 76,(8), 57.

Anon 2002a. The Control of Substances Hazardous to Health Regulations 2002, Stationary Office.

Anon. 2002b. Guidelines for public health management of meningococcal disease in the UK. Commun Dis Public Health. 5,(3), 187-204.

Anon. 2002c. Pyrosequencing and Mayo Clinic collaborate on diagnostic tests. Expert Rev Mol Diagn. 2,(1), 5.

Anon 2003. British National Formulary. London, British Medical Association and the Royal Pharmaceutical Society of Great Britain.

Arhin, F. F., F. Moreau, J. W. Coulton and E. L. Mills. 1998. Sequencing of porA from clinical isolates of Neisseria meningitidis defines a subtyping scheme and its genetic regulation. Can J Microbiol. 44,(1), 56-63.

Arreaza, L., B. Alcala, C. Salcedo and J. A. Vazquez. 2001. Interruption of siaD in a meningococcal carrier isolate mediated by an insertion sequence. Clin Diagn Lab Immunol. 8,(2), 465-466.

Ballada, D., G. Ventura, M. Tumbarello, R. Cauda, L. Toti and L. Ortona. 1991. Meningococcal meningitis in Italy: 1887-1986. Eur J Epidemiol. 7,(4), 431-433.

Ballada, D., Ventura, G., Tumbarello, M., Cauda, R., Toti, l., and Ortona, L., 1990. Meningococcal meningitidis in Italy: 1887 - 1986. Medicina (Firenze). 10,(2), 129-133.

Balmer, P., R. Borrow and E. Miller. 2002. Impact of meningococcal C conjugate vaccine in the UK. J Med Microbiol. 51,(9), 717-722.

Balmer, P. and E. Miller. 2002. Meningococcal disease: how to prevent and how to manage. Curr Opin Infect Dis. 15,(3), 275-281.

Barata Rde, C., 1988. Epidemiology of meningococcal disease, 1970/1977. Onset and dissemination of the epidemic process. Rev Saude Publica. 22,(1), 16-24.

Barker, R. M., R. M. Shakespeare, A. J. Mortimore, N. A. Allen, C. L. Solomon and J. M. Stuart. 1999. Practical guidelines for responding to an outbreak of meningococcal disease among university students based on experience in Southampton. Commun Dis Public Health. 2,(3), 168-173.

Barrett, S. J. and P. H. Sneath. 1994. A numerical phenotypic taxonomic study of the genus Neisseria. Microbiology. 140 (Pt 10), 2867-2891.

Bart, A., I. G. Schuurman, M. Achtman, D. A. Caugant, J. Dankert and A. van der Ende. 1998. Randomly amplified polymorphic DNA genotyping of serogroup A meningococci yields results similar to those obtained by multilocus enzyme electrophoresis and reveals new genotypes. J Clin Microbiol. 36,(6), 1746-1749.

Bavykin, S. G., Akowski, J.P., Zakhariev, V.M., Barsky, V.E., Perov, A.N., and Mirzabekov, A.D., 2001. Portable system for microbial sample preparation and oligonucleotide microarray analysis. Appl Environ Microbiol. 67, 922-928.

Beavis, R. C., and Chait, B., 1996. Matrix assisted laser desorption ionisation mass-spectrometry of proteins. Methods Enzymol. 270, 519.

Begg, N. (1995). Outbreak management. Meningococcal Disease. K. Cartwright. Chichester, John Wiley and Sons: 285-307.

Bellete, B., L. E. Cuevas, P. Kazembe, G. Mughogho, D. Sunderland and C. A. Hart. 1994. Meningococcal disease in Malawi: studies on the genetic relatedness of the bacteria. Ann Trop Med Parasitol. 88,(1), 59-64.

Blondeau, J. M., F. E. Ashton, M. Isaacson, Y. Yaschuck, C. Anderson and G. Ducasse. 1995. *Neisseria meningitidis* with decreased susceptibility to penicillin in Saskatchewan, Canada. J Clin Microbiol. 33,(7), 1784-1786.

Borrow, R., 2001. The development of DNA microarrays with regards *Neisseria meningitidis*. Personal Communication.

Borrow, R., H. Claus, U. Chaudhry, M. Guiver, E. B. Kaczmarski, M. Frosch and A. J. Fox. 1998. *siaD* PCR ELISA for confirmation and identification of serogroup Y and W135 meningococcal infections. FEMS Microbiol Lett. 159,(2), 209-214.

Borrow, R., H. Claus, M. Guiver, L. Smart, D. M. Jones, E. B. Kaczmarski, M. Frosch and A. J. Fox. 1997. Non-culture diagnosis and serogroup determination of meningococcal B and C infection by a sialyltransferase (*siaD*) PCR ELISA. Epidemiol Infect. 118,(2), 111-117.

Borrow, R., Guiver, M., Sadler, F., Kaczmarski, E.B., and Fox, A.J., 1998. False positive diagnosis of meningococcal infection by the IS1106 PCR ELISA. FEMS Microbiol Lett. 162, 215-218.

Bougnoux, M. E., S. Morand and C. d'Enfert. 2002. Usefulness of multilocus sequence typing for characterization of clinical isolates of Candida albicans. J Clin Microbiol. 40,(4), 1290-1297.

Bovre, K. (1984). Family VIII. Neisseriaceae Prevot, The Williams & Wilkins co., Baltimore.

Boxer, M., 2000. Molecular techniques: divide or share. J Clin Pathol. 53, 19-21.

Boyd, E. F., F. S. Wang, T. S. Whittam and R. K. Selander. 1996. Molecular genetic relationships of the salmonellae. Appl Environ Microbiol. 62,(3), 804-808.

Braude, A. I., 1953. A brief outline of the specific use of antibiotics and sulfonamides. Med Bull (Ann Arbor). 19,(7), 169-177.

Bygraves, J. A. and M. C. Maiden. 1992. Analysis of the clonal relationships between strains of *Neisseria meningitidis* by pulsed field gel electrophoresis. J Gen Microbiol. 138 (Pt 3), 523-531.

Capecchi, B., D. Serruto, J. Adu-Bobie, R. Rappuoli and M. Pizza. 2004. The genome revolution in vaccine research. Curr Issues Mol Biol. 6,(1), 17-27.

Cardenosa, N., A. Dominguez, A. Orcau, H. Panella, P. Godoy, S. Minguell, N. Camps and J. A. Vazquez. 2001. Carriers of *Neisseria meningitidis* in household contacts of meningococcal disease cases in Catalonia (Spain). Eur J Epidemiol. 17,(9), 877-884.

Cartwright, K. (1995). Meningococcal disease. Chichester, England, John Wiley & Sons, Inc.

Cartwright, K., R. Morris, H. Rumke, A. Fox, R. Borrow, N. Begg, P. Richmond and J. Poolman. 1999. Immunogenicity and reactogenicity in UK infants of a novel meningococcal vesicle vaccine containing multiple class 1 (PorA) outer membrane proteins. Vaccine. 17,(20-21), 2612-2619.

Cartwright, K., N. Noah and H. Peltola. 2001. Meningococcal disease in Europe: epidemiology, mortality, and prevention with conjugate vaccines. Report of a European advisory board meeting Vienna, Austria, 6-8 October, 2000. Vaccine. 19,(31), 4347-4356.

Cartwright, K., S. Reilly, D. White and J. Stuart. 1992. Early treatment with parenteral penicillin in meningococcal disease. Bmj. 305,(6846), 143-147.

Caugant, D. A., K. Bovre, P. Gaustad, K. Bryn, E. Holten, E. A. Hoiby and L. O. Froholm. 1986. Multilocus genotypes determined by enzyme electrophoresis of *Neisseria meningitidis* isolated from patients with systemic disease and from healthy carriers. J Gen Microbiol. 132 (Pt 3), 641-652.

Chan, M. S., Maiden, M. C., and Spratt, B. G., 2001. Database-driven multi-locus sequence typing (MLST) of bacterial pathogens. Bioinformatics. 17,(11), 1077-1083.

Clarke, S. C., 2002a. Nucleotide sequence-based typing of bacteria and the impact of automation. Bioessays. 24, 858-862.

Clarke, S. C., S. Brownlie, B. C. Denham, J. Mooney, P. Christie, and G. F. S Edwards. 2002b. Meningococcal Disease in Scotland 2000. SCIEH Weekly Report. 36,(2002/40), 2-5.

Clarke, S. C. and M. A. Diggle. 2002. Automated PCR/sequence template purification. Mol Biotechnol. 21,(3), 221-224.

Clarke, S. C., M. A. Diggle and G. F. S. Edwards. 2001a. Automated non-culture-based sequence typing of meningococci from body fluids. Br J Biomed Sci. 58,(4), 230-234.

Clarke, S. C., M. A. Diggle and G. F. S. Edwards. 2001b. Semiautomation of multilocus sequence typing for the characterization of clinical isolates of *Neisseria meningitidis*. J Clin Microbiol. 39,(9), 3066-3071.

Clarke, S. C., M. A. Diggle, P. Molling, M. Unemo and P. Olcen. 2003. Analysis of PorA variable region 3 in meningococci: implications for vaccine policy? Vaccine. 21,(19-20), 2468-2473.

Clarke, S. C., M. A. Diggle, J. A. Reid, L. Thom and G. F. Edwards. 2001c. Introduction of an automated service for the laboratory confirmation of meningococcal disease in Scotland. J Clin Pathol. 54,(7), 556-557.

Clarke, S. C., and M.A. Diggle. 2002c. Automation PCR/sequence template purification. Mol Biotechnol. 21, 221-224.

Clarke, S. C., Diggle, M. A., and G. F. S. Edwards. 2002d. Multilocus sequence typing and *porA* gene sequencing differentiates strains of *Neisseria meningitidis*. Br J Biomed Sci. 59, 160-162.

Clarke, S. C., J. Reid, L. Thom, B. C. Denham and G. F. S. Edwards. 2002. Laboratory confirmation of meningococcal disease in Scotland, 1993-9. J Clin Pathol. 55,(1), 32-36.

Claus, H., U. Vogel, M. Muhlenhoff, R. Gerardy-Schahn and M. Frosch. 1997. Molecular divergence of the sia locus in different serogroups of *Neisseria meningitidis* expressing polysialic acid capsules. Mol Gen Genet. 257,(1), 28-34.

Clewley, J. P., 1999. Automation of the polymerase chain reaction. Part 4. Detecting and characterising the product. Commun Dis Public Health. 2,(4), 299-300.

Clewley, J. P., 2000. DNA microarrays. Commun Dis Public Health. 3, 71-72.

Connolly, M. and N. Noah. 1999. Is group C meningococcal disease increasing in Europe? A report of surveillance of meningococcal infection in Europe 1993-6. European Meningitis Surveillance Group. Epidemiol Infect. 122,(1), 41-49.

Coovadia, Y. M., J. Van den Ende and Z. Solwa. 1989. Comparison of gram stain and latex agglutination for diagnosis of meningococcal meningitis. Lancet. 2,(8664), 677.

Counts, G. W. and R. G. Petersdorf. 1980. 'The wheel within a wheel': meningococcal trends. JAMA. 244,(19), 2200-2201.

Cowan, S. W., T. Schirmer, G. Rummel, M. Steiert, R. Ghosh, R. A. Paupit, J. N. Jansonius, and J. P. Rosenbusch., 1992. Crystal structures explain functional properties of two *E. coli* porins. Nature. 358, 727-733.

Danelli, M. G., N. M. Batoreu, M. D. Lacerda, C. R. Ferreira, J. D. Cardoso, J. M. Peralta and C. E. Frasch. 1995. Surface antigen analysis of group B *Neisseria meningitis outer* membrane by monoclonal antibodies: identification of bactericidal antibodies to class 5 protein. Curr Microbiol. 31,(3), 146-151.

Davidson, K., L., Crowcroft, N.S., Ramsay, M.E., Begg, N.T.,Kaczmarski, E.B., Stuart, J.M., White, J.M, Orr., H, on behalf of the Enhanced Surveillance of Meningococcal Disease Project Group., 2002. Enhanced surveillance scheme for suspected meningococcal disease in five regional health authorities in England: 1998. Commun Dis Public Health. 5,(3), 205-212.

de Kleijn, E. D., R. de Groot, J. Labadie, A. B. Lafeber, G. van den Dobbelsteen, L. van Alphen, H. van Dijken, B. Kuipers, G. W. van Omme, M. Wala, R. Juttmann and H. C. Rumke. 2000a. Immunogenicity and safety of a hexavalent meningococcal outer-membrane-vesicle vaccine in children of 2-3 and 7-8 years of age. Vaccine. 18,(15), 1456-66.

de Kleijn, E. D., R. de Groot, A. B. Lafeber, J. Labadie, K. C. van Limpt, J. Visser, G. A. Berbers, L. van Alphen and H. C. Rumke. 2000b. Immunogenicity and safety of monovalent p1.7(h),4 meningococcal outer membrane vesicle vaccine in toddlers: comparison of two vaccination schedules and two vaccine formulations. Vaccine. 19,(9-10), 1141-1148.

Demina, A. A. and E. V. Orlova. 1981. Improved method of isolating meningococci from nonsterile material. Lab Delo.(3), 186-187.

Derrick, J. P., R. Urwin, J. Suker, I. M. Feavers and M. C. Maiden. 1999. Structural and evolutionary inference from molecular variation in Neisseria porins. Infect Immun. 67,(5), 2406-2413.

Diggle, M. A., C. M. Bell and S. C. Clarke. 2003a. Nucleotide sequence-based typing of meningococci directly from clinical samples. J Med Microbiol. 52,(6), 505-508.

Diggle, M. A. and S. C. Clarke. 2002a. Rapid assignment of nucleotide sequence data to allele types for multi-locus sequence analysis (MLSA) of bacteria using an adapted database and modified alignment program. J Mol Microbiol Biotechnol. 4,(6), 515-517.

Diggle, M. A. and S. C. Clarke. 2002b. What a load of old sequence!!! J. Clin. Microbiol. 40, 2707.

Diggle, M. A. and S. C. Clarke. 2003a. Detection and genotyping of meningococci using a nested PCR approach. J Med Microbiol. 52,(Pt 1), 51-57.

Diggle, M. A. and S. C. Clarke. 2003c. A novel method for the preparation of single stranded DNA for pyrosequencing. Mol Biotechnol. 24, 221-224.

Diggle, M. A. and S. C. Clarke. 2004a. Genotypic characterisation of *Neisseria meningitidis* using Pyrosequencing™. Mol Biotechnol. 28, 139-145.

Diggle, M. A. and S. C. Clarke. 2004b. Pyrosequencing™ - sequence typing at the speed of light. Mol Biotechnol. 28, 129-138.

Diggle, M. A. and S. C. Clarke. 2003. Detection and genotyping of meningococci using a nested PCR approach. J Med Microbiol. 52, 51-57.

Diggle, M. A., G. F. S. Edwards and S. C. Clarke. 2001a. Automation of fluorescence-based PCR for confirmation of meningococcal disease. J Clin Microbiol. 39,(12), 4518-4519.

Diggle, M. A., G. F. S. Edwards and S. C. Clarke. 2001b. Developments in the diagnosis of meningococcal disease and the characterisation of *Neisseria meningitidis*. Rev Med Microbiol. 12,(4), 211-217.

Diggle, M. A., K. Smith, E. K. Girvan and S. C. Clarke. 2003b. Evaluation of a fluorescence-based PCR method for identification of serogroup A meningococci. J Clin Microbiol. 41,(4), 1766-1768.

Dingle, K. E., F. M. Colles, R. Ure, J. A. Wagenaar, B. Duim, F. J. Bolton, A. J. Fox, D. R. Wareing and M. C. Maiden. 2002. Molecular Characterization of *Campylobacter jejuni* Clones: A Basis for Epidemiologic Investigation. Emerg Infect Dis. 8,(9), 949-955.

Dingle, K. E., F. M. Colles, D. R. Wareing, R. Ure, A. J. Fox, F. E. Bolton, H. J. Bootsma, R. J. Willems, R. Urwin and M. C. Maiden. 2001. Multilocus sequence typing system for *Campylobacter jejuni*. J Clin Microbiol. 39,(1), 14-23.

Dobson, R., 2000. Smoking in parents increases meningococcal disease risk. Bmj. 321,(7257), 319A.

Drouin, G., F. Prat, M. Ell, and GD, Clarke., 1999. Detecting and characterising gene conversions between multigene family members. Mol Biol Evol. 10,(16), 1369-1390.

Eldridge, J., Sutcliffe, E.M., Abbott, J.D., and Jones, D.M. 1978. Serological grouping of meningococcal and detection of antigen in cerebrospinal fluid by coagglutination. Med. Lab. Sci. 35, 130-133.

Ellis-Pegler, R. B., 2003. Meningococcal disease: treatable but still terrifying. Intern Med J. 33,(1-2), 56.

Emonts, M., J. A. Hazelzet, R.de Groot and P. W. Hermans. 2003. Host genetic determinants of *Neisseria meningitidis* infections. Lancet Infect. Dis. 3,(9), 565-577.

Enright, M. C., N. P. Day, C. E. Davies, S. J. Peacock and B. G. Spratt. 2000a. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J Clin Microbiol. 38,(3), 1008-1015.

Enright, M. C., K. Knox, D. Griffiths, D. W. Crook and B. G. Spratt. 2000b. Molecular typing of bacteria directly from cerebrospinal fluid. Eur J Clin Microbiol Infect Dis. 19,(8), 627-630.

Enright, M. C. and B. G. Spratt. 1998. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiology. 144 (Pt 11), 3049-60.

Enright, M. C. and B. G. Spratt. 1999. Multilocus sequence typing. Trends Microbiol. 7,(12), 482-487.

Enright, M. C., B. G. Spratt, A. Kalia, J. H. Cross and D. E. Bessen. 2001. Multilocus sequence typing of *Streptococcus pyogenes* and the relationships between emm type and clone. Infect Immun. 69,(4), 2416-2427.

Enting, R. H., L. Spanjaard, D. van de Beek, E. F. Hensen, J. de Gans and J. Dankert. 1996. Antimicrobial susceptibility of *Haemophilus influenzae*, *Neisseria meningitidis* and *Streptococcus pneumoniae* isolates causing meningitis in The Netherlands, 1993-1994. J Antimicrob Chemother. 38,(5), 777-786.

Eugene, E., I. Hoffmann, C. Pujol, P. O. Couraud, S. Bourdoulous and X. Nassif. 2002. Microvilli-like structures are associated with the internalization of virulent capsulated *Neisseria meningitidis* into vascular endothelial cells. J Cell Sci. 115,(Pt 6), 1231-1241.

Fakhrai-Rad, H., N. Pourmand and M. Ronaghi. 2002. Pyrosequencing trademark: An accurate detection platform for single nucleotide polymorphisms. Hum Mutat. 19,(5), 479-485.

Fallon, R. J., 1988. Meningococcal infections in Scotland. J Med Microbiol. 26,(3), 168-172.

Fallon, R. J., W. M. Brown and W. Lore. 1984. Meningococcal infections in Scotland 1972-82. J Hyg (Lond). 93,(2), 167-180.

Faoagali, J. L., 1995. Carriage of *Neisseria meningitidis*. N Z Med J. 108,(1009), 415.

Feavers, I. M., A. J. Fox, S. Gray, D. M. Jones and M. C. Maiden. 1996. Antigenic diversity of meningococcal outer membrane protein PorA has implications for epidemiological analysis and vaccine design. Clin Diagn Lab Immunol. 3,(4), 444-450.

Feavers, I. M., S. J. Gray, R. Urwin, J. E. Russell, J. A. Bygraves, E. B. Kaczmarski and M. C. Maiden. 1999. Multilocus sequence typing and antigen gene sequencing in the investigation of a meningococcal disease outbreak. J Clin Microbiol. 37,(12), 3883-3887.

Feavers, I. M. and M. C. Maiden. 1998. A gonococcal *porA* pseudogene: implications for understanding the evolution and pathogenicity of *Neisseria gonorrhoeae*. Mol Microbiol. 30,(3), 647-656.

Feil, E., G. Carpenter and B. G. Spratt. 1995. Electrophoretic variation in adenylate kinase of *Neisseria meningitidis* is due to inter- and intraspecies recombination. Proc Natl Acad Sci U S A. 92,(23), 10535-10539.

Feil, E. J., E. C. Holmes, D. E. Bessen, M. S. Chan, N. P. Day, M. C. Enright, R. Goldstein, D. W. Hood, A. Kalia, C. E. Moore, J. Zhou and B. G. Spratt. 2001. Recombination within natural populations of pathogenic bacteria: short-term empirical estimates and long-term phylogenetic consequences. Proc Natl Acad Sci U S A. 98,(1), 182-187.

Feil, E. J., M. C. Maiden, M. Achtman and B. G. Spratt. 1999. The relative contributions of recombination and mutation to the divergence of clones of *Neisseria meningitidis*. Mol Biol Evol. 16,(11), 1496-502.

