A Study of Fatal Cases of Group B Meningococcal Disease Using Non-culture Multilocus Sequence Typing and *porA* Sequence Typing

Anthony Dutton Carr

This thesis is submitted for Masters Degree in the School of Biology, Chemistry and Health Science, Faculty of Science and Engineering, the Manchester Metropolitan University.

February 2010

Collaborating Establishment:

Manchester Health Protection Agency Laboratory

Manchester Royal Infirmary

Manchester.

Declaration

With the exception of any statements to the contrary, all data presented in this report are the results of my own efforts. No part of this report has been copied from other sources. In addition, no portion of the work included in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning

Anthony Dutton Carr

Abstract

Since the introduction of the UK MenC vaccine programme in November 1999 there has been a 97% decrease in the number of confirmed group C meningococcal disease cases. However, in the year of introduction and the subsequent two years an increase in the numbers of group B cases was noted.

Meningococcal infection may be microbiologically confirmed by culture and non culture methods. The introduction by the Meningococcal Reference Unit (MRU) of diagnostic polymerase chain reaction (PCR) testing in 1996 dramatically increased the number of cases ascertained solely by PCR, currently over 50%. Routinely cases determined by PCR only are confirmed to serogroup level and are not characterised further.

This study was carried out to investigate whether the application of non culture DNA sequence typing which had recently been developed by the Heath Protection Agency could improve surveillance of meningococcal disease, and to determine if the increase in the number of observed group B cases was associated with particular meningococcal clones.

The non culture DNA sequence typing of the *porA* antigen gene and of the seven genes of the Multilocus sequence typing (MLST) scheme was undertaken. Archived samples from one year before and two years following the immunisation programme, 96 samples in total, were analyzed using nested amplification and DNA sequence typing. The non culture group B fatal case data were also compared to other available molecular typing datasets.

Results showed that there was no significant change in predominating group B clones and therefore no significant epidemiological shift in the meningococci causing group B disease, including fatalities, in the years 1999-2001. It was found by the combination of culture and non culture data that the two predominating clonal complexes causing fatal disease were ST41/44 lineage3 (36.3% pre and 30.8% post immunisation) and ST269 (22.4% pre and 31.9% post immunisation). This provided reassurance that the epidemiology of group B meningococcal disease was not impacted by the group C immunisation programme, and in particular that there was no noted proliferation of the hyper invasive ST11 clonal complex among group B disease (there were no fatal ST11 group B cases pre and 4.4% post immunisation). The *porA* and MLST data is important for group B vaccine development, which is informed by the monitoring of population shift among group B meningococci.

In addition the non culture sequence typing methods described here have subsequently been applied to case cluster and outbreak investigation by the MRU. Incidents involving patients diagnosed by both non culture and culture methods are now able to be resolved.

Acknowledgements

I wish to thank my supervisors, Professor Valerie Edwards-Jones and Professor Andrew J. Fox, for their help and support throughout the duration of this study. Professor Edwards-Jones's patience has been immeasurable and her direction essential in progressing the write-up.

Dr Andrew Birtles, whose help was invaluable in understanding the methods and beginning the write up.

My colleagues at the Meningococcal Reference Unit, particularly Dr Steve Gray, for their encouragement and support.

Mr. Andrew Walker, Manchester HPA Lab, for photographic expertise.

And finally my parents for their unswerving belief in me.

For

My children Brendan George and Melanie Frances Carr

Contents

Tables

Figures

Abbreviations

A	Adenine
bp	Base pairs
C	Cytosine
CCDC	Consultant in Communicable Disease Control
CDSC	Communicable Disease Surveillance Centre
Chemicals:-	
$MgCl_2$	Magnesium Chloride
Na_2EDTA	Disodium Ethylene Diamine Tetra acetic Acid
NaCl	Sodium Chloride
NaOAc	Sodium acetate
SO_2	Sulphur dioxide
Tris-HCl	Tris-Hydrochloric acid
CPS	Capsular Polysaccharide
CSF	Cerebrospinal Fluid
CT	The cycle number at which the measured fluorescent signal exceeds a calculated background threshold identifying amplification of the target sequence on the ABI 7700. The lower the value the greater the number of copies of the target sequence added to the PCR; a value of >45.0 is deemed to be negative amplification.
dNTP	Deoxynucleotide triphosphate
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
DNA	Deoxyribonucleic acid
DTCS	Dye Terminator Cycle Sequencing
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ESMD	Enhanced Surveillance of Meningococcal Disease
EMGM	European monitoring group for meningococci
ET	Electrophoretic type

FAM	6-carboxy-Fluorescein
G	Guanine
GCNS	Non selective gonnococcal agar
Genes:-	
abcZ	Putative ABC transporter gene
adk	Adenylate Kinase gene
aroE	Shikimate dehydrogenase gene
ctrA	Capsule transfer gene A
fetA	Iron-regulated outer-membrane protein
fumC	Fumarate hydratase gene
gdh	Glucose-6-phosphate dehydrogenase gene
pdhC	Pyruvate dehyrogenase Subunit gene
ply	Pneumolysin gene
porA	Meningococcal Class 1 protein gene
porB	Meningococcal Class 2/3 protein gene
pgm	Phosphoglucomutase gene
siaD	Sialic acid synthesis gene
Hib	*Haemophilus influenzae* type b
HPA	Health Protection Agency
HPU	Health Protection Unit
LPS	Lipopolysaccharides
LP	Lumbar puncture
Mab	Monoclonal Antibody
MCC	Meningococcal C Conjugate
MenC	Meningococcal C conjugate vaccine
MeNZB™	Meningococcal B vaccine for New Zealand
MLEE	Multi Locus Enzyme Electrophoresis
MLST	Multi Locus Sequence Typing
MRU	Meningococcal Reference Unit
NCAM	Human neuro cell adhesion molecule
NG	Non-groupable
NT	Non-typeable

OMP	Outer-membrane Protein
ONS	Office of National Statistics
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
REA	Restriction endonuclease analysis
RNA	Ribonucleic Acid
rRNA	ribosomal Ribonucleic Acid
SMPRL	Scottish Meningococcus and Pneumococcus reference Laboratory
ST	Sequence type
T	Thymine
TAMRA	6-carboxy-tetramethylrodamine
Taq	*Thermus aquaticus*
TBE	45 mM Tris borate, 1mM EDTA
TE buffer	10 mM Tris HCl, 1 mM disodium EDTA; pH 7.6
UK	United Kingdom
UV	Ultra-violet
VR	Variable region

INTRODUCTION

1.1 Meningococcal disease

Meningococcal disease is caused by invasive infection in the human population by the bacterium *Neisseria meningitidis*. This organism is carried in the nasopharynx of between 10 and 20% of the population in an asymptomatic carrier state (Caugant *et al,* 1994; Cartwright*,* 1995). It can cause invasive disease in some individuals by entering the bloodstream, causing bacteraemia. The spectrum of disease progression can then vary considerably from transient bacteraemia to fulminant septicaemia with or without meningitis, or meningitis alone (Steven and Wood, 1995).

Fulminant or invasive meningococcal disease can cause serious clinical sequelae with mortality and morbidity rates of between 5% and 13%. For meningococcal disease caused by *N. meningitidis* serogroup C, which caused approximately 40% of the disease in the United Kingdom prior to introduction of conjugate vaccine, the mortality rate was between 10 and 13%, whilst for group B disease, which caused 60% of disease prior to and >90% post immunisation, mortality was between 5 and 7% (Goldacre *et al*, 2003; Jensen *et al*, 2006).

Traditional microbiological confirmation of meningococcal infection is by isolation of the organism from blood culture, cerebrospinal fluid (CSF) or other sites such as throat swab or joint fluid. Commercially produced latex agglutination kits such as the Pastorex^{TM} Meningitis (BIO-RAD, France) may be used for the direct detection of meningococcal polysaccharide antigen

from clinical samples. Since the early 1980's, the Meningococcal Reference Unit (MRU) in Manchester has offered a national service of phenotypic characterisation of isolates, involving ascertainment of serogroup, serotype and serosubtype. Microbiology laboratories throughout England and Wales submit isolates identified biochemically as *N. meningitidis to* the MRU. The isolates are phenotypically characterised using both co-agglutination and enzyme-linked immunosorbent assay (ELISA) methods. This information is used in the epidemiological study of the disease including case cluster, outbreak investigation and endemic disease monitoring.

1.2 *Neisseria meningitidis*

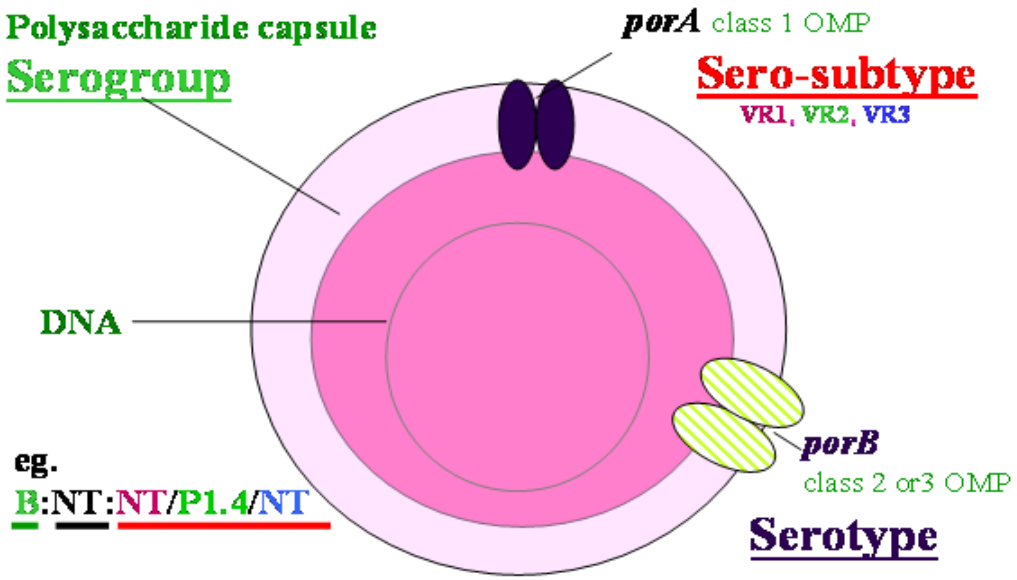
The organism is a Gram negative diplococcus which is oxidase positive. *Neisseria sp.* can be isolated from a range of both animal and human hosts, some as harmless commensals (*Neisseria lactamica*) or as exclusive human pathogens (*N. meningitidis* and *Neisseria gonorrhoeae*). *N. meningitidis* can cause invasive disease such as meningitis and septicaemia but can also be found colonising the nasopharyngeal mucous membranes in asymptomatic carriage in 10-20% of the population. Occasionally *N. meningitidis* can be isolated from sites such as the urethral mucosa and the eye (Cartwright, *et al* 1987).

1.3 Antigenic characterisation of *N. meningitidis*

The meningococcus has several surface structures which can be used for serological characterisation and typing of the organism (Figure 1.1). The extracellular polysaccharide capsule and outer membrane proteins are characterised by the MRU using dot-blot ELISA for all submitted isolates. The serogroup of meningococci is determined by the polysaccharide capsule produced extracellularly. The capsule is an important pathogenic determinant of the meningococcus and may be characterised as one of 13 serogroups: A, B, C, D, X, Y, Z, 29E, W135, H, I, K and L which have been described (Davis *et al,* 1980). Groups B, C, Y and W135 are responsible for invasive disease in the UK, whilst group A is responsible for the majority of disease globally. The other groups rarely cause invasive disease. Sialic acid is found in the polysaccharide capsule of all meningococcal serogroups apart from serogroup A, which contains mannosaminephosphate (Poolman *et al,* 1995).

The differing chemical composition of the polysaccharide capsule enables the serological classification of meningococci into one of the thirteen serogroups. Serogroup is routinely determined in the MRU using dot blot ELISA assays for serogroup A, B, C and W135, if negative in these assays, co-agglutination reagents produced in-house are used to further test by co-agglutination.

Figure 1.1 Diagram of meningococcal cell structure



This diagram of the cell structure shows the polysaccharide capsule and outer membrane proteins (OMP) used in phenotypic characterisation in the MRU. (personal communication, Dr S.J. Gray)

Reagents for co-agglutination serogroup testing are made using polyclonal anti-polysaccharide antibodies raised in rabbits which are bound to killed *Staphylococcus aureus* cells. Serogroup A, B, C, X, Y, Z, 29E and W135 can be ascertained (Eldridge *et al,* 1978). Serotype and serosubtype of meningococci are determined by antigenic outer membrane proteins of various classes expressed in the bacterial cell membrane. Serotype is determined by class 2 or class 3 *porB* proteins and sero sub-type by class 1 *porA* proteins.

Dot blot ELISA assays are used in the MRU to screen for the more commonly expressed *porA* and *porB* proteins but the great diversity of protein epitopes, particularly in group B organisms, and limited monoclonal antibody reagent panel leads to many isolates being reported as “not typeable” (NT) by phenotypic means. The phenotypic data (Table 1.1) is used for the epidemiological monitoring of meningococcal disease in England and Wales.

Table 1.1 *N. meningitidis* serogroups, serotypes and serosubtypes identified by dot blot ELISA.

	porA-sero-subtypes, VR1, 2 and 3.			*porB*
Serogroup	VR1	VR2	VR3	Sero-types
A	P1.5	P1.1	P1.6	3.1
B	P1.7	P1.2		2.2a
C	P1.12	P1.3		2.2b
W135	P1.19	P1.4		3.4p
		P1.9		3.4z
		P1.10		2.11
		P1.13		3.14
		P1.14		3.15
		P1.15		3.21
		P1.16		2.22

Table 1.1 illustrates the four serogroups, 15 serosubtypes (VR1, VR2 and VR3) and 10 serotypes which can be identified by dot blot ELISA used to serologically phenotype all *N. meningitidis* isolates received at the MRU (HPA, Manchester, UK).

1.4 Laboratory diagnosis of meningococcal disease

In routine microbiology laboratories confirmation of meningococcal disease is by culture of the meningococcus from normally sterile sites such as the CSF, blood or synovial fluid. The organism can be isolated from nose or throat swabs in a patient with disease, but if isolated from the throat the organism's presence may indicate asymptomatic carriage. Meningococci may also be isolated incidentally from other sites such as the genito-urinary tract and the respiratory tract. (Cartwright,1995).

Microbiology laboratories identify isolates as *N. meningitidis* using biochemical identification systems such as API NH (Biomerieux, France) and carry out antibiotic susceptibility testing to assist clinicians in the acute treatment of the patient. Some laboratories will also ascertain the serogroup of the organism using commercially produced latex agglutination kits such as Pastorex™ Meningitis (Bio-Rad Marnes-la-Coquette, France).

Public health action such as contact tracing, administration of prophylactic antibiotics, or immunisation of contacts, will be informed by the serogroup of an organism in a case of disease and is undertaken by the local Health Protection Unit (HPU) (Stuart *et al*, 2002). All laboratories in England and Wales submit isolates to the MRU for characterisation. These isolates are then included in the national culture archive to enable epidemiological monitoring of the disease, and to provide material for vaccine development.

1.5 Molecular detection of *N. meningitidis*

In the mid 1990s a greater awareness of meningococcal disease, clinical reluctance to perform lumbar puncture (LP) in infants due to the possibility of coning (brain damage caused by rapid release of high intracranial pressure) (Nadel, 2001), and the much earlier administration of antibiotics (Cartwright *et al*, 1992; Strange and Paugh, 1992) led to a decrease in laboratory confirmed cases. Notification of possible or probable cases remained at similar levels (Ragunathan *et al*, 2000), and the inability to grow the causative organism led to the consideration of non-culture methods. The development of polymerase chain reaction (PCR) based assays was undertaken in order to microbiologically confirm those cases where no isolate was available (Corless *et al*, 2001). Samples can be rendered sterile very rapidly following instigation of antibiotic therapy, in a matter of hours for CSF (Kanegaye *et al*, 2001) and case ascertainment by PCR would allow cases without culture confirmation to be included in surveillance of the disease (Hackett *et al*, 2002a).

Following trials of the PCR testing on a national basis the service was offered as a case confirmation service for England and Wales in 1996 (Kaczmarski *et al*, 1998). The sample types suitable for analysis include peripheral whole ethylenediaminetetraacetic acid (EDTA) blood, CSF, joint fluids, serum from clotted blood and other liquid samples. The proportion of cases ascertained solely by PCR detection has risen to around 50% (Figure 1.2).

Diagnosis by PCR testing involves an initial nucleic acid extraction process followed by PCR testing for a deoxyribonucleic acid (DNA) target specific to meningococci. The first chosen target was an insertion sequence, IS1106, of which there are several copies in the meningococcal genome, enhancing sensitivity of the assay. However following a small number of false positives (Borrow *et al*, 1998b) caused by IS1106 occasionally being found in other bacterial species, a PCR test for the meningococcal specific capsule transfer gene (*ctrA*) was developed and put into routine use by the MRU (Guiver *et al*, 2000).

Samples which tested positive using this meningococcal DNA screening PCR assay were further tested to ascertain the serogroup B or C which were the most common serogroups in the UK. The serogroup specific PCR assays target the sialic acid synthesis gene (*siaD*) responsible for polysaccharide capsule production (Borrow *et al*, 1997). Assays for the ascertainment of group Y and group W135 have also been introduced (Borrow *et al*, 1998a), and are used when samples are positive for meningococcal DNA but negative for group B and C. An assay for confirmation of serogroup A is also available.

Figure 1.2 Laboratory confirmed cases 1984 onwards, all serogroups.

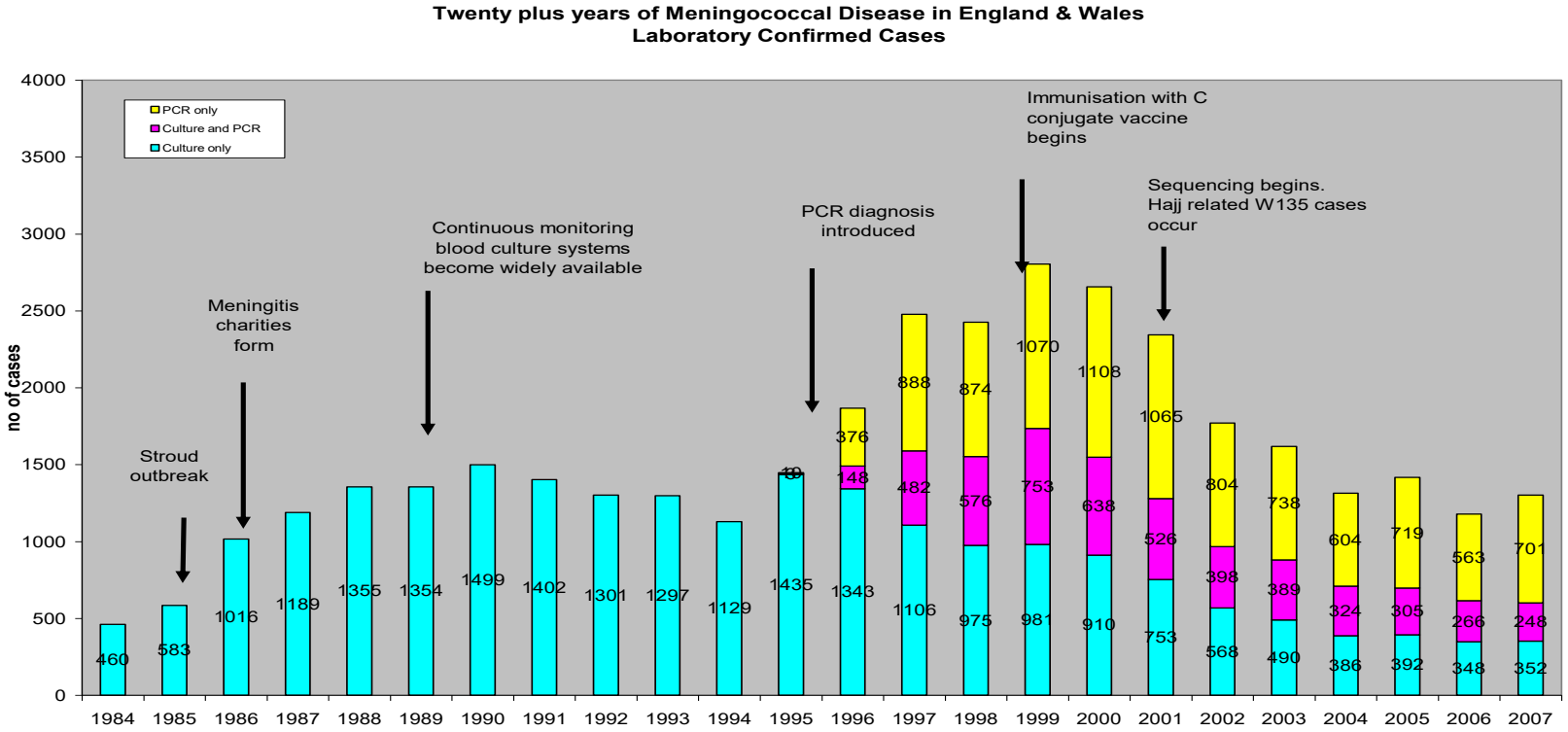


Figure 1.2 shows laboratory confirmed cases of meningococcal disease 1984-2007 with significant events highlighted. Data for groups B and C in appendices H and I. (Produced by Mr Richard Mallard from MRU data).

1.6 Molecular characterisation of *N. meningitidis.*

Several methods for molecular characterisation of meningococci have been described (Yakubu *et al*, 1994; Yakubu and Pennington, 1995; Swaminathan *et al*, 1996) including restriction endonuclease analysis (REA) and pulsed field gel electrophoresis (PFGE). These methods involve sizing cleaved DNA fragments separated by gel electrophoresis and comparison by fingerprint patterns using fragment sizing software programmes. The fragments can be hybridised with specific gene probes to allow greater discrimination of the patterns obtained. Multi locus enzyme electrophoresis (MLEE) was used to designate meningococci into clonal complex groups for population epidemiology, where organisms are related irrespective of their phenotypic properties. Such techniques have not been employed by the MRU for routine characterisation of meningococci due to the complex nature of the procedures involved.

In 1998, multi locus sequence typing (MLST) was described as a portable approach to designating pathogenic micro organisms into clonal complexes for epidemiological study (Maiden *et al*, 1998). The scheme includes methodology for meningococci, and uses DNA sequence analysis of a series (seven) of constitutive gene loci. For meningococci these loci are a putative ABC transporter (*abcZ*), adenylate kinase (*adk*), shikimate dehydrogenase (*aroE*), fumarate hydratase (*fumC*), glucose-6-phosphate dehydrogenase (*gdh*), pyruvate dehydrogenase subunit (*pdhC*) and phosphoglucomutase (*pgm*). The target genes are distributed over the

whole meningococcal genome and are not under immune selective pressure.

The PCR primers for amplification of these loci are described and products are processed in sequencing reactions. The fragments of the seven loci produced for MLST sequence analysis vary in size from 433 to 501 base pairs (bp). Sequence data are processed using DNA sequence editing software packages such as Sequencher™ and the base sequences submitted to the website www.mlst.net. For each organism a seven number code relating to the seven loci is produced which can then be allocated a sequence type and clonal complex on the website (Figure1.3).

Surface antigen genes which are under immune selective pressure may also be used for molecular typing; *por*A sequence typing is used for molecular characterisation of meningococci (Jolley *et al*, 2007; Russell *et al*, 2004). The limited panel of monoclonal antibodies in the dot-blot ELISA for typing and sub-typing means some isolates are reported as “not typeable”. Sequence typing commonly gives a result and hence more data for isolates where the ELISA method does not give a complete phenotype. The MRU targets variable regions one (VR1) and two (VR2) and data can be submitted on the website www.neisseria.org to obtain results after software analysis of the base sequence data.

Figure 1.3 mlst.net website allelic profile enquiry.

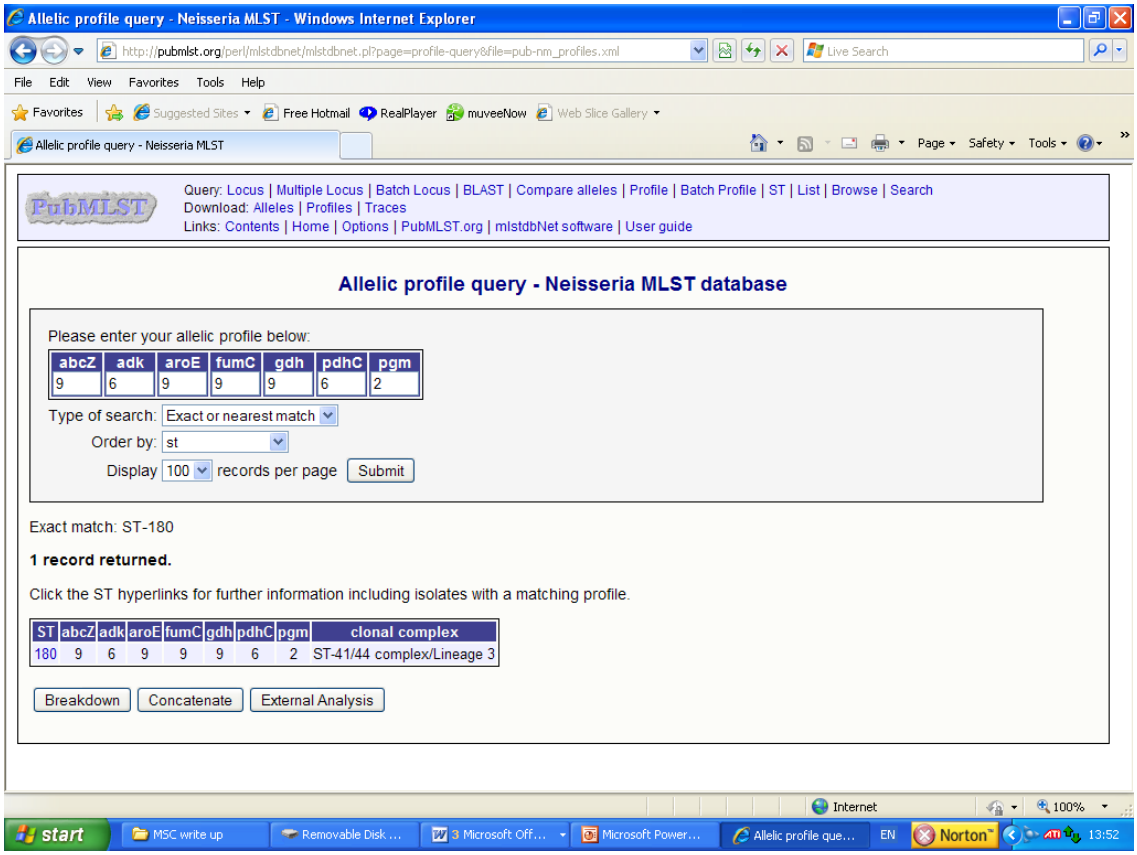


Figure 1.3 shows the pubmlst website being used to designate sequence type and clonal complex by submitting a seven locus allelic profile.

1.7 Non-culture molecular characterisation

Approximately 50% of cases of meningococcal disease are ascertained by PCR testing alone (Figure 1.2). In these patients no isolate is available for phenotypic characterisation and therefore serogroup is the only available epidemiological information. For study of possible case clusters, outbreaks and for longer term epidemiological study of the disease, it is important to have more information available (Caugent, 1998; Taha, 2002). Assays using enhanced DNA amplification from clinical samples have been reported on a limited number of samples (Kriz *et al*, 2002; Diggle and Clarke, 2003; Diggle *et al*, 2003). The MRU have previously designed assays in house to provide DNA sequence typing data direct from clinical samples (Birtles *et al*, 2005). These nested PCR assays have been applied in this study.

If laboratory diagnosis of meningococcal disease was by PCR alone, the new assays could be used to provide additional epidemiological data. Two rounds of amplification with different primer sets were carried out (Birtles *et al*, 2005). DNA products for subsequent DNA sequencing reactions for the seven gene targets of the MLST scheme, and for *por*A regions of the genome were produced. The sequence data could then be used along with phenotypic and genotypic data from isolated organisms in order to provide more complete surveillance data using both culture confirmed and non-culture cases.

In the epidemiological investigation of possible clusters and outbreaks of meningococcal disease cases may be related by close contact between

patients, such as in a school, nursery or tertiary education setting, or by close geographical relatedness. The information described can show whether the cases are caused an indistinguishable organism or to be unrelated sporadic cases of disease. The appropriate public health action can then be taken by Health Protection Unit infection control staff informed by the results from the MRU.

In clusters or outbreaks of group B disease the only action possible is the administration of prophylactic oral antibiotics to case contacts. The prophylaxis, with oral rifampicin or ciprofloxacin, eliminates nasopharyngeal carriage of meningococci in close contacts of a case or cases, and removes the infective organism from the at risk population. The risk of further cases is reduced by this action, but at the present time immunisation is not available as there are no polysaccharide group B vaccines available and multicomponent group B vaccines suitable for the diversity of UK group B infection are at the development stage only (Lewis *et al,* 2009).

For contacts of group C, Y or W135 cases immunisation is offered in addition to prophylaxis. Either the conjugate group C vaccine or the quadrivalent A.C.Y.W135 polysaccharide vaccine is given as appropriate (on a named patient basis).

1.8 Notification of meningococcal disease.

Meningococcal disease is notifiable, so clinicians treating a patient presenting with possible or probable meningococcal infection have a statutory duty to notify a “Proper Officer” under the Public Heath legislation of 1988. Notification is to the Health Protection Unit covering the area of the patient’s home post code, or to the HPU local to where the patient presents. This enables the Health Protection team, Consultants in communicable disease control (CCDC’s) and infection control specialist nursing staff, to take appropriate measures such as contact tracing (Stuart *et al*, 2002). Contacts of a case would be considered for antibiotic prophylaxis or immunisation, dependent upon laboratory confirmation of meningococcal disease and further epidemiological information provided by the MRU.

Cases of disease thought to have a link or connection to others by close “kissing” contact or geographical proximity may be further investigated both by the HPU and MRU to ascertain if public health action is necessary. For instance more than one case in a school or nursery would be investigated to see if the causative organisms were distinguishable, and several cases within a close geographical area may be investigated to look for the possibility of endemic disease.

As group C disease was the cause of a majority of the case clusters prior to the success of the immunisation campaign there have been fewer investigations in the recent past. Case clusters or related cases with group B disease are less common, but investigations are still called for. In any

investigation the proportion of cases which are confirmed microbiologically by PCR detection alone will be approximately 50%, and without non-culture sequence typing data on these cases only the serogroup will be known. Further information is important to inform public health action in dealing with case clusters.

Both the Health Protection Agency (HPA) and the Office of National Statistics (ONS) are involved in the collection of data for meningococcal disease nationally. This includes laboratory confirmed versus reported cases, distribution of cases by serogroup, mortality and morbidity rates and disease by age group.

1.9 Meningococcal vaccination programmes.

Before the development and introduction of the conjugate group C vaccine in the UK only plain polysaccharide meningococcal vaccines were available (Frasch, 1995). The success of the programme in the UK (Miller *et al*, 2001; Balmer *et al*, 2002; Trotter *et al*, 2002) has led to national vaccination programmes in other countries such as Holland (De Greef et al, 2003) and Canada (Bettinger *et al*, 2009). Conjugation had previously proved successful for *Haemophilus influenzae* type b (Hib) in many countries (Laval *et al*, 2003) and subsequently for *Streptococcus pneumoniae* (Trotter *et al,* 2008). Some adjustments have been made to the programmes following surveillance to optimise long term protection (Cameron and Peabody, 2006).

Other areas of the World have problems with epidemic group A, W135 and X meningococcal disease, particularly in the African meningitis belt where epidemics can reach very high incidence (Harrison *et al*, 2009). Vaccination with group A (Marc Laforce *et al*, 2009) and quadrivalent (A, C, Y and W135) conjugate vaccines (Zimmer and Stephens 2004; Pace *et al*, 2009) may prove effective if successfully introduced.

Meningococcal group B polysaccharide is not a suitable candidate for immunisation, even when conjugated. This is largely due to its low immunogenicity; it is chemically very similar to human neuro cell adhesion molecule (NCAM) (Lewis *et al* 2009). This has led to the study of other antigens as possible vaccine candidates for immunisation against group B disease. In New-Zealand epidemic group B disease caused by a particular clone (Martin *et al,* 1998) has been addressed using a vaccine incorporating antigens from the prevalent strain (Ameratunga *et al,* 2005; Dyet *et al,* 2005). Other work includes study of the possibility of using *Neisseria lactamica* as a vaccine candidate (Gorringe *et al*, 2005).

In the UK, disease causing group B meningococci are antigenically diverse, and vaccines using combinations of outer membrane proteins are under trial at the present time (Lewis *et al* 2009). Studies being undertaken includes ascertaining if any cross protection against other clones is instigated (Morley *et al*, 2001). At the present time there is still no group B vaccine programme in the UK.

1.10 Surveillance of meningococcal disease.

Following the licensing and introduction of the new conjugated group C (MenC) vaccines in November 1999 (Salisbury, 2001; Trotter *et al*, 2002), a marked fall in group C cases was noted (Figure 1.4). Prior to immunisation the burden of group C disease had been estimated to inform the strategy (Davison *et al*, 2002). The vaccine was introduced into the infant immunisation programme with three doses at 2, 4 and 6 months, following which a catch up programme for up to age 17 years was also implemented. This was then extended up to age 25 years. One dose of the vaccine is sufficient to provide protection in immunocompetent children and adults (Miller *et al*, 2001).

Any laboratory confirmed group C cases in patients in the target age range were followed up to ascertain immunisation history and any underlying immunological pathology. This would enable immunisation failures to be identified and the efficacy of the vaccine to be informed. The enhanced surveillance was carried out by the communicable disease surveillance centre CDSC (Shigematsu *et al*, 2002). In the same timescale, an increase in the numbers of group B meningococcal disease cases was noted (Figure 1.5). The increase may have been the result of the emergence of more invasive or hyper virulent clones of group B meningococci or by increased use of non-culture detection methods.

Figure 1.4 Meningococcal cases by serogroup 1997-2008

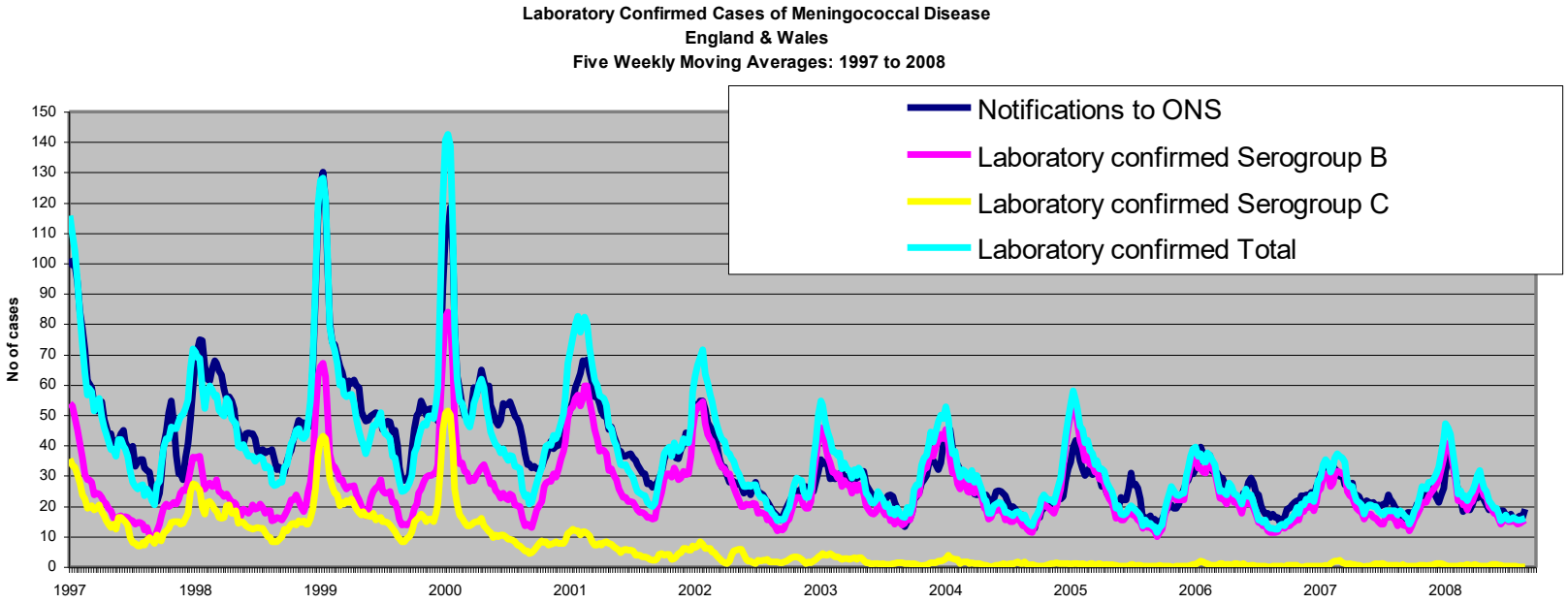


Figure 1.4 shows the five week moving averages for serogroup B and C laboratory confirmed meningococcal disease cases from 1997 to 2008 in England and Wales. The marked reduction of serogroup C cases following the immunisation programme is demonstrated (Produced by Mr Richard Mallard from MRU data).

Figure 1.5 Group B cases by calendar Year 1998-2006

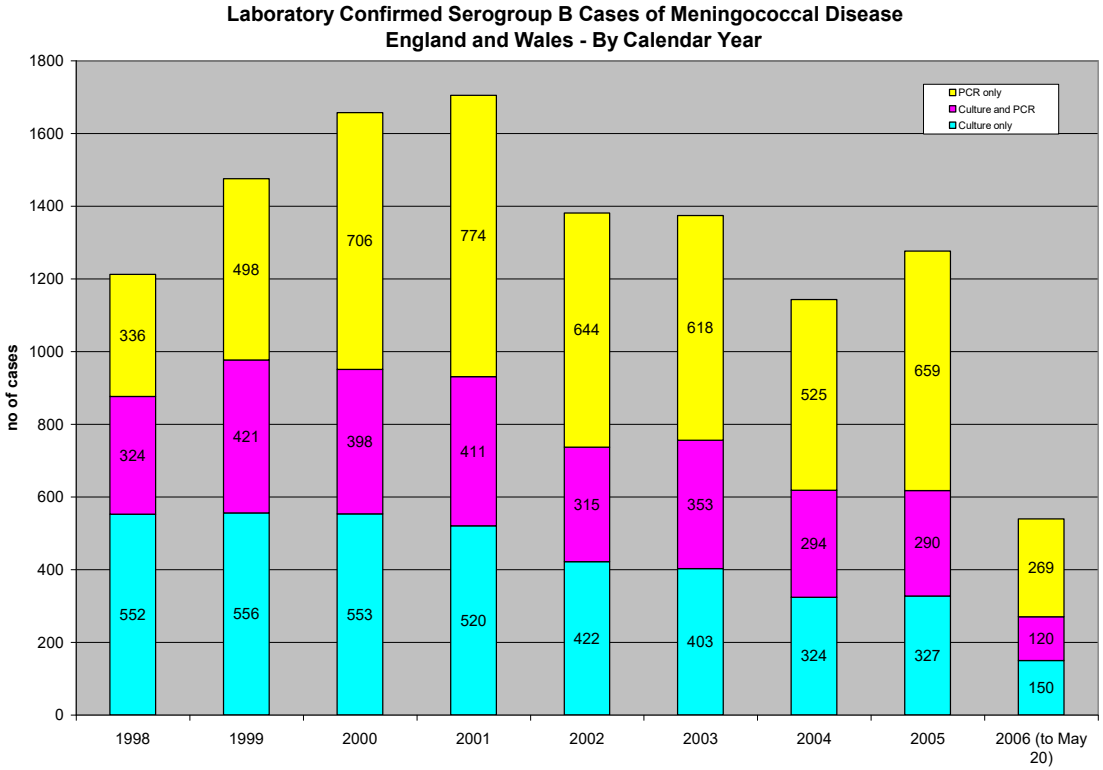


Figure 1.5 shows the increase in group B cases following introduction of MenC vaccine in November 1999, MRU data. (Appendix E)

A reason for the perceived increase in group B cases has yet to be ascertained. The possibility of capsular switching (Swartley *et al* 1997, Stefanelli *et al* 2003,Tsang *et al* 2005, Lancellotti *et al* 2006) which could lead to such a population shift was considered in the lead up to the immunisation programme.

1.11 Impact of surveillance on group B meningococcal vaccination.

Group B meningococcal genotypes and antigenic types tend to show cycling over time even without such factors as immunisation pressure (Russell *et al*, 2008). For instance the endemic group B disease in the Stroud area in the 1980's, which led to the formation of the "meningitis" charities, was caused by clonal complex sequence type (ST) 32/ electrophoretic type (ET) 5 group B meningococci. This clone has become less common over time whilst others (particularly ST41/44Lineage3 and ST269) have increased (Gray *et al*, 2006). Herd immunity (where a population is protected against an infectious disease by the immunity of some individuals causing less spread of the infectious agent) against common antigenic types may be responsible for these phenomena. There is a contrast with group C disease which was largely caused by the hyper invasive ST11/ET37 complex, phenotypically group C, type 2a, subtype P1.5; P1.2. As shown in Figure 3.5 the group C isolates in the year prior to immunisation were largely ST11/ET37 (78.5%), with only ST8clusterA4 complex having any significant number (15.3%) apart from this. Within the ST11/ET37 complex 91% were sequence type ST11. In contrast the diversity of group B isolates is shown. Three clonal

complexes contributed significantly (ST 41/44lineage3 complex being 37.6% and ST269 complex 22.0%. ST32/ET5 complex showed 14.2%) but a wide range of clonal complexes were found. Within these major complexes there was great diversity of sequence type, for 1998-1999 among the ST41/44Lineage3 complex 110 distinct sequence types were identified. Different sequence types within a clonal complex may differ by as little as one base pair, and for this reason ST is not used epidemiologically.

The difference in epidemiology impacts significantly upon group B vaccine design (Urwin *et al*, 2004) particularly as group B polysaccharide is not a suitable vaccine candidate. Group C polysaccharide has been successfully conjugated and the vaccine is effective against all Group C strains, whilst group B vaccine research is ongoing (Perrett and Pollard, 2005; Lewis *et al*, 2009), with no introduction in the UK to date. To cover a significant proportion of group B meningococcal disease in the UK multicomponent vaccines are being researched and trialled (Holst *et al*, 2009; Lewis *et al*, 2009).

There has been some use of narrower spectrum group B vaccines particularly in New Zealand where a national immunisation campaign with a vaccine (MeNZB™) against a particular epidemic strain of the ST41/44 lineage3 complex has been implemented (Ameratunga *et al,* 2005; Dyet *et al,* 2005). This New Zealand epidemic strain has a phenotype of B:4:P1.4 and a *porA* genotype of VR1 7-2,VR2 4. The vaccine consists of outer membrane vesicles and is not conjugated. A different vaccine has been

used in the Seine-Martime department in France where an ST32/ET5 complex epidemic strain with the phenotype B:14:P1.7,16 and a *porA* genotype of VR1 7, VR2 16 was targeted (Rouaud *et al*, 2006). The vaccine used in this programme was the MenBvac® which was developed and used in Norway (Bjune *et al*, 1991; Hoiby *et al*, 1991) in the late 1980s to address a high incidence of group B meningococcal disease (120/100,000) with a case fatality ratio of 10%. This was the highest incidence of meningococcal disease in Europe at the time, and was due to an ST32/ET5 group B organism with a phenotype of B:15:P1.7,16. This meningococcus differs in phenotype from the French epidemic strain by serotype (14 rather than 15). The French programme of immunisation followed initial antibiotic prophylaxis of 8000 people as a control measure for one particular case cluster, and an increase to 3.4/100,000 in the incidence of meningococcal disease for the area (Rouaud *et al*, 2006). Assessment of the impact of the programme is awaited.

This Msc study of fatal serogroup B cases was instigated due to a rise in group B cases subsequent to the group C immunisation programme. One thousand four hundred and seventy five cases were seen in 1999, 1659 in 2000 and 1710 in 2001. There was concern that this rise could be attributable to a shift in the epidemiology of group B disease and possibly emergence of more hyper virulent clones of group B meningococci (Urwin *et al*, 2004). The plasticity of the meningococcal genome does lead to antigenic shift, but this usually takes place over extended time periods (Morelli *et al*, 1997;Kyaw *et al*, 2002). An event such as the MenC

immunisation programme, having such an impact, could alter disease epidemiology. Capsule switching among meningococci has been proposed (Swartley *et al*, 1997; Vogel *et al*, 2000a; Stefanelli *et al*, 2003; Tsang *et al*, 2005; Lancellotti *et al*, 2006), although a single organism with the capability to produce more than one polysaccharide capsule has yet to be found. A particular concern in the lead-up to the immunisation programme was that capsular switching from C to B among the ST11/ET37 complex would, if it became apparent, significantly undermine the programme. This form of group B has been seen historically in the UK (Russell *et al*, 2008) but is uncommon, only 0.7% (1998-99) and 1.0% (2000-01) of the group B isolates included in figure 3.5 were ST11/ET37 complex.

A factor which could impact upon immunisation for meningococcal disease is the global transport of strains unfamiliar to the indigenous population, and hence not covered by national immunisation policy. This was observed in 2000 and 2001 when serogroup W135 meningococcal disease of ST11/ET37 complex was disseminated from Mecca in Saudi Arabia following the Hajj pilgrimage (WHO 2001; Hahne *et al*, 2002). This organism causes disease in the African meningitis belt (Nicolas *et al*, 2005) but is rarely isolated in the UK. High case fatality was noted and secondary cases became apparent as the outbreak progressed. As the causative organism has, apart from serogroup, the same phenotype as the ST11/ET37 group C clone found in the UK prior to MenC immunisation, it would become a serious issue if it became endemic. The MRU used non culture sequence typing to designate W135 cases as Hajj related (Birtles *et al*, 2005) if no

organism was isolated. An indigenous serogroup W135 organism is found in the UK which is antigenically distinct (*porA* VR1 18-1, VR2 3) and of a distinct clonal complex (ST22). ST11/ET37 group W135 meningococci had been seen in the UK in the 1970s (Russell *et al*, 2008) but has been uncommon since then. Following a change in immunisation advice for pilgrims travelling to Hajj post 2001 (Wilder-Smith and Memish, 2003) the imported ST11/ET37 serogroup W135 organism was rarely seen. The importation of meningococci from Hajj had been seen previously (Jones and Sutcliffe, 1990) when group A disease was involved. The earlier immunisation advice was for pilgrims to have A/C polysaccharide vaccine, and the change in 2001 was for the quadrivalent (ACYW135) vaccine to be recommended.

Multicomponent group B vaccines currently under trial in the UK are designed to provide broad spectrum cover. They may, however, not cover all the observed antigenic variants causing group B disease. Clones not covered by the vaccines could proliferate. This is being observed with the multivalent pneumococcal vaccines, where those pneumococcal types not covered are beginning to be seen in larger numbers than pre-immunisation (Singleton *et al*, 2007). The importance of enhanced surveillance around the introduction of group B meningococcal vaccines is highlighted by this possibility.

A large proportion of group C meningococcal cases in England and Wales prior to the immunisation programme were caused by a particular group C

clone. The phenotype was recognisable as C:2a:P1.5,P1.2, and these organisms belonged to the hyper virulent ST11/ET37 clone (Gray *et al* 2006). Other hyper virulent group C clones have been observed such as ST11/ET15 in Canada (Tyler and Tsang, 2004) and have also been observed to cause high fatality rates (Smith *et al*, 2006). The group B meningococcal population is much more diverse, (Ashton and Caugent, 2002) showing a wide range of sequence types with a distribution which changes with time (Kyaw *et al*, 2002)

1.12 National carriage study.

Carriage of meningococci has been studied in the past and used to investigate the epidemiology of the disease (Cartwright *et al*, 1987; Caugent *et al*, 1994; Ala'Aldeen *et al*, 2000). A national meningococcal carriage study was undertaken both pre and post immunisation to assess the effects of the MenC vaccine programme on carriage of meningococci in the UK. The study involved six centres nationally and throat swabs were obtained from adolescents. The carriage rate of meningococci is between 10 and 20% of which 1% is serogroup C. therefore a large number of swabs (over 13,000 each year) were collected (Maiden *et al* 2002).

Swabbing was undertaken at secondary schools and sixth form colleges and was done prior to the immunisation programme (in 1999), and again in 2000 and 2001. All the pupils should have been immunised as part of the catch up programme with one dose of the new conjugate group C vaccine. Swabs were anonymised and all meningococci isolated were sent to the MRU for

phenotypic characterisation. The study showed a reduction in group C carriage by 70%, indicating that as well as reducing group C disease vaccination had altered the carriage population of meningococci (Maiden *et al*, 2002; Trotter *et al*, 2005). The niche created by this phenomenon could be exploited by any shift to more hypervirulent group B meningococcal strains (De Filippis and Vincente, 2005). Non-culture molecular characterisation methods applied before and after the vaccination programme will further enhance surveillance of group B meningococcal disease and, along with data obtained from isolates, enable reasons for observed phenomena to be investigated.

1.13 Aims.

This study aimed to enhance the surveillance of group B meningococcal disease around the introduction of new conjugate group C vaccines in a national immunisation programme, which began in November 1999, by the application of non-culture multilocus and *porA* sequence typing.

To provide further epidemiological data on non-culture (PCR) confirmed fatal cases of group B meningococcal disease in the years 1999-2001.

To determine whether the strain composition amongst fatal group B cases changed during the period of study.

To examine other MRU data from the same time period.

Chapter 2: Materials and Methods

2.1 Stored material from meningococcal positive PCR samples.

Under the specimen retention policy for the Meningococcal Reference Unit nucleic acid extracts, obtained by Generation® capture column methodology (EDTA blood) or DNAzol extraction (CSF and serum), were archive stored at –20°C. Original samples were also archived and meningococcal DNA positive samples were stored long-term at -80°C. Isolates of meningococci were also archived using a commercial glycerol broth (Microbank, Prolab Diagnostics, Canada) stored indefinitely at -80°C, and a National collection of case isolates with material dating back to the late 1970s was maintained.

The retention policy aims to retain all positive material for confirmatory re-testing, prospective testing of alternative test methods, and retrospective testing for further epidemiological markers (as part of case cluster or outbreak investigations), long term epidemiological study of meningococcal disease and approved research projects.

2.2 Provision of demographic data.

The Communicable Disease Surveillance Centre (CDSC) provided data on laboratory confirmed fatal group B meningococcal disease cases for 1999 (n=79), 2000 (n=88) and 2001 (n=111). Table 2.1 below describes the diagnostic confirmation details and method involved per year.

Table 2.1 Laboratory confirmation in the three years of study

Year	Fatal group B cases	Culture confirmed	PCR only	Percentage PCR only
1999	79	48	31	39
2000	88	51	37	42
2001	111	65	46	41

This information was gathered retrospectively as reported cases of probable and possible meningococcal disease were followed up to ascertain clinical outcome. Morbidity and mortality/case fatality ratio figures were obtained. At a time of reducing disease burden due to the success of the group C immunisation campaign the observed trend of increasing numbers of group B cases was of particular interest.

All the positive results, by isolate and PCR testing (or both) are held on a secondary database at the MRU which can be interrogated to provide further data. This is part of the National epidemiological monitoring of meningococcal disease. Some of the samples from the study period were

missing. This was because the sample was all used for analysis as a result of small initial sample volume or having been removed for an approved study involving human genomics (Read *et al* 2009). Six, eight and three samples were missing from 1999, 2000 and 2001 respectively and were not available for inclusion in this study.

2.3 Phenotypic sero-typing and serogrouping methods routinely employed in the MRU for *N. meningitidis* isolates.

2.3.1 Dot-blot ELISA.

A suspension of *N. meningitidis* from the surface of a non selective *Neisseria gonorrhoeae* agar (GCNS) plate with 10% (v/v) lysed horse blood (Oxoid Ltd, Basingstoke, Hampshire, UK code PB1052A) was made in 3 ml of 0.85% (w/v) saline (Oxoid Ltd, Basingstoke, Hampshire, UK code EB0334B) to MacFarland standard 2.0 inside a class 2 safety cabinet. The suspensions were then placed in a water bath at 60^{o}C (Grant Instruments (Cambridge) Ltd, Royston, Hertfordshire, UK) for 60 minutes. The suspensions were then stored at 4^{o}C for up to 1 week after heat treatment if not used immediately. A nitrocellulose strip (2mm by 11cm) (Bio-Rad, Hemel Hempstead, Hertfordshire, UK) was labelled for each of the 29 available monoclonal antibodies, comprising serogroups, serotypes and serosubtypes.

1μl of meningococcal suspension was spotted onto each strip and allowed to air dry for 5 minutes; if more than one isolate was being typed the suspensions from the different organisms were spaced evenly down the strip. The strips were then placed in divided trays (one division for each

monoclonal antibody) and 2ml of a 4% (w/v) skimmed milk powder in PBS blocking solution was added to each division and rocked on a platform (Camlab Ltd, Over, Cambridge, UK) for 30 minutes. The strips were then washed twice with 5 ml of 0.85% (w/v) saline. Using a separate 1 ml graduated pastette (Alpha Laboratories, Eastleigh, Hampshire, UK) for each one, 1 ml of diluted serotyping or serogrouping monoclonal antibody (mab) was added to the corresponding marked strip and mixed by rocking for 60 minutes. The strips were washed twice with 5 ml of 0.85% (w/v) saline. A 1 ml aliquot of diluted protein A-peroxidase conjugate (www.sigma-aldrich.com) was added to the serotyping and sub-typing strips, 1 ml of diluted anti-mouse IgG conjugate (www.sigma-aldrich.com) was added to the serogroup A and C strips and 1 ml of diluted goat anti-mouse IgM conjugate (AbD serotech www.ab-direct.com) was added to the serogroup B strips. The substrate was removed after 60 minutes mixing and the strips washed twice with 5 ml of 0.85% (w/v) saline. 1 ml of colour developer substrate was added to each strip and mixed by rocking for 5 minutes. The substrate was removed and the strips washed with 10 ml of tap water. The strips were then examined, a dark purple/black spot representing a positive reaction for a particular serogroup or serotype/sub-type. An example of a completed assay is shown in figure 2.1.

2.3.2 Serogrouping of *N. meningitidis* using co-agglutination.

A suspension of the *N. meningitidis* was harvested from growth on a GCNS base agar with 10% (v/v) lysed horse blood plate (Oxoid Ltd, Basingstoke, Hampshire, UK ref PB1052A) to approximately MacFarland standard 2.0 in 3 ml of 0.85% (w/v) saline (Oxoid Ltd, Basingstoke, Hampshire, UK ref EB0334B) in a class 2 microbiological safety cabinet. Three drops of 38% formaldehyde (WWR International, Poole UK Prod 101134A) coloured with crystal violet stain (Pro-Lab diagnostics UK) was added and the suspension left to stand for 5 minutes. A single drop (≈ 40 µl) of each serogroup suspension B, C, X, Y, Z, 29E and W135 was added to the well corresponding to that serogroup in large well haemaglutination trays using a 1 ml graduated pastette. For more than one test organism the suspensions were added in columns. A single drop (≈ 40 µl) of the *N. meningitidis* suspension was added to each well in a row. The tray was gently rocked/ tapped for up to 2 minutes in the class 2 safety cabinet and the co-agglutination read over a black surface using side illumination from a bench lamp. (Figure 2.2)

Figure 2.1 Dot blot ELISA assay.

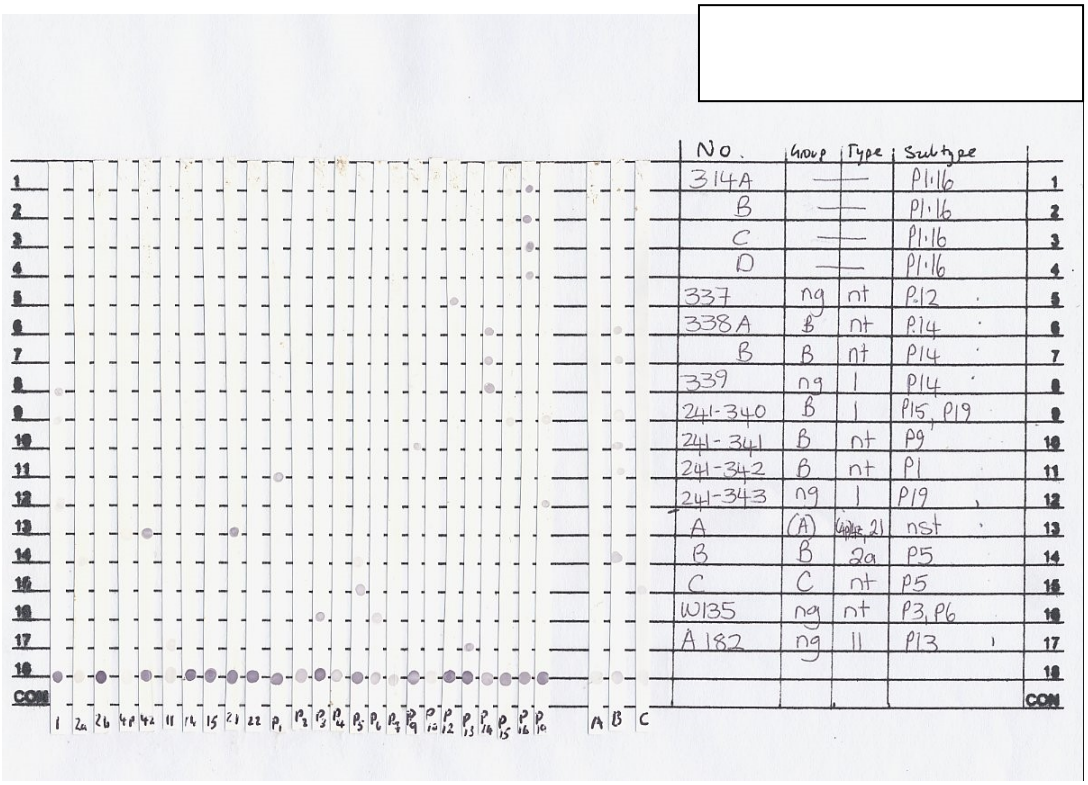


Figure 2.1 shows a completed Dot-Blot ELISA assay showing group, type and subtype results (prior to the introduction of group W135 by ELISA)

Figure 2.2 Co-agglutination to determine serogroup of meningococci

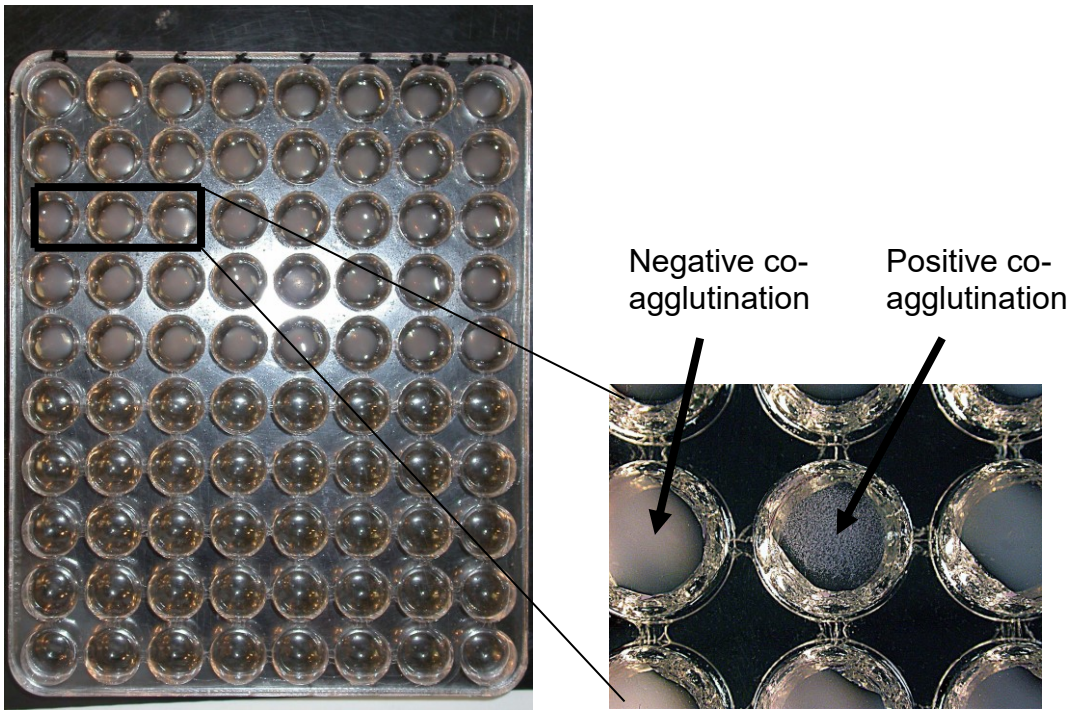


Figure 2.2 shows co-agglutination grouping of five isolates. This method was used if dot-blot ELISA assays failed to identify the serogroup.

2.4 Nucleic acid extraction methods from clinical samples in the MRU.

2.4.1 EDTA blood samples.

A Generation® capture column was placed into a 1.5 ml Generation® waste collection tube, 200 µl of EDTA blood sample was then added to the centre of the column and incubated for 1 minute at room temperature. Next, 400 µl of Generation® nucleic acid purification solution was added to the capture column, which was left to incubate for a further minute at room temperature. The column was centrifuged at 12,631 xg for 10 seconds in a Biofuge Primo R (Kendro Laboratory Products, Hanau, Germany) after which the waste collection tube was replaced with a fresh one. A further 400 µl of nucleic acid purification solution was added to the column followed by incubation and centrifugation as described above, 200 µl of Generation® nucleic acid elution solution was then added to the column which was centrifuged immediately at 12,641 xg for 10 seconds. The capture column was transferred to a nucleic acid collection tube and 200 µl of nucleic acid elution solution added and incubated at 99^{o}C for ten minutes on a QBT2 heating block (Grant Instruments Ltd, Cambridge, UK) heating block. The sample was centrifuged at 12,641 xg for 20 seconds. The capture column was discarded leaving the nucleic acid in the collection tube ready for analysis or storage at -20^{o}C (according to the MRU specimen retention policy).

2.4.2 Cerebrospinal fluid and serum.

A 1 ml aliquot of DNAzol (Invitrogen, Paisley, Scotland) was placed in a 1.5 ml micro centrifuge tube (Sarstedt, Beaumont Leys, Leicester, UK), 100 µl of CSF sample or standardized bacterial suspension was then added. After vortexing using a MS2 minishaker (IKA®, Staufen, Germany) the samples were left to incubate at room temperature for 5 minutes. Next 0.5ml of 95% ethanol (v/v) (Sigma-Aldrich, Gillingham, Dorset, UK) was added to each sample and the samples left to incubate for a further 10 minutes at room temperature. The samples were then centrifuged at 13,684 xg in a Biofuge Primo R (Kendro Laboratory Products, Hanau, Germany) for 10 minutes after which the supernatant was removed using a extended pastette (Alpha laboratories, Eastleigh, Hampshire, UK) to leave a pellet. After adding 1 ml of 75% cold ethanol (v/v) to each sample and centrifuging for 5 minutes at 13,684 xg the supernatant was removed using a extended pastette and the pellet re-suspended in 50 µl of molecular grade water (Sigma-Aldrich, Gillingham, Dorset, UK). The pellet was dissolved by incubating at 60^{o}C for 10 minutes in a QBT2 heating block (Grant Instruments Ltd, Cambridge, UK); the DNA sample was then ready for analysis or storage at -20^{o}C (according to the MRU specimen retention policy).

2.5 Real-Time PCR detection and genogrouping of *N. meningitidis* offered as a national diagnostic test at the MRU.

2.5.1 Detection of *N. meningitidis* and *S. pneumoniae* using the ABI Prism® 7700 sequence detection system.

Detection of *N. meningitidis* and *S. pneumoniae* DNA was carried out as a duplex real-time PCR assay as developed by Corless *et al*. (2001), using the *ctrA* gene target for *N. meningitidis* and the *ply* gene target for *S. pneumoniae* (the pneumococcal result is only reported if positive or if specifically requested by the sender). For a single PCR, a reaction mix was prepared as follows: 2.5µl of *N. meningitidis* primer mix (6 µM concentration of both forward and reverse *ctrA* primers (*ctrA*-F and *ctrA*-R), 2.5 µl of *S. pneumoniae* primer mix (6 µM concentration of both forward and reverse *ply* primers (*ply*-F and *ply*-R), 2.5 µl of probe mix (2 µM concentration of both *ctrA* and *ply* probes (*ctrA*-PROBE and *ply*-PROBE), 12.5 µl of 2X TaqMan® Universal Master mix (Applied Biosystems, Warrington, Cheshire, UK) consisting of 5.5 mM $MgCl_2$, 200 µM each of deoxynucleoside triphosphates (dNTP), dATP, dCTP, dGTP, dUTP and 0.125 units of AmpliTaq GOLD™. Finally 3 µl of molecular grade water (Sigma-Aldrich, Gillingham, Dorset, UK) was added and the reaction mix was inoculated with 2 µl of the DNA extract of interest. The reaction mix sample was placed into a well of a MicroAmp® Optical 96 well reaction plate with bar code (Applied Biosystems, Warrington, Cheshire, UK) and sealed with ABI Prism™ optical caps (Applied Biosystems, Warrington, Cheshire, UK). The plate was placed on an ABI Prism® 7700 Sequence Detection system and subjected to the thermal cycling conditions listed in Table 2.2. The results were analyzed

using ABI Prism® Sequence Detection software v1.7 (Applied Biosystems, Warrington, Cheshire, UK).

TaqMan® *ctrA* gene target primers and probe, incorporating 6-carboxy-fluorescein (FAM) reporter dye quenched by 6-carboxy-tetramethylrodamine (TAMRA), for detection of *N. meningitidis* DNA:

ctrA-F 5'-GCTGCGGTAGGTGGTTCAA-3'

ctrA-R 5'-TTGTCGCGGATTTGCAACTA-3'

ctrA-PROBE 5'-FAM-CATTGCCACGTGTCAGCTGCACAT-TAMRA-3'

TaqMan® *ply* gene target primers and probe, VIC™ reporter dye (Applied Biosystems, Warrington, Cheshire, UK) quenched by TAMRA, for detection of *S. pneumoniae* DNA:

ply-F 5'-TGCAGAGCGTCCTTTGGTCTAT-3'

ply-R 5'-CTCTTACTCGTGGTTTCCAACTTGA-3'

ply-PROBE 5'-VIC-TGGCGCCCATAAGCAACACTCGAA-TAMRA-3'

Table 2.2 Thermal cycling conditions for the ABI Prism® 7700 sequence detection system.

Stage	Temp (°C)	Time (Min:Sec)	No of cycles
1	50	2:00	1
2	95	10:00	1
3	95 60	00:15 1:00	45

Table 2.1 illustrates the thermal cycling conditions for dual labelled probe detection of *N. meningitidis* and *S. pneumoniae* as well as genogrouping of *N. meningitidis* using the ABI Prism® 7700 sequence detection system.

2.5.2 Genogrouping of *N. meningitidis* using the ABI Prism® 7700 sequence detection system.

Genogrouping of *N. meningitidis* DNA was carried out as a single target real-time PCR assay for meningococcal groups B and C and as a duplex real-time PCR assay for meningococcal groups Y and W135 (Taha *et al*., 2005), with all primer and probe sets using the *siaD* gene target to determine genogroup. The primers and probes for all four meningococcal groups are listed below. For a single PCR to identify meningococcal groups B or C, a reaction mix was prepared as follows: 2.5 µl of forward primer (3 µM concentration), 2.5 µl of reverse primer (3 µM concentration), 2.5 µl of probe (1 µM concentration of probe), 12.5 µl of 2X TaqMan® Universal Master mix (Applied Biosystems, Warrington, Cheshire, UK), 3 µl of molecular grade water and inoculated with 2 µl of the DNA extract of interest.

For a single duplex PCR to identify meningococcal types Y and W135 a reaction mix was prepared as follows: 1.25 µl of forward primer (identical primer used for both Y and W135 *siaD* targets) (4 µM concentration), 1.25 µl of *siaD* (Y) reverse primer (4 µM concentration), 1.25 µl of *siaD* (W135) reverse primer (4 µM concentration),1.25 µl *siaD* (W135)-probe (2 µM concentration of probe), 1.25 µl of *siaD (*W135)-probe (2 µM concentration of probe), 12.5 µl of 2X TaqMan® Universal Master mix (Applied Biosystems, Warrington, Cheshire, UK), 4.25 µl of molecular grade water and inoculated with 2 µl of the DNA extract of interest.

A reaction mix sample was then placed into a well of a MicroAmp® Optical 96 well reaction plate with bar code (Applied Biosystems, Warrington, Cheshire, UK) and sealed with ABI Prism™ optical caps (Applied Biosystems, Warrington, Cheshire, UK). The plate was then placed on an ABI Prism® 7700 Sequence Detection system and subjected to the thermal cycling conditions listed in Table 2.1. The results were then analyzed using ABI Prism® Sequence Detection software v1.7.

TaqMan® *siaD* gene target Primers and probes, incorporating 6-carboxy-fluorescein (FAM) or VIC™ (Applied Biosystems, Warrington, Cheshire, UK) reporter dyes both quenched by TAMRA, for detection of *N. meningitidis* genogroups B, C, W135 and Y:

N. meningitidis genogroup B

siaD (B)-F 5'-TGCATGTCCCCTTTCCTGA-3'
siaD (B)-R 5'-AATGGGGTAGCGTTGACTAACAA-3'
siaD (B)-PROBE
5'- FAM –TGCTTATTCCTCCAGCATGCGCAAA-TAMRA-3'

N. meningitidis genogroup C

siaD(C)-F 5'-GATAAATTTGATATTTTGCATGTAGCTTTC-3'
siaD(C)-R 5'-TGAGATATGCGGTATTTGTCTTGAAT-3'
siaD(C)-PROBE
5'- FAM –TTGGCTTGTGCTAATCCCGCCTGA-TAMRA -3'
N meningitidis genogroup Y

siaD(Y/W135)-F 5'-GGTGAATCTTCCGAGCAGGA-3'

siaD(Y)-R 5'-GGGATATCGTACACCATACCCTCTAG-3'

siaD(Y)-PROBE

5'-FAM –AGCCTTCGCTTTGAGACGTCATGATTAGGATATCTG-TAMRA -3'

N. meningitidis genogroup W135

siaD(Y/W135) 5'-GGTGAATCTTCCGAGCAGGA-3'

siaD (W135) 5'-GAATATCATACACCATGCCTTCCATA-3'

siaD (W135)-PROBE

5'-VIC-ATCCCTCACTTTCTGATGTCATGATCAGGATATCTG-TAMRA-3'

2.6 Nested PCR amplification for molecular characterisation on group B positive nucleic acid extracts from clinical samples.

2.6.1 *PorA* PCR amplification of *N. meningitidis*.

For the *porA* PCRs the total volume of PCR mixture was 50 μl and contained 1.5 μl of 50 mM $MgCl_2$ (Invitrogen, Paisley, Scotland), 10 μl of a 1mM dNTP mix containing dATP, dGTP, dCTP, dTTP (Amersham Pharmacia Biotech, Little Chalfont, UK), 5 μl of a 5 μM concentration of primers 210 and 211 (Urwin, 2000), 5 μl of 10X buffer, 1 μl of 1% (v/v) W1 (Invitrogen, Paisley, Scotland), 1 μl of extracted DNA sample, 21.25 μl of molecular grade water (Sigma-Aldrich, Gillingham, Dorset, UK) and 0.25 μl (5 U/μl) of *Taq* DNA polymerase (Invitrogen, Paisley, Scotland).

The first round of the nested *porA* PCR carried out on clinical sample DNA extracts used the same reaction mix as for the isolate *porA* PCR listed

above except that the amount of DNA extract added was increased from 1 μl to 5 μl and the volume of sterile water decreased to 17.25 μl. For the second round of the nested *porA* PCR two separate PCRs were prepared, each PCR mixture was 50 μl in volume and contained 1.5 μl of 50 mM $MgCl_2$ (Invitrogen, Paisley, Scotland), 10 μl of a 1 mM dNTP mix containing dATP, dGTP, dCTP, dTTP (Amersham Pharmacia Biotech, Little Chalfont, UK), 5 μl of a 5 μM concentration of VR1A and VR2B for amplification of variable region 1, primers VR2C and VR2E for amplification of variable region 2, 5 μl of 10X buffer, 1 μl of 1% (v/v) W1 (Invitrogen, Paisley, Scotland), 1 μl of first round *porA* PCR, 21.25 μl of molecular grade water (Sigma-Aldrich, Gillingham, Dorset, UK) and 0.25 μl (5 U/μl) of *Taq* DNA polymerase (Invitrogen, Paisley, Scotland).

Thermal cycling was carried out using a MJ PTC 200 thermal cycler (GRI, Braintree, UK) using the thermal cycling conditions listed in Table 2.3 and primer sequences are listed in Table 2.2.

2.6.2 MLST PCR amplification of *N. meningitidis* DNA direct from clinical samples.

For the first round of the nested touchdown MLST PCR for group B positive extracts, each reaction mixture was 50 μl in volume and contained 10 μl of a 1mM dNTP mix containing dATP, dGTP, dCTP, dTTP (Amersham Pharmacia Biotech, Little Chalfont, UK), 5 μl of 10X buffer (containing 15 mM of $MgCl_2$)(Qiagen, Crawley, UK), 10 μl of Q-Solution (Qiagen, Crawley, UK), 5 μl of a 1 μM concentration of each primer, 0.5 μl (5 U/μl) of HotStarTaq™ DNA

polymerase (Qiagen, Crawley, UK),9.5 μl of molecular grade water (Sigma-Aldrich, Gillingham, Dorset, UK) and 5 μl of specimen DNA extract.

The second round MLST nested PCR for DNA direct from clinical samples used the same reaction mix concentrations and final volume as the first apart from the fact that 1 μl of PCR product from the first round reaction was added along with 13.5 μl of sterile water.

The PCR primers for both 1st and 2nd round nested protocol and their thermal cycling conditions are listed in Tables 2.3. 2.4 and 2.5. Thermal cycling was carried out using a MJ PTC 200 thermal cycler (GRI, Braintree

Table 2.3 Thermal cycling conditions for *N. meningitidis porA* and MLST (nested PCR).

*por*A N1				*por*A N2				N-MLST N1				N-MLST N2			
Stage	Temp (°C)	Time (Min)	No of cycles	Stage	Temp (°C)	Time (Min)	No of cycles	Stage	Temp (°C)	Time (Min)	No of cycles	Stage	Temp (°C)	Time (Min)	No cycles
1	94	2	1	1	94	5	1	1	95	15	1	1	95	15	1
2	94	1		2	94	1		2	94	1		2	94	1	
	68	1	40		46	1	40		63	1	30		65	1	30
									-0.5 per cycle				-0.5 per cycle		
	72	2			72				72	2			72	2	
3	72	2	1	3	72		1	3	94	1		3	94	1	
4	4	forever		4	4	forever			48	1	10		50	1	10
									72	2			72	2	
								4	72	2	1	4	72	2	1
								5	4	forever		5	4	forever	

Table 2.1 lists the thermal cycling conditions for *porA* PCR from isolates as well as 1st and 2nd round nested *porA* PCR along with the 1st round nested MLST PCR for gene targets *adk, aroE, fumC, gdh, pdhC* and *pgm* as well as 2nd round MLST nested/semi-nested PCR for gene targets *abcZ, aroE, fumC, gdh and pdhC*.

Table 2.4 Thermal cycling conditions for *N. meningitidis* MLST (nested PCR).

N-MLSTN3				N-MLSTN4				N-MLSTN5				N-MLSTN6			
Stage	Temp (ºC)	Time (Min)	No of cycles	Stage	Temp (ºC)	Time (Min)	No of cycles	Stage	Temp (ºC)	Time (Mins)	No of cycles	Stage	Temp (ºC)	Time (Min)	No of cycles
1	95	15	1	1	95	15	1	1	95	15	1	1	95	15	1
2	94	1		2	94	1		2	94	1		2	94	1	
	67	1	30		63	1	30		60	1	30		66	1	30
	-0.5 per cycle				-0.3 per cycle				-0.5 per cycle				-0.3 per cycle		
	72	2			72	2			72	2			72	2	
3	94	1		3	94	1		3	94	1		3	94	1	
	52	1	10		53	1	10		45	1	10		56	1	10
	72	2			72	2			72	2			72	2	
4	72	2	1	4	72	2	1	4	72	2	1	4	72	2	1
5	4	forever		5	4	forever		5	4	forever		5	4	forever	

Table 2.3 lists the thermal cycling conditions for the 1st round nested MLST PCR for gene targets *abcZ* as well as 2nd round MLST nested/semi-nested PCR for gene targets *adk, fumC and pgm*.

Table 2.5 Oligonucleotide primers for *N. meningitidis* MLST and *porA* nested/semi-nested PCR.

Gene	Protocol	Forward primer	Reverse primer	Amplicon Size (bp)	Thermal cycling conditions
abcZ	*abcZ*-CS1st	*abcZ*-P1d 5'-GCTGGCGGCGCAGYTCTTCC-3'	*abcZ*-P2d 5'-ATGGGCGGCATCATTATTGTTTCC-3'	985	MLSTN3
	abcZ-CS2nd	*abcZ*-P1C 5'-TGTTCCGCTTCGACTGCCAAC-3'	*abcZ*-SNP1A 5'-CGGTAAAATCCAAACGGTAACTG-3'	802	MLSTN1
adk	*adk*-CS1st	*adk*-P1b 5'-CCAAGCCGTGTAGAATCGTAAACC-3'	*adk*-P2b 5'-TGCCCAATGCGCCCAATAC-3'	708	MLSTN1
	adk-CS2nd	*adk*-SNP1 5'-GCATTCCGCAAATCTCTACCG-3'	*adk*-P2b 5'-TGCCCAATGCGCCCAATAC-3'	570	MLSTN4
aroE	*aroE*-CS1st	*aroE*-P1 5'-ACGCATTTGCGCCGACATC-3' [c]	*aroE*-P2 5'-ATCAGGGCTTTTTTCAGGTT-3' [c]	911	MLSTN1
	aroE-CS2nd	*aroE*-P1b 5'-TTTGAAACAGGCGGTTGCGG-3'	*aroE*-P2b 5'-CAGCGGTAATCCAGTGCGAC-3'	835	MLSTN2
fumC	*fumC*1-CS1st	*fumC*-A1 5'-CACCGAACACGACACGATGG-3'[d]	*fumC*-A2 5'-ACGACCAGTTCGTCAAACTC-3' [d]	1350	MLSTN2
	*fumC*1-CS2nd	*fumC*-P1b 5'-TCCCCGCCGTAAAAGCCCTG-3'	*fumC*-P2b 5'-GCCCGTCAGCAAGCCCAAC-3'	820	MLSTN2
	*fumC*2-CS1st	*fumC*-P1b 5'-TCCCCGCCGTAAAAGCCCTG-3'	*fumC*-P2b 5'-GCCCGTCAGCAAGCCCAAC-3'	820	MLSTN2
	*fumC*2-CS2nd	*fumC*-SNP1 5'-GTCAAAATCGGCCGCACCCAC-3'	*fumC*-P2b 5'-GCCCGTCAGCAAGCCCAAC-3'	798	MLSTN6
gdh	*gdh* CS1st	*gdh*-P2 5'-GGTTTTCATCTGCGTATAGAG-3'	*gdh*-P1 5'-ATCAATACCGATGTGGCGCGT-3'	678	MLSTN1
	gdh CS2nd	*gdh*-P2 5'-GGTTTTCATCTGCGTATAGAG-3'	*Gdh*-P2b 5'-TGTTGCGCGTTATTTCAAAGAAGG-3'	726	MLSTN1
pdhC	*pdhC*-CS1st	*pdhC*-P1b 5'-CCGGCCGTACGACGCTGAAC-3'	*pdhC*-P2b 5'-GATGTCGGAATGGGGCAAACAG-3'	818	MLSTN2
	pdhC-CS2nd	*pdhC*-NP1A 5'-TGCGCCGTATGTATGCCAATAATG-3'	*pdhC*-NP2A 5'-ACAGGCCGTCTGAAACATCAATCA-3'	662	MLSTN1
pgm	*pgm*-CS1st	*pgm*-P2 5'-CGGATTGCTTTCGATGACGGC-3'c	*pgm*-P1 5'-CTTCAAAGCCTACGACATCCG-3'c	963	MLSTN2
	pgm-CS2nd	*pgm* -NP1A 5'-GGCTTTGAATTGGTTTTGAATCC-3'	*pgm* -NP2A 5'-AATCGGCYTGGCGTTTGAC-3'	796	MLSTN5
porA	*porA*-CS1st	210 5′-ATGCGAAAAAAACTTACCGCCCTC-3′	211 5′-AATGAAGGCAAGCCGTCAAAAACA-3′	1148	*porA*N1
	porA-VR1	VR1A 5′-CTTACCGCCCTCGTATTG-3′	VR1B 5′-GGCAACGGATACGTCTTG-3′	300	*porA*N2
	porA-VR2	VR2C 5′-TGGCTTCGCAATTGGGTA-3′	VR2E 5′-ACCGGCATAATACACATC-3′	250	*porA*N2

Table 2.4 illustrates the PCR primers for the nested/semi-nested *N. meningitidis* MLST as well as the *porA* PCR primers and provides their thermal cycling conditions and the amplicon size.

2.7 Visualisation of PCR products.

All PCR products were electrophoresed on a 2% (v/v) agarose gel, consisting of 3 g agarose (Oncor Appligene, Durham, UK), 150 ml 1X Tris Borate EDTA (Sigma, Dorset, UK) and 15 μl (500 μg/ml) ethidium bromide (Sigma, Dorset, UK). 5μl of PCR product was mixed with 2 μl of loading buffer (70% (w/v) sterile injectable water (Phoenix Pharmaceuticals, Gloucester, UK), 29.5% (w/v) Glycerol (Sigma, Dorset, UK) and 0.5% (w/v) Bromphenol Blue (Bio-Rad, Hertfordshire, UK)) on a Parafilm® sheet (Sigma, Dorset, UK) and loaded into the gel. In order to determine the size of the PCR products 2 μl of loading buffer was also added to 5 μl of 100 base pair ladder (1 μg/μl) (Amersham Pharmacia Biotech, Buckinghamshire, UK) and loaded onto the gel. The gel was electrophoresed for 45 minutes at 130 v/cm in a Horizon 11.14 electrophoresis tank (Gibco Life Technologies Paisley, Scotland) which contained 1litre of 1x Tris Borate EDTA buffer (Sigma, Dorset, UK) and 10 μl (500 μg/ml) ethidium bromide (Sigma, Dorset, UK). The gel was imaged using a Gel Doc 3000 (Bio-Rad, Hertfordshire, UK) Ultra Violet (UV) imaging system (Figure 2.3, 2.4). Each image was saved on the laboratory network in a named folder. (For examples of gel images see Figure 2.3 and 2.4)

For any channel indicating no product for either *porA* or MLST allele the amplification was repeated in duplicate in order to obtain a product if possible.

Figure 2.3 MLST and *PorA* products compared on the same gel.

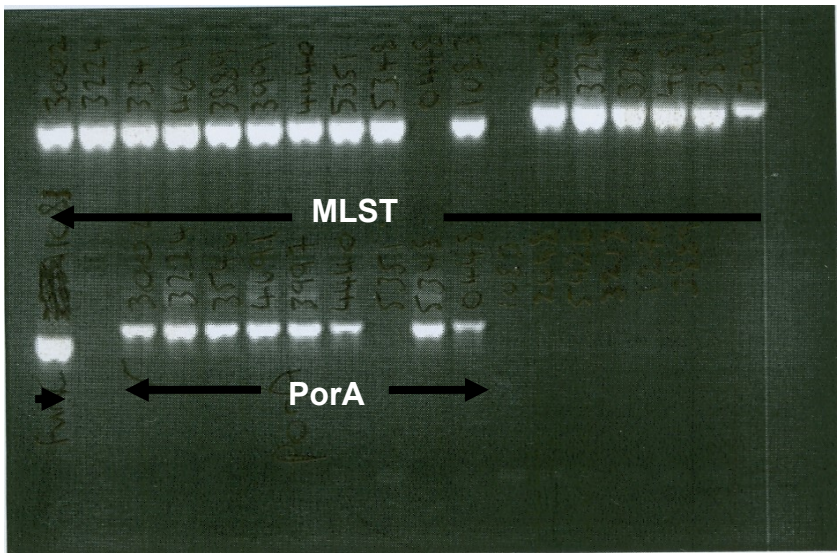
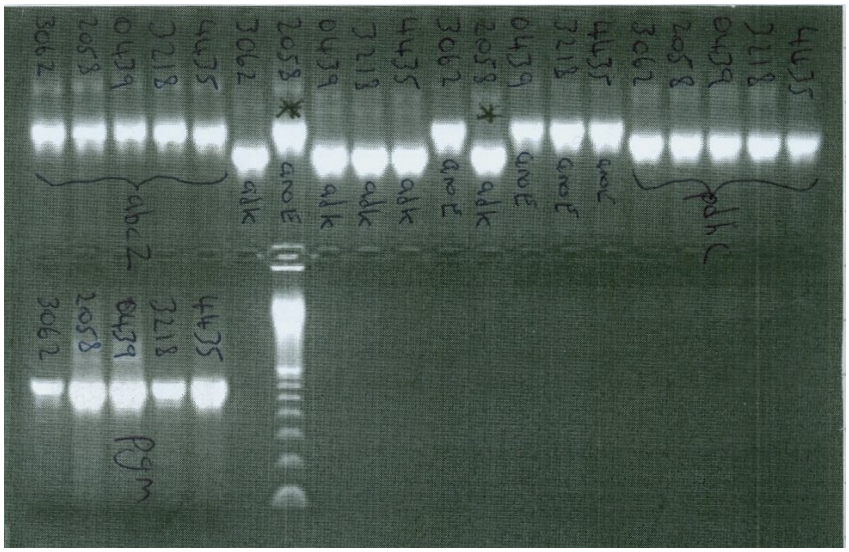


Figure 2.3 demonstrates that MLST nested PCR produces more product than *porA*.

Figure 2.4 MLST nested PCR products run against a 100bp ladder.



Showing different sized amplicons for different MLST targets.

Figure 2.3 and 2.4 show examples of the gel images obtained for both *porA* and MLST products.

2.8 PCR Cleanup via Montage PCR$_{96}$ plate from solution.

The PCR product was loaded into a well on a Montage PCR$_{96}$ plate (Millipore (U.K.) Ltd, Watford, UK), any wells not in use were covered in sealing tape and the plate placed on the multiscreen vacuum manifold. A vacuum was then applied at 20 inches Hg for 20 minutes. After the vacuum was removed, 50 µl (for porA) or 100µl (for MLST) of molecular grade water (Sigma-Aldrich, Gillingham, Dorset, UK) was added to the well and the plate transferred to a Wellmix 3 mini shaker (Denley Ltd, Cambridge, Cambridgeshire, UK) and mixed for 20 minutes at maximum speed. The re-suspended and cleaned PCR product was then removed to a 96 well flat bottomed Costar® plate (Corning, www.corning.com/lifesciences) for analysis and storage at -20^{o}C.

2.9 Sequencing reactions using Beckman Coulter® chemistry for MLST and porA gene fragments for *N. meningitidis*

Sequencing reactions were prepared using the reduced volume protocol described by Azadan *et al*., (2002). The final volume of the sequencing reactions was 10 µl containing 4 µl of Dye Terminator Cycle Sequencing (DTCS) Quick Start master mix (Beckman Coulter, High Wycombe, Buckinghamshire, UK), 1 µl of sequencing primer (10 µM concentration), 4.5 µl of molecular grade water (Sigma-Aldrich, Gillingham, Dorset, UK) and 1 µl of PCR product template which had been diluted with molecular grade water to approximately 16 ng/µl. Reaction mixtures were placed on a MJ PTC 200 thermal cycler (GRI, Braintree, Essex, UK) and amplified using the following thermal cycling conditions (Table2.6).

Table 2.6 Sequencing thermal cycling conditions.

Stage	Temperature (°C)	Time (minutes: seconds)	Cycles
1	96 50 60	00:20 00:20 04:00	40
2	4	indefinately	1

Table 2.5 presents the optimal thermal cycling conditions for the used in Beckman Coulter® Dye Terminator Cycle Sequencing kits incorporating WellRed Dyes.

The sequencing primers used for *N. meningitidis* MLST from cultured microorganisms and directly from clinical samples (Maiden *et al*, 1998; Feavers *et al*, 1999) are listed below:

abcZ-S1 5'-AATCGTTTATGTACCGCAGG-3'

abcZ-S2 5'-GAGAACGAGCCGGGATAGGA-3'

adk-S1 5'-AGGCTGGCACGCCCTTGG-3'

adk-S2 5'-CAATACTTCGGCTTTCACGG-3'

aroE-S1 5-GCGGTCAAYACGCTGATT-3

aroE-S2 5-ATGATGTTGCCGTACACATA-3

fumC-S1 5-TCCGGCTTGCCGTTTGTCAG-3

fumC-S2 5-TTGTAGGCGGTTTTGGCGAC-3

gdh-S3 5'-CCTTGGCAAAGAAAGCCTGC-3'

gdh-S2 5-GCGCACGGATTCATATGG-3

pdhC-S1 5'-TCTACTACATCACCCTGATG-3'

pdhC-S2 5'-ATCGGCTTTGATGCCGTATTT-3'

pgm-S1 5'-CGGCGATGCCGACCGCTTGG-3'

pgm-S2 5'-GGTGATGATTTCGGTTGCGCC-3'

Identification of ET-15 clone of *N. meningitidis* required sequencing of the *fumC* allele with the following specifically designed primer (Vogel *et al.,* 2000b):

fumC-P3 5'-CGTAAAAGCCCTGCGCGAC-3'

The sequencing primers used for *N. meningitidis porA* variable regions 1 and 2 subtyping are listed below:

porA-VR1

VR1A 5′-CTTACCGCCCTCGTATTG-3′

VR1B 5′-GGCAACGGATACGTCTTG-3′

porA-VR2

VR2C 5′-TGGCTTCGCAATTGGGTA-3′

VR2E 5′-ACCGGCATAATACACATC-3′

2.10 Clean up of sequencing products amplified using Beckman Coulter® chemistry for porA and MLST gene fragments for *N. meningitidis.*

Stop solution (2.5 μl), consisting of 1 μl of 1.5 M NaOAc pH-5.2 (Sigma-Aldrich, Gillingham, Dorset, UK), 1 μl of 50 mM Na_2EDTA pH-8.0 (Sigma-Aldrich, Gillingham, Dorset, UK), 0.5 μl of 20 mg/mL glycogen (Beckman Coulter, High Wycombe, Buckinghamshire, UK) followed by 30 μl of -20ºC 95% (v/v) ethanol (Sigma-Aldrich, Gillingham, Dorset, UK) was added and the samples placed at -20ºC for 10 minutes. The samples were centrifuged

at 3000 rpm for 30 min at 4°C in an Allegra™ 21R Centrifuge (Beckman Coulter, High Wycombe, Buckinghamshire, UK). The plate was inverted and the ethanol poured off, the plate was then blotted twice on absorbent paper. The samples were rinsed twice with 200 μl of -20°C 70% ethanol (v/v) (Sigma-Aldrich, Gillingham, Dorset, UK). After each rinse the samples were inverted, the ethanol was poured off and the plate was then blotted twice on absorbent paper as before. The plate was centrifuged, inverted, on absorbent paper for 15 seconds at 300 rpm to further remove residual ethanol. The plates were vacuum dried for 20 minutes and the sequencing products re-suspended in sample loading solution (Beckman Coulter), overlayed with mineral oil (Beckman Coulter, High Wycombe, Buckinghamshire, UK) and sequenced on a CEQ™ 8000XL DNA Genetic Analysis system (Beckman Coulter, High Wycombe, Buckinghamshire, UK) using Genetic analysis system software version 4.3.9 (Beckman Coulter, High Wycombe, Buckinghamshire, UK). Pre-programmed sequencing methodology (LFR1) and raw data analysis protocol (Default sequence analysis parameter) were used to produce the analyzed sequence data for further use (Figure 2.5).

Figure 2.5 Analyzed data produced by the Beckman Sequencer.

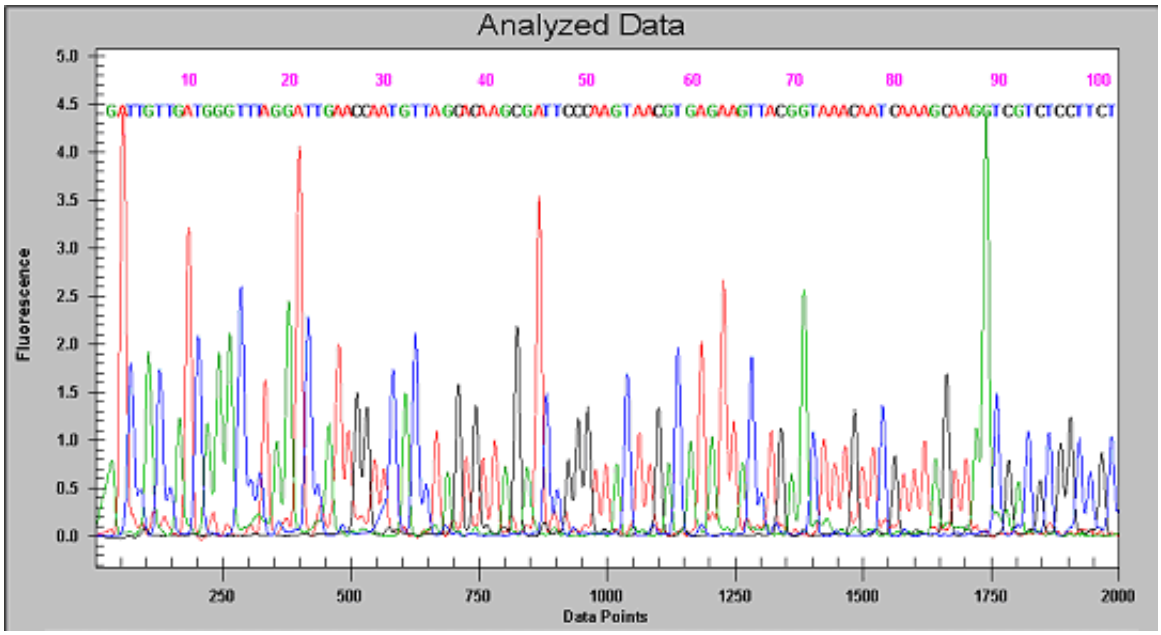


Figure 2.5 shows base sequence data produced by the Beckman CEQ™ 8000 Genetic Analysis system

2.11 Analysis of *porA* and MLST sequence data.

The forward and reverse sequences for the two *porA* regions (VR1 and VR2), four per sample, and the seven MLST gene fragments, fourteen per sample for were assembled from the resultant chromatograms using Sequencher™ sequence analysis software (Gene Codes Corporation, MI, USA) (Figure 2.6). The edited sequences for the *porA* and the different MLST loci were queried against the relevant *N.meningitidis* databases (Jolley, 2007). Sequence data for *porA* were compared to published known sequences for *porA* variable regions one and two (VR1 and VR2) on the www.neisseria.org website (Figure 2.7) to determine the designated variant for each locus. For MLST the sequence data was compared to known alleles on www.mlst.net.

In order to designate a sequence type and clonal complex a seven number allelic profile was in turn submitted on the mlst.net website. For partial profiles (with missing alleles) it was possible to designate clonal complex but not sequence type on most occasions.

Figure 2.6 Forward and reverse sequences assembled in Sequencher™

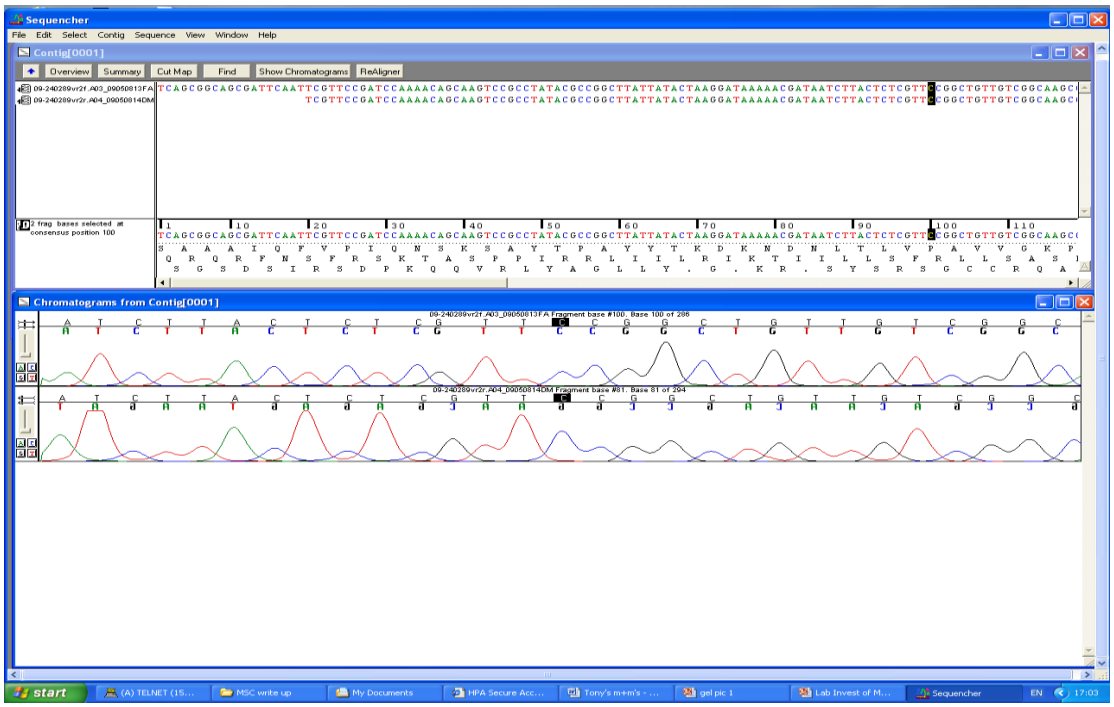


Figure 2.6 demonstrates a porA VR2 (Peptide sequence YYTKDKNDNLTLVP) VR2=16-3 on the Sequencher™ sequence editing software package.

Figure 2.7 Use of the *Neisseria.org.* website.

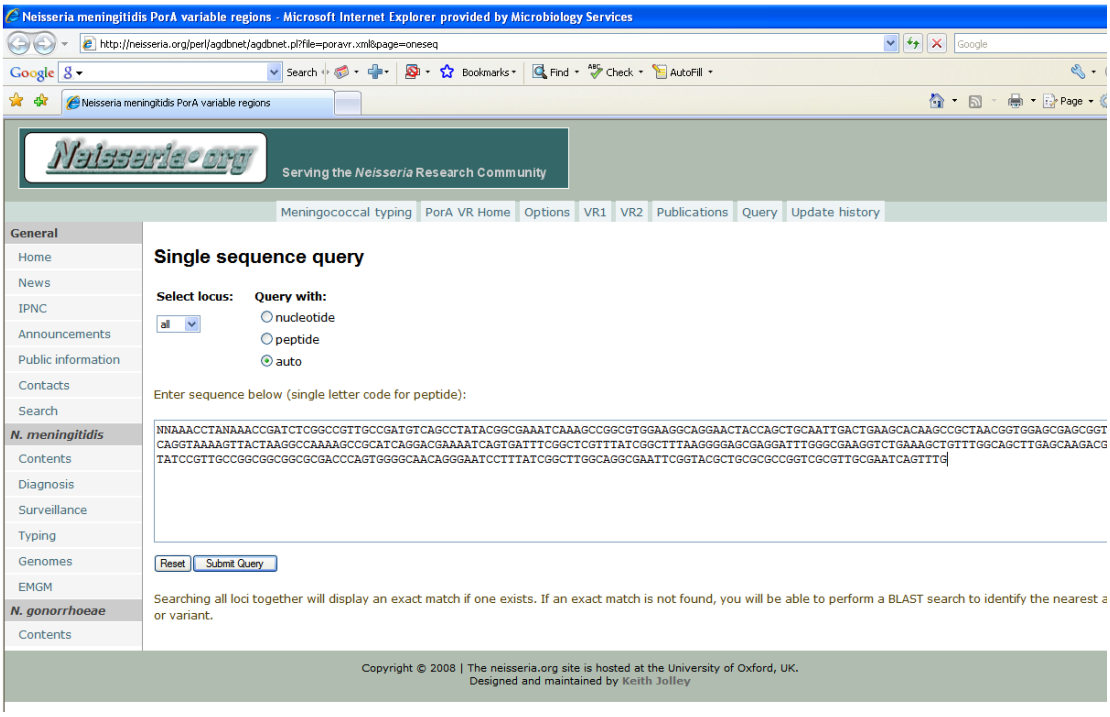


Figure 2.7 demonstrates a base sequence copied and pasted from Sequencher™ into the neisseria.org website. Click on “Submit Query” to identify the target.

In summary, for each clinical sample included in the study, two-round nested PCR was carried out for *porA* and for the seven different MLST targets, 16 PCR reactions in total for each sample. If products were obtained four sequencing reactions for *porA* and fourteen for MLST were carried out, a total of 18 sequencing reactions per sample.

Over the three years, a total of 96 samples were available for study. 1536 PCR reactions were carried out, with a maximum of 1728 sequencing reactions (forward and reverse for each target) ensuing. A maximum of 864 sequences for analysis using Sequencher^{TM} sequence analysis software were produced.

The sequence data obtained from 1999, 2000 and 2001 non-culture confirmed fatal group B meningococcal disease cases were compared with data from culture confirmed cases, to ascertain any epidemiological shift in the disease. Comparison with other data available within the MRU was included in the study to enhance epidemiological findings. This is indicated throughout the text where relevant.

2.12 Statistical Analysis

Where appropriate statistical comparison of data was undertaken using the paired *t* test at www.graphpad.com/quickcalcs/ttest1 and chi squared analysis at www.udel.edu/~mcdonald/statfishers (online Handbook of Biological Statistics, University of Delaware).

Chapter 3: Results

3.1 Sample types for non-culture group B fatal cases.

The majority (86.8%) of the 96 clinical samples included in the study were blood samples, this included Ethylenediaminetetraacetic (EDTA) blood, serum (from clotted blood), plasma and one heparinised blood sample. CSF samples made up 11.4% of the total and other sample types (1.75%) included a tracheal aspirate and a single nucleic acid extract. The nucleic acid extract came from a laboratory which performs in-house meningococcal PCR testing, one of only two in England to do so at the time, and was sent to the MRU for confirmatory testing. The extract and the tracheal aspirate were among the samples which were missing from the sample archive. Data for sample types is included in appendices A, B and C.

3.2 Application of nested PCR for non-culture fatal group B cases 1999, 2000 and 2001.

Ninety six DNA extracts from blood and/or CSF samples were available for study, 25 from 1999, 28 from 2000 and 43 from 2001. All were processed by nested PCR to amplify the *porA* region of the meningococcal genome and the seven gene targets for MLST before DNA sequencing reactions were carried out. The DNA extracts were from patients who had a positive meningococcal group B real time PCR test, no isolate, and whose disease outcome was fatal.

A positive PCR reaction on the TaqMan® platform was indicated by the Cross-Threshold (CT) value (Figure 3.1), this is the number of thermal

cycles before the trace crosses a calculated threshold value to be flagged as positive. The MRU uses a 45 cycle protocol for meningococcal PCR testing, so a CT of 45 indicates a negative PCR test (no amplification). The mean CT values for the *ctrA* gene for the samples included in the study are shown in appendices A, B and C and are summarised in Table 3.1.The table also includes non-fatal samples from the same time period (the non fatal samples were not included in the study).

3.3 Sensitivity of the non-culture sequence typing methods.

The percentage of samples which produced sequence data to enable *porA* VR1,2, sequence type (ST) and clonal complex (CC) to be ascertained are shown in Table 3.2. Of the 96 samples, 78 (81%) gave a *porA* result, 80 (83%) samples yielded an MLST clonal complex and 63 (66%) an MLST sequence type. Seven samples (7.3%) gave neither sequence type nor clonal complex. For *porA,* six of the samples with no result had CT values greater than 35, three had no CT value recorded, and seven a CT value of less than 35. Of the seven samples with neither ST nor CC three had a CT of greater than 35 and four a CT less than 35. Thirty-five samples (36%) did not give a full MLST profile due to missing allele data.

A full data set is available in Tables 3.3, 3.4 and 3.5.

Table 3.1 Summary of the cross-threshold (CT) values for group B meningococcal PCR positive samples for the three years of the study.

Year	Number of samples (CSF)		No. with no CT value entered		Data (n=)		Mean CT value (*ctrA* gene)	
	Fatal	**Non-fatal**	**Fatal**	**Non-fatal**	**Fatal**	**Non-fatal**	**Fatal**	**Non-fatal**
1999	25 (6)	417	7	133	18	284	29.0	31.5
2000	28 (1)	615	1	39	27	576	28.0	31.3
2001	43 (6)	659	0	17	43	642	24.3	30.6
Overall	**96 (13)**	**1691**	**8**	**189**	**88**	**1502**	**27.1 ***	**31.1 ***

Table 3.1 shows the CT values for the meningococcal positive (*ctrA* gene) PCR tests on the non-culture (PCR) confirmed group B cases for the three years of the study. Subsequent analysis was carried out on the 96 fatal case samples. Fatal case results included in appendices A, B and C. CT data from MRU secondary archive database.

*Overall the mean CT value for fatal cases was significantly lower by Paired *t* test (p= <0.0001) than for non-fatal cases.

Table 3.2 Summary of sequence data obtained from the samples studied.

Year	Sample numbers	Number giving *porA* (VR1,2)	Number with complete MLST	Number allowing ST designation	Number allowing CC designation
1999	25	19 (76%)	10 (40%)	13 (52%)	18 (72%)
2000	28	22 (78%)	19 (68%)	19 (68%)	24 (84%)
2001	43	37 (86%)	32 (75%)	31 (72%)	38 (89%)
Total	**96**	**78 (81%)**	**62 (65%)**	**63 (66%)**	**80 (83%)**

ST= sequence type, CC= clonal complex. VR1,2= variable regions 1 and 2.

Table 3.2 shows the percentage of positive data obtained over the three years using the non-culture molecular characterisation methods (*porA* and MLST). All included samples initially tested positive for meningococcal DNA by *ctrA* PCR and were confirmed as serogroup B by *siaD* PCR.

Table 3.3 *PorA* and MLST results for fatal group B non culture samples 1999

Study number	Sample type	Group	Ctvalue	*PorA* VR1	*PorA* VR2	*abcZ*	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*	MLST	Clonal Complex
0036	edta	B	29	19*	15	8	10	77	4	6	3	np	UA	ST32 ET5
0114	plasma	B	na	22	9	rptx2	35	2	5*/rpt	38	11	9	UA	ST269
0140	csf	B	na	7-2*	4	3*	5	9	5	9	6	9	3754	ST41/44Lin3
0180	plasma	B	36	np	rpt	4	35	15	17	8	11	17	479	ST269
0207	plasma	B	30	7-2	4	3	6	9	17	9	6	9	482	ST41/44Lin3
0286	plasma	B	21	19-1*	15-11	4*	rpt/rpt	15	9	8	11	9	UA	ST269
0587	blood	B	na	np	np	4	rpt/rpt	6	np	38	np	np	UA	ST269
0907	plasma	B	na	5-2*	10	9	6	9	9	9	6	2	180	ST41/44Lin3
1193	edta	B	na	21	16	6	7	6	17	26	21	8	840	UA
0963	plasma	B	na	rpt	10	rptx2	10	6	4	5	3	2	74	ST32 ET5
2614	plasma	B	26	7-9	13-1	8	10	5	4	5	3	8	34	ST32 ET5
3498	csf	B	23	5	2	17	5	19	17	3	26	2	60	UA
4540	plasma	B	21	19-1	15-11	4	np	15	9	8	11	rpt/9*	UA	ST269
4863	csf	B	28	7-2*	4	4	6	9	rpt/rpt	9	6	9	UA	ST41/44Lin3
6036	edta	B	30	7-2	4	3	6	9	rpt/5*	9	6	9	41	ST41/44Lin3
6643	plasma	B	28	7-2	4	3	6	9	rpt/5*	9	6	9	41	ST41/44Lin3
7304	csf	B	38	np	np	rptx2	rpt/np	9	5	rpt	np	np	UA	UA
9630	serum	B	34	np	np	rpt/np	np	np	rpt	np	rpt	rpt		NP

Table 3.3 continued. *PorA* and MLST results for fatal group B non culture samples 1999

Study number	Sample type	Group	Ctvalue	*PorA* VR1	*PorA* VR2	*abcZ*	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*	MLST	Clonal Complex
9798	plasma	B	30	22	9	4	rpt/rpt	2	rpt/5*	38	11	rpt/9	UA	UA
5223	csf	B	na	19-1*	15-11	4	10	15	9	8	11	9	269	ST269
5608	serum	B	26	22-1	14	4	10	161	9	11	21	3	UA	UA
5945	serum	B	25	7-2*	4	4	6	9	5	9*/9*	6	9	5098	ST41/44Lin3
8269	plasma	B	32	22	14	7	5	1	13	36	rpt	15	3496	ST213
8808	csf	B	na	5-1	10-4	42	np	46	24	6	rpt	17	UA	UA
4383	plasma	B	36	np	np	3	6	9	rpt	9	22	np	UA	ST41/44Lin3

UA=unassigned, np= no product (amplification repeated), *=single strand only. Rpt=sequence data obtained but not resolvable, sequencing reaction repeated and data still unresolvable. Na=Ct value not recorded in laboratory reporting system.

Table 3.4 *PorA* and MLST results for fatal group B non culture samples 2000

Study number	Sample type	Group	Ctvalue	*PorA* VR1	*PorA* VR2	*abcZ*	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*	MLST	Clonal Complex
0082	serum	B	31	7-2	13-1	3	6	9	5	9	22	9	40	ST41/44Lin3
0306	plasma	B	na	21	16	3	6	9	60	9	6	9	170	ST41/44Lin3
0624	plasma	B	na	np	np	3	6	9	5	9	6	9	41	ST41/44Lin3
0737	edta	B	21	5	10-5	2	3	7	2	34	5	2	153	ST8 Cluster4
0880	edta	B	27	7*	16	4	10	5	40	6	3	8	259	ST32 ET5
1065	serum	B	24	19-1*	15-11	4	10	15	9	8	11	9	269	ST269
1100	plasma	B	27	19-1*	15-11	4	10	15	9	8	11	9	269	ST269
1248	serum	B	37	np	np	np	np	np	rpt	np	np	np		NP
1388	plasma	B	26	5-2*	10	8	10	5	4	5	3	8	34	ST32 ET5
1510	edta	B	30	7-2*	16	4	10	4	4	6	3	8	2145	ST32 ET5
1840	plasma	B	23	7-2*	4	3	6	9	17	9	6	9	482	ST41/44Lin3
2148	plasma	B	28	7-2*	4	3	6	19*/19*	5	3	6	9	340	ST41/44Lin3
2705	plasma	B	30	7-2	4	3	6	9	5	9	6	9	41	ST41/44Lin3
2982	edta	B	37	np	np	rpt/np	6	9*/np	np	rpt	np	rpt	UA	UA
3115	serum	B	ins											M
3497	serum	B	24	7-2*	4	3	rpt/rpt	rpt/np	5	9	6	9	UA	ST41/44LIN3
4306	edta	B	24	7-2*	4	3	6	9	5	9	6*	9	41	ST41/44Lin3
5644	edta	B	21	22	14-6	28*/np	6	9	9	9	21	2	3919	ST41/44Lin3
5844	edta	B	27	7-2	4	3	6	9	5	9	6	2	2314	ST41/44Lin3
6090	edta	B	27	7-1	14-6	17	5	19	17	3	26	2	60	ST60

Table 3.4 continued. *PorA* and MLST results for fatal group B non culture samples 2000

LABnumber	Sample type	Group	Ctvalue	*PorA* VR1	*PorA* VR2	*abcZ*	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*	MLST	Clonal Complex
280950	edta	B	32	7-2*	4	3	np	9	5	9	6	9	UA	ST41/44Lin3
280958	edta	B	28	7-2*	4	3	rpt/rpt	9	5*/np	9	rpt/6	9	UA	ST41/44Lin3
270234	serum	B	missing											M
277192	csf	B	36	np	np	np	np	np	np	np	np	np		NP
283545	extract	B	missing											M
283818	edta	B	27	7-2	4	10	6	9	5*	9	6	9	42	ST41/44Lin3
284001	edta	B	26	22	9	4	rpt	34	5	38	11	9	UA	ST269
284650	edta	B	24	19-1*	15-11	4	10	15	9	8	11*	17	283	ST269
284927	plasma	B	38	np	np	rpt	6	9	np	11	6*	46	UA	ST41/44Lin3
285320	edta	B	28	7-2	4	3	6	19	5	3	6*	9	340	UA
286218	serum	B	26	np	np	3	6	rpt	23	9	6	9	UA	ST41/44Lin3

UA=unassigned, np= no product (amplification repeated), *=single strand only, rpt=sequence data obtained but not resolvable (Sequencing reaction repeated). Na=Ct value not recorded in laboratory reporting system.

Table 3.5 *PorA* and MLST results for fatal group B non culture samples 2001

Study number	Sample type	Group	Ctvalue	*PorA* VR1	*PorA* VR2	*abcZ*	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*	MLST	Clonal Complex
4230	csf	B	29	19-1	15-11	np	10	rpt	9	8	11	9	UA	ST269
4505	serum	B	22	7-2*	4	3	6	9*	5	9	6	9	41	ST41/44Lin3
0113	serum	B	16	7-2	4	3	6	9	17	9	6	9	482	ST41/44Lin3
0878	edta	B	24	19-1	15-11	4	rpt/rpt	6	9	8	11	9	1092	ST269
0888	edta	B	21	5	10-4	42	2	rpt	24	6	20	17	1164	UA
1050	heparin	B	25	np	np	np	rpt/np	rpt	np	rpt	rpt	9	UA	UA
4532	edta	B	24	5-1	10-1	2	3	4	3	8	4	6	11	ST11 ET37
4602	edta	B	20	5-1	10-1	2	3	4	3	8	4	6	11	ST11 ET37
1393	edta	B	22	21	4*	?3	6	9*	9	9	6	9	303	ST41/44Lin3
1471	csf	B	29	19-1*	15-11	4	10	15	9	8	11	9	269	ST269
2404	edta	B	34	np	np	np	np	rpt	np	np	np	rpt		NP
2702	edta	B	22	np	np	7	8	10	np	10	1	2	UA	ST18
4209	edta	B	26	7-2*	4	3	6	9	5	9	6	9	41	ST41/44Lin3
4513	edta	B	23	19-1*	15-11	4	35	15	9	8	11	9	393	ST269
5200	edta	B	22	np	np	np	np	np	rpt/np	np	rpt	rpt		NP
3054	csf	B	27	7-2	16	4	10	15	9	8	11	17	283	ST269
3098	edta	B	22	22	9	4	10	2	5	38	11	9	275	ST269
3585	edta	B	24	7-2*	16	4	np	15	9	8	11	17	UA	ST18
3834	edta	B	24	21	16	17	5	19	17	3	26	2	UA	ST60
2098	edta	B	20	7-2	16	4	10	15	9	8	11	17	283	ST269
1463	edta	B	28	7-2*	4	3	6	9	5	9	6	9	41	ST41/44Lin3
5835	edta	B	23	22	14	7	8	10	19	38	1	2	UA	ST18
6350	edta	B	20	7-2*	4	17	5	19	17	3	26	17	3014	ST60

Table 3.5 continued. *PorA* and MLST results for fatal group B non culture samples 2001

Study number	Sample type	Group	Ctvalue	*PorA* VR1	*PorA* VR2	*abcZ*	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*	MLST	Clonal Complex
8632	edta	B	26	21*	16	6	7	6	17	26	21	8	840	ST167
9680	edta	B	25	18-1*	3	3	6	34	5*/5*	11	6	9	UA	ST41/44Lin3
2327	plasma	B	23	7-2	4	3	6	19	5	3	6	9	340	ST41/44Lin3
9700	plasma	B	18	19-1	15-11	4	10	15	9	8	11	17	283	ST269
2764	edta	B	20	7-2	34	3	6	9*	5*/5*	9	6	9	41	ST41/44Lin3
2865	edta	B	26	7-2*	4	3	6	9*	5*/rp	9	6	9	41	ST41/44Lin3
3556	serum	B	26	rpt	4	3	6	19	5	3	6	9	340	ST41/44Lin3
4393	serum	B	27	np	np	15	3	rpt	2	8	19	15	UA	UA
4462	edta	B	26	18-7*	9-5	8	4	rpt	4	5	18	9	UA	ST103
2764	edta	B	34	19-1	15-11	4	10	15	5*/5*	8	11	9	354	ST269
4958	edta	B	23	22*	9	4	10	2	9	38	11	16	5372	ST269
5059	csf	B	29	19-1	15-11	4	10	15	9*/rpt	8	11	9	269	ST269
3459	edta	B	26	7-2	4	3	6	9	5	9	6	9	41	ST41/44Lin3
3528	edta	B	32	19	13-1	rpt/rpt	10	15	9	8	11	rpt	UA	ST269
3954	edta	B	23	7-2	4	3	6	12	5*/5*	9	6	9	4965	ST41/44LIN3
4440	edta	B	26	19	13-1	12	5	12	35	60	22	17	461	ST461
5257	csf	B	26	19-1	30-1	4	10	15*	9	8	11	17	283	ST269
0124	csf	B	16	22*	9	4	10	34	5*/5*	38	11	9	1161	ST269
0128	serum	B	19	22*	9	4	10	2	5	38	11	9	275	ST269
0422	plasma	B	27	19-1	15-11	4	10	15	17	8	11	9	1049	ST269

UA=unassigned, np= no product, *=single strand only, rpt=sequence data obtained but not resolvable-repeated.

3.4 *PorA* genotypes for the non-culture fatal group B cases.

Two *porA* genotypes were prevalent from the non-culture fatal group B cases in each of the three years (1999, 2000 and 2001) (Figure 3.2). The VR1 7-2, VR2 4 genotype and the VR1 19-1, VR2 15-11 genotypes combined made up 53, 62 and 46% of the types ascertained for the three years respectively. In 1999 and 2000 in addition to the two prevalent types only singleton genotypes were identified, with three being common to both years. In 2001 an additional eleven *porA* types were identified as well as the two prevalent ones, of which seven were singletons.

The two prevalent *porA* genotypes show a strong association with particular MLST clonal complexes. Over all three years, 23 of the VR1 7-2, VR2 4 types were from samples designated ST41/44 lineage3 clonal complex, whilst one was unassigned and one associated with the ST60 clonal complex. All of the VR1 19-1, VR2 15-11 *porA* genotypes were associated with ST269 clonal complex (data in Table 3.6)

Table 3.6 *PorA* and clonal complex for the non culture group B samples 1999, 2000 and 2001

1999 PorA VR1	1999 PorA VR2	1999 CC	2000 *PorA* VR1	2000 *PorA* VR2	2000 CC	2001 *PorA* VR1	2001 *PorA* VR2	2001 CC	2001 *PorA* VR1	2001 *PorA* VR2	2001 CC
19	15	ST32ET5	7-2	13-1	ST41/44L3	19-1	15-11	ST269	18-7	9-5	ST103
22	9	ST269	21	16	ST41/44L3	7-2	4	ST41/44L3	19-1	15-11	ST269
7-2	4	ST41/44L3	5	10-5	ST8CL4	7-2	4	ST41/44L3	22	9	ST269
7-2	4	ST41/44L3	7	16	ST32ET5	19-1	15-11	ST41/44L3	19-1	15-11	ST269
19-1	15-11	ST269	19-1	15-11	ST269	5	10-4	UA	7-2	4	ST41/44L3
5-2	10	ST41/44L3	19-1	15-11	ST269	5-1	10-1	ST11ET37	19	13-1	ST269
21	16	UA	5-2	10	ST32ET5	5-1	10-1	ST11ET37	7-2	4	ST41/44L3
7-9	13-1	ST32ET5	7-2	16	ST32ET5	21	4	ST41/44L3	19	13-1	ST461
5	2	UA	7-2	4	ST41/44L3	19-1	15-11	ST269	19-1	30-1	ST269
19-1	15-11	ST269	7-2	4	ST41/44L3	7-2	4	ST41/44L3	22	9	ST269
7-2	4	ST41/44L3	7-2	4	ST41/44L3	19-1	15-11	ST269	22	9	ST268
7-2	4	ST41/44L3	7-2	4	ST41/44L3	7-2	16	ST269	19-1	15-11	ST269
7-2	4	ST41/44L3	7-2	4	ST41/44L3	22	9	ST269			
22	9	UA	22	14-6	ST41/44L3	7-2	16	ST18			
19-1	15-11	ST269	7-2	4	ST41/44L3	21	16	ST60			
22-1	14	UA	7-1	14-6	ST60	7-2	16	ST269			
7-2	4	ST41/44L3	7-2	4	ST41/44L3	7-2	4	ST41/44L3			
22	14	ST213	7-2	4	ST41/44L3	22	14	ST16			
5-1	10-4	UA	7-2	4	ST41/44L3	7-2	4	ST60			
			22	9	ST269	21	16	ST167			
			19-1	15-11	ST269	18-1	3	ST41/44L3			
			7-2	4	ST41/44L3	7-2	4	ST41/44L3			
						19-1	15-11	ST269			
						7-2	34	ST41/44L3			
						7-2	4	ST41/44L3			

Figure 3.1 Percentages of *porA* genotypes for fatal group B cases.

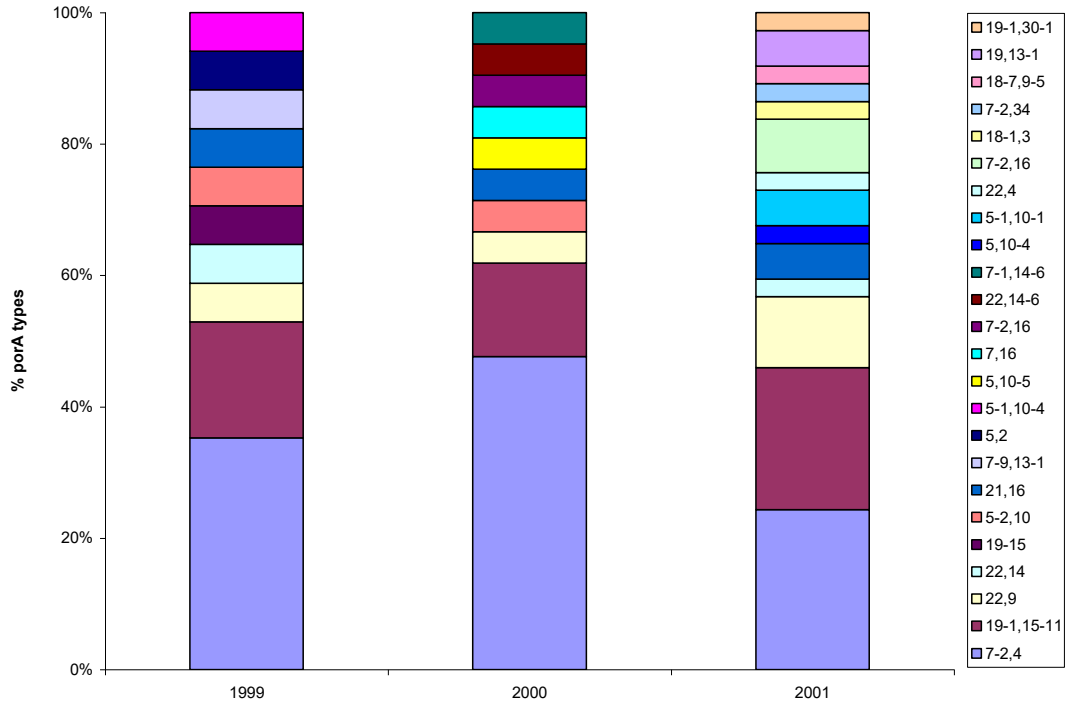


Figure 3.2 shows the percentages of *porA* genotypes identified from the PCR positive samples for the three years of the study (data from appendices A, B and C).

Statistical analysis was not valid for this data due to the small sample sizes.

3.5 MLST genotyping for the non-culture fatal group B cases.

The MLST data obtained from the non-culture fatal group B samples is presented in figure 3.3. Of the 18 samples designated a clonal complex for 1999 only four distinct complexes were noted; ST41/44Lineage3 (33.4%) was the most common, with ST269 (25.0%), ST32/ET5 (25.0%) and ST213 (4.2%). 25% of the samples were unassigned. For 2000, 24 samples yielded five distinct complexes; ST41/44Lineage3 (57.8%), ST269 (15.4%), ST32/ET5 (11.6%). ST8 and ST60 were singleton data, and 7.7% were unassigned. For 2001, 38 samples were observed in 8 distinct complexes ST41/44Lineage3 (30%), ST269 (39%), ST18 (7.3%) and the remainder (ST60, ST8, ST11/ET37, ST137 and ST461 either less than 5% or singleton data. 7.3% were unassigned.

The two main clonal complexes represented in the fatal group B cases, ST41/44 lineage3 and ST269, were the two most common among group B disease complexes in general over this timescale. This is shown in figure 3.5 where sample sizes for the isolates analyzed were larger (for 1998-99 n=933 and for 2000-01 n=934).

Figure 3.2 Clonal complex distribution of non-culture fatal group B cases.

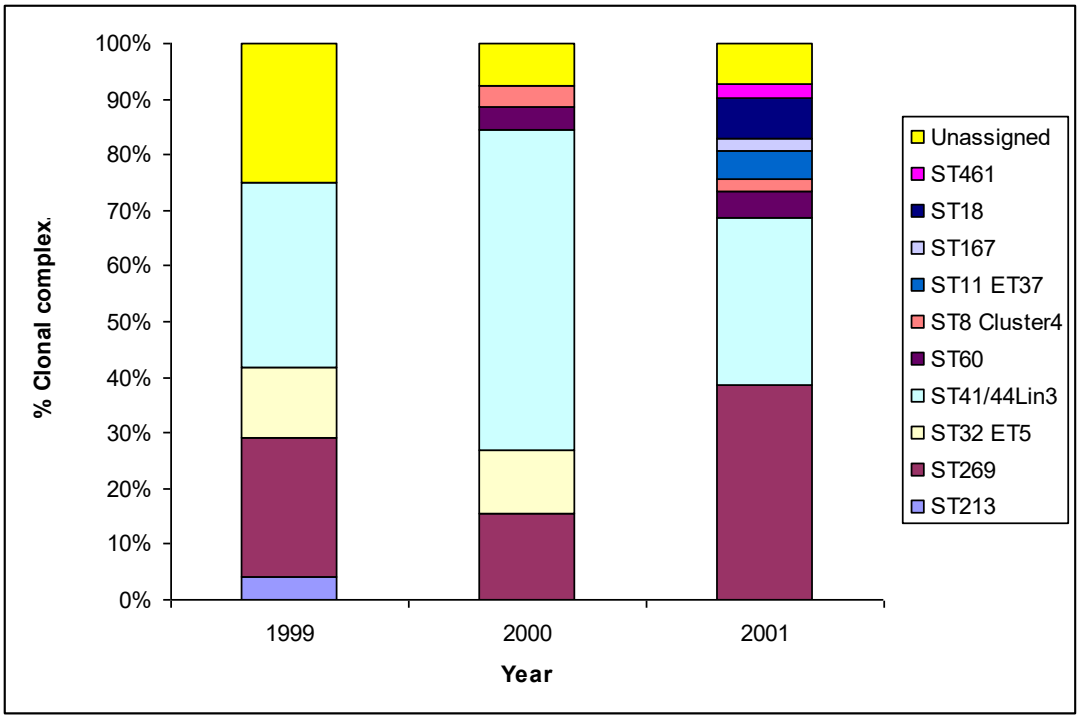


Figure 3.3 shows the clonal complex percentages for the non-culture fatal group B cases included in the study (data from appendices A, B and C).

Statistical analysis was not valid for this data due to the small sample sizes.

3.6 Fatal group B cases, comparison of combined culture and non-culture data for 1999 and 2001.

In order to provide as complete epidemiological data for fatal group B disease cases as possible the data from the study was combined with data from case isolates. The combined clonal complex data are summarised in Figure 3.4. It was not possible to include the year 2000 as isolates for that year (with the exception of those from November and December) did not have MLST designation available.

The distribution of clonal complexes obtained from both culture and non culture MLST sequence typing on fatal group B cases shows that two major complexes (ST41/44 lineage3 and ST269) were prevalent in 1999. Other common complexes (ST32, ST18 and ST60) were also seen.

In 1999, pre-immunisation, fatal cases were associated with 41/44Lineage3 complex (36.3%) compared to 30.8% in 2001 (post immunisation). The percentage distribution for other clonal complexes in the same two years were; 22.4% and 31.9% for ST269; 17.6 and 6.6% for ST32; 4.8 and 4.4% for ST18 and 3.2 and 3.3% for ST60. ST11/ET37 complex was only observed in 2001, causing 4.4% of fatal group B cases. This distribution of clonal complexes was found not to be significantly different ($p=0.179$) between the two years.

Figure 3.3 Combined culture and non culture fatal group B cases 1999 and 2001.

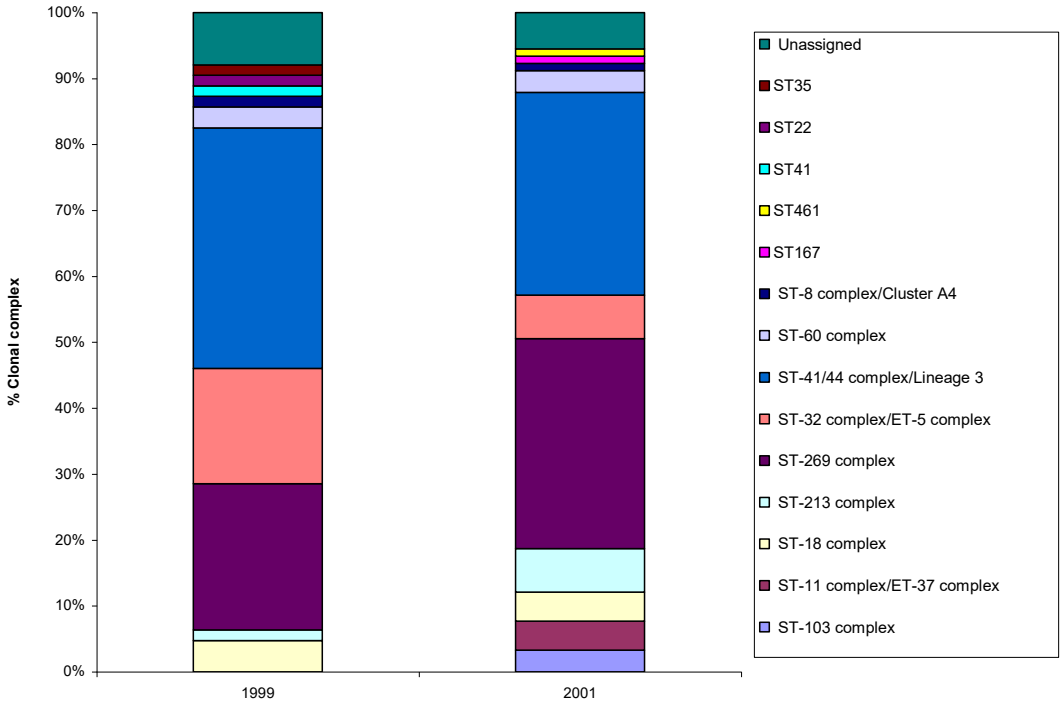


Figure 3.4 shows clonal complex percentages for culture and non-culture confirmed fatal group B cases pre (1999) and post (2001) MenC immunisation.

Using chi-squared analysis there was no significant difference (p=0.179) between the clonal complex distribution for the two years.

MLST determination of the cultures was carried out at Oxford University as part of an MRU collaborative study.

3.7 Meningococcal disease in England and Wales for one calendar year prior to the MenC immunisation programme. (Nov 1998-Nov 1999).

At the time of this study MLST analysis was routinely carried out by the MRU on every tenth *N. meningitidis* isolate. In order to provide a more comprehensive surveillance around the introduction of serogroup C vaccine MLST was carried out on every isolate for one calendar year prior to immunization (November 1998 to November 1999). There were 933 group B isolates submitted to the MRU during this period, all had MLST analysis carried out at the department of Zoology, Oxford University as part of an MRU collaborative study. The information is included in this study for comparison and to put the fatal case non culture results in epidemiological context. 406 (37.6%) were found to belong to clonal complex ST41/44 lineage3, 237 (22.0%) to clonal complex ST269 and 95 (14.2%) to clonal complex ST32. In total 18 different clonal complexes were identified among the group B isolates for this pre immunization year, with 45 (7.8%) being unassigned. Within each of the two major clonal complexes a range of sequence types were identified. Within CC ST41/44 lineage3, 110 different sequence types were identified, many were singletons or in very low (<10) numbers. The most common ST found was ST41 (n=185, 45.6%) with ST154 (n=17, 4.2%), ST180 (n=12, 3.0%), ST1097 (n=12, 3.0% and ST340 (n=11, 2.7%) others STs were present at n=<10. Within CC ST269 36 different sequence types were identified. For CC ST41/44 lineage3, many were singletons, or found in low numbers. The most common sequence type was ST 269 (n=108, 45.6%) with ST283 (n=26, 11.0%) and ST275 (n=24, 10.1%) being the only other STs significantly represented.

There were 655 group C isolates submitted to the MRU during this period and were also assigned at the department of Zoology, Oxford University. 544 (83.1%) were found to belong to clonal complex ST11, 73 (11.1%) to CC ST8, 10 (1.5%) to CC ST41/44 lineage3 and 8 (1.2%) to CC ST269. In total 13 different clonal complexes were identified, with 7 (1.1%) of the isolates being unassigned to a complex. Of the 544 CC ST11, 495 (91.0%) were identified as sequence type ST11, and 11 (2.0%) as ST67. Of the 73 CC ST8 58 (79.5%) were sequence type ST8, 5 (6.9%) were ST66 and 5 (6.6%) were ST4047.

Data from MLST analysis of MRU isolates, carried out at the department of Zoology, Oxford University, are in appendices E and F. Figure 3.5 shows a summary of the results relating to clonal complex distribution.

Figure 3.4 Group B and Group C clonal complex distribution for *N meningitidis* isolates November 1998- November 1999.

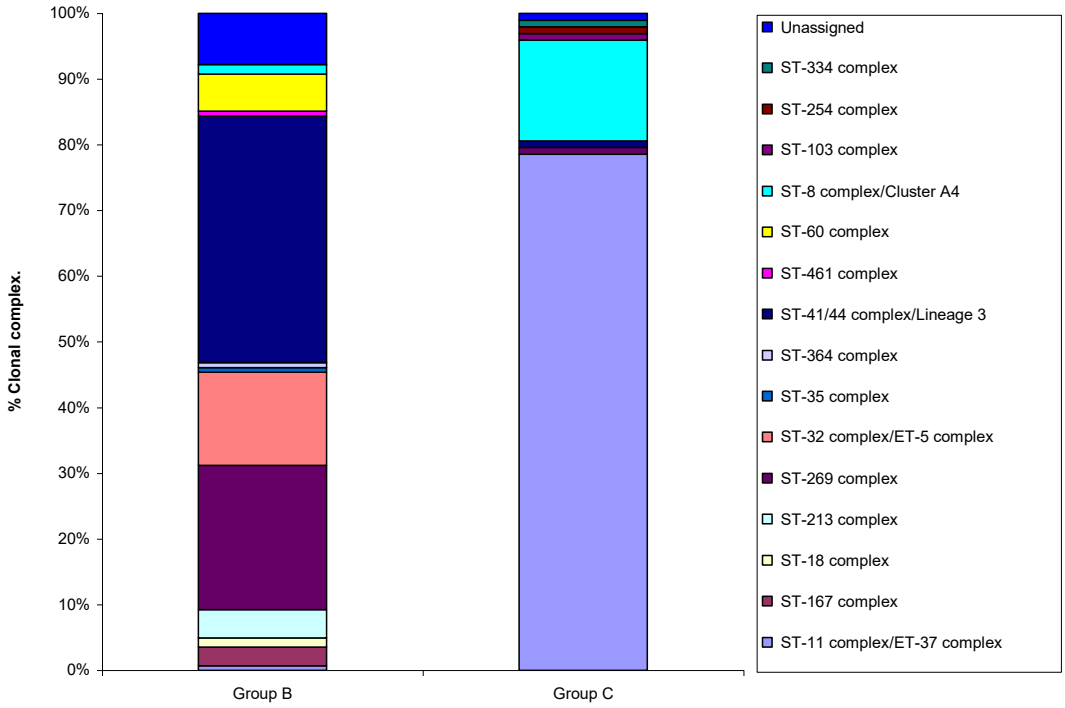


Figure 3.5 shows the distribution of clonal complexes among group B and group C isolates causing meningococcal disease in England and Wales prior to the MenC immunisation programme.

MLST designation of the isolates was carried out at the University of Oxford Zoology department, as part of an MRU collaborative study.

3.8 Group B meningococcal disease pre and post MenC immunization, culture confirmed totals.

The epidemiology of group B disease for one calendar year before immunization was compared to group C in the same timescale in figure 3.6. A further calendar year, November 2000 to November 2001, was also studied by MLST sequence typing of isolates from the MRU. This year was chosen, leaving out November 1999 to November 2000, to give the immunization programme sufficient time to be implemented nationally, and to allow a full calendar year of meningococcal disease data post immunisation to be included.

Figure 3.6 compares the group B isolates already included in figure 3.5, from 1998-1999, to those from 2000-2001 by percentage clonal complex distribution. The total numbers of isolates from the two calendar years are remarkably similar (1998-1999; n=933 and 2000-2001; n=934), the numbers of distinct clonal complexes identified for each year were the same (18) though three complexes were found exclusively in either year in small numbers (three as singleton data). There were five and seven percent unassigned to a complex respectively. The percentage clonal complex distribution was not significantly different (p=0.112) for the two years. MLST data of MRU isolates determined at Oxford University is presented in appendix E and G.

Figure 3.5 Group B isolate Clonal complex distribution pre and post immunization.

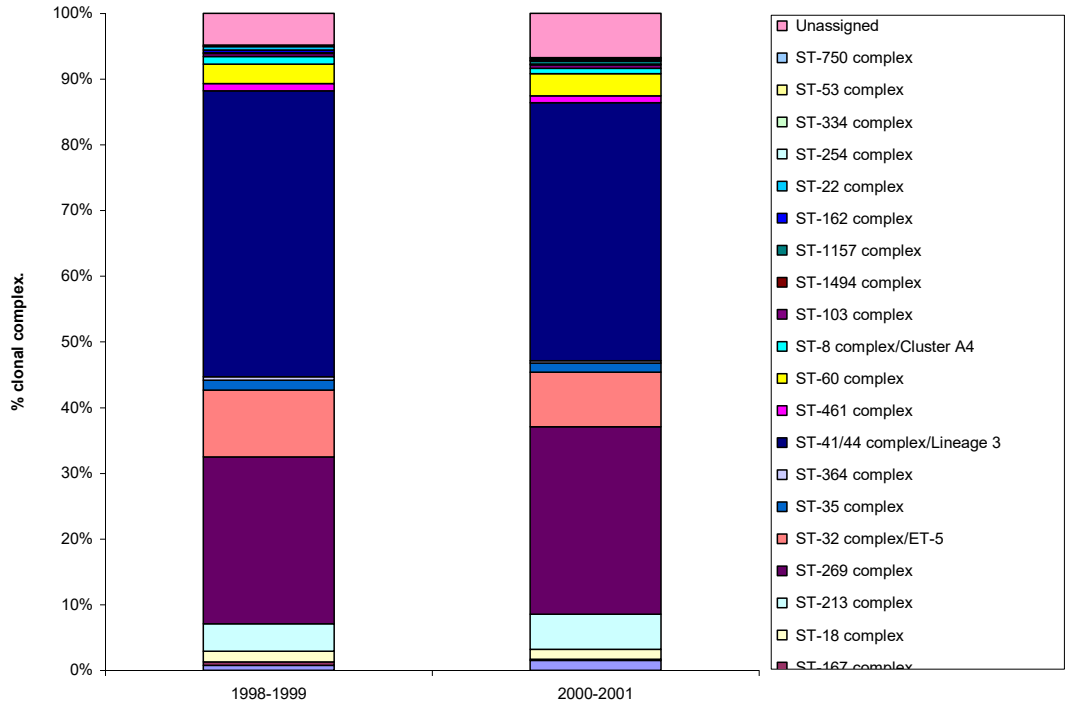


Figure 3.6 shows the distribution of clonal complexes among group B isolates causing meningococcal disease in England and Wales prior to (Nov1998-Nov1999) and post (Nov2000-Nov2001) the MenC immunisation programme.

Using chi-squared analysis there was no significant difference (p=0.112) between the clonal complex distribution for the two years.

MLST determination of the isolates was carried out at the University of Oxford Zoology department as part of an MRU collaborative study.

Chapter 4. Discussion and further work

This study intended to enhance the epidemiology of group B meningococcal disease in England and Wales by the implementation of non-culture DNA sequencing methods developed in the MRU. In particular the provision of epidemiological data on non culture confirmed fatal group B disease cases. The period around the introduction of the national immunisation programme with conjugated group C vaccines was studied. In the MRU cases confirmed by *ctrA* PCR and with no organism isolated (defined as non-culture cases) had the serogroup determined by *siaD* PCR. PCR only cases now represent almost 50% of laboratory confirmed meningococcal disease. Further epidemiological information is necessary to provide a more complete epidemiological picture of meningococcal disease and to enable a more accurate study both of case clusters and longer term disease trends.

DNA sequence typing from isolates is relatively straightforward but does involve significant resourcing for the MRU, for this reason all isolates have had *porA* sequence typing performed routinely since October 2007 but MLST is currently only applied to isolates submitted during January and July each year for molecular surveillance. Previous studies have shown that complex procedures are required to obtain comparable typing information from the meningococcal DNA detected in positive PCR assays, the non culture confirmed cases. A nested PCR approach is necessary for positive PCR samples, as a single round PCR reaction fails to give products (for sequence analysis) with any consistency, even for strongly positive samples (Birtles *et al* 2005).

The samples included in this study were predominantly (86.8%) blood samples, indicating that either the fatal cases studied were the result of septicaemia rather than meningitis, that lumber puncture was unable to be performed due to the severity of the patient's presentation, or a clinical decision not to perform lumbar puncture was taken. The progression of meningococcal disease is normally from an initial bacteraemic spread, followed by septicaemia and/or meningitis (Steven and Wood 1995). Peripheral blood is therefore a good sample to obtain even in a patient presenting clinically with meningitis as the infective organism may often be detected without lumbar puncture being necessary. EDTA blood is the preferable sample type as nucleic acid extraction from whole blood will enable detection of intracellular organisms. PCR testing of serum from clotted blood samples could give a false negative result. The MRU will advise on specimen types but will test most samples submitted as on occasion EDTA blood has not been obtained. Samples need to be taken as soon as possible because previous studies have found that meningococcal PCR, following the initial administration of antibiotics, remains positive for as little as 12 hours in some patients (Hackett *et al* 2002a). For these reasons all meningococcal PCR negative laboratory reports include the phrase "a negative PCR result does not exclude meningococcal disease", this is due to negative predictive values of ≈75% (Carrol *et al* 2000).

In order to assess and examine the possibility of implementing the new non-culture sequence typing methods, in the time scale available for this study, it

was decided to study only fatal group B cases. This yielded a manageable sample number (96 samples available in total) upon which to apply the non-culture sequence typing techniques. Also the study of fatal cases would highlight any antigenic or population shift in the group B meningococci causing the most serious clinical outcome. For the three years of the study 5.4, 5.3 and 6.5% of group B cases were fatal, so non fatal cases (hence sample numbers), would be too numerous to include in the study (1691 non-fatal samples compared to the 96 from fatal cases).

Previous studies have shown a correlation between the bacterial load at presentation with meningococcal disease and the clinical outcome (Hackett *et al*, 2002b; Darton *et al*, 2009) and data from this study was from patients with a fatal disease outcome. When compared with non fatal non culture group B cases over the same time period, the positivity of the fatal cases included in this study was significantly ($p=<0.0001$: t test) greater. The difference in the mean CT values of four cycles represented a sixteen-fold difference in amplified PCR product. The fatal case positive PCR samples would therefore be expected to produce sequence data due to their strong positivity. It was previously reported that where a positive sample gave a CT value greater than 35 it was difficult to obtain sequence data (Birtles *et al*, 2005). This study included some such samples, and some MLST sequence data was obtained, but no *porA* data. In the application of non-culture *porA* sequencing techniques to study case clusters the MRU will attempt sequence typing on more weakly positive samples (CT>35) and results have been obtained on occasion by replicating the nested PCR up to six times. A

targeted approach in the use of non-culture *porA* sequencing is employed by the MRU, as sequence data would be difficult to obtain for all non-culture cases. General meningococcal epidemiology (with the exception of the serogroup, which is routinely determined on non culture cases) is reliant upon molecular characterisation of isolates rather than samples from non-culture cases.

The non culture sequence typing methods employed in this study successfully provided both *porA* and MLST data, giving results for 81 and 83% of the samples respectively. Ascertaining a sequence type was more difficult, as some samples did not give sequence data for all seven MLST alleles, and only 66% of the samples gave a sequence type result. All these samples were previously confirmed as group B meningococcal DNA positive by PCR, but no further epidemiological information was available. Without further data these cases could not be included in either medium to long term epidemiological study or in case cluster investigation. None of the samples tested with a CT value of >35 gave sequence data for *porA* typing and sensitivity of the assay was therefore unsatisfactory. The product yield for *porA* is lower than for MLST (Figure 2.3) and further optimisation of the nested amplification for *porA* may be necessary. All targets where no product was obtained had the nested PCRs repeated, and those samples with non resolvable sequence were also repeated. Despite this repeat testing some targets still remained unresolved. 18% of *porA* investigation yielded no data and 12.5% remained unresolved for MLST alleles. This may be due to the visual quantification and optimisation of DNA concentration, by

running the products in agarose gel, for subsequent DNA sequencing. Fully quantitative methods are available and their implementation may improve the yield of sequence data, bur would incur further costs. Three samples gave no data despite CT values <35 (34, 25 and 22), suggesting that they may have deteriorated in storage.

PorA genotype data showed common (VR1 7-2, VR2 4 and VR1 19-1, VR2 15-11) types represented (Figure 3.2). Small sample sizes led to many singleton data, and statistical analysis of this data was not possible. Similarly percentage clonal complex distribution results were affected by small sample sizes, but the common (ST41/44lineage3 and ST269) complexes did predominate (Figure 3.3). The two non-culture fatal case ST11/ET37 results seen in 2001 were both from patients who were part of a case cluster studied by the MRU. One culture confirmed non-fatal case was also part of this cluster; the patients were two 12 year old males who attended different schools and a 57 year old male who all used the same bus to travel daily to school or work. Apart from this one case cluster there were no other fatal group B cases caused by ST11/ET37 group B meningococci in the three years studied. This indicated no proliferation of this particular more invasive clone of group B following the immunisation programme, and that capsule switching from C to B has not been apparent. The data from non-culture cases obtained in the study, combined with data from isolates, showed that fatal cases of group B disease over the time period studied were caused by a similar diversity of group B clones as general group B disease, within the limitations of the sample sizes available.

Group C immunisation seems to have had little impact on group B disease in the short term, surveillance by the MRU continues.

The most meaningful data, using the results from this study, was obtained in combination with culture confirmed cases to provide a more complete data set of fatal group B case epidemiology. Figure 3.4 shows that fatal group B cases were being caused by the major clonal complexes prevalent at the time (ST41/44Lineage3 and ST269) with a range of other clonal complexes, and that there was no significant difference (p=0.179 : chi squared) between 1999 and 2001 (pre and post MenC immunisation).The more comprehensive data for all culture confirmed group B cases (including fatal cases) shown in Figure 3.6 also shows no significant difference in the clonallity of group B disease pre and post immunisation (p=0.112 : chi squared). No shift in group B epidemiology had been caused by the MenC vaccination programme.

The *porA* sequence results from this study were characterised by submission to www.neisseria.org, where it can be seen that to date, 11 VR1 and 20 VR2 families have been identified, with many variants of each type including 104 variants of the P1.16 family alone. The Neisseria.org website does not include *porA* VR3 sequences as they have been characterised and curated by the Scottish Meningococcal and Pneumococcal Reference Laboratory in Glasgow (Clarke *et al* 2003). The sequences characterised to date are available on the website www.smprl.scot.nhs.uk where the limited variability of the VR3 region is indicated as only thirteen genotypes are

included. Since the time of this study the VR2 amplicon, when the stated primers are used, has been found to include the VR3 region.

Since completion of this study further work has been undertaken and non-culture *porA* sequence typing has been implemented in the study of case clusters. In 2009 the technique has been used in ten case cluster investigations, including a large study of all meningococcal disease cases in Leicestershire. Data on ten cases was provided solely by non-culture techniques, and along with the data from isolates, was instrumental in determining that an increase in disease incidence was not caused by a particular strain but was sporadic group B disease. A similar investigation in Cumbria found group B disease with a higher than expected incidence of *porA* VR1 19-1, VR2 15-11 genotype. In 2008 an investigation of cases in Cornwall found sporadic disease, but with a case cluster among family contacts (four cases separated geographically but with epidemiological contact) which was *porA* genotype 22, 14. This type of investigation is usually instigated by the Health Protection Unit covering the area where high incidence or possible connected cases of meningococcal disease are noted, and the information provided used to inform any public health action subsequently taken. Recently a Spanish exchange student who became unwell soon after entering the UK was diagnosed as having group C meningococcal disease by PCR testing in the MRU. As the causative meningococcus was not isolated, non-culture *porA* sequence typing was undertaken at the request of the Spanish MRU. The result showed *porA* indistinguishable from three other group C cases in the region the patient

had travelled from. The previous three cases had been reported as an outbreak in the Canary Islands (personal communication, Dr Julio Vazquez, Spanish MRU).

Other antigen genes have been proposed for inclusion in the molecular epidemiology of meningococcal disease, in particular *fetA* (Thompson *et al*, 2003; Russell *et al*, 2008) which is already being included in the analysis of isolates in some of the European meningococcal reference laboratories (personal communication, Dr S.J Gray from 2009 European Monitoring Group for Meningococci (EMGM) conference). More information on the EMGM (Trotter *et al* 2006) can be found on the EMGM website via www.neisseria.org. The latest initiative is to propose a move to a new "fine typing" scheme for meningococci (Elias *et al* 2006, Fox *et al* 2007) which involves ascertaining serogroup, *porA* VR1 and VR2 (VR3 is not included as it fails to provide additional discrimination within common antigenic types), *fetA* and MLST (if required, for clonal complex) for meningococcal isolates. A *fetA* variable region database has already been created on the www.neisseria.org. Website. The region has been sub divided into six different families and a total of 295 variants have been included to date. The PCR and sequencing primers for use with isolates are published on the website, and the MRU intend to include *fetA* analysis in the near future. In order to include *fetA* for non-culture samples a nested PCR will be needed. An additional primer for this purpose has been described (Fox *et al* 2007). The MRU intend to implement the "fine typing" scheme. *FetA* non-culture sequence typing may be included to further enhance case cluster analysis.

Changes in methodology and legislation have taken place since completion of this study which may impact on similar studies in the future. The molecular department carrying out the meningococcal PCR testing has become significantly larger as more molecular assays are developed and put into use. The manual extraction methods used at the time of this study have been superseded by automation based methods to allow large batch processing. The MagNA Pure™ platform (Roche Applied Sciences, Indianapolis Ind) initially replaced the manual extraction methods but was then replaced by the MDX Biorobot (Quiagen LTD, Crawley UK) of which two are in use. A NucliSens® easyMAG™ (Biomerieux, Marcy l'Etoile, France) and an EZ1 Biorobot (Qiagen LTD, Crawley UK) are also in use. At the time of the study two ABI Prism® 7700 (Applied Biosystems, Warrington, Cheshire, UK) Sequence Detection system platforms were in use. At present eleven platforms are in use, eight ABI Prism® 7500, one ABI Prism® 7900 (both Applied Biosystems, Warrington, Cheshire, UK) and two M2000RT platforms (Abbott Diagnostics Maidenhead, Berkshire, UK). These PCR detection platforms have a faster completion time of 42 minutes compared to 2 hours and 9 minutes, allowing for a faster turnaround time for PCR assays. Due to the large increase in PCR testing the meningococcal testing has become a smaller part of the molecular department, which has impacted on turnaround times. The robotic extraction methods tend to use a larger sample volume and produce a smaller final elution volume for the extract, which may mean volume of extract for subsequent sequence analysis is limited.

Automation has been used in sequence typing (Clarke *et al*, 2001a; Sullivan *et al*, 2006) and in non culture sequence typing (Clarke et al 2001b). A Biomek® 2000 (Beckman Coulter, High Wycombe, Buckinghamshire, UK) liquid handling platform was available in the MRU and, following the practical work of this study, was trialed to perform any part of the processing of the non-culture sequencing work. As the MLST amplification gave higher amplicon yields (Figures 2.3 and 2.4) the platform was programmed to carry out the clean up procedure following this amplification. The platform was set up with a vacuum manifold and with a final elution volume of 100µl for all the products (gel visualization of products was not carried out prior to robotic clean-up). The platform proved useful when whole plates (96 products) were processed, and good sequence data was obtained. Liquid handling robotics would be useful in large batch testing, but is not used for the *porA* non-culture work currently undertaken by the MRU.

A more recent change in methodology has seen the MRU cease to use the CEQ™ 8000XL DNA Genetic Analysis system (Beckman Coulter, High Wycombe, Buckinghamshire, UK) which has been replaced by the ABI 3130XL Genetic Analyzer, used with ABI PRISM® BigDye™ terminator sequencing chemistry (Applied Biosystems Inc Foster City, CA, USA). The ABI chemistry produces a better quality sequence data, including enhanced read length, and this allows the *porA* sequencing to be performed with two primers rather than four. The reassembled double stranded sequence incorporates VR1, VR2 and VR3 regions. This halves the number of

sequencing reactions needed to obtain *porA* data, a saving in reagents and staff time for this work.

The legislative change impacting on further work is the Human Tissue act of 2004. This act was brought about by the retention of organs from fatal case pediatric patients at the Alder Hey children's hospital without parental consent (Ellis *et al* 2003); the act was implemented in 2006. Under the legislation samples from fatal cases containing human cells, which includes whole blood and CSF samples, must be either destroyed or returned to the sending laboratory, and cannot be stored beyond initial testing. Serum samples, which are sub optimal for PCR testing, can be retained as can the nucleic acid extracts. The extracts do contain human genomic DNA, but to date it is believed that they can be retained. The MRU has not yet applied to establish a tissue bank of samples (McHale *et al* 2007) but this would be a possibility to archive samples. As samples are invariably taken from patients who are still alive at the time of sampling, and are often used retrospectively in case cluster analysis, this legislation has implications for some of the work of the MRU.

In summary this study illustrates the successful use of non-culture MLST and *porA* sequence typing to enhance the surveillance of group B meningococcal disease by the MRU. Epidemiological information, previously unavailable without an isolate of *N. meningitidis* is made available by the techniques applied. Non-culture cases, routinely having only serogroup ascertained, are able to be included in both case cluster investigation and

long term disease surveillance by the DNA sequence based typing as described and discussed in this study. Using a set of clinical specimens from fatal cases over three years, it was possible to add to the data obtained from isolates to give a more complete data set. Without non-culture sequence data the 50% of clinical cases without culture confirmation cannot be included. Using the combined data and other results from MRU isolates it can be shown that during the time period studied there was no antigenic shift among group B meningococci causing invasive disease, including fatality. The implementation of the MenC immunisation programme appears to have had no discernable short term effect on the epidemiology of group B disease in the study period, and the proposed phenomena of capsule switching was not apparent. The non-culture *porA* techniques have been implemented by the MRU in the investigation of case clusters, but resourcing issues have so far prevented more extensive implementation of the methods.

References

Ala'Aldeen, D. A. A., Neal, K., Ait-Tahar, J. S., Nguten-Van-Tam, J. S., English, A., Falla, T. J., Hawkey, P. M. and Slack, R.C.B. (2000). Dynamics of Meningococcal Long-Term Carriage among University Students and Their Implications for Mass Vaccination. *Journal of Clinical Microbiology*, **38**, 2311-2316.

Ameratunga, S., Macmillan, A., Stewart, J., Scott, D., Mulholand, K and Crengle, S. (2005). Evaluating the post-licensure effectiveness of a group B meningococcal vaccine in New Zealand: a multi-faceted strategy. *Vaccine*, **23**, 2231-2234.

Ashton, F. E. and Caugant, D. A. (2002). The panmictic nature of *Neisseria meningitidis* serogroup B during a period of endemic disease in Canada. *Canadian Journal of* Microbiology, **47**, 283-289.

Azadan, R. J., Fogleman, J. C. and Danielson, P. B. (2002). Capillary electrophoresis sequencing: maximum read length at minimal cost. *Biotechniques*, **32**, 24-26.

Balmer, P., Borrow, R. and Miller, E. (2002). Impact of meningococcal C conjugate vaccine in the UK. *Journal of Medical Microbiology,* **51**,717-722.

Bettinger, J. A., Scheifele, D. W., Le Saux, N., Halperin, S. A., Vaudry, W., Tsang, R. Canadian Immunisation Monitoring Programme (IMPACT). (2009). The impact of childhood meningococcal serogroup C conjugate vaccine programmes in Canada. *Journal of Paediatric Infectious Diseases*, **28**, 220-224.

Birtles, A., Hardy, K., Gray, S. J., Handford, S., Kaczmarski, E. B., Edwards-Jones, V. and Fox, A. J. (2005). Multilocus Sequence Typing of *Neisseria meningitidis* Directly from Clinical Samples and Application of the Method to the Investigation of Meningococcal Disease Case Clusters. *Journal of Clinical Microbiology,* **43**, 6007-6014.

Bjune, G., Høiby, E. A., Grønnesby, J. K., Arnesen, O., Fredriksen, J. H., Halstensen, A., Holten, E., Lindbak, A. K., Nøkleby, H. and Rosenqvist E. (1991). Effect of outer membrane vesicle vaccine against group B meningococcal disease in Norway. *The Lancet.* **338**, 1093-1096.

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

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

Borrow, R., Guiver, M., Sadler, F., Kaczmarski, E. B. and Fox, A. J. (1998b). False positive diagnosis of meningococcal infection by the IS1106 PCR ELISA. *Federation of European Microbiological Societies Microbiology Letters*, **162**, 215-218.

Cameron, C. and Peabody, R. (2006). Introduction of pneumococcal conjugate vaccine to the UK childhood immunisation programme, and changes to the meningitis C and Hib schedules. *European surveillance*, **11**, E060302.4

Carrol, E. D., Thopson, A. P. J., Shears, P., Kaczmarski, E. B. and Hart, C. A. (2000). Performance characteristics of the polymerase chain reaction assay to confirm clinical meningococcal disease. *Archive of Childhood Disease*, **83**, 271-273.

Cartwright, K. A. V., Stