A molecular epidemiological analysis of meningococcal isolates within Scotland 1972-1998

Christopher B Sullivan

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Division of Infection and Immunity

Institute of Biomedical Life Science

University of Glasgow

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Abstract

Neisseria meningitidis is an important cause of meningitis and bacteraemia worldwide and is associated with high case-fatality rates. Meningococcal disease continues to remain a public health issue in Scotland and the rest of Europe. Typing methods are used for epidemiological purposes to investigate outbreaks and the spread of meningococci and to examine the population structure of the organism in order to better understand its variation and evolution. Reference institutes have employed such methods for a number of decades for the diagnosis and detection of meningococci. However, phenotypic methods for serogrouping, serotyping and serosubtyping meningococci, although providing good strain information, can lead to endemic strains appearing identical using these methods when they are in fact quite different. More recently methods have been developed to further characterise bacteria. These methods have included PCR for the detection of meningococcal disease within blood, serogrouping and sequencing of housekeeping genes (MLST) and antigen genes such as PorA. These molecular epidemiological methods were used for the retrospective typing of invasive meningococci in Scotland, 1972-1998, using a fully automated procedure. The results of these were then analysed using statistical packages to examine the population structure of the organism.

In total there were 2517 invasive isolates, received by the Scottish Meningococcus and Pneumococcus Reference Laboratory (SMPRL) from the start of 1972 to the end of 1998. Serogroup distribution changed from year to year during the time period 1972-1998 but serogroups B and C were dominant throughout this period. Serogroup B was the dominant serogroup throughout the seventies and early eighties until serogroup C became dominant during the mid 1980s.

This increase in dominance of serogroup C has been found in this study not to be associated with one particular sequence type (ST) but is associated with a number of STs, which include ST-8, ST-11, ST-206 and ST-334. This is in contrast to the increase in serogroup C disease in the 1990s that was due to the ST-11 clonal complex. While there was much diversity in the STs (309 different STs among the 2517 isolates), only ten accounted for 1562 isolates (59.9%). These were ST-11, ST-8, ST-41, ST-153, ST-1, ST-32, ST-33, ST-269, ST-334 and ST-60. There were 177 new STs found during the time period. The STs were further differentiated into 31 clonal complexes, with 57 singleton types. As with the STs, although there was much diversity in the clonal complexes, only seven accounted for 1993 isolates.

It was found that with PorA variable region (VR) types there were certain combinations significantly more common than others. There was a strong link with PorA type and ST and more so with clonal complex. This link was evident with the PorA type 5, 2-1, 36-2, which occured in 70 isolates representing the ST-11 complex and in all but two isolates representing ST-11. Similarly PorA type 18-3, 1, 35-1 was associated with 15 isolates belonging to the ST41-44 complex. However, this was not the case with all PorA combinations as the PorA type 19, 15, 36 was associated with 10 different complexes. There was some association between serogroup and PorA VR types. There was strong evidence of certain VR1, 2 and 3 regions being associated with certain serogroups, although this was not definitive. For example, of 192 isolates with PorA type 19, 15, 36, 85.4% were associated with serogroup B. Genosubtyping of the *porA* gene has been shown to increase the power of differentiation within clonal meningococcal populations. For, example, seven isolates that had the same serogroup, ST, VR1 and VR2 could be differentiated by their VR3 type.

Using cluster detection software SaTScan to analyse all isolates, it was found there were 29 clusters in Scotland, from 1972-1998. These clusters included 63 cases, which accounted for 2.5% of all cases. A range of different strains caused the clusters that were identified in this study, some caused by hypervirulent strains. These strain types were responsible for a number of cases throughout the world as well as in Scotland during the period of this study. However it was also shown that there were clusters identified in this study caused by lesser-known strain types that were not responsible for many cases and that appear to be unique to Scotland or the UK. This study is the first to look at the detection of clusters over a time period of 26 years and to identify clusters that would have previously been unidentified due to lack of suitable characterisation techniques.

The results in this study indicate that the multivalent preparation produced by the Netherlands Vaccine Institute (Nonavalent vaccine) had the potential, based on the PorA types that it contains, to prevent the majority of serogroup B infection that had occurred in Scotland, from 1972-1998. It also had the potential, although not to the same extent as serogroup B, to protect against other serogroups. For the age groups that would potentially have been the first to be immunised with any vaccine as part of the childhood vaccination programme, the 0-4 years old group, the potential coverage was over 92% which is comparable with the coverage seen with the serogroup C meningococcal conjugate (MCC) vaccine, of approximately 90%.

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Abbreviations

AA	Ayrshire & Arran
abcZ	putative ABC transporter
AC	Argyll & Clyde
ACDP	Advisory Committee on Dangerous Pathogens
adk	adenylate kinase
AFLP	amplified fragment length polymorphism
AMOVA	Analysis of molecular variance
APS	ammonium persulphate
aroE	shikimate dehydrogenase
BR	Borders
CDC	Centers for Disease Control and Prevention
CO_2	Carbon dioxide
COSHH	Control of substances hazardous to health
CPA	Clinical Pathology Accreditation
Cps	capsule-synthesis
CSF	cerebrospinal fluid
ctrA	capsular transferase gene
DG	Dumfries & Galloway
DLV	double locus variant
DNA	deoxyribonucleic acid
eBURST	Based Upon Related Sequence Types
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay

ET	electrophoretic type
EU-IBIS	European Union Invasive Bacterial Infection Surveillance Network
FbpA	ferric binding protein
FetA	iron-regulated outer-membrane protein
FF	Fife
fumC	fumarate hydratase
FV	Forth Valley
GG	Greater Glasgow
GR	Grampian
gdh	glucose-6-phosphate dehydrogenase
HCl	hydrochloric acid
HexaMen	hexavalent PorA OMV vaccine
HG	Highland
Hib	Haemophilus influenzae type b
HitA	ferric-binding protein from *Haemophilus influenzae*
I_A	Index of Association
IgG	immunoglobulin G
IgM	immunoglobulin M
IMD	invasive meningococcal disease
K	is the number of loci at which two individuals differ
LN	Lanarkshire
LO	Lothian
LOS	lipooligosaccharide
LPA	linear polyacrylamide

LPS	lipopolysaccharides
Mabs	monoclonal antibodies
MCC	serogroup C meningococcal conjugate
MCV-4	quadrivalent conjugate vaccines
MLEE	multi-locus enzyme electrophoresis
MLST	multi-locus sequence typing
NHS	National Health Service
N. meningitidis	*Neisseria meningitidis*
NonaMen	nonavalent PorA OMV vaccine
NPHI	National Public Health Institutes
NVI	Netherlands Vaccine Institute
OMP	outer-membrane protein
OMV	outer-membrane vesicle
Opa	class 5 outer-membrane protein
Opc	class 5 outer-membrane protein
OR	Orkney
PCR	polymerase chain reaction
pdhC	pyruvate dehydrogenase subunit
PFGE	pulsed-field gel electrophoresis
pgm	phosphoglucomutase
PorA	porin, class I outer-membrane protein
porA	gene encoding PorA protein
RFLP	restriction fragment length polymorphism
RIVM	National Institute for Public Health and the Environment
RMP	the gene for the class 4 OMP

siaD	polysialyltransferase gene
SfuA	ferric-binding protein from *Serratia marcescens*
SH	Shetland
SLV	single locus variant
SMPRL	Scottish Meningococcus and Pneumococcus Reference Laboratory
ST	sequence type
START	sequence type analysis and recombinational tests
Tris	2-Amino-2hydroxymethyl-propane-1,3-diol
TY	Tayside
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
V_E	expected variance of K
V_0	observed variance of K
VR	variable region
WI	Western Isles
WWW	World Wide Web

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Finally, I would like to dedicate my thesis to the memory of all the family members I have lost over the course of my PhD. Dad, Gran, Nana and Uncle you are all sadly missed.

Author’s declaration

The work presented in this thesis was performed entirely by the author except as acknowledged. This thesis has not been previously submitted for a degree at this or any other institution.

Christopher Sullivan

Chapter 1

Introduction

1.1 Meningococcal disease

Neisseria meningitidis 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, a stiff neck, fever and can lead to confusion and coma and may be rapidly fatal (Ferguson *et al.*, 2002). Meningitis is notable for its rapid development and sometimes sporadic or epidemic presentation in both industrialised and developing countries. Meningococcal disease is associated with high case fatality rates (5%-15%) even when good medical treatment is available. Up to 20% of meningococcal meningitis survivors may have neurological sequelae including mental retardation and hearing loss. The risk of acquiring meningococcal disease has been shown to decrease with age. The natural habitat and reservoir of *N. meningitidis* is the upper respiratory nasopharyngeal mucosal membranes. Meningococci colonise the nasopharynx of 10-25% of the general population and this carriage may be intermediate or prolonged.

The prevalence of carriage varies broadly and does not directly predict disease, although, without meningococcal carriage, there is no meningococcal disease. Transmission is by direct contact with or inhalation of meningococci in large droplet nuclei that are acquired through very close contact with respiratory secretions and

saliva. Close contact with a colonised individual can result in transmission of the organism to a susceptible individual and this may result in colonisation and lead to invasive disease. Meningococcal disease usually occurs 1–14 days after acquisition. Once meningococci reach human epithelial cells, a series of interactions occurs, leading to effacement of the epithelial surface, microcolony formation and/or epithelial cell invasion (Stephens & McGee, 1983). In humans, meningococcal carriage can be prolonged (months) in around 25% of individuals, be brief (days to several weeks) in about 33% of individuals, be temporary in around 30-40% of individuals, or not occur (Stephens, 1999).

Meningococcal carriage increases in settings of closed populations, such as schools, universities, military establishments and Hajj pilgrims. Carriage is low in young children (*Neisseria lactamica* predominates) and highest in adolescents. Cofactors that are thought to increase the incidence of carriage and meningococcal disease include coinfections such as influenza and other respiratory viral infections, smoking, and environmental damage to the upper respiratory tract (Artenstein *et al.*, 1967; Young *et al.*, 1972; Moore *et al.*, 1990). In a large UK study, social behaviour such as attendance at pubs/clubs, intimate kissing and cigarette smoke or exposure to passive smoke were most highly associated with the risk of meningococcal carriage but not age or sex (MacLennan *et al.*, 2006).

The unexpected appearance of meningococcal disease in previously healthy children and young adults has resulted in a great deal of public as well as medical attention. This has led to a large amount of research concerning diagnosis, characterisation, therapy and vaccine design (van der Ende *et al.*, 1995; Richmond *et al.*, 2001; Pollard

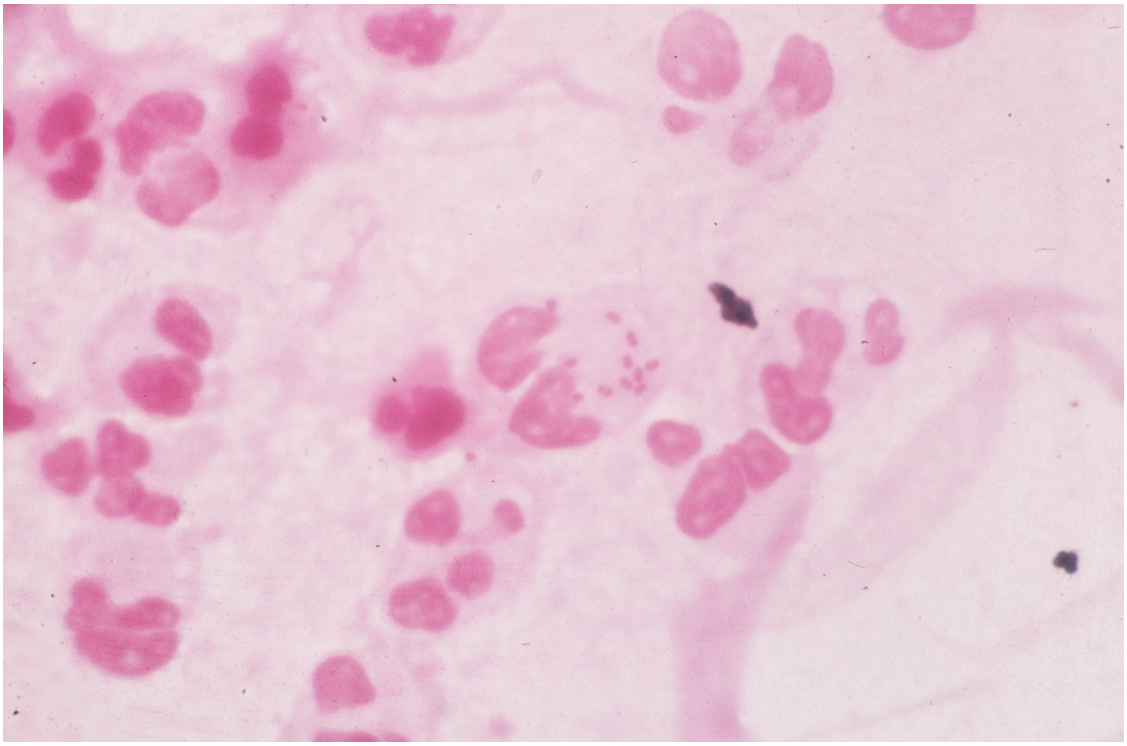
& Moxon, 2002; Riordan *et al.*, 2002; Welch & Nadel, 2003). The development of bacteriology and science in general over the late 19th century culminated in microbiologists making repeated attempts to isolate organisms from patients who had died from suspected meningitis (Knapp, 1988). In the 1880s, in Vienna, Anton Weichselbaum made a breakthrough with the isolation of a coccoid bacterium from meningeal exudates. This coccoid bacterium was later termed *Diplococcus intracellularis meningitides* and then *Neisseria meningitidis*. 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 chains of Gram-positive cocci similar to streptococci (Weichselbaum, 1887). Because of low discrimination techniques between cultures during this period there was confusion over what was isolated and this continued for some time. It was another eight years after these initial findings that meningococci were isolated from lumbar punctures for the first time from patients clinically confirmed with meningitis (Knapp, 1988). 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.

1.2 Characteristics of *Neisseria meningitidis*

Neisseria are Gram-negative, aerobic diplococci that have an oxidative metabolism, are susceptible to drying and their growth is inhibited by free fatty acids (Figure 1.1). Within the genus *Neisseria* there are the species: *Neisseria meningitidis, Neisseria gonorrhoeae, Neisseria lactamica, Neisseria animalis, Neisseria*

bacilliformis, Neisseria canis, Neisseria cinerea, Neisseria elongata, Neisseria dentrificans, Neisseria dentiae, Neisseria flava, Neisseria flavescens, Neisseria iguanae, Neisseria macacae, Neisseria mucosa, Neisseria perflava, Neisseria pharyngis, Neisseria polysaccharea, Neisseria sicca, Neisseria subflava and *Neisseria weaveri*. Different species can be identified by the sets of sugars from which they will produce acid. For example, *N. gonorrheae* makes acid from glucose only whereas *N. meningitidis* produces acid from both glucose and maltose.

Figure 1.1 *N. meningitidis* in cerebrospinal fluid stained using the Gram protocol. In this film, numerous polymorphonuclear leukocytes are evident. Within one, numerous diplococci with the characteristic appearance of bacteria of the genus *Neisseria* can be seen.



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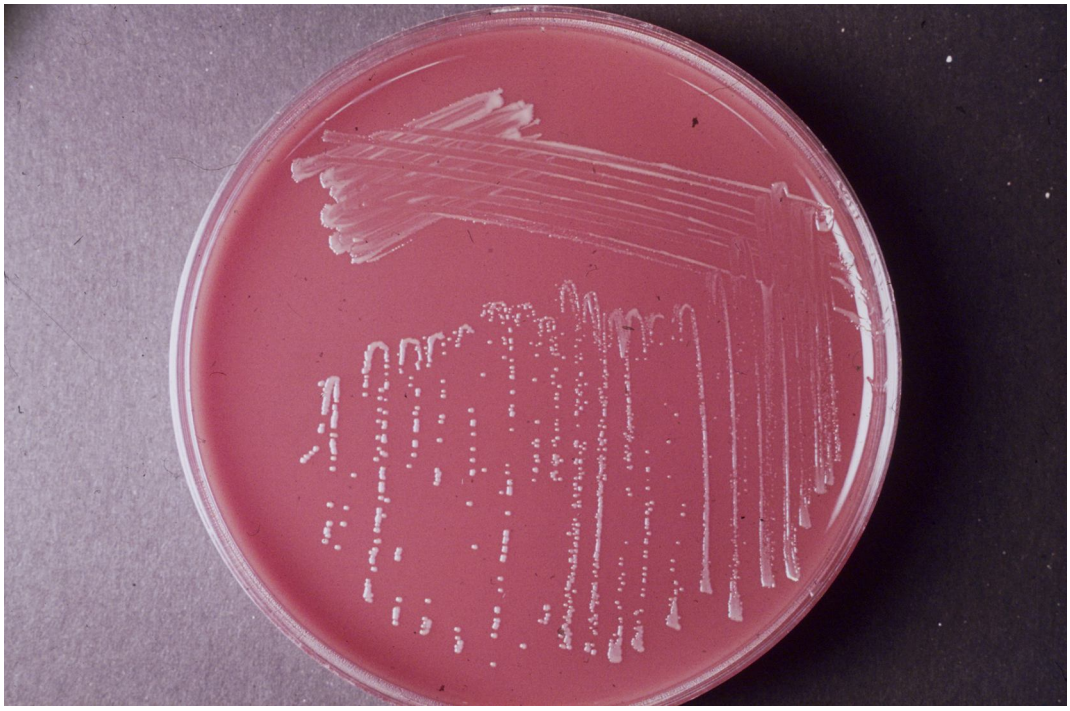
http://www.bioscience.heacademy.ac.uk/imagebank/

Colonies of *N. meningitidis* are non-pigmented and smooth, and after 18-24 hours of incubation, are around 1-2mm in diameter (Figure 1.2).

Figure 1.2 *N. meningitidis* growing on blood agar.



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Encapsulated, piliated meningococci adhere selectively to the microvilli on non-ciliated epithelial cells (Rayner *et al.*, 1995). To facilitate attachment to the epithelial cell layer, pathogenic meningococci possess class I and class II pili. Pili mediating attachment have been found among both the pathogenic strains of *N. meningitidis* as well as some of the non-pathogenic strains. Non-capsulated strains 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 in the respiratory tract and one

possible reason for 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 colonisation (Virji *et al.*, 1996). In general, the major attributes of pathogenic neisseriae for interaction with non-phagocytic cells are pilus-mediated adhesion factors, pilin antigenic variation and modulation of adhesiveness through the formation of bundles of pili for cell-binding specificity, opacity proteins, the capsule and lipo-oligosaccharide (LOS).

1.2.1 Capsule synthesis

Serogroups of the organism are defined by immunochemical differences among meningococcal capsules (Jarvis & Vedros, 1987). Thirteen serogroups based on different capsular polysaccharide structure are known but only six serogroups, A, B, C, Y, W135 and more recently X, are currently associated with significant disease potential (Rosenstein *et al.*, 2001; Djibo *et al.*, 2003; Apicella, 2005). There are five regions within the meningococcal capsule-synthesis (*cps*) gene cluster: region A consists of the genes that are required for polysaccharide synthesis (Edwards *et al.*, 1994; Swartley *et al.*, 1998), region B consists of the genes responsible for lipid modification (Frosch & Muller, 1993), region C contains the *ctr* genes required for polysaccharide transport (Frosch *et al.*, 1991; Frosch *et al.*, 1992), region D is involved in lipopolysaccharide synthesis (Hammerschmidt *et al.*, 1994) and region E is of unknown function. Region A contains genes which are serogroup specific with variants of the *siaD* gene required for synthesis of the sialic-acid-containing capsules (B, C, Y and W-135) and the *myn* genes necessary for the expression of a serogroup A capsule (Claus *et al.*, 1997; Swartley *et al.*, 1997). Because of the role of capsule expression in invasive disease, the *ctrA* gene (region C) is conserved in most

meningococcal isolates from patients (Frosch *et al.*, 1992) and is used as a target for the detection of meningococci in clinical specimens (Guiver *et al.*, 2000).

Serogroups B, C, Y, and W135 boast a polysaccharide capsule that is composed of polysialic acid and are biochemically very similar (Lewis *et al.*, 2003). The capsules of serogroups B and C contain α2-8 linked sialic acid and α2-9 linked sialic acid, respectively, whereas the capsules of serogroups Y and W135 contain *N*-acetylneuraminic acid and D-glucose and *N*-acetylneuraminic acid and D-galactose, respectively. The gene cassettes for all the capsules contain four open reading frames (ORFs), (*siaA*, *siaB*, *siaC* and *siaD*) which code for different sialytransferase genes. The *siaD* sequences (Borrow *et al.*, 1997; Claus *et al.*, 1997; Vogel *et al.*, 1997; Borrow *et al.*, 1998; Arreaza *et al.*, 2001) of serogroups B and C are highly conserved but are less conserved among serogroups Y and W135 where there is a variable region towards the end of the first third of the gene in which abundant nucleotide differences occur (Borrow *et al.*, 1997; Claus *et al.*, 1997; Vogel *et al.*, 1997; Borrow *et al.*, 1998).

The capsules of members of *N. meningitidis* serogroup A differ from those of members of serogroups B, C, Y, and W135, the other disease-associated serogroups. The capsular polysaccharide of serogroup A isolates is composed of repeating units of (16)-linked-*N*-acetyl-D-mannosamine-1-phosphate (Swartley *et al.*, 1998). In contrast, the capsular polysaccharides of isolates of serogroups B, C, Y, and W135 are all composed of, or contain, sialic acid (Claus *et al.*, 1997). The capsular polysaccharide of *N. meningitidis* serogroup A is encoded by an operon of four genes, *mynA*, *-B*, *-C*, and *-D* (formerly known as open reading frames 1 to 4) (Swartley *et al.*, 1998).

Confusingly, these are also known as *sacA-D*. 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.

1.2.2 Outer-membrane proteins

The prominent outer-membrane proteins (OMP) of *N. meningitidis* have been designated class 1 through class 5 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. The class 4 OMP is antigenically stable (Munkley *et al.*, 1991) and appears to be the most highly conserved between meningococcal strains. Although cellular function is unknown, it shares sequence homology with *Escherichia 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 OMPs, the heat modifiable class 5 opacity proteins, Opa and Opc are hypervariable (Stephens & McGee, 1983; Tinsley & Heckels, 1986) both in their 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 monoclonal antibodies (Mabs) (Zollinger *et al.*, 1984).

1.2.2.1 PorA

PorA is a class 1 outer membrane protein (OMP) that has been well studied and is used as a phenotypic marker in the characterisation of meningococci for the serosubtype. This protein is variable because it is an antigen exposed on the meningococcal cell surface and some regions of the protein are hypervariable. A two-dimensional structural model has been predicted for the meningococcal PorA protein which consists of eight surface exposed loops (I-VIII) (van der Ley *et al.*, 1991). Most variation occurs 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). The two PorA variable regions VR1 and VR2 are especially important because they elicit bactericidal antibodies in humans. Consequently, a number of meningococcal vaccines under development contain the PorA protein as a major component. Point mutations in the coding region may result in meningococci without PorA expression (van der Ende *et al.*, 2000). PorA expression may also be absent because of deletion of the complete *porA* gene (van der Ende *et al.*, 1999) or insertion of an insertion sequence element in the *porA* coding region (Newcombe *et al.*, 1998).

Nucleotide sequence analyses of *porA* genes from a large collection of meningococcal isolates recognised that the panel of sero-subtyping Mabs was not comprehensive. Therefore, meningococci were frequently only partially serosubtyped or classified as non-serosubtypable because either PorA was not expressed or because a variant was not recognised by Mabs. Characterising the PorA VR1 and VR2 amino acid sequence from nucleotide sequence data has overcome this. To accommodate subtypes identified on the basis of sequence data alone, the scheme originally developed for

Mab reactivity data was modified so that VR families and variants were assigned on the basis of amino acid sequence relationships rather than reactivity with specific Mabs (Russell *et al.*, 2004). VR amino acid sequences containing ≥80% identity to each other were grouped into VR families. The VR epitope recognised by an existing Mab raised against PorA or the first defined amino acids sequence of a VR family was subjectively designated as the prototype VR for that particular family (Russell *et al.*, 2004). The VR3 (loop V) has also been described as variable and a number of its genosubtypes have also been described (Riesbeck *et al.*, 2000; Clarke *et al.*, 2003). Therefore the VR3 region can also be sequenced along with VR1 and VR2 to allow for further characterisation that can aide in distinguishing between strains, especially in an outbreak situation. These regions of PorA are likely to be exposed to continual selection imposed by host immune responses, and VR families might evolve over time into different families. An example of this may be the similarity of the P1.2 and P1.10 VR2 families, which is perhaps due to the relatively recent divergence of one VR family into two.

1.2.2.2 PorB

Meningococcal strains have the ability to possess either PorB2 (class 2) or PorB3 (class 3) OMPs. These are predominant proteins of the outer membrane, which are expressed by alternative alleles (*porB2* or *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 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 domains have been postulated. As would be expected, the antigenically variable epitopes targeted by the host immune response were found to reside in the most exposed loops (McGuinness *et al.*, 1990; Maiden *et al.*, 1991).

1.2.2.3 FetA

Meningococcal FetA, an iron-regulated outer-membrane protein and vaccine component, has been shown to be highly diverse (Thompson *et al.*, 2003) and has been investigated as a vaccine candidate for both the meningococcus and gonococcus (Ala'Aldeen *et al.*, 1994; Beucher & Sparling, 1995). The FetA protein is a component of some meningococcal vaccines which have undergone phase III trials and appears to elicit an immune response in vaccinees (Wedege *et al.*, 1998). As with the PorA VRs, the sequences of the FetA VRs are highly divergent, and the peptide sequences can be resolved into distinct families. The FetA VR has been successfully used as a portable typing scheme for meningococcal disease surveillance in conjuction with serogroup, PorA VRs and MLST (Jolley *et al.*, 2007). Perhaps a useful role for FetA is as a component of outer-membrane vesicle (OMV) vaccines, along with other antigens such as PorA (Urwin *et al.*, 2004). These vaccines elicit bactericidal responses in people, including infants, but the immunity induced is strain-specific, probably as a consequence of antigenic diversity of their components (Martin *et al.*, 2000).

1.3 Detection of *N. meningitidis*

Detection or suspicion of meningococcal disease in the clinical setting requires laboratory confirmation whenever possible as this information is critical for managing the individual patient, outbreak management, epidemiological purposes and for

vaccine evaluation. Providing the laboratory with appropriate information can aid the process, as information regarding which test is required and diagnostic questions can be crucial in directing the optimal handling and reporting of the specimens in the laboratory. The diagnosis of *N. meningitidis* has progressed within the last 15 years. However, culture still forms the backbone of diagnosis in spite of the major improvements in non-culture diagnosis. Culture is very important because the availability of an isolate growing in the laboratory will allow species designation, antibiotic susceptibility testing and characterisation of an isolate for public health and epidemiological purposes. An evident factor of importance is also that every microbiological laboratory can perform cultures for meningococci. A great number of blood-culture systems with different indicator systems are in everyday use, with most of them utilising bottles containing culture media into which the blood is inoculated (Weinstein, 1996; Mylotte & Tayara, 2000). In patients with signs or symptoms suggesting meningitis or meningoencephalitis, a lumbar puncture is usually performed (Stephenson, 1998). The best possible site from which to take a swab for culture of meningococci in patients and healthy carriers is not generally acknowledged. However, with good selective culture media it is clear that carriers with or without local symptoms carry meningococci on the tonsils more often than in the nasopharnyx (Olcen *et al.*, 1979).

In combination with biochemical characterisation, basic techniques are still used for diagnosis. Light microscopy of Gram-stained specimens identifies meningococci as pink-stained Gram-negative diplococci arranged in pairs. *N.meningitidis* utilises glucose and maltose, which is used as a standard biochemical identification of the bacterium. The utilization of other sugars such as lactose and sucrose can identify

other *Neisseria* species such as *Neisseria lactamica* and *Neisseria gonorrhoeae*. False 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.

The further characterisation of organisms is vitally important for the development of improved therapies, vaccines, epidemiology and public health interventions. The main technique used for serological characterisation of meningococci over the decades has been based on the enzyme-linked immunosorbent assay (ELISA) (Eldridge *et al.*, 1978; Frasch *et al.*, 1985). The ELISA technique has been preferentially chosen because it is safe, simple, sensitive, reproducible and specific. Meningococcal serology has been used in the field of vaccinology to evaluate candidate vaccines and quantify individuals' immune responses. Attention has also been focused on the class of antibody responses to meningococcal polysaccharides. Certain studies have shown the presence of serogroup B IgM isotype antibodies in healthy individuals (Leinonen & Frasch, 1982; Devi *et al.*, 1991) whereas other studies have shown these to be absent. Low levels of serogroup B polysaccharide-specific IgG have been detected in sera from healthy individuals (Leinonen & Frasch, 1982; Devi *et al.*, 1991) but not in convalescent sera from patients recovering from serogroup B disease. Antibody isotype responses following serogroup B infection are largely of the IgM isotype (Leinonen & Frasch, 1982; Devi *et al.*, 1991). For serogroup C, both IgG and IgM antibodies have been demonstrated following disease (Kayhty *et al.*, 1981). Serogroup C specific antibodies are also induced following oropharyngeal carriage of serogroup C meningococci (Goldschneider *et al.*, 1969) or following vaccination with serogroup C polysaccharide or conjugate (Balmer *et al.*, 2002) vaccines. Serogroup A and C

specific antibodies may be quantified utilising a standard reference serum (Holder *et al.*, 1995) but there is no such reference serum currently available for serogroup B specific antibodies. In-house assays are often used (Jones & Mallard, 1993) employing different criteria to differentiate serogroups B and C.

Outer-membrane vesicle (OMV) based ELISAs have largely been utilised to evaluate meningococcal OMV based vaccines (Zollinger *et al.*, 1979) and have been employed by some workers to confirm meningococcal disease in the absence of a positive culture result (Jones & Mallard, 1993; Saunders *et al.*, 1997). However, caution must be applied with interpretation of positives owing to the oropharyngeal carriage of meningococci or other *Neisseria* species (often commensal) including antibodies that may cross-react and may indeed be protective against meningococcal disease (Goldschneider *et al.*, 1969). Meningococcal serology can be a useful addition for corroborative evidence but by its nature is a retrospective investigation. Ideally the serological tests should be performed on paired sera, one acute sample and one convalescent sample taken at least 10 days after onset. Serological evidence alone is not suitable to define cases of meningococcal disease without strong clinical evidence.

Traditional phenotypic methods, such as latex agglutination, co-agglutination and ELISA (Eldridge *et al.*, 1978; Frasch *et al.*, 1985) have been successful over the years and have provided important information. More recently, molecular methods of characterisation have come to the fore. Recent developments in DNA analysis, together with the natural limitations of phenotypic methods, have resulted in a natural evolution towards genotypic procedures (Clarke, 2002). The polymerase chain reaction (PCR) method has been introduced into laboratory diagnostics where, in

general, PCR is performed in a commercial thermocycler followed by visualisation of PCR products on a gel-based system. PCR allows for the rapid detection of infection when cultures have not been grown. It also allows for the further characterisation of organisms, for example determination of serogroup.

Non-culture diagnosis has become essential in maximising case ascertainment of disease due to *N. meningitidis* (Clarke *et al.*, 2001b). Although sero-diagnosis is available for confirmation, results are often inconclusive (Borrow *et al.*, 1997). Amplification of meningococcal DNA by polymerase chain reaction (PCR) provides a rapid, highly sensitive, and specific method for detecting meningococcal DNA from clinical samples. The first meningococcal PCR assay was based upon the insertion sequence element IS1106 (Ni *et al.*, 1992). This PCR was designed specifically for the detection of meningococci within clinical samples. Insertion sequence elements were chosen as gene targets for non-culture diagnosis of bacterial infections owing to the presence of multiple copies within the bacterial genome, which it was hoped would increase sensitivity (Zhou *et al.*, 1995). However, there was a problem with the inherent genetic mobility of these elements (Hernandez Perez *et al.*, 1994) which means they can transfer among species and genera. Therefore, during an evaluation period of the IS1106 PCR ELISA, a number of false-positive results were caused by organisms other than *N. meningitidis* (Borrow *et al.*, 1998). As a result of this the focus has switched to the *ctrA* gene (Frosch *et al.*, 1992). The *ctrA* gene is an ideal target for detection of meningococci by PCR as it occurs exclusively in *N.meningitidis* and not in other pathogenic or nonpathogenic *Neisseria* species. Conserved regions of this gene have therefore been exploited, enabling the amplification of a product from all clinically significant serogroups, thereby providing an initial screening test for all

samples. Serogroup-specific sequences within the *siaD* gene have also been exploited in designing PCR tests for the identification and discrimination of operons encoding serogroups B, C, Y and W135 (Borrow *et al.*, 1997; Borrow *et al.*, 1998). Standard and fluorescence-based PCR assays have been developed for the identification of serogroup A meningococci by detection of the *mynA* gene (Diggle *et al.*, 2003b).

1.4 MLST

There are a number of molecular techniques which are available for the characterisation of organisms, including pulsed-field gel electrophoresis (PFGE) (Bygraves & Maiden, 1992; Suzuki *et al.*, 1993; Popovic *et al.*, 2001), amplified fragment length polymorphism (AFLP) (Duim *et al.*, 1999; van Eldere *et al.*, 1999; Goulding *et al.*, 2000), multi-locus enzyme electrophoresis (MLEE) (Caugant *et al.*, 1986a; Lefevre *et al.*, 1993) and multi-locus sequence typing (MLST) (Maiden *et al.*, 1998). All of these have their own advantages and disadvantages but MLST has become the standard when characterising *N. meningitidis*.

1.4.1 Development of MLST

MLST was developed as an alternative to MLEE, which recognised relatively invariant genes by the electrophoretic mobilities of the enzymes they encode (Maiden *et al.*, 1998). MLEE has its disadvantages as the technique indirectly measures genetic variation and is of relatively low-resolution, requiring large numbers of loci (up to 20) to be examined. The identification of allelic variants also required an extensive collection of reference isolates and the technique was relatively complex and time consuming, although high throughput could be achieved in laboratories with the

appropriate equipment. MLEE is also considered laborious and it can be difficult to compare results between laboratories. This was a major reason behind the development of MLST (Maiden *et al.*, 1998; Maiden, 2006), which enables the transfer of digital data between laboratories and is an essential component of a successfully implemented worldwide typing system.

With MLST, the number of alleles assigned per locus is much higher than in MLEE. This is due to the direct and unambiguous assignment of alleles based on nucleotide sequence determination of internal fragments from multiple housekeeping genes. This allows high levels of discrimination between isolates by using half the loci that are typically required for MLEE. The length of DNA fragment used in MLST was chosen as it can be sequenced accurately on both strands using a single pair of primers and, in most bacterial pathogens, it provides sufficient variation to identify many different alleles within the population (Maiden *et al.*, 1998; Enright & Spratt, 1999). MLST was validated using *Neisseria meningitidis* (Maiden *et al.*, 1998) because it is a species in which recombinational replacements are frequent (Feil *et al.*, 1999; van der Ende *et al.*, 1999). A collection of 107 meningococcal isolates from invasive disease and healthy carriers that had been previously characterised by MLEE were used. Initially 10 loci were chosen (Maiden *et al.*, 1998) but a subset of seven was selected on the basis of its discriminatory power. Approximately 450-500 bp internal fragments of each gene were used, as they could be accurately sequenced on both strands using an automated DNA sequencer. For each housekeeping gene, the different sequences present within a bacterial species were assigned as distinct alleles and, for each isolate, the alleles at each of the loci define the allelic profile and sequence type (ST). Each isolate of a species is therefore unequivocally characterised

by a series of seven integers, which correspond to the alleles at the housekeeping loci. Housekeeping genes are used in MLST because they vary in nucleotide sequence slowly over time. For MLST, the number of nucleotide differences between alleles is generally ignored and sequences are given different allele numbers whether they differ at a single nucleotide site or at many sites. Most bacterial species have sufficient variation within housekeeping genes to provide many alleles per locus, allowing an almost infinite amount of distinct allelic profiles to be distinguished using the chosen housekeeping loci. The sequence data obtained from MLST can then be analysed using the MLST database (http://pubmlst.org/neisseria/) (Jolley *et al.*, 2004).

1.4.2 Advantages and disadvantages of MLST

The great advantage of MLST is that the sequence data are unambiguous and the allelic profiles of isolates can easily be compared to those in a large central database via the Internet. This is in contrast to most typing procedures which involve comparing DNA fragment sizes on gels. It is also possible to create your own internal database or intranet, which needs to be maintained by regularly updating, but allows a speedier and regularly accessible form of analysis than waiting for slow Internet access at peak times. A further advantage of MLST is that the allelic profiles of isolates can be obtained from clinical material by PCR amplification of the house-keeping loci directly from cerebrospinal fluid (CSF) or blood (Enright *et al.*, 2000; Clarke *et al.*, 2001b; Diggle *et al.*, 2003a). Thus isolates can be precisely characterised even when they cannot be cultured from clinical material. It also means that laboratories do not need to obtain reference isolates of each of the important clones of a pathogen. A further advantage is that killed cell suspensions or purified DNA can be used for MLST, thus eliminating the need to transfer live bacterial

species, such as meningococci, between laboratories (Tzanakaki *et al.*, 2001). MLST has been applied to a large number of human pathogens including *N. meningitidis* (Maiden *et al.*, 1998), *Streptococcus pneumoniae* (Zhou *et al.*, 2000), *Staphylococcus aureus* (van Belkum, 2000), *Campylobacter jejuni* (Dingle *et al.*, 2001), *Streptococcus pyogenes* (Enright *et al.*, 2001), *Haemophilus influenzae (Meats et al., 2003), Helicobacter pylori* (Platonov *et al.*, 2000) and *Bordetella pertussis* (van Loo *et al.*, 2002).

One of the biggest disadvantages of MLST, like other methods, is that it can be time consuming if performed manually. There is a limit on the number of samples that can be performed depending on whether the MLST is performed manually or automated and which type of DNA sequencer is available. The only realistic way to provide high throughput is for the system to be automated (Clarke *et al.*, 2001a; Clarke *et al.*, 2001b; Clarke *et al.*, 2001c). This allows a large volume of samples to be processed with the minimum of set-up. It also increases the reliability and reproducibility of results, as there is less scope for human error. In addition, it allows better time management as, during the automated stages, other work can be carried out by the user.

1.4.3 Applications of MLST

MLST can be used for epidemiological surveillance and adds greatly to our knowledge of the genetic variation that can occur within a species. The sequence data obtained from MLST can also be used to determine population structures by analysing the extent of linkage disequilibrium between alleles and to look for recombination by the non-congruence of gene trees (Boyd *et al.*, 1996) and by the presence of

significant mosaic structure. For highly clonal species, the phylogenetic relationship between isolates can be inferred from the dendrogram derived from the pairwise differences between STs and independently from a consensus tree constructed from the gene sequences. In the case of weakly clonal species such as the meningococcus, MLST is very useful for the identification of the currently circulating hyper-virulent lineages because these are recognised as clusters of isolates with identical or very similar sequence types (Maiden *et al.*, 1998).

MLST is also an ideal method for performing population and evolutionary analysis in large-scale epidemiological studies. One such study where MLST has been used is in the carriage of meningococci in the Czech Republic. In this study, 218 meningococci isolated from healthy young adults during 1993 were analysed using MLST together with the characterisation of the *siaD* gene (Jolley *et al.*, 2000)**.** This study provided much needed information about meningococcal carriage and this would not have been possible without the various molecular techniques employed. This has not been the only large-scale epidemiological study that has used MLST. A UK study has looked at how meningococci have changed before, during and after the introduction of the Men C vaccine (Diggle & Clarke, 2005; Gray *et al.*, 2006; Trotter *et al.*, 2006). This study used a number of techniques to characterise the organisms found including MLST, PorA and *siaD* analyses. These examples illustrate the increasing role that MLST has had on better understanding the evolutionary biology of pathogenic bacteria and this role looks set to increase in the future. Such studies provide early information relating to public health practice and also help inform vaccine policy.

Although nucleotide differences between alleles are normally ignored in MLST, such differences can actually be used to determine the evolution of given alleles using appropriate analyses. To put this into a real life context, case clusters of meningococcal disease can be examined using MLST to determine whether they are caused by the same strain or caused by a different strain (Feavers *et al.*, 1999; Gilmore *et al.*, 1999; Clarke *et al.*, 2002a). It can also be used to compare the differences between bacterial strains causing disease in different parts of the world or even whether there has been a shift in the common types of alleles found throughout the years (Jolley *et al.*, 2000; Nicolas *et al.*, 2000; Smith *et al.*, 2000; Nicolas *et al.*, 2001), as well as providing obvious public health benefits.

1.4.4 MLST analysis

Most bacterial species have sufficient variation within housekeeping genes to provide many alleles per locus, allowing an almost infinite amount of distinct allelic profiles to be distinguished using the chosen housekeeping loci. The analysis of data can be separated into different categories: lineage assignment, recombination tests and tests for selection.

Lineage assignment is used as a way of displaying the relationships between closely related isolates of a bacterial species or population. It is used to provide a hypothesis about the way each clonal complex may have emerged and diversified. This is important as it allows for the comparison of different lineages to see how closely related they are. This can be useful for understanding the transmission and population dynamics within patients or in healthy carriers. For lineage assignment there are a number of different methods that are available. Distance-matrix methods such as

Neighbour-Joining or Unweighted Pair Group Method with Arithmetic Mean (UPGMA), which calculate genetic distance from multiple sequence alignments, are the simplest to put into practice, but do not invoke an evolutionary model.

Neighbour-Joining is based on the topology that gives the least total branch length preferred at each step of the algorithm. Neighbour-Joining may not find the true tree topology with least total branch length because it is a demanding algorithm that constructs the tree in a step-wise fashion. However it has been extensively tested and usually finds a tree that is quite close to the optimal tree. Neighbour-Joining like most tree drawing algorithims only work when there is not much recombination disrupting the phylogeny. The main advantage of Neighbour-Joining is its efficiency. It is a polynomial-time algorithm that can be used on very large data sets for which other means of phylogenetic analysis (e.g. maximum likelihood) are computationally not viable. Unlike the UPGMA algorithm for phylogenetic tree reconstruction, Neighbour-Joining does not assume that all lineages evolve at the same rate (the tree does not assume an evolutionary clock) and produces an unrooted tree. Furthermore, Neighbour-Joining is statistically consistent under many models of evolution. Hence, given data of sufficient length, neighbor-joining will reconstruct the true tree with high probability.

UPGMA (Unweighted Pair Group Method with Arithmetic Mean) (Sneath & Sokal, 1973a) is a basic 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). With this method it is important, therefore, not to draw phylogenetic inferences from the clustering pattern seen,

although it may prove useful as a rapid guide to identifying similar isolates. It is commonly not considered a good algorithm for construction of phylogenetic trees as it relies on the rates of evolution among different lineages to be approximately equal. In the study of bacterial population biology this is likely not to be the case, so the method should not be relied upon to cluster strains without artefacts. BURST (Based Upon Related Sequence Types) is a web-implemented clustering algorithm, designed for use on MLST data sets. The approach explicitly examines the relationships between very closely related genotypes within clonal complexes where the relationships between different clonal complexes are ignored (Feil *et al.*, 2001). This had its limitations in that it was unable to cope with very large data sets and the graphical outputs displayed were unsophisticated. This led to the introduction of eBURST (Feil *et al.*, 2004), which differs from BURST slightly in the way the algorithms display the relationships between STs. BURST and eBURST are important as epidemiological tools as they are designed for examining clonal diversification over short evolutionary timescale. Therefore antibiotic-resistant strains provide a good sample case, as these are unlikely to predate the introduction of the antibiotics to which they show resistance and should have diversified little from their primary founder within this very short period of time.

eBURST incorporates an uncomplicated model of bacterial evolution in which strains increasing in frequency diversify to form clusters of similar genotypes descended from the founding strain. MLST isolates of an expanding founding ST initially have the same allelic profile, but diversification results in the appearance of variants in which one of the MLST loci has changed (single locus variants; SLVs), either as the result of mutation or recombination. Further diversification generates double locus variants (DLVs) and then triple locus variants (TLVs) of the founding ST, to result in

a cluster of closely-related STs descended from the founding ST. The BURST algorithm identifies these clonal complexes within bacterial populations, deduces the founding ST of each clonal complex, and displays the probable pattern of recent evolutionary descent of all STs within the clonal complex from this predicted founder (Feil *et al.*, 2004). Founder STs are assigned as the ST in an eBURST group that is linked to the greatest number of SLVs, with bootstrapping providing evaluated confidence in this assignment (Feil *et al.*, 2004). The nature of the allelic change (mutation versus recombination) is unimportant for discerning patterns of descent among related STs within a clonal complex. Therefore, for exploring recent ancestry, eBURST is uninfluenced by recombination, in contrast to most methods that use the nucleotide sequences themselves (Feil *et al.*, 2004).

Split Decomposition (a technique for tree reconstruction) (Bandelt & Dress, 1992) is a non-approximative method that can detect groupings of species in the data that are caused by common ancestry, convergence, or systematic or random errors. Evolutionary data is most often presented as a phylogentic tree, the underlying assumption being that evolution is a branching process. The programme splitstree produces splits-graphs from distance matrices or sequence data.

ClonalFrame is for the inference of bacterial microevolution using multilocus sequence data (Didelot & Falush, 2007). The ClonalFrame method estimates the clonal relationships between the members of a sample, while also estimating the chromosomal position of homologous recombination events that have disrupted the clonal inheritance. ClonalFrame takes account of both mutation and recombination events and weights these components accordingly in generating its tree. It is probably,

therefore, more robust in its lineage assignment than other methods which either assume bifurcating tree-like phylogeny (caused by mutation) or they ignore the differences between alleles, assuming that most change is through recombination. The main disadvantage is that it is computationally intensive and, because of its probabilistic nature, multiple runs should be performed and tested for convergence.

Maximum likelihood attempts to estimate the actual amount of change according to the evolutionary model in place. Maximum likelihood works with a prior nucleotide substitution model to compute a likelihood score for each tree given the original data. Before beginning, either an evolutionary model must be specified that can account for the change of one sequence into another or parameters must be selected that can be estimated from the data. Then the maximum likelihood approach evaluates the probability that the selected evolutionary model will have generated the observed sequences. The trees yielding the highest likelihood are used to infer phylogeny. The substitution model should be optimised to fit the observed data as modifying the substitution parameters modifies the likelihood of the data being associated with particular trees.

The 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 (Maynard- Smith *et al.*, 1993.). The Index of Association (I_A) is calculated as follows: $I_A = V_0/V_E - 1$ if V_0 is the observed variance of K and V_E is the expected variance of K, where K is the number of loci at which two individuals differ. If there is linkage equilibrium because of

frequent recombination events, the expected value of IA is zero. Clonal populations are identified by an IA value that differs significantly from zero.

Selection is the process whereby the implementation of conditions allows them discriminate between specific isolates displaying a required phenotype/genotype. Nucleotide substitutions within these genes that encode for proteins can either be synonymous (do not change amino acid) or non-synonymous (changes amino acid). 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. Usually, most non-synonymous changes are 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. Tests for selection include d_S/d_N ratios.

1.5 Global epidemiology and surveillance

The documentation of meningococcal disease has highlighted major global outbreaks at the time of both World War I and II. Since World War II, the largest epidemics of meningococcal disease have affected mainly sub-Saharan countries and periodically the disease has devastated some of these countries. More than 340,000 cases and more than 53,000 deaths were reported during the period 1951-1960 from this part of the world where the total population was 35 million. However, epidemic meningococcal disease is a worldwide problem and can affect any country regardless of variations in climate. Whereas, in the 1960s, meningococcal disease was considered to be a large

health problem in some tropical countries and no longer a serious health problem in North American and European countries, this opinion changed in the 1970s (WHO, 1998).

During this time, meningococcal disease epidemics occurred all over the world and the disease incidence increased in a number of countries of the Americas, Asia and Europe showing an epidemiological pattern characterised by recurrent epidemics and persistent endemic disease with sporadic cases. Outbreaks of meningococcal meningitis were reported from Finland, Mongolia and the USSR (1973/1974), Norway (from 1975 throughout the 1980s), Algeria and Chile (1979), Vietnam and Rwanda (1977/1978) (WHO, 1998). In the 1980s, an epidemic wave of meningococcal disease spread over territories in Asia; India with 6,133 cases in 1985 and Nepal Katmandu Valley in 1982-1984 with 103 cases per 100,000 population (WHO, 1998). Group B meningococcal disease epidemics were reported from Cuba in 1982-1984 and from Chile in 1986 and 1993 (WHO, 1998).

Epidemics of meningococcal disease occur in the same period of the year as the seasonal upsurge observed in endemic conditions: in winter/spring in temperate zones, and in the dry season in tropical countries. 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.*, 2002b). 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. 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 meningococcal disease peaks at the end of the dry season and virtually disappears at the start of the rainy season (Achtman, 1995; Riedo *et al.*, 1995). Since the 1980s, no evident periodicity has been observed; intervals between epidemics became more irregular, maybe as a result of extensive traffic and mixing of populations (WHO, 1998).

Worldwide, patterns of meningococcal disease vary considerably. There are areas of lower endemicity, for example the industrially developed countries with an annual attack rate of 1 to 12 per 100,000 (Pollard & Frasch, 2001). This pattern contrasts strongly with annual attack rates as high as 25 per 100,000 seen in parts of the developing world (Tikhomirov, 1987). In large areas of Africa, meningococcal meningitis is a highly endemic disease. In a region extending from Gambia in the west to Sudan in the east, and from the Sahara in the north to the tropical rain forests of Central Africa in the south, the incidence of meningitis has been so high that this part of Africa has been dubbed 'the Meningitis Belt' (Moore *et al.*, 1989). Major epidemics arise very rapidly, peaking within a few weeks. Incidence rates often remain elevated for 1 to 2 years after the appearance of an epidemic. During an epidemic, the age distribution shifts from young children to teenage children and young adults. Wherever this disease is endemic, there is a risk of periodic epidemics. During those epidemics, attack rates can reach 1,000 per 100,000 (Greenwood, 1984). The serogroups responsible can also shift. Although serogroup A is the main cause of epidemics, other serogroups, such as C, W135 and Y, can cause infection in highly endemic regions (Griffiss & Brandt, 1979; Greenwood, 1984)

A combination of risk factors must exist for an epidemic to occur. In the Meningitis

Belt, meningococcal outbreaks tend to be seasonal and it has been suggested that one such risk factor may be low humidity, which alters the pharyngeal mucosal barrier, resulting in invasive disease (Moore *et al.*, 1989). Socio-economic factors appear to be significant, with higher rates of infection occurring among the lower socio-economic groups. For example, in recent epidemics in the US, the poorest minorities were most affected irrespective of racial origin. Contact with cases is a significant factor in disease transmission. Crowded conditions also predispose towards infection and household clustering of cases is frequent. During an epidemic in northern Nigeria, 10% of patients gave a history of close contact with an infected person (Moore *et al.*, 1989). Household contacts may be expected to exhibit a disease incidence that is 100 to several thousand times that of the general population (Broome, 1986; Cooke *et al.*, 1989; Stuart *et al.*, 1989).

One reason suggested for the periodicity of meningococcal epidemics in sub-Saharan Africa is waning herd immunity. If non-capsular antigens are important in creating natural immunity against meningococci, introduction of a new serogroup, such as serogroup A with different OMP antigens, may place a previously resistant population at risk of an epidemic, as shown by the outbreaks of serogroup A meningococcal disease in the Meningitis Belt (Moore *et al.*, 1989). The World Health Organisation has estimated that in the two decades following World War II, the average incidence of meningococcal infection in the Meningitis Belt was about 70 cases per 100,000.

Major epidemics have occurred in the Meningitis Belt every 5 to 10 years since the beginning of the century, with three such waves in the last 30 years (Crowe *et al.*, 1987). Large outbreaks have also occurred recently in East African countries that are

becoming popular holiday destinations. Over several years, bacterial studies showed serogroup A as the predominant cause of large epidemics in Africa (Peltola, 1983). In the 1970s, however, several outbreaks were reported citing other serogroups. One study in Zaire, Nigeria, reported that, of those patients studied, 63% had a serogroup C infection compared with 36% with serogroup A. It was also reported that serogroup C infection was more severe than serogroup A infection, resulting in higher overall mortality (Evans-Jones *et al.*, 1977). Epidemics due to serogroup B meningococci occurred in Brazil (1971/72), Cuba (1982-84), Chile (1986), and an epidemic due to serogroup A in Brazil (1974). In Cuba due to the high incidence of meningococcal disease and the large epidemic between 1982-1984, mainly caused by serogroup B, a bivalent B/C vaccine was developed by Finlay, including OMPs from a B4: P1.15 strain (predominant in Cuba) and serogroup C capsule polysaccharide (Sierra *et al.*, 1991). This vaccine is now implemented into the routine immunisation schedule for children.

Parts of the Middle East are considered highly endemic for meningococcal meningitis. One outbreak in the Middle East occurred in August 1987 during the annual pilgrimage (Hajj) to Mecca, Saudi Arabia, and was caused by a single meningococcal serogroup A clone designated AIII-I. Epidemiological investigations and enzyme typing indicated that this clone was carried to Saudi Arabia by pilgrims from South Asia (Moore *et al.*, 1989). Subsequently, pilgrims who became carriers returned to their homes and serogroup A meningococcal disease was later reported in neighbouring Gulf States, Saudi Arabia, Egypt, Sudan, in sub-Saharan Africa, in the US and in Europe among the pilgrims and their contacts (Moore *et al.*, 1988; Jones & Sutcliffe, 1990). In Yemen and Sudan, epidemic spread occurred following

importation. Since then, some countries such as Egypt, Saudi Arabia and Sudan immunise high risk groups, and the health authorities of Saudi Arabia request health certificates showing a valid immunisation against meningococcal disease from all pilgrims (Geneva, 1998). Following the Hajj in 2000, meningococcal disease mainly due to serogroups A and W-135 was reported in pilgrims returning to other parts of the world including Europe, (Anon, 2000; Taha *et al.*, 2000; Hahne *et al.*, 2002; Gold, 2003; Khan, 2003).

1.6 European epidemiology and surveillance

Although meningococcal infection is endemic in most western European countries, in such communities the attack rates are usually low or moderate and only modest variations in incidence are observed from year to year. However, the high case fatality rate, even in countries with a well developed health service of about 10% for meningitis and up to 50% for septicaemia, as well as persistent sequelae following acute meningitis (about 20%), is of concern for the public and the health authorities. In many countries, clusters of meningococcal disease have been reported. However, large epidemics of meningococcal disease are now unusual although they have occurred in the past. Serogroup A epidemics were recorded during the war years 1914-1918 and 1939-1945, which were times of great social disturbance, crowding and extensive transfer of military personnel into high endemicity areas (Peltola, 1983). In the 1970s and 1980s, epidemics were reported from northern European countries. A surveillance system to assess the impact and changing epidemiology of invasive meningococcal disease in Europe was set up in 1987. Since about 1991, contributors from national reference laboratories, national communicable disease surveillance centres and institutes of public health in 35 European countries (as well

as from Australia and New Zealand) provided information on reported cases of meningococcal disease in their country (Connolly & Noah, 1999). In 2002 the incidence of culture-confirmed meningococcal disease in Europe varied between 0.3 and 4.7 per 100,000. Meningococcal disease was highest in young children, although there was a secondary peak in incidence observed in teenagers. Serogroup B is the most common cause of invasive meningococcal disease, followed by serogroup C. Serogroup C incidence can vary quite considerably between countries in Europe and there has been a reduction in serogroup C disease in those countries which have implemented the serogroup C conjugate vaccine. Case fatality rate is on average 7-8% although this varies by age and serogroup. The incidence of serogroup B in Europe is highest in children under one year old, with a small secondary peak in 15-19 year olds. However, serogroup B disease remains low in older ages. The incidence of serogroup C in Europe is also highest in children under one year old, with a small secondary peak in 15-19 year olds. Amongst the other serogroups that cause invasive disease serogroup W135 is the most common followed by serogroup Y.

There are differences in major serotypes of serogroup C and serogroup B within Europe. There has been an increase in serogroup C serotype 2a infection in many countries that have not implemented the serogroup C conjugate vaccine. This raises concerns, in view of the association of this serotype and strains of the hypervirulent ST-11 complex (Claus *et al.*, 2005). Variation in serogroup B strains is seen across the whole of Europe with no one dominant strain.

1.7 Meningococcal epidemiology and surveillance in the UK

In the UK, as in most European countries, meningococcal disease due to serogroup B and C infection has a seasonal peak with most cases occurring in the first quarter of the year (Jones & Mallard, 1993). The incidence of infection is greatest in infants and pre-school children and more cases are observed in males (Jones & Mallard, 1993). Epidemics of meningococcal disease occurred during the first and second world wars with annual totals of notified cases reaching 3500 in 1915 and 12,775 in 1940. Since then, two periods of increased case reports have been observed, but annual numbers of notified cases remained well below the totals observed in the wartime epidemics. The first of these hyperendemic periods occurred between 1972 and 1975 and was associated with a short-lived increase in infections with a specific serogroup B strain (Jones & Kaczmarski, 1995). The second period began in 1985 and several serotypes have been implicated (Jones & Kaczmarski, 1995). During late 1995 and 1996 a large number of clusters of serogroup C infections were reported (Kaczmarski & Cartwright, 1995) and led to polysaccharide meningococcal AC vaccine being given to pupils at several schools and within the local communities. A similar increase in reported cases had previously been observed in 1989/1990 in England and Wales, and this was in association with an epidemic of influenza (Cartwright, 1995). Studies have suggested that influenza may predispose to meningococcal infection (Cartwright *et al.*, 1991), but in late 1995 the increase in meningococcal disease preceded the rise in influenza activity. Similar increases in meningococcal disease, with a shift to more serogroup C infection and to older age groups, have been observed in recent years in Scotland (Fallon, 1988), Canada (Whalen *et al.*, 1995), and the United States (Jackson *et al.*, 1995). The increase in Canada was attributed by electrophoretic typing to a new

clone of serogroup C *N. meningitidis* ET-15, which became prevalent in the community (Kertesz *et al.*, 1998).

1.7.1 Scottish epidemiology and surveillance

Meningococcal disease trends within Scotland are similar to those experienced by England and Wales (Fallon *et al.*, 1984; Fallon, 1988; Clarke *et al.*, 2002b; Kyaw *et al.*, 2002). Meningococcal infections in Scotland have been under detailed bacteriological surveillance since 1970. The increased prevalence of infection in 1974 was shown by the number of cultures received. As in the rest of the UK, the highest number of notifications is usually in the first quarter of the year and is a useful indicator of how the pattern is likely to develop for the rest of the year. Hence the increasing level of infection seen in 1985 and 1986 was heralded by the highest numbers in the first quarters of those years than in the same periods in the previous years. The increase in prevalence of disease in Scotland during the mid-eighties was first noticed in Lanarkshire in 1984. The increased prevalence of infection in 1974-76 was mainly due to serogroup B that predominated in succeeding years. However, although serogroup B infection was prevalent in the latter part of 1984, serogroup C strains had increased in prevalence since that time and began to dominate for a few years. This was the first time that this had been seen in Scotland since the laboratory surveillance of infection had begun. This pattern occurred again in the late nineties when the incidence of disease had increased in many parts of Europe (Connolly & Noah, 1999) and, in Scotland, much of this increase resulted from the emergence of serogroup C strains after the decline in serogroup B disease in the early 1990s (Clarke, 1999; Smart, 1999). Many of the serogroup C strains that occurred in Scotland belonged to the ET-37/ST-11 clonal complex, (Wang *et al.*, 1993; Clarke, 1999)

which was often associated with clusters or outbreaks (Vogel *et al.*, 2000), although the incidence of serogroup C disease has decreased in individuals from those age groups that have received immunisation with serogroup C meningococcal conjugate (MenC) vaccine. A study looked at the relationship between meningococcal genotype and capsular polysaccharide by investigating carried meningococci isolated from 8000 children and young adults in Bavaria, Germany (Claus *et al.*, 2005). It found that the rate of capsule gene expression did not vary with age of carrier or meningococcal genotype, except for serogroup C, for which increased expression was associated with the ST-11 complex (Claus *et al.*, 2005). Therefore, it was concluded that serogroup C capsule expression during carriage may contribute to the invasive character of the ST-11 complex and to the high efficacy of serogroup C meningococcal conjugate (MenC) vaccine (Claus *et al.*, 2005). Since the introduction of the MenC vaccines, laboratory confirmation and serogroup determination of the infecting organism has become more important (Maiden & Spratt, 1999).

1.8 Spatio-temporal analysis of meningococcal isolates

In the developed world most cases of invasive meningococcal disease (IMD) are sporadic but when outbreaks of IMD occur it becomes a public health emergency because of the disease's unpredictability, serious symptoms and sudden mortality. A cluster is defined as two or more cases of meningococcal disease occurring in the same preschool group, school, or college/university within a four-week period (Stuart, 2006). In most instances over the years, and during the period of 1972-1998, detection of increases in cases that were closely grouped within space and time relied on the alertness of public health officials due to the lack of more objective methods.

Many methods of computer-assisted cluster analysis have been developed (Kulldorff, 1997; Hoebe *et al.*, 2004; Ranta *et al.*, 2004) and have helped to identify and statistically evaluate increases in the incidence of IMD, thus providing valuable information for public health investigation and intervention. A stochastic model (a tool for estimating probability distributions of potential outcomes by allowing for random variation in one or more inputs over time) has been applied to predict outbreaks of meningococcal disease in closed communities such as military cohorts (Ranta *et al.*, 2004). Clusters of IMD in the Netherlands were evaluated statistically using space-time nearest neighbour analysis (Hoebe *et al.*, 2004). In Germany there have also been studies of clusters of meningococci that have used SaTScan (Elias *et al.*, 2006a; Elias *et al.*, 2006b). SaTScan (Kulldorff, 1997) is software that analyses spatial, temporal and space-time data using the spatial, temporal, or space-time scan statistics. SaTScan can also be used to test whether a disease is randomly distributed over space, over time or over space and time. It can also evaluate the statistical significance of disease cluster alarms and perform repeated time-periodic disease surveillance for early detection of disease outbreaks. The programme works by applying a likelihood function to circular windows (the program draws circles with variable size to define the potential cluster area) originating at defined locations of increasing size and compares observed and expected case numbers inside and outside the scan window to detect clusters that are least likely to have occurred by chance. Monte Carlo hypothesis testing is used to obtain the statistical significance for each cluster, i.e., results of the likelihood function are compared for a large number of random replications of the dataset generated under the null hypothesis.

1.9 Vaccines

The problems faced by vaccine developers are designing meningococcal vaccines that are safe, comprehensive and effective in the age groups most susceptible to disease. Meningococcal disease surveillance data show that, in principle, the development and implementation of vaccines against the meningococcus should be relatively straightforward. Possession of one of the six disease-associated capsules (A, B, C, Y, W135 and X) is an absolute requirement for pathogenesis. Evidence of the importance of horizontal genetic exchange in the generation of meningococcal antigenic diversity is provided by the mosaic structure of the genes and operons that encode major cell surface structure (Pollard & Maiden, 2001). This has major implications for both the development and the evaluation of vaccine candidates, as well as for the implementation of vaccination programmes, as it provides a mechanism for the reassortment of antigen-encoding genes among meningococcal clones and increases the prospect of meningococci evading host immunity. In addition the expression of many antigen genes is tightly regulated so that critical antigens are not continuously expressed *in vivo*. Meningococci can be considered as commensals that rarely cause disease rather than a strict pathogen as carriage occurs a great deal more than disease.

Meningococcal disease has always been a particular problem in the young. This poses a problem for vaccine developers as carbohydrate antigens such as capsular polysaccharides or lipopolysaccharides (LPS) are poorly immunogenic in the very young and frequently mimic host cell structures. Therefore, the question arises as to whether it is possible to enhance immunity to a carbohydrate in infants and would a vaccine elicit an autoimmune response. Protein vaccines are generally considered

better immunogens than carbohydrates but they have other problems associated with them. The more immunogenic meningococcal surface protein antigens suffer from the disadvantage that they are also antigenically highly variable.

1.9.1 Polysaccharide vaccines

Meningococcal vaccine development began in the 1930s with killed whole-cell and exotoxin vaccines, and it was established in the late 1960s that vaccines against meningococcal capsules can protect against infection (Gotschlich *et al.*, 1969). These vaccines are very safe and systemic reactions have been extremely rare. The most common adverse reactions are erythema and slight pain for one or two days at the site of injection. The recognised short-term efficacy levels of both serogroup A and serogroup C polysaccharides are 85%–100% in older children or in adults. In infants of three months, neither of the polysaccharide vaccines reliably elicits protective antibodies. Serogroup C polysaccharide vaccines given to children below two years of age are not reliably immunogenic and may lead to tolerance to serogroup C antigen in later years. In children aged two years and above serogroup Y and W135 polysaccharides have been proved to be safe and immunogenic.

The greatest challenge for vaccine developers is an effective serogroup B vaccine. Meningococcal polysaccharide antigens of serogroups A, C, Y and W135 do not provide any protection against serogroup B meningococci, which in some countries are the leading cause of endemic meningococcal disease. The use of capsular polysaccharide as the basis of a vaccine for prevention of serogroup B diseases has had problems associated with it. Serogroup B polysaccharide is poorly immunogenic, even when conjugated to a protein carrier. This has been attributed to the similarity of

the serogroup B polysaccharide to antigens of the central nervous system. The serogroup B capsular polysaccharide is identical to a widely distributed human carbohydrate [(238)N-acetyl neuraminic acid or polysialic acid], which, being a self-antigen, is a poor immunogen in humans. Furthermore, use of this polysaccharide in a vaccine may elicit auto-antibodies (Hayrinen *et al.*, 1989).

1.9.2 Conjugate vaccines

Conjugate vaccines are produced by covalently attaching a poor antigen to a carrier protein. Human immune systems respond much more strongly to proteins than to sugars, so conjugate vaccines trigger a long-lasting immune response and are effective in babies as young as two months of age. For this reason, the meningococcal serogroup C conjugate vaccine is a big improvement over the previous polysaccharide vaccine, which provided protection only for about three years and did not provide protection in children under two years old. Through conjugation of serogroup C-specific meningococcal polysaccharide to a protein carrier, a thymus-dependent immune response is achieved. Internationally there are three serogroup C meningococcal conjugate (MCC) vaccines currently licensed. In some vaccines the polysaccharide is linked to a non-toxic mutant of diphtheria toxin (CRM 197) whereas in one vaccine, tetanus toxoid is used as the carrier protein. The conjugate vaccines induce enhanced levels of IgG anti-capsular antibodies and memory B-cells. In 1999, in the United Kingdom, immunisation against serogroup C meningococcal disease using MCC vaccines became part of the national childhood immunisation programme. The incidence of meningococcal serogroup C disease at that time was approximately 2 per 100 000 population. Infants were vaccinated at two, three and four months of age and children aged 4–13 months and children under the age of 18 years offered

'catch-up' vaccination. Afterwards, several countries introduced national mass MCC vaccination campaigns. These included Ireland, Spain, the Netherlands and Iceland. Large-scale studies in the UK showed that, 16 months after vaccination with one single dose of the MCC vaccine, 88% of children aged one to two years still had protective antibody levels whereas among adolescents of 15–17 years 96% had protective levels (WHO, 2002). Surveillance has so far shown no evidence of changes of the prevalent serogroups and serotypes among invasive meningococcal isolates since the MCC program was launched in the United Kingdom. A major protective effect of the C conjugate vaccines is by herd immunity (Ramsay *et al.*, 2003). The United Kingdom experience confirms that the current MCC vaccine's safety profile is excellent.

A serogroup A, C, Y and W-135 polysaccharide-protein conjugate meningococcal vaccine was recently introduced into the US for adolescents (Bilukha *et al.*, 2007). The control of major serogroup A and serogroup C epidemics throughout the world has been achieved by the implementation of mass immunisation campaigns. In addition to their use in emergency mass campaigns, meningococcal vaccines are also recommended for groups in which a particularly high risk of disease has been documented. These include those attending army units, training camps, or boarding schools, travellers to epidemic areas, and persons with immunological predisposition to meningococcal disease (such as asplenia and inherited immunological deficiencies).

1.9.3 Vaccine development

The implementation of effective vaccination programmes is seen as the solution to controlling meningococcal disease. One such method is to develop a conjugate vaccine. Conjugate vaccines induce immune memory and are immunogenic in children aged under 2 years in contrast with polysaccharide vaccines. Conjugate vaccines were first developed for *Haemophilus influenzae* type b (Hib). The success of the Hib conjugate vaccination programme (Peltola, 2000) influenced the use of the same approach for meningococcal vaccines, which led to the development and implementation of the aforementioned MCC vaccines. Since September 2006, a combination MCC–Hib vaccine (conjugated to tetanus toxoid) has also been available in the UK, as a booster at 12 months of age (Chief Medical Officer, 2006).

Protection against serogroups A, C, W135 and Y can be provided by quadrivalent conjugate vaccines (MCV-4), which have recently been recommended for use in adolescents in the USA (Centers for Disease Control and Prevention (CDC), 2005). This is because of an increase in the USA of serogroup Y meningococcal disease which has been on the increase in the USA over the last decade (Rosenstein *et al.*, 1999; Kimmel, 2005). As previously mentioned, serogroup A has caused few cases of disease in Scotland over the past twenty years but epidemic serogroup A disease is still common in other parts of the world, particularly sub-Saharan Africa. Serogroup W135 has emerged as an important cause of disease in Africa (Mueller *et al.*, 2006).

Unfortunately, as previously mentioned, there is currently no generally effective vaccine against the most common serogroup, serogroup B. A vaccine based on the serogroup B polysaccharide has not been developed because the serogroup B

polysaccharide is poorly immunogenic. Therefore, conjugate serogroup B vaccines could not be introduced because overcoming the apparent immune tolerance to this self-antigen carries the hypothetical risk of inducing autoimmunity (Finne *et al.*, 1983). However, there have been no reported severe adverse effects to natural or vaccine-induced anti-B capsular polysaccharide antibody (Stein *et al.*, 2006). Therefore, other methods have had to been employed to develop other vaccine targets. The focus for vaccines against serogroup B has therefore moved to non-capsular antigens. Vaccines based on outer-membrane vesicles (OMVs) from single strains of serogroup B meningococci have been developed for use in Cuba, Norway, and New Zealand (Oster *et al.*, 2005). These vaccines have been shown both to elicit serum bactericidal antibody responses and to protect against developing meningococcal disease in clinical trials (de Moraes *et al.*, 1992b).

PorA, as previously mentioned, is an OMP that is expressed by almost all meningococci and has been identified as a major inducer of, and target for, serum bactericidal antibodies. However, eliciting an immune response against one PorA antigen does not offer protection against strain types with different PorA antigens, as there are a large number of PorA proteins with different antigenic specificities. Thus, OMV vaccines are strain-specific vaccines that can be used against clonal disease outbreaks but are not beneficial for prevention of sporadic disease caused by diverse strains. Two of the most extensively studied OMV vaccines are from Norway (MenBvac™) (Rosenqvist *et al.*, 1995) and Cuba (VA-MENGOC-BC®) (Sierra *et al.*, 1991) and were produced in response to national outbreaks. The Cuban meningococcal vaccine against serogroups B and C (VA-MENGOC-BC®) was developed and it is manufactured by Finlay Institute. VA-MENGOC-BC® is a

bivalent vaccine of capsular polysaccharide of *N. meningitidis* serogroup C, and outer membrane vesicles of serogroup B meningococccus that includes PorA, PorB, Opa, Opc, Tbp, NspA, high molecular weight proteins and other proteins (Uli *et al.*, 2006). This vaccine was licensed in 1989, and successfully used for epidemic control in Cuba, Brazil, Colombia and Uruguay (Azeredo *et al.*, 1994; Rodriguez *et al.*, 1999; Pirez *et al.*, 2004). Since 1991 VA-MENGOC-BC® has formed part of the Cuban infant vaccination schedule and after mass vaccination campaigns, there was a swift fall in the incidence of meningococcal disease in all age groups. The incidence rate of this disease in Cuba remains at 0.3 per 100,000 inhabitants during the last 4 years, lower than the pre-epidemic period (Dominguez *et al.*, 2006). Although there have been outbreaks of meningococcal disease in the areas where this vaccine has been administered, some outbreaks were caused by different strains. But this vaccine has also shown the potential to provide protection against some serogroup B meningococcal strains other than the vaccine type-strain (de Moraes *et al.*, 1992a).

The Norwegian vaccine (MenBvac) is an OMV vaccine, based on a serogroup B strain (B: 15:P1.7, 16) representative of the epidemic that started in Norway in 1974 and was developed in collaboration between the Norway Institute of Public Health and Chiron. A combination of the Norwegian OMV MenBvac vaccine with a conjugate MenC vaccine was studied in adult volunteers and shown to be immunogenic with regard to both serogroup B and C meningococci (Aaberge *et al.*, 2005).

Novartis scientists have used "reverse vaccinology" to develop the Novartis MenB vaccine. Reverse vaccinology uses bioinformatic approaches to screen the entire

genome to find genes. The genes are then filtered for desirable attributes that would make good vaccine targets, such as outer membrane proteins (Rappuoli, 2000). The major advantage for reverse vaccinology is finding vaccine targets quickly and efficiently. The disadvantage is that only proteins can be targeted using this process. Normal vaccinology approaches can find other biomolecular targets such as polysaccharides.

By first decoding the entire genetic makeup of a pathogenic meningococcal serogroup B strain, Novartis discovered 580 vaccine candidate antigens. Reproduced through genetic engineering for further investigation, antigens were selected that had the ability to stimulate the immune system to kill bacteria from a panel of strains of meningococcal serogroup B representative of global and temporal diversity. New data show that the Novartis Meningitis B vaccine may be the first to protect infants six months and older against multiple strains of meningococcal serogroup B. In a recent study, more than 95% of infants aged six to 12 months generated a protective immune response as early as one month post-second dose against strains representing multiple antigens included in the vaccine (Snape, 2008). Novartis MenB vaccine is the first potentially broad coverage meningitis B vaccine to reach phase III clinical testing, which began in the first quarter of 2008 (Snape, 2008).

In New Zealand the epidemic of systemic serogroup B meningococcal disease was dominated by a single subtype and therefore, the best option for its control was the use of a strain-specific vaccine (Oster *et al.*, 2005). A PorA vaccine was developed by the Norwegian NPHI and Chiron, in partnership with the New Zealand Ministry of Health and the University of Auckland. The resulting tailor-made vaccine, MeNZB™,

is a meningococcal serogroup B outer membrane vesicle vaccine for intramuscular injection in a three-dose regimen, intended to provide immunity against serious systemic disease caused by *N. meningitidis* serogroup B subtype P1.7b, 4 (Martin *et al.*, 1998; Sexton *et al.*, 2004). This subtype accounted for 86% of all group B meningococci isolated from cases of disease, from 1990 to 2003 in New Zealand (Martin & McDowell, 2004). The seed stock for this tailor-made vaccine, MeNZB™, is derived from strain NZ98/254, which was chosen as representative of the New Zealand epidemic. This strain was isolated from a 15-year-old boy from New Zealand diagnosed with meningitis in October 1998.

A recombinant hexavalent PorA OMV vaccine (HexaMen) was developed at the National Institute for Public Health and the Environment (RIVM), Netherlands. The vaccine formulation contains two OMVs, each expressing three different PorAs (P1.7, 16; P1.5-1, 2-2; P1.19, 15-1; P1.5-2, 10; P1.12-1, 13; P1.7-2, 4) (van der Ley *et al.*, 1995; Claassen *et al.*, 1996). This hexavalent OMV vaccine formulation was safe, well tolerated and immunogenic in infants, toddlers and schoolchildren (Cartwright *et al.*, 1999; de Kleijn *et al.*, 2000). The vaccine research activities of the RIVM on PorA-based meningococcal B vaccines are now in the Netherlands Vaccine Institute (NVI). The NVI is a public institute of the Ministry of Health devoted to the vaccines for the Netherlands Vaccination Programme. In order to provide an even broader protection, the hexavalent vaccine developed by RIVM has now been extended to a nonavalent PorA OMV vaccine (NonaMen) vaccine, which contain three additional PorA OMPs, P1.22, 14; P1.7-1, 1; P1.18-1, 3, 6. Adding a third trivalent OMV to cover the nine most frequently occurring subtypes in the developed countries has

achieved this. The third trivalent OMV is called HP1416 and expresses P1.22, 14; P1.7-1, 1 and P1.18-1, 3,6 (Van den Dobbelsteen *et al.*, 2004).

The main problem with targeting non-capsular antigens, such as PorA, is that they are antigenically diverse and exhibit geographical and temporal variability. Sequence variation could also affect effectiveness of a multivalent OMV vaccine (Findlow *et al.*, 2005). Multivalent OMV vaccines are being developed (Borrow *et al.*, 2006), which target the most common OMPs associated with serogroup B, with the benefit that the efficacy will not be restricted to serogroup B strains, as sub-capsular antigens are targeted. The potential benefit from OMP vaccines depends on the main strains causing disease in a particular country.

1.10 Project background

Due to the high prevalence of serogroup C in Scotland and the availability of a serogroup C polysaccharide conjugate meningococcal (MenC) vaccine, MenC vaccination was implemented in 1999. The vaccine was highly effective in reducing the incidence of serogroup C meningococcal disease and associated mortality, with no adverse effects on other serogroups (Mooney *et al.*, 2004; Diggle & Clarke, 2005). However, the long-term effectiveness of the vaccine remains unknown. It is therefore vital that the surveillance of meningococcal disease is continued. In order to improve our understanding of the current situation, long-term retrospective data is also essential as the current information is post implementation of MenC vaccines. Although data were available on meningococcal serogroups dating back to the 1970s, and serogroup and serotype data were available from the 1990s, there was little

molecular data on meningococci circulating from the 1970s to present. Although sequence type data were available in Scotland (Diggle & Clarke, 2005), this only includes strains from 1999 onwards and therefore there is only one year of data prior to the implementation of MenC vaccines. There was a need to perform retrospective analysis on meningococcal isolates using MLST and *por*A gene sequencing. This was only possible because SMPRL were in a unique position in having a large collection of disease-associated meningococci isolated within Scotland since 1972, which accounted for all known invasive cases.

In total there were 2607 invasive isolates, i.e. isolates from blood and CSF that had caused meningitis and septicaemia, received by the SMPRL from the start of 1972 to the end of 1998. All the samples originated in Scotland covering all regions and they were initially identified as *N. meningitidis* and then serogrouped. In the early 1980s other tests were introduced namely antibiotic resistance tests and sero-subtyping. Once all tests were complete the isolates were freeze-dried in order to preserve them. This freeze-dried method was replaced in 1996 when the samples were stored in Protect beads and 20% glycerol. However, not all of these isolates have survived and 2517 isolates were available for further characterisation.

1.11 Aims of the project

- Molecular epidemiology and analysis of 2517 isolates of invasive *Neisseria meningitidis* isolated in Scotland, 1972-1998. To characterise and analyse isolates at seven housekeeping loci (*abcZ, adk, aroE, fumC, gdh, pdhC, pgm*) by multilocus sequence typing (MLST) and at the *porA* locus, which encodes a major surface antigen and vaccine candidate.

- To determine the clusters of disease that occurred in Scotland, 1972-1998, using SaTScan software and to investigate where these clusters occurred geographically, the strains responsible and the patients involved.

- To use data generated by this study, on isolates from the period 1972-1998, to estimate the potential coverage, within Scotland, of possible serogroup B meningococcal OMV vaccines: Cuba (VA-MENGOC-BC®), Norway (MenBvac™), New Zealand (MeNZB™), hexavalent (HexaMen) and nonavalent (NonaMen). Also to determine if there are other variants which might be included in these types of vaccine.

Chapter 2

Materials and Methods

2.1 Health and safety

N. meningitidis has been reported as a laboratory-acquired infection (Sejvar *et al.*, 2005) therefore all procedures were performed with the necessary precautions according to health and safety guidelines. As the specific risk factors for laboratory-acquired infection are likely associated with exposure to droplets or aerosols containing *N. meningitidis* (Sejvar *et al.*, 2005) appropriate safety wear and class II biological safety cabinets were used when appropriate. All chemicals were handled with care and the required precautions taken in accordance 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). All chemicals and materials were disposed off in the appropriate containers.

2.2 Bacterial strains

In total there were 2607 invasive isolates, i.e. isolates from blood and CSF that had caused meningitis and septicaemia, received by the SMPRL from the start of 1972 to the end of 1998 (Table 2.1). All the samples originated in Scotland covering all regions and they were identified initially as *N. meningitidis* and then serogrouped. In the early 1980s other tests were introduced, namely antibiotic resistance tests and sero-subtyping. Once all tests were complete the isolates were freeze-dried in order to

preserve them. This freeze-dried method was then replaced in 1996 when the samples were stored in Protect beads and 20% glycerol. However, due to the ravages of time, not all of these isolates survived with 2517 isolates available for further characterisation.

Table 2.1 Serogroup and time period of the 2607 invasive isolates

	Time period		
Serogroup	**1972-1979**	**1980-1989**	**1990-1998**
A	119	25	3
B	348	448	650
C	59	350	403
Y	7	10	20
W135	40	12	15
X	3	1	6
Z	0	2	3
29e	2	3	1
Non-groupable	6	5	8
Unknown	33	10	15

2.3 Growth conditions

N. meningitidis isolates were cultured on Columbia horse blood agar (contains 5% horse blood) (Oxoid, Basingstoke, UK) and incubated overnight at 37°C in an atmosphere of 5% CO_2. Freeze-dried meningococci, stored within glass vials, were resuscitated. A diamond was used to score a line around the glass vial, which allowed for the vial to be easily snapped in half. Drops of Mueller Hinton broth were added to rehydrate the freeze-dried culture and then transferred to a Columbia horse blood agar plate. The plate was streaked out and incubated overnight at 37°C in an atmosphere of 5% CO_2.

2.4 Storage of bacterial strains

From an overnight culture on a Columbia horse blood agar plate a heavy scraping of *N. meningitidis* was suspended in the support medium in Protect beads (Technical Service Consultants Ltd, Heywood, Lancashire, UK). The vial was gently mixed to coat the beads and the liquid removed. The protect beads were marked with the appropriate strain designation and then stored at -70^0C. Bacterial strains were recovered by taking a sterile loop containing a single bead, plating it onto a Columbia horse blood agar plate and incubating as described in section 2.3.

2.5 Sterilisation

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

2.6 Miscellaneous Reagents

2.6.1 Agarose gel

For 1.5% agarose gel, 0.75g of hi-pure low electroendosmosis (EEO) agarose (BioGene, Kimbolton, Cambridgeshire, UK) was added to 50ml of 1 x electrophoresis (ELFO) buffer in a glass bottle. This was heated until the solution was completely dissolved. To this solution, 2µl of 1mg/ml ethidium bromide (SIGMA, Gillingham, Dorset, UK) was added and the gel was poured into the gel mould. After use, the gels were discarded in plastic drums designed for disposal of toxic ethidium bromide.

2.6.2 Production of 5% carbon dioxide atmosphere

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

2.6.3 ELFO buffer x10

Into a 2 litre conical flask, 9.3g of EDTA, 27.5g of boric acid (SIGMA, Gillingham, Dorset, UK) and 162g of Trizma base were placed. One litre of 18MΩ distilled water was then added to the flask, which was placed on a heated plate with a magnetic stirrer and mixed at 56^{0}C until dissolved. The pH was adjusted to pH 7.2 and the solution was transferred into two 500ml Duran bottles and stored at 4^{0}C.

2.6.4 Ethidium bromide

A 10mg/ml solution of ethidium bromide was prepared by adding 1g of ethidium bromide powder to 100ml of de-ionised water and mixing to ensure that the powder had dissolved completely. The container was wrapped with aluminium foil to keep out light and stored at 4^{o}C.

2.6.5 20% Glycerol

Twenty millilitres of glycerol (SIGMA, Gillingham, Dorset, UK) were added to 80ml of 18mΩ distilled water and mixed.

2.6.6 15% Hydrochloric acid

Hydrochloric acid (HCl) (810ml) was slowly added to 1,190ml of de-ionised water and mixed gently. The mixture was then allowed to cool. This was transferred to a 5 litre plastic container. This procedure was carried out in a fume hood.

2.6.7 Mueller-Hinton broth

Twenty one grams of Mueller-Hinton broth (Oxoid, Basingstoke, Hampshire, UK) were suspended in 1 litre of de-ionised water and mixed to dissolve. Two millilitre aliquots were distributed into bijoux bottles and sterilised at 121^0C for 15 min.

2.6.8 MegaBace LPA buffer x1

Fifty millilitres of 10x MegaBace linear polyacrylamide (LPA) buffer (Amersham Biosciences, Little Chalfont, UK) was diluted by adding 450ml of deionised water. This was then stored at 4°C for use with MegaBace sequencing runs.

2.6.9 Orange G

A 0.1% solution of orange G in distilled water was prepared.

2.6.10 TBE long read run buffer (x10)

Trizma base (126g), boric acid (27.5g) and EDTA (9.3g) were added to a two litre conical flask. One litre of deionised water was then added. The solution was heated to 50°C until dissolved and the pH adjusted to 8.3. This was then stored at 4°C.

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2.6.11 TRIS buffer x50

Two hundred and forty-two grams of Trizma base (SIGMA, Gillingham, Dorset, UK) and 18.61g of EDTA (ethylenediaminetetraacetic acid di-sodium salt) (SIGMA, Gillingham, Dorset, UK) were dissolved in 900ml of deionised water. The pH was adjusted to 7.7 with approximately 50ml of glacial acetic acid (BDH lab supplies, Poole, UK) and the solution made up to a final volume of 1 litre with deionised water.

2.6.12 2% Virkon

Two grams of Virkon powder (Antee International, Sudbury, Suffolk, UK) was added to 100ml of distilled water in a sterilised bottle and mixed until dissolved.

2.7 DNA preparation

From overnight cultures, 5-10 single colonies were resuspended into 0.5 ml of 18 MΩ distilled water and heated to 100°C for 15 min. The suspension was centrifuged at 15000 g for 2 min and the supernatant was used as a DNA source for subsequent PCR amplifications.

2.8 Phenotypic characterisation

There was a number of biochemical methods required for the characterisation of *N. meningitidis*, which were used by the SMPRL to perform tests previous to 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) (Eldridge *et al.*, 1978; Frasch *et al.*,

1985) for typing and, in cases where identification was not conclusive, confirmation by API NH was carried out (BioMérieux UK Ltd Basingstoke).

2.9 Determination of capsular serogroups by PCR

All previous non-groupable isolates were characterised again using genotypic methods. A representative number of isolates were taken from the dataset to analyse the accuracy of previous serogrouping methods.

2.9.1 SiaD (Serogroups B, C, Y and W135)

Each PCR reaction was performed in a final volume of 25µl using 1.1x Reddymix PCR master mix (ABgene, Epsom, UK). Each reaction mix consisted of 20µl of PCR master, 1µl of forward and reverse primer for each serogroup (B, C, Y and W135) (MWG Biotech, Milton Keynes, UK) (Table 2.2), at a working concentration of 50pmol, and 3µl of sample DNA from each isolate. Three microlitres of the positive controls (laboratory reference strains) and negative controls (deionised water) for each of the serogroups were also added. PCR reactions were carried out in a thermocycler (MWG Biotech, Milton Keynes, UK). The PCR conditions were 95°C for 10 min, 60 cycles of 95°C for 15 s, 60°C for 1 min and 75°C for 5 min (Lewis & Clarke, 2003; Lewis *et al.*, 2003). Twenty microlitres of the samples were then loaded on a 1.5% agarose gel for serogroup determination. 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.

Table 2.2 Primers used for the determination of sergroups B, C, Y and W135

Primer Name	Primer Sequence
B Forward	5'-TGCATGTCCCCTTTCCTGA-3
B Reverse	5'-AATGGGGTAGCGTTGACTAACAA-3'
C Forward	5'-GATAAATTTGATATTTTGCATGTAGCTTC-3'
C Reverse	5'-TGAGATAGCGGTATTTGTCTTGAAT-3'
Y/W135Forward	5'-GGTGAATCTTCCGAGCAGGA-3'
Y Reverse	5'-GGGATATCGTACACCATACCCTCTAG-3'
W135 Reverse	5'-GAATATCATACACCATGCCTTCCATA-3'

2.9.2 *MynA* (Serogroup A)

Each PCR reaction was performed in a final volume of 25µl using 1.1x Reddymix PCR master mix (ABgene, Epsom, UK). Each reaction mix consisted of 20µl of PCR master, 1µl each of forward and reverse *mynA* primer (MWG Biotech, Milton Keynes, UK) (Table 2.3), containing 50pmol, and 3µl of sample DNA from each isolate. Three microlitres of the positive controls (laboratory reference strains) and negative controls (deionised water) for each of the serogroups were also added. PCR reactions were carried out in a thermocycler (MWG Biotech, Milton Keynes, UK). The PCR conditions were 95°C for 2 min, 50 cycles of 95°C for 1 min, 53°C for 1 min 30 s 72°C for 30 s and 72°C for 2 min (Clarke *et al.*, 2003). Twenty microlitres of the

samples were then loaded on a 3% agarose gel. 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.

Table 2.3 Primers used for determination of serogroup A

Primer Name	Primer Sequence
mynA Forward	5'-AACCCAACCAGAGCCTACAAG-3'
mynA Reverse	5'- CTGTTGGCCACATTGAAGCAG-3'

2.9.3 Capsule null locus (*cnl*)

Each PCR reaction was performed in a final volume of 25µl using 1.1x Reddymix PCR master mix (ABgene, Epsom, UK). Each reaction mix consisted of 20µl of PCR master, 1µl of each forward and reverse *cnl* primer (MWG Biotech, Milton Keynes, UK) (Table 2.4), containing 50pmol, and 3µl of sample DNA from each isolate. Three microlitres of the positive controls (laboratory reference strains) and negative controls (deionised water) for each of the serogroups were also added. PCR reactions were carried out in a thermocycler (MWG Biotech, Milton Keynes, UK). The PCR conditions were; 95°C for 2 minutes, 45 cycles of 95°C for 25 s, 56°C for 20 s 72°C for 30 s and 72°C for 5 min (Claus *et al.*, 2002). Twenty microlitres of the samples were then loaded on a 1.5% agarose gel. 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.

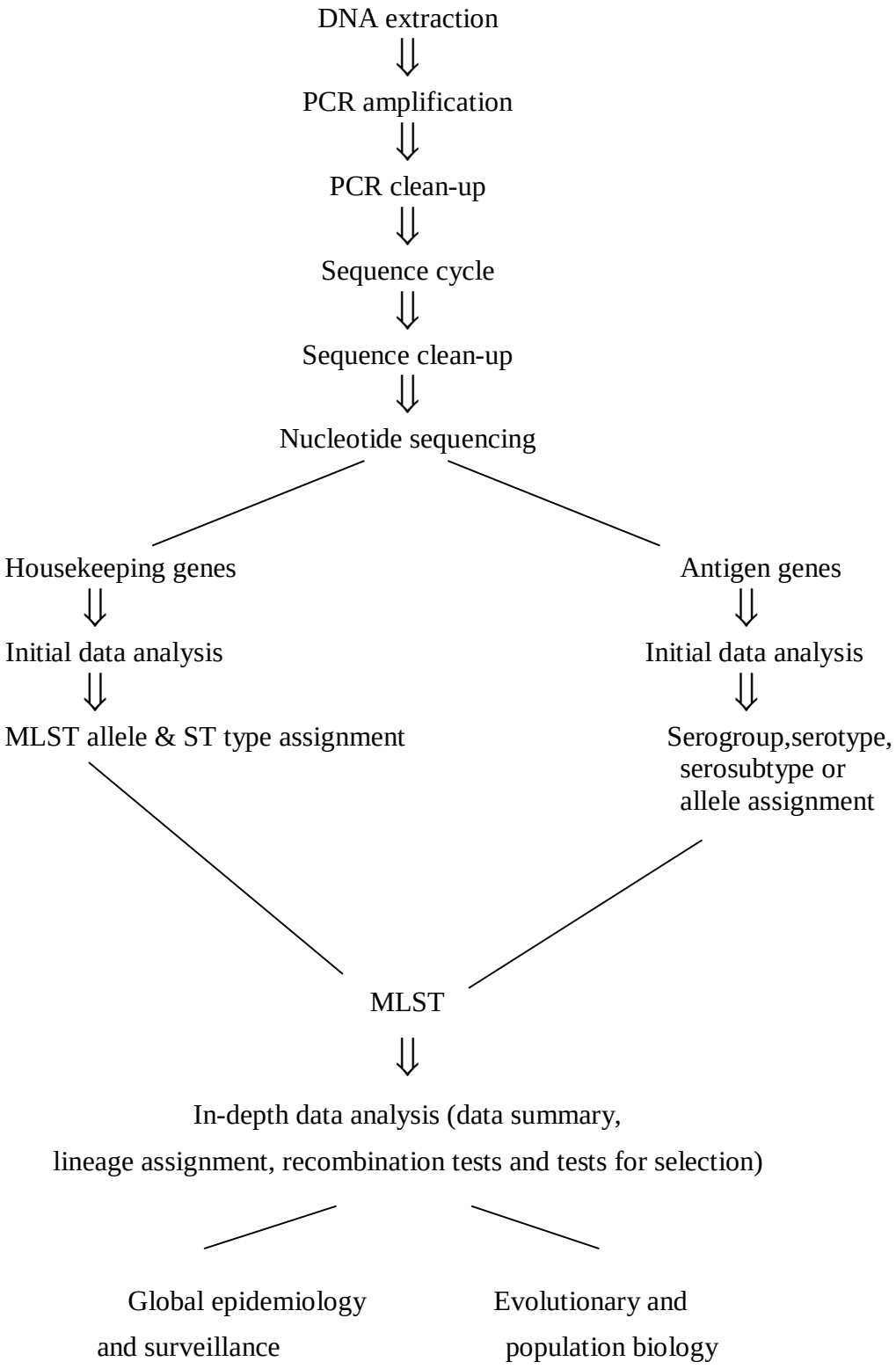
Table 2.4 Primers used for determination of capsule null locus

Primer Name	Primer Sequence
cnl Forward	5'-CGCGCCATTTCTGCC-3'
cnl Reverse	5'-GGTCGTCTGAAAGCTTGCCTTGCT C-3'

2.10 MLST and antigen gene sequencing

MLST and antigen gene sequencing utilised two robotic liquid handling systems, the RoboSEQ 4200 SE (MWG Biotech, Milton Keynes, UK) and THEONYX (MWG Biotech, Milton Keynes, UK) and an automated capillary system DNA sequencer, the MegaBACE 1000 (Amersham Biosciences, Little Chalfont UK). This project developed a procedure for MLST using a third generation liquid handling robot (THEONYX) (Sullivan *et al.*, 2006). This allowed for the automation of all the procedures required for DNA amplification and sequencing. For each semi-automated procedure one batch constituted 24 samples. Each sample had eight genes sequenced, seven housekeeping (*abcZ, adk, aroE, fumC, gdh, pdhC, pgm*) and one antigen gene (*porA*). A flow diagram showing the stages involved in MLST is shown in Figure 2.1 (Sullivan *et al.*, 2005). Another DNA sequencer the LI-COR L4200-L2 was also used for sequencing smaller numbers of samples.

Figure 2.1 Flow diagram of the stages involved in MLST Analysis



2.10.1 PCR amplification

This was based on a procedure described by Clarke *et al.* (Clarke *et al.*, 2001a) and Sullivan *et al.* (Sullivan *et al.*, 2006). The RoboSEQ 4200 SE and THEONYX liquid handling systems were programmed according to the manufacturer's specifications (MWG Biotech, Milton Keynes, UK). All PCR reagents were maintained at 4°C on the platform within the specified reagent rack. For the THEONYX liquid handling system all procedures in the automated procedure used washable tips. For the RoboSEQ 4200 SE, disposable or washable tips were used depending on the procedure. Each PCR reaction was performed in a final volume of 25µl using 1.1x Reddymix PCR master mix, containing 1.25U of TAQ DNA polymerase (ABgene, Epsom, UK), 75mM Tris-HCl (pH 8.8 at 25°C), 20mM $(NH_4)_2$, 1.5mM $MgCl_2$, 0.01% (V/V) Tween 20, 0.2mM each of dATP, dCTP, dGTP and dTTP and red dye for gel electrophoresis (ABgene, Epsom, UK). For a 25µl reaction, 20µl of PCR master mix and 1µl of each forward and reverse primer (for each housekeeping or antigen gene) (MWG Biotech, Milton Keynes, UK) (Table 2.5), containing 50pmol, were added to produce a master mix of 22µl, for each gene. These pre-prepared master mixes were placed on the refrigerated reagent rack. DNA extracted samples were placed within a separate 96-well thermosprint plate (Web Scientific, Crewe, UK) and placed upon another refrigerated platform. 22µl of master mix was automatically added to the appropriate wells within a refrigerated 96-well thermosprint plate. Advanced pipetting parameters for the RoboSEQ 4200 SE were using a disposable tip prefill with 21µl master mix at a speed of 50µl per second. A further 1µl of master mix was added at 50µl/s. This 22µl volume was dispensed into the appropriate well at a speed of 100µl/s. During the transfer a transport airgap of 2µl

Table 2.5 Amplification primers for housekeeping genes and *porA*

Primer Name	Primer Sequence
abcZ Forward	5'-AATCGTTTATGTACCGCAGG-3'
abcZ Reverse	5'-GTTGATTTCTGCCTGTTCGG-3'
adk Forward	5'-ATGGCAGTTTGTGCAGTTGG-3'
adk Reverse	5'-GATTTAAACAGCGATTGCCC-3'
aroE Forward	5'-ACGCATTTGCGCCGACATC-3'
aroE Reverse	5'-ATCAGGGCTTTTTTCAGGTT-3'
fumC Forward	5'-CACCGAACACGACACGATGG-3'
fumC Reverse	5'-ACGACCAGTTCGTCAAACTC-3'
gdh Forward	5'-ATCAATACCGATGTGGCGCGT-3'
gdh Reverse	5'-GGTTTTCATCTGCGTATAGAG-3'
pdhC Forward	5'-GGTTTCCAACGTATCGGCGAC-3'
pdhC Reverse	5'-ATCGGCTTTGATGCCGTATTT-3'
pgm Forward	5'-CTTCAAAGCCTACGACATCCG-3'
pgm Reverse	5'-CGGATTGCTTTCGATGACGGC-3'
porA Forward	5'-ATGCGAAAAAAACTTACCGCCCTC-3'
porA Reverse	5'-AATGAAGGCAAGCCGTCAAAAACA-3'

was maintained. Advanced pipetting parameters for THEONYX were using washable tips for each of the genes the tips were washed before first step, aspiration of 22µl master mix at a speed of 250µl/s and dispension into the appropriate well at a speed of 400µl/s. During the transfer a transport airgap of 2µl was maintained. After PCR master mix distribution, 3µl of DNA from each sample were added to each of the wells. Advanced pipetting parameters for the RoboSEQ 4200 SE were using a

disposable tip aspirated at a speed of 100µl/s with liquid mixing repeated three times and DNA dispense at 200µl/s with liquid mixing repeated twice. Advanced pipetting parameters for THEONYX were the tips were washed before each step, aspirated at a speed of 250µl/s with liquid mixing repeated three times and DNA dispensed at 400µl/s with liquid mixing repeated twice. During the transfer, a transport airgap of 2µl was maintained. The final reaction volume was 25µl. To perform more than twelve samples an additional plate was used. This was transferred from the refrigerated stacker onto an available 96 well-refrigerated position. The initial plate was then transferred from the platform into the refrigerated stacker and maintained at 4°C. These plates were placed into MWGBiotech thermocyclers. The PCR conditions were modified from Clarke *et al.* (Clarke *et al.*, 2001a). 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 followed by 3 cycles at 94°C for 1 min, 58°C for 1 min and 72°C for 2 min followed by 3 cycles at 94°C for 1 min, 56°C for 1 min and 72°C for 2 min followed by 20 cycles at 94°C for 1 min, 54°C for 1 min and 72°C for 2 min followed by and finally 72°C for 10 min.

2.10.2 PCR product purification for sequencing on MegaBace 1000

For the RoboSEQ 4200 SE, disposable tip units were removed and replaced with washable tips before the procedure continued. For THEONYX washable tips were used throughout. The total reaction volume of 25µl was transferred from each well into a Multiscreen™ 384-PCR plate (Millipore,Hertfordshire,UK) situated on the vacuum manifold. Advanced pipetting parameters for the RoboSEQ 4200 SE were; 25µl/s aspiration and 200µl/s dispension. Advanced pipetting parameters for THEONYX were; wash tips before each step, 250µl/s aspiration and 400µl/s

dispension. During the transfer, a transport airgap of 2µl was maintained. Washable tips for the RoboSEQ 4200 SE were removed and replaced with disposable tips before the next procedure. A vacuum was applied at a pressure of 450mbar for 20 min. The sequence setup was then performed with a sequence reaction of 10µl, this consists of 3µl of sequence mix (DYEnamic ET Terminator sequence premix, Amersham Biosciences, Little Chalfont, UK) and 1µl of each MLST or antigen gene primer (5pmol/µl) (MWG Biotech, Milton Keynes, UK) (Table 2.6) added to produce a master mix volume of 4µl. These pre-prepared master mixes were placed on the refrigerated reagent rack. The 96-well thermosprint plates were then transferred from the cool stacker to the refrigerated positions for the sequence mix distribution where 4 µl of the mixes were aliquoted into the appropriate wells. Advanced pipetting parameters for RoboSEQ 4200 SE were prefill of 2µl at 50µl/s, aspiration of 1µl at 50 µl/s with one mix of 4µl and final dispension of 400µl/s. Advanced pipetting parameters for THEONYX were; for each gene wash tips before first step aspiration of 4µl at 250µl/s with one mix of 4µl and final dispension of 200µl/s. During the transfer a transport airgap of 2µl was maintained.

Table 2.6 Sequencing primers for housekeeping genes and *porA*

Primer Name	Primer Sequence
abcZ Forward	5'-AATCGTTTATGTACCGCAGG-3'
abcZ Reverse	5'-GAGAACGAGCCGGGATAGGA-3'
adk Forward	5'-AGGCTGGCACGCCCTTGG-3'
adk Reverse	5'-CAATACTTCGGCTTTCACGG-3'
aroE Forward	5'-GCGGTCAACYTACGCTGATT-3'
aroE Reverse	5'-ATGATGTTGCCGTACACATA-3'
fumC Forward	5'-TCGGCACGGGTTTGAACAGC-3'
fumC Reverse	5'-CAACGGCGGTTTCGCGCAAC-3'
gdh Forward	5'-CCTTGGCAAAGAAAGCCTGC-3'
gdh Reverse	5'-GCGCACGGATTCATATGG-3'
pdhC Forward	5'-TCTACTACATCACCCTGATG-3'
pdhC Reverse	5'-ATCGGCTTTGATGCCGTATTT-3'
pgm Forward	5'-CGGCGATGCCGACCGCTTGG-3'
pgm Reverse	5'-GGTGATGATTTCGGTTGCGCC-3'
porA Forward	5'-AACGGATACGTCTTGCTC-3'
porA Reverse	5'-TCCGTACGCTACGATTCTCC-3

2.10.3 PCR re-elution for sequencing on MegaBace 1000

Washable tips for the RoboSEQ 4200 SE were removed and replaced with disposable tips before the next procedure. For the RoboSEQ 4200 SE, each well of the Multiscreen™ 384-PCR plate was re-eluted 50 times with 40µl of 18MΩ water contained on the reagent rack. Advanced parameters were: prefill 10µl at 100µl/s,

aspiration 30µl at 50µl/s, dispension at 100µl/s followed by 50 repeat mixes of 20µl/s. For THEONYX, 50µl of 18 MΩ deionised water were transferred to each well of the Multiscreen™ 384-PCR plate with advanced pipetting parameters of: wash tips before each step, 250µl/s aspiration and 400µl/s dispension. Then, for the re-elution stage, 20µl were transferred in and out of the same wells in order to perform re-elution. The advanced parameters were: wash tips before each step, aspiration at 250µl/s then 20 repeat mixes of 20µl at 250µl/s for aspiration and dispension, followed by a final dispension at 400µl/s. During the transfer, a transport airgap of 2µl was maintained. After re-elution, 6µl of the clean DNA were transferred into the appropriate wells in a 96-well thermosprint plate containing sequence mix. Advanced pipetting parameters for RoboSEQ 4200 SE were: aspiration 6µl at 50µl/s, dispension 50µl/s of 5µl. Advanced pipetting parameters for THEONYX were: wash tips before each step, aspiration 6µl at 250µl/s, dispension at 400µl/s with two 5µl mixes at 100µl/s. During the transfer, a transport airgap of 2µl was maintained.

2.10.4 Sequence reaction for sequencing on MegaBace 1000

After the sequence reaction setup, the 96-well thermosprint plates were placed into the MWG thermocyclers for the sequencing reaction. The sequence cycle conditions were 95°C for 2 min, 30 cycles of 95°C for 20s, 50°C for 15s and 60°C for 1 min.

2.10.5 Sequence clean up for sequencing on MegaBace 1000

The used Multiscreen™ 384-PCR plate was replaced on the vacuum manifold with a Multiscreen™ 384-SEQ plate (Millipore, Hertfordshire,UK). The total sequence reaction volumes were transferred to the Multiscreen™ 384-SEQ plate. Advanced

pipetting parameters for the RoboSEQ 4200 SE were: aspiration 25µl at 50µl/s, dispension 200µl/s. Advanced pipetting parameters for THEONYX were: wash tips before each step, aspiration 25µl at 250µl/s, dispension 400µl/s. During the transfer, a transport airgap of 2µl was maintained. The vacuum was automatically switched on for 20 min at 250mbar. Twenty microlitres of 18MΩ deionised water were then added to each well of the Multiscreen™ 384-SEQ plate, and the vacuum was applied for a further 20 min, to wash the sequence products. Advanced pipetting parameters for the RoboSEQ 4200 SE were: aspiration 10µl at 50µl/s, dispension 200µl/s. Advanced pipetting parameters for THEONYX were: wash tips before each step, aspiration 20µl at 250µl/s, dispension 400µl/s. During the transfer transport airgap of 2µl was maintained. For the RoboSEQ 4200 SE, each well of the Multiscreen™ 384-SEQ was re-eluted 50 times with 50µl of 18MΩ deionised water. Advanced pipetting parameters prefill 40µl/ at 100µl/s, aspiration 10µl at 50µl/s and dispension 100µl/s with 50 repeat mixes of 40µl. For THEONYX, 60µl of 18MΩ deionised water was transferred into each well of the Multiscreen™ 384-SEQ plate with advanced pipetting parameters of 250µl/s aspiration and 400µl/s dispension. During the transfer, a transport airgap of 2µl was maintained. Then, for the re-elution stage, 20µl was transferred in and out of the same wells in order to perform re-elution. The advanced parameters were: wash tips before each step, aspiration at 250µl/s then 15 repeat mixes of 20µl at 250µl/s for aspiration and dispension, followed by a final dispension at 400µl/s. Twenty microlitres of the re-eluted sequence product was then transferred into a 96-well skirted plate on a refrigerated 96-well position. The plate had previously been stored in the refrigerated stacker. Advanced pipetting parameters for the RoboSEQ 4200 SE were: aspiration 20µl at 50µl/s, dispension 100µl/s. Advanced

pipetting parameters for THEONYX were: wash tips before each step, aspiration 20µl at 250µl/s, dispension 400µl/s. During the transfer, a transport airgap of 2µl was maintained. Once complete it was returned to the refrigerated stacker ready for loading on the DNA sequencer. For 24 samples, four 96-well plates were generated.

2.10.6 DNA sequencing on MegaBace 1000

Sequencing was performed using the automated MegaBace 1000 96-capillary sequencer according to the manufacturer's instructions. The skirted 96-well plate, which contained the cleaned sequence products, was loaded into the sequencer and the samples injected at a voltage of 3kV for 40s. A run voltage of 9kV for 120min was applied. This separates the dye-labeled DNA fragments generated in the sequence reaction.

2.10.7 Sequence interpretation of housekeeping gene fragments

The MegaBace sequence software using the integrated Cimaron v1.53 Slim Phredify base caller automatically reads the sequence data. The raw data for each sequence were viewed as an electropherogram (example shown in figure 2.2) and converted to FASTA (text) format using the MegaBace sequence analyser software. The FASTA files were downloaded into a local database of meningococcal MLST alleles (Diggle & Clarke, 2002) **(Figure 2.3)** in which each sequence was automatically compared against all other similar alleles to produce an allele number. After sequence comparisons of all 7 MLST genes, the alleles were entered into the MLST website http://pubmlst.org/neisseria/ to produce a sequence type (ST). When sequence data resulted in a new allele or ST, the information was sent to the curator of the MLST database who assigns the appropriate allele number or numbers and then a new ST.

Figure 2.2: Part of a DNA sequence displayed as an electropherogram

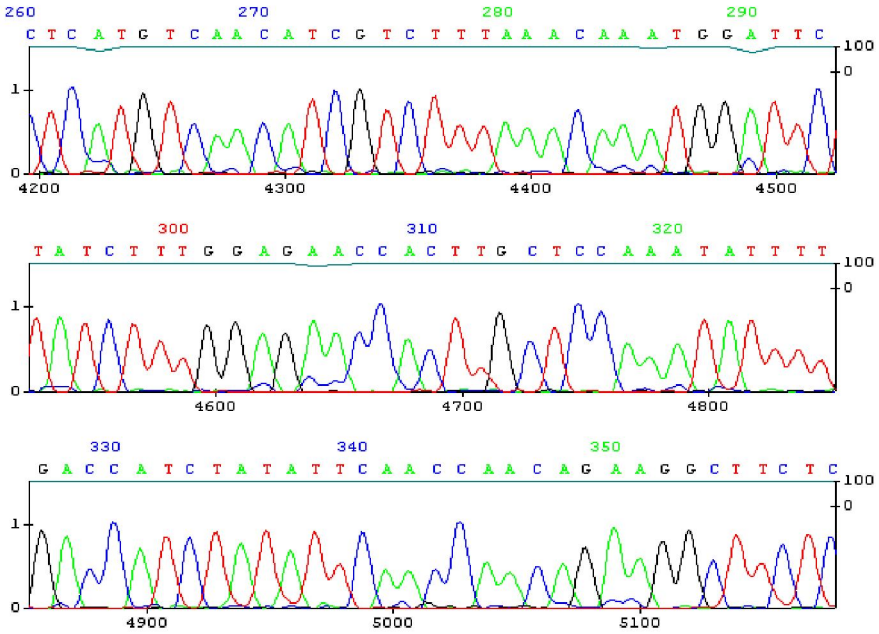
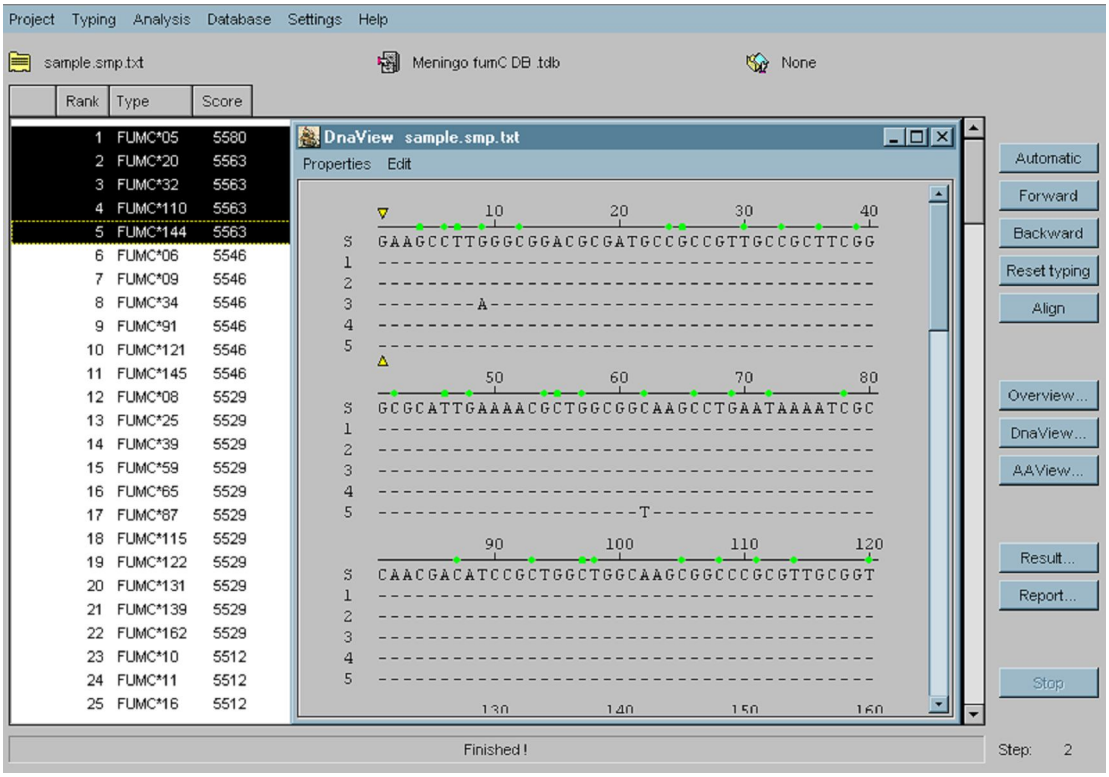


Figure 2.3 Internal databases developed for nucleotide sequence analysis.



2.10.8 Sequence interpretation of antigen gene fragments

The MegaBace sequence software automatically generated the sequence data. Meningococcal isolates have the *porA* gene, which has hypervariable regions, three of which (VRs 1, 2 and 3) were analysed to provide genosubtype information. The raw data were viewed for each sequence as an electropherogram and converted to FASTA (text) format using the MegaBace sequence analyser software. After sequence confirmation, the sequence was copied onto the TRANSLATE nucleotide website (http://au.expasy.org/tools/dna.html) and the nucleotide sequence was converted into an amino acid sequence. The correct variant types were assigned for VR1 **(**Appendix A1) and VR2 **(**Appendix A2**)** using the *porA* variable region database (http://neisseria.org/nm/typing/pora/). To assign the variant type for VR3 (Appendix A3**)** the SMPRL website (http://www.show.scot.nhs.uk/smprl/) was used.

2.10.9 Liquid phase PCR purification for sequencing on LI-COR L4200-L2

From the PCR purification onwards the procedures for sequencing on LI-COR L4200-L2 were different to those for the MegaBace 1000. After amplification, 5µl of PCR product was added to 2µl of Exosapit (Amersham Biosciences, Little Chalfont, UK) and placed in the thermocycler. Conditions were 37°C for 15min and 80°C for 15min.

2.10.10 PCR sequence labeling for sequencing on LI-COR L4200-L2

A three-microlitre aliquot of each purified PCR product was transferred into a 96-well skirted plate. A pre-dilution was performed by adding 24µl of 18MΩ deionised water and 1.5 µl of both forward and reverse sequencing primers specific for each PCR product (MWG Biotech, Milton Keynes, UK) (Table 2.7). All forward sequencing primers were tagged with 700-nm infrared dye, and all reverse sequencing primers

were tagged with 800-nm infrared dye. Four microlitre of each prediluted sequence mix was distributed into appropriate wells of another 96-well skirted plate, containing 1µl (each) of A and C and 2µl (each) G and T from a Thermo Sequenase fluorescence-labeled primer cycle sequencing (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

Table 2.7 Sequencing primers for LI-COR L4200-L2

Primer Name	Primer Sequence
abcZ Forward	5'-GAGAACGAGCCGGGATAGGA-3'
abcZ Reverse	5'-GAGAACGAGCCGGGATAGGA-3'
adk Forward	5'-AGGCTGGCACGCCCTTGG-3'
adk Reverse	5'-CAATACTTCGGCTTTCACGG-3'
aroE Forward	5'-GCGGTCAACYTACGCTGATT-3'
aroE Reverse	5'-ATGATGTTGCCGTACACATA-3'
fumC Forward	5'-TCGGCACGGGTTTGAACAGC-3'
fumC Reverse	5'-CAACGGCGGTTTCGCGCAAC-3'
gdh Forward	5'-CCTTGGCAAAGAAAGCCTGC-3'
gdh Reverse	5'-GCGCACGGATTCATATGG-3'
pdhC Forward	5'-TCTACTACATCACCCTGATG-3'
pdhC Reverse	5'-ATCGGCTTTGATGCCGTATTT-3'
pgm Forward	5'-CGGCGATGCCGACCGCTTGG-3'
pgm Reverse	5'-GGTGATGATTTCGGTTGCGCC-3'
porA Forward	5'-AACGGATACGTCTTGCTC-3'
porA Reverse	5'-TCCGTACGCTACGATTCTCC-3'

Finally, one drop of Chill-out 14 liquid wax (Genetic Research Instruments, Braintree, Essex, UK) was added to each well. The plate was placed into the thermocycler. The sequence cycle conditions were 96°C for 2 min, 30 cycles of 96°C for 30s, 50°C for 30s and 70°C for 1 min. Afterwards the plate was removed from the thermocycler and placed on a refrigerated block. A 4µl aliquot of formamide loading dye-stop solution (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) was added to all 96 wells of the plate and then the plate was placed into a thermocycler at 65°C for 10 min.

2.10.11 DNA sequencing on LI-COR L4200-L2

A 0.2mm thick sequencing gel was cast using two 41cm plates separated by two 0.2mm strips. The gel matrix contained 7.5ml of RapidGelXL (40% concentrate) (USB Corporation, Cleveland, Ohio, US), 4ml of formamide (SIGMA, Gillingham, Dorset, UK), 21g of urea (SIGMA, Gillingham, Dorset, UK), 5ml of X10 Tris buffer and 28ml of 18MΩ deionised water and these were added to a 100ml glass duran bottle. The contents were then mixed until dissolved using a magnetic stirrer. During this time 1.5ml of 18MΩ deionised water was added to a serum tube along with the contents of an ammonium persulphate (APS) capsule (SIGMA, Gillingham, Dorset, UK) to make 10 % APS solution. Seventy-five microlitres of TEMED and 350µl of APS were added to the gel mixture. Thirty millilitres of the gel mixture was taken up into a plastic syringe and used for the pouring of the gel. The gel mixture was injected slowly between the glass plates from the top. Careful attention had to be taken so that there were no bubbles formed within the gel. This was done by repeatedly tapping the glass as the gel passed between the plates. Once the gel mixture had reached the bottom the plates were placed on a level surface and a comb was inserted. A further

75µl of TEMED (SIGMA, Gillingham, Dorset, UK) and 350µl of APS were added to the gel mixture still remaining in the syringe and this was then added to the area surrounding the comb to seal the gel. The gel was then allowed to set for at least two hours at room temperature. The prepared gel plate was then loaded vertically into the sequencer and the top buffer tank, filled with one litre X1 Tris buffer inserted. The pre-run protocol was performed following the displayed commands. After the pre-run protocol was complete one microlitre of each sample was added to the wells and then the sequence run was started.

2.10.12 Sequence interpretation using LI-COR L4200-L2

The sequence data were automatically read from the LI-COR sequencer using the integrated image analysis and data collection software. The data were then analysed as previously described (2.10.7 and 2.10.8).

2.11 Data analysis

After sequence comparisons the alleles that have been assigned, as previously described, were entered into the MLST website http://pubmlst.org/neisseria/ to produce a sequence type (ST). STs were also grouped into clonal complexes by their similarity to a central allelic profile (genotype). The START (sequence type analysis and recombinational tests) package was used as it brings together many of the preliminary analyses that can be performed on MLST data (Jolley *et al.*, 2001). This package is separated into four main categories: the summary of data, lineage assignment, recombination tests and tests for selection.

2.11.1 Summary of data - allele frequencies

Allele frequencies show how common an allele was within a dataset. Allele frequency analysis was performed once the *N. meningitidis* allelic profiles had been entered. A table was displayed showing the frequencies of each allele at each locus.

2.11.2 Summary of data - Profile Frequencies

Profile frequencies show how common an ST was within a dataset. Once the allelic profiles were entered, profile frequency analysis was performed. A table was displayed showing the frequencies of each profile ST in the dataset in frequency order.

2.11.3 Summary of data - Polymorphism Frequencies

Polymorphism frequencies show the different nucleotide changes present within a dataset. To determine polymorphism frequencies, both the allelic profiles and the allele sequences were loaded into the software. The allele sequences were obtained from the MLST website. First a sequence map, showing the positions and identities of all polymorphic sites at each locus were displayed. Below this was a table that displayed the number of alleles in the dataset that had a particular nucleotide at each polymorphic site.

2.11.4 Lineage assignment – eBURST (Based Upon Related Sequence Types)

This was used to specifically examine the relationships within clonal complexes while the relationships between different clonal complexes were ignored. eBURST is a web-implemented clustering algorithm and is designed for use on MLST data sets from bacterial pathogens. The website to access the software is http://eburst.mlst.net/.

2.11.5 Lineage assignment – UPGMA

UPGMA (Unweighted Pair Group Method with Arithmetic Mean) (Sneath & Sokal, 1973b) is a straightforward method of tree construction. The algorithm used utilised 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. The analysis was performed using the START program. Output displays were in the form of a tree. Allelic profiles were displayed along with the isolate numbers.

2.11.6 Tests for recombination - Index of Association

Index of Association (I_A) 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 (Maynard-Smith *et al.*, 1993). 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 differs significantly from zero. This analysis required only the allelic profiles.

2.11.7 Tests for selection - *d*S/*d*N ratio

Nucleotide substitutions in genes encoding for proteins can be either synonymous (do not change amino acid), alternatively called silent substitutions, or non-synonymous (changes amino acid). Usually, most non-synonymous changes are 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. An 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.

2.12 Analysis of molecular variance (AMOVA)

Significant genetic differentiation among groups of isolates was assessed by AMOVA (Excoffier *et al.*, 1992) as implemented in Arlequin software (version 2.0) (Schneider *et al.*, 2000). This program computed an *F* statistic (F_{ST}s) (Wright, 1943; Wright, 1951) by applying a permutation test to assess statistical significance. AMOVAs were performed on the data as grouped by health board regions. F_{ST}s for the allelic profiles, *porA* and concatenated locus sequences were performed.

2.13 Spatio-temporal analysis

Analysis was performed using SaTScan version 5.1.1 software, which is available at http://www.satscan.org. The programme applies a likelihood function to circular windows originating at defined locations of increasing size and compares observed and expected case numbers inside and outside the scan window to detect clusters that are least likely to have occurred by chance. The statistical significance for each cluster is obtained through Monte Carlo hypothesis testing, i.e., results of the likelihood

function are compared for a large number of random replications of the dataset generated under the null hypothesis. In this study, cases were assumed to be Poisson distributed in each location and the programme's space-time scan statistic was applied. The date of specimen sampling was defined as time of illness and the county of residence, derived from the postcode, was used as place. Spatio-temporal scanning was initiated within the 15 health boards. The temporal settings were defined as a maximal temporal window of 30 days as this should detect most of the existing clusters. Each strain present more than once within the dataset was individually entered into the SaTScan program to identify clustering of IMD. Strains were defined by serogroup, MLST and *porA*.

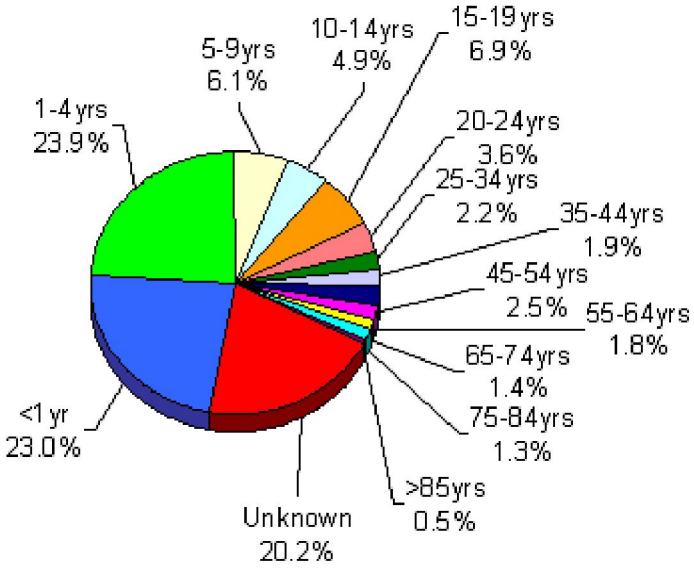
Chapter 3

Results

3.1 Source of isolates

With regard to the patient's age, as with most studies of meningococcal disease it was found that disease was most common amongst infants under 4 years of age (Figure 3.1) accounting for 46.9% of all isolates. When these data were analysed for different time periods i.e. 1970s, 1980s and 1990s, there was little change in percentages for the age groups (data not shown).

Figure 3.1 The percentage of invasive isolates associated with each age group, Scotland 1972-98.



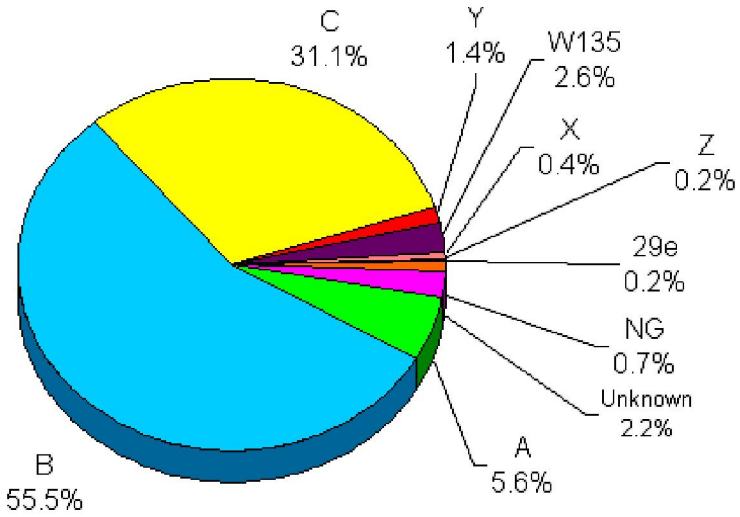
There was not much difference between the sexes over the time period with males accounting for 47% of isolates, females 41% and 12% unknown. The sites for the isolates were 913 blood isolates (35%), 1434 CSF isolates (55%) and 260 from other

invasive sites (10%). The geographical spread of isolates will be discussed in greater detail within the next chapter.

3.1.1 Serogroup characterisation

All isolates had been previously characterised for serogroup. However, some isolates were non-groupable so these isolates were characterised using genotypic methods. A representative number of isolates was taken from the total number of isolates to analyse the accuracy of previous serogrouping methods. From these results there were no discrepancies between previously serogrouped isolates and with those repeated. Serogroup distribution included 147 serogroup A isolates (5.6%), 1446 serogroup B isolates (55.5%), 812 serogroup C isolates (31.1%), 37 serogroup Y isolates (1.4%), 67 W135 isolates (2.6%), 10 serogroup X isolates (0.4%), 5 serogroup Z isolates (0.2%) and 6 serogroup 29e isolates (0.2%) (Figure 3.2).

Figure 3.2 The percentage of invasive isolates associated with each serogroup, Scotland 1972-98.

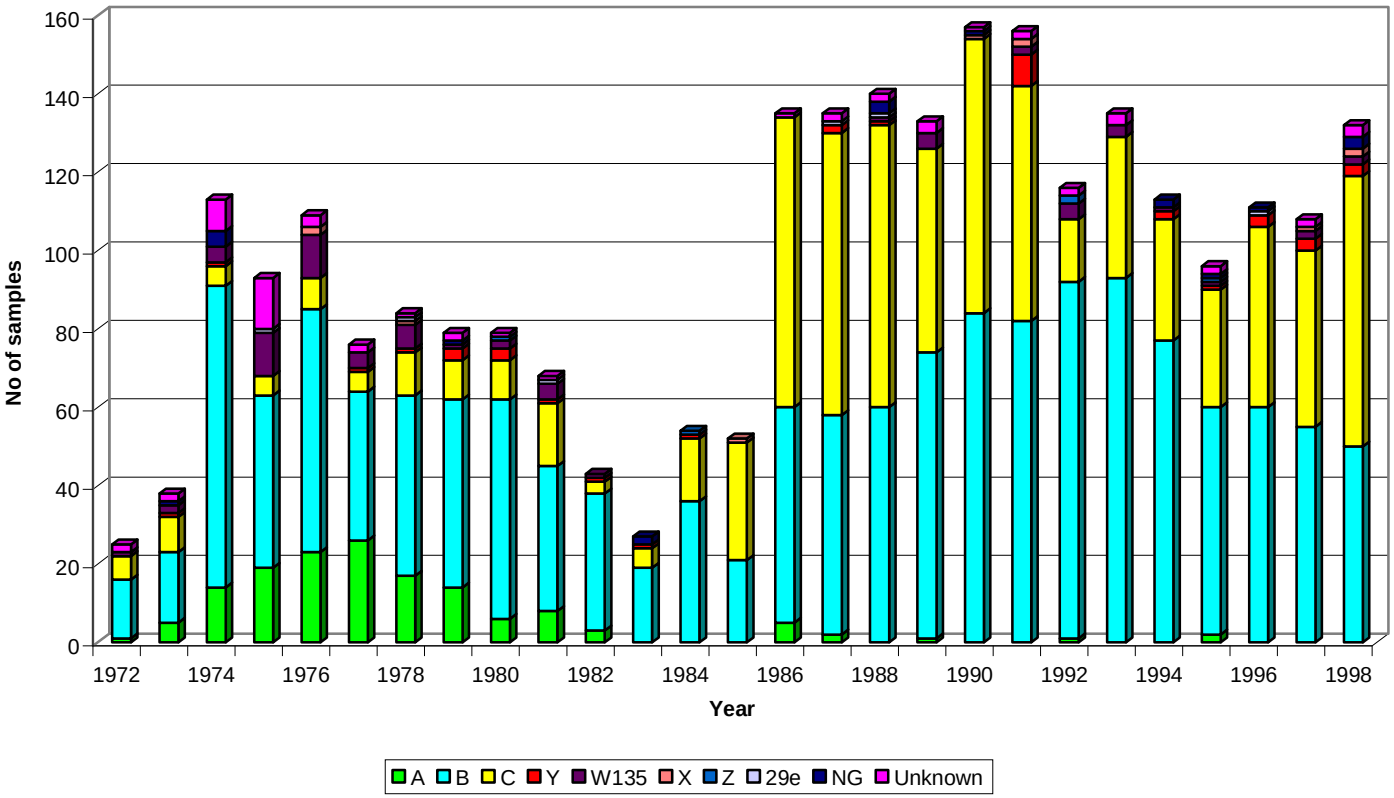


There were also 19 isolates which remained non-groupable (0.7%) and 58 isolates (2.2%) were not available to perform any characterisation including serogrouping, MLST or *porA* sequencing. There had been 82 non-groupable isolates using latex agglutination but PCR based techniques reduced that number to 19.

Serogroup distribution changed from year to year during the time period 1972-1998. However, serogroups B and C were evidently the dominant serogroups over this period (Figure 3.3). Serogroup B was the dominant serogroup throughout the 1970s and early 1980s but, during the early to mid 1980s, there was an increase in serogroup C disease. For example during 1984, 16 of 52 isolates were serogroup C (30.8%), an increase of 11% on the previous year whereas, in 1985, 30 of 52 isolates were serogroup C (57.7%), an increase of 26.9% on the previous year, making serogroup C the dominant serogroup. This change in serogroup distribution also coincided with the emergence of the ST-11 strain. Serogroup C remained dominant over the next three years, 1986 (53.8%), 1987 (52.6%) and 1988 (51.1%) until 1989 when there was a decrease to 38.6% of isolates. At this time serogroup B had become the dominant serogroup again with a total of 73 of 132 isolates (55.3%), an increase of 11.5% on the previous year.

The number of serogroup C isolates fluctuated during the 1990s, with a low of 16 of 132 isolates (13.8%) in 1992. Subsequently, the numbers of serogroup C isolates increased over the next six years 1993 (26.7%), 1994 (27.4%), 1995 (29.0%), 1996 (41.8%) 1997 (41.0%) and 1998 (51.5%). This was prior to the introduction of the MenC vaccine. Serogroup A was the second most common serogroup during the 1970s, but numbers began to decline into the 1980s. Serogroups Y, WI35, X, Z and

Figure 3.3 Distribution of invasive isolates according to serogroup, Scotland from 1972-98.



29E were also present in Scotland over this time period but in significantly smaller numbers. Together these serogroups represented only 4.8% of isolates

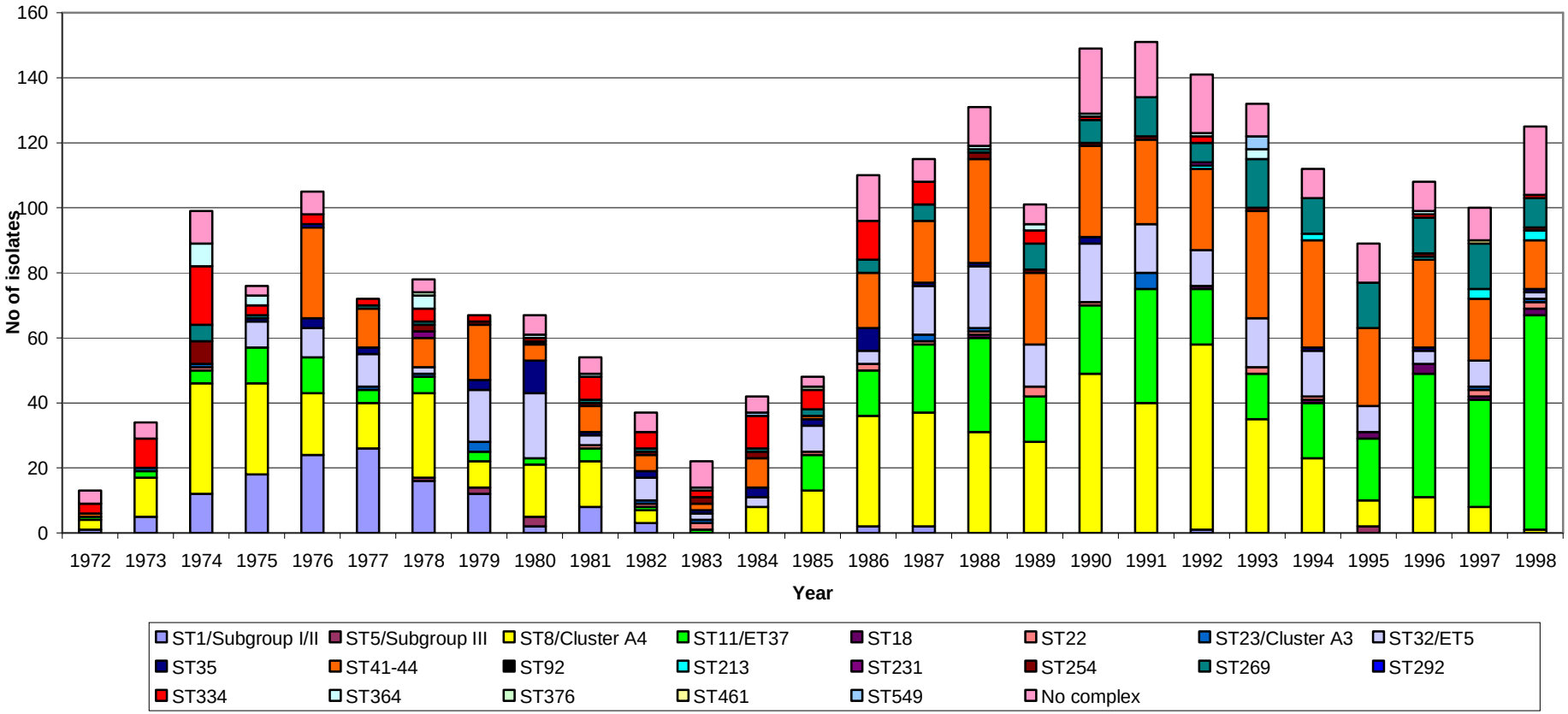
3.2 MLST analysis

All isolates were characterised at seven housekeeping loci by multilocus sequence typing (MLST) (Maiden *et al.*, 1998). There were 309 different sequence types (STs) among the 2607 isolates. While there was much diversity in the STs (309 types), only ten accounted for 1562 isolates (59.9%). These were ST-11 (374 isolates, 14.3%), ST-8 (369 isolates, 14.2%), ST-41 (210 isolates, 8.1%), ST-153 (141 isolates, 5.4%), ST-1 (128 isolates, 4.9%), ST-32 (102 isolates, 3.9%), ST-33 (75 isolates, 2.9%), ST-269 (64 isolates, 2.5%), ST-334 (50 isolates, 1.9%) and ST-60 (49 isolates, 1.9%). Using BURST analysis the STs were further differentiated into 31 distinct lineages, with 67 singleton types. There were 177 new STs found which accounted for 253 samples or 9.7% of the total number of isolates (Appendix A4). As with the STs although there was much diversity in clonal complexes, only seven accounted for 1993 isolates (76.4%) (Table 3.1, Figure 3.4). The ST-8 complex was the most prevalent with 567 isolates (21.7%) and these were divided into 21 different STs, 369 were ST-8 (65.1%), 141 were ST-153 (24.9%) a *gdh* locus variant of ST-8, 18 were ST-66 (3.2%) a *fumC* locus variant of ST-8 and the remaining STs were present in single figures with 9 represented by a single isolate.

Table 3.1 Association of STs with predominant clonal complexes from Scotland 1972-98.

Clonal complexes	Number of isolates	Number of different STs	STs present in double figures within clonal complex and the percentage with which they occurred				
ST-8	567	21	ST-8 (65.1%)	ST-153 (24.9%)	ST-66 (3.2%)		
ST-41/ST-44	422	51	ST-41 (49.7%)	ST-206 (12.1%)	ST-180 (6.4%)	ST-43 (5.2%)	ST-1362 (4.7%)
ST-11	393	12	ST-11 (95.1%)				
ST-32	239	21	ST-32 (42.7%)	ST-33 (31.4%)	ST-259 (8.0%)	ST-343 (6.3%)	
ST-1	133	5	ST-1 (96.2%)				
ST-269	132	26	ST-269 (48.5%)	ST-275 (24.2%)			
ST-334	107	23	ST-334 (49.5%)	ST-189 (14.0%)	ST-415 (12.1%)		

Figure 3.4 Clonal complexes of invasive meningococcal isolates from Scotland 1972-98.



There were 7 new STs present within the ST-8 complex. The ST-41/44 complex accounted for 422 isolates (16.2%) and included 51 different STs, ST-41 (49.7%), ST-206 (12.1%), ST-180 (6.4%), ST-43 (5.2%), and ST-1362 (4.7%). The remaining STs were present in single figures with 31 represented by a single isolate. There were 29 new STs present within the ST-41/44 complex. The ST-11 complex accounted for 393 isolates (15.1%) and included 12 different STs. The most common ST was ST-11 (95.1%). The remaining STs were present in single figures with 6 represented by a single isolate. There were 7 new STs present within the ST-11 complex. The ST-32 complex accounted for 239 isolates (9.2%) and these included 21 different STs. The most common were ST-32 (42.7%), ST-33 (31.4%, an *abcZ* locus variant of ST-32), ST-259 (8.0%, a *fumC* locus variant of ST-32), ST-343 (6.3% a *pgm* locus variant of ST-32). The remaining STs were present in single figures with 10 represented by a single isolate. There were 11 new STs present within the ST-32 complex. The ST-1 complex accounted for 133 isolates (5.1%) and these included five different STs. The most common was ST-1 (96.2%). The remaining STs were present in single figures with three represented by a single isolate. There were 4 new STs present within the ST-1 complex, ST-2512 a *pgm* locus variant of ST-1, ST-2517 an *abcZ* locus variant of ST-1, ST-4570 a *gdh* locus variant of ST-1 and ST-4570 a *fumC* locus variant of ST-1. The ST-269 complex accounted for 132 isolates (5.1%) and these included 26 different STs. The most common were ST-269 (48.5%) and ST-275 (24.2%). The remaining STs were present in single figures with 18 represented by a single isolate. There were 16 new STs present within the ST-269 complex. The ST-334 complex accounted for 107 isolates (4.1%) and these included 23 different STs. The most common were ST-334 (49.5%), ST-189 (14.0%, a *pgm* locus variant of ST-334) and ST-415 (12.1%). The remaining STs were present in single figures with 13

represented by a single isolate. The clonal complexes varied in their genetic diversity. The ST1/Subgroup I/II, ST23/Cluster A3 and ST-364 complexes were the most conserved, with each containing only one major ST, with ST-1 and ST-23 having the central ST of the clonal complex. However, the majority of complexes were more diverse containing multiple STs, in particular ST41-44/Lineage 3 complex.

From Figure 3.4 it can be observed that the distribution of clonal complexes has changed from year to year. These changes were also observed with STs. The *P*-value for STs was calculated for year on year changes (Table 3.2). A significant difference was noted in 7 of 26 years ($P < 0.05$). The results for this show the highest P-value (0.991) for the years 1990-1991 and the lowest *P*-value (0.001) for the years 1988-1989. The years 1990-1991 corresponds to the period where ST-8, predominant during the 1980s, was replaced by similar strains of the same complex.

Table 3.2 Calculated *P*-values to determine how similar the ST distribution is between periods.

Year	***P*-value**	**Year**	***P*-value**
1972-1973	0.608	1985-1986	0.774
1973-1974	0.622	1986-1987	0.078
1974-1975	0.830	1987-1988	0.376
1975-1976	0.213	1988-1989	0.001
1976-1977	0.021	1989-1990	0.024
1977-1978	0.004	1990-1991	0.991
1978-1979	0.236	1991-1992	0.880
1979-1980	0.810	1992-1993	0.208
1980-1981	0.142	1993-1994	0.002
1981-1982	0.516	1994-1995	0.020
1982-1983	0.503	1995-1996	0.657
1983-1984	0.801	1996-1997	0.154
1984-1985	0.352	1997-1998	0.045

3.2.1 Diversity of housekeeping genes and sequence types

Analysis of allele frequency from 1972 to 1998 showed that the number of alleles present at each locus varied between 16 for *adk* (adenylate kinase) and 44 for *pdhC* (pyruvate dehydrogenase) (Table 3.3).

Table 3.3 Genetic diversity in meningococci between 1972 and 1998

Locus	Length (bp)	No. alleles	No. polymorphic sites	d_N/d_S [Note]
abcZ	432	31	88	0.0504
adk	465	16	31	0.0176
aroE	489	37	174	0.2858
fumC	465	39	39	0.0209
gdh	501	36	42	0.0481
pdhC	480	44	86	0.0671
pgm	450	26	79	0.1101

[Note] A d_N/d_S ratio of <1 indicates that nonsynonymous nucleotide sequence changes are deleterious and are selected out of the population. A d_N/d_S ratio of 1 implies that sequences are evolving neutrally. A d_N/d_S ratio of >1 indicates that the gene is under diversifying selection, with nonsynonymous changes being selected.

This mirrors the data present on the MLST website which shows that *adk* has the least number of alleles present. The number of polymorphic sites present at each locus for each year ranged between 31 for *adk* and 174 for *aroE*. The d_N/d_S ratios ranged from 0.0176-0.2858 for *adk* and *aroE* respectively. All d_N/d_S ratios are less than one suggesting that the genes are subject to selective constraint. However, because these ratios are averaged across the sequence, any positive selection occurring at a particular site, might be offset by surrounding highly constrained regions. The d_N/d_S ratios for each allele were comparable throughout the 28-year period (results not shown). These data were comparable to invasive meningococci contained within the global data set found on the MLST website.

To determine what changes occurred to *N.meningitidis* in Scotland over time, analysis was performed on the data, which was split into the following time periods 1972-1979, 1980-1989 and 1990-1998. From 1972 to 1979 there were 616 isolates and these accounted for 54 STs (Figure 3.5). There were 20 different clonal complexes and 5 singletons. The largest complex was ST-8, which had 146 isolates, and divided into two different STs, 144 were ST-8 (98.6%) and two were ST-2174 (1.4%). Analysis of allele frequency from 1972 to 1979 showed that the number of alleles present at each locus varied between 7 for *adk* and 18 for *pdhC*. The number of polymorphic sites present at each locus for each year ranged between 15 (3.2% of sites for *adk*) and 122 (24.9% of sites for *aroE*).

From 1980 to 1989 there were 845 isolates and these accounted for 182 STs (Figure 3.6). There were 25 different clonal complexes and 39 singletons. The largest complex was ST-8, which had 192 isolates, and consists of 14 different STs. 135 were ST-8 (70.1%), 37 were ST-153 (19.3%), five were ST-9 (2.6%), three were ST-1380 (1.6%), three were ST-4609 (1.6%), two were ST-4612 (1.0%), two were ST-4819 (1.0%) and the rest all had only one isolate associated with them. The most varied complex was the ST-41-44 complex, which had 126 isolates spread amongst 30 STs. Analysis of allele frequency from 1980 to 1989 showed that the number of alleles present at each locus varied between 16 for *adk* and 34 for *pdhC*. The number of polymorphic sites present at each locus for each year ranged between 38 (8.1% of sites for *adk* and *fumC*) and 171 (34.9% of sites for *aroE*).

Figure 3.5 UPGMA tree of *N meningitidis* STs and clonal complexes present in Scotland 1972-1979

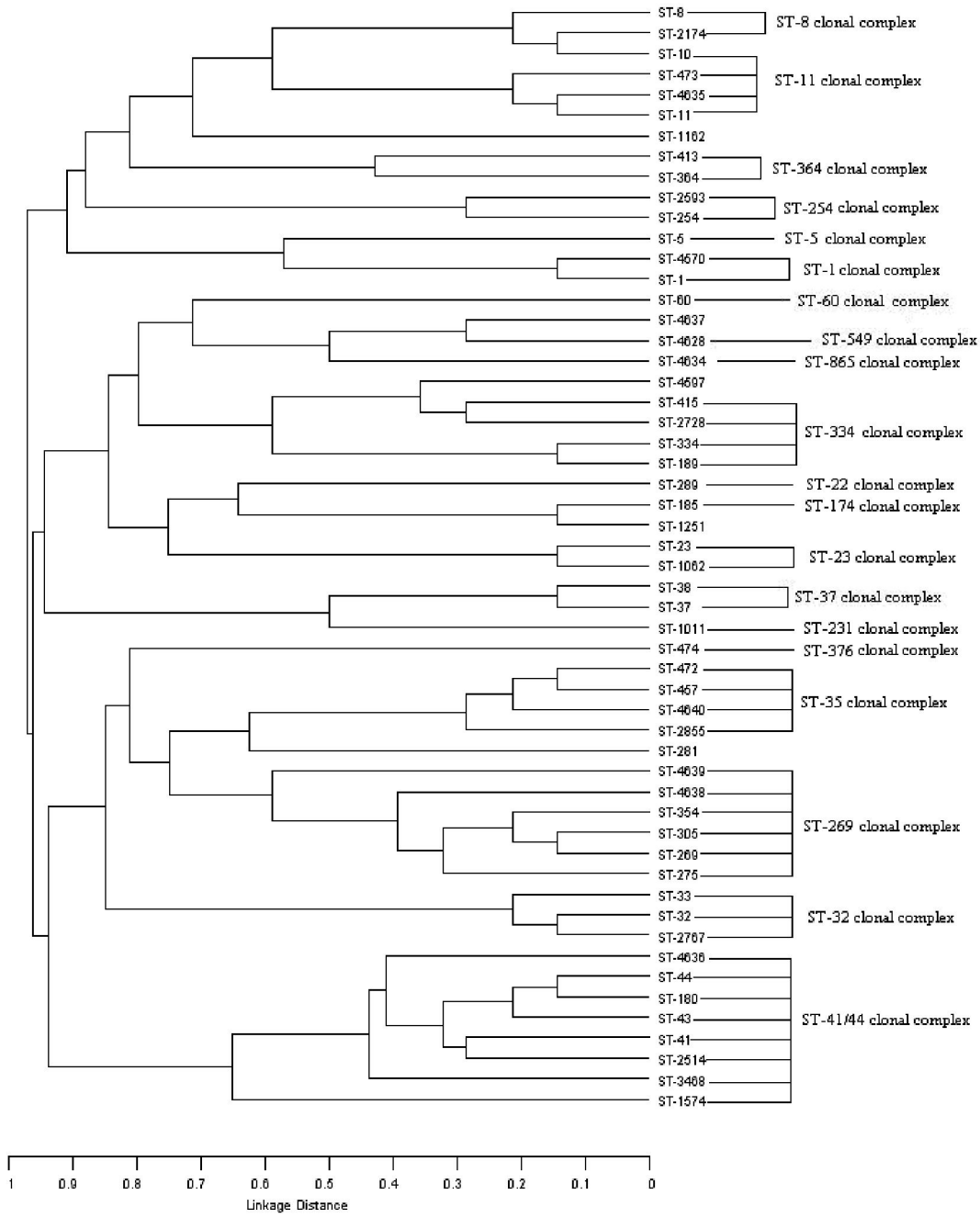
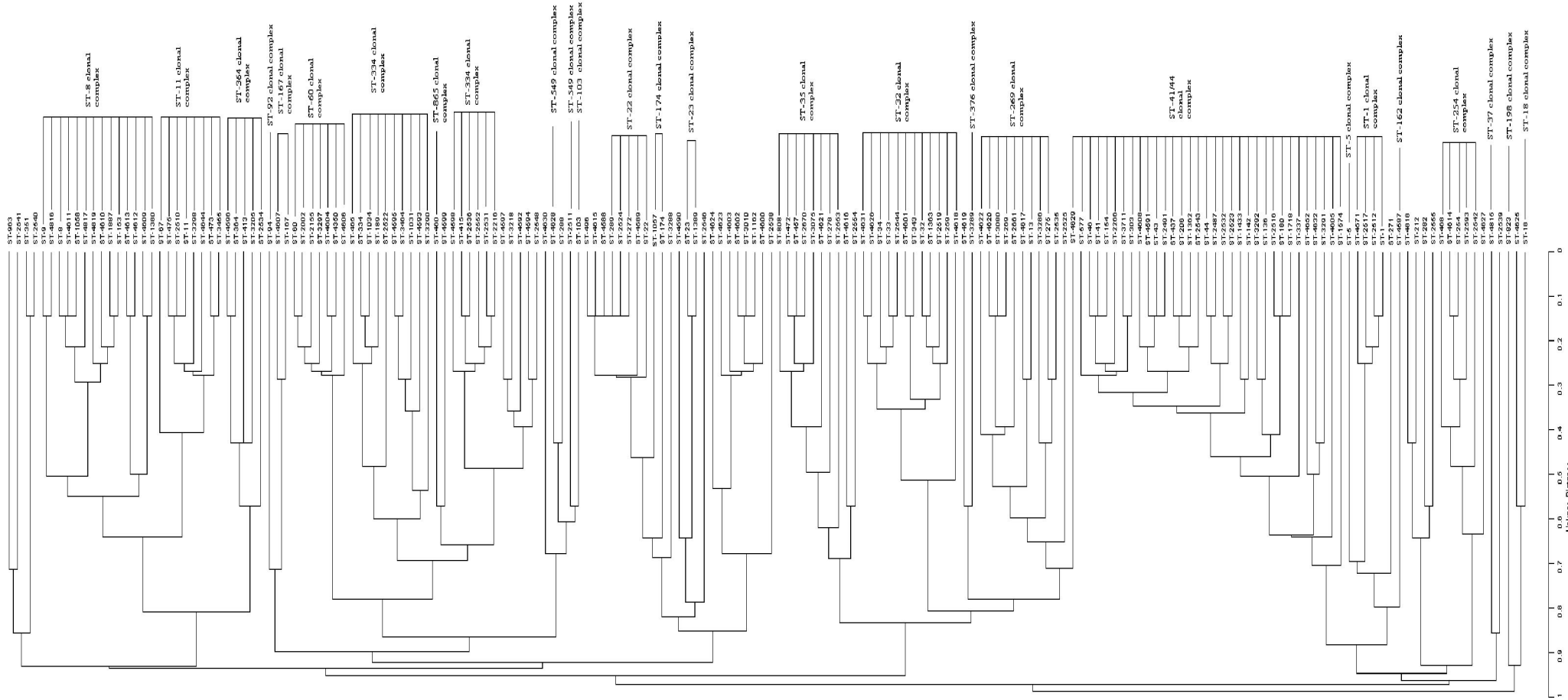


Figure 3.6 UPGMA tree of *N meningitidis* STs and clonal complexes present in Scotland 1980-1989.



From 1990 to 1998 there were 1146 isolates and these accounted for 160 STs (Figure 3.7). There were 27 different clonal complexes and 31 singletons. The largest complex was ST-11, which had 254 isolates representing 6 STs. The majority of isolates were ST-11 (97.2%). The most varied complex was the ST-41-44 complex, which had 222 isolates and 33 STs. Analysis of allele frequency from 1990 to 1998 showed that the number of alleles present at each locus varied between 13 for *adk* and 34 for *pdhC*. The number of polymorphic sites present at each locus for each year ranged between 30 (6.5% of sites for *adk* and *fumC*) and 136 (27.8% of sites for *aroE*).

There were differences in the STs and complexes circulating during the 1970s, 1980s and 1990s. While there are similar patterns between the 1980s and 1990s i.e. at least 25 complexes that included a number of different STs and at least 30 singletons, the similarities to the 1970s are less apparent. In the 1970s there was not the same diversity of STs causing disease. There were fewer clonal complexes present compared to the other decades and these complexes consisted only of one or two major STs accounting for the majority of the samples within the complex and one or two minor STs.

As mentioned previously, there were 177 new STs found in this study, which accounted for 253 isolates. These STs were spread over the whole time period from 1973 to 1998. Using BURST analysis they were further differentiated into nineteen distinct lineages, with 50 singleton types (Figure 3.8). The ST-41/44 complex accounted for the majority of the new STs with 38 isolates. These were divided into 27 STs, five were ST-231, four were ST-2523, three were ST-2516, two

Figure 3.7 UPGMA tree of *N meningitidis* STs and clonal complexes present in Scotland 1990-1998

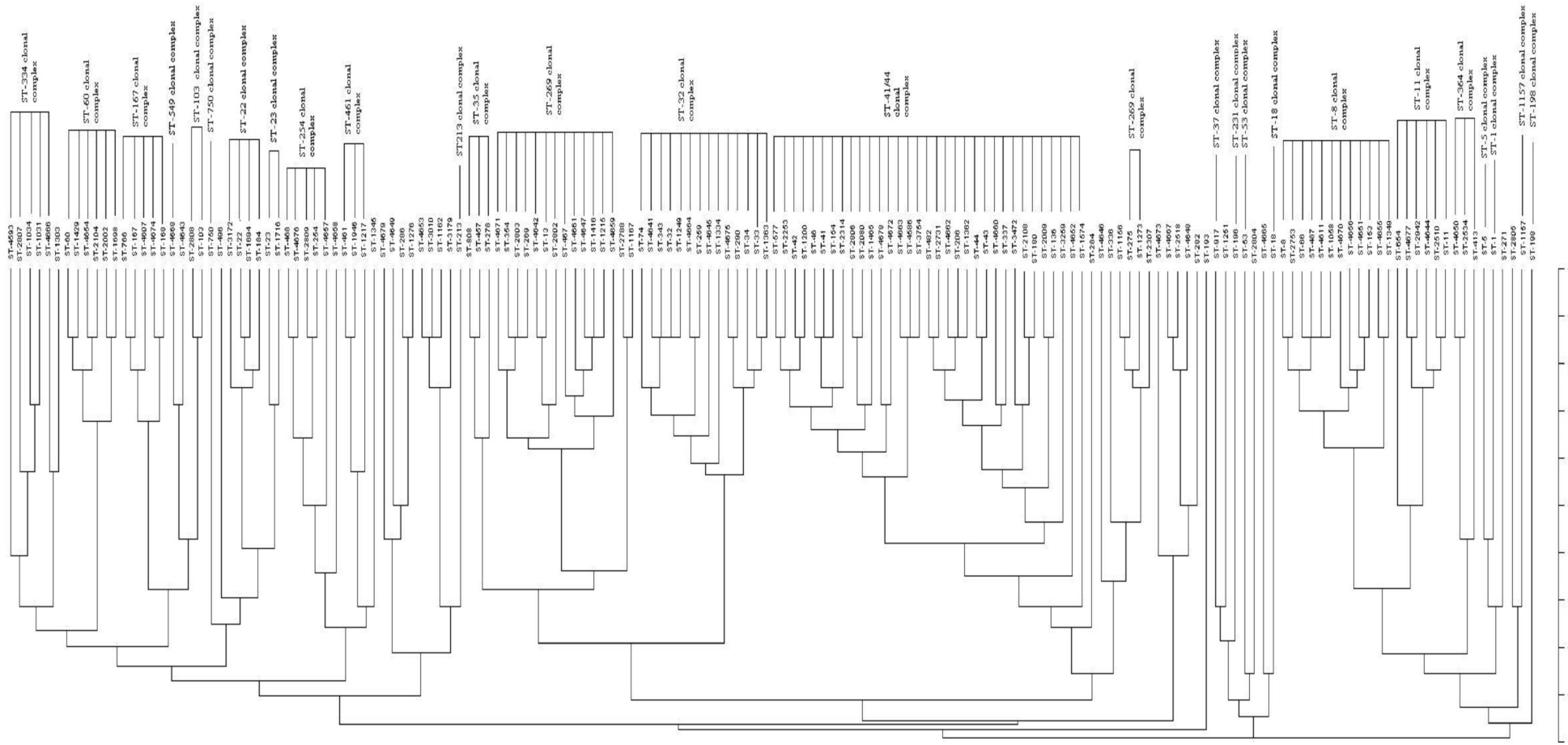
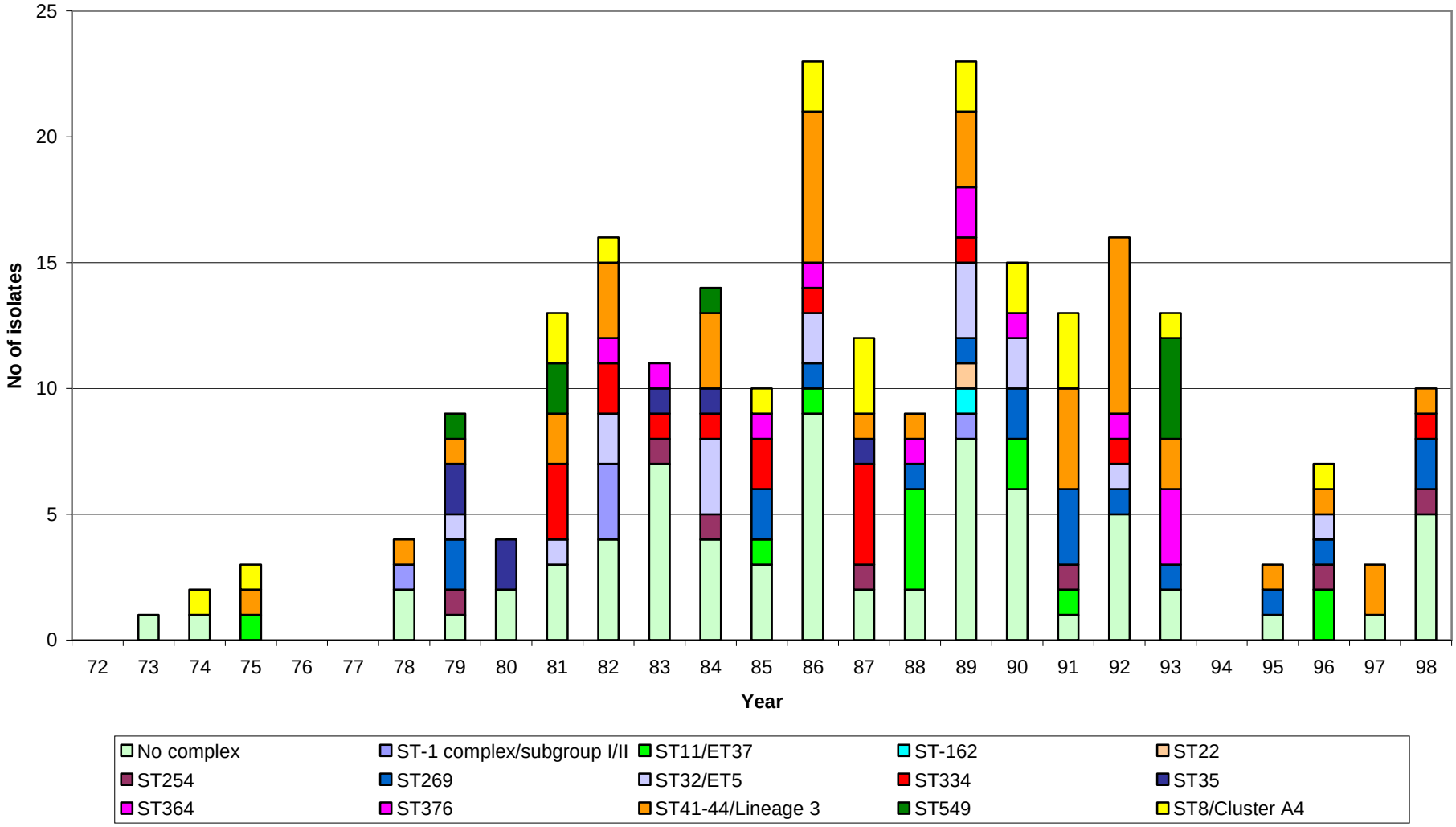


Figure 3.8 Distribution of clonal complexes, per year for new STs, Scotland 1972-98.



were ST-2543, two were ST-2731 and the remaining STs were represented by a single isolate. Serogroups B and C were associated with the majority of new STs (Figure 3.9). The significance of these new STs is that they have occurred in the past (i.e. 1972-1998) and have caused disease but have not been present in recent years. Therefore it is possible that they could occur again.

3.2.2 MLST analysis within serogroup

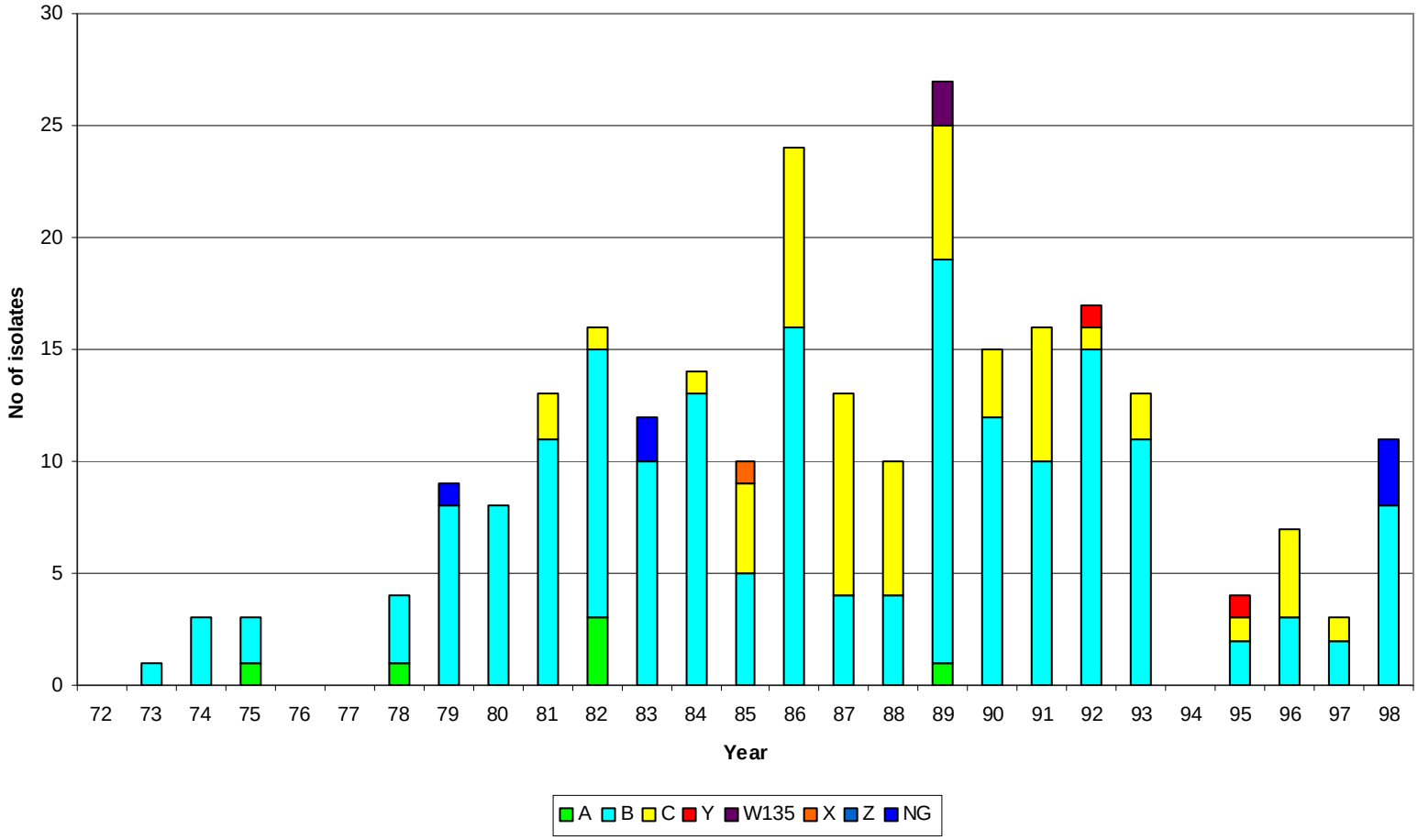
Certain STs showed association with certain serogroups although this was not definitive. For example ST-8 included 206 serogroup C (55.8%) and 160 serogroup B (43.4%) isolates whereas ST-33 and ST-41 were entirely serogroup B.

Serogroup B isolates could be differentiated into 218 different STs. Using BURST analyses they were further differentiated into twenty distinct lineages, with 54 singleton types. However, three lineages accounted for 888 isolates. The ST-41/44 complex had the largest number of isolates (333, 23.0%) and these were divided into 42 different STs, 208 were ST-41 (62.5%), 22 were ST-43 (6.6%), 21 were ST-180 (6.3%) and the remaining STs were present in single figures with 25 represented by a single isolate. The ST-8 complex accounted for 321 isolates (22.2%) and they were divided into 15 different STs, 160 were ST-8 (49.8%), 130 were ST-153 (40.5%) and the remaining STs were present in single figures with 5 represented by a single isolate. The ST-32 complex accounted for 234 isolates and they were divided into 20 different STs, 102 were ST-32 (43.6%), 74 were ST-33 (31.6%), 19 were ST-259 (8.1%), 15 were ST-343 (6.4%) and the remaining STs were present in single figures with 10 represented by a single isolate.

Serogroup C isolates could be differentiated into 84 different STs. Using BURST analyses they were further differentiated into thirteen distinct lineages, with 11 singleton types. However three lineages accounted for 664 isolates. The ST-11 complex accounted for the majority of isolates (328, 40.4%), these were divided into 8 STs, 314 were ST-11 (95.7%), 3 were ST-2510, 3 were ST-4644, 2 were ST-3298 and ST-67, ST-2942, ST-3455 and ST-4677 were represented by a single isolate. The ST-8 complex accounted for 243 isolates (29.9%) and they were divided into 9 different STs, 208 were ST-8 (85.6%), 15 were ST-66 (6.2%), 13 were ST-153 (5.3%) and the remaining STs were present in single figures with 4 represented by a single isolate. ST-41/44 complex accounted for 93 isolates (11.5%) and these were divided into 14 different STs, 45 were ST-206 (48.4%), 16 were ST-1362 (17.2%), 6 were ST-180 (6.5%), 5 were ST-41 (5.4%) and the remaining STs were present in single figures with 9 represented by a single isolate.

Serogroup A isolates could be differentiated into 9 different STs. Using BURST analyses they were further differentiated into three distinct lineages, with two singleton types. ST-1 complex accounted for the majority with 132 isolates, these were divided into 4 STs, 127 were ST-1 (97.7%) and ST-2517, ST-4570 and ST-4671 were represented by a single isolate. The ST-5 complex accounted for 8 isolates, all were ST-5. There was also one isolate associated with ST-8 complex, which was ST-2174.

Figure 3.9 Distribution of serogroups amongst new STs, Scotland 1972-98.



Serogroup Y isolates could be differentiated into 10 different STs. Using BURST analyses they were further differentiated into three distinct lineages, with four singleton types. ST-23 complex accounted for the majority with 19 (51.4%) of all the isolates, these were divided into four STs, 16 were ST-23 (84.2%) and ST-1062, ST-1389 and ST-1716 were represented by a single isolate. ST-766 and ST-167 belonged to no complex although they only differed by the *gdh* allele. Invasive serogroup Y meningococcal disease was associated mostly with the young or old. Although serogroup Y meningococcal disease was uncommon and a rare cause of invasive disease in Scotland between 1972 and 1998 it is essential that microbiologists are aware of its potential for increasing in incidence due to the introduction of the MenC vaccine, and its increased incidence in the USA (Pollard & Scheifele, 2001).

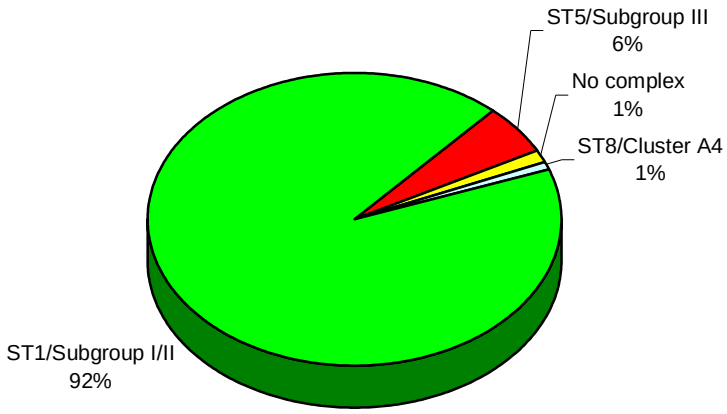
Serogroup W135 isolates could be differentiated into 11 different STs. Using BURST analyses they were further differentiated into four distinct lineages, with two singleton types. ST-11 complex accounted for the majority with 42 isolates, these were divided into two STs, 41 were ST-11 (97.6%) and ST-473 represented by a single isolate.

3.2.3 Disappearance of serogroup A

Serogroup A meningococci caused 143 cases of invasive disease in Scotland between 1972 and 1998. Of these 134 isolates (94%) occurred between 1973-1982, thereafter there were three cases in 1986, two cases in 1987, one case in 1989, one case in 1992 and two cases in 1995. There were 9 STs associated with serogroup A meningococci (ST-1, ST-5, ST-60, ST-2002, ST-2174, ST-2152, ST-2517, ST-4570 and ST-4571). However, 88.7% of isolates were of ST-1. The only other ST that occurred more than once was ST-5 with 5.67%. The STs belonged to three complexes the ST-1/Subgroup

I/II, ST-5/Subgroup III and ST-8/Cluster A4 (Figure 3.10). There were also two STs that did not belong to a complex. Serogroup A was therefore shown to be highly clonal with only 9 different STs present, the most common being ST-1 which has been responsible for epidemics and sporadic cases in Africa since the 1960s.

Figure 3.10 Distribution of clonal complexes amongst serogroup A isolates, Scotland 1972-98.



Of all the isolates, 91.5% belonged to the ST-1/Subgroup I/II complex. There were three new STs found within Scotland that belonged to serogroup A: ST-2517, ST-4570 and ST-4571 which all belonged to the ST-1/Subgroup I/II complex. The ST-1/Subgroup I/II and ST-5/Subgroup III complexes are almost exclusively associated with serogroup A throughout the world. Of the STs found amongst serogroup A only ST-60 has been present within Scotland in the last five years and has been associated with both serogroup A and serogroup B.

3.2.4 Emergence of serogroup C ST-11 meningococci

The data have shown the emergence of serogroup C ST-11 meningococci during the 1980s within Scotland. This is important as this strain has a worldwide distribution and is classed as a hypervirulent strain. From retrospective analysis, the first recorded example of serogroup C ST-11 was in 1976 in Brazil and became prominent in Canada and Europe during the 1990s. In Scotland, the rate of serogroup C meningococcal disease decreased during the early 1990s and then increased slowly through the second half of the 1990s due to an increase in ST-11. Although this endemic strain is associated with serogroup C disease, there have been 26 examples among the total number of isolates where the ST-11 were serogroup B, 41 that were serogroup W135, 1 that was serogroup X and one that was serogroup 29e. These ST-11 strains with serogroups other than serogroup C had also been present during the 1970s. This may suggest evidence of capsular switching as the *porA* data for serogroup W135 ST-11 strains from the 1970s had the combinations of 5, 2, 36-2 or 5-1, 10-4, 36-2 and these combinations have been commonly found within serogroup C ST-11s. ST distribution amongst serogroups B and C showed an interesting pattern. Serogroup B isolates could be differentiated into a large number of STs (216 different STs) whereas serogroup C isolates did not show nearly the same diversity (84 different STs) and with the majority of strains representing the aforementioned ST-11 and ST-8.

The ST-11 strain could represent the ET-37 or ET-15 variant (that emerged in Canada, Greece and the Czech Republic in the late 1980s/early 1990s). The ET-15 variant is associated with a higher case: fatality rate. The two can be differentiated by

a mutation in the *fumC* gene that falls outside of the locus used for MLST and by insertion sequence IS1301 (Elias & Vogel, 2007). Unfortunately the analysis to determine which variant the ST-11 strains in Scotland belonged to was not performed. When examining the PorA variants VR1 and VR2, 62% of the ST-11 strains were 5,2. This combination has been found in both the ET-37 and ET-15 variants. However, there were also 14 cases of PorA variants VR1 and VR2 5-1,10-8 which is the genosubtype shown by the hypervirulent ET-15 variant of the ST-11/ET-37 complex, which has affected a region of Spain (Perez-Trallero *et al.*, 2002). Interestingly, of these 14 cases, one occurred in 1997 and the other 13 in 1998. There was also one case of PorA variants VR1 and VR2 7,1 in 1993 and the strain has been identified as an ET-15 variant that has been linked to an outbreak in Quebec, Canada (Tsang *et al.*, 2004). However, these strains of meningococci were not isolated in Canada in large numbers prior to 2001 (Tsang *et al.*, 2004).

3.2.5 Genetic changes within the meningococcal ST-8 complex/cluster A4 strains

It was also found that meningococci of ST-8, which were predominant during the 1980s, were replaced by similar strains of the same complex in the early 1990s. These clones included ST-153, ST-1349 and ST-66. ST-153 differed from ST-8 by one nucleotide in the *gdh* gene and ST-66 differed from ST-8 by five nucleotides in the *fumC* gene. The nucleotide differences between ST-8 and ST-153 and between ST-8 and ST-66 were all at synonymous sites and did not result in protein changes. However, ST-1349 differed from the ST-8 at three loci, fumC 2→9, gdh 8→34 and *pgm* 2→8. These nucleotide differences result in amino acid substitution. The *pgm* change is at 22 nucleotides and when analysed using the Max Chi Squared Test there

is a significant mosaic in the *pgm* at position 159 with a Max Chi Squared value of 36.5916 (p=0.005). This is strong evidence that these changes came about by a recombination event.

3.2.6 Recombination

In a regularly recombining organism there is no single phylogenetic tree for a collection of isolates. Recombination leads to different phylogenies at different positions in the genome. The frequency of recombination determines the extent to which these trees are correlated. Therefore, the degree of incongruence between phylogenetic trees at distinct loci is a way to quantify the extent of recombination in a population. To quantify the effect of recombination on phylogenetic congruence, a subset of 30 out of a global sample of 107 predominantly disease-causing meningococci (Maiden *et al.*, 1998) were analysed (Holmes *et al.*, 1999; Feil *et al.*, 2001). Under the null hypothesis of complete linkage in the absence of recombination, all loci share the same phylogenetic tree topology. For each MLST locus a maximum likelihood (ML) tree was estimated. To test for congruence between the ML tree topology at each locus and all the others, the difference in log likelihood was calculated, having re-optimised the branch lengths for the other trees. A null distribution for the difference in log likelihood was produced using 200 bifurcating topologies simulated uniformly at random. The results demonstrated that the extent of recombination in *N. meningitidis* is therefore sufficient to create phylogenetically incongruent trees within a 450bp sequence (Feil *et al.*, 2001).

The thirty most diverse strains observed in the MLST analysis were chosen as representative of the phylogenetic history of *N. meningitidis* in Scotland. The UPGMA generated from the STs of the 30 more distantly related members of the

strain collection is shown in Figure 3.11 combined with a table showing the alleles for each locus. The index of association test for linkage disequilibrium was performed on the 7 loci for all isolates (I_A=0.978) and the 30-strain subset (I_A=0.303) showing evidence for linkage in the former but not the latter. It can be shown that, for each locus, identical alleles were distributed among distantly related isolates. Examples included *abcZ* allele 2, which occurred in six isolates, *adk* allele 5, present in nine isolates and *pgm* allele 2, found in five isolates at various locations on the dendrogram.

The ML phylogenetic trees for the seven loci are presented in Figure 3.12. All phylogenetic trees used in the congruence analysis were reconstructed by using the maximum likelihood (ML) method available in the PAUP* package (version 4). The HKY85 model of DNA substitution was used with the optimal ratio of transitions to transversions (Ts/Tv) and the α parameter, which describes the extent of rate variation among nucleotide sites assuming a discrete gamma distribution with eight categories, both estimated from the empirical data during tree reconstruction.

First, for each gene, the differences in log likelihood (Δ-ln *L*) were computed between the ML tree for that gene and the ML trees constructed on the other genes, but with branch lengths optimised to maximize the likelihood of this topology on the reference data. Values for Ts/Tv and α were also reoptimised. To determine whether these differences in log likelihood are significantly different (they will not be if the gene trees are congruent), 200 random trees were created for each gene. The likelihoods of these trees were then estimated, again by optimizing branch lengths and Ts/Tv and α values, and the differences in log likelihood between these random trees and the ML

tree for each gene were computed. These can then be considered as a null distribution of Δ-ln *L* values, as would be obtained when there is no more similarity in topology among gene trees than expected by chance. If the Δ-ln *L* values for the comparisons among the different ML trees fall within the 99th percentile of this null distribution, then we may say that they are significantly different and hence incongruent. The results of the maximum likelihood analysis of congruence are presented in Table 3.4.

This shows that there is a lack of congruence with only a small number of gene tree comparisons showing levels of topological similarity greater than the random expectation, and even in these cases the trees were very dissimilar, with likelihood differences falling only marginally outside the 99th percentile of the random distribution (Figure 3.12). The lack of congruence among gene trees can be explained as the consequence of a legacy of relatively frequent recombinational exchanges that over time have almost eliminated the phylogenetic signal in each tree. High rates of recombination have been shown previously from estimates of linkage disequilibrium (Smith *et al.*, 1993), the presence of frequent mosaic structure in housekeeping genes (Zhou *et al.*, 1997) and the lack of congruence between gene trees (Feil *et al.*, 1996; Holmes *et al.*, 1999; Feil *et al.*, 2001).

Figure 3.11 Alleles present at each locus and the relationship of isolates by UPGMA analysis drawn on the basis of the alleles present at each of the 7 loci in the subset of the 30 most diverse meningococcal isolates. A UPGMA dendrogram together with the alleles present at each locus for each isolate is shown.

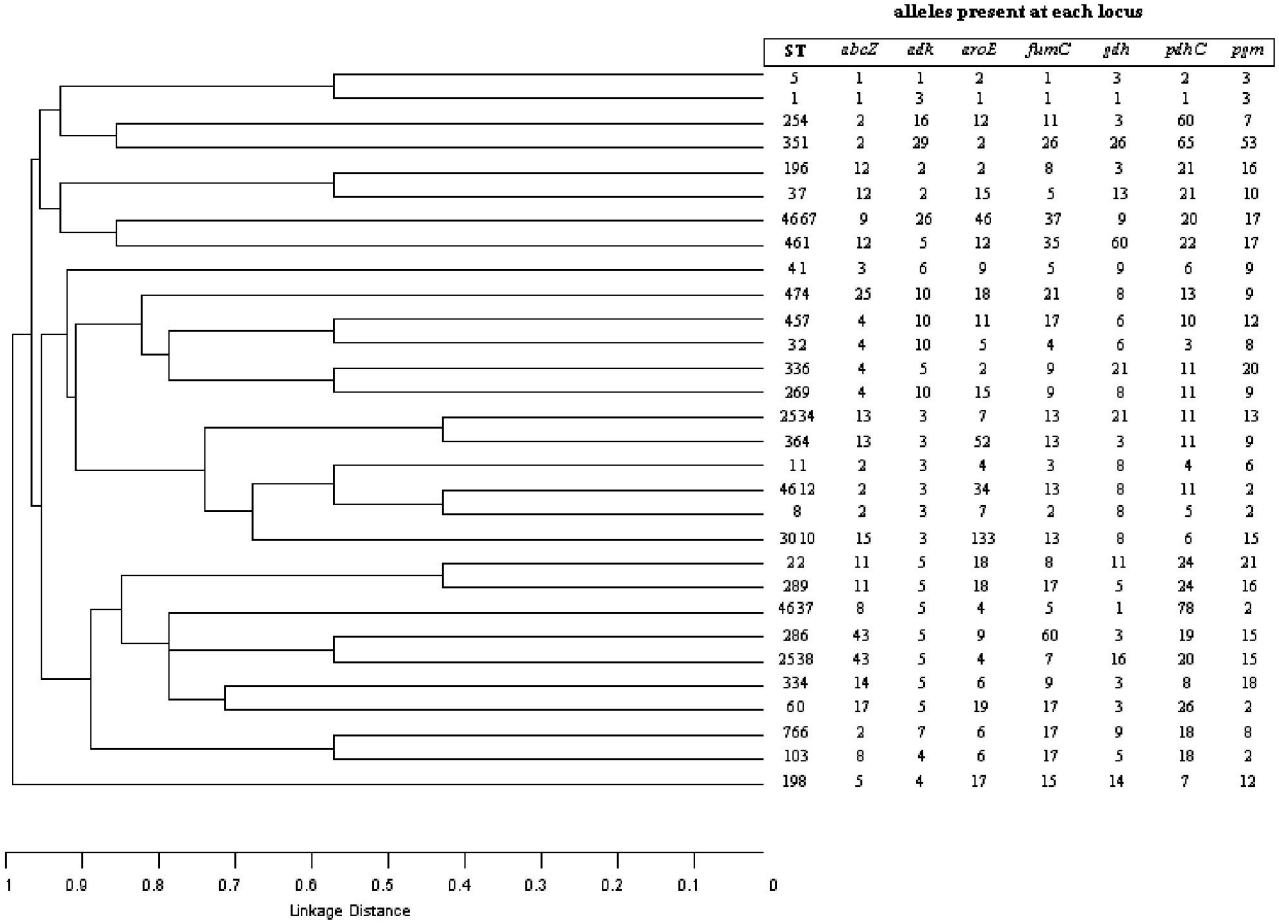
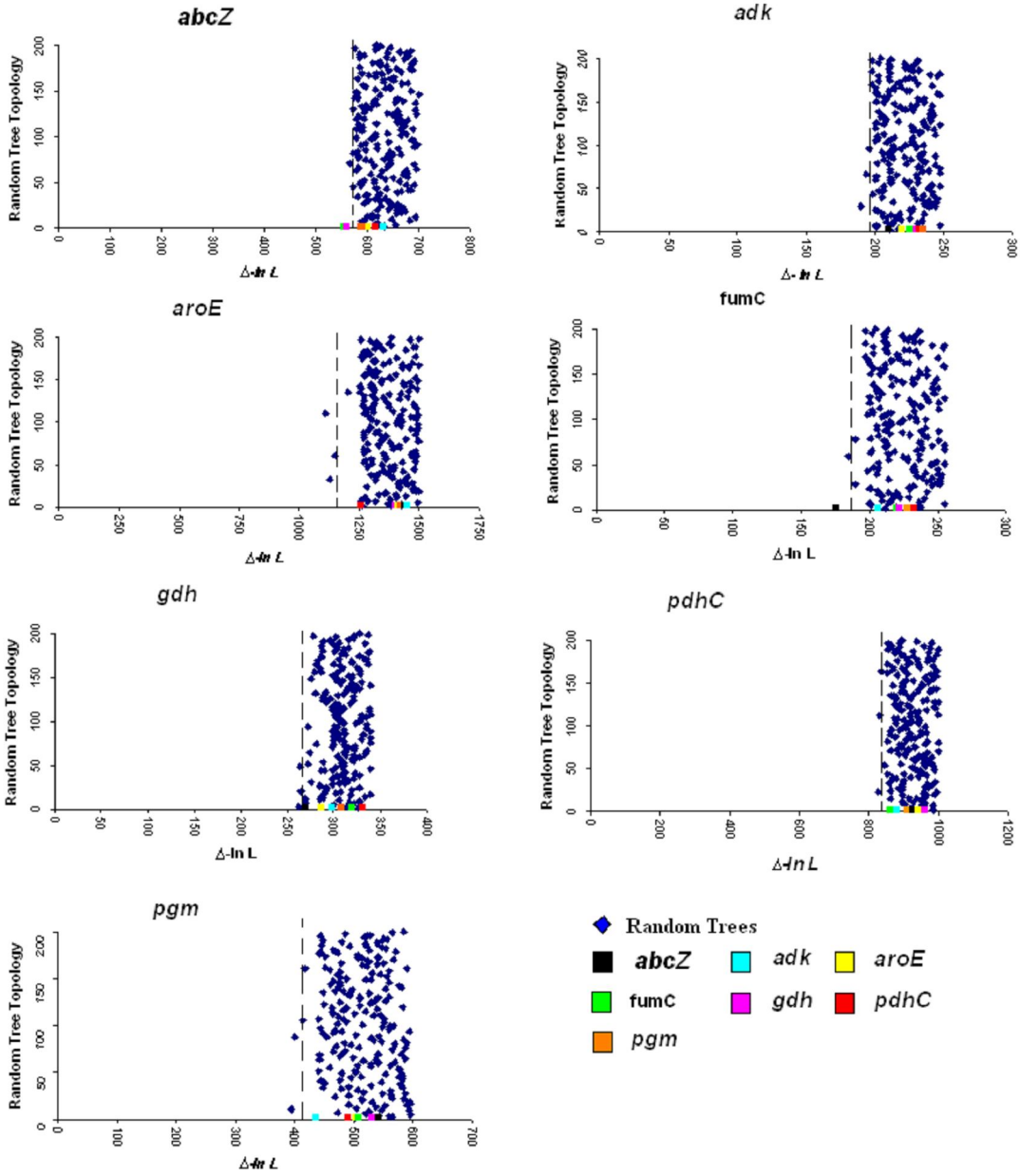


Table 3.4 Maximum likelihood analysis of congruence between seven housekeeping genes.

Gene	bp	Δ-ln *L* of ML tree	Δ-ln *L* of ML trees from other gene	99th percentile in Δ-ln *L* in random trees	Loci outside 99th percentile of random trees
abcZ	433	1251.638	566.278-682.457	571.681	*fumC, gdh*
adk	465	844.767	211.291-245.980	195.601	__
aroE	490	1798.894	1163.242-1472.311	1153.215	__
fumC	465	968.303	170.153-225.727	187.726	*abcZ*
gdh	501	993.788	270.244-331.562	268.147	__
pdhC	480	1592.003	799.284-960.181	835.687	__
pgm	450	1235.102	440.650-542.987	414.564	__

Figure 3.12 Maximum likelihood analysis of congruence amongst MLST loci for the 30 most diverse *N. meningitidis* isolates within Scotland. The ML tree of each locus is compared with the ML trees from the other six loci. The differences in likelihood (Δ- In *L*) are shown between loci (coloured squares) and between each locus and 200 trees of random topology (diamonds). The 99th percentile of the likelihood differences between the ML tree for each gene and the 200 random tree topologies is indicated by the dotted line.



3.2.7 Analysis of molecular variance (AMOVA)

Assessing the genetic structure of the population and ascertaining whether distinct subpopulations occur in specific areas can be performed using the analysis of MLST allelic profile and sequence data. Population subdivision can be measured using the *F*-statistic (F_{ST}). Wright's *F*-statistic (Wright, 1943; Wright, 1951) measures the extent and/or presence of genetic variation and subdivision by comparing alleles within subpopulations (e.g., health board regions) and alleles within the total population. If there is a high rate of gene flow in the total population and no subdivision is apparent then F_{ST} is zero. However, if there is subdivision within the population, then F_{ST} is greater than zero. In the Scottish disease isolates, calculations of F_{ST}s for the allelic profiles, *porA* and concatenated locus sequences were carried out using AMOVA (Excoffier *et al.*, 1992) as implemented in Arlequin software (version 2.0) (Schneider *et al.*, 2000). The results (Table 3.5) show the presence of gene flow between certain neighbouring health board regions. For example, between AC (Argyll and Clyde) and AA (Ayrshire and Arran) with a F_{ST} value of 0.00072 and between FF (Fife) and FV (Forth Valley) with a F_{ST} value of 0.00006.

The results for the health board regions of Shetland (SH), Western Isles (WI) and Borders (BR) do show higher F_{ST} values than average, which could suggest the presence of structure among certain health board regions. However, these regions have low number of isolates associated with them (SH has one isolate, WI has five isolates and BR has 25 isolates), which may skew results.

Table 3.5 F_{ST} values of pair-wise comparisons for the allelic profiles, *porA* and concatenated locus sequences of the 15 different health boards within Scotland. F_{ST} Measures the extent of structuring in a population by comparing the alleles within sub population and alleles within the total population. An F_{ST} value of 0 indicates no subdivision and therefore high gene flow in the population. A value greater than 0 indicates the presence of structure.

	AA	AC	BR	DG	FF	FV	GG	GR	HG	LN	LO	SH	TY
AC	0.00072												
BR	0.02584	0.01818											
DG	0.00496	0.00934	0.03007										
FF	0.00953	0.00976	0.00026	0.00291									
FV	0.00715	0.00460	0.01271	0.00176	0.00006								
GG	0.00010	0.00098	0.01604	0.00795	0.00836	0.00476							
GR	0.00131	0.00410	0.01299	0.00158	0.00216	0.00123	0.00258						
HG	0.00380	0.00141	0.02355	0.01010	0.01321	0.00746	0.00219	0.00407					
LN	0.00983	0.00754	0.02012	0.01355	0.01155	0.00656	0.00751	0.00902	0.00961				
LO	0.00350	0.00368	0.00476	0.00447	0.00054	0.00207	0.00264	0.00108	0.00618	0.00807			
SH	0.08559	0.06599	0.07667	0.10020	0.07221	0.07248	0.06797	0.06792	0.05165	0.06577	0.06221		
TY	0.00080	0.00187	0.01356	0.00418	0.00467	0.00521	0.00023	0.00006	0.00248	0.00699	0.00073	0.07158	
WI	0.09090	0.06717	0.05583	0.10012	0.06561	0.07551	0.07319	0.06991	0.05611	0.07106	0.06862	0.10000	0.07744

3.3 *PorA* analysis

All isolates were characterised at the *porA* locus, which encodes a major surface antigen and vaccine candidate PorA (Russell *et al.*, 2004). The first variable region (VR1) was represented by 34 different variable types, within nine distinct families (5, 7, 12, 17, 18, 19, 20, 21 and 22). The first variable region was represented in the majority from the P1.5 family (1299 isolates). Within this family to date there are 27 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. The four VR1 types represented in Scotland were 5 (572 isolates), 5-1 (394 isolates), 5-2 (330 isolates) and 5-3 (three isolates).

The second variable region (VR2) was represented by 44 different variable types within 15 distinct families (1, 2, 3, 4, 9, 10, 13, 14, 15, 16, 23, 25, 26, 28 and 30) with 33.7% of isolates characterised as having variable regions 2, 2-1 and 2-2. This variable region contains more variant types in comparison to the other two. For example, within the type 2-family there are, to date, 59 different variants. This family has variants of from one to six amino acids either by substitution, insertion or mutation from the original fifteen amino acid sequence. The six VR2 types represented in Scotland were 2 (515 isolates), 2-1 (77 isolates), 2-2 (287 isolates), 2-4 (one isolate), 2-12 (one isolate) and 2-16 (one isolate).

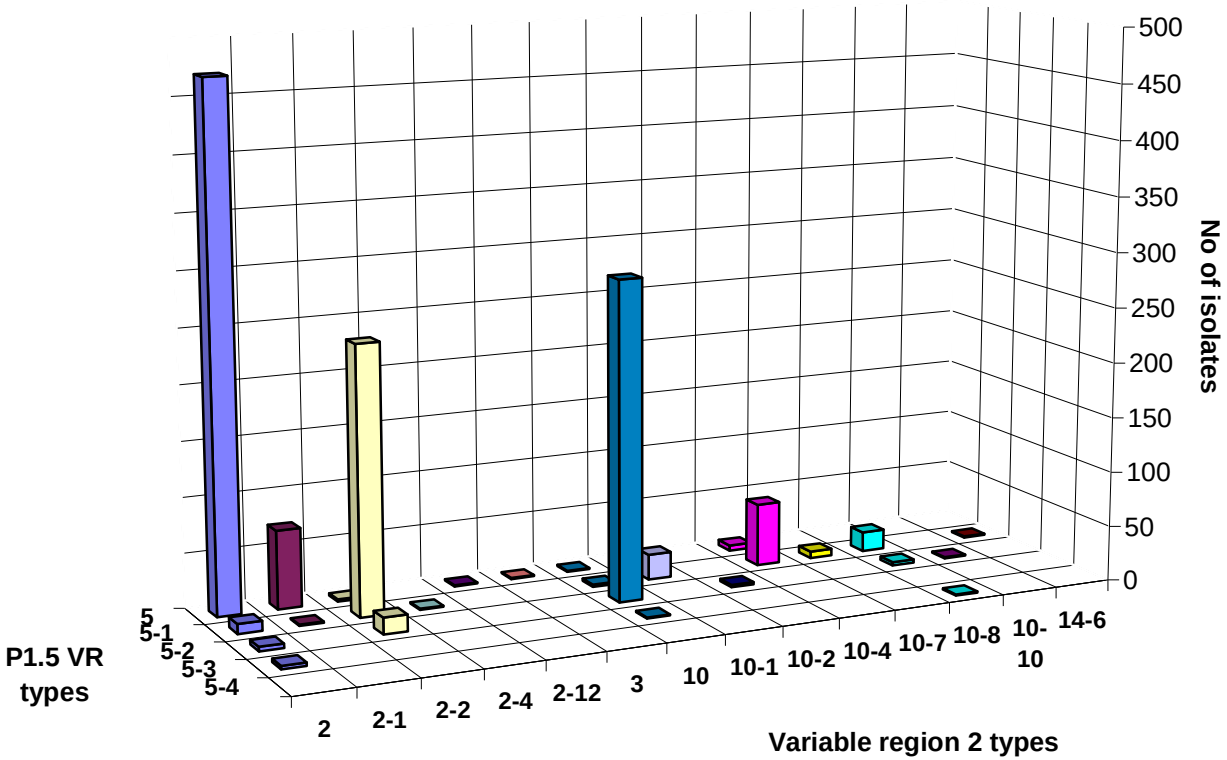
The third variable region (VR3) was represented by nine different variable types within four distinct families (35, 36, 37, and 38) with 38.6% of isolates characterised

as having variable region 36-2. Within the type 36-family there are to date three different variants. The three VR3 types represented in Scotland were 36 (319 isolates), 36-1 (8 isolates) and 36-2 (1007 isolates). No new variable types were found within any of the variable regions.

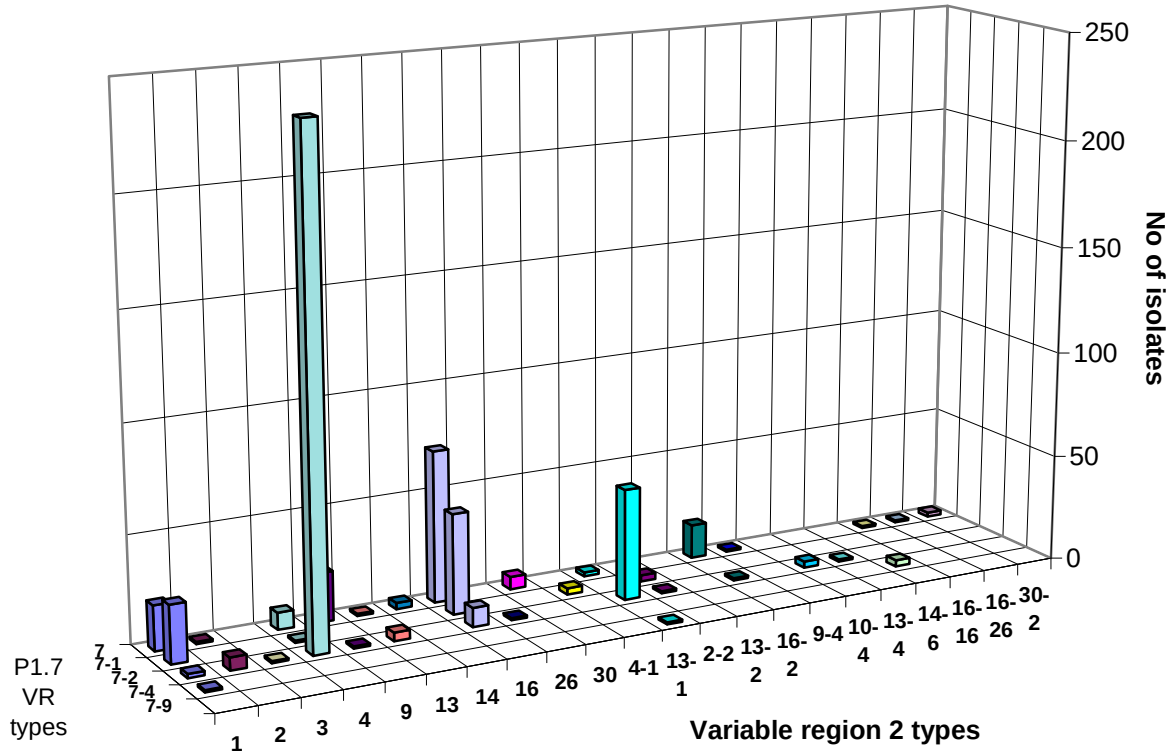
Certain combinations of VRs were more common than others. The combination 5, 2, 36-2 occurred 466 times (17.9%) and 19, 15, 36 occurred 192 times (7.4%). Figures 3.13-3.15 shows the different combinations of variable region 1 and variable region 2 present within the three most common variable region 1s (which accounts for 83.5% of all isolates) present within Scotland 1972-1998. There was a link between *porA* and ST and also clonal complex. This is evident with the combination 5, 2-1, 36-2, which occurs 70 times all belonging to the ST-11 complex and all but two isolates were ST-11. Also 15 isolates that were 18-3, 1, 35-1 all belonged to the ST41-44/Lineage 3 complex. However, this was not the case with all *porA* combinations as the combination 19, 15, 36 had 10 different complexes associated with it and 41 different STs.

As previously mentioned, before genotyping, the phenotypic methods most frequently employed to identify subtypes consisted of using panels of monoclonal antibodies (Abdillahi & Poolman, 1988). Complete serosubtyping of meningococci can rarely be performed and strains that cannot be serosubtyped or only partially serosubtyped are

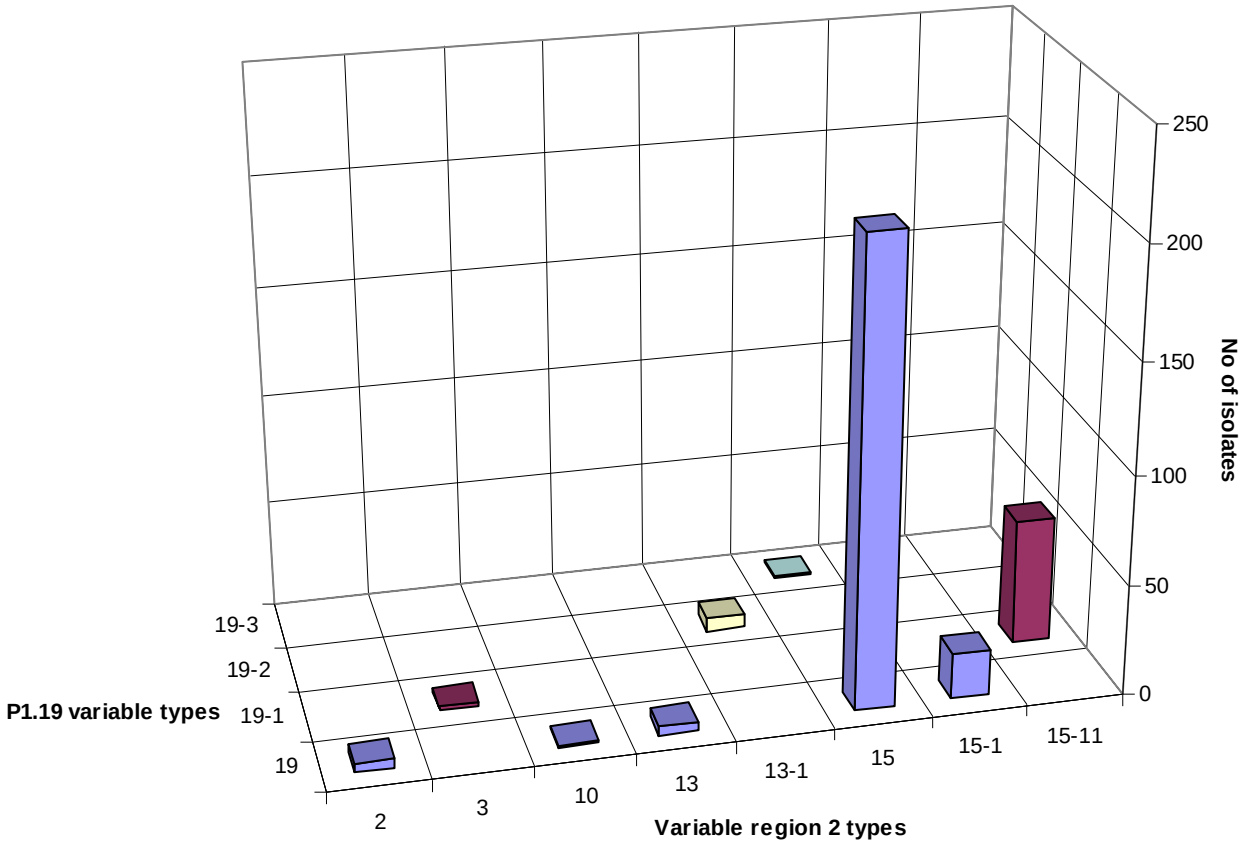
Figures 3.13 The different combinations of variable region 1 and variable region 2 present within the P1.5 family, Scotland 1972-1998.



Figures 3.14 The different combinations of variable region 1 and variable region 2 present within the P1.7 family, Scotland 1972-1998.



Figures 3.15 The different combinations of variable regions 1 and 2 present within the P1.19 family, Scotland 1972-1998.



frequently found. This limitation is attributed to the use of incomplete sets of monoclonal antibodies, a lack of PorA expression and the absence of reactivity of the monoclonal antibodies used with some of the numerous VR1 and VR2 variants of the prototype strains (Sacchi *et al.*, 2000; Vogel & Claus, 2003). Genotypic techniques that analyse the VR1 and VR2 sequences of the *porA* gene overcome these limitations and allow complete identification (Feavers *et al.*, 1992; Sacchi *et al.*, 1998; Diggle & Clarke, 2003). Isolates from the 1990s were previously serosubtyped using panels of monoclonal antibodies. A comparison of strains with over 50 samples from the 1990s and their resulting genotyping are shown in Table 3.6.

Table 3.6 Distribution of genosubtypes and serosubtypes for genosubtypes with >50 isolates during the 1990s.

VR1	VR2	VR3	No of samples (%)	Subtype	No of samples
5	2	36-2	288 (25.1%)	NST*	87 (7.6%)
				P1.2	123 (10.7%)
				P1.2, P1.5	61 (5.3%)
				P1.5	17 (1.5%)
7-2	4	37	153 (13.3%)	NST	78 (6.8%)
				P1.4	61 (5.3%)
				P1.7	14 (1.2%)
5-2	10	37-1	119 (10.4%)	NST	27 (2.4%)
				P1.10	91 (7.9%)
				P1.5	1 (0.1%)
19	15	36	66 (5.8%)	NST	12 (1.1%)
				P1.15	54 (4.7%)
21	16	37-1	55 (4.8%)	NST	11 (1.0%)
				P1.16	44 (3.8%)

***NST = Non-subtypeable**

Of the 681 isolates from Table 3.6, 215 isolates (31.5%) could not be identified by serosubtyping. Genosubtyping identified the VR1 type of 589 strains that could not be identified by serosubtyping. Moreover, genosubtyping identified the VR2 type of 353 strains that could not be identified by serosubtyping. The results of the present study, like those of previous studies (Sacchi *et al.*, 2000; Clarke *et al.*, 2003), demonstrate the advantages of genosubtyping over serosubtyping. Genosubtyping achieves complete identification of both VR1 and VR2 and discriminates the variants among strains (Feavers *et al.*, 1992; McGuinness *et al.*, 1993).

Certain VR1, 2 and 3 regions were associated with certain serogroups, although this was not definitive, examples included the *porA* combination 5, 2, 36-2 which had 354 isolates (76.0%) associated with serogroup C, although this is not surprising as the *porA* combination is highly associated with ST-8 and ST-11. Of the 192 isolates with *porA* combination 19, 15, 36, 85.4% were associated with serogroup B. When serogroup and the porA variable regions 1, 2 and 3 were compared, there did seem to be a pattern of certain variable regions being associated with certain serogroups. However, there were also some VRs that have been observed in all serogroups.

3.3.1 Serogroup C isolates

The first variable region was represented by 22 different variable types, within eight distinct families (5, 7, 12, 17, 18, 19, 21 and 22). The P1.5 family contained the largest number of isolates, 449 isolates were 5, 136 isolates were 5-1, 29 isolates were 5-2 and two isolates were 5-3. The second variable region was represented by 31 variable types within 13 families, namely 1, 2, 3, 4, 9, 10, 13, 14, 15, 16, 25, 26 and 28. 46.5% were allocated to variable region 2. The third variable region was

represented by nine different variable types within four distinct families (35, 36, 37 and 38). 70.2% were allocated to variable region 36-2.

3.3.2 Serogroup B isolates

The first variable region was represented by 26 different variable types, within eight distinct families (5, 7, 12, 17, 18, 19, 21 and 22). However, two families (5 and 7) were associated with 69% of serogroup B isolates. The P1.7 family contained the largest number of isolates, 93 isolates were 7, 74 isolates were 5-1, and 302 isolates were 7-2. This was closely followed by the P1.5 family where 75 isolates were 5, 206 isolates were 5-1, 160 isolates were 5-2 and one isolate was 5-3. The second variable region was represented by 31 variable types. These were contained within 13 families (1, 2, 3, 4, 9, 10, 13, 14, 15, 16, 25, 26 and 28). 46.5% were allocated to variable region 2. The third variable region was represented by nine different variable types within four distinct families, namely 35, 36, 37 and 38, with 70.2% allocated to variable region 36-2.

3.3.3 Serogroup A isolates

Six different variable types, within four distinct families (5, 18, 20 and 21) represented the first variable region, however 5-2 was responsible for 89.9% of serogroup A isolates. The other variable types present were one isolate was 5, two isolates were 5-1, one isolate was 18-1, nine isolates were 20 and two isolates were 21. The second variable region was represented by six variable types; these were contained within 5 families, namely 2, 3, 9, 10 and 16. However, variable type 10 was responsible for 90% of serogroup A isolates and all but one of these also had 5-2 for its first variable region. The third variable region was represented by four different

variable types 35-1 (9 isolates), 36-2 (three isolates), 37-1 (136 isolates) and 38 (one isolate). All but one of the isolates with third variable region 37-1 had 5-2 for its first variable region and all but two of the isolates with third variable region 37-1 had 10 for its second variable region.

3.3.4 Serogroup Y isolates

Four different variable types, within two distinct families (5 and 7) represented the first variable region. However, 5-1 was responsible for 81.1% of serogroup Y isolates. The other variable types present were: one isolate was 5, three isolates were 5-2 and one isolate was 7-1. The second variable region was represented by seven variable types; these were contained within three families (2, 10 and 16). However, variable type 10 was responsible for 72.2% of serogroup Y isolates. The third variable region was represented by three different variable types, namely 35 (one isolate), 36-1 (one isolates) and 36-2 (33 isolates).

3.3.5 Serogroup W135 isolates

Six different variable types, within four distinct families (5, 12, 18 and 22) represented the first variable region. The variable type 5 was responsible for 43 of serogroup W135 isolates. The other variable types present were: five isolates were 5-1, one isolate was 5-2, one isolate was 12-1, 15 isolates was 18-1 and one isolate was 22-1. The second variable region was represented by nine variable types within five families (2, 3, 10, 13 and 14). However, 2 was responsible for 41 isolates. The third variable region was represented by four different variable types 35 (one isolate), 36-2 (46 isolates), 37-1 (one isolate), and 38 (16 isolates). The most common profile within the W135 isolates was 5, 2, 36-2 and this occurred 41 times (61.2%). All of

these isolates with this profile belonged to the ST-11 complex and, apart from one isolate from 1995, all occurred before 1983. This *porA* profile and ST-11 complex is more commonly associated with serogroup C. However, the complex worldwide has more recently been associated with serogroup W135 outbreaks in Africa and also with those returning from the Hajj pilgrimage.

3.3.6 Serogroup X isolates

Four different variable types, within four distinct families: 5 (one isolate), 19 (one isolate), 21 (four isolates) and 22 (one isolate), represented the first variable region. The second variable region was represented by four variable types within four families and these were, 2-1 (one isolate), 9 (one isolate), 15 (one isolate) and 16 (14 isolates). The third variable region was represented by four different variable types 35-1 (one isolate), 36 (one isolate), 36-2 (one isolate), and 37-1 (four isolates).

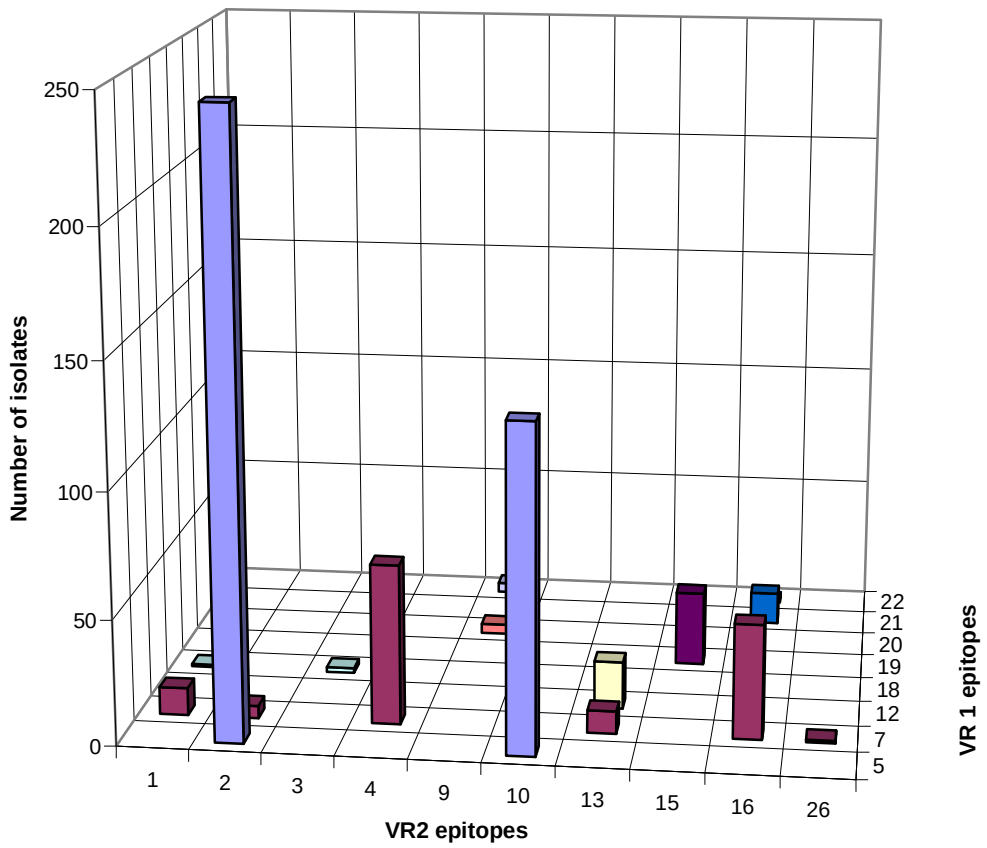
3.3.7 Miscellaneous serogroup isolates

These included serogroups Z (five isolates) and 29e (6 isolates). 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. Unfortunately, of these isolates, only one serogroup Z isolate and two serogroup 29e isolates were available for *porA* sequencing. The serogroup Z isolate had variable regions 7-4, 1 and 35-1 and this profile was not present within any other isolate in Scotland. Variable regions 5, 2 and 36-2 and 21, 16 and 37-1 were present in the two serogroup 29e isolates.

3.3.8 PorA types present in *N. meningitidis* strains 1972-1979

The first variable region (VR1) was represented by 14 different variable types, within eight distinct families (5, 7, 12, 18, 19, 20, 21 and 22). The first variable region which had the largest number associated with it was the P1.5 family (356 isolates). The three variants of the P1.5 family represented in Scotland during the 1970s were: 5 (50 isolates, 14.1%), 5-1 (180 isolates, 50.6%) and 5-2 (126 isolates, 35.4%). The second largest was the P1.7 family (131 isolates). The three variants of the P1.7 family represented in Scotland during the 1970s were: 7 (34 isolates, 26.0%), 7-1 (18 isolates, 13.7%) and 7-2 (79 isolates, 60.3%). The third largest was the P1.19 family (28 isolates). The second variable region (VR2) was represented by 17 different variable types within 10 distinct families (1, 2, 3, 4, 9, 10, 13, 15, 16, and 26). The second variable region which had the largest number associated with it was variable regions 2 (55 isolates) and 2-2 (179 isolates). The second largest was the variable region 10 (123 isolates). The three variants represented in Scotland during the 1970s were: 10 (115 isolates, 93.5%), 10-1 (two isolates, 1.6%) and 10-4 (6 isolates, 4.9%). The third variable region was represented by seven different variable types 35 (43 isolates, 7.0%), 35 (49 isolates, 8.0%), 36 (32 isolates, 5.2%), 36-2 (229 isolates, 37.2%), 37 (59 isolates, 9.6%), 37-1 (141 isolates, 22.9%) and 38 (two isolates, 0.3%). Figure 3.16 shows the different combinations of VR1 and VR2 present in *N. meningitidis* isolates in Scotland 1972-1979.

Figure 3.16 The different combinations of variable region 1 and variable region 2 present in Scotland 1972-1979



3.3.9 PorA types present in *N. meningitidis* strains 1980-1989

The first variable region was represented by 27 different variable types, within nine distinct families (5, 7, 12, 17, 18, 19, 20, 21 and 22). As with the previous decade the first variable region, which had the largest number, associated with it was the P1.5 family (372 isolates). Variable types within this family were 5 (196 isolates, 52.7%), 5-1 (100 isolates, 26.9%) 5-2 (72 isolates, 19.3%) and 5-3 (four isolates, 1.1%). Compared to the previous decade though there was a much higher percentage of variable type 5 during the 1980s. However, this is no surprise when the MLST data is also analysed as there was an increase in both ST-11 and ST-8 isolates which both are often associated with this variable type. The second largest was the P1.7 family (146 isolates). The four variants of the P1.7 family represented in Scotland during the 1980s were: 7 (37 isolates, 25.3%), 7-1 (41 isolates, 28.1%), 7-2 (67 isolates, 45.9%) and 7-4 (one isolate, 0.7%). The third largest was the P1.19 family (145 isolates). The three variants of the P1.19 family represented in Scotland during the 1980s were: 19 (136 isolates, 93.8%), 19-1 (8 isolates, 5.5%) and 19-3 (one isolate, 0.7%).

The second variable region was represented by 38 different variable types within 14 distinct families (1, 2, 3, 4, 9, 10, 13, 14, 15, 16, 25, 26, 28 and 30). The second variable region which had the largest number associated with it was variable region 2 (288 isolates). The five variants represented in Scotland during the 1980s were: 2 (156 isolates, 54.2%), 2-1 (49 isolates, 17.0%), 2-2 (81 isolates, 28.1%), 2-4 (one isolate 0.35%) and 2-16 (one isolate, 0.35%). The second largest was the variable regions 15 (140 isolates). The three variants represented in Scotland during the 1980s were: 15 (116 isolates, 82.9%), 15-1 (16 isolates, 11.4%) and 15-11 (8 isolates, 5.7%).

In the previous decade the variable region 15 was only present 28 times (4.6%). The third largest was the variable region 10 (123 isolates). The three variants represented in Scotland during the 1970s were: 10 (115 isolates, 93.5%), 10-1 (two isolates, 1.6%) and 10-4 (6 isolates, 4.9%). The third variable region was represented by nine different variable types: 35 (40 isolates, 4.7%), 35-1 (125isolates, 14.8%), 36 (128 isolates, 15.1%), 36-1 (two isolates, 0.2%), 36-2 (331 isolates, 39.2%), 37 (35 isolates, 4.1%), 37-1 (108 isolates, 12.8%), 38 (44 isolates, 5.2%) and 38-1 (7 isolates, 0.8%). Figure 3.17 shows the different combinations of VR1 and VR2 present in *N. meningitidis* isolates in Scotland 1980-1989.

3.3.10 PorA types present in *N. meningitidis* strains 1990-1998

The first variable region was represented by 29 different variable types, within nine distinct families (5, variants of the P1.7 family 7, 12, 17, 18, 19, 20, 21 and 22). As with the previous two decades, the P1.5 family had the largest number of isolates within the first variable region (530 isolates). Variable types within this family were: 5 (306 isolates, 57.7%), 5-1 (100 isolates, 18.9%) and 5-2 (124 isolates, 23.4%). The second largest was the P1.7 family (259 isolates). The four variants of the P1.7 family represented in Scotland during the 1990s were: 7 (54 isolates, 20.8%), 7-1 (27 isolates, 10.4%), 7-2 (176 isolates, 68.0%) and 7-4 (two isolates, 0.8%). The third largest was the P1.19 family (134 isolates). The three variants of the P1.19 family represented in Scotland during the 1990s were: 19 (77 isolates, 57.5%), 19-1 (51 isolates, 38.1%) and 19-3 (6 isolates, 4.4%). The second variable region was represented by 31 different variable types within 14 distinct families (1, 2, 3, 4, 9, 10, 13, 14, 15, 16, 25, 26, 28 and 30). The second variable region which had the largest number associated with it was variable region 2 (328 isolates). The three variants

represented in Scotland during the 1980s were: 2 (288 isolates, 87.8%), 2-1 (22 isolates, 6.7%) and 2-2 (18 isolates, 5.5%). The second largest was the variable regions 10 (205 isolates). The six variants represented in Scotland during the 1990s were 10 (120 isolates, 58.5%), 10-1 (17 isolates, 8.3%), 10-2 (one isolate, 0.5%), 10-4 (45 isolates, 22.0%), 10-7 (one isolate, 0.5%) and 10-8 (21 isolates, 10.2%). The third variable region was represented by nine different variable types: 35 (51 isolates, 4.5%), 35-1 (118 isolates, 10.3%), 36 (145 isolates, 12.7%), 36-1 (6 isolates, 0.5%), 36-2 (417 isolates, 36.4%), 37 (149 isolates, 13.0%), 37-1 (179 isolates, 15.6%), 38 (33 isolates, 2.9%) and 38-1 (one isolates, 0.1%). Figure 3.18 shows the different combinations of VR1 and VR2 present in *N. meningitidis* isolates in Scotland 1990-1998.

Figure 3.17 The different combinations of variable region 1 and variable region 2 present in Scotland 1980-1989

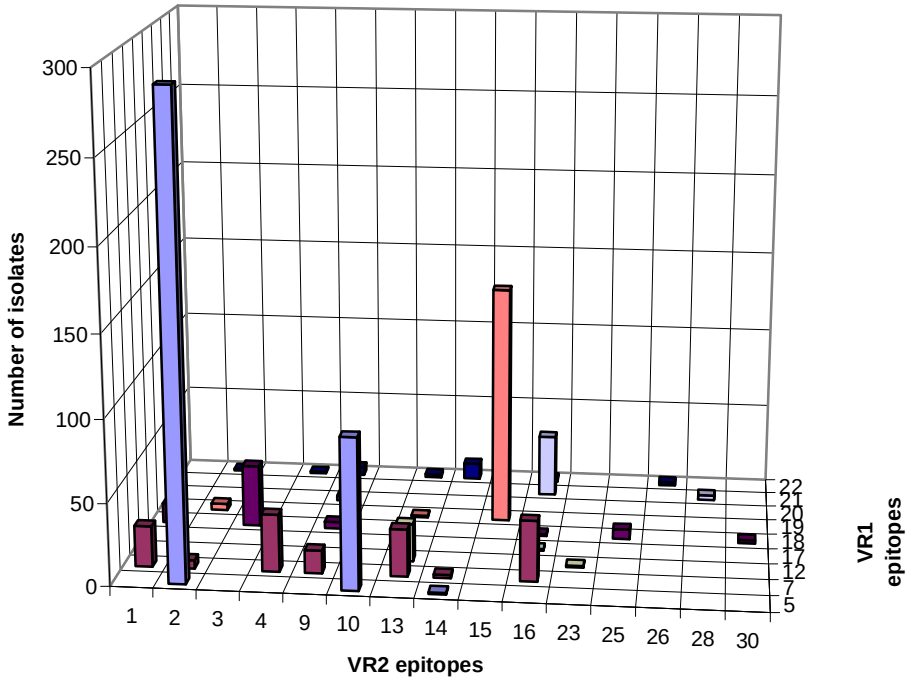
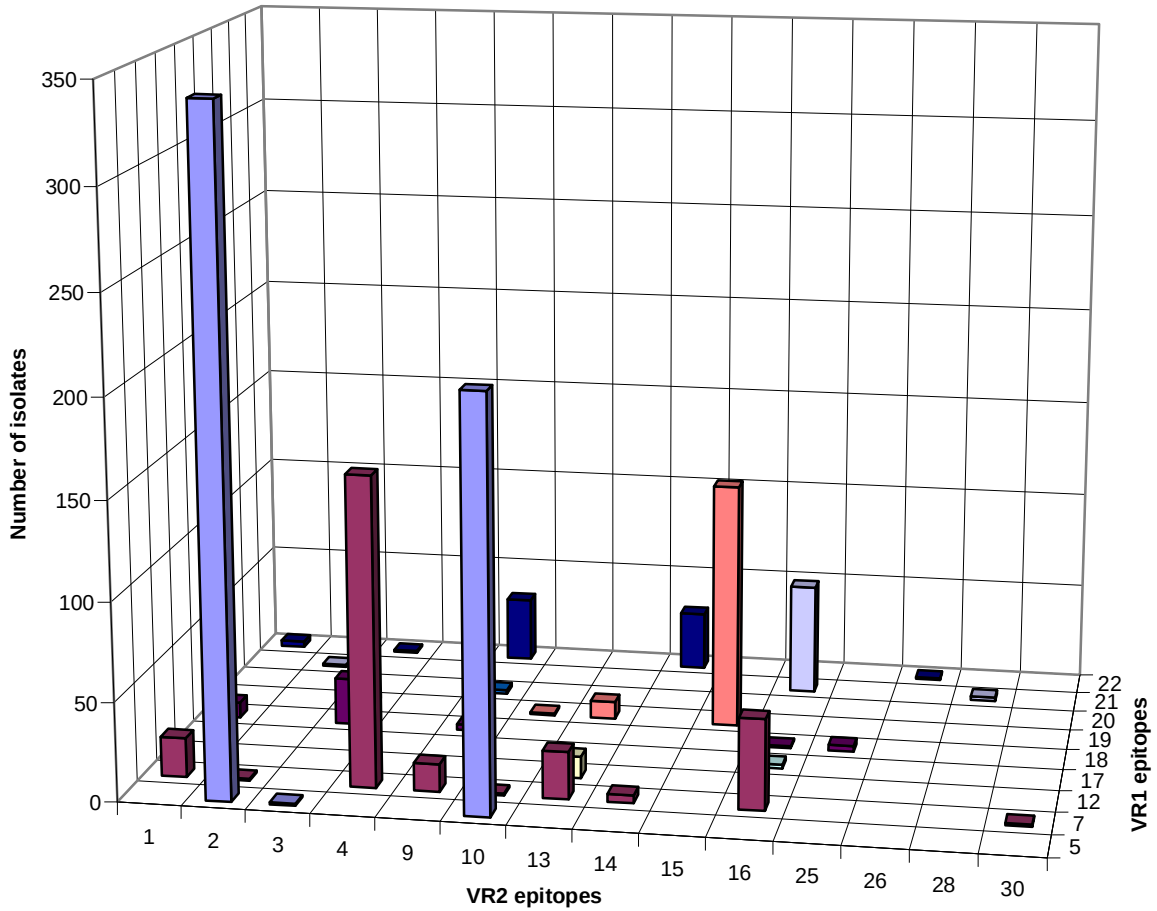


Figure 3.18 The different combinations of variable region 1 and variable region 2 present in Scotland 1990-1998



3.3.11 Comparison of phylogenies obtained from antigen and housekeeping genes

A maximum–likelihood (ML) method was used to construct phylogenetic trees. A total of 159 isolates, representative of the seven most prevalent clonal complexes present in Scotland 1972-1998 were chosen for this analysis. These included five isolates from the ST-1 complex, 21 isolates from the ST-8 complex, 12 isolates from the ST-11 complex, 21 isolates from the ST-32 complex, 51 isolates from the ST-41/44 complex, 26 isolates from the ST-269 complex and 19 isolates from the ST-334 complex. The first tree was constructed with concatenated housekeeping genes and clustered the isolates by clonal complex (Figure 3.19). As expected, the isolates formed clusters related with their clonal complex. The second tree was constructed from the concatenated sequences of the antigen gene *porA* (Figure 3.20). This tree did not show the same clustering affect as the previous tree. There was the serogroup A cluster that was present in the previous tree and there were some clustering of clonal complexes, most notably ST-8 and ST-11 clonal complexes. However, the majority of isolates did not show the same pattern of clustering by clonal complex. Instead there were clusters with different clonal complexes but with the same serogroup. A similar analysis had been performed previously by Urwin *et al.* (Urwin *et al.*, 2004). However they were able to perform a more extensive examination with regards to the tree constructed from the concatenated sequences of the antigen gene *porA* as they were also able to include two other antigen genes *fetA* and *porB*. Unfortunately, the data for *fetA* and *porB* was not available for use in this study. The results from Urwin *et al.* (Urwin *et al.*, 2004) showed that similar clusters of isolates were observed in both the ML tree constructed from the concatenated sequences of the three antigen genes and the ML tree constructed from the concatenated housekeeping genes.

Figure 3.19 Phylogenetic analysis of 159 invasive meningococci isolates, representative of the seven most prevalent clonal complexes present in Scotland 1972-1998 by using seven housekeeping gene sequences. Each isolate is colour coded according to clonal complex, as defined by MLST.

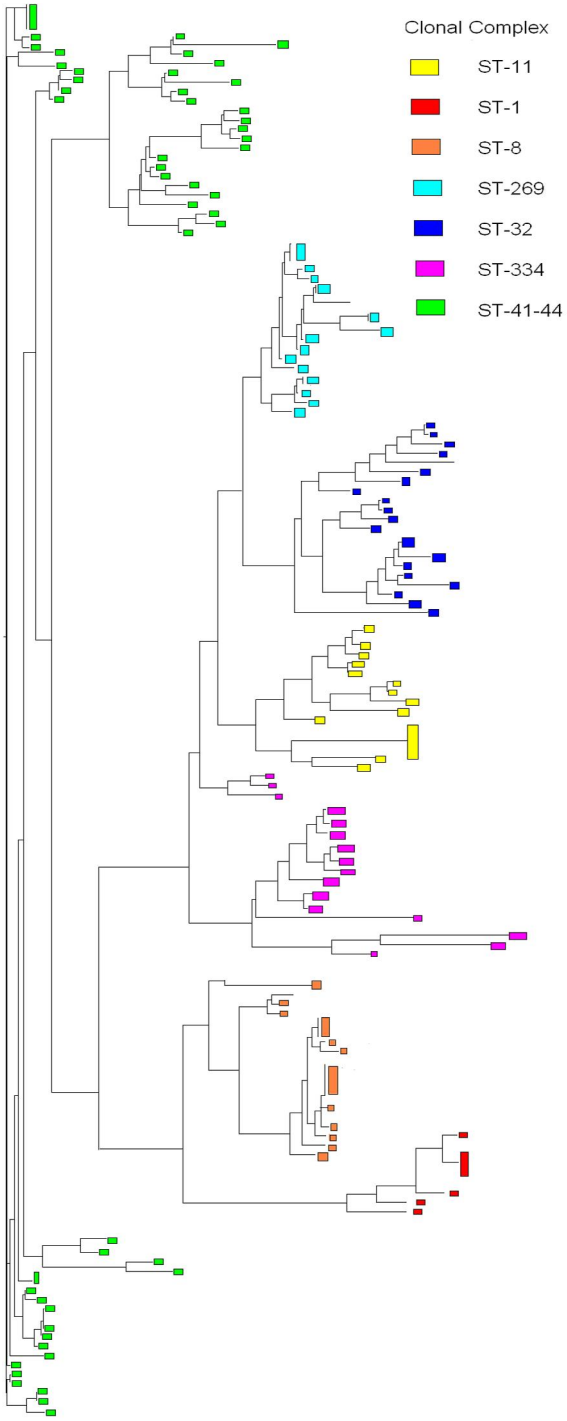
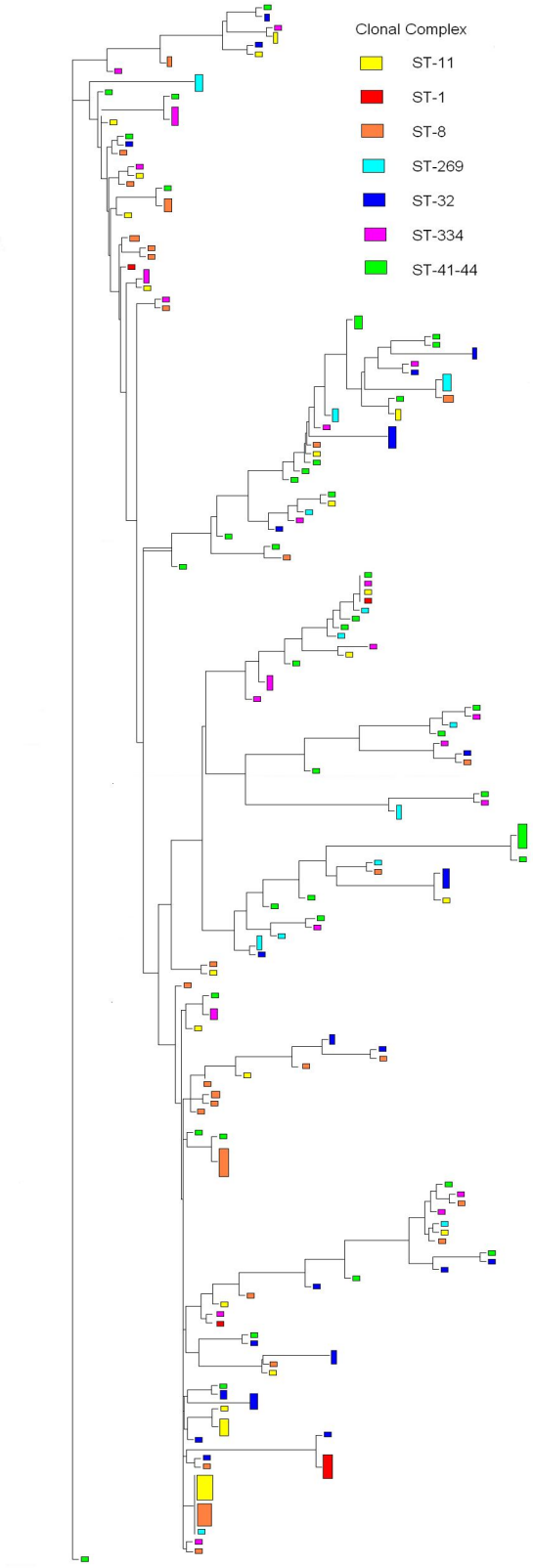


Figure 3.20 Phylogenetic analysis of 159 invasive meningococci isolates, representative of the seven most prevalent clonal complexes present in Scotland 1972-1998 from the concatenated sequences of the antigen gene *porA*. Each isolate is colour coded according to clonal complex, as defined by MLST.

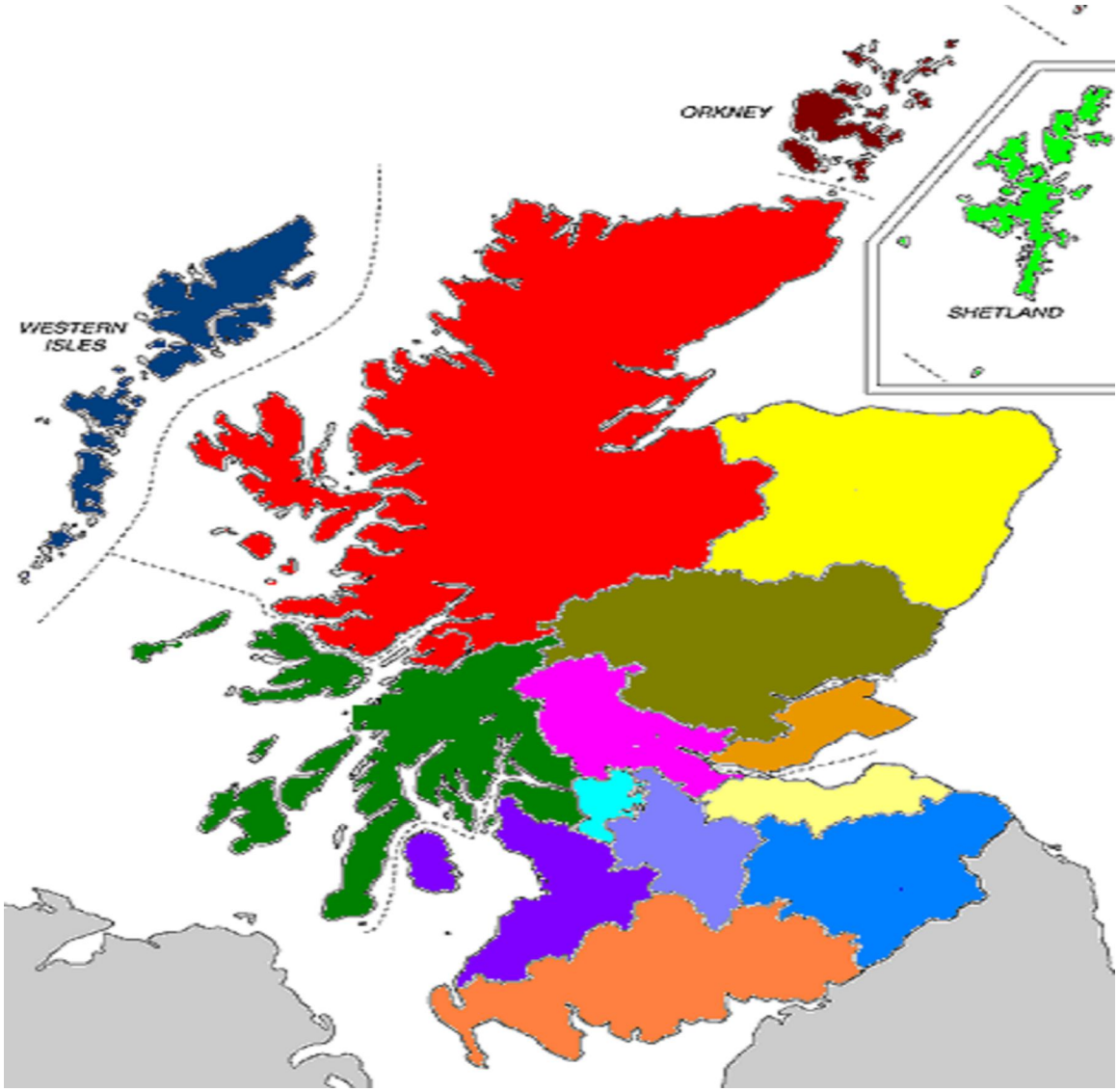


3.4 Geographical distribution of isolates

Between 1972-1998 Scotland contained 15 NHS health boards (NHS Argyll and Clyde is now defunct. Its responsibilities were transferred to NHS Highland and NHS Greater Glasgow on 1 April 2006). These NHS boards differ in both geographical size and population size (Figure 3.21 and Table 3.7). Geographically, the largest land area is Highland but it is only tenth in terms of size of population. The estimated population in Scotland under surveillance was just under 5.1 million. It is important to state that rates of incidence for individual NHS boards may be based on small numbers, and can vary substantially from year to year. The overall average rate of incidence for Scotland between 1972-1998 (1.8 cases per year per 100,000 population) gives a more accurate and consistent view of meningococcal disease activity.

The number of cases per 100,000 population differed markedly between NHS health boards between 1972 and 1998. The Orkney NHS Board had no reported cases of IMD between 1972 and 1998. The Greater Glasgow NHS Board had the highest average prevalence of IMD with 2.8 cases per year per 100,000 population, followed by Tayside with 2.2 cases per year per 100,000 population (Table 3.7, Figure 3.22).

Figure 3.21 Map showing the 15 different health boards within Scotland. Key contains the name of the health board and its corresponding colour.



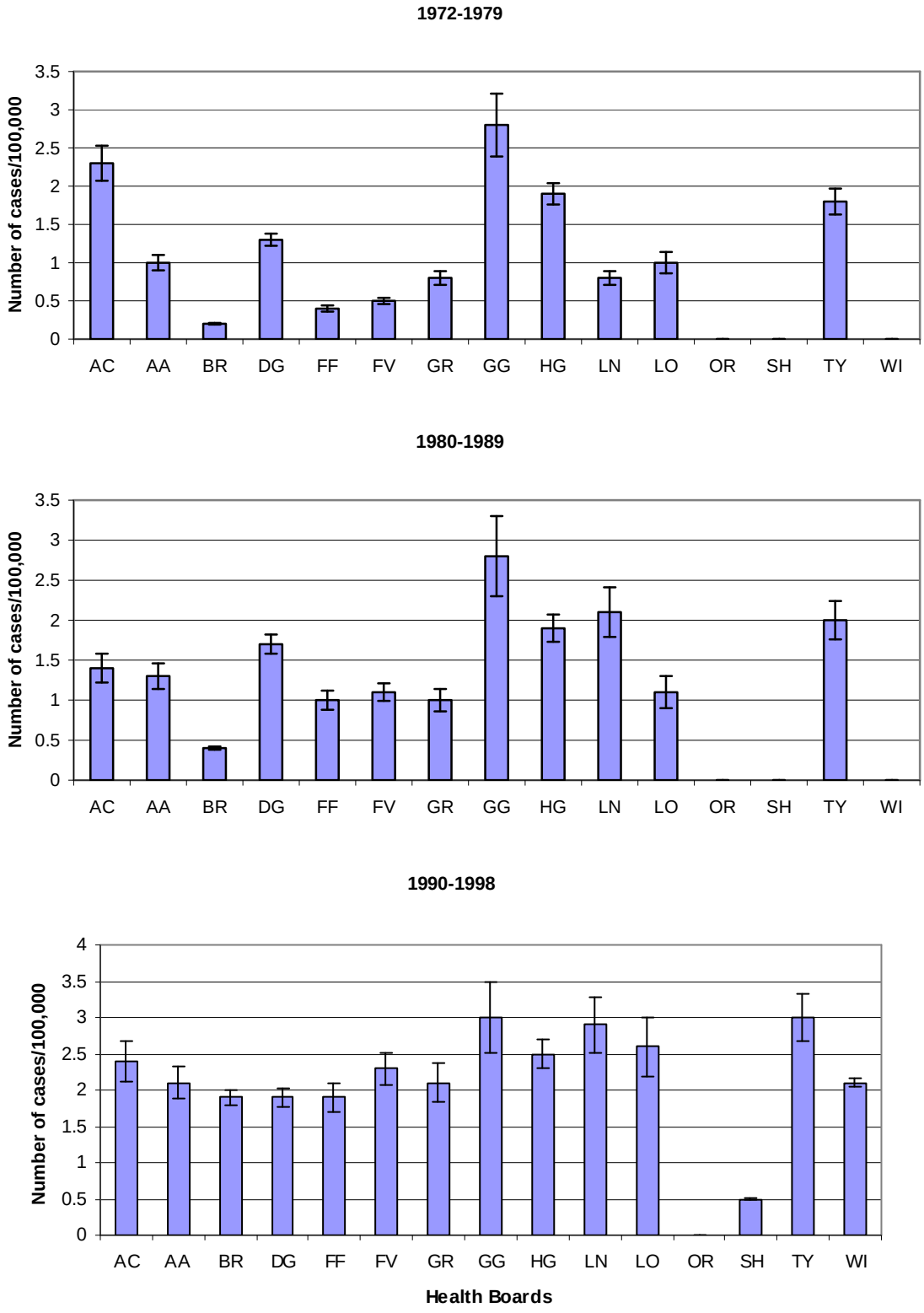
Key:

- Argyll and Clyde
- Ayrshire and Arran
- Borders
- Dumfries and Galloway
- Fife
- Forth Valley
- Grampian
- Greater Glasgow
- Highland
- Lanarkshire
- Lothian
- Orkney
- Shetlands
- Tayside
- Western Isles

Table 3.7 Number of cases of IMD and the number of cases per year per 100,000 population in NHS Health Boards in Scotland between 1972-1998.

Health Boards	**Population Size**	**Number of cases**	**Number of cases/100,000 1972 to 1979**	**Number of cases/100,000 1980 to 1989**	**Number of cases/100,000 1990 to 1998**	**Number of cases/100,000 1972 to 1998**
Argyll & Clyde (AC)	415,658	146	2.3	1.4	2.4	2.0
Ayrshire & Arran (AA)	367,590	226	1.0	1.3	2.1	1.5
Borders (BR)	109,270	25	0.2	0.4	1.9	0.8
Dumfries & Galloway (DG)	147,930	64	1.3	1.7	1.9	1.6
Fife (FF)	354,519	109	0.4	1.0	1.9	1.1
Forth Valley (FV)	281,764	101	0.5	1.1	2.3	1.3
Grampian (GR)	524,020	185	0.8	1	2.1	1.3
Greater Glasgow (GG)	867,083	665	2.8	2.8	3.0	2.8
Highland (HG)	211,340	121	1.9	1.9	2.5	2.1
Lanarkshire (LN)	556,114	297	0.8	2.1	2.9	2.0
Lothian (LO)	787,504	337	1	1.1	2.6	1.6
Orkney (OR)	19,500	0	0	0	0	0
Shetland (SH)	21,940	1	0	0	0.5	0.2
Tayside (TY)	387,908	235	1.8	2	3.0	2.2
Western Isles (WI)	26,260	5	0	0	2.1	0.7
	Total population Size	**Total number of cases**	**Average number of cases/100,000 1972 to 1979**	**Average number of cases/100,000 1980 to 1989**	**Average number of cases/100,000 1990 to 1998**	**Average number of cases/100,000 1972 to 1980**
	5,078,400	2517	1.0	1.2	2.1	1.4

Figure 3.22 Number of cases per year per 100,000 population in NHS Health Boards in Scotland between 1972-1998 (95% confidence intervals included in graphs).



The number of cases per year per 100,000 population could also fluctuate from decade to decade as well as from year on year within the same health board. An example of this is Lanarkshire, which had an average of 0.8 cases per 100,000 population from 1972 to 1979 which increased to 2.9 cases per 100,000 population in the 1990 to 1998 period. However, the incidence in Greater Glasgow remained fairly constant during the three decades at 2.8 to 3.0 cases per 100,000 population. All NHS boards showed their highest average prevalence during the 1990 to 1998 period.

The highest prevalence per 100,000 population recorded in a single year was within the Highlands in 1974 with 6.6 cases per 100,000 population. This was a huge increase on the previous year within the Highlands where, in 1973, there was only 1.0 case per 100,000 population. In 1975 the prevalence dropped to 2.8 cases per 100,000 population. The average prevalence within the Highlands during the 1970s was 1.9 cases per 100,000 population and between 1972 and 1998 was 2.1 cases per 100,000 population. The next highest prevalence per 100,000 population recorded in a single year was within Forth Valley in 1992 with 6.1 cases per 100,000 population. This was an increase on the previous year within Forth Valley where, in 1991, there were only 1.1 cases per 100,000 population. In 1993 the prevalence dropped to 2.1 cases per 100,000 population. The average prevalence within Forth Valley during the 1990s was 2.3 cases per 100,000 population and between 1972 and 1998 was 1.3 cases per 100,000 population. The next highest prevalence per 100,000 population recorded in a single year was within Greater Glasgow in 1990 with 5.9 cases per 100,000 population. This was an increase on the previous year within Greater Glasgow where, in 1989, there were 4.5 cases per 100,000 population. In 1991 the prevalence dropped to 3.8 cases per 100,000 population. After 1991 for the remaining years up to 1998,

except for 1993 (3.2 cases per 100,000 population), the prevalence was below 2.8 cases per 100,000 population. The average prevalence for Greater Glasgow remained constant between the different decades with 2.8 cases per 100,000 population during 1972 to 1980 and 1980 to 1990 with just a small increase to 3.0 cases per 100,000 population during 1990 to 1998 due to the aforementioned high prevalence in 1990.

3.5 Spatio-temporal analysis of meningococcal isolates within Scotland 1972-1998

This study retrospectively calculated the number of IMD cases that occurred in clusters in Scotland from 1972-1988. Analysis was performed using SaTScan (version 5.1.1 software), which is available at http://www.satscan.org. SaTScan was selected, in the present study, as it is the most comprehensively evaluated software for detecting spatio-temporal clusters of infectious diseases. SaTScan has been used in spatio-temporal analyses of methicillin-resistant *Staphylococcus aureus* infection (Tirabassi *et al.*, 2005), listeriosis (Sauders *et al.*, 2003), gonorrhoea (Jennings *et al.*, 2005) and pediatric pneumonia (Andrade *et al.*, 2004). It has also been used for national bioterrorism syndromic surveillance (Yih *et al.*, 2004). The date of specimen sampling was defined as the time of illness and the county of residence, derived from the postcode, was used as the place of origin. Spatio-temporal scanning was initiated within the 15 health boards. These vary in size and population (19,500 to 867,083 inhabitants/region). The temporal settings were defined based on work carried out by earlier retrospective cluster studies. A study in Germany calculated a maximal temporal window of 30 days, which was able to detect most existing clusters (Elias *et al.*, 2006b). This was based on a number of different previous studies, including one

from England and Wales, which estimated the median intervals between the index case and the second case to be 1.5, 5, or 23 days, depending on the setting of the cluster (Hastings *et al.*, 1997), a French study which found 72% of secondary cases occurred in the first week after the first case (Olivares & Hubert, 1992), and an American study which found that 73% of secondary cases appeared ≤14 days after the index case (Zangwill *et al.*, 1997). Therefore, a maximal temporal window of 30 days was used, as this should detect most of the existing clusters, although the time between the first and the second case may infrequently exceed this temporal limit. Each strain type present more than once within the dataset was individually entered into the SaTScan program to identify clustering of IMD. Strain types were defined by serogroup, ST and *porA*. Clusters were considered significant for *P*-values ≤0.05. Due to constant interaction with host immunity, there are temporal and spatial changes to the different *porA* types of meningococci (Harrison *et al.*, 2006). *PorA* types are expected to fluctuate to a greater degree over time, compared with STs. The application of *porA* for cluster analysis is justified because of its stability within clusters that appear for days or weeks (Elias *et al.*, 2006b).

3.5.1 Spatio-temporal analysis

Twenty-nine clusters were found to have occurred within Scotland from 1972-1998 and there included 63 cases (2.5% of all cases) (Table 4.2, Figure 3.23). The maximum number of patients per cluster was four. The median duration of the clusters was 8 days with the duration ranging from 1 to 27 days. In 86.2% of the clusters there were only two cases assigned to each cluster. The health board that had the highest number of clusters was Greater Glasgow with five different clusters. This is not surprising as this health board has the greatest population of all health boards.

Not all health boards were represented, with Borders, Highlands, Orkney, Shetland and the Western Isles not having any clusters associated with them, which is not surprising, as these areas are not densely populated with a low ratio of person to square mile. Spatio-temporal proximity could be shown for up to four patients (cluster 29, Table 3.8). However, similar to findings of other studies (Hastings *et al.*, 1997; Elias *et al.*, 2006b) most clusters had only two patients.

There were only two serogroups associated with the clusters identified within Scotland, namely serogroups B and C. Serogroup B was associated with 21 clusters (containing 44 cases) and serogroup C with 8 clusters (containing 19 cases). A serogroup C ST-11 strain was responsible for 5 different clusters within the 1990s. The age breakdown for these clusters showed that 11 of the 13 cases were from patients under the age of fifteen. Four of these strain types had the *porA* variable types 5, 2, 36-2 and these accounted for 11 cases and one had the *porA* variable types 5, 2-1, 36-2 and this accounted for two cases. These clusters occurred in different regions. A serogroup B strain type (ST-153, 5-2, 10, 37-1) was also associated with five different clusters that contained 11 cases from 1987 to 1996. In all but one case the patients were under the age of four years old. These clusters also occurred in different regions. The strain type B ST-41, 7-2, 4, 37 was responsible for four clusters that accounted for 8 cases. The strain type B, ST-457, 19, 15, 36 was responsible for two clusters that accounted for two cases each. The clusters occurred in separate regions.

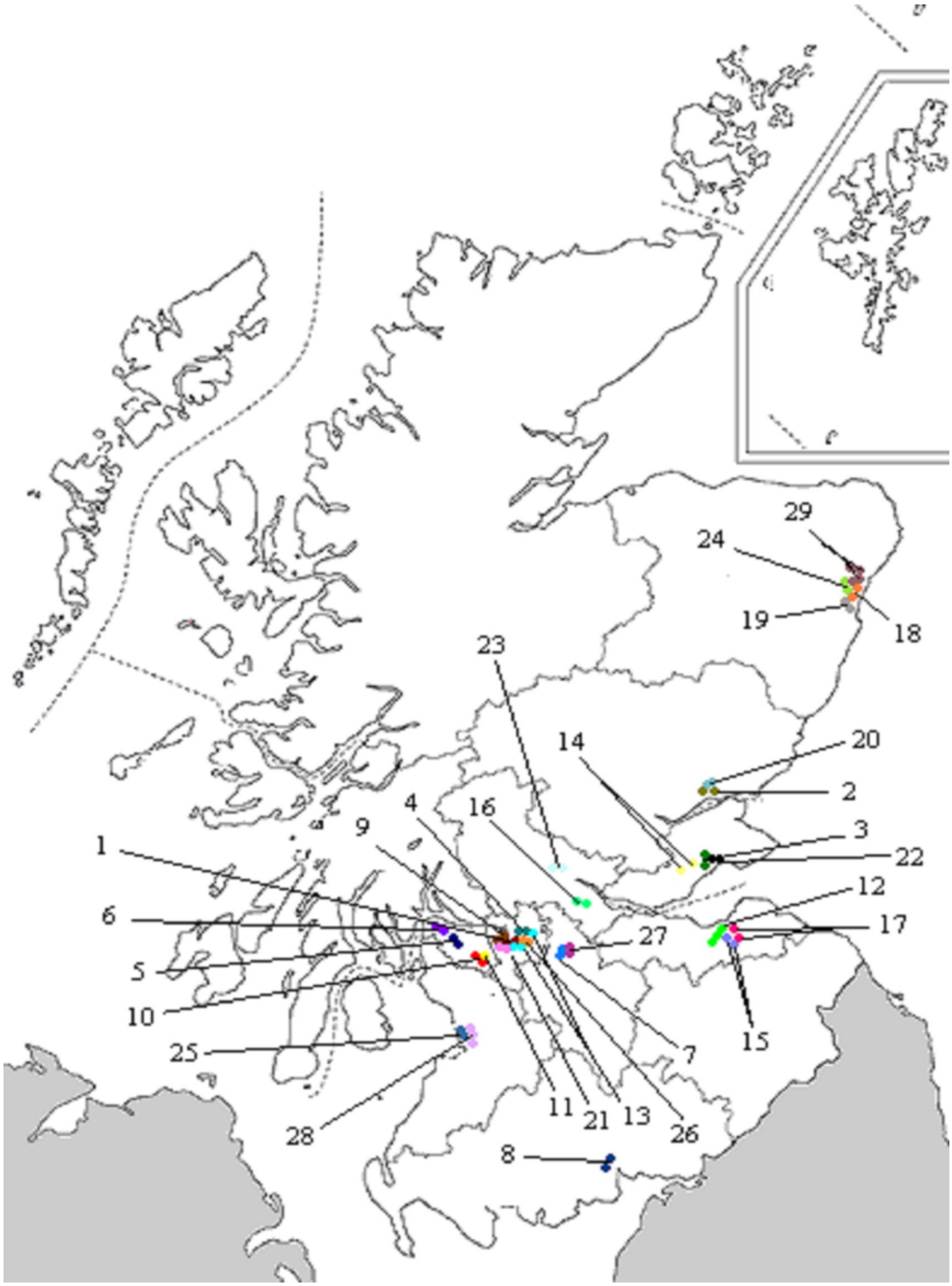
Of the clusters that were identified in this study, some had been identified previously when the samples first had been characterised. However when the SaTScan analysis was performed, these previously identified clusters were still included in the scan to

act as a control. The clusters that had not been identified before included the clusters from the 1970s and early eighties, which could not have been assessed as a cluster at the time with any certainty due to the fact that only serogrouping was performed on the sample. Some of the clusters, however, might have been suspected due to proximity or case association, although from the data available this was not the case. Clusters were also found amongst family members as well as amongst neighbours.

Table 3.8 Clusters of IMD detected by SaTScan analysis in Scotland 1972-1998

Cluster	Strain types	Cases	Health boards	Population	Year	Duration	*p* value
1	B, ST-415, 7, 1, 35-1	2	Greater Glasgow	867,083	1974	1 day	0.008
2	B, ST-60, 5, 2, 36-2	2	Tayside	387,908	1974	16 days	0.027
3	B, ST-1162, 5-1, 2-2, 36-2	2	Greater Glasgow	867,083	1976	1 day	0.008
4	B, ST-1162, 5-1, 2-2, 36-2	2	Fife	354,519	1976	19 days	0.007
5	B, ST-41, 7-2, 4, 37	2	Argyll & Clyde	415,658	1976-1977	7 days	0.009
6	B, ST-457, 19, 15, 36	2	Argyll & Clyde	415,658	1977	27 days	0.041
7	B, ST-3010, 5-1, 2-2, 36-2	2	Lanarkshire	556,114	1981	1 day	0.017
8	B, ST-343, 7, 16-2, 35	2	Dumfries & Galloway	147,930	1985	4 days	0.010
9	B, ST-457, 19, 15, 36	2	Greater Glasgow	867,083	1986	1 day	0.009
10	C, ST-206, 18-3, 1, 35-1	2	Argyll & Clyde	415,658	1986	6 days	0.031
11	C, ST-8, 5, 2, 36-2	2	Argyll & Clyde	415,658	1986	6 days	0.034
12	B, ST-153, 5-2, 10, 37-1	3	Lothian	787,504	1987	12 days	0.006
13	B, ST-32, 7-1, 16, 35-1	3	Greater Glasgow	867,083	1987	19 days	0.007
14	B, ST-153, 5-2, 10, 37-1	2	Fife	354,519	1989	13 days	0.034
15	C, ST-11, 5, 2-1, 36-2	2	Lothian	787,504	1991	7 days	0.045
16	B, ST-41, 7-2, 4, 37	2	Ayrshire & Arran	367,590	1992	1 day	0.009
17	C, ST-11, 5, 2, 36-2	2	Forth Valley	281,764	1992	5 days	0.008
18	C, ST-66, 5, 2, 36-2	2	Lothian	787,504	1992	1 day	0.023
19	B, ST-275, 22, 9, 35-1	2	Grampian	524,020	1993	10 days	0.005
20	B, ST-153, 5-2, 10, 37-1	2	Grampian	524,020	1993	1 day	0.012
21	B, ST-153, 5-2, 10, 37-1	2	Tayside	387,908	1993	1 day	0.006
22	B, ST-33, 19, 15, 36	2	Greater Glasgow	867,083	1993	1 day	0.005
23	C, ST-11, 5, 2, 36-2	2	Fife	354,519	1993	22 days	0.007
24	B, ST-41, 7-2, 4, 37	2	Forth Valley	281,764	1994	7 days	0.009
25	B, ST-41, 7-2, 4, 37	2	Grampian	524,020	1994	1 day	0.009
26	B, ST-269, 19-1, 15-11, 36	2	Greater Glasgow	867,083	1994	22 days	0.028
27	B, ST-153, 5-2, 10, 37-1	2	Lanarkshire	556,114	1996	1 day	0.006
28	C, ST-11, 5, 2, 36-2	3	Ayrshire & Arran	367,590	1997	2 days	0.009
29	C, ST-11, 5, 2, 36-2	4	Grampian	524,020	1998	7 days	0.017

Figure 3.23 Map of clusters of IMD detected by SaTScan analysis in Scotland 1972-1998



Key:

1 2 3 4 5 6 7 8 9 10

11 12 13 14 15 16 17 18 19 20

21 22 23 24 25 26 27 28 29

Tables 3.9 and 3.10 shows information on the age groups and sex of the patients involved in the clusters, details on the number of cases relating to the strain type that caused the cluster and whether it was responsible for any other cluster.

There is also some additional information relating to certain clusters of interest. The first cluster involved the strain type B, ST-415, 7, 1, 35-1. This strain type was responsible only for this cluster with only ten other cases of this strain type present within the dataset. These cases all occurred between 1972-1974 with 70% of cases occurring in the Greater Glasgow area and all of these cases occurring in patients ages 0-4 years old. This is an example of a strain type that has seemed to disappear in Scotland with no cases since 1974. The second cluster involved the strain type B, ST-60, 5, 2, 36-2. This strain type was not responsible for any other clusters and in addition there were only two other cases of this strain type within the dataset. These cases occurred in 1994 and 1995 in the Ayrshire and Arran and Greater Glasgow areas, respectively, with both patients under the age of one year old. Although there were only two other cases of this particular strain type there were 32 cases of a strain type that only differed in its PorA, B, ST-60, 21, 16, 37-1. This strain type occurred from 1974-1997.

The fifth cluster involved the strain B, ST-41, 7-2, 4, 37. This strain type was responsible for three other clusters, cluster 16, cluster 24, and cluster 25. In total there were 202 cases of this strain type and these were spread throughout the 26-year period and the geographical areas.

Table 3.9 Information relating to clusters 1-15 of IMD detected by SaTScan analysis in Scotland 1972-1998

Clusters 1-15	Strain types	Age groups of cases	Sex of patients	Number of other cases involving strain type	Other clusters involving strain type
1	B, ST-415, 7, 1, 35-1	>1 year , >1 year	Male, Female	10	No other cluster
2	B, ST-60, 5, 2, 36-2	1-4 years, 1-4 years	Male, Male	2	No other cluster
3	B, ST-1162, 5-1, 2-2, 36-2	1-4 years, Unknown	Female, Male	18	Cluster 4
4	B, ST-1162, 5-1, 2-2, 36-2	1-4 years, 1-4 years	Male, Female	18	Cluster 3
5	B, ST-41, 7-2, 4, 37	10-14 years, 5-9 years	Male, Male	202	Clusters 16, 24, 25
6	B, ST-457, 19, 15, 36	1-4 years, 5-9 years	Male, Male	23	Cluster 9
7	B, ST-3010, 5-1, 2-2, 36-2	Unknown, Unknown	Male, Female	1	No other cluster
8	B, ST-343, 7, 16-2, 35	Unknown, Unknown	Unknown, Unknown	13	No other cluster
9	B, ST-457, 19, 15, 36	>1 year , >1 year	Female, Female	23	Cluster 6
10	C, ST-206, 18-3, 1, 35-1	>1 year, 5-9 years	Female, Male	12	No other cluster
11	C, ST-8, 5, 2, 36-2	5-9 years ,1-4 years	Male, Female	146	No other cluster
12	B, ST-153, 5-2, 10, 37-1	1-4 years , 1-4 years, 75-84 years	Male, Male, Female	125	Clusters 14, 20, 21, 27
13	B, ST-32, 7-1, 16, 35-1	1-4 years , 1-4 years , 1-4 years	Male, Male, Male	46	No other cluster
14	B, ST-153, 5-2, 10, 37-1	1-4 years, Unknown	Male, Female	125	Clusters 12, 20, 21, 27
15	C, ST-11, 5, 2-1, 36-2	1-4 years, >1 year	Female, Male	66	No other cluster

The sixth cluster involved the strain type B, ST-457, 19, 15, 36. This strain type was responsible for one other cluster, cluster 9, and in total was responsible for 23 cases spanning 1975-1994 and covering ten of the different health board regions. ST-457 is part of the ST-35 complex. The seventh cluster involved the strain type B, ST-3010, 5-1, 2-2, 36-2. There was only one more case of this strain type within the dataset in 1993 in the Forth Valley area and there was only one other case on the MLST website again in Scotland in 2003. Therefore, this strain type appears to be unique to Scotland and this ST is not associated with any clonal complex.

The twelfth cluster involved the strain type B, ST-153, 5-2, 10, 37-1. This cluster had been identified previously as there had been known contact between the patients. The strain type involved was also the cause of four other clusters, cluster 14, cluster 20, cluster 21 and cluster 27. In total it was responsible for 125 cases from 1986-1997 within 12 different regions. The fifteenth cluster involved the strain type C, ST-11, 5, 2-1, 36-2, which was the first time ST-11 was involved in a cluster. This was the only cluster that involved this particular strain type, although ST-11 was involved in other clusters, the PorA for this strain type differed to the other clusters. However, the strain type did cause a total of 66 cases from 1985-1998 within 11 different regions.

Table 3.10 Information relating to clusters 16-29 of IMD detected by SaTScan analysis in Scotland 1972-1998

Clusters 16-29	Strain types	Age groups of cases	Sex of patients	Number of other cases involving strain type	Other clusters involving strain type
16	B, ST-41, 7-2, 4, 37	>1 year , >1 year	Male, Male	202	Clusters 5, 24, 25
17	C, ST-11, 5, 2, 36-2	1-4 years , 1-4 years	Female Female	185	Clusters 23, 28, 29
18	C, ST-66, 5, 2, 36-2	1-4 years , 1-4 years	Male, Male	12	No other cluster
19	B, ST-275, 22, 9, 35-1	1-4 years , 1-4 years	Female, Female	28	No other cluster
20	B, ST-153, 5-2, 10, 37-1	1-4 years , 1-4 years	Male, Female	125	Clusters 12, 14, 21, 27
21	B, ST-153, 5-2, 10, 37-1	>1 year , >1 year	Male, Male	125	Clusters 12, 14, 20, 27
22	B, ST-33, 19, 15, 36	>1 year , 1-4 years	Male, Female	67	No other cluster
23	C, ST-11, 5, 2, 36-2	15-19 years, 15-19 years	Male, Male	185	Clusters 17, 28, 29
24	B, ST-41, 7-2, 4, 37	>1 year, 75-84 years	Male, Male	202	Clusters 5, 16, 25
25	B, ST-41, 7-2, 4, 37	1-4 years , 1-4 years	Male, Female	202	Cluster 5, 16, 24
26	B, ST-269, 19-1, 15-11, 36	>1 year , >1 year	Male, Female	45	No other cluster
27	B, ST-153, 5-2, 10, 37-1	1-4 years, >1 year	Male, Female	125	Clusters 12, 14, 20, 21
28	C, ST-11, 5, 2, 36-2	35-44 years, 20-24 years, 20-24 years	Female, Female, Male	185	Clusters 17, 23, 29
29	C, ST-11, 5, 2, 36-2	5-9 years, 35-44 years, 5-9 years, 1-4 years	Female, Female, Male, Male	185	Clusters 17, 23, 28

The seventeenth cluster involved the strain type C, ST-11, 5, 2, 36-2. In total, this strain type was responsible for 185 cases between 1983-1998 and it was responsible for three more clusters, cluster 23, cluster 28 and cluster 29. The eighteenth cluster involved the strain type C, ST-66, 5, 2, 36-2. ST-66 is part of the ST-8 complex and only differs from the ST-8 by five nucleotides in the *fumC* housekeeping gene and this resulted in a synonymous change but no protein change. The twenty-seventh cluster involved the strain type B, ST-153, 5-2, 10, 37-1. There were two patients involved in this cluster, a sister and brother aged less than one year old and two years old, respectively. The sister was admitted to hospital one day before her brother and both had septicaemia.

3.5.2 Seasonal distribution

In terms of seasonal distribution of IMD in Scotland between 1972-1998, the patterns that were observed matched the trends normally associated with the disease. These are a higher number of cases in the winter months with a decrease in the number of cases during summer. From the data here, these trends were observed during the 1970s, 1980s and 1990s (Figure 3.24). For all three time periods the month that had the most number of cases was January. The least number of cases for the 1980s and 1990s occurred in September and for the 1970s in August. This pattern was repeated with the number of cases of serogroups B and C (Figure 3.25). Both serogroup B and C had the most number of cases in January and the least number of cases in September. When examining the average temperature for these months (Figure 3.24) January is the coldest month of the year in all three time periods and September is the month of the year where summer turns into autumn and the temperature is fairly mild. The three other main serogroups A, Y and W135 show different patterns to

serogroups B and C (Figure 3.25). Serogroup A had the maximum number of cases in March and the least in October. There was also an unexplained increase in the month of November, which was not linked to any cluster or outbreak of disease and was not due to a high prevalence in a particular year. Serogroup Y, like serogroup A had the maximum number of cases in March but the least number of cases occurred in July when there was not a single case. Serogroup W135 showed its highest number of cases in December and the least number of cases in April. This pattern of the major number of cases in winter was repeated with the three most common clonal complexes (Figure 3.26). For the three most common clonal complexes the maximum number of cases occurred in January and the least in July for the ST-11 and ST-44 complex and September for the ST-8 complex.

Figure 3.24 Average number of IMD cases per month and average temperatures in Scotland 1972-1998. The average number of cases and average temperatures for each month for 1972-1979, 1980-1989 and 1990-1998 were calculated.

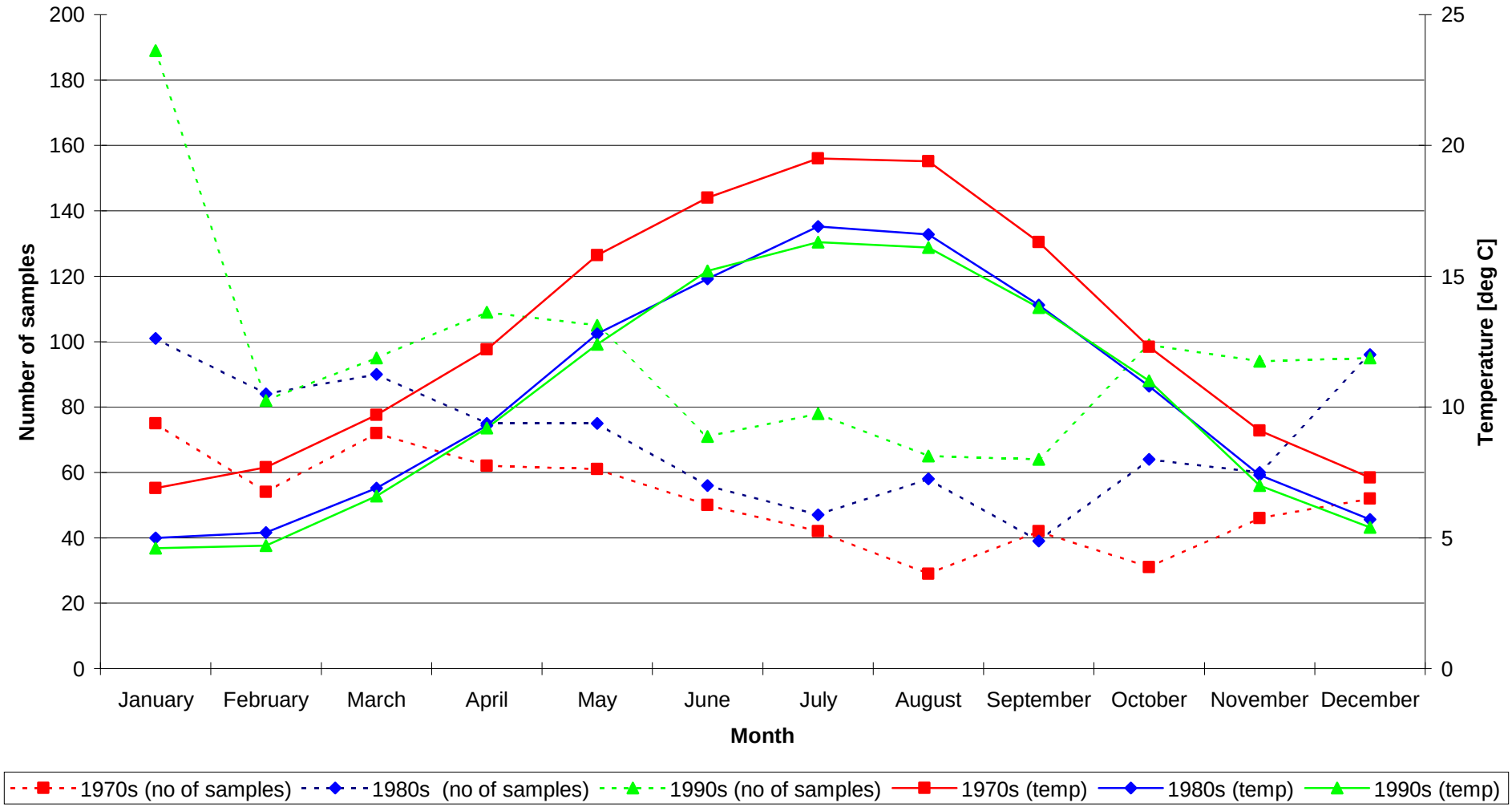
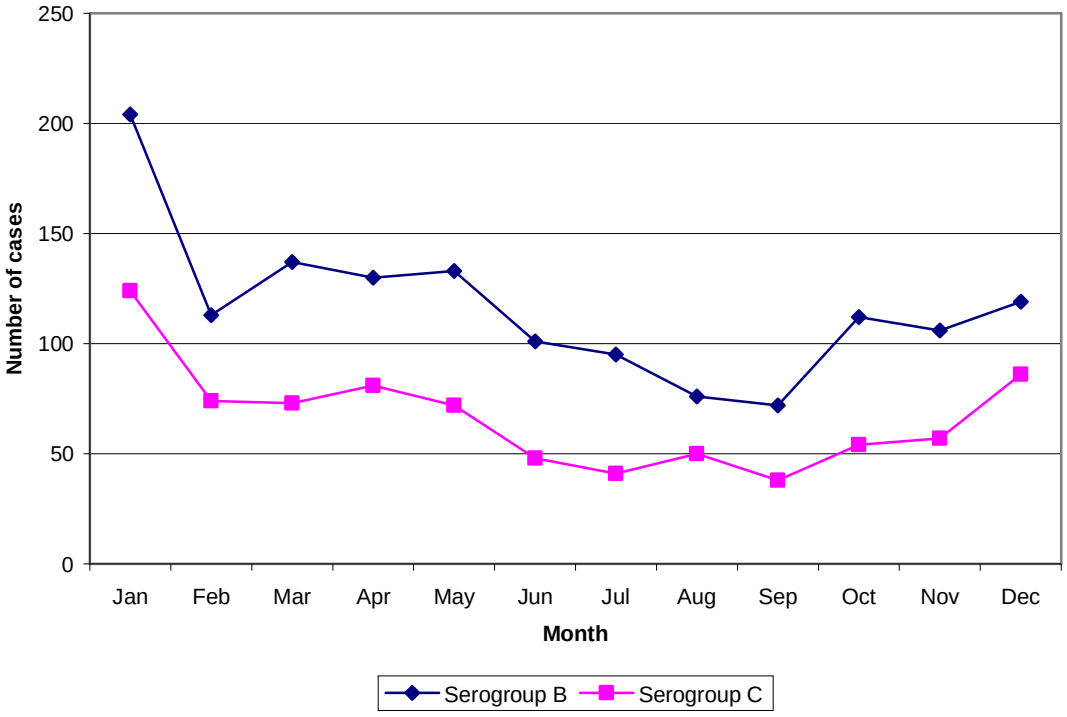


Figure 3.25 Average number of IMD cases per serogroup, per month, in Scotland 1972-1998. The average number of cases for each month for 1972-1998 were calculated.



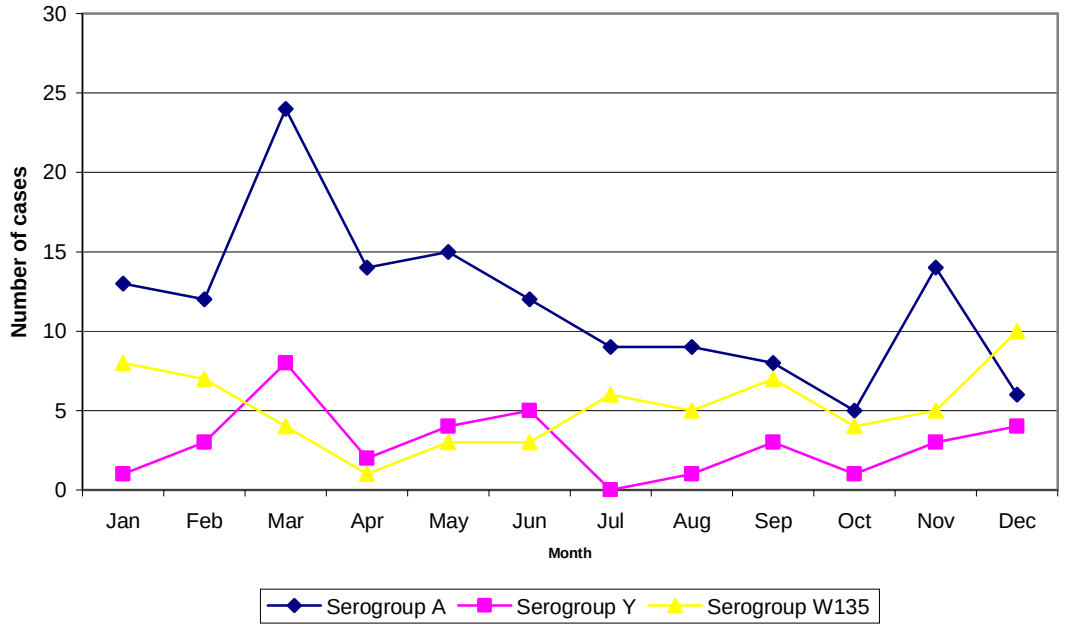
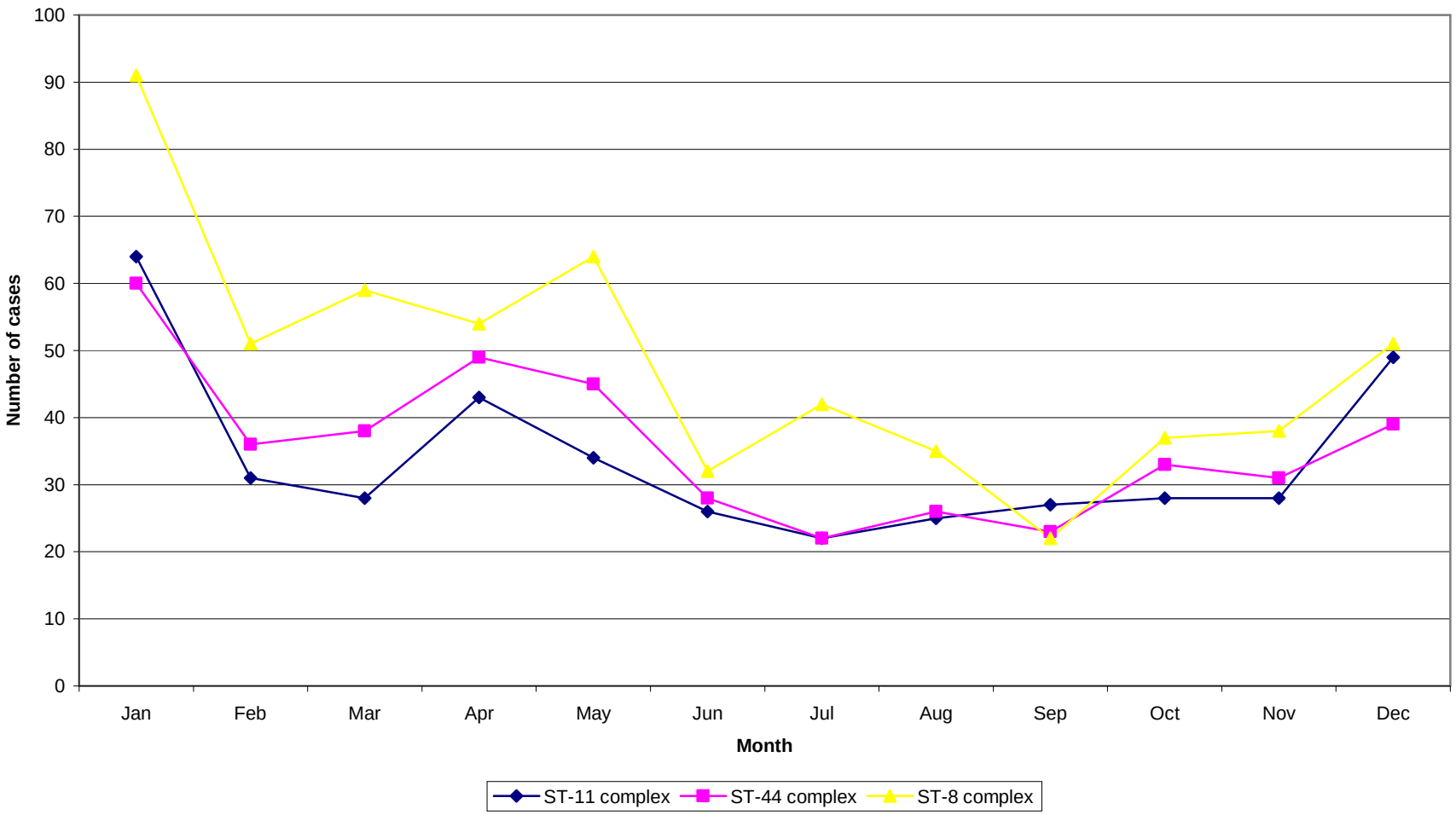


Figure 3.26 Average number of IMD cases for the 3 most common clonal complexes, per month, present in Scotland 1972-1998. The average number of cases for each month for 1972-1979, 1980-1989 and 1990-1998 were calculated.



3.6 Potential coverage of meningococcal vaccines

Using data generated by this study from the time period 1972-1998 the potential coverage has been estimated, within Scotland, of the major OMV vaccines described: Cuba (VA-MENGOC-BC®), Norway (MenBvac™), New Zealand (MeNZB™), hexavalent (HexaMen) and nonavalent (NonaMen) (Jodar *et al.*, 2002; Oster *et al.*, 2005; Borrow *et al.*, 2006). This will provide useful information to determine what affect these vaccines would have had on the meningococcal population in Scotland.

3.6.1 Potential vaccine coverage

Potential coverage was estimated for five vaccines based on OMVs developed to help protect against serogroup B. These were the Cuban vaccine (P1.15), Norwegian (P1.7, 16), New Zealand (P1.7, 4), Hexavalent (P1.7, 16; P1.5-1, 2-2; P1.19, 15-1; P1.5-2, 10; P1.12-1, 13; P1.7-2, 4) and Nonavalent (P1.7, 16; P1.5-1, 2-2; P1.19, 15-1; P1.5-2, 10; P1.12-1, 13; P1.7-2, 4; P1.22, 14; P1.7-1, 1; P1.18-1, 3, 6) (Borrow *et al.*, 2006). Using PorA information for all isolates from 1972-1998 the potential coverage was estimated (Figure 3.27). This indicates that the Cuban (P1.15) vaccine would have had a coverage of less than 10%. This was followed by the Norwegian (P1.7, 16) and New Zealand (P1.7, 4) vaccines with 9.7% and 15.1% coverage respectively. The vaccines with the most coverage were the vaccines produced by the Netherlands Vaccine institute (Hexavalent and Nonavalent vaccines). The Hexavalent vaccine would have had 60.8% coverage and the Nonavalent vaccine probably would have prevented 65% of meningococcal infection in Scotland.

The potential coverage, of all the vaccines, for the three decades 1970s, 1980s and 1990s were also estimated to see how the coverage would have changed over the years (Figures 3.28). The Cuban (P1.15) vaccine for two of the three time periods was below 10% coverage. It was only during the 1980s that the coverage rose to 14%. The Norwegian (P1.7, 16) vaccine's coverage remained constant during the 1970s, 1980s and 1990s. The New Zealand (P1.7, 4) vaccine showed a different pattern to the Cuban (P1.15) vaccine in that its coverage was lowest during the 1980s. At 8.4% it was half the value of 16.9% that it had during the 1970s.

The two vaccines with the best potential coverage were the Hexavalent and Nonavalent vaccines. However, in both cases coverage would have reduced through the decades, this was particularly the case for the Hexavalent vaccine. From coverage of 88.6% in the 1970s the Hexavalent vaccine coverage was reduced to 62.0% in the 1980s and then to 56.0% in the 1990s. So in a 26-year period the coverage of the Hexavalent vaccine reduced by 32%. The Nonavalent vaccine showed a similar pattern to the Hexavalent vaccine, with coverage of 95.2% in the 1970s reduced to 76.3% in the 1980s and then to 68.7% in the 1990s. The three additional PorA OMPs (P1.22, 14; P1.7-1, 1; P1.18-1, 3, 6) present in the Nonavalent vaccine occurred in different numbers over the three decades. In total, they occurred three times in the 1970s, 52 times in the 1980s and 51 times in the 1990s. This could account for the reduced coverage for the Hexavalent vaccine (which does not contain these three additional PorA OMPs) in the 1980s and 1990s.

Figure 3.27 Potential coverage of candidate outer membrane vesicle vaccines, for all isolates from Scotland 1972-1998.

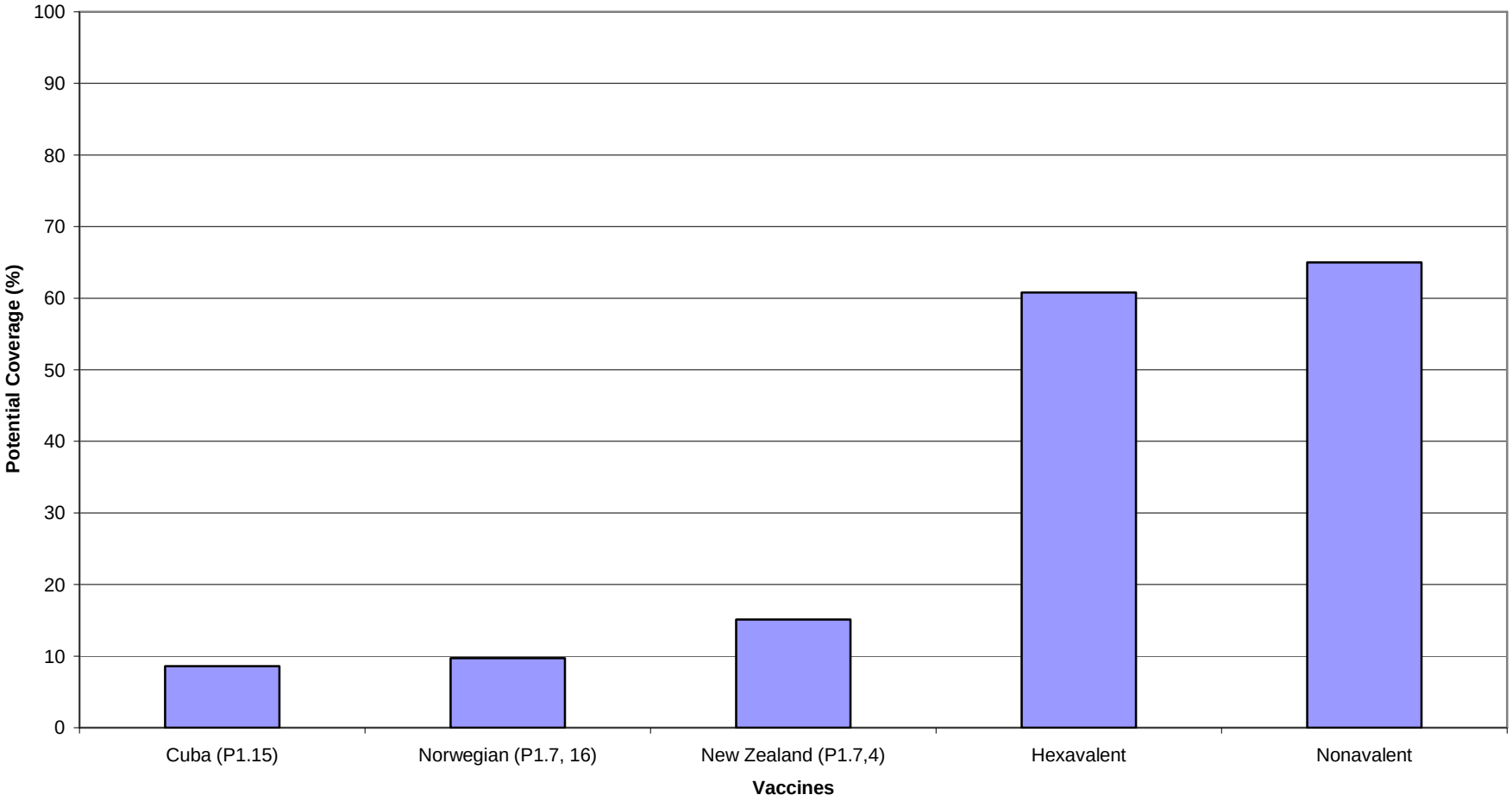
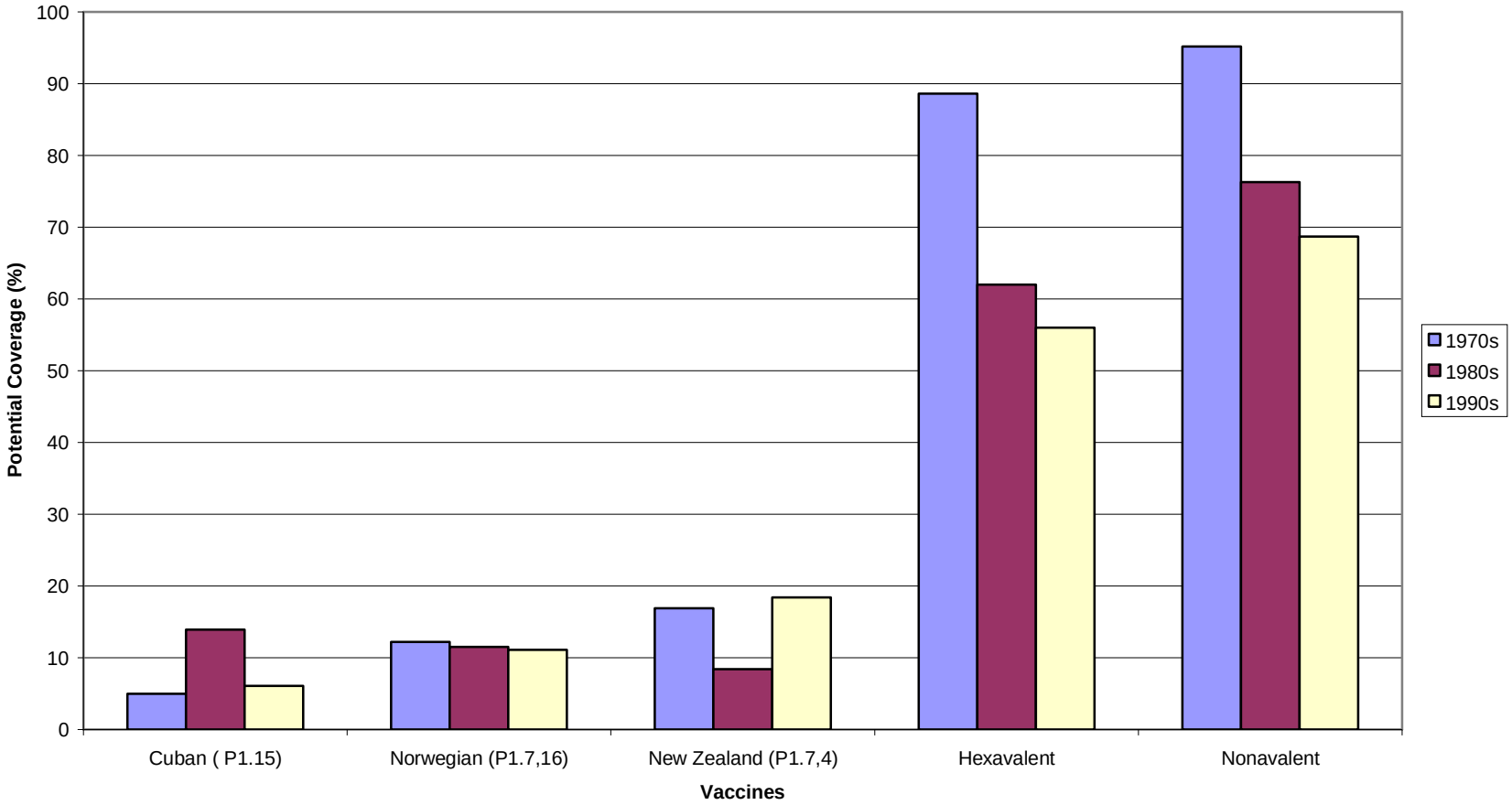


Figure 3.28 Potential coverage of candidate outer membrane vesicle vaccines, for all isolates from Scotland 1972-1979, 1980-1989 and 1990-1998.



However, this does not explain the reduction in coverage in the Nonavalent vaccine. These data would suggest that the potential coverage reduced over the decades due to the increase in different PorA types not covered in the vaccine and that this decrease could continue, further decreasing the usefulness of the vaccine. There were a number of different PorA types present in small numbers that were found in the 1980s and 1990s but that had not occurred in the 1970s, but one particular PorA type, 5, 2, increased from 245 isolates in the 1980s to 290 isolates in the 1970s and 341 isolates in the 1990s. This PorA type is associated with serogroup C, which also increased in number over the decades. Therefore, to answer the question 'Why was there a decrease in the coverage over the decades?', it is important to look at how potentially effective these vaccines would have been against the different serogroups.

As mentioned previously these vaccines have been designed specifically to protect against the serogroup B strain but they do also protect against other serogroups containing the same PorA types. Figure 3.29 shows that, for the five main serogroups, over the 26-year period, there were different levels of coverage. For the main purpose of the vaccines, to protect against serogroup B, the coverage was high for both the Hexavalent (80.2%) and Nonavalent (94.8%) vaccines. Figure 3.30 shows that between the three decades there was little fluctuation between the coverage for serogroup B for the Hexavalent and Nonavalent vaccines. For serogroup A the coverage was also high and showed little difference in values between Hexavalent (93.8%) and Nonavalent (94.6%) vaccines. For serogroup Y the coverage was also high for both Hexavalent (94.0%) and Nonavalent (97.0%) vaccines. However, with serogroup C and serogroup W135, the vaccine did not show the same high levels of coverage as with the other serogroups. For serogroup C the coverage was 39.8% for

the Hexavalent vaccine and 46.0% for the Nonavalent vaccine. For serogroup W135 the coverage was lower than serogroup C with 11.7% for the Hexavalent vaccine and 38.3% for the Nonavalent vaccine.

The three other vaccines did not offer coverage for all of the five main serogroups. The Cuban (P1.15) vaccine only showed coverage for serogroup B (11.6%) and serogroup C (5.1%) and showed no coverage for serogroups A, Y and W135. The Norwegian (P1.7, 16) vaccine did not show coverage for serogroup W135 but did show coverage for the other four serogroups. The New Zealand (P1.7, 4) vaccine, like the Cuban (P1.15) vaccine, only showed coverage for serogroup B (23.6%) and serogroup C (4.8%) and showed no coverage for serogroups A, Y and W135.

These results help to explain the reasons for the decrease in potential coverage mentioned earlier between the decades. As mentioned in previous chapters, serogroup C was not around in such high numbers during the 1970s as in the 1980s and particularly the 1990s. So, the increase in number of serogroups C isolates during this time period in combination with the fact the main PorA types associated with serogroup C were not included in the vaccines resulted in an overall decrease in the potential coverage by the vaccine.

Figure 3.29 Potential coverage of vaccines by serogroup, Scotland 1972-1998.

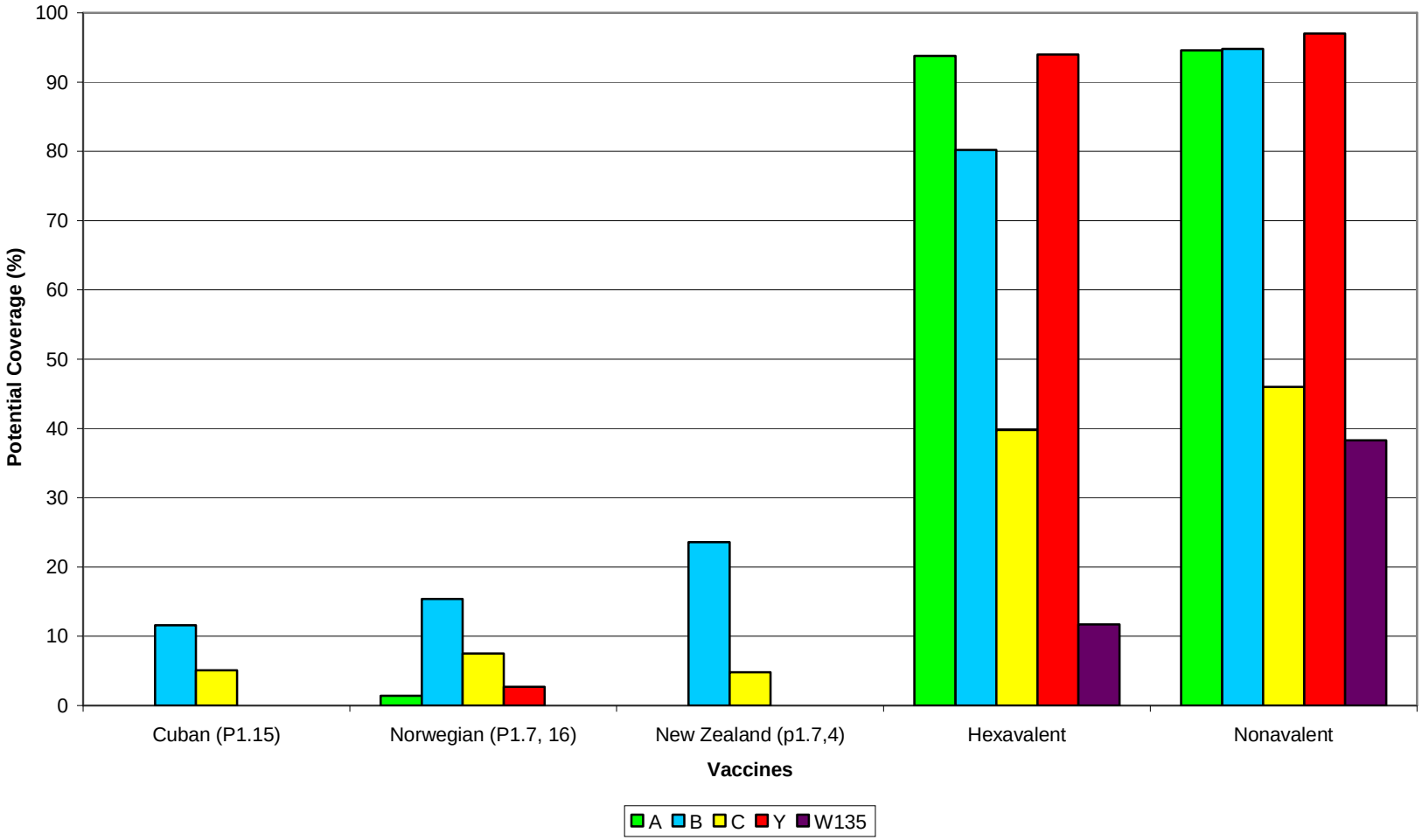
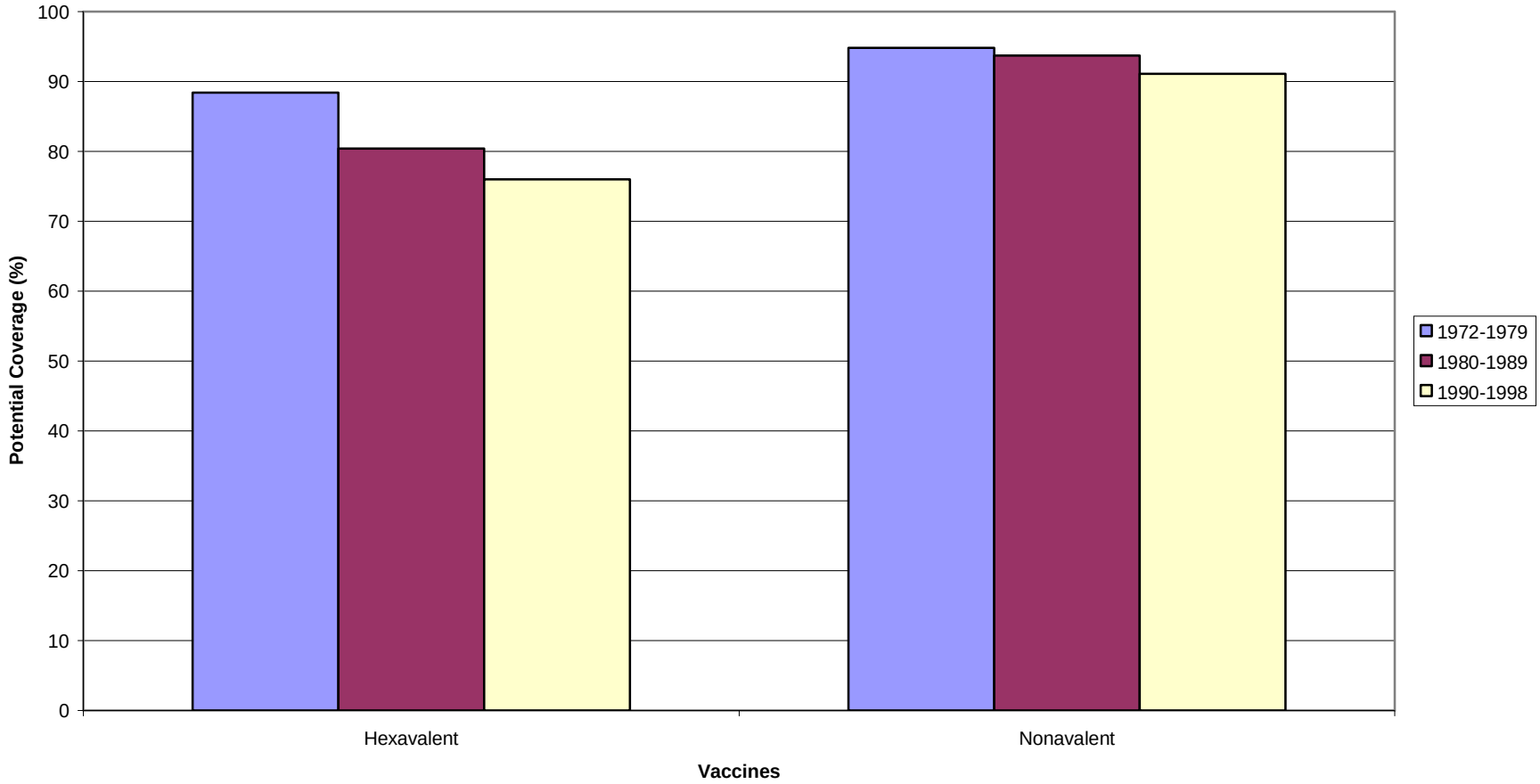


Figure 3.30 Potential coverage of candidate outer membrane vesicle vaccines, for all serogroup B isolates from Scotland 1972-1979, 1980-1989 and 1990-1998.



3.6.2 Potential vaccine coverage within age groups

It was also important to look at what the potential coverage of these vaccines would have been within different age groups, as age is a risk factor for meningococcal disease. If it was the case, for example, that < 1yrs showed low coverage by the vaccines then this would greatly affect the suitability of the vaccine as a viable protection option. From the data (Figure 3.31), there were fluctuations in the coverage between the age groups for all the vaccines. For the Cuban (P1.15) vaccine the largest coverage was observed within the 10-14 years age group (13.7%) and the smallest was within the 65-74 years age group (0%). For the age groups of <1 years and 1-4 years, an important age group because any vaccine against serogroup B would hopefully be included as part of the childhood vaccination programme, the coverage was 6.7% and 6.8% respectively. For the Norwegian (P1.7, 16) vaccine the largest coverage was observed within the 25-34 years age group (17.1%) and the smallest was within the >75 years age group (6.8%). Unlike the Cuban (P1.15) vaccine the Norwegian (P1.7, 16) vaccine showed coverage for all age groups. For the age groups of <1 years and 1-4 years the coverage was 13% and 9.6% respectively. For the New Zealand (P1.7, 4) vaccine the largest coverage was observed within the 55-64 years age group (19.1%) and the smallest within the 45-54 years age group (4.6%). For the age groups of <1 years and 1-4 years the coverage was 18.3% and 16.1%, respectively. For the Hexavalent vaccine the largest coverage was for the 20-24 years age group with 71.6% and the smallest was 53.1% for the 25-34 years age group. For the age groups of <1 years and 1-4 years the coverage was 67.4% and 66% respectively. For the Nonavalent vaccine the highest coverage was for the 35-44 years

with 90.2% and the lowest was the 45-54 years age groups with 66.2%. For the age groups of <1 years and 1-4 years the coverage was 81.8% and 76.8% respectively.

The above coverage values are for protection against all serogroups. It is important to see what the values are for serogroup B, as this is the serogroup that the vaccines have been developed to combat (Figure 3.32). For the Cuban (P1.15) vaccine the largest coverage was observed within the 10-14 years age group (28.6%) and the smallest was within the 65-74 years age group (0%). For the age groups of <1 years and 1-4 years the coverage was 7.4% and 8% respectively. For the Norwegian (P1.7, 16) vaccine the largest coverage was observed within the 25-34 years age group (26.1%) and the smallest was within the >75 years age group (0%). This value for the >75 years age group shows that the coverage estimated from Figure 3.31 was for serogroups other than serogroup B. For the age groups of <1 years and 1-4 years the coverage was 15% and 11.5% respectively. For the New Zealand (P1.7, 4) vaccine the largest coverage was observed within the 55-64 years age group (47.1%) and the smallest was within the 45-54 years age group (4.4%). For the age groups of <1 years and 1-4 years the coverage was 24.5% and 24.3% respectively. For the Hexavalent vaccine the largest coverage was for the >75 years with 88.9% and the smallest was 56.5% for the 45-54 years age group. For the age groups of <1 years and 1-4 years the coverage was 77% and 79.5% respectively. For the Nonavalent vaccine the highest coverage was for the 20-24 years with 97.6% and the lowest was the 65-74 years with 60%. For the age groups of <1 years and 1-4 years the coverage was 95.7% and 92.5% respectively. The coverage though remains high for the Nonavalent vaccine with 9 of the 12 age groups having a value greater the 88%.

Figure 3.31 Potential coverage of vaccines in terms of age groups, for all isolates from Scotland 1972-1998.

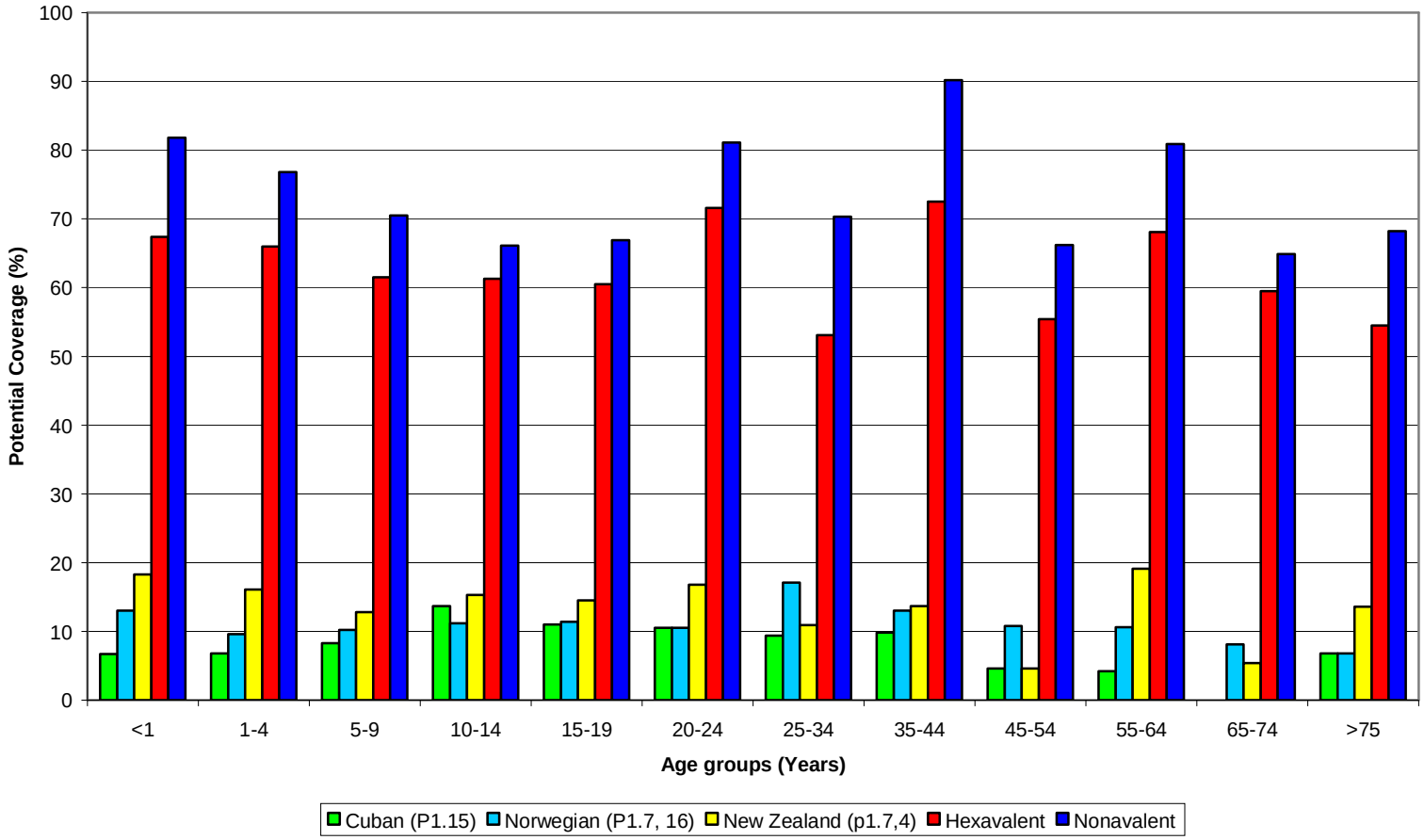
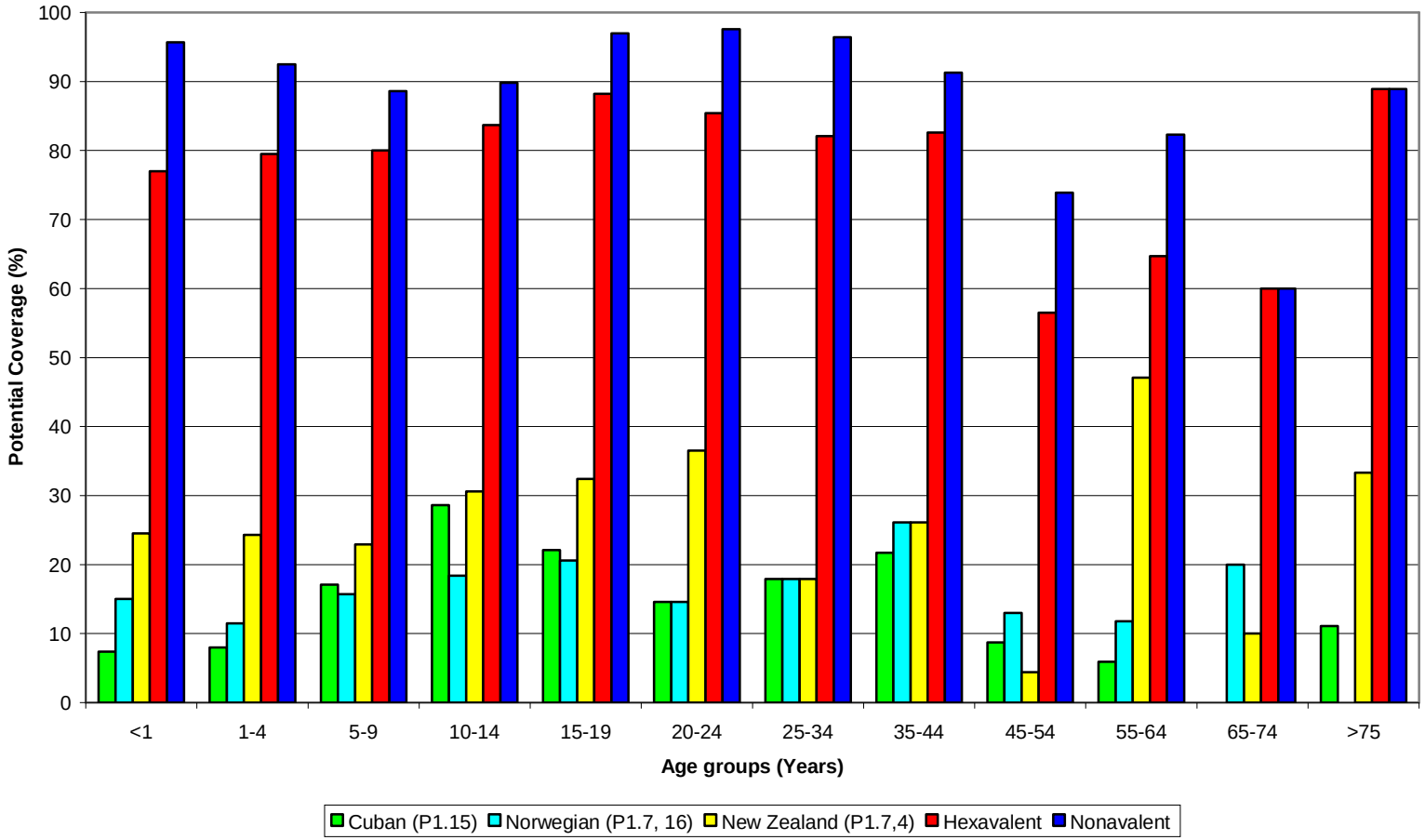


Figure 3.32 Potential coverage of vaccines amongst serogroup B and in terms of age groups, Scotland 1972-1998.



Chapter 4

Discussion

4.1 Introduction

For over 100 years, *N. meningitidis* has been acknowledged as a cause of meningitis and septicaemia and as a cause of disease of rapid onset with high mortality and morbidity if not treated. Even with all the developments in diagnosis, vaccination and treatment this statement is still true today. For understanding bacterial pathogens that colonise and infect only humans, *Neisseria meningitidis* has become a model organism (Stephens *et al.*, 2007). With advances in molecular techniques, analysis systems and decades of research, *N. meningitidis* has become better understood. Further investigations of the genetics and pathogenicity of this organism will help our understanding about how the meningococcus evolved, causes disease and spreads.

This study aimed to further our understanding of *N. meningitidis* by examining how it has evolved and changed in one country over a thirty-year period. This was only possible due to the development of a high-throughput automated nucleotide sequencing system that performed MLST and *porA* sequencing from extracted DNA (Sullivan *et al.*, 2006). This allowed the genotypic analysis of 2517 meningococcal isolates recovered in Scotland from 1972-1998. Detailed analyses were performed to characterise and compare a large number of nucleotide sequences from disease isolates. This would ultimately highlight a realistic picture of the dynamics of pathogenic meningococci within the human population during these years. This involved the development and optimisation of automated liquid handling robots to achieve high-throughput MLST and *porA* sequencing.

4.2 Semi-automation: a requirement for large-scale genotypic analysis

Over the last two decades there has been a change in emphasis in meningococcal typing, with phenotypic approaches being increasingly replaced by molecular techniques aimed at determining genotypes (Maiden & Frosch, 2001). These new methods, in particular MLST, provide much greater information for bacterial identification and discrimination. As time has progressed, MLST has become widely implemented globally and the cost of analysing bacteria to a DNA level has decreased. Therefore, high-throughput typing has become more standardised, reproducible, and available. The portability of the techniques and availability of information has been greatly enhanced by curated databases via the Internet.

Semi-automation of MLST has been highly successful and essential where high numbers of isolates are analysed. As a consequence, it has enabled reproducibility and reduced the costs. There are many advantages in using a sequence-based system for typing organisms such as *N. meningitidis* (Maiden *et al.*, 1998; Enright & Spratt, 1999; Maiden & Frosch, 2001). MLST has provided a very dependable means of identifying hyperinvasive meningococci (Brehony *et al.*, 2007; Buckee *et al.*, 2008), but does not necessarily provide sufficient discrimination for the unambiguous identification of disease clusters representing outbreaks (Bygraves *et al.*, 1999; Feavers *et al.*, 1999). The addition of PorA and FetA VRs enables further discrimination (Jolley *et al.*, 2007) and these have been employed in the identification of outbreak clusters in Germany (Elias *et al.*, 2006b). The use of MLST and antigen gene sequencing with a semi-automated procedure enables quick, reliable and reproducible production of results (Clarke, 2002; Sullivan *et al.*, 2006). The data

generated enable epidemiological analysis at local, national and global levels and academic and applied research into the biology and control of this globally important pathogen. The continuing use of these systems will enable the scientific community to monitor the detailed genetic profiles of the meningococcal population.

This project developed a procedure for MLST using a third generation liquid handling robot (THEONYX) (Sullivan *et al.*, 2006). The success of this procedure has been shown by the quality of the results analysed. As a result, the vast majority of samples were assigned MLST types. Manual set-up is considered quite time consuming, monotonous and laborious and must be performed during normal working hours. Automation of MLST provides an accurate, efficient, reproducible and faster method than its manual equivalent. Without this automated procedure it would have been impossible to perform MLST on such a large number of samples, in an efficient, effective manner. Consequently, this allowed additional time for detailed analysis of the vast amount of sequence data, time which would have normally been spent on the manual set-up. This automated process can be adapted for a number of different organisms providing an MLST procedure has been developed. Although DNA sequencing and therefore MLST is considered relatively expensive, the cost of equipment and materials has significantly reduced over the last few years. However, if an institution had only a few samples requiring characterisation then it would be more cost effective to give them to a laboratory with a sequencing service.

4.3 Genotypic characterisation

A number of studies have been performed with collections of meningococci (Maiden *et al.*, 1998; Feil *et al.*, 1999; Jolley *et al.*, 2000; Tzanakaki *et al.*, 2001; Diggle & Clarke, 2005; Ferreira *et al.*, 2006; Brehony *et al.*, 2007; Trotter *et al.*, 2007). The work carried out by these authors showed similar patterns to those seen in this study. In Scotland, 432 *N. meningitidis* isolates recovered from patients (between 1999 and 2002) with invasive meningococcal disease were analysed by MLST (Diggle & Clarke, 2005). It was found that the genetic diversity of disease-causing meningococci significantly increased after the introduction of the MenC vaccine and that this increase reflected a significant decrease in serogroup C ET-37/ST-11 meningococci. This has not been accompanied by an increase in serogroup B meningococci of the same clonal complex (Diggle & Clarke, 2005). The diversity of the current disease-causing meningococcal population is due, however, to the presence of new combinations of alleles arising from recombination, thus pertaining to new ST profiles. It has been reported that the increase in recombination between 1999 and 2002 may have been due to natural selection and the increased ability of non-ST-11 meningococci to fill the position left by ST-11 (Diggle & Clarke, 2005). There was no hyperendemic clone present in the United Kingdom that had directly replaced the ST-11 clone. However, some clones, such as the ST-269 clone, must be monitored closely, as they have increased year by year since 1999. Of the ST-269 isolates that occurred between 1972 and 1998, 74% of the isolates occurred after 1990.

A study on carriage of meningococci in the Czech Republic analysed 218 isolates from healthy young adults during 1993 (Jolley *et al.*, 2000) and showed that the bacterial population was highly diverse, comprising 71 different sequence types

(STs), which were assigned to 34 distinct complexes. Three previously identified hyperinvasive lineages were present, namely the ST-41, ST-11, and ST-32 complexes. The data were consistent with the view that most nucleotide sequence diversity resulted from the reassortment of alleles by horizontal genetic exchange (Jolley *et al.*, 2000). One of the most recent published studies was the EU-MenNet project (Brehony *et al.*, 2007; Trotter *et al.*, 2007; Trotter & Ramsay, 2007). Over 4000 European disease isolates were analysed from the 18 countries involved in the project for the 3 years 2000–2002. Its findings were similar to this study. There was much diversity in the STs (≈1000 types), although only ten accounted for half of the isolates. The STs resolved into 31 distinct clonal complexes, the most prevalent being the ST-41/44 complex (1014 isolates, 25%), ST-11 complex (901 isolates, 22%), ST-32 complex (706 isolates, 17%), ST-8 complex (273 isolates, 7%) and ST-269 complex (256 isolates, 6%). These major disease-associated complexes which were highlighted as the complexes associated with the majority of disease in Scotland 1972-1998, have also been found world-wide and have occurred over a number of years. The ST-11 complex was the predominant complex found in the EU-MenNet project and it accounted for most serogroup C disease. As previously stated it has also been associated with outbreaks of disease in Europe, Australia, the USA and Canada. The complex worldwide has more recently also been associated with serogroup W135 outbreaks in Africa and also with those returning from the Hajj pilgrimage.

Prior to the 1970s, the ST-32 complex (which is mainly linked with serogroup B) was rarely associated with epidemic disease. Since then, however, it has spread world-

wide to cause raised levels of disease in Europe, South Africa, South America and the USA. Apart from in European countries, the ST-41/44 complex has been found to cause disease in various countries such as the USA and New Zealand where it has been responsible for an epidemic since 1991 (Oster *et al.*, 2005). The ST-8 complex is associated with serogroups B and C and has been known to have caused cases of disease world-wide since the 1970s. Although there have been few cases of the ST-8 complex in Scotland and also the rest of the UK from the late 1990s onwards it still remains a problem in some countries in the world including Portugal, Spain and Germany. The range of prevalence of the ST-8 complex varied from 0% (Czech Republic, Iceland, Sweden, Finland) to 39% (Portugal) (Brehony *et al.*, 2007). The ST-269 complex associated with serogroup B disease has recently emerged in Quebec, Canada (Law *et al.*, 2006).

Overall the EU-MenNet project found there were no major changes in the distribution of types over the 3 year study period; however, there was a decrease in the ST-8 complex from 9% to 5% of isolates over this period. In Spain there was a sizeable decrease in ST-8 complex from 27% to 7% over the 3 years. At the individual country level, the ST-11 complex increased in some countries (Germany, Netherlands, France), while it declined in prevalence in others, particularly those that had implemented MCC vaccination programmes during this time (UK, Republic of Ireland, Belgium).

Other areas where the findings from the EU-MenNet study compare with this project include diversity within clonal complexes. In both this project and the EU-MenNet study some complexes were more diverse than others in terms of the number of

different STs observed. In the EU-MenNet study, the ST-11 complex was the least diverse with the central genotype accounting for 90% of isolates. The other major hyperinvasive complexes, i.e., ST-41/44, ST-32, ST-8 and ST-269, were much more diverse in comparison. Both this project and the EU-MenNet study also show a link between clonal complex and serogroup. For example in the EU-MenNet study, the ST-32, ST-41/44 and ST-269 complexes were associated with serogroup B and this was mostly true for Scottish isolates. In the EU-MenNet study the ST-11 and ST-8 complexes were mainly associated with serogroup C. ST-11 showed similar results in Scotland but ST-8 in Scotland did have a large number of serogroup B isolates.

In both this project and the EU-MenNet study there was also some association between genotype and the antigenic gene *porA* variable region (VR) types. In the EU-MenNet study there were strong relationships between the ST-11 complex with 5,2 and ST-8 complex with 5,2 (80%) and this was mirrorred with Scottish isolates. The EU-MenNet project showed the emergence of the ST-213 clonal complex, which was localized mainly in the UK, especially in Scotland, in 2000-2002. In Scotland the ST-213 complex was present at a higher prevalence (10%) than in most other countries. This typically has a phenotype of B: P1.22, 14. This clonal complex was also found during the present study and occurred eight times, all during the 1990s. The first case was in 1992, followed by a case in 1994, one case in 1996, two cases in 1997 and three cases in 1998. All cases were strain B: ST-213: 22,14,36, except the last strain in 1998 which was B: ST-213: 22-3,14,36-2. ST213 was the only ST belonging to the ST-213 clonal complex that was present within this study. The present study has examined 2517 invasive isolates over a 26-year period within a single country and the

data were consistent with previous studies that have indicated dynamic behaviour in meningococcal populations (Yazdankhah *et al.*, 2004; Harrison *et al.*, 2006).

Serogroup distribution changed from year to year in Scotland during the time period 1972-1998, but serogroups B and C were the dominant serogroups over this period. Serogroup B was the dominant serogroup throughout the seventies and early eighties until serogroup C became dominant during the mid 1980s. This increase in dominance of serogroup C was not found to be associated with one particular ST. Thus, serogroup C was associated with ST-8, ST-11, ST-206 and ST-334. This is in contrast to the increase in serogroup C disease in the 1990s that was due to a single complex, the ST-11 genetic complex. Serogroup C ST-11 meningococci emerged during the 1980s within Scotland and were a major contributing factor for the increase in serogroup C at the time. ST-11 strains with serogroups other than serogroup C had also been present during the 1970s. This may suggest evidence of a capsular switch as the PorA data for serogroup W135 ST-11 strains from the 1970s had the same combinations of PorA VRs that have been commonly found within serogroup C ST-11s. However to confirm the hypothesis of capsule switch a more complete antigen repertoire (e.g. PorB, FetA.) would have to be tested for theses isolates which was not possible due to financial restraints.

Although the exact mechanism of capsular switching is not yet known, it is thought to involve some recombination event(s) within the meningococcal *cps* locus (Tyler & Tsang, 2004). The process of capsule switching may arise spontaneously (Frosch & Meyer, 1992) or as a result of some selective advantage conferred on the organisms that have their capsules replaced (Alcala *et al.*, 2002; Perez-Trallero *et al.*, 2002; Stefanelli *et al.*, 2003). With increasingly widespread use of both the A, C, Y, W135

polysaccharide vaccine and the conjugate meningococcal C vaccine in many countries, one of the continuing epidemiological concerns is the effect of vaccination on serogroup replacement in *N. meningitidis*. The new progeny organisms always bear some resemblance to their parent strains (eg, the same serotype and/or serosubtype antigens, identical or similar ST type and PFGE profile (Alcala *et al.*, 2002; Perez-Trallero *et al.*, 2002; Stefanelli *et al.*, 2003).

The emergence of serogroup C ST-11 meningococci coincided with a reduction in the number of ST-8 strains, which were predominant during the 1980s. The ST-8 strains were replaced by strains of the same complex in the early 1990s. These clones included the ST-153, ST-1349 and ST-66. Isolates of the ST-8 complex were reduced from 52 isolates in 1990 to one isolate by 1998. Over the same period the ST-11 complex increased from 21 isolates in 1990 to 66 isolates in 1998.

Although there was significant diversity in the STs (309 types) of the 2517 isolates recovered from 1972-1998, ten of these STs accounted for 1562 isolates (59.9%). There is extensive evidence for the persistence of particular multilocus genotypes among meningococcal disease isolates (Caugant *et al.*, 1986b; Caugant, 1998). Similar observations have been made with other bacteria (Feil *et al.*, 2001; Enright *et al.*, 2002). In this present study, clonal complexes associated with seven lineages accounted for 1993 isolates (76.4%). Thus, although there were cases associated with novel STs, the majority of disease was associated with the same predominant disease-associated complexes that have been found world wide and over a number of years (Achtman, 1995; Brehony *et al.*, 2007). The common disease-associated complexes found in this study included the ST-8, ST-41/44, ST-11, ST-32, ST-1 and ST-269 complexes. One hundred and seventy seven new STs were found and these accounted

for 9.7% of the total number of isolates. Although these STs are defined as new in terms of the fact that they have not been described before they are actually STs that occurred a number of years ago. These new STs occurred throughout the time period from 1974 to 1998 with some occurring only once but others occurring more than once and with many years apart. This indicates that these strains have been present in the population but have rarely caused disease. In any large study, new STs will be found. These are often found at low frequency and do not spread geographically, probably because they are less robust than the central genotypes that they have emerged from.

The general consensus is that populations of carried meningococci comprise numerous lineages (or clonal complexes), whereas collections of meningococcal isolates from cases of invasive disease typically contain a limited subset of lineages (Yazdankhah *et al.*, 2004; Jolley *et al.*, 2005). The clonal complex information allowed for the identification of hyperinvasive lineages. This enables the monitoring of trends in meningococcal disease over time and comparison with other datasets (Brehony *et al.*, 2007). These hyperinvasive lineages are known to be persistent, having spread worldwide over many decades (Caugant, 1998). The incidence of disease rises and falls with the presence of hyperinvasive lineages in the carried population of meningococci. The antigens associated with particular clonal complexes consequently rise and fall over time, as predicted by models of pathogen strain structuring by immunological selection (Gupta & Maiden, 2001), although there appear to be differences in the stability of the lineages with regard to different antigens. For example, the ST-1 clonal complex is very strongly associated with particular antigenic variants, including capsule and subcapsular antigens (Suker *et al.*, 1994; Urwin *et al.*, 2004) and serogroup A.

Serogroup A had been shown in this study to be present within the 1970s and early 1980s. Serogroup A caused 134 cases of IMD between 1973-1982, thereafter there were three cases in 1986, two cases in 1987, one case in 1989, one case in 1992 and two cases in 1995. 89% of serogroup A isolates were of ST-1. Only one other ST occurred more than once (ST-5 with 5.67%). Serogroup A in Scotland was highly clonal as it was represented by only 9 different STs. Despite a small number of cases since the 1970s and early 1980s (e.g. ST-5 complex meningococci in the 1990s) serogroup A meningococci have, to date, not re-established themselves as a cause of disease in the UK (Jones & Sutcliffe, 1990).

In terms of PorA VR types it was found that there were certain combinations of VR types that were significantly more common than others. The first variable region was represented in the majority from the type 5-family or the P1.5 family (1299 isolates). The second variable region had over 33.7% characterised as variable regions 2, 2-1 and 2-2. The third variable region had over 38.6% allocated to variable region 36-2. The combination 5, 2, 36-2 occurred 466 times (17.9%) and 19, 15, 36 occurred 192 times (7.4%). There was a strong link with *porA* and ST and more so with clonal complex. This association has been observed in other meningococcal populations (Bygraves *et al.*, 1999; Urwin *et al.*, 2004). This link is evident with the PorA type 5, 2-1, 36-2, which occured in 70 isolates representing the ST-11 complex and in all but two isolates representing ST-11. Similarly PorA type 18-3, 1, 35-1 was associated with 15 isolates belonging to the ST41-44/lineage 3 complex. However, this was not the case with all PorA combinations as the PorA type 19, 15, 36 was associated with 10 different complexes. There was some association between serogroup and PorA VR types. There was strong evidence of certain VR1, 2 and 3 regions being associated

with certain serogroups, although this was not definitive. For example, PorA type 5, 2, 36-2 occurred in 354 serogroup C isolates (76.0%), although this is not surprising as the PorA combination is strongly associated with ST-8 and ST-11. Similarly, of 192 isolates with PorA type 19, 15, 36, 85.4% were associated with serogroup B. Genosubtyping of the *porA* gene has been shown to increase the power of differentiation within clonal meningococcal populations. For, example, seven isolates that had the same serogroup, ST, VR1 and VR2 could be differentiated by their VR3 type. Although this is of note retrospectively, this could be useful when looking at an outbreak in a real-time situation.

4.4 Spatio-temporal analysis

Using the cluster detection software SaTScan, 29 clusters were identified in Scotland from 1972-1998; these included 63 cases and comprised 2.5% of all cases. A range of different strain types was associated with clusters identified in this study. Predictably, there were clusters caused by strain types that are known to be hypervirulent, i.e. strain types which were responsible for a number of cases throughout the world as well as in Scotland (e.g. strain types of serogroup C, ST-11, 5, 2, 36-2). However, clusters were also identified in this study that were caused by lesser-known strain types that were not responsible for many cases. These include strain types B, ST-3010, 5-1, 2-2, 36-2, and B, ST-343, 7, 16-2, 35. This shows again that although a few strain types are associated with the majority of disease, other less common strain types are capable of causing disease and clusters of disease under appropriate circumstance.

The results of this analysis on meningococcal clusters compares with the findings of other studies. Hoebe *et al.* (2004) found statistical evidence for clustering in 6 of 25 clusters reported by the Dutch Inspectorate of Health Care by applying a global clustering test (space-time nearest-neighbour analysis) to different serosubtypes of meningococci. However this approach was different from the present study as only phenotypic typing was performed and therefore analysis was limited by a number of non-typeable isolates. In Germany, 26 clusters were detected in 42 months from December 2001 to June 2005 with the proportion of patients involved in clusters being 4.2% (Elias *et al.*, 2006b). In France, 28 clusters were identified in 1987 and 1988, and this accounted for 8% of total cases (Olivares & Hubert, 1992). This compares with the findings in the present study, where 29 clusters were found to have occurred within Scotland from 1972-1998. These clusters included 63 cases, which accounted for 2.5% of all cases. In England and Wales, a total of 114 clusters were reported in preschool and school settings in England and Wales between 1 April 1995 and 31 March 2001 or approximately 20 clusters per year (Davison *et al.*, 2004). The majority (86%) of these clusters had only two cases, which was the same as our findings. This study is the first to look at the detection of clusters over a time period of 26 years and to identify clusters that previously would have been unidentified due to lack of suitable characterisation techniques.

This comprehensive investigation was only feasible due to highly discriminatory characterisation techniques for *N. meningitidis*, availability of data regarding time and place of occurrence of IMD and free availability of the cluster detection software SaTScan. However there were limitations of the SaTScan software (Kulldorff, 1997).

These include the assumption that the clusters are cylindrically shaped and the constraints that are attributable to centroids and the edge effects of the scan method. There is also no direct link with geographic information system software, which would help with capturing, storing, analyzing, managing and presenting data that is spatially referenced. One limitation of performing cluster analyses using the patient's residence as the source of acquisition of IMD is that this may not always reflect the area where acquisition occurred. With the amount of travelling people do in their everyday lives infection might be contracted at locations other than the one suggested by where they live, e.g., at gatherings outside the county of residence. Thus, a few clusters out with places of residence might have been missed by this approach but there would be no way to detect these anyway unless you knew all movement and contacts of the patients.

The health board area which had the highest number of clusters associated with it was Greater Glasgow with five different clusters; this area has the greatest population size. Not all areas were represented, since five regions did not have any clusters. Most clusters were associated with only two patients which was similar to the findings of other studies (Hastings *et al.*, 1997; Elias *et al.*, 2006b). The maximum number of patients per cluster was four, but this occurred only once. Two serogroups were responsible for all the clusters identified within Scotland, namely serogroups B and C. This again highlights the dominance of these two serogroups in causing disease within Scotland over the 26-year period. This study is the first to look at the detection of clusters within one country over a time period of 26 years and to identify clusters that would have previously been unidentified due to lack of suitable characterisation techniques. 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.*, 2002b). This study found the same pattern with the highest number of cases in the winter months and the lowest number of cases during summer.

4.5 Vaccine coverage

In cases of invasive meningococci, a limited repertoire of antigen variants is persistent over time and these tend to be associated with particular invasive lineages (Russell *et al.*, 2008). Combinations of subcapsular antigens reappear over time, sometimes associated with different lineages, perhaps in response to increases and decreases of herd immunity against particular strain types (Russell *et al.*, 2008). This leads to the possibility that appropriately composed component vaccines may be able to protect human populations from meningococcal disease over periods of time sufficient to warrant their development and implementation (Russell *et al.*, 2008). Current approaches in the development of a comprehensive meningococcal vaccine can be separated into two distinctive strategies: vaccines to enhance the immune response to conserved antigens, and vaccines based on highly immunogenic yet variable antigens. While not excluding the effectiveness of vaccines based on conserved antigens, the results from this study support the development of multivalent vaccines consisting of variable antigens. This is also consistent with clinical studies of OMV vaccines, which have repeatedly highlighted the importance of PorA for vaccine-induced immunity. In terms of clinical development, the most advanced multivalent vaccines consist of OMVs produced from genetically-modified strains expressing multiple variants of PorA (van den Dobbelsteen *et al.*, 2007). The results in the present study indicate that the multivalent preparations produced by the Netherlands Vaccine Institute

(Nonavalent vaccine) have the potential to prevent the majority of serogroup B infections in Scotland as well as having the potential, although not to the same extent as with serogroup B, to protect against other serogroups. The data also show that the potential coverage against serogroup B did not fluctuate greatly over a thirty-year period, even after the introduction of the MCC vaccine. Although there was a reduction in the potential coverage over the three decades, this was not due to any particular PorA type being present in large numbers but instead was due to a number of different PorA types present in small numbers. Therefore there was no candidate for an additional PorA type that could be added to any potential vaccine that could greatly improve coverage. It was also shown that, for the age groups that would potentially be the age group that would be the first to be immunised with any vaccine as part of the childhood vaccination programme (the 0-4 years old group) the potential coverage was over 92%. This is comparable with the coverage seen with the MCC vaccine, of approximately 90%. These data have therefore provided important information for informing vaccine development, providing a rational approach for deciding which variants should be included in this type of vaccine. In the Netherlands, the potential cost-effectiveness of a vaccine containing nine OMVs plus pneumococcal conjugate has been assessed (Bos *et al.*, 2006) and it was concluded that this combined vaccine was likely to be cost-effective in that setting and could prevent 201 cases of meningococcal B meningitis per year. Similar conclusions have also been reached in another study from England and Wales, which also examined FetA VRs (Russell *et al.*, 2008).

4.6 Further work

With reference to this study, there are options available for further work to be performed. Little information was available pertaining to patient outcome after disease. These data are collected by a different organisation and, when combined with the data from this study, could be used to look at the trends involved in case mortality. Previous studies have shown that the outcome of meningococcal disease is associated with the phenotype, age and clinical presentation with the clonal complex also appearing to be an indicator of virulence (Spanjaard *et al.*, 1987; Scholten *et al.*, 1994; Iversen & Aavitsland, 1996; Trotter *et al.*, 2002; Jensen *et al.*, 2003; Gottfredsson *et al.*, 2006). Therefore, analysis of case fatality and its association with clonal complexes, age and geographical location and estimating the chances of death by clonal complexes could be examined. Another aspect that could be examined is a more through investigation of how the data have changed over time. This would require some specialist statistical analysis such as logistic regression. There was also very little data regarding antibiotic resistance due to the fact this was not performed regularly until the 1990s. These data could have been used to look at the extent of antibiotic resistance in Scotland 1972-1998, as well as its association with particular lineages, ages and geographical areas.

In order to improve our understanding of the population structure of *N. meningitidis*, it is important to examine carriage. Previous studies using MLST analysis have shown the widespread diversity of the carried meningococci, but have also provided indications that meningococcal populations from healthy carriers comprise a number

of successful clones that are geographically prevalent (Maiden & Stuart, 2002; Yazdankhah & Caugant, 2004; Caugant *et al.*, 2007). It has also been shown that hypervirulent STs and complexes are rare among strains from carriers (Yazdankhah & Caugant, 2004; Caugant *et al.*, 2007). The hypervirulent clones have been shown to vary greatly in their capability to establish a commensal relationship with their host. For example, the ST-11 complex has been described as being a poor coloniser but the ST-23 complex appear to be tailored to a commensal relationship with the host (Caugant *et al.*, 2007). There was a national multi-centre carriage study to assess the effect of the introduction of the MCC vaccine in the United Kingdom on those under 19 years of age (Maiden *et al.*, 2008). The impact of this intervention on asymptomatic carriage of meningococci was investigated to establish whether serogroup replacement or protection by herd immunity occurred. Multicenter surveys of carriage were conducted throughout the UK, including Scotland, during vaccine introduction and on two successive years. A reduction in serogroup C carriage was observed that lasted at least two years with no evidence of serogroup replacement. Vaccination had a disproportionate impact on the carriage of the ST-11 complex serogroup C meningococci. The high impact on the carriage of ST-11 complex serogroup C could be attributed to high levels of capsule expression. The impact of vaccination with MCC vaccine on the prevalence of carriage of serogroup C meningococci was consistent with herd immunity (Maiden *et al.*, 2008).

Surveillance is an area, which has seen developments over the last few years but there are still opportunities for improvement. Data gathered from numerous countries can help monitor and identify emerging problems faster than surveillance from one country. These data can also be used to inform the impact of mass vaccination and

show the differences between countries, providing data for future vaccine programmes and strategies. This has already been shown to be a success with a surveillance network for meningococcal disease in Europe. Beginning in 1999, the European Union Invasive Bacterial Infection Surveillance Network (EU-IBIS) has 27 European countries involved submitting case reports of meningococcal disease with the data added to the EU-IBIS database (http://www.euibis.org/meningo/meningo_statistics.htm).

The publication of complete genomes of a number of different *N. meningitidis* isolates (Rappuoli, 2000) has provided a broad profile of the genetic complement of this important organism. However, the genome sequences highlight inadequacies in our understanding of meningococcal pathogenesis, as it is not known which genes are involved in the disease process. Therefore, with the increased accessibility of genome sequencing methods of high-throughput analysis of gene function are needed to take advantage of the vast amount of information being generated. Molecular typing techniques, such as MLST, only allow the examination of a limited number of loci, giving no information either of genome-wide variation or of the full impact of horizontal gene transfer (Ochman *et al.*, 2000). The introduction of high-throughput sequencing and the associated development of analytical tools have considerably changed the field. This has lead to the development of DNA-array technology (DNA microarrays) (Joyce *et al.*, 2003; Bryant *et al.*, 2004), which can transform our understanding of meningococcal population genetics on a genome-wide scale. DNA microarrays enable a 'bird's-eye view' of all the genes absent or present in a given genome compared with the reference genome on the microarray (Claus *et al.*, 2007). Neisseria research has been in a fortunate position in that a variety of genomes have

been publicly available since the year 2000, (Parkhill *et al.*, 2000; Tettelin *et al.*, 2000). Thus there has been a number of Neisseria microarray studies (Grifantini *et al.*, 2002; Grifantini *et al.*, 2004; Snyder *et al.*, 2004; Ducey *et al.*, 2005; Stabler *et al.*, 2005; Bartolini *et al.*, 2006). DNA microarray technology has already made very useful contributions to the understanding of meningococcal genome diversity, population genetics and pathogenesis.

The limitations of such new technology include cost and the practicality within laboratories with limited resources and technical support. The results are highly dependent on the set-up, where a number of different variables are present, like chip design, hybridisation conditions, the choice of strains, and appropriate data analysis. Another major limitation of the technology is that only the distribution of already known genes can be assessed. Although laborious and time-consuming, and therefore not appropriate to larger strain collections, genome comparison by representational difference analysis might still be an alternative approach that enables the identification of novel sequences of DNA (Tinsley & Nassif, 1996; Claus *et al.*, 2007). In the end, it is only by whole-genome sequencing of as many pathogenic as well as non-pathogenic strains as possible that we might be able to have a true insight into the as yet unknown virulence-associated genes that might contribute to the meningococcal gene pool (Maiden *et al.*, 1996).

4.7 Conclusions

This study has provided a unique insight into the molecular epidemiology of meningococci causing invasive disease within Scotland in the period 1972-1998. This long-term, nation-wide study looked at evolutionary trends of invasive meningococcal isolates in a well-defined setting. It has shown that serogroup distribution changed from year to year during the time period 1972-1998. However, serogroups B and C were evidently the dominant serogroups over this period. The increase in serogroup C disease in the 1990s was due to the ET-37/ST-11 clonal complex. The mid-1980s saw the emergence of ST-11, which is strongly associated with serogroup C disease. Although there were not a huge number of cases, over the next decade the number of strains of ST-11 did increase to such an extent that it became the dominant hypervirulent ST causing disease. This also coincided with a dramatic decrease in strains of ST-8, which are now rarely seen. ST-8 had caused a large incidence of invasive disease during the early 1980s. ST-8 strain was associated with serogroups B and C. Serogroup A was present within the 1970s and early 1980s before disappearing.

While there was much diversity in the STs (309 types), only ten accounted for 1562 isolates (59.9%). Only seven lineages accounted for 1993 isolates (76.4%). There were certain combinations of *porA* VR types which were significantly more common than others and there was some association between genotype and *porA* VR type

The results of analysis on meningococcal clusters compares with the findings of other studies. Twenty-nine clusters were found to have occurred within Scotland from 1972-1998. These clusters included 63 cases, which accounted for 2.5% of all cases.

This study is the first to look at the detection of clusters over a time period of 26 years and to identify clusters that would have previously been unidentified due to lack of suitable characterisation techniques.

Multivalent vaccines have the potential to prevent the strains of the disease not covered by other vaccines i.e. serogroup B disease. The results in this study indicate that the multivalent preparation produced by the Netherlands Vaccine Institute (Nonavalent vaccine) had the potential to prevent the majority of serogroup B infection in Scotland and has the potential to do so in the future. The data have also shown that the vaccine had the potential to protect against other serogroups although not to the same extent as serogroup B.

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Appendix

Appendix Table A.1: ***PorA*** **Variable Region 1 peptide sequences**

Variant	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
5-11	NIQQPQVTKR
5-12	PLPKIQPQVTKR
5-13	PLQNIIQQPQVTKR
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
7-17	AQAANGGVSGQVKVTKA
7-18	AQATNGGASGQVKVTKA
7-19	AQAANGRASGQVKVTKVTKA
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
12-11	KPSSTNAKPGNKVEVTKA
12-12	QPSNTNGKTSNKVEVTKA
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
18-12	PPSKGQTGNKVTKR
18-13	PPLQGQTGNKVTKG
18-14	PPSKAQTGNKVTKG
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
19-18	PPSKSQPQVKKSQPQVKVTKA
19-19	PSSKSQLQVKVTKA

19-20	PPSKSQSKVKVTKA
P1.20 family	
20	QPQTANTQQGGKVKVTKA
20-1	QPQIANTQQGGKVKVTKA
20-2	QPQTANTQQGRKVKVTKA
20-3	QPQAANTQQGGKVKVTKA
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
21-9	QPQVTNGAQGNQVKVTKA
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
22-8	QPSKDQGQTNNQVKVTKA
22-9	WPSKAQGQTNNQVKVTKA
22-10	QPSSTQGQTSNQVKVTKV
22-11	QPSSTQGQTSNQVKVTKA
P1.31 family	
31	PPSSNQGKNQAQTGNTVTKA

Appendix Table A.2: ***PorA*** **Variable Region 2 peptide sequences**

Variant	Peptide sequence
P1.1 family	
1	YVAVENGVAKKVA
1-1	YVAVENGATKKVA
1-2	YVAVENGVVKKVA
1-3	YVAVENGVAKKVT
1-4	YVAVENGVTKKVA
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
2-30	HLVQQTPQSQPTLVP
2-31	HFVKSQPTLVP
2-32	HFVQQTPKSQPTPKSQPTLVP
2-33	HFVQQASQSQPTLVP
2-34	HFVQQTPQSQPKSQPTLVP
2-35	HFVQQTPKSQPILVP

2-36	HFVQQTPQIQPTLVP
2-37	HFVQQKPTSNPTLVP
2-38	HFVQQTPKRQPTLVP
2-39	HFVQQTPQGQPTLVP
2-40	HFVQQISKSQSTLVP
P1.3 family	
3	TLANGANNTIIRVP
3-1	TVANGANNTIIRVP
3-2	TLANGANDTIIRVP
3-3	TLANGADNTIIRVP
3-4	TPANGANNTIIRVP
3-5	TLAKGANNTIIRVP
3-6	TLANGATNTIIRVP
3-7	TLATLANGANNTIIRVP
3-8	TLANNTIIRVP
3-9	TLANGANNTP (missing IIRVP motif)
P1.4 family	
4	HVVVNNKVATHVP
4-1	HVVVNNNVATHVP
4-2	HVVVNNKVATHVPAKVATHVP
4-3	HVVVNNKVTTHVP
4-4	HVVVNNKVATPHVP
4-5	HVVVNNKV (missing THVP motif)
4-6	HVVVNNRVATHVP
4-7	HVVVKVATHVP
4-8	HVVVNNQVATHVP
4-9	VNNKVATHVP
4-10	HVVNNKVATHVP
4-11	HVVVNNKVAPHVP
4-12	HVHVVVNNKVATHVP
4-13	HVVVNNKVA (missing THVP motif)
4-14	HFVVNNKVATHVP
4-15	HVVVNNEVATHVP
4-16	HVVVNNVVVNNKVATHVP
4-17	SVVVNNKVATHVP
4-18	HVVVNNKVAT (missing HVP motif)
P1.9 family	
9	YVDEQSKYHA
9-1	YVDSKYHA
9-2	YVGEQSKYHA
9-3	YVDEQSKDHA
9-4	YVDKQSKYHA
9-5	YVDEQSEYHA
9-6	YVDEQSQYHA
9-7	YVDEQRKYHA
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
10-27	HFVRNKQNQRPTLVP
10-28	HFVQNQNKQNQPPTLVP
10-29	HFVQNKQNQSPTLVP
10-30	HFVQNKQNQRPTLV (missing P from end motif)
10-31	HFVPNKQNQRPTLVP
10-32	HFVQNKQDQRPTLVP
10-33	HFVQNKQNKQPTLVP
10-34	YFVQNKQNKQNQPPTLVP
10-35	HFVQNKQNQQNQQPTLVP
10-36	HFVQDKKGQLPTLVP
10-37	HFVQNKQNQPLTLVP
10-38	HFVQDKQDQLPTLVP
10-39	HFVQNKQNPPPTLVP
10-40	HFVQNKQKQPPTLVP
10-41	HFVQNKQNQRLTLVP
10-42	HFVQNKQNQQNQPSTLVP
10-43	HFVQNKQNKLPTLVP
10-44	HFVQDKKGQSPTLVP
10-45	HFVQNKQNQRP (missing TLVP end motif)
10-46	HFVQNKQNKQNKPPTLVP
10-47	HFVQNKQSQQPTLVP

10-48	HFVQNKPTLVP
10-49	HFVQNKQDQPPTLVP
10-50	HFVRNKQNQQSTLVP
10-51	HFVQNKQNVQNKQNQPPTLVP
10-52	HFVQNKQNKQNKQNKQNQPPTLVP
10-53	HFVQNKQNQQNQQNQSPTLVP
10-54	HFVQNKQSQLPTLVP
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
13-9	YWTTVNTGSATTFVP
13-8	YWITVNTGSATTTTFVP
13-10	YWTTVNTGSVTTTTFVP
13-11	YWTTVNTGSAATTTTFVP
13-12	YWTAVNAGSATTTFVP
13-13	YWTTVNNGNATTTTFVP
13-14	YWTTVNTSSATTTTTFVP
13-15	YWTTVNTGSATTTTTTTFVP
13-16	VNTGSATTTFVP (missing YWT motif)
13-17	YWTTVNTGNATTTTFVP
13-18	YWTTVNTSSATTTTFVP
13-19	YWTTVNTGSATTPFVP
13-20	YYTTVTKGNATTTTFVP
13-21	YWTTVNTGSAT (missing TTTTFVP motif)
13-22	YWTTVNNGNATTTFVP
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	YVDETKMVHA
14-8	YVDEKRMVHA
14-9	YVDAKKMVHA
14-10	YVDEKGMVHA
14-11	YVDEKRVSH
14-12	YVNEKKMVHA
14-13	YVDEEQVSHA
14-14	YVDERKMVHA

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
15-22	HYTRQNNYTRQNNIDVFVP
15-23	HYTSQNNADVFVP
15-24	HYTRQNDTDVFVP
15-25	HYTKQNNTDVFVP
15-26	HYTGQNYIDVFVP
15-27	HYTRKNNADVFVP
15-28	HTRQNNADVFVP
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 (missing YYTK motif)
16-21	YYTKDTKNNLTLVP
16-22	YYTKDTNNILTLVP
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	YYTKHTNNNPTLVP
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	YYNTKDTKNNLTLVP
16-41	YYTKDTNNKLTLVP
16-42	YYTKDTNYTKDTNNNLTLVP
16-43	YYTKDTNNNLTNNNLTLVP
16-44	YYTNTNDNLTLVP
16-45	YYTNYTKDTNNNLTLVP
16-46	YYTKDTKDTNNNLTLVP
16-47	YYTKDTSNNLTLVP
16-48	YYTKDRNNNLTLVP
16-49	YYTKDKNDDLTLVP
16-50	YYTKDTNNNL (missing TLVP motif)
16-51	YYKDTNNNLTLVP
16-52	YYTKDKNDALTLVP
16-53	YTNTNNNLTLVP (missing first Y)
16-54	YYTKVINNNRTLVP
16-55	YYTKDTNNNLNNLTLVP
16-56	YYTKDTNNNLTHNLTLVP
16-57	YYTKDKDTNNNLTLVP
16-58	YYTKDTDNNLTLVP
16-59	YYTKGTNNNLTLVP
16-60	YYTKDKNDNLTPVP
16-61	YYTKKDKNDNLTLVP
16-62	YYTKDINNNLTLVP

16-63	YYTKNTNNKNTNNNLTLVP
16-64	YYTKDTNNPLVP
16-65	YYTKYTNNNLTLVP
16-66	YTKDTNNNLTLVP (missing first Y)
16-67	YYTKDKTDNLTLVP
16-68	YYTNTNNNL (missing TLVP motif)
16-69	YYTKDTNNNTKDTNNNLTLVP
P1.23 family	
23	HWNTVYNTNGTTTTFVP
23-1	HWNTVYNTNGTTTTTTTFVP
23-2	HWNTVYNTNGTTTTTFVP
23-3	HWNTVYNTNGTTTTTTFVP
23-4	HWTTVYNTNGTTTFVP
23-5	HWTTVYNTNGTTATFVP
23-6	HWNTVYNTNGTTTFVP
23-7	HWNTVYNTNGTTTTTTTTFVP
23-8	HWTTVYNTNGTTTTTFVP
23-9	HWNTVYNTNGTTTTTTTTTFVP
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
25-14	TYTVNSSSVVTPVP
25-15	TYTVDSSSVVTPVP
25-16	SSGVFTPVP (missing TYTVDS motif)
25-17	TYVDSSGVVTPVP
P1.26 family	
26	HFVADSQGKITRVP
26-1	HFVADSQGEITRVP
26-2	YFTADPNDQNKITRVP
26-3	HFVADSQDKITRVP
P1.28 family	
28	YYYTTATNSSTSTTFVP
28-1	YYYTTATNSSTSTTATNSSTSTTFVP
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
30-8	HYTTVYNATTTTFVP
30-9	HYTTVYNATTTTTTTTTTTTFVP
P1.34 family	
34	YVDDQGKVKGP
34-1	YVDDQKVKGP
34-2	YVDDQGKVKG
P1.42 family	
42	HLVLDGQGKITQVP
42-1	HLVSDGQGKITQVP
42-2	DGQGKITQVP (missing HLV[X] motif)
42-3	HLVSDGQGEITQVP
P1.43 family	
43	TFTLESNQMKPVP

Appendix Table A.3: ***PorA*** **Variable Region 3 peptide sequences**

Variant	Peptide Sequence
35	LIGSGSDQ
35-1	LLGSGSDQ
36	LLGSTSDE
36-1	LLGSTSDQ
36-2	LLGSGSDE
36-3	LLGSASDE
37	LIGSATSDQ
37-1	LIGSATSDE
38	LLGRIGDDDE
38-1	LLGRIGEDDE
39	LLGSGSDG
40	LLGRSGDDDE
41	LLGRGSDE

<u>Appendix Table A.4</u>: New Sequence Types first identified during this study and deposited on the MLST database.

ST	Serogroup	Profile	ST	Serogroup	Profile
2147	B	13,5,4,40,59,8,18	4605	B	17,6,9,2,26,6,2
2155	C	17,6,19,17,3,26,2,	4606	C	17,5,19,2,3,26,2
2174	B	2,3,7,2,8,15,2	4607	W135	2,7,6,26,9,18,8
2253	B	2,6,9,5,9,6,9	4608	C	2,6,9,2,9,6,9
2307	B	4,5,2,5,38,11,9	4609	C	2,3,175,2,8,160,2
2314	B	3,6,9,5,9,6,2	4610	B	2,3,7,4,34,5,2
2510	C	9,3,4,3,8,4,6	4611	C	2,3,7,17,8,5,2
2511	B	8,30,6,16,3,17,2	4612	B, C	2,3,34,13,8,11,2
2512	A	1,3,1,1,1,1,9	4613	C	2,3,34,13,9,11,2
2516	B	9,6,9,56,9,6,2	4614	C	2,16,12,11,3,60,30
2517	A	12,3,1,1,1,1,3	4615	C	3,5,18,17,5,24,16
2518	B	9, 26,46,9,9,20,17	4616	B	4,2,11,5,58,10,21
2519	B	4,10,5,4,13,3,8	4617	C	4,10,15,2,8,11,18
2522	C	14,5,6,9,11,6,16	4618	B	4,10,5,4,8,4,2
2523	B	1,6,46,9,9,6,9	4619	B	4,5,2,5,38,11,15
2524	B	20,5,18,17,5,24,16	4620	B	4,10,15,9,43,11,9
2525	B	4,10,2,17,16,6,20	4621	B	4,3,11,55,6,10,12
2531	B	4,5,6,40,3,8,18	4622	B	4,29,135,9,8,11,9
2532	B	1,6,9,9,9,6,9	4623	B	43,3,133,13,8,19,15
2534	B	13,3,7,13,21,11,13	4624	B	43,5,9,13,6,19,15
2535	B	4,5,9,9,21,11,20	4625	B	7,4,10,116,10,15,20
2536	B	13,19,6,40,59,8,18	4626	B	8,10,5,4,6,3,7
2538	B	43,5,4,7,16,20,15	4627	B	8,16,12,17,43,39,7
2541	B	6,3,2,12,6,24,14,	4628	B	8,5,6,5,26,78,2
2542	B	2,6,12,11,26,14,7	4629	B	8,10,9,9,70,6,9
2543	B	9,6,9,10,9,6,9	4630	B	8,10,6,5,58,86,12
2544	B	8,26,5,4,6,3,8	4631	B	8,10,5,4,6,3,17
2546	B	2,5,1,37,16,6,17	4632	B	9,6,25,8,58,6,9
2548	B	111,5,4,40,3,8,2	4634	B	8,5,15,6,1,44,2
2552	B	13,5,6,40,5,8,18	4635	B	1,3,4,3,8,4,6
2553	B	14,10,11,9,6,10,2	4637	B	8,5,4,5,1,78,2
2554	B	4,29,11,17,5,10,62	4638	B	4,2,4,5,8,11,9
2555	NG	8,2,46,15,26,20,17	4639	B	4,6,7,9,8,11,20
2802	B	4,10,15,55,8,11,18	4641	B	4,10,5,4,6,3,11
2803	B	4,10,15,59,8,11,9	4642	B	4,10,15,7,8,11,2
2804	NG	110,2,9,5,26,120,46	4643	B	6,5,6,17,26,68,2
2805	B	100 ,4,7,11,77,26,13	4644	C	2,3,4,3,43,4,6
2806	B	10,6,9,5,9,18,9	4646	B	4,6,2,1,8,124,20
2807	B	3,6,52,9,3,8,18	4647	C	4,10,15,9,21,11,16
2808	NG	8,4,6,17,5,18,6	4648	B	9,26,46,37,9,20,18
2809	NG	2,16,12,11,3,14,7	4649	B	43,5,9,76,215,11,21

ST	Serogroup	Profile	ST	Serogroup	Profile
3010	B	15,3,133,13,8,6,15	4650	B	13,3,7,13,21,11,2
3286	B	4,10,6,5,8,11,9	4651	B	2,3,7,2,34,4,2
3288	B	11,5,185,35,5,171,129	4652	B, C	2,6,4,85,26,6,9
3289	X	25,10,18,9,8,13,9	4653	B	15,3,133,13,8,16,15
3290	B	20,5,124,9,3,20,18	4654	B	6,5,19,17,3,26,2
3291	B	9,6,4,32,9,6,9	4655	B	2,3,7,3,34,5,8
3292	C	2,6,9,17,9,6,16	4656	C	2,3,7,2,46,5,2
3297	C	9,5,19,17,3,26,2	4657	B	8,6,12,11,3,22,7
3298	C	2,3,4,3,8,4,9	4658	C	2,5,12,143,29,285,7
3464	C	13,5,62,9,9,8,18	4659	B	4,10,15,9,8,6,16
4570	A	1,3,1,1,13,1,3	4660	C	12,5,9,17,9,6,9
4571	A	1,3,1,9,1,1,3	4661	B	4,10,15,9,21,11,6
4586	B	3,5,9,5,11,6,9	4662	C	26,6,9,17,9,6,9
4587	B	1,6,13,53,26,223,3	4663	B	3,5,9,5,10,6,9
4588	B	10,5,18,17,5,24,16	4664	B	4,10,5,40,6,3,2
4589	B	11,5,18,17,5,24,19	4665	B	37,2,53,259,259,116,2
4590	B	12,5,4,85,13,9,53	4666	B	6,5,6,9,3,21,2
4591	C	12,6,9,2,9,6,9	4667	B	9,26,46,37,9,20,17
4592	C	12,5,4,40,59,8,18	4668	B, C	8,5,6,17,26,78,2
4593	C	13,5,6,9,9,9,18	4670	B	2,3,7,17,34,5,2
4594	B	13,5,4,40,3,8,16	4671	C	4,10,15,5,8,6,9
4595	C	13,5,87,9,9,8,18	4672	B	6,6,9,60,9,6,9
4596	B	13,3,52,9,3,11,9	4673	B	14,6,46,24,118,20,18
4597	B	13,5,4,40,26,8,18	4674	Y	2,16,6,17,9,18,9
4598	B	13,141,6,40,3,8,18	4675	B	8,10,5,4,1,3,8
4599	C	13,5,15,6,3,8,15	4676	C	2,10,12,11,8,39,7
4600	B	15,3,133,21,8,19,15	4677	C	2,3,4,11,8,4,6
4601	B, C	15,10,5,4,6,3,2	4678	B	6,6,9,5,10,6,9
4602	B	15,3,133,13,8,18,15	4679	B	43,5,274,7,215,20,15
4603	C	15,3,133,13,11,19,15			
4604	B	17,5,19,17,13,26,2			