Feil, E. J. and B. G. Spratt. 2001. Recombination and the population structures of bacterial pathogens. Annu Rev Microbiol. 55, 561-590.

Ferguson, L. E., M. D. Hormann, D. K. Parks and R. J. Yetman. 2002. *Neisseria meningitidis*: Presentation, treatment, and prevention. J Pediatr Health Care. 16,(3), 119-124.

Finn, A. and R. Lakshman. 2002. Meningococcal serogroup C conjugate vaccine. Expert Opin Biol Ther. 2,(1), 87-96.

Finne, J., D. Bitter-Suermann, C. Goridis and U. Finne. 1987. An IgG monoclonal antibody to group B meningococci cross-reacts with developmentally regulated polysialic acid units of glycoproteins in neural and extraneural tissues. J Immunol. 138,(12), 4402-4407.

Fischbacher, C. M., J. E. Lally and N. M. Black. 2001. Improving the quality of communicable disease control: the example of meningococcal disease. Commun Dis Public Health. 4,(4), 268-272.

Fischer, M., K. Hedberg, P. Cardosi, B. D. Plikaytis, F. C. Hoesly, K. R. Steingart, T. A. Bell, D. W. Fleming, J. D. Wenger and B. A. Perkins. 1997. Tobacco smoke as a risk factor for meningococcal disease. Pediatr Infect Dis J. 16,(10), 979-983.

Fornara, P., 1968. Historical notes on purulent meningitis. G Mal Infett Parassit. 20,(5), 403-406.

Fox, A. J., D. M. Jones, S. J. Gray, D. A. Caugant and N. A. Saunders. 1991. An epidemiologically valuable typing method for *Neisseria meningitidis* by analysis of restriction fragment length polymorphisms. J Med Microbiol. 34,(5), 265-270.

Frasch, C. E., 1985. Status of a group B *Neisseria meningitidis* vaccine. Eur J Clin Microbiol. 4,(6), 533-536.

Frasch, C. E., W. D. Zollinger and J. T. Poolman. 1985. Serotype antigens of *Neisseria meningitidis* and a proposed scheme for designation of serotypes. Rev Infect Dis. 7,(4), 504-510.

Frosch, M., Müller, D., Bousset, K., and Müller, A., 1992. Conserved outer membrane protein of *Neisseria meningitidis* involved in capsule expression. Infect Immun. 60, 798-803.

Galimand, M., Gerbaud, G., Guibourdenche, M., Roou, J-Y., and Courvalin, P., 1998. IHigh-level chloramphenicol resistance in *Neisseria meningitidis*. N Engl J Med. 339, 868-874.

Garcia, C. A., A. Ahmadian, B. Gharizadeh, J. Lundeberg, M. Ronaghi and P. Nyren. 2000. Mutation detection by pyrosequencing: sequencing of exons 5-8 of the p53 tumor suppressor gene. Gene. 253,(2), 249-257.

Gharizadeh, B., M. Kalantari, C. A. Garcia, B. Johansson and P. Nyren. 2001. Typing of human papillomavirus by pyrosequencing. Lab Invest. 81,(5), 673-679.

Gill, M. J., 1991. Serotyping *Neisseria gonorrhoeae*: a report of the Fourth International Workshop. Genitourin. Med. 67, 53-57.

Goldstein, D. B., 2003. Pharmacogenetics in the laboratory and the clinic. N Engl J Med. 348,(6), 553-556.

Gomez-Herruz, P., R. Gonzalez-Palacios, J. Romanyk, J. A. Cuadros and J. Ena. 1995. Evaluation of the Etest for penicillin susceptibility testing of *Neisseria meningitidis*. Diagn Microbiol Infect Dis. 21,(2), 115-117.

Gordon, M. H., and Murray E.G., 1915. Identification of the meningococcus. J R Army Med Corps. 25, 411-423.

Gotschlich, E. C., 1985. The status of vaccines to meningococcal and gonococcal disease. Ann Inst Pasteur Microbiol. 136B,(3), 341-355.

Goulding, J. N., J. V. Hookey, J. Stanley, W. Olver, K. R. Neal, D. A. Ala'Aldeen and C. Arnold. 2000. Fluorescent amplified-fragment length polymorphism genotyping of *Neisseria meningitidis* identifies clones associated with invasive disease. J Clin Microbiol. 38,(12), 4580-4585.

Goulding, J. N., J. Stanley, W. Olver, K. R. Neal, D. A. Ala'Aldeen and C. Arnold. 2003. Independent subsets of amplified fragments from the genome of *Neisseria meningitidis* identify the same invasive clones of ET37 and ET5. J Med Microbiol. 52,(Pt 2), 151-154.

Granoff, D. M., Y. E. McHugh, H. V. Raff, A. S. Mokatrin and G. A. Van Nest. 1997. MF59 adjuvant enhances antibody responses of infant baboons immunized with *Haemophilus influenzae* type b and *Neisseria meningitidis* group C oligosaccharide-CRM197 conjugate vaccine. Infect Immun. 65,(5), 1710-1715.

Gray, S. J., M. A. Sobanski, E. B. Kaczmarski, M. Guiver, W. J. Marsh, R. Borrow, R. A. Barnes and W. T. Coakley. 1999. Ultrasound-enhanced latex immunoagglutination and PCR as complementary methods for non-culture-based confirmation of meningococcal disease. J Clin Microbiol. 37,(6), 1797-1801.

Green, S. and G. Tillotson. 1997. Use of ciprofloxacin in developing countries. Pediatr Infect Dis J. 16,(1), 150-162.

Greenwood, B., 1999. Manson Lecture: Meningococcal meningitis in Africa. Trans R Soc Trop Med Hyg. 93, 341-353.

Greenwood, B. M., I. S. Blakebrough, A. K. Bradley, S. Wali and H. C. Whittle. 1984. Meningococcal disease and season in sub-Saharan Africa. Lancet. 1,(8390), 1339-1342.

Greenwood, B. M., A. K. Bradley and R. A. Wall. 1985. Meningococcal disease and season in sub-Saharan Africa. Lancet. 2,(8459), 829-830.

Greisen, K., Loeffelholz, M., Purhoit, A., and Leong, D., 1994. PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. J Clin Microbiol. 32, 335-351.

Guibourdenche, M., M. Y. Popoff and J. Y. Riou. 1986. Deoxyribonucleic acid relatedness among *Neisseria gonorrhoeae, N. meningitidis, N. lactamica, N. cinerea* and *Neisseria polysaccharea*. Ann Inst Pasteur Microbiol. 137B,(2), 177-185.

Guiver, M., R. Borrow, J. Marsh, S. J. Gray, E. B. Kaczmarski, D. Howells, P. Boseley and A. J. Fox. 2000. Evaluation of the Applied Biosystems automated Taqman polymerase chain reaction system for the detection of meningococcal DNA. FEMS Immunol Med Microbiol. 28,(2), 173-179.

Hackett, S. J., E. D. Carrol, M. Guiver, J. Marsh, J. A. Sills, A. P. Thomson, E. B. Kaczmarski and C. A. Hart. 2002. Improved case confirmation in meningococcal disease with whole blood Taqman PCR. Arch Dis Child. 86,(6), 449-452.

Hahne, S. J., S. J. Gray, F. Jean, Aguilera, N. S. Crowcroft, T. Nichols, E. B. Kaczmarski and M. E. Ramsay. 2002. W135 meningococcal disease in England and Wales associated with Hajj 2000 and 2001. Lancet. 359,(9306), 582-583.

Hardy, J., S., Christodoulides, M., Weller, R, O., and Heckels, J, E., 2000. Interations of *Neisseria meningitidis* with cells of the human meninges. Mol Microbiol. 36,(4), 817-829.

Harrison, L. H., C. W. Armstrong, S. R. Jenkins, M. W. Harmon, G. W. Ajello, G. B. Miller, Jr. and C. V. Broome. 1991. A cluster of meningococcal disease on a school bus following epidemic influenza. Arch Intern Med. 151,(5), 1005-1009.

Hart, C. A. and L. E. Cuevas. 1997. Meningococcal disease in Africa. Ann Trop Med Parasitol. 91,(7), 777-785.

Hayrinen, J., H. Jennings, H. V. Raff, G. Rougon, N. Hanai, R. Gerardy-Schahn and J. Finne. 1995. Antibodies to polysialic acid and its N-propyl derivative: binding properties and interaction with human embryonal brain glycopeptides. J Infect Dis. 171,(6), 1481-1490.

Hefler, L. A., C. B. Tempfer, M. T. Bashford, G. Unfried, R. Zeillinger, C. Schneeberger, H. Koelbl, F. Nagele and J. C. Huber. 2002. Polymorphisms of the angiotensinogen gene, the endothelial nitric oxide synthase gene, and the interleukin-1beta gene promoter in women with idiopathic recurrent miscarriage. Mol Hum Reprod. 8,(1), 95-100.

Hegde, P., Qi, R., Abernathy, K., Gay, C., Dharap, S., Gaspard, R., Earle-Hughes, J., Snesrud, E., Lee, N., and Quackenbush, J., 2000. A concise guide to cDNA microarray analysis. Biotechniques. 29, 548-562.

Hitchcock, J., 1989. Unified nomenclature for pathogenic *Neisseria* species. Clin Microbiol Rev. 2, S64-S65.

Hitchcock, P. J., L. Leive, P. H. Makela, E. T. Rietschel, W. Strittmatter and D. C. Morrison. 1986. Lipopolysaccharide nomenclature--past, present, and future. J Bacteriol. 166,(3), 699-705.

Hjetland, R., D. A. Caugant, T. Hofstad, L. O. Froholm and R. K. Selander. 1990. Serogroup A *Neisseria meningitidis* of clone III-1 present in western Norway as early as 1969-73. Scand J Infect Dis. 22,(2), 241-242.

Hoehn, G. T., and Clark, V.L., 1990. Distribution of a protein antigenically related to the major anacrobically induced gonococcal outer membrane protein among other *Neisseria* species. Infect Immun. 58, 3929-3933.

Holmes, E. C., R. Urwin and M. C. Maiden. 1999. The influence of recombination on the population structure and evolution of the human pathogen *Neisseria meningitidis*. Mol Biol Evol. 16,(6), 741-749.

Homan, W. L., D. Tribe, S. Poznanski, M. Li, G. Hogg, E. Spalburg, J. D. Van Embden and R. J. Willems. 2002. Multilocus Sequence Typing Scheme for *Enterococcus faecium*. J Clin Microbiol. 40,(6), 1963-1971.

Horby, P., A. Rushdy, C. Graham and M. O'Mahony. 2001. PHLS overview of communicable diseases 1999. Commun Dis Public Health. 4,(1), 8-17.

Hughes, J. H., D. J. Biedenbach, M. E. Erwin and R. N. Jones. 1993. E test as susceptibility test and epidemiologic tool for evaluation of *Neisseria meningitidis* isolates. J Clin Microbiol. 31,(12), 3255-3259.

Iles, K., N. K. Poplawski and R. T. Couper. 2001. Passive exposure to tobacco smoke and bacterial meningitis in children. J Paediatr Child Health. 37,(4), 388-391.

Il'ina, T. V., 1992. Assessment of the sensitivity of *Neisseria meningitidis* strains to benzylpenicillin using the disc diffusion method. Antibiot Khimioter. 37,(10), 32-34.

Imrey, P. B., L. A. Jackson, P. H. Ludwinski, A. C. England, 3rd, G. A. Fella, B. C. Fox, L. B. Isdale, M. W. Reeves and J. D. Wenger. 1995. Meningococcal carriage, alcohol consumption, and campus bar patronage in a serogroup C meningococcal disease outbreak. J Clin Microbiol. 33,(12), 3133-3137.

Isenberg, H. D. (1992). Clinical Microbiology Procedures Handbook. Washington, DC., American Society of Microbiology.

Jackson, L. A., Schuchat, A., Reeves, M.W., Wenger., J.D., and The Meningococcal Disease Study Group., 1994. Prevalence of *Neisseria meningitidis* relaitively resistant to penicillin in the United States, 1991. J Infect Dis. 169, 438-441.

Jackson, L. A. and J. D. Wenger. 1993. Laboratory-based surveillance for meningococcal disease in selected areas, United States, 1989-1991. MMWR CDC Surveill Summ. 42,(2), 21-30.

Jacobsson, S., M. Issa, M. Unemo, A. Backman, P. Molling, N. Sulaiman and P. Olcen. 2003. Molecular characterisation of group A *Neisseria meningitidis* isolated in Sudan 1985-2001. Apmis. 111,(11), 1060-1066.

Jeanteur, D., J. H. Lakey, and F. Pattus., 1991. The bacterial proin superfamily: sequence alignment and structure prediction. Mol Microbiol. 5, 2153-2164.

Jolley, K., A., Feil, E. J., Chan, M-S. and M. C. J. Maiden. 2001. Sequence type analysis and recombinational tests (START). Bioinformatics. 17, 1230-1231.

Jolley, K. A., J. Kalmusova, E. J. Feil, S. Gupta, M. Musilek, P. Kriz and M. C. Maiden. 2000. Carried meningococci in the Czech Republic: a diverse recombining population. J Clin Microbiol. 38,(12), 4492-4498.

Jonasson, J., M. Olofsson and H. J. Monstein. 2002. Classification, identification and subtyping of bacteria based on pyrosequencing and signature matching of 16S rDNA fragments. Apmis. 110,(3), 263-272.

Kertesz, D. A., S. K. Byrne and A. W. Chow. 1993. Characterization of *Neisseria meningitidis* by polymerase chain reaction and restriction endonuclease digestion of the porA gene. J Clin Microbiol. 31,(10), 2594-2598.

Kiefer, F., 1896. Zur differentialdiagnose des erregersder epidemischen cerebrospinalmeningitis und der gonorrhoe. Berl Klin Woch. 33, 628-630.

Kijas, J. M., R. K. Juneja, S. Gafvert and L. Andersson. 2000. Detection of the causal mutation for canine leukocyte adhesion deficiency (CLAD) using pyrosequencing. Anim Genet. 31,(5), 326-328.

King, S. J., J. A. Leigh, P. J. Heath, I. Luque, C. Tarradas, C. G. Dowson and A. M. Whatmore. 2002. Development of a multilocus sequence typing scheme for the pig pathogen *Streptococcus suis*: identification of virulent clones and potential capsular serotype exchange. J Clin Microbiol. 40,(10), 3671-3680.

Klugman, K. P., E. C. Gotschlich and M. S. Blake. 1989. Sequence of the structural gene (rmpM) for the class 4 outer membrane protein of *Neisseria meningitidis*, homology of the protein to gonococcal protein III and *Escherichia coli* OmpA, and construction of meningococcal strains that lack class 4 protein. Infect Immun. 57,(7), 2066-2071.

Knapp, J. S., 1988. Historical perspectives and identification of Neisseria and related species. Clin Microbiol Rev. 1,(4), 415-431.

Knight, A. I., H. Ni, K. A. Cartwright and J. J. McFadden. 1992. Identification and characterization of a novel insertion sequence, IS1106, downstream of the porA gene in B15 *Neisseria meningitidis*. Mol Microbiol. 6,(11), 1565-1573.

Kotetishvili, M., O. C. Stine, A. Kreger, J. G. Morris, Jr. and A. Sulakvelidze. 2002. Multilocus sequence typing for characterization of clinical and environmental salmonella strains. J Clin Microbiol. 40,(5), 1626-1635.

Krause, G., C. Blackmore, S. Wiersma, C. Lesneski, C. W. Woods, N. E. Rosenstein and R. S. Hopkins. 2001. Marijuana use and social networks in a community outbreak of meningococcal disease. South Med J. 94,(5), 482-485.

Kremastinou, J., G. Tzanakaki, A. Kansouzidou, A. Pagalis, V. Danielides, G. Kouppari, E. Lada, P. Kriz, M. Musilek, D. M. Weir and C. C. Blackwell. 1999a. Recent emergence of serogroup C meningococcal disease in Greece. FEMS Immunol Med Microbiol. 23,(1), 49-55.

Kremastinou, J., G. Tzanakaki, A. Pagalis, M. Theodondou, D. M. Weir and C. C. Blackwell. 1999b. Detection of IgG and IgM to meningococcal outer membrane proteins in relation to carriage of *Neisseria meningitidis* or *Neisseria lactamica*. FEMS Immunol Med Microbiol. 24,(1), 73-78.

Kriz, P., M. Bobak and B. Kriz. 2000. Parental smoking, socioeconomic factors, and risk of invasive meningococcal disease in children: a population based case-control study. Arch Dis Child. 83,(2), 117-121.

Kyaw, M. H., J. C. Bramley, S. Clark, P. Christie, I. G. Jones and H. Campbell. 2002a. Prevalence of moderate penicillin resistant invasive *Neisseria meningitidis* infection in Scotland, 1994-9. Epidemiol Infect. 128,(2), 149-156.

Kyaw, M. H., P. Christie, I. G. Jones and H. Campbell. 2002b. The changing epidemiology of bacterial meningitis and invasive non-meningitic bacterial disease in scotland during the period 1983-99. Scand J Infect Dis. 34,(4), 289-298.

Kyaw, M. H., S. C. Clarke, P. Christie, I. G. Jones and H. Campbell. 2002c. Invasive meningococcal disease in Scotland, 1994 to 1999, with emphasis on group B meningococcal disease. J Clin Microbiol. 40,(5), 1834-1837.

Lapeyssonnie, M., 1963. La meningite cerebro-spinale en Afrique. Bull World Health Organ. 28, 3-114.

Lehmann, A. K., A. Halstensen, I. S. Aaberge, J. Holst, T. E. Michaelsen, S. Sornes, L. M. Wetzler and H. Guttormsen. 1999. Human opsonins induced during meningococcal disease recognize outer membrane proteins PorA and PorB. Infect Immun. 67,(5), 2552-2560.

Lewis, C., M. A. Diggle and S. C. Clarke. 2003. Nucleotide sequence analysis of the sialyltransferase genes of meningococcal serogroups B, C, y and W135. J Mol Microbiol Biotechnol. 5,(2), 82-86.

Lewis, R., N. Nathan, L. Diarra, F. Belanger and C. Paquet. 2001. Timely detection of meningococcal meningitis epidemics in Africa. Lancet. 358,(9278), 287-293.

Lindsay, A. P., V. Hope, R. J. Marshall and J. Salinger. 2002. Meningococcal disease and meteorological conditions in Auckland, New Zealand. Aust N Z J Public Health. 26,(3), 212-218.

Lingappa, J. R., N. Rosenstein, E. R. Zell, K. A. Shutt, A. Schuchat and B. A. Perkins. 2001. Surveillance for meningococcal disease and strategies for use of conjugate meningococcal vaccines in the United States. Vaccine. 19,(31), 4566-4575.

Livak, K. J., Flood, S.J., Marmaro, J., Giusti, W., and K. Deetz. 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridisation. PCR Methods Appl. 4, 357-362.

Lowe, C. A., M. A. Diggle and S. C. Clarke. 2004. Rapid screening of a single nucleotide polymorphism in the *Neisseria meningitidis fumC* gene using MALDI-TOF. Br J Biomed Sci. 61,(1), 8-10.

Maiden, M. C., J. A. Bygraves, E. Feil, G. Morelli, J. E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D. A. Caugant, I. M. Feavers, M. Achtman and B. G. Spratt. 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci U S A. 95,(6), 3140-3145.

Maiden, M. C., J. A. Bygraves, J. McCarvil and I. M. Feavers. 1992. Identification of meningococcal serosubtypes by polymerase chain reaction. J Clin Microbiol. 30,(11), 2835-41.

Maiden, M. C. and I. M. Feavers. 1994. Meningococcal typing. J Med Microbiol. 40,(3), 157-158.

Maiden, M. C., J. Russell, J. Suker and I. M. Feavers. 1999. *Neisseria meningitidis* subtype nomenclature. Clin Diagn Lab Immunol. 6,(5), 771-772.

Maiden, M. C. and J. M. Stuart. 2002. Carriage of serogroup C meningococci 1 year after meningococcal C conjugate polysaccharide vaccination. Lancet. 359,(9320), 1829-1831.

Maiden, M. C., J. Suker, A. J. McKenna, J. A. Bygraves and I. M. Feavers. 1991. Comparison of the class 1 outer membrane proteins of eight serological reference strains of *Neisseria meningitidis*. Mol Microbiol. 5,(3), 727-736.

Marchiafava, N., Celi, A., 1884. Sopra i micrococchi della meningite cerebrospinale epidemica. Gazetta degli Ospitali. 8, 59-60.

Markman, A. F., 1993. The polymerase chain reaction: a tool for molecular medicine. Br Med J. 306, 1637-1640.

Marshall, S. A., P. R. Rhomberg and R. N. Jones. 1997. Comparative evaluation of etest for susceptibility testing *Neisseria meningitidis* with eight antimicrobial agents. An investigation using U.S. Food and Drug Administration regulatory criteria. Diagn Microbiol Infect Dis. 27,(3), 93-97.

Martin, J. E., Sammuels, S. B., Peacok, W. l., and Thayer, J. D., 1964. *Neisseria gonorrhoeae* and *Neisseria meningitidis* sensitivity to spectomycin, lincomycin and penicillin G. Antimicrob Agents Chemother. 5, 366-368.

Martin, S. L., R. Borrow, P. van der Ley, M. Dawson, A. J. Fox and K. A. Cartwright. 2000. Effect of sequence variation in meningococcal PorA outer membrane protein on the effectiveness of a hexavalent PorA outer membrane vesicle vaccine. Vaccine. 18,(23), 2476-2481.

McGuinness, B., A. K. Barlow, I. N. Clarke, J. E. Farley, A. Anilionis, J. T. Poolman and J. E. Heckels. 1990. Deduced amino acid sequences of class 1 protein (PorA) from three strains of *Neisseria meningitidis*. Synthetic peptides define the epitopes responsible for serosubtype specificity. J Exp Med. 171,(6), 1871-82.

McGuinness, B. T., P. R. Lambden and J. E. Heckels. 1993. Class 1 outer membrane protein of *Neisseria meningitidis*: epitope analysis of the antigenic diversity between strains, implications for subtype definition and molecular epidemiology. Mol Microbiol. 7,(4), 505-514.

McNeely, T., 2003. Pyrosequencing AB. Pharmacogenomics. 4,(2), 217-221.

McNeil, G. and M. Virji. 1997. Phenotypic variants of meningococci and their potential in phagocytic interactions: the influence of opacity proteins, pili, PilC and surface sialic acids. Microb Pathog. 22,(5), 295-304.

Meats, E., E. J. Feil, S. Stringer, A. J. Cody, R. Goldstein, J. S. Kroll, T. Popovic and B. G. Spratt. 2003. Characterization of Encapsulated and Noncapsulated *Haemophilus influenzae* and Determination of Phylogenetic Relationships by Multilocus Sequence Typing. J Clin Microbiol. 41,(4), 1623-1636.

Mele, J. A., 3rd, S. Linder and A. Capozzi. 1997. Treatment of thromboembolic complications of fulminant meningococcal septic shock. Ann Plast Surg. 38,(3), 283-290.

Meyers, L. A., B. R. Levin, A. R. Richardson and I. Stojiljkovic. 2003. Epidemiology, hypermutation, within-host evolution and the virulence of *Neisseria meningitidis*. Proc R Soc Lond B Biol Sci. 270,(1525), 1667-1677.

Miller, E., D. Salisbury and M. Ramsay. 2001. Planning, registration, and implementation of an immunisation campaign against meningococcal serogroup C disease in the UK: a success story. Vaccine. 20 Suppl 1, S58-67.

Molesworth, A. M., M. C. Thomson, S. J. Connor, M. P. Cresswell, A. P. Morse, P. Shears, C. A. Hart and L. E. Cuevas. 2002. Where is the meningitis belt? Defining an area at risk of epidemic meningitis in Africa. Trans R Soc Trop Med Hyg. 96,(3), 242-249.

Molling, P., A. Backman, P. Olcen and H. Fredlund. 2001. Comparison of serogroup w-135 meningococci isolated in Sweden during a 23-year period and those associated with a recent hajj pilgrimage. J Clin Microbiol. 39,(7), 2695-2699.

Molling, P., S. Jacobsson, A. Backman and P. Olcen. 2002. Direct and rapid identification and genogrouping of meningococci and *porA* amplification by LightCycler PCR. J Clin Microbiol. 40,(12), 4531-4535.

Molling, P., M. Unemo, A. Backman and P. Olcen. 2000. Genosubtyping by sequencing group A, B and C meningococci; a tool for epidemiological studies of epidemics, clusters and sporadic cases. Apmis. 108,(7-8), 509-516.

Monstein, H., S. Nikpour-Badr and J. Jonasson. 2001. Rapid molecular identification and subtyping of *Helicobacter pylori* by pyrosequencing of the 16S rDNA variable V1 and V3 regions. FEMS Microbiol Lett. 199,(1), 103-107.

Morin, P. A., Saiz, R., and, A. Monjazeb. 1999. High-throughput single nucleotide polymorphism genotyping by fluorescent 5' exonuclease assay. Biotechniques. 27, 538-540.

Morley, S. L. and A. J. Pollard. 2001. Vaccine prevention of meningococcal disease, coming soon? Vaccine. 20,(5-6), 666-687.

Morse, S. A., and J. S. Knapp (1992). The Genus *Neisseria*. The prokaryotes. H. G. T. A. Balows, M. Dworkin, W. Harder, and K-H. Schleifer. New York, N.Y, Springer-Verlag: 2495-2559.

Moura, A. S., A. Pablos-Mendez, M. Layton and D. Weiss. 2003. Epidemiology of meningococcal disease, New York City, 1989-2000. Emerg Infect Dis. 9,(3), 355-361.

Mullis, K., F. Faloona, S. Scharf, R. Saiki, G. Horn and H. Erlich. 1986. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harb Symp Quant Biol. 51 Pt 1, 263-273.

Munkley, A., C. R. Tinsley, M. Virji and J. E. Heckels. 1991. Blocking of bactericidal killing of *Neisseria meningitidis* by antibodies directed against class 4 outer membrane protein. Microb Pathog. 11,(6), 447-452.

Murray, P. R. (1999). Manual of Clinical Microbiology. Washington DC, ASM Press.

Myburg, A. A., D. L. Remington, D. M. O'Malley, R. R. Sederoff and R. W. Whetten. 2001. High-throughput AFLP analysis using infrared dye-labeled primers and an automated DNA sequencer. Biotechniques. 30,(2), 348-352, 354, 356-357.

Nallapareddy, S. R., R. W. Duh, K. V. Singh and B. E. Murray. 2002. Molecular typing of selected *Enterococcus faecalis* isolates: pilot study using multilocus sequence typing and pulsed-field gel electrophoresis. J Clin Microbiol. 40,(3), 868-876.

Nassif, X. 1999. Interaction mechanisms of encapsulated meningococci with eucaryotic cells: what does this tell us about the crossing of the blood-brain barrier by *Neisseria meningitidis*? Curr Opin Microbiol. 2,(1), 71-77.

Nassif, X., S. Bourdoulous, E. Eugene and P. O. Couraud. 2002. How do extracellular pathogens cross the blood-brain barrier? Trends Microbiol. 10,(5), 227-232.

Nelson, S. J., A. Charlett, H. J. Orr, R. M. Barker, K. R. Neal, C. Taylor, P. N. Monk, M. R. Evans and J. M. Stuart. 2001. Risk factors for meningococcal disease in university halls of residence. Epidemiol Infect. 126,(2), 211-217.

Newcombe, J., K. Cartwright, W. H. Palmer and J. McFadden. 1996. PCR of peripheral blood for diagnosis of meningococcal disease. J Clin Microbiol. 34,(7), 1637-1640.

Newcombe, J., S. Dyer, L. Blackman, K. Cartwright, W. H. Palmer, J. McFadden and L. Blackwell. 1997. PCR-single-stranded confirmational polymorphism analysis for non-culture-based subtyping of meningococcal strains in clinical specimens. J Clin Microbiol. 35,(7), 1809-1812.

Newcombe, J., L. J. Eales-Reynolds, L. Wootton, A. R. Gorringe, S. G. Funnell, S. C. Taylor and J. J. McFadden. 2004. Infection with an avirulent *phoP* mutant of *Neisseria meningitidis* confers broad cross-reactive immunity. Infect Immun. 72,(1), 338-344.

Ni, H., Knight, A.I., Cartwright, K., Palmer, W.H., and J. McFadden. 1992. Polymerase chain reaction for diagnosis of meningococcal meningitis. Lancet. 340, 1432-1434.

Nicholls, H., 2003. Impoving drug response with parmacogenomics. Drug Discov Today. 8,(7), 281-282.

Nicolas, P., L. Decousset, V. Riglet, P. Castelli, R. Stor and G. Blanchet. 2001. Clonal Expansion of Sequence Type (ST-)5 and Emergence of ST-7 in Serogroup A Meningococci, Africa. Emerg Infect Dis. 7,(5), 849-854.

Noah, N., Henderson, B. 2001. Surveillance of bacterial meningitis in Europe 1999/2000.

Nolan, J. and R. Sinclair. 2001. Review of management of purpura fulminans and two case reports. Br J Anaesth. 86,(4), 581-586.

Noller, A. C., M. C. McEllistrem, O. C. Stine, J. G. Morris, Jr., D. J. Boxrud, B. Dixon and L. H. Harrison. 2003. Multilocus sequence typing reveals a lack of diversity among *Escherichia coli* O157:H7 isolates that are distinct by pulsed-field gel electrophoresis. J Clin Microbiol. 41,(2), 675-679.

Norheim, G., E. Rosenqvist, P. Aavitsland and D. A. Caugant. 2000. Meningococcal disease in Africa - epidemiology and prevention. Tidsskr Nor Laegeforen. 120,(15), 1735-1739.

Nygren, M., Reizenstein, E., Ronaghi, M., and Lundeberg, J., 2000. Polymorphism in the pertussis toxin promoter region affecting the DNA-based diagnosis of *Bordetalla* infection. J Clin Microbiol. 38,(1), 55-60.

Oberti, J., N. T. Hoi, R. Caravano, C. M. Tan and J. Roux. 1981. An epidemic of meningococcal infection in Vietnam (southern provinces). Bull World Health Organ. 59,(4), 585-590.

Olcen, P., and H. Fredlund. (2001). Isolation, Culture, and Identification of meningococci from Clinical Specimens`. Meningococcal Disease. A. J. Pollard, and Maiden, C. J. Totowa, New Jersey, Humana Press: 9-22.

Olcen, P., Kjellander, J., Danielsson, D., and B. L. Linquist 1979. Culture diagnosis of meningococcal carriers. J Clin Pathol. 32, 1222-1225.

Olive, D. M., and P. Bean. 1999. Principles and applications of methods for DNA based typing of microbial organisms. J. Clin. Microbiol. 37,(6), 1661-1669.

Olyhoek, T., B. A. Crowe and M. Achtman. 1987. Clonal population structure of *Neisseria meningitidis* serogroup A isolated from epidemics and pandemics between 1915 and 1983. Rev Infect Dis. 9,(4), 665-692.

O'Meara, D., K. Wilbe, T. Leitner, B. Hejdeman, J. Albert and J. Lundeberg. 2001. Monitoring resistance to human immunodeficiency virus type 1 protease inhibitors by pyrosequencing. J Clin Microbiol. 39,(2), 464-473.

Oostenbrink, R., M. Maas, K. G. Moons and H. A. Moll. 2002. Sequelae after bacterial meningitis in childhood. Scand J Infect Dis. 34,(5), 379-382.

Oppenheim, B. A., 1997. Antibiotic resistance in *Neisseria meningitidis*. Clin Infect Dis. 24 Suppl 1, S98-101.

Peeters, C. C., H. C. Rumke, L. C. Sundermann, E. M. Rouppe van der Voort, J. Meulenbelt, M. Schuller, A. J. Kuipers, P. van der Ley and J. T. Poolman. 1996. Phase I clinical trial with a hexavalent PorA containing meningococcal outer membrane vesicle vaccine. Vaccine. 14,(10), 1009-1015.

Peltola, H., 1983. Meningococcal disease: still with us. Rev Infect Dis. 5, 71-91.

Perkins, B. A., K. Jonsdottir, H. Briem, E. Griffiths, B. D. Plikaytis, E. A. Hoiby, E. Rosenqvist, J. Holst, H. Nokleby, F. Sotolongo, G. Sierra, H. C. Campa, G. M. Carlone, D. Williams, J. Dykes, D. Kapczynski, E. Tikhomirov, J. D. Wenger and C. V. Broome. 1998. Immunogenicity of two efficacious outer membrane protein-based serogroup B meningococcal vaccines among young adults in Iceland. J Infect Dis. 177,(3), 683-691.

Picard, B. 2000. Molecular epidemiology of large bacterial endemics in Sub-Saharan Africa. Bull Soc Pathol Exot. 93,(3), 219-223.

Pitt, T. L. 2000. Molecular bacteriology: a diagnostic tool for the millennium. J Clin Pathol. 53, 71-75.

Pizza, M., V. Scarlato, V. Masignani, M. M. Giuliani, B. Arico, M. Comanducci, G. T. Jennings, L. Baldi, E. Bartolini, B. Capecchi, C. L. Galeotti, E. Luzzi, R. Manetti, E. Marchetti, M. Mora, S. Nuti, G. Ratti, L. Santini, S. Savino, M. Scarselli, E. Storni, P. Zuo, M. Broeker, E. Hundt, B. Knapp, E. Blair, T. Mason, H. Tettelin, D. W. Hood, A. C. Jeffries, N. J. Saunders, D. M. Granoff, J. C. Venter, E. R. Moxon, G. Grandi and R. Rappuoli. 2000. Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. Science. 287,(5459), 1816-1820.

Pollard, A. J. and M. C. Maiden. 2003. Epidemic meningococcal disease in sub-Saharan Africa--towards a sustainable solution? Lancet Infect Dis. 3,(2), 68-70.

Pollard, A. J. and E. R. Moxon. 2002. The meningococcus tamed? Arch Dis Child. 87,(1), 13-17.

Pollard, A. J. and D. Scheifele. 2001. Meningococcal disease and vaccination in North America. J Paediatr Child Health. 37,(5), 20-27.

Poolman, J. T., 1995. Development of a meningococcal vaccine. Infect Agents Dis. 4,(1), 13-28.

Poolman, J. T., I. Lind, K. Jonsdottir, L. O. Froholm, D. M. Jones and H. C. Zanen. 1986. Meningococcal serotypes and serogroup B disease in North-West Europe. Lancet. 2,(8506), 555-558.

Popovic, T., S. Schmink, N. A. Rosenstein, G. W. Ajello, M. W. Reeves, B. Plikaytis, S. B. Hunter, E. M. Ribot, D. Boxrud, M. L. Tondella, C. Kim, C. Noble, E. Mothershed, J. Besser and B. A. Perkins. 2001. Evaluation of pulsed-field gel electrophoresis in epidemiological investigations of meningococcal disease outbreaks caused by *Neisseria meningitidis* serogroup C. J Clin Microbiol. 39,(1), 75-85.

Porritt, R. J., J. L. Mercer and R. Munro. 2003. Ultrasound-enhanced latex immunoagglutination test (USELAT) for detection of capsular polysaccharide antigen of *Neisseria meningitidis* from CSF and plasma. Pathology. 35,(1), 61-64.

Probert, W. S., S. L. Bystrom, S. Khashe, K. N. Schrader and J. D. Wong. 2002. 5' exonuclease assay for detection of serogroup Y *Neisseria meningitidis*. J Clin Microbiol. 40,(11), 4325-4328.

Pujol, C., E. Eugene, L. de Saint Martin and X. Nassif. 1997. Interaction of *Neisseria meningitidis* with a polarized monolayer of epithelial cells. Infect Immun. 65,(11), 4836-4842.

Ragunathan, L., M. Ramsay, R. Borrow, M. Guiver, S. Gray and E. B. Kaczmarski. 2000. Clinical features, laboratory findings and management of meningococcal meningitis in England and Wales: report of a 1997 survey. Meningococcal meningitis: 1997 survey report. J Infect. 40,(1), 74-79.

Ramsay, M., E. Kaczmarski, M. Rush, R. Mallard, P. Farrington and J. White. 1997. Changing patterns of case ascertainment and trends in meningococcal disease in England and Wales. Commun Dis Rep CDR Rev. 7,(4), R49-54.

Ramsay, M. E., N. Andrews, E. B. Kaczmarski and E. Miller. 2001. Efficacy of meningococcal serogroup C conjugate vaccine in teenagers and toddlers in England. Lancet. 357,(9251), 195-196.

Ramsay, M. E., N. J. Andrews, C. L. Trotter, E. B. Kaczmarski and E. Miller. 2003. Herd immunity from meningococcal serogroup C conjugate vaccination in England: database analysis. Bmj. 326,(7385), 365-366.

Rappuoli, R., 2000. Reverse vaccinology. Curr Opin Microbiol. 3,(5), 445-450.

Rappuoli, R., 2001a. Conjugates and reverse vaccinology to eliminate bacterial meningitis. Vaccine. 19,(17-19), 2319-2322.

Rappuoli, R., 2001b. Reverse vaccinology, a genome-based approach to vaccine development. Vaccine. 19,(17-19), 2688-2691.

Rayner, C. F., A. Dewar, E. R. Moxon, M. Virji and R. Wilson. 1995. The effect of variations in the expression of pili on the interaction of *Neisseria meningitidis* with human nasopharyngeal epithelium. J Infect Dis. 171,(1), 113-121.

Richmond, P., R. Borrow, J. Findlow, S. Martin, C. Thornton, K. Cartwright and E. Miller. 2001. Evaluation of De-O-acetylated meningococcal C polysaccharide-tetanus toxoid conjugate vaccine in infancy: reactogenicity, immunogenicity, immunologic priming, and bactericidal activity against O-acetylated and De-O-acetylated serogroup C strains. Infect Immun. 69,(4), 2378-2382.

Richter, S. S., K. A. Gordon, P. R. Rhomberg, M. A. Pfaller and R. N. Jones. 2001. *Neisseria meningitidis* with decreased susceptibility to penicillin: report from the SENTRY antimicrobial surveillance program, North America, 1998-99. Diagn Microbiol Infect Dis. 41,(1-2), 83-88.

Riedo, F. X., B. D. Plikaytis and C. V. Broome. 1995. Epidemiology and prevention of meningococcal disease. Pediatr Infect Dis J. 14,(8), 643-657.

Riesbeck, K., P. Orvelid-Molling, H. Fredlund and P. Olcen. 2000. Long-term persistence of a discotheque-associated invasive *Neisseria meningitidis* group C strain as proven by pulsed-field gel electrophoresis and porA gene sequencing. J Clin Microbiol. 38,(4), 1638-1640.

Ringuette, L., M. Lorange, A. Ryan and F. Ashton. 1995. Meningococcal infections in the Province of Quebec, Canada, during the period 1991 to 1992. J Clin Microbiol. 33,(1), 53-57.

Riordan, F. A., O. Marzouk, A. P. Thomson, J. A. Sills and C. A. Hart. 2002. Prospective validation of the Glasgow Meningococcal Septicaemia Prognostic Score. Comparison with other scoring methods. Eur J Pediatr. 161,(10), 531-537.

Robertson, G. A., V. Thiruvenkataswamy, H. Shilling, G. P. Price, F. Huygens, F. A. Henskens and P. M. Gifford. 2004. Identification and iterogation of highly informative single nucleotide polymorhism sets defined by bacterial multilocus sequence typing databases. J. Med. Microbiol. 53,(1), 35-45.

Robinson, P., K. Taylor and T. Nolan. 2001. Risk-factors for meningococcal disease in Victoria, Australia, in 1997. Epidemiol Infect. 127,(2), 261-268.

Rockowitz, J. and A. R. Tunkel. 1995. Bacterial meningitis. Practical guidelines for management. Drugs. 50,(5), 838-853.

Ronaghi, M., 2001. Pyrosequencing sheds light on DNA sequencing. Genome Res. 11,(1), 3-11.

Ronagi, M., and Elahi, E., 2002. Pyroseuencing for microbial typing. J Chromatogr B Analyt Technol Biomed life Sci. 782,(1-2), 67-72.

Rosenqvist, E., E. A. Hoiby, E. Wedege, K. Bryn, J. Kolberg, A. Klem, E. Ronnild, G. Bjune and H. Nokleby. 1995. Human antibody responses to meningococcal outer membrane antigens after three doses of the Norwegian group B meningococcal vaccine. Infect Immun. 63,(12), 4642-4652.

Rosenqvist, E., E. Wedege, E. A. Hoiby and L. O. Froholm. 1990. Serogroup determination of *Neisseria meningitidis* by whole-cell ELISA, dot-blotting and agglutination. Apmis. 98,(6), 501-506.

Rosenstein, N. E., M. Fischer and J. W. Tappero. 2001a. Meningococcal vaccines. Infect Dis Clin North Am. 15,(1), 155-169.

Rosenstein, N. E., B. A. Perkins, D. S. Stephens, T. Popovic and J. M. Hughes. 2001b. Meningococcal disease. N Engl J Med. 344,(18), 1378-1388.

Round, A., M. R. Evans, R. L. Salmon, I. K. Hosein, A. K. Mukerjee, R. W. Smith and S. R. Palmer. 2001. Public health management of an outbreak of group C meningococcal disease in university campus residents. Eur J Public Health. 11,(4), 431-436.

Rouppe van der Voort, E., M. Schuller, J. Holst, P. de Vries, P. van der Ley, G. van den Dobbelsteen and J. Poolman. 2000. Immunogenicity studies with a genetically engineered hexavalent PorA and a wild-type meningococcal group B outer membrane vesicle vaccine in infant cynomolgus monkeys. Vaccine. 18,(14), 1334-1343.

Rouppe van der Voort, E. M., B. Kuipers, H. F. Brugghe, L. M. van Unen, H. A. Timmermans, P. Hoogerhout and J. T. Poolman. 1997. Epitope specificity of murine and human bactericidal antibodies against PorA P1.7,16 induced with experimental meningococcal group B vaccines. FEMS Immunol Med Microbiol. 17,(3), 139-148.

Rudel, T., A. Schmid, R. Benz, H. A. Kolb, F. Lang, and T. F. Meyer. 1996. Modulation of Neisseria porin (PorB) by cytosolic ATP/GTP of target cells: parallels between pathogen accomodation and mitochondrial endosymbiosis. Cell. 85, 391-402.

Sacchi, C. T., A. P. Lemos, M. E. Brandt, A. M. Whitney, C. E. Melles, C. A. Solari, C. E. Frasch and L. W. Mayer. 1998a. Proposed standardization of *Neisseria meningitidis* PorA variable-region typing nomenclature. Clin Diagn Lab Immunol. 5,(6), 845-855.

Sacchi, C. T., A. P. Lemos, A. M. Whitney, C. A. Solari, M. E. Brandt, C. E. Melles, C. E. Frasch and L. W. Mayer. 1998b. Correlation between serological and sequencing analyses of the PorB outer membrane protein in the *Neisseria meningitidis* serotyping system. Clin Diagn Lab Immunol. 5,(3), 348-354.

Saez Nieto, J. A., J. A. Vazquez and C. Marcos. 1990. Meningococci moderately resistant to penicillin. Lancet. 336,(8706), 54.

Salcedo, C., L. Arreaza, B. Alcala, L. De La Fuente and J. A. Vazquez. 2003. Development of a Multilocus Sequence Typing Method for Analysis of *Listeria monocytogenes* Clones. J Clin Microbiol. 41,(2), 757-762.

Salih, M. A., A. A. Ahmed, H. Sid Ahmed and P. Olcen. 1995. An ELISA assay for the rapid diagnosis of acute bacterial meningitis. Ann Trop Paediatr. 15,(4), 273-278.

Sambrook, J., MacCallum, P., Russel, D. (2000). Molecular cloning: a laboratory manual. New York, Cold Spring Harbour Laboratory Press, Cold Spring Harbour.

Sarkar, R., S. Sharma, R. Natarajan and D. S. Agarwal. 1987. Sulphadiazine resistant group 'A' meningococci isolated during the meningitis outbreak in Delhi (1985). Indian J Med Res. 85, 612-616.

Saunders, N. B., Zollinger, W.D., and Rao, V.B., 1993. A rapid and sensitive PCR strategy emplyed for amplification and sequencing of *porA* from a single colony-forming unit of *Neisseria meningitidis*. Gene. 137, 153-162.

Sawyer, S., 1989. Statistical test for detecting gene conversion. Mol Biol Evol. 5,(6), 526-38.

Scheld, W. M. and G. L. Mandell. 1984. Landmark perspective. Sulfonamides and meningitis. Jama. 251,(6), 791-794.

Scholten, R. J., B. Kuipers, H. A. Valkenburg, J. Dankert, W. D. Zollinger and J. T. Poolman. 1994. Lipo-oligosaccharide immunotyping of *Neisseria meningitidis* by a whole-cell ELISA with monoclonal antibodies. J Med Microbiol. 41,(4), 236-243.

Schwartz, B., P. S. Moore and C. V. Broome. 1989. Global epidemiology of meningococcal disease. Clin Microbiol Rev. 2 Suppl, S118-24.

Selander, R. K., D. A. Caugant, H. Ochman, J. M. Musser, M. N. Gilmour and T. S. Whittam. 1986. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl Environ Microbiol. 51,(5), 873-884.

Sclander, R. K., J. M. Musser, D. A. Caugant, M. N. Gilmour and T. S. Whittam. 1987. Population genetics of pathogenic bacteria. Microb Pathog. 3,(1), 1-7.

Seward, R. J. and K. J. Towner. 2000. Use of an automated DNA analysis system (DARAS(R)) for sequence-specific recognition of *Neisseria meningitidis* DNA. Clin Microbiol Infect. 6,(1), 29-33.

Shalon, D., Smith, S.J., Brown, P.O., 1996. A DNA microarray system for analysing comple DNA samples using two-colour fluorescent probe hybridisation. Genome Res. 6, 639-645.

Shenep, J. L., 1986. Antibiotic-induced bacterial cell lysis: a therapeutic dilemma. Eur J Clin Microbiol. 5,(1), 11-12.

Sherrard, J. S., and J. S. Bingham. 1995. Gonorrhoea now. Int J STD AIDS. 6, 162-166.

Shi, M. M., 2001. Enabling large-scale pharmacogenetic studies by high-throughput mutation detection and genotyping technologies. Clin Chem. 47,(2), 164-172.

Shigematsu, M., K. L. Davison, A. Charlett and N. S. Crowcroft. 2002. National enhanced surveillance of meningococcal disease in England, Wales and Northern Ireland, January 1999-June 2001. Epidemiol Infect. 129,(3), 459-470.

Simmons, G., N. Jones and L. Calder. 2000. Equivalence of ceftriaxone and rifampicin in eliminating nasopharyngeal carriage of serogroup B *Neisseria meningitidis*. J Antimicrob Chemother. 45,(6), 909-911.

Simmons, G., D. Martin, J. Stewart, N. Jones, L. Calder and D. Bremner. 2001. Carriage of *Neisseria meningitidis* among household contacts of patients with meningococcal disease in New Zealand. Eur J Clin Microbiol Infect Dis. 20,(4), 237-242.

Singhi, P., M. Kaushal, S. Singhi and P. Ray. 2002. Seven days vs. 10 days ceftriaxone therapy in bacterial meningitis. J Trop Pediatr. 48,(5), 273-279.

Skalova, R., 1984. Epidemiology of meningococcal meningitis in Sahel and Mongolia. Infection. 12 Suppl 1, S35-43.

Smart, L. E. and S. J. O'Brien. 1997. Meningococcal infection in Scotland, 1995 to 1997. Euro Surveill. 2,(10), 76-77.

Smith, J. M., 1992. Analysing the mosaic structure of genes. J Mol Evol. 2,(34), 126-129.

Smith, J. M., N. H. Smith, M. O'Rourke and B. G. Spratt. 1993. How clonal are bacteria? Proc Natl Acad Sci U S A. 90,(10), 4384-4388.

Sneath, P. H., and R.R Sokal. (1973). Numerical Taxonomy - The principles and practice of numerical classification. San Francisco, W.H.Freeman.

Sobanski, M. A., R. Vince, G. A. Biagini, C. Cousins, M. Guiver, S. J. Gray, E. B. Kaczmarski and W. T. Coakley. 2002. Ultrasound enhanced detection of individual meningococcal serogroups by latex immunoassay. J Clin Pathol. 55,(1), 37-40.

Southern, E. M., 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol. 98, 503-511.

Speers, D. J. and J. Jelfs. 1997. Typing *of Neisseria meningitidis* by restriction analysis of the amplified porA gene. Pathology. 29,(2), 201-5.

Spratt, B. G., 1999. Multilocus sequence typing: molecular typing of bacterial pathogens in an era of rapid DNA sequencing and the internet. Curr Opin Microbiol. 2,(3), 312-316.

Squires, S. G., L. Pelletier, M. Mungai, R. Tsang, F. Collins and J. Stoltz. 2000. Invasive meningococcal disease in Canada, 1 January 1997 to 31 December 1998. Can Commun Dis Rep. 26,(21), 177-182.

Stefanelli, P., C. Fazio and P. Mastrantonio. 2001. Typing of *Neisseria meningitidis* isolates from patients with invasive disease by molecular analysis of porin genes. New Microbiol. 24,(2), 149-155.

Stephens, D. S. and Z. A. McGee. 1983. Association of virulence of *Neisseria meningitidis* with transparent colony type and low-molecular-weight outer membrane proteins. J Infect Dis. 147,(2), 282-292.

Struelens, M. J., 1996. Consensus guidelines for appropriate use and evaluation of microbial epidemiological typing systems. Clin Microbiol Infect. 2, 2-11.

Stuart, J. M., N. Middleton and D. J. Gunnell. 2002. Socioeconomic inequality and meningococcal disease. Commun Dis Public Health. 5,(4), 327-328.

Suker, J., I. M. Feavers, M. Achtman, G. Morelli, J. F. Wang and M. C. Maiden. 1994. The porA gene in serogroup A meningococci: evolutionary stability and mechanism of genetic variation. Mol Microbiol. 12,(2), 253-265.

Suker, J., I. M. Feavers and M. C. Maiden. 1993. Structural analysis of the variation in the major outer membrane proteins of *Neisseria meningitidis* and related species. Biochem Soc Trans. 21,(2), 304-306.

Sun, Y. H., S. Bakshi, R. Chalmers and C. M. Tang. 2000. Functional genomics of *Neisseria meningitidis* pathogenesis. Nat Med. 6,(11), 1269-1273.

Sutcliffe, E. M., Jones, D.M., El-sheikh, S., and Percival, A., 1988. Penicillin-insensitive meningococci in the UK. Lancet. 657-658.

Swaminathan, B., G. M. Matar, M. W. Reeves, L. M. Graves, G. Ajello, W. F. Bibb, L. O. Helsel, M. Morales, H. Dronavalli, M. el-Swify, W. DeWitt and S. B. Hunter. 1996. Molecular subtyping of *Neisseria meningitidis* serogroup B: comparison of five methods. J Clin Microbiol. 34,(6), 1468-1473.

Swartley, J. S., L. J. Liu, Y. K. Miller, L. E. Martin, S. Edupuganti and D. S. Stephens. 1998. Characterization of the gene cassette required for biosynthesis of the (alpha1>6)-linked N-acetyl-D-mannosamine-1-phosphate capsule of serogroup A *Neisseria meningitidis*. J Bacteriol. 180,(6), 1533-1539.

Taha, M. K., 2000. Simultaneous approach for nonculture PCR-based identification and serogroup prediction of *Neisseria meningitidis*. J Clin Microbiol. 38,(2), 855-857.

Taha, M. K., A. E. Deghmane, A. Antignac, M. L. Zarantonelli, M. Larribe and J. M. Alonso. 2002. The duality of virulence and transmissibility in *Neisseria meningitidis*. Trends Microbiol. 10,(8), 376-382.

Tait, R. C., 1999. The application of molecular biology. Current Issues in Molecular Biology. 1,(1), 1-12.

Tappero, J. W., R. Lagos, A. M. Ballesteros, B. Plikaytis, D. Williams, J. Dykes, L. L. Gheesling, G. M. Carlone, E. A. Hoiby, J. Holst, H. Nokleby, E. Rosenqvist, G. Sierra, C. Campa, F. Sotolongo, J. Vega, J. Garcia, P. Herrera, J. T. Poolman and B. A. Perkins. 1999. Immunogenicity of 2 serogroup B outer-membrane protein meningococcal vaccines: a randomized controlled trial in Chile. Jama. 281,(16), 1520-1527.

Tarafdar, K., S. Rao, R. A. Recco and M. M. Zaman. 2001. Lack of sensitivity of the latex agglutination test to detect bacterial antigen in the cerebrospinal fluid of patients with culture-negative meningitis. Clin Infect Dis. 33,(3), 406-408.

Tärnberg, M., Jakobsson, T., Jonasson, J and Forsum, U., 2002. Identification of randomly selected colonies of lactobacilli from normal vaginal fluid by pyrosequeincg of the 16S rDNA variable VR1 and VR3 regions. Apmis. 110,(11), 801-810.

Tinsley, C. and X. Nassif. 2001. Meningococcal pathogenesis: at the boundary between the pre- and post-genomic eras. Curr Opin Microbiol. 4,(1), 47-52.

Tinsley, C. R. and J. E. Heckels. 1986. Variation in the expression of pili and outer membrane protein by *Neisseria meningitidis* during the course of meningococcal infection. J Gen Microbiol. 132 (Pt 9), 2483-90.

Tondella, M. L., T. Popovic, N. E. Rosenstein, D. B. Lake, G. M. Carlone, L. W. Mayer and B. A. Perkins. 2000. Distribution of *Neisseria meningitidis* serogroup B serosubtypes and serotypes circulating in the United States. The Active Bacterial Core Surveillance Team. J Clin Microbiol. 38,(9), 3323-3328.

Tondella, M. L., M. W. Reeves, T. Popovic, N. Rosenstein, B. P. Holloway and L. W. Mayer. 1999. Cleavase fragment length polymorphism analysis of *Neisseria meningitidis* basic metabolic genes. J Clin Microbiol. 37,(8), 2402-2407.

Tondella, M. L., N. E. Rosenstein, L. W. Mayer, F. C. Tenover, S. A. Stocker, M. W. Reeves and T. Popovic. 2001. Lack of evidence for chloramphenicol resistance in *Neisseria meningitidis*, Africa. Emerg Infect Dis. 7,(1), 163-164.

Townsend, R., L. Goodwin, T. M. Stevanin, P. B. Silcocks, A. Parker, M. C. Maiden and R. C. Read. 2002. Invasion by *Neisseria meningitidis* varies widely between clones and among nasopharyngeal mucosae derived from adult human hosts. Microbiology. 148,(Pt 5), 1467-1474.

Tsai, C. M., C. E. Frasch and L. F. Mocca. 1981. Five structural classes of major outer membrane proteins in *Neisseria meningitidis*. J Bacteriol. 146,(1), 69-78.

Tsang, R. S., D. Law and S. G. Squires. 2001. Two serologically non-groupable *Neisseria meningitidis* strains from clinical specimens identified by molecular method as serogroup B meningococci. Can Commun Dis Rep. 27,(1), 9-12.

Tsend, N., B. Enkhbold, N. P. Deviatkina, V. Martynov Iu and A. A. Demina. 1992. The evolution of meningococcal infection in Mongolia. Zh Mikrobiol Epidemiol Immunobiol.(11-12), 27-30.

Tunkel, A. R. and W. M. Scheld. 2002. Treatment of Bacterial Meningitis. Curr Infect Dis Rep. 4,(1), 7-16.

Tyagi, S., and Kramer, F.R., 1996. Molecular beacons: probes that fluoresce upon hybridisation. Nat Biotechnol. 14, 303-308.

Tzanakaki, G., C. C. Blackwell, J. Kremastinou, C. Kallergi, G. Kouppari and D. M. Weir. 1992. Antibiotic sensitivities of *Neisseria meningitidis* isolates from patients and carriers in Greece. Epidemiol Infect. 108,(3), 449-455.

Tzanakaki, G., R. Urwin, M. Musilek, P. Kriz, J. Kremastinou, A. Pangalis, C. C. Blackwell and M. C. Maiden. 2001. Phenotypic and Genotypic Approaches to Characterization of Isolates of *Neisseria meningitidis* from Patients and Their Close Family Contacts. J Clin Microbiol. 39,(4), 1235-1240.

Unnerstad, H., Ericsson, H., Alderborn, A., Tham, W., Danielsson-Tham, M.L., and Mattsson, J.G., 2001. Pyrosequencing as a method for grouping *Liseria monocytogenes* strains on the basis of single-nucleotide polymorphism in the inIB gene. Appl Environ Microbiol. 67,(11), 5339-5342.

Urwin, R., I. M. Feavers, D. M. Jones, M. C. Maiden and A. J. Fox. 1998a. Molecular variation of meningococcal serotype 4 antigen genes. Epidemiol Infect. 121,(1), 95-101.

Urwin, R., A. J. Fox, M. Musilek, P. Kriz and M. C. Maiden. 1998b. Heterogeneity of the PorB protein in serotype 22 *Neisseria meningitidis*. J Clin Microbiol. 36,(12), 3680-3682.

Van der Ende, A., C. T. P. Hopman and J. Dankert. 2000. Multiple mechanisms of phase variantion of *porA* in *Neisseria meningitidis*. Infect and Immun. 68,(12), 6685-6690.

Van Belkum, A., S. Scherer, W. Van Alplen and H. Verbrugh. 1998. Short-sequence DNA repeats in prokcryotic genomes. Microbiol. Mol. Biol. Rev. 62,(2), 275-293.

Van Belkum, A., S. Scherer, W. Van Leeuwen, D. Willease, C. Van Alplen and H. Verbrugh. 1997. Variable number of tandem repeats in clinical strains of *Haemophilus influenzae*. Infect and Immun. 65,(12), 5017-5027.

Van der Ende, A., C. T. Hopman, W. C. Keijzers, L. Spanjaard, E. B. Lodder, P. H. van Keulen and J. Dankert. 2003. Outbreak of meningococcal disease caused by PorA-deficient meningococci. J Infect Dis. 187,(5), 869-871.

Van der Ende, A., I. G. Schuurman, C. T. Hopman, C. A. Fijen and J. Dankert. 1995. Comparison of commercial diagnostic tests for identification of serogroup antigens of *Neisseria meningitidis*. J Clin Microbiol. 33,(12), 3326-3327.

Van der Ley, P., J. E. Heckels, M. Virji, P. Hoogerhout and J. T. Poolman. 1991. Topology of outer membrane porins in pathogenic *Neisseria spp*. Infect Immun. 59,(9), 2963-2971.

Van Der Ley, P. and J. T. Poolman. 1992. Construction of a multivalent meningococcal vaccine strain based on the class 1 outer membrane protein. Infect Immun. 60,(8), 3156-3161.

Van der Voort, E. R., P. van der Ley, J. van der Biezen, S. George, O. Tunnela, H. van Dijken, B. Kuipers and J. Poolman. 1996. Specificity of human bactericidal antibodies against PorA P1.7,16 induced with a hexavalent meningococcal outer membrane vesicle vaccine. Infect Immun. 64,(7), 2745-2751.

Van Deuren, M., P. Brandtzaeg and J. W. van der Meer. 2000. Update on meningococcal disease with emphasis on pathogenesis and clinical management. Clin Microbiol Rev. 13,(1), 144-66.

Van Deuren, M. and J. F. Meis. 2001. Meningococcal disease. N Engl J Med. 345,(9), 699.

Van Esso, D., Fontanals, D., Uriz, S., Morera, M.A., Juncosa, T., Latorre, C., and Duran, M., 1987. *Neisseria meningitidis* strains with decreased susceptibility to penicillin. Pediatr Infect Dis J. 6, 438-439.

Van Loo, I. H., K. J. Heuvelman, A. J. King and F. R. Mooi. 2002. Multilocus Sequence Typing of *Bordetella pertussis* Based on Surface Protein Genes. J Clin Microbiol. 40,(6), 1994-2001.

Van Looveren, M., D. A. Caugant, S. Chapelle, F. Carion and H. Goossens. 2001. Interpreting the rising incidence of meningococcal disease in Belgium: the contribution of molecular typing. J Med Microbiol. 50,(11), 986-990.

Verdu, M. E., P. Coll, J. A. Vazquez, F. March, D. Fontanals, S. Berron, I. Pons, D. Van Esso and G. Prats. 2001. Association between asymptomatic carriage and sporadic (endemic) meningococcal disease in an open community. Epidemiol Infect. 127,(2), 245-259.

Vermont, C. L. and G. P. Van Den Dobbelsteen. 2003. Meningococcal serogroup B infections: a search for a broadly protective vaccine. Expert Rev Vaccines. 2,(5), 673-681.

Vieusseux, M., 1806. Memoire sur la maladie qui regne a Geneve au printemps de 1805. J de Medecine, de Chirurgie et de Pharmacologie. 11, 163-182.

Vimont-Vicary, P. and F. Rogerie. 1983. Epidemic of Neisseria meningitidis cerebrospinal meningitis in the Ruhengeri health district (Rwanda). Med Trop (Mars). 43,(2), 155-161.

Virji, M., 1996. Meningococcal disease: epidemiology and pathogenesis. Trends Microbiol. 4,(12), 466-9; discussion 469-470.

Virji, M., K. Makepeace, I. R. Peak, D. J. Ferguson, M. P. Jennings and E. R. Moxon. 1995. Opc- and pilus-dependent interactions of meningococci with human endothelial cells: molecular mechanisms and modulation by surface polysaccharides. Mol Microbiol. 18,(4), 741-754.

Virji, M., K. Makepeace, I. R. Peak, D. J. Ferguson and E. R. Moxon. 1996. Pathogenic mechanisms of *Neisseria meningitidis*. Ann N Y Acad Sci. 797, 273-276.

Viscidi, R. P., and R.G. Yolken. 1987. Molecular diagnosis of infectious disease by nucleic acid hybridisation. Mol Cell Probes. 1, 3-14.

Vogel, U., H. Claus and M. Frosch. 2000. Rapid serogroup switching in *Neisseria meningitidis*. N Engl J Med. 342,(3), 219-220.

Wainwright, M. (1990). Miracle Cure: The story of penicillin and the golden age of antibiotics. Oxford, Basil Blackwell.

Wall, R. A., 2001. Meningococcal disease - some issues in treatment. J Infect. 42,(2), 87-99.

Wang, J., G. A. Jarvis, M. Achtman, E. Rosenqvist, T. E. Michaelsen, A. Aase and J. M. Griffiss. 2000. Functional activities and immunoglobulin variable regions of human and murine monoclonal antibodies specific for the P1.7 PorA protein loop of *Neisseria meningitidis*. Infect Immun. 68,(4), 1871-1878.

Ward, M. J., P. R. Lambden and J. E. Heckels. 1992. Sequence analysis and relationships between meningococcal class 3 serotype proteins and other porins from pathogenic and non-pathogenic *Neisseria species*. FEMS Microbiol Lett. 73,(3), 283-289.

Wasson, J., G. Skolnick, L. Love-Gregory and M. A. Permutt. 2002. Assessing allele frequencies of single nucleotide polymorphisms in DNA pools by pyrosequencing technology. Biotechniques. 32,(5), 1144-6, 1148, 1150.

Wedege, E. and T. E. Michaelsen. 1987. Human immunoglobulin G subclass immune response to outer membrane antigens in meningococcal group B vaccine. J Clin Microbiol. 25,(8), 1349-1353.

Weichselbaum, A., 1887. Ueber die aetiologie der akuten meningitis cerebrospinalis. Fortschr Med. 5, 573-583, 620-626.

Weiss, D. P., P. Coplan and H. Guess. 2001. Epidemiology of bacterial meningitis among children in Brazil, 1997-1998. Rev Saude Publica. 35,(3), 249-255.

Welch, S. B. and S. Nadel. 2002. Treating meningococcal infections in children. Hosp Med. 63,(5), 268-273.

Westin, L., Miller, C., Vollmer, D., Canter, D., Radtkey, R., Nerenberg, M., and O'Connell, J.P., 2001. Antimicrobial resistance and bacterial identification utilising a microelectronic chip array. J Clin Microbiol. 39, 1097-1104.

Wheeler, J. S., B. J. Anderson and T. M. De Chalain. 2003. Surgical interventions in children with meningococcal purpura fulminans - a review of 117 procedures in 21 children. J Pediatr Surg. 38,(4), 597-603.

WHO (1998). Control of epidemic meningococcal disease., World Health Organisation.

Wilcox, M. H. and N. Modi. 2000. Meningococcal disease in healthcare workers. Ceftriaxone may be helpful. Bmj. 320,(7229), 248-249.

Wilder-Smith, A., K. T. Goh, T. Barkham and N. I. Paton. 2003. Hajj-associated outbreak strain of *Neisseria meningitidis* serogroup W135: estimates of the attack rate in a defined population and the risk of invasive disease developing in carriers. Clin Infect Dis. 36,(6), 679-683.

Wildes, S. S. and A. R. Tunkel. 2002. Meningococcal vaccines: a progress report. BioDrugs. 16,(5), 321-329.

Williams, D. W., Wilson, M.J., and Lewis, M.A.O., 1999. Deoxyribonucleic acid typing methods for medically important microorganisms. Br J Biomed Sci. 56, 56-65.

Woods, C. R., A. L. Smith, B. L. Wasilauskas, J. Campos and L. B. Givner. 1994. Invasive disease caused by *Neisseria meningitidis* relatively resistant to penicillin in North Carolina. J Infect Dis. 170,(2), 453-456.

Wright, P. A., and Wynford-Thomas, D., 1990. The polymerase chain reaction: miricale or mirage? A critical review of its uses and limitations in diagnosis and research. Journal of pathology. 162, 99-117.

Wylie, P. A., D. Stevens, W. Drake, 3rd, J. Stuart and K. Cartwright. 1997. Epidemiology and clinical management of meningococcal disease in west Gloucestershire: retrospective, population based study. Bmj. 315,(7111), 774-779.

Yakubu, D. E., F. J. Abadi and T. H. Pennington. 1999. Molecular typing methods for *Neisseria meningitidis*. J Med Microbiol. 48,(12), 1055-1064.

Yakubu, D. E. and T. H. Pennington. 1995. Epidemiological evaluation of *Neisseria meningitidis* serogroup B by pulsed-field gel electrophoresis. FEMS Immunol Med Microbiol. 10,(3-4), 185-189.

Yusuf, H. R., R. W. Rochat, W. S. Baughman, P. M. Gargiullo, B. A. Perkins, M. D. Brantley and D. S. Stephens. 1999. Maternal cigarette smoking and invasive meningococcal disease: a cohort study among young children in metropolitan Atlanta, 1989-1996. Am J Public Health. 89,(5), 712-717.

Zagursky, R. J., S. B. Olmsted, D. P. Russell and J. L. Wooters. 2003. Bioinformatics: how it is being used to identify bacterial vaccine candidates. Expert Rev Vaccines. 2,(3), 417-436.

Zollinger, W. D., E. E. Moran, H. Connelly, R. E. Mandrell and B. Brandt. 1984. Monoclonal antibodies to serotype 2 and serotype 15 outer membrane proteins of Neisseria meningitidis and their use in serotyping. Infect Immun. 46,(1), 260-266.

Zollinger, W. D., E. E. Moran, S. J. Devi and C. E. Frasch. 1997. Bactericidal antibody responses of juvenile rhesus monkeys immunized with group B Neisseria meningitidis capsular polysaccharide-protein conjugate vaccines. Infect Immun. 65,(3), 1053-1060.

Appendix

Figure 6.1 List of all porin A variable region 1 (VR1) types. These are represented in peptide sequence format.

VARIANT NAME	VR1 PEPTIDE SEQUENCE
P1.5 FAMILY	
5	PLQNIQPQVTKR
5-1	PLQNIQQPQVTKR
5-2	PLPNIQPQVTKR
5-3	PLQNIKQPQVTKR
5-4	PLQNIQKPQVTKR
5-5	PLQNIQPSVTKR
5-6	LLQNIQQPQVTKR
5-7	PLSNIQPQVTKR
5-8	PIQNIQQPQVTKR
5-9	PLLNIQPQVTKR
5-10	PFQNIQPQVTKR
P1.7 FAMILY	
7	AQAANGGASGQVKVTKVTKA
7-1	AQAANGGAGASGQVKVTKVTKA
7-2	AQAANGGASGQVKVTKA
7-3	AQAANGGARASGQVKVTKVTKA
7-4	AQAANGGAGASGQVKVTKA
7-5	AQAANGGAVASGQVKVTKVTKA
7-6	AQAANGGASDQVKVTKA
7-7	AQSANGGASGQVKVTKVTKA
7-8	AQAANGGAGASGQVKVTKVTKVTKA
7-9	AQAANGGASGANGGASGQVKVTKA
7-10	AQAANGGVSGQVKVTKVTKA
7-11	AQAANGGASGQVKVTKVTKVTKA
7-12	AQAANGGARGQVKVTKVTKA
7-13	AQAANGGARGQVKVTKVTKVTKA
7-14	AQAVNGGASGQVKVTKA
7-15	AQAANGGAGASGQVKVTKVTKV
7-16	AQAANDGASGQVKVTKVTKA
P1.12 FAMILY	
12	KLSSTNAKTGNKVEVTKA
12-1	KPSSTNAKTGNKVEVTKA
12-2	KPSSTKAKTGNKVEVTKA
12-3	KPSSTNAKTGNKVKVTKA
12-4	KSSNTNAKTSNKVEVTKA
12-5	KPSSTNPKTGNKVEVTKA
12-6	QPSNTNGKTGNKVEVTKA
12-7	KPSSTNANSSTNAKTGNKVEVTKA
12-8	KPSSTNAKTSNEVEVTKA
12-9	KPSSTNATTGNKVEVTKA
12-10	KPSSTNAKTDNKVEVTKA

VARIANT NAME	VR1 PEPTIDE SEQUENCE
P1.17 FAMILY	
17	PPQKNQSQPVVTKA
17-1	PPPKNQSQPVVTKA
17-2	PPQKNQSQPLVTKA
P1.18 FAMILY	
18	PPSKGQTGNKVTKG
18-1	PPSQGQTGNKVTKG
18-2	PPSKSQTGNKVTKG
18-3	PPSKGQTGNKVTKA
18-4	PPSKGQTGNKVIKG
18-5	PPSKGQVGNKVTKG
18-6	QLSKGQVGNKVTKG
18-7	QPSKGQVGNKVTKG
18-8	PPSKGQTGNKVTNG
18-9	PPPKDQTGNKVTKG
18-10	PPSEGQTGNTVTKA
18-11	PPSQGQTGNKVTKA
P1.19 FAMILY	
19	PPSKSQPQVKVTKA
19-1	PPSKSQSQVKVTKA
19-2	PPSKSQLQVKVTKA
19-3	PRSKSQPQVKVTKA
19-4	PPSNSQPQVKVTKA
19-5	PLSKSQPQVKVTKA
19-6	PPLKSQPQVKVTKA
19-7	PSSKSQPQVKVTKA
19-8	PPPKSQPQVKVTKA
19-9	PPSKSQPQVKVTQVKVTKA
19-10	PHSKSQPQVKVTKA
19-11	PPSRSQPQVKVTKA
19-12	PSSKSQSQVKVTKA
19-13	PPSKSQTQVKVTKA
19-14	PPSKSQHQVKVTKA
19-15	PPSESQPQVKVTKA
19-16	PLSKSQSQVKVTKA
19-17	PLSKSQHQVKVTKA
P1.20 FAMILY	
20	QPQTANTQQGGKVKVTKA

VARIANT NAME	VR1 PEPTIDE SEQUENCE
P1.21 FAMILY	
21	QPQVTNGVQGNQVKVTKA
21-1	QPNGVQGNQVKVTKA
21-2	QPQATNGVQGGQQGNQVKVTKA
21-3	QPQVTKGVQGNQVKVTKA
21-4	QPQVPNGVQGNQVKVTKA
21-5	QPQVPNSVQGNQVKVTKA
21-6	QPQATNGVQGGRQGNQVTVTKA
21-7	QLQVTNGVQGNQVKVTKA
21-8	QPQVTTGVQGNQVKVTKA
P1.22 FAMILY	
22	QPSKAQGQTNNQVKVTKA
22-1	QPSRTQGQTSNQVKVTKA
22-2	QPSRTQAQTSNQVKVTKA
22-3	QPSKAKGQTNNQVKVTKA
22-4	QLSKAQGQTNNQVKVTKA
22-5	QPSKAQGQTNNQVKVTKR
22-6	QPSRTQGQTRNQVKVTKA
22-7	QPSKAQGQTNNQVEVTKA
P1.31 FAMILY	
31	PPSSNQGKNQAQTGNTVTKA

Figure 6.2 List of all porin A variable region 2 (VR2) types. These are represented in peptide sequence format.

VARIANT NAME	VR2 PEPTIDE SEQUENCE
P1.1 FAMILY	
1	YVAVENGVAKKVA
1-1	YVAVENGATKKVA
1-2	YVAVENGVVKKVA
1-3	YVAVENGVAKKVT
P1.2 FAMILY	
2	HFVQQTPKSQPTLVP
2-1	HFVQQPPKSQPTLVP
2-2	HFVQQTPQSQPTLVP
2-3	HFVQQPPKSQLTLVP
2-4	HFVQQTPQSRPTLVP
2-5	HFVQQIPQSQPTLVP
2-6	HFVQQTPTLVP
2-7	HFVQQTSKSQPTLVP
2-8	HFVQQTTKSQPTLVP
2-9	HFVQQTPQSKPTLVP
2-10	HFVQQAPQSQSTLVP
2-11	HFVLQTPQSQPTLVP
2-12	HFVQQIPKSQPTLVP
2-13	YFVQQTPQSQPTLVP
2-14	HFVQQKLASKPTLVP
2-15	HFVQQKSTSKPTLVP
2-16	HFVQQKPTSKPTLVP
2-17	HFVQQQPTSEPTLVP
2-18	HFVQQIPKSQPILVP
2-19	HFVQQTSQSQPTLVP
2-20	HFVQQTPIVQQTPKSQPTLVP
2-21	HFVQQTHQSQPTLVP
2-22	HSVQQTPKSQPTLVP
2-23	HFVQQTPKSQPPLVP
2-24	HFVQQTPTHFVQQTPKSQPTLVP
2-25	HFVQQTPKSVP
2-26	HFVQQTPQRQPTLVP
2-27	HFVQQTPNSQPTLVP
2-28	PQSQPTLVP (MISSING HFVQQ MOTIF)
2-29	HFVQQTPQSQTPQSQPTLVP
P1.3 FAMILY	
3	TLANGANNTIIRVP
3-1	TVANGANNTIIRVP
3-2	TLANGANDTIIRVP
3-3	TLANGADNTIIRVP
3-4	TPANGANNTIIRVP

VARIANT NAME	VR2 PEPTIDE SEQUENCE
3-5	TLAKGANNTIIRVP
3-6	TLANGATNTIIRVP
3-7	TLATLANGANNTIIRVP
P1.4 FAMILY	
4	IIVVVNNKVATHVP
4-1	HVVVNNNVATHVP
4-2	HVVVNNKVATHVPAKVATHVP
4-3	HVVVNNKVTTHVP
4-4	HVVVNNKVATPHVP
4-5	HVVVNNKV (MISSING THVP MOTIF)
4-6	HVVVNNRVATHVP
P1.9 FAMILY	
9	YVDEQSKYHA
9-1	YVDSKYHA
9-2	YVGEQSKYHA
9-3	YVDEQSKDHA
9-4	YVDKQSKYHA
9-5	YVDEQSEYHA
9-6	YVDEQSQYHA
P1.10 FAMILY	
10	HFVQNKQNQRPTLVP
10-1	HFVQNKQNQPPTLVP
10-2	HFVQDKKGQPPTLVP
10-3	HFVQNKQNQQPTLVP
10-4	HFVQNKQNKQNQPPTLVP
10-5	HFVQNKQSQRPTLVP
10-6	HFVQNKQNQQNQQNQPPTLVP
10-7	HFVQNKQNKPPTLVP
10-8	HFVQNKQNQQNQPPTLVP
10-9	HFVQNKQNKQNQLPTLVP
10-10	HFVQNKQNKQNKQNQPPTLVP
10-11	HFVQNKQNQRSTLVP
10-12	HFVQNKQNQLPTLVP
10-13	HFVQNKQNKKNQPPTLVP
10-14	HFVQNKQHQPPTLVP
10-15	HFVQNKQNQPSTLVP
10-16	HFVQNKQNQWSTLVP
10-17	HFVQNKQNQTPTLVP
10-18	HFVQNKQSQPPTLVP
10-19	HFVQNKQNKQKQPPTLVP
10-20	HFVQNKQNQWLTLVP
10-21	HFVPDKKGQPPTLVP
10-22	HFVQNKQNKQNQQNQPPTLVP
10-23	HFVQNKQNQWPTLVP
10-24	HFVKNKQNQRPTLVP
10-25	HFVQDKKGQP (missing PTLVP motif)
10-26	HFVQNKQNKPNQPPTLVP

VARIANT NAME	VR2 PEPTIDE SEQUENCE
10-27	HFVRNKQNQRPTLVP
10-28	HFVQNQNKQNQPPTLVP
10-29	HFVQNKQNQSPTLVP
10-30	HVPNKQNQRPTLVP
10-31	HFVQNKQDQRPTLVP
10-32	HFVQNKQNKQPTLVP
10-33	YFVQNKQNKQNQPPTLVP
10-34	HFVQNKQNQQNQQPTLVP
10-35	HFVQNKQNQQNQQPTLVP
10-36	HFVQDKKGQLPTLVP
P1.13 FAMILY	
13	YWTTVNTGSATTTTTFVP
13-1	YWTTVNTGSATTTTFVP
13-2	YWTTVNTGSATTTFVP
13-3	YWTTVNTGSATITTFVP
13-4	YYTTVTQGSATTTTFVP
13-5	YWTTVNTGSATTTTTTTTTFVP
13-6	YWTTVNTGSATTTTTTTTFVP
13-7	YWTTVNTGSATTTTTTFVP
13-8	YWITVNTGSATTTTFVP
13-9	YWTTVNTGSATTFVP
13-10	YWTTVNTGSVTTTTFVP
13-11	YWTTVNTGSAATTTTFVP
13-12	YWTAVNAGSATTTFVP
13-13	YWTTVNNGNATTTTFVP
13-14	YWTTVNTSSATTTTTFVP
13-15	YWTTVNTGSATTTTTTTTFVP
13-16	VNTGSATTTFVP (MISSING YWT MOTIF)
13-17	YWTTVNTGNATTTTFVP
P1.14 FAMILY	
14	YVDEKKMVHA
14-1	YVDEKKKMVHA
14-2	YVDEKKKVHA
14-3	YVDEKNMVHA
14-4	YVDENKMVHA
14-5	YVDKEQVSHA
14-6	YVDEKQVSHA
14-7	YVDETKMVIIA
14-8	YVDEKRMVHA
14-9	YVDAKKMVHA
14-10	YVDEKGMVHA
14-11	YVDEKRVSH
14-12	YVNEKKMVHA

VARIANT NAME	VR2 PEPTIDE SEQUENCE
P1.15 FAMILY	
15	HYTRQNNADVFVP
15-1	HYTRQNNTDVFVP
15-2	HYTRQNNNNTDVFVP
15-3	HYTRPNNTDVFVP
15-4	HYNTRQNNADVFVP
15-5	HYTRQNSADVFVP
15-6	HYTRQNYADVFVP
15-7	HYTRQNNANVFVP
15-8	HYTRQNNAGVFVP
15-9	HYTRQNNTRQNNADVFVP
15-10	HYTGQNNADVFVP
15-11	HYTRQNNIDVFVP
15-12	HYNTRQNNIDVFVP
15-13	HYTRQNNQNNIDVFVP
15-14	HYTNTRQNNIDVFVP
15-15	HYTRQSNTDVFVP
15-16	HYTRQNNADFVP
15-17	HYTRQNNAYVFVP
15-18	HYTRQNNDTRQNNADVFVP
15-19	HTRQNNIDVFVP
15-20	HYTRQNNAAVFVP
15-21	HYTRQNDADVFVP
P1.16 FAMILY	
16	YYTKDTNNNLTLVP
16-1	YYTKGKNNALTLVP
16-2	YYTKNTNNNLTLVP
16-3	YYTKDKNDNLTLVP
16-4	YYTKDKNDKLTLVP
16-5	YYTKDTNNNNNLTLVP
16-6	YYTKHTNNNLTLVP
16-7	YYTKDTNTKDTNNNLTLVP
16-8	YYTKDKNNALTLVP
16-9	YYTKDTNDLTLVP
16-10	YYTNNNLTLVP
16-11	YYTTDTNNNLTLVP
16-12	YYTKDTNDNLTLVP
16-13	YYTEDTNNNLTLVP
16-14	YYTKDTNTNLTLVP
16-15	YYNTKDTNNNLTLVP
16-16	YYTKDTNNNPTLVP
16-17	YYTKDTNNTNNNLTLVP
16-18	YYTKDTNTNNNLTLVP
16-19	YYTKDTNNNLTHTKDTNNNLTLVP
16-20	KDTNNNLTLVP
16-21	YYTKDTKNNLTLVP
16-22	YYTKDTNNILTLVP

VARIANT NAME	VR2 PEPTIDE SEQUENCE
16-23	YYTKDNKNDNLTLVP
16-24	YYTKVENDNLTLVP
16-25	YYTKDTNNNLNLTLVP
16-26	YYTNTNNNLTLVP
16-27	YYTKDTNNNLTLVS
16-28	YYTKVKNDNLTLVP
16-29	YYTKGTNNDLTLVP
16-30	YYTKDKNDNRTLVP
16-31	YYTKTNNNPTLVP
16-32	YYTKVTNNNLTLVP
16-33	YYTNTKDTNNNLTLVP
16-34	YYTKDTNNNLKDTNNNLTLVP
16-35	YYTKHANNNLTLVP
16-36	YYTKGTNNNPTLVP
16-37	YYTKVTDNNLTLVP
16-38	YYTKDTNNNLPLVP
16-39	YYTKDTTNNNLTLVP
16-40	YYNTKDTKNNLTLVPA
16-41	YYTKDTNNKLTLVP
16-42	YYTKDTNYTKDTNNNLTLVP
16-43	YYTKDTNNNLTNNNLTLVP
16-44	YYTNTNDNLTLVP
16-45	YYTNYTKDTNNNLTLVP
P1.23 FAMILY	
23	HWNTVYNTNGTTTTFVP
23-1	HWNTVYNTNGTTTTTTTFVP
23-2	HWNTVYNTNGTTTTTFVP
23-3	HWNTVYNTNGTTTTTTFVP
23-4	HWTTVYNTNGTTTFVP
P1.25 FAMILY	
25	TYTVDSSGVVTPVP
25-1	TYTVDSSGVFTPVP
25-2	TYTEGSSGVFTPVP
25-3	TYTVDSSGVVTPLP
25-4	TYTVGSRDVVTPVP
25-5	TYTVDSSNVVTPVP
25-6	TYTVDSGVVTPVP
25-7	YTVDSSGVVTPVP
25-8	TYTVDSSGVP
25-9	TYTVDNSSVVTPVP
25-10	TYTVDSSRVVTPVP
25-11	TYTVDSSDVVTPVP
25-12	TYTVDSSSVVTVPV
25-13	TYTVDSMDSSGVVTPVP

VARIANT NAME	VR2 PEPTIDE SEQUENCE
P1.26 FAMILY	
26	HFVADSQGKITRVP
26-1	HFVADSQGEITRVP
26-2	YFTADPNDQNKITRVP
P1.28 FAMILY	
28	YYYTTATNSSTSTTFVP
P1.30 FAMILY	
30	HYTTVYNATTTTTTFVP
30-1	HYTTVYNATTTTTFVP
30-2	HYTTVYNATTTTTTTFVP
30-3	HYTTVYNATTTTTTTTFVP
30-4	HYTTVYNATTTTTTTTTFVP
30-5	HYTTVYNATTTTTTTTTTFVP
30-6	HYTTVYNATTTTTTTTTTTFVP
30-7	HYTTVYNATTTTTTTTTTTTTFVP
P1.34 FAMILY	
34	YVDDQGKVKGP
34-1	YVDDQKVKGP
P1.42 FAMILY	
42	HLVLDGQGKITQVP
42-1	HLVSDGQGKITQVP
P1.43 FAMILY	
43	TFTLESNQMKPVP

Figure 6.3 List of all porin A variable region 3 (VR3) types. These are represented in peptide sequence format.

VARIANT NAME	VR3 PEPTIDE SEQUENCE
35 FAMILY	
35	LIGSGSDQ
35-1	LLGSGSDQ
35-2	SLGSGSDQ
36 FAMILY	
36	LLGSTSDE
36-1	LLGSTSDQ
36-2	LLGSGSDE
36-3	LLGSASDE
37 FAMILY	
37	LIGSATSDQ
37-1	LIGSATSDE
38 FAMILY	
38	LLGRIGDDDE
38-1	LLGRIGEDDE
39 FAMILY	
39	LLGSGSDG
40 FAMILY	
40	LLGRSGDDDE
41 FAMILY	
41	LLGRGSDE

Figure 6.4 Polymorphic sites associated with each allelic variant within each housekeeping gene from meningococci isolated in 1999 (year 1). Each housekeeping gene is represented by its abbreviated initials followed by the total number of different alleles characterised followed by the number of polymorphic sites. These are displayed within the nucleotide sequence following this information. A consensus sequence is displayed for each housekeeping gene and if a variation within the sequence is identified the alternative base pair is displayed directly below the consensus nucleotide sequence base pair.

abcZ

14 alleles, 60 polymorphic sites

```
   1       10        20        30        40        50
   TTTGATACCGTTGCCGAAGGTTTGGGTGAAATTCGCGATTTATTGCGCCG
            T                  CA        T

  51       60        70        80        90       100
   TTACCACCGCGTCGGCCATGAGTTGGAAAACGGTTCGGGTGAGGCTTTGT
      T  T AT    A T      T      GC TAA    AAAC  C TC  A

 101      110       120       130       140       150
   TGAAAGAACTCAACGAATTACAACTTGAAATCGAAGCGAAGGACGGCTGG
    A     G           G      C CG                T

 151      160       170       180       190       200
   AAGCTGGATGCGGCAGTCAAGCAGACTTTGGGGGAACTCGGTTTGCCGGA
     AT          G  G                C

 201      210       220       230       240       250
   AAACCAAAAAATCGGCAACCTTTCCGCCGCTCAGAAAAAGCGTGTCGCCT
      T                  C                      C

 251      260       270       280       290       300
   TGGCGCAGGCTTGGGTGCAGAAGCCCGACGTATTGCTGCTGGACGAACCG
       T               A                     C  T  G

 301      310       320       330       340       350
   ACCAACCATTTGGATATCGACGCGATTATTTGGCTGGAAAATCTGCTCAA
                  C  T           C   T       C

 351      360       370       380       390       400
   AGCGTTTGAAGGCAGCTTGGTTGTGATTACCCACGACCGCCGTTTTTTGG
   G                C    C               T

 401      410       420       430
   ACAATATCGCCACGCGGATTGTCGAACTCGATC
    T        T     C  C  T     T  C
```

adk

8 alleles, 14 polymorphic sites

```
  1        10         20         30         40         50
  GAAGCGAAAAAAATCATTGACGAAGGCGGCTTGGTGCGCGACGACATCAT
                          T

 51        60         70         80         90        100
  TATCGGCATGGTCAAAGAACGCATCGCGCAAGACGACTGCAAAAACGGTT

101       110        120        130        140        150
  TCCTGTTCGACGGTTTCCCGCGCACATTGGCACAAGCCGAAGCGATGGTT
    T     T                   GC        G

151       160        170        180        190        200
  GAAGCAGGCGTGGATTTGGATGCAGTCGTTGAAATCGACGTGCCTGACAG
                                                T

201       210        220        230        240        250
  CGTGATTGTCGACCGTATGAGCGGCCGCCGCGTGCATTTGGCTTCCGGCC
                     C

251       260        270        280        290        300
  GTACTTACCACGTTACCTACAACCCGCCCAAAGTTGAAGGCAAAGACGAC

301       310        320        330        340        350
  GTAACCGGCGAAGATTTGATTCAGCGCGACGACGACAAAGAAGAAACCGT
           A

351       360        370        380        390        400
  GAAAAAACGCCTTGCCGTTTACCACGAGCAAACCGAAGTTTTGGTCGATT
                                                    T

401       410        420        430        440        450
  TTTACAGCAAACTGGAAGGCGAACACGCGCCTAAATACATCAAAGTTGAC

451       460
  GGCACTCAGCCGGTA
        C  AG A
```

aroE

18 alleles, 122 polymorphic sites

```
   1        10         20         30         40         50
  TATCGGTTTGGCCAACGACATCACGCAGGTCAAAAACATTGCCATCGAAG
   T G       AT GG    T   AGT A     GC  C T CA AA  T  G
                                    T

  51        60         70         80         90        100
  GCAAAACCATCTTGCTTTTGGGCGCGGGCGGCGCGGTGCGCGGCGTGATT
        AT  TC AT GC C      A      T

 101       110        120        130        140        150
  CCTGTTTTGAAAGAACACCGTCCTGCCCGTATCGTCATTGCCAACCGCAC
    G                   C             A              T

 151       160        170        180        190        200
  CCACGCCAAAGCCGAAGAATTGGCGCGGCTTTTCGGCATTGAAGCCGTCC
    GT            G       T   A

 201       210        220        230        240        250
  CGATGGCGGATGTGAACGGCGGTTTTGATATCATCATCAACGGCACGTCC
            CT A                                    A  G

 251       260        270        280        290        300
  GGCGGCTTGAGCGGTCAGCTTCCTGCCGTCAGTCCTGAAATTTTCCTCGG
       TC A A        A    G ATA TCAG GC  T      T GAAA
                          C       CC              A

 301       310        320        330        340        350
  CTGCCGCCTTGCCTACGATATGGTTTACGGCGACGCGGCGCAGGAGTTTT
      GCG                  G   A  TGA     AA ACC

 351       360        370        380        390        400
  TGAACTTTGCCCAAAGCAACGGTGCGCCCGAAGTTTCAGACGGACTGGGT
   AG T      A GGCAATCG  C T AAAA  ACCG C     C     C
                             C

 401       410        420        430        440        450
  ATGCTGGTCGGTCAAGCGGCGGCTTCCTACGCCCTCTGGCGCGGATTTAC
       A         G  T  C  C       CATA T        G

 451       460        470        480        490
  GCCCGATATCCGCCCTGTTATCGAATACATGAAAGCCATG
     TA          CA                    C A
```

fumC

14 alleles, 36 polymorphic sites

```
  1        10         20         30         40         50
  GAAGCCTTGGGCGGACGCGATGCCGCCGTTGCCGCTTCGGGCGCATTGAA
       AC A                              T            C

 51        60         70         80         90        100
  AACGCTGGCGGCAAGCCTGAATAAAATCGCCAACGACATCCGCTGGCTGG
     AT                   C      T                T

101       110        120        130        140        150
  CAAGCGGCCCGCGCTGCGGTTTGGGCGAAATCAAAATCCCCGAAAACGAG
                 T                                T

151       160        170        180        190        200
  CCGGGTTCGTCCATCATGCCGGGCAAAGTCAACCCGACCCAATGCGAAGC
                  T                    T  A

201       210        220        230        240        250
  GATGACCATGGTGTGCTGCCAAGTGTTCGGCAACGACGTTACCATCGGCA
  A      G            T                              T

251       260        270        280        290        300
  TGGCGGGCGCGTCGGGCAATTTCGAGCTGAACGTCTATATGCCCGTTATC
      A      A                                       C

301       310        320        330        340        350
  GCCTACAACCTCTTGCAATCCATCCGCCTGTTGGGCGACGCGTGCAACAG
                               AT  C         T

351       360        370        380        390        400
  CTTCAACGAACACTGCGCCGTCGGCATCGAACCCGTACCGGAAAAAATCG
                        A        T         G

401       410        420        430        440        450
  ACTATTTCCTGCACCATTCCCTGATGCTGGTTACTGCGTTAAACCGTAAA
                                C      C  AC G      C

451       460
  ATCGGCTACGAAAAC
       T
```

gdh

13 alleles, 25 polymorphic sites

```
   1        10         20         30         40         50
   ATGTTCGAGCCGCTGTGGAACAATAAATACATCGAAAGCGTGCAGTTGAC
                  T                  T                  C

  51        60         70         80         90        100
   CATCGCCGAACAGCTCGGTGTGGAAGAGCGCGGCGAGTTTTACGACATTA
             G    T G   C          A      T          T

 101       110        120        130        140        150
   CTGGCGCCTTGCGCGATATGGTGCAAAACCACTTGATGCAAATGCTGTGC
    C      G                          T   T          G    T

 151       160        170        180        190        200
   ATGACTGCGATGGAAGCCCCCGCCAGCTTGGATGCCGACGCGGTGCGCGA

 201       210        220        230        240        250
   TGAAAAAGTCAAAGTCATCAAGTCATTGAAGCCGCTGACCGTCGAGTCTG
                                                      A

 251       260        270        280        290        300
   TCAATGAAAACGTCGTGCGCGGACAATATACCGCCGCCAGAGGCATGAAC
             T                                   A

 301       310        320        330        340        350
   GGCTATCTTGAAGAAATCAACGTTCCGCAAGACAGCTTTACCGAAACCTA

 351       360        370        380        390        400
   CGTCGCCATTAAAGCCGAAATCGAAAACGAACGCTGGAAGGGCGTTCCCT

 401       410        420        430        440        450
   TCTACCTGCGTACCGGCAAACGCATGGCGGGCAAAGTGGCGGAAATCGTT
                        G        A     T

 451       460        470        480        490        500
   TTGAACTTCAAAGATTTGAACAGCCATATTTTTGAAGGCAGCCGCACCGC
                                            A     T    G

 501
   G
```

pdhC

16 alleles, 71 polymorphic sites

```
  1        10         20         30         40         50
  ATGCCCGAAGGTGCGGAACAAGACATCTTGAAAGGTATGTACCTGCTGAA
               C  A              C               T

 51        60         70         80         90        100
  AGCCGGCGGCAAAGGCGACAAGAAAGTCCAACTGATGGGTTCCGGTACGA
                       T         T   T        C         C

101       110        120        130        140        150
  TTTTGCAAGAAGTGATTGCCGGTGCCGAGCTGCTGAAAGCCGACTTCGGC
   CC           CG  A  A        AT
                                 T

151       160        170        180        190        200
  GTGGAAGCAGACATTTGGTCTTGCCCATCTTTCAACCTGTTGCATCGCGA
  T A            C            G  C          C    C

201       210        220        230        240        250
  TGCTATCGAAGCAGAACGTTTCAACCGCCTGAATCCTTTGGAAACTGCAA
  C  CG    GATC      C              C    GC     G C A

251       260        270        280        290        300
  AAGTACCGTTTGTTACTTCTCAACTGCAAGGTCATGACGGCCCGGTGATT
      G  T  C     C  CA  T                   T     T  C

301       310        320        330        340        350
  GCCGCTACCGACTATATCCGTAGCTATGCTGACCGCATCCGTGCCTACAT
                 C     C         C     T     C

351       360        370        380        390        400
  CCCTAACGACTACCACGTCTTGGGTACTGACGGCTTCGGCCGCTCCGACA
     G                       C         T         T

401       410        420        430        440        450
  GCCGTGCCAACCTGCGTAGCTTCTTCGAAGTTGACCGCTACAACGTTGCC
   T  C            CC        T     G  TT

451       460        470        480
  GTTGCTGCATTGAGCGCATTGGCCGATCAA
    G  A       GC          G  A
    A  C
```

pgm

12 alleles, 59 polymorphic sites

```
  1        10         20         30         40         50
GTGGTTACCAAAGACGGCAACATTATTTATCCCGACCGCCAACTGATGCT
C A  C                    C  C     G          T A   T
                                   A

 51        60         70         80         90        100
GTTCGCCCAAGACGTTTTGAACCGCAATCCCGGCGCGAAAGTCATTTTCG
T     G         G  A G          TAAA  A     G  C
                                     C     A

101        110        120        130        140        150
ACGTGAAGTCCACCCGCCTGCTTGCGCCTTGGATTAAAGAACACGGCGGC
 T  C  A         T     GA T  G           C
                         C

151        160        170        180        190        200
AAAGCCATAATGGAAAAAACCGGCCACAGCTTTATCAAATCCGCCATGAA
G  C TG G                                      T

201        210        220        230        240        250
AGAAACCGGCGCGCCGGTTGCCGGCGAAATGAGCGGACACATCTTCTTCA
 A             T

251        260        270        280        290        300
AAGAACGCTGGTTCGGCTTCGACGACGGTCTGTACGCCGGCGCACGCCTC

301        310        320        330        340        350
TTGGAAATCCTGTCTGCCTCCGATAATCCGTCCGAAGTGTTAAACAACCT
               C                          G

351        360        370        380        390        400
GCCGCAAAGCATTTCCACGCCCGAACTCAACATCGCCCTGCCCGAAGGCA
          A         T  G           T A      G

401        410        420        430        440        450
GCAACGGCCATCAGGTTATCGACGAACTCGCCGCCAAAGCCGAATTTGAA
           CA A  G  T  A  G  G  T     T   A G  C
```

Figure 6.5 Polymorphic sites associated with each allelic variant within each housekeeping gene from meningococci isolated in 2000 (year 2). Each housekeeping gene is represented by its abbreviated initials followed by the total number of different alleles characterised followed by the number of polymorphic sites. These are displayed within the nucleotide sequence following this information. A consensus sequence is displayed for each housekeeping gene and if a variation within the sequence is identified the alternative base pair is displayed directly below the consensus nucleotide sequence base pair.

abcZ

14 alleles, 69 polymorphic sites

```
  1        10        20        30        40        50
  TTTGATACTGTTGCCGAAGGTTTGGGCGAAATTCGCGATTTATTGCGCCG
           C                  TA G      T         A

 51        60        70        80        90       100
  TTATCATCATGTCAGCCATGAGTTGGAAAATGGTTCGAGTGAGGCCTTAT
     C   C GC    G T      T       GC CAA    ACAC   C TT   G

101       110       120       130       140       150
  TGAAAGAGCTCAACGAATTGCAACTTGAGATCGAAGCGAAGGACGGCTGG
   A      A      T      A      C CA                T

151       160       170       180       190       200
  AAGTTGGATGCGGCGGTGAAGCAGACTTTGGGCGAACTCGGTTTGCCGGA
    AC            A  C                 G

201       210       220       230       240       250
  AAACGAAAAAATCGGCAACCTCTCCGGCGGTCAGAAAAAGCGCGTCGCCT
     T                   T                      T

251       260       270       280       290       300
  TGGCGCAGGCTTGGGTGCAGAAGCCCGACGTATTGCTGCTCGATGAACCG
      T                 A                      G   C   G

301       310       320       330       340       350
  ACCAACCATTTGGACATCGACGCGATTATTTGGTTGGAAAACCTGCTCAA
                  T   T   T         C    C        T      T

351       360       370       380       390       400
  AGCGTTTGAAGGCAGCCTGGTTGTGATTACCCACGACCGCCGTTTTTTGG
  G                 T     C                 T          C

401       410       420       430
  ACAATATCGCCACGCGGATTGTCGAACTCGATC
   T   C   T   T   A   C   C   T      T   C
```

adk

10 alleles, 23 polymorphic sites

```
  1        10         20         30         40         50
  GAAGCGAAAAAAATCATTGACGAAGGCGGCTTGGTGCGCGACGACATCAT
                          T

 51        60         70         80         90        100
  TATCGGCATGGTCAAAGAACGCATCGCGCAAGACGACTGCAAAAACGGTT

101       110        120        130        140        150
  TCCTGTTTGACGGTTTCCCGCGCACGCTGGCACAGGCCGAAGCGATGGTT
    T     C                    AT        A

151       160        170        180        190        200
  GAAGCAGGCGTGGATTTGGATGCAGTCGTTGAAATCGACGTGCCTGACAG
                                                T

201       210        220        230        240        250
  CGTGATTGTCGACCGCATGAGCGGCCGCCGCGTGCATTTGGCTTCCGGCC
                    T

251       260        270        280        290        300
  GTACTTACCACGTTACCTACAACCCGCCCAAAGTTGAAGGCAAAGACGAC
                               T         G  C            T

301       310        320        330        340        350
  GTAACCGGCGAAGATTTGATTCAGCGCGACGACGACAAAGAAGAAACCGT
           A      C

351       360        370        380        390        400
  GAAAAAACGCCTTGCCGTTTACCACGAGCAAACCGAAGTTTTGGTTGATT
                        G                        GC     C

401       410        420        430        440        450
  TTTACAGCAAACTGGAAGGCGAACACGCGCCTAAATACATCAAAGTTGAC
                                    G

451       460
  GGCACTCAGCCGGTA
        C  AG A
```

aroE

18 alleles, 127 polymorphic sites

```
  1        10         20         30         40         50
TATCGGTTTGGCCAACGACATCACGCAGGTCAAAAACATTGCCATCGAAG
 T G       AT GG    T   AGT A     GC  C T CA AA  T  G
                                   T

 51        60         70         80         90        100
GCAAAACCATCTTGCTTTTGGGCGCGGGCGGCGCGGTGCGCGGCGTGATT
       AT  TC AT GC C      A      T                A

101       110        120        130        140        150
CCTGTTTTGAAAGAACACCGTCCTGCCCGTATCGTCATTGCCAACCGCAC
  G  G        G       C              A  G           T

151       160        170        180        190        200
CCACGCCAAAGCCGAAGAATTGGCGCGGCTTTTCGGCATTGAAGCCGTCC
 TGT            G       T   A

201       210        220        230        240        250
CGATGGCGGATGTGAACGGCGGTTTTGATATCATCATCAACGGCACGTCC
           CT A            C                     A  G

251       260        270        280        290        300
GGCGGCTTGAGCGGTCAGCTTCCTGCCGTCAGTCCTGAAATTTTCCTCGG
      TC A A        A    G ATA TCAG GC  T      T GAAA
                         C       CC             A

301       310        320        330        340        350
CTGCCGCCTTGCCTACGATATGGTTTACGGCGACGCCGCCGCAGGAGTTTT
     GCG                   G   A  TGA      AA ACC

351       360        370        380        390        400
TGAACTTTGCCCAAAGCAACGGTGCGGCCGAAGTTTCAGACGGACTGGGT
 AG T      A GGCAATCG  C   AAAA  ACCG C      C      C
                                 C

401       410        420        430        440        450
ATGCTGGTCGGTCAAGCGGCGGCTTCCTACGCCCTCTGGCGCGGATTTAC
      A        G  T  C  C       TATA T        G
                                 C

451       460        470        480        490
GCCCGATATCCGCCCTGTTATCGAATACATGAAAGCCATG
   TA           CA                    C A
```

fumC

13 alleles, 36 polymorphic sites

```
  1       10        20        30        40        50
GAAGCCTTGGGCGGACGCGATGCCGCCGTTGCCGCTTCGGGCGCATTGAA
      AC A                          T           C

 51       60        70        80        90       100
AACGCTGGCGGCAAGCCTGAATAAAATCGCCAACGACATCCGCTGGCTGG
   AT                  C     T              T

101       110       120       130       140       150
CAAGCGGCCCCGCGCTGCGGTTTGGGCGAAATCAAAATCCCCGAAAACGAG
               T                               T

151       160       170       180       190       200
CCGGGTTCGTCCATCATGCCGGGCAAAGTCAACCCGACCCAATGCGAAGC
                T                    T  A

201       210       220       230       240       250
GATGACCATGGTGTGCTGCCAAGTGTTCGGCAACGACGTTACCATCGGCA
A     G           T                                T

251       260       270       280       290       300
TGGCGGGCGCGTCGGGCAATTTCGAGCTGAACGTCTATATGCCCGTTATC
     A     A                                      C

301       310       320       330       340       350
GCCTACAACCTCTTGCAATCCATCCGCCTCTTGGGCGACGCGTGCAACAG
                            AT  G         T

351       360       370       380       390       400
CTTCAACGAACACTGCGCCATCGGCATCGAACCCGTACCGGAAAAAATCG
                      G       T         G

401       410       420       430       440       450
ACTATTTCCTGCACCATTCCCTGATGCTCGTTACCGCGTTAAACCGCAAA
                              G     T  AC G     T

451       460
ATCGGTTACGAAAAC
      C
```

gdh

15 alleles, 32 polymorphic sites

```
  1        10        20        30        40        50
  ATGTTCGAGCCGCTGTGGAACAATAAATACATCGAAAGCGTGCAGTTGAC
                   T                                C

 51        60        70        80        90       100
  CATCGCCGAACAGCTCGGTGTGGAAGAGCGCGGCGAGTTTTACGACATTA
            G    T G   C         A                T

101       110       120       130       140       150
  CTGGCGCCTTGCGCGATATGGTGCAAAACCACTTGATGCAAATGCTGTGC
   C      G                        T   T         G    T T   T

151       160       170       180       190       200
  ATGACTGCGATGGAAGCCCCCGCCAGCTTGGATGCCGACGCGGTGCGCGA
                     A   TT A   T             T

201       210       220       230       240       250
  TGAAAAAGTCAAAGTCATCAAGTCATTGAAGCCGCTGACCGTCGAGTCTG
                                                    A

251       260       270       280       290       300
  TCAATGAAAACGTCGTGCGCGGACAATATACCGCCGCCAGAGGCATGAAC
             T                                  A    G

301       310       320       330       340       350
  GGCTATCTTGAAGAAATCAACGTTCCGCAAGACAGCTTTACCGAAACCTA

351       360       370       380       390       400
  CGTCGCCATTAAAGCCGAAATCGAAAACGAACGCTGGAAGGGCGTTCCCT

401       410       420       430       440       450
  TCTACCTGCGTACCGGCAAACGCATGGCGGGCAAAGTGGCGGAAATCGTT
                       G       A     T

451       460       470       480       490       500
  TTGAACTTCAAAGATTTGAACAGCCATATTTTTGAAGGCAGCCGCACCGC
                                              A     T    G

501
  G
```

pdhC

17 alleles, 78 polymorphic sites

```
   1        10        20        30        40        50
   ATGCCCGAAGGTGCGGAACAAGACATCTTGAAAGGTATGTACCTGCTGAA
         G      C TA              C C            TT
         T

  51        60        70        80        90       100
   AGCCGGCGGCAAAGGCGACAAGAAAGTCCAACTGATGGGTTCCGGTACGA
                     T         T   T        C         C

 101       110       120       130       140       150
   TTTTGCAAGAAGTGATTGCCGGTGCCGAGCTGCTGAAAGCCGACTTCGGC
    CC           CG  A  A        AT                   T
                                  T

 151       160       170       180       190       200
   GTGGAAGCAGACATTTGGTCTTGCCCATCTTTCAACCTGTTGCATCGCGA
   T A            C           G  C          C    C

 201       210       220       230       240       250
   TGCTATCGAAGCAGAACGTTTCAACCGCCTGAATCCTTTGGAAACTGCAA
   C  CG     GATC      C             C    GC A   G C A

 251       260       270       280       290       300
   AAGTACCGTTTGTTACTTCTCAACTGCAAGGTCATGACGGCCCGGTGATT
       G  T  C     C  CA  T                   T  A  T  C

 301       310       320       330       340       350
   GCCGCTACCGACTATATCCGTAGCTATGCTGACCGCATCCGTGCCTACAT
                        C         C     T     C     T

 351       360       370       380       390       400
   CCCTAACGACTACCACGTCTTGGGTACTGACGGCTTCGGCCGCTCCGACA
      G     T                 C         T         T

 401       410       420       430       440       450
   GCCGTGCCAACCTGCGTAGCTTCTTCGAAGTTGACCGCTACAACGTTGCC
    T  C           CC        T     G  T

 451       460       470       480
   GTTGCTGCATTGAGCGCATTGGCCGATCAA
     G  A       GC          G  A
     A  C
```

pgm

12 alleles, 67 polymorphic sites

```
   1        10         20         30         40         50
   GTGGTTACCAAAGACGGCAACATTATTTATCCCGACCGCCAACTGATGCT
   C A  C                   C  C     G          T A   T
                                     A

  51        60         70         80         90        100
   GTTCGCCCAAGACGTTTTGAACCGCAATCCCGGCGCGAAAGTCATTTTCG
   T     G          G  A G      C  TAAA  A      G  C
                                          C      A

 101       110        120        130        140        150
   ACGTGAAGTCCACCCGCCTGCTTGCGCCTTGGATTAAAGAACACGGCGGC
    T  C  A          T      GA T  G           C
                              C

 151       160        170        180        190        200
   AAAGCCATAATGGAAAAAACCGGCCACAGCTTTATCAAATCCGCCATGAA
   G  C TG G                            C         GA
                                                   T

 201       210        220        230        240        250
   AGAAACCGGCGCGCCGGTTGCCGGCGAAATGAGCGGACACATCTTCTTCA
    A           A TC  C

 251       260        270        280        290        300
   AAGAACGCTGGTTCGGCTTCGACGACGGTCTGTACGCCGGCGCACGCCTC
                     A           C

 301       310        320        330        340        350
   TTGGAAATCCTGTCTGCCTCCGATAATCCGTCCGAAGTGTTAAACAACCT
                   C                                 G

 351       360        370        380        390        400
   GCCGCAAAGCATTTCCACGCCCGAACTCAACATCGCCCTGCCCGAAGGCA
             A          T  G            T A       G

 401       410        420        430        440        450
   GCAACGGCCATCAGGTTATCGACGAACTCGCCGCCAAAGCCGAATTTGAA
               CA A  G  T  A  G  G  T     T   A G  C
```

Figure 6.6 Polymorphic sites associated with each allelic variant within each housekeeping gene from meningococci isolated in 2001 (year 3). Each housekeeping gene is represented by its abbreviated initials followed by the total number of different alleles characterised followed by the number of polymorphic sites. These are displayed within the nucleotide sequence following this information. A consensus sequence is displayed for each housekeeping gene and if a variation within the sequence is identified the alternative base pair is displayed directly below the consensus nucleotide sequence base pair.

abcZ

16 alleles, 66 polymorphic sites

```
  1        10         20         30         40         50
  TTTGATACCGTTGCCGAAGGTTTGGGTGAAATTCGCGATTTATTGCGCCG
           T                  A  CA        T             A

 51        60         70         80         90        100
  TTACCACCGCGTCGGCCATGAGTTGGAAAACGGTTCGGGTGAGGCTTTGT
     T  T AT  TA T      T      GC TAA   AAAC  C TC  A

101        110        120        130        140        150
  TGAAAGAACTCAACGAATTACAACTTGAAATCGAAGCGAAGGACGGCTGG
   A     G            G     C CG                T

151        160        170        180        190        200
  AAGCTGGATGCGGCAGTCAAGCAGACTTTGGGGGAACTCGGTTTGCCGGA
    AT           G  G     A         C

201        210        220        230        240        250
  AAACGAAAAAATCGGCAACCTTTCCGGCGGTCAGAAAAAGCGTGTCGCCT
     T                  C                       C     T

251        260        270        280        290        300
  TGGCGCAGGCTTGGGTGCAGAAGCCCGACGTATTGCTGCTGGACGAACCG
      T               A                        C  T  G

301        310        320        330        340        350
  ACCAACCATTTGGATATCGACGCGATTATTTGGCTGGAAAATCTGCTCAA
                 C  T           C   T       C

351        360        370        380        390        400
  AGCGTTTGAAGGCAGCTTGGTTGTGATTACCCACGACCGCCGTTTTTTGG
  G               C    C              T

401        410        420        430
  ACAATATCGCCACGCGGATTGTCGAACTCGATC
   T  C     T     C  C  T     T  C
```

adk

13 alleles, 26 polymorphic sites

```
1         10         20         30         40         50
  GAAGCGAAAAAAATCATTGACGAAGGCGGCTTGGTGCGCGACGACATCAT
                       T

 51         60         70         80         90        100
  TATCGGCATGGTCAAAGAACGCATCGCGCAAGACGACTGCAAAAACGGTT

101        110        120        130        140        150
  TCCTGTTCGACGGTTTCCCGCGCACATTGGCACAAGCCGAAGCGATGGTT
    T    T                   GC        G

151        160        170        180        190        200
  GAAGCAGGCGTGGATTTGGATGCAGTCGTTGAAATCGACGTGCCTGACAG
                                                T

201        210        220        230        240        250
  CGTGATTGTCGACCGTATGAGCGGCCGCCGCGTGCATTTGGCTTCCGGCC
               T  C

251        260        270        280        290        300
  GTACTTACCACGTTACCTACAACCCGCCCAAAGTTGAAGGCAAAGACGAC
                           T        G  C           T

301        310        320        330        340        350
  GTAACCGGCGAAGATTTGATTCAGCGCGACGACGACAAAGAAGAAACCGT
          A     C

351        360        370        380        390        400
  GAAAAAACGCCTTGCCGTTTACCACGAGCAAACCGAAGTTTTGGTCGATT
                      G                      GC    T

401        410        420        430        440        450
  TTTACAGCAAACTGGAAGGCGAACACGCGCCTAAATATATCAAAGTTGAC
             G                       G     C

451        460
  GGCACTCAGCCGGTA
       C  AG A
```

aroE

17 alleles, 123 polymorphic sites

```
  1        10         20         30         40         50
TATCGGTTTGGCCAACGACATCACGCAGGTCAAAAACATTGCCATCGAAG
 T G       AT GG    T  AGT A     GC  C T CA AA  T  G
                                  T

 51        60         70         80         90        100
GCAAAACCATCTTGCTTTTGGGCGCGGGCGGCGCGGTGCGCGGCGTGATT
       AT  TC AT GC C      A      T                A

101       110        120        130        140        150
CCTGTTTTGAAAGAACACCGTCCTGCCCGTATCGTCATTGCCAACCGCAC
  G           G       C                  G           T

151       160        170        180        190        200
CCACGCCAAAGCCGAAGAATTGGCGCGGCTTTTCGGCATTGAAGCCGTCC
  GT            G       T   A

201       210        220        230        240        250
CGATGGCGGATGTGAACGGCGGTTTTGATATCATCATCAACGGCACGTCC
           CT A                                   A  G

251       260        270        280        290        300
GGCGGCTTGAGCGGTCAGCTTCCTGCCGTCAGTCCTGAAATTTTCCTCGG
      TC A A        A    G ATA TCAG GC  T      T GAAA
                         C       CC              A

301       310        320        330        340        350
CTGCCGCCTTGCCTACGATATGGTTTACGGCGACGCGGCGCAGGAGTTTT
     GCG                   G   A  TGA      AA ACC

351       360        370        380        390        400
TGAACTTTGCCCAAAGCAACGGTGCGGCCGAAGTTTCAGACGGACTGGGT
  AG T     A GGCAATCG  C   AAAA  ACCG C     C      C
                               C

401       410        420        430        440        450
ATGCTGGTCGGTCAAGCGGCGGCTTCCTACGCCCTCTGGCGCGGATTTAC
      A         G  T  C  C       CATA T         G

451       460        470        480        490
GCCCGATATCCGCCCTGTTATCGAATACATGAAAGCCATG
   TA           CA                    C A
```

fumC

17 alleles, 37 polymorphic sites

```
  1        10         20         30         40         50
  GAAGCCTTGGGCGGACGCGATGCCGCCGTTGCCGCTTCGGGCGCATTGAA
        AC A                            T            C

 51        60         70         80         90        100
  AACGCTGGCGGCAAGCCTGAATAAAATCGCCAACGACATCCGCTGGCTGG
     AT                   C      T               T

101       110        120        130        140        150
  CAAGCGGCCCCGCGCTGCGGTTTGGGCGAAATCAAAATCCCCGAAAACGAG
                 T                                T

151       160        170        180        190        200
  CCGGGTTCGTCCATCATGCCGGGCAAAGTCAACCCGACCCAATGCGAAGC
                  T                    T  A

201       210        220        230        240        250
  GATGACCATGGTGTGCTGCCAAGTGTTCGGCAACGACGTTACCATCGGTA
  A      G            T                              C

251       260        270        280        290        300
  TGGCGGGCGCGTCGGGCAATTTCGAGCTGAACGTCTATATGCCCGTCATC
       A      A                                      T

301       310        320        330        340        350
  GCCTACAACCTCTTGCAATCCATCCGCCTGTTGGGCGACGCGTGCAACAG
            T                  AT  C         T

351       360        370        380        390        400
  CTTCAACGAACACTGCGCCGTCGGCATTGAACCCCTACCGGAAAAAATCG
                       A        C         G

401       410        420        430        440        450
  ACTATTTCCTGCACCATTCCCTGATGCTCGTTACCGCGTTAAACCGCAAA
                                 G      T  AC G      T

451       460
  ATCGGTTACGAAAAC
        C
```

gdh

16 alleles, 34 polymorphic sites

```
   1        10        20        30        40        50
ATGTTCGAGCCGCTGTGGAACAATAAATACATCGAAAGCGTGCACTTGAC
                 T                 T                 C

  51        60        70        80        90       100
CATCGCCGAACAGCTCGGTGTGGAAGAGCGCGGCGAGTTTTACGACATTA
          G    T G   C         A     T         T

 101       110       120       130       140       150
CTGGCGCCTTGCGCGATATGGTGCAAAACCACTTGATGCAAATGCTGTGC
  C     G                       T  T        G    T T  T

 151       160       170       180       190       200
ATGACTGCGATGGAAGCCCCCGCCAGCTTGGATGCCGACGCGGTGCGCGA
                   A  TT A                T

 201       210       220       230       240       250
TGAAAAAGTCAAAGTCATCAAGTCATTGAAGCCGCTGACCGTCGAGTCTG
                                                   A

 251       260       270       280       290       300
TCAATGAAAACGTCGTGCGCGGACAATATACCGCCGCCAGAGGCATGAAC
           T                                 A   G

 301       310       320       330       340       350
GGCTATCTTGAAGAAATCAACGTTCCGCAAGACAGCTTTACCGAAACCTA

 351       360       370       380       390       400
CGTCGCCATTAAAGCCGAAATCGAAAACGAACGCTGGAAGGGCGTTCCCT
                                                  T

 401       410       420       430       440       450
TCTACCTGCGTACCGGCAAACGCATGGCGGGCAAAGTGGCGGAAATCGTT
                     G  T   A    T

 451       460       470       480       490       500
TTGAACTTCAAAGATTTGAACAGCCATATTTTTGAAGGCAGCCGCACCGC
                                           A    T

 501
G
```

pdhC

16 alleles, 75 polymorphic sites

```
  1        10         20         30         40         50
  ATGCCCGAAGGTGCGGAACAAGACATCTTGAAAGGTATGTACCTGCTGAA
       G      C  A              C C            T

 51        60         70         80         90        100
  AGCCGGCGGCAAAGGCGACAAGAAAGTCCAACTGATGGGTTCCGGTACGA
                     T         T   T        C         C

101       110        120        130        140        150
  TTTTGCAAGAAGTGATTGCCGGTGCCGAGCTGCTGAAAGCCGACTTCGGC
    CC           C             A AT                   T
                                 T

151       160        170        180        190        200
  GTGGAAGCAGACATTTGGTCTTGCCCATCTTTCAACCTGTTGCATCGCGA
  T A            C            G  C          C    C

201       210        220        230        240        250
  TGCTATCGAAGCAGAACGTTTCAACCGCCTGAATCCTTTGGAAACTGCAA
  C  CG     GATC      C              C    GC A   G C A

251       260        270        280        290        300
  AAGTACCGTTTGTTACTTCTCAACTGCAAGGTCATGACGGCCCGGTGATT
      G  T  C     C  CA  T                   T  A  T  C

301       310        320        330        340        350
  GCCGCTACCGACTATATCCGTAGCTATGCTGACCGCATCCGTGCCTACAT
                C     C         C     T     C     T

351       360        370        380        390        400
  CCCTAACGACTACCACGTCTTGGGTACTGACGGCTTCGGCCGCTCCGACA
     G     T               C         T         T

401       410        420        430        440        450
  GCCGTGCCAACCTGCGTAGCTTCTTCGAAGTTGACCGCTACAACGTTGCC
    T  C           CC        T     G  T

451       460        470        480
  GTTGCTGCATTGAGCGCATTGGCCGATCAA
    G  A      GC          G  A
    A  C
```

pgm

14 alleles, 68 polymorphic sites

```
  1        10        20        30        40        50
GTGGTTACCAAAGACGGCAACATTATTTATCCCGACCGCCAACTGATGCT
  A  C                    C  C     G          T A  AT
                                   A

 51        60        70        80        90       100
GTTCGCCCAAGACGTTTTGAACCGCAATCCCGGCGCGAAAGTCATTTTCG
T     G         G  A G      C  TAAA  A     G  C
                                     C     A

101       110       120       130       140       150
ACGTGAACTCCACCCGCCTGCTTGCGCCTTGGATTAAAGAACACGGCGGC
 T  C  A         T      GA T  G           C
                             C

151       160       170       180       190       200
AAAGCCATAATGGAAAAAACCGGCCACAGCTTTATCAAATCCGCCATGAA
G  C TG G                              C        GA
                                                 T

201       210       220       230       240       250
AGAAACCGGCGCGCCGGTTGCCGGCGAAATGAGCGGACACATCTTCTTCA
 A           A TC  C

251       260       270       280       290       300
AAGAACGCTGGTTCGGCTTCGACGACGGTCTGTACGCCGGCGCACGCCTC
                   A                   A

301       310       320       330       340       350
TTGGAAATCCTGTCTGCCTCCGATAATCCGTCCGAAGTGTTAAACAACCT
                  C                              G

351       360       370       380       390       400
GCCGCAAAGCATTTCCACGCCCGAACTCAACATCGCCCTGCCCGAAGGCA
          A          T  G            T A       G

401       410       420       430       440       450
GCAACGGCCATCAGGTTATCGACGAACTCGCCGCCAAAGCCGAATTTGAA
            CA A  G  TA A  G  G  T     T   A G  C
```

Figure 6.7 Polymorphic sites associated with each allelic variant within each housekeeping gene from meningococci isolated in 2002 (year 4). Each housekeeping gene is represented by its abbreviated initials followed by the total number of different alleles characterised followed by the number of polymorphic sites. These are displayed within the nucleotide sequence following this information. A consensus sequence is displayed for each housekeeping gene and if a variation within the sequence is identified the alternative base pair is displayed directly below the consensus nucleotide sequence base pair.

abcZ

17 alleles, 74 polymorphic sites

```
  1       10        20        30        40        50
  TTTGATACCGTTGCCGAAGGTTTGGGTGAAATTCGCGATTTATTGCGCCG
          T                 A  CA G     T         A

 51       60        70        80        90       100
  TTACCACCGCGTCGGCCATGAGTTGGAAAACGGTTCGGGTGAGGCTTTGT
     T  T AT  TA T      T      GC TAA   AAAC  CATC  A

101       110       120       130       140       150
  TGAAAGAACTCAACGAATTACAACTTGAAATCGAAGCGAAGGACGGCTGG
   A     G     T     G     C CG                T

151       160       170       180       190       200
  AAGCTGGATGCGGCAGTCAAGCAGACTTTGGGGGAACTCGGTTTGCCGGA
    AT           G  G     A        C

201       210       220       230       240       250
  AAACGAAAAAATCGGCAACCTTTCCGGCGGTCAGAAAAAGCGTGTCGCCT
     T                  C                       C     T

251       260       270       280       290       300
  TGGCGCAGGCTTGGGTGCAGAAGCCCGACGTATTGCTGCTGGACGAACCG
      T               A                     C  T  G

301       310       320       330       340       350
  ACCAACCATTTGGATATCGACGCGATTATTTGGCTGGAAAATCTGCTCAA
                 C  T  T        C   T       C     T

351       360       370       380       390       400
  AGCGTTTGAAGGCAGCTTGGTTGTGATTACCCACGACCGCCGTTTTTTGG
  G               C    C  A           T         C

401       410       420       430
  ACAATATCGCCACGCGGATTGTCGAACTCGATC
   T     T  T  A  C  C  T     T  C
```

adk

10 alleles, 32 polymorphic sites

```
  1        10         20         30         40         50
  GAAGCGAAAAAAATCATTGACGAAGGCGGCTTGGTGCGCGACGACATCAT
                     C  T

 51        60         70         80         90        100
  TATCGGCATGGTCAAAGAACGCATCGCGCAAGACGACTGCAAAAACGGTT
        T                                  T

101       110        120        130        140        150
  TCCTGTTCGACGGTTTCCCGCGCACATTGGCACAAGCCGAAGCGATGGTT
    T     T                    GC         G

151       160        170        180        190        200
  GAAGCAGGCGTGGATTTGGATGCAGTCGTTGAAATCGACGTGCCTGACAG
       C  T                 C  G  C          T  A  C

201       210        220        230        240        250
  CGTGATTGTCGACCGTATGAGCGGCCGCCGCGTGCATTTGGCTTCCGGCC
         A     T  A
                  C

251       260        270        280        290        300
  GTACTTACCACGTTACCTACAACCCGCCCAAAGTTGAAGGCAAAGACGAC
                           T           C           T

301       310        320        330        340        350
  GTAACCGGCGAAGATTTGATTCAGCGCGACGACGACAAAGAAGAAACCGT
          A

351       360        370        380        390        400
  GAAAAAACGCCTTGCCGTTTACCACGAGCAAACCGAAGTTTTGGTCGATT
                                                GC     T

401       410        420        430        440        450
  TTTACAGCAAACTGGAAGGCGAACACGCGCCTAAATACATCAAAGTTGAC
             G

451       460
  GGCACTCAGCCGGTA
       C  AG A
```

aroE

20 alleles, 128 polymorphic sites

```
  1        10         20         30         40         50
  TATCGGTTTGGCCAACGACATCACGCAGGTCAAAAACATTGCCATCGAAG
   T G       AT GG    T   AGT A     GC   C T CA AA   T   G
                                    T

 51        60         70         80         90        100
  GCAAAACCATCTTCCTTTTGGGCGCGGGCGGCGCGGTGCGCGGCGTGATT
        AT   TC AT GC C      A      T

101       110        120        130        140        150
  CCTGTTTTGAAAGAACACCGTCCTGCCCGTATCGTCATTGCCAACCGCAC
    G           G       C              A  G           T

151       160        170        180        190        200
  CCACGCCAAAGCCGAAGAATTGGCGCGGCTTTTCGGCATTGAAGCCGTCC
    GT             G       T   A             C

201       210        220        230        240        250
  CGATGGCGGATGTGAACGGCGGTTTTGATATCATCATCAACGGCACGTCC
             CT A             C  C   G              A  G

251       260        270        280        290        300
  GGCGGCTTGAGCGGTCAGCTTCCTGCCGTCAGTCCTGAAATTTTCCTCGG
  A     TC A A        A     G ATA TCAG GC  T      T GAAA
                             C        CC             A

301       310        320        330        340        350
  CTGCCGCCTTGCCTACGATATGGTTTACGGCGACGCGGCGCAGGAGTTTT
      GCG                   G   A  TGA      AA ACC

351       360        370        380        390        400
  TGAACTTTGCCCAAAGCAACGGTGCGGCCGAAGTTTCAGACGGACTGGGT
   AG T      A GGCAATCG  C   AAAA  ACCG C      C      C
                                 C

401       410        420        430        440        450
  ATGCTGGTCGGTCAAGCGGCGGCTTCCTACGCCCTCTGGCGCGGATTTAC
       A         G  T  C  C       CATA T         G

451       460        470        480        490
  GCCCGATATCCGCCCTGTTATCGAATACATGAAAGCCATG
     TA          CA                    C A
```

fumC

17 alleles, 46 polymorphic sites

```
  1        10         20         30         40         50
  GAAGCCTTGGGCGGACGCGATGCCGCCGTTGCCGCTTCGGGCGCATTGAA
       AC A                              T      A       C

 51        60         70         80         90        100
  AACGCTGGCGGCAAGCCTGAATAAAATCGCCAACGACATCCGCTGGCTGC
     AT                    C      T                 T

101       110        120        130        140        150
  CAAGCGGCCCGCGCTGCGGTTTGGGCGAAATCAAAATCCCCGAAAACGAG
          T      T                                T         A

151       160        170        180        190        200
  CCGGGTTCGTCCATCATGCCGGGCAAAGTCAACCCGACCCAATGCGAAGC
                   T                     T   A

201       210        220        230        240        250
  GATGACCATGGTGTGCTGCCAAGTGTTCGGCAACGACGTTACCATCGGCA
  A      G             T                                A T

251       260        270        280        290        300
  TGGCGGGCGCGTCGGGCAATTTCGAGCTGAACGTCTATATGCCCGTTATC
      A      A                                        C

301       310        320        330        340        350
  GCCTACAACCTCTTGCAATCCATCCGCCTGTTGGGCGACGCGTGCAACAG
           T    C          G    AT   C          T

351       360        370        380        390        400
  CTTCAACGAACACTGCGCCGTCGGCATCGAACCCGTACCGGAAAAAATCG
             A          AC    T   T          G

401       410        420        430        440        450
  ACTATTTCCTGCACCATTCCCTGATGCTGGTTACTGCGTTAAACCGTAAA
                                 C      C   AC G      C

451       460
  ATCGGCTACGAAAAC
        T
```

gdh

16 alleles, 36 polymorphic sites

```
  1        10         20         30         40         50
  ATGTTCGAGCCGCTTTGGAACAATAAATACATCGAAAGCGTGCAGCTGAC
                 G                  T            A   T

 51        60         70         80         90        100
  CATCGCCGAGCAGTTGGGCGTGGAAGAACGCGGCGAGTTTTACGACATTA
           A   C C  T         G      T         T

101       110        120        130        140       150
  CCGGCGCGTTGCGCGATATGGTGCAAAACCACTTGATGCAAATGCTGTGC
   T     C                        T  T         G   T T  T

151       160        170        180        190       200
  ATGACTGCGATGGAAGCCTCCGCCAGCTTGGATGCCGACGCGGTGCGCGA
                     AC TT A

201       210        220        230        240       250
  TGAAAAAGTCAAAGTCATCAAGTCATTGAAGCCGCTGACCGTCGAATCTG
                    G                                G

251       260        270        280        290       300
  TCAATGAAAATGTCGTGCCCGGACAATATACCGCCGCCAAAGGCATCAAC
             C                                 G

301       310        320        330        340       350
  GGCTATCTTGAAGAAATCAACGTTCCGCAAGACAGCTTTACCGAAACCTA

351       360        370        380        390       400
  CGTCGCCATTAAAGCCGAAATCGAAAACGAACGCTGGAAGGGCGTTCCCT
                                                   T

401       410        420        430        440       450
  TCTACCTGCGTACCGGCAAGCGCATGGCGGGCAAAGTGGCGGAAATCGTT
                      A  T    A     T

451       460        470        480        490       500
  TTGAACTTCAAAGATTTGAACAGCCATATTTTTGAAGCCAGTCGCACCGC
                                              A    C   G

501
  G
```

pdhC

16 alleles, 77 polymorphic sites

```
  1        10         20         30         40         50
  ATGCCCGAAGGTGCGGAACAAGACATCTTGAAAGGTATGTACCTGCTGAA
       G      C TA              C C            TT
       T

 51        60         70         80         90        100
  AGCCGGCGGCAAAGGCGACAAGAAAGTCCAACTGATGGGTTCCGGTACGA
                     T         T   T        C         C

101       110        120        130        140        150
  TTTTGCAAGAAGTGATTGCCGGTGCCGAGCTGCTGAAAGCCGACTTCGGC
    CC           C    A           AT                   T
                                    T

151       160        170        180        190        200
  GTGGAAGCAGACATTTGGTCTTGCCCATCTTTCAACCTGTTGCATCGCGA
  T A            C            G  C          C     C

201       210        220        230        240        250
  TGCTATCGAAGCAGAACGTTTCAACCGCCTGAATCCTTTGGAAACTGCAA
  C  CG     GATC      C              C     GC A    G C A

251       260        270        280        290        300
  AAGTACCGTTTGTTACTTCTCAACTGCAAGGTCATGACGGCCCGGTGATT
       G  T  C      C  CA  T                  T  A  T  C

301       310        320        330        340        350
  GCCGCTACCGACTATATCCGTAGCTATGCTGACCGCATCCGTGCCTACAT
                 C      C         C      T      C      T

351       360        370        380        390        400
  CCCTAACGACTACCACGTCTTGGGTACTGACGGCTTCGGCCGCTCCGACA
     G      T                 C         T         T

401       410        420        430        440        450
  GCCGTGCCAACCTGCGTAGCTTCTTCGAAGTTGACCGCTACAACGTTGCC
    T  C            CC        T      G  T

451       460        470        480
  GTTGCTGCATTGAGCGCATTGGCCGATCAA
    G  A       GC          G  A
    A  C
```

pgm

15 alleles, 64 polymorphic sites

```
   1        10         20         30         40         50
GTGGTTACCAAAGACGGCAACATTATTTATCCCGACCGCCAACTGATGCT
  A  C                    C  C     G          T A   T
                                    A

  51        60         70         80         90        100
GTTCGCCCAAGACGTTTTGAACCGCAATCCCGGCGCGAAAGTCATTTTCG
T     G          G  A G       C  TAAA  A      G  C
                                        C      A

 101       110        120        130        140        150
ACGTGAAGTCCACCCGCCTGCTTGCGCCTTGGATTAAAGAACACGGCGGC
  T  C  A          T      GA C  G            C

 151       160        170        180        190        200
AAAGCCATAATGGAAAAAACCGGCCACAGCTTTATCAAATCCGCCATGAA
G  C TG G                                C         GA
                                                    T

 201       210        220        230        240        250
AGAAACCGGCGCGCCGGTTGCCGGCGAAATGAGCGGACACATCTTCTTCA
 A           A TC  C

 251       260        270        280        290        300
AAGAACGCTGGTTCGGCTTCGACGACGGTCTGTACGCCGGCGCACGCCTC
                   A                    A

 301       310        320        330        340        350
TTGGAAATCCTGTCTGCCTCCGATAATCCGTCCGAAGTGTTAAACAACCT
                  C

 351       360        370        380        390        400
GCCGCAAAGCATTTCCACGCCCGAACTCAACATCGCCCTGCCCGAAGGCA
                     T  G            T A      G

 401       410        420        430        440        450
GCAACGGCCATCAGGTTATCAACGAACTCGCCGCCAAAGCCGAATTTGAA
           CA A   G   TG A   G   G   T     T   A G
```

Figure 6.8 Genbank nucleotide submission of a Neisseria meningitidis *shikimate dehydrogenase* (*aroE*) gene allele 225.

```
LOCUS       AY348556                 490 bp    DNA     linear   BCT
20-AUG-2003
DEFINITION  Neisseria meningitidis AroE (aroE) gene, aroE-225 allele,
partial
            cds.
ACCESSION   AY348556
VERSION     AY348556.1  GI:33694224
KEYWORDS    .
SOURCE      Neisseria meningitidis
  ORGANISM  Neisseria meningitidis
            Bacteria; Proteobacteria; Betaproteobacteria;
Neisseriales;
            Neisseriaceae; Neisseria.
REFERENCE   1  (bases 1 to 490)
  AUTHORS   Lawie,D.I., Diggle,M.A. and Clarke,S.C.
  TITLE     Neisseria meningitidis aroE allele 225
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 490)
  AUTHORS   Lawie,D.I., Diggle,M.A. and Clarke,S.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-JUL-2003) SMPRL, Stobhill Hospital,
Balornock Road,
            Glasgow, Lanarkshire G21 3UW, Scotland
FEATURES             Location/Qualifiers
     source          1..490
                     /organism="Neisseria meningitidis"
                     /mol_type="genomic DNA"
                     /db_xref="taxon:487"
     gene            <1..>490
                     /gene="aroE"
                     /allele="225"
     CDS             <1..>490
                     /gene="aroE"
                     /allele="225"
                     /codon_start=2
                     /transl_table=11
                     /product="AroE"
                     /protein_id="AAQ24851.1"
                     /db_xref="GI:33694225"
```

Figure 6.9 Genbank nucleotide submission of a *Neisseria meningitidis* porin A class 1 outer membrane protein variable region 3 (VR3) variant 35.

```
LOCUS       AF489496                909 bp    DNA     linear   BCT
27-MAR-2002
DEFINITION  Neisseria meningitidis strain 590-60 class 1 outer
membrane protein
            (porA) gene, partial cds.
ACCESSION   AF489496
VERSION     AF489496.1  GI:19744840
KEYWORDS    .
SOURCE      Neisseria meningitidis
  ORGANISM  Neisseria meningitidis
            Bacteria; Proteobacteria; Betaproteobacteria;
Neisseria_es;
            Neisseriaceae; Neisseria.
REFERENCE   1  (bases 1 to 909)
  AUTHORS   Clarke,S.C., Diggle,M.A., Molling,P., Unemo,M. and
Olcen,P.
  TITLE     Analysis of PorA variable region 3 in meningococci has
implications
            for vaccine policy
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 909)
  AUTHORS   Molling,P. and Unemo,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (01-MAR-2002) National Reference Laboratory for
            Pathogenic Neisseria, Department of Clinical
Microbiology, Orebro
            University Hospital, Orebro SE-701 85, Sweden
FEATURES             Location/Qualifiers
     source          1..909
                     /organism="Neisseria meningitidis"
                     /mol_type="genomic DNA"
                     /strain="590-60"
                     /db_xref="taxon:487"
                     /note="genosubtype P1.7,16-29,35
                     isolated in 2001
                     serogroup: C"
     gene            1..>909
                     /gene="porA"
     CDS             1..>909
                     /gene="porA"
                     /note="porin; PorA; variable regions 1,2,3"
                     /codon_start=1
                     /transl_table=11
                     /product="class 1 outer membrane protein"
                     /protein_id="AAL96679.1"
                     /db_xref="GI:19744841"
```

Figure 6.10 Genbank nucleotide submission of a *Neisseria meningitidis* porin A class 1 outer membrane protein variable region 3 (VR3) variant 37.

```
LOCUS       AF489497                 885 bp    DNA     linear   BCT
27-MAR-2002
DEFINITION  Neisseria meningitidis strain 90-108 class 1 outer
membrane protein
            (porA) gene, partial cds.
ACCESSION   AF489497
VERSION     AF489497.1  GI:19744842
KEYWORDS    .
SOURCE      Neisseria meningitidis
  ORGANISM  Neisseria meningitidis
            Bacteria; Proteobacteria; Betaproteobacteria;
Neisseriales;
            Neisseriaceae; Neisseria.
REFERENCE   1  (bases 1 to 885)
  AUTHORS   Clarke,S.C., Diggle,M.A., Molling,P., Unemo,M. and
Olcen,P.
  TITLE     Analysis of PorA variable region 3 in meningococci has
implications
            for vaccine policy
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 885)
  AUTHORS   Molling,P. and Unemo,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (01-MAR-2002) National Reference Laboratory for
            Pathogenic Neisseria, Department of Clinical
Microbiology, Orebro
            University Hospital, Orebro SE-701 85, Sweden
FEATURES             Location/Qualifiers
     source          1..885
                     /organism="Neisseria meningitidis"
                     /mol_type="genomic DNA"
                     /strain="90-108"
                     /db_xref="taxon:487"
                     /note="genosubtype P1.7-2,4,37; former
P1.7b,4,37
                     isolated in 1997
                     serogroup: B"
     gene            1..>885
                     /gene="porA"
     CDS             1..>885
                     /gene="porA"
                     /note="porin; PorA; variable regions 1,2,3"
                     /codon_start=1
                     /transl_table=11
                     /product="class 1 outer membrane protein"
                     /protein_id="AAL96680.1"
                     /db_xref="GI:19744843"
```

Figure 6.11 Genbank nucleotide submission of a *Neisseria meningitidis* porin A class 1 outer membrane protein variable region 3 (VR3) variant 39.

```
LOCUS       AF481699                 493 bp    DNA     linear   BCT
21-MAR-2002
DEFINITION  Neisseria meningitidis strain 00-1008 outer membrane
porin (porA)
            gene, partial cds.
ACCESSION   AF481699
VERSION     AF481699.1  GI:19568837
KEYWORDS    .
SOURCE      Neisseria meningitidis
  ORGANISM  Neisseria meningitidis
            Bacteria; Proteobacteria; Betaproteobacteria;
Neisseriales;
            Neisseriaceae; Neisseria.
REFERENCE   1  (bases 1 to 493)
  AUTHORS   Clarke,S.C., Diggle,M.A. and Edwards,G.F.S.
  TITLE     Semiautomation of multilocus sequence typing for the
            characterization of clinical isolates of Neisseria
meningitidis
  JOURNAL   J. Clin. Microbiol. 39 (9), 3066-3071 (2001)
  MEDLINE   21417403
   PUBMED   11526130
REFERENCE   2  (bases 1 to 493)
  AUTHORS   Diggle,M.A. and Clarke,S.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (05-FEB-2002) SMPRL, Stobhill Hospital,
Balornock Road,
            Glasgow G21 3UW, Scotland
FEATURES             Location/Qualifiers
     source          1..493
                     /organism="Neisseria meningitidis"
                     /mol_type="genomic DNA"
                     /strain="00-1008"
                     /db_xref="taxon:487"
                     /note="subgroup: C"
     gene            <1..>493
                     /gene="porA"
     CDS             <1..>493
                     /gene="porA"
                     /codon_start=1
                     /transl_table=11
                     /product="outer membrane porin"
                     /protein_id="AAL91932.1"
                     /db_xref="GI:19568838"
```

Figure 6.12 Genbank nucleotide submission of a *Neisseria meningitidis* porin A class 1 outer membrane protein variable region 3 (VR3) variant 40.

```
LOCUS       AY192543                 156 bp    DNA     linear   BCT
04-FEB-2003
DEFINITION  Neisseria meningitidis porin A variable region 3 variant
40 gene,
            partial cds.
ACCESSION   AY192543
VERSION     AY192543.1  GI:28207736
KEYWORDS    .
SOURCE      Neisseria meningitidis
  ORGANISM  Neisseria meningitidis
            Bacteria; Proteobacteria; Betaproteobacteria;
Neisseriales;
            Neisseriaceae; Neisseria.
REFERENCE   1  (bases 1 to 156)
  AUTHORS   Diggle,M.A. and Clarke,S.C.
  TITLE     Novel porin A variable region three
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 156)
  AUTHORS   Diggle,M.A. and Clarke,S.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (10-DEC-2002) SMPRL, Stobhill Hospital,
Balornock Road,
            Glasgow G21 3UW, Scotland
FEATURES             Location/Qualifiers
     source          1..156
                     /organism="Neisseria meningitidis"
                     /mol_type="genomic DNA"
                     /db_xref="taxon:487"
     CDS             <1..>156
                     /codon_start=1
                     /transl_table=11
                     /product="porin A variable region 3 variant 40"
                     /protein_id="AAO31978.1"
                     /db_xref="GI:28207737"
```

Figure 6.13 Genbank nucleotide submission of a *Neisseria meningitidis* porin A class 1 outer membrane protein variable region 3 (VR3) variant 41.

```
LOCUS       AY192542                 150 bp    DNA     linear   BCT
04-FEB-2003
DEFINITION  Neisseria meningitidis porin A variable region 3 variant
41 gene,
            partial cds.
ACCESSION   AY192542
VERSION     AY192542.1  GI:28207734
KEYWORDS    .
SOURCE      Neisseria meningitidis
  ORGANISM  Neisseria meningitidis
            Bacteria; Proteobacteria; Betaproteobacteria;
Neisseriales;
            Neisseriaceae; Neisseria.
REFERENCE   1  (bases 1 to 150)
  AUTHORS   Diggle,M.A. and Clarke,S.C.
  TITLE     Novel porin A variable region three
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 150)
  AUTHORS   Diggle,M.A. and Clarke,S.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (10-DEC-2002) SMPRL, Stobhill Hospital,
Balornock Road,
            Glasgow G21 3UW, Scotland
FEATURES             Location/Qualifiers
     source          1..150
                     /organism="Neisseria meningitidis"
                     /mol_type="genomic DNA"
                     /db_xref="taxon:487"
     CDS             <1..>150
                     /codon_start=1
                     /transl_table=11
                     /product="porin A variable region 3 variant 41"
                     /protein_id="AAO31977.1"
                     /db_xref="GI:28207735"
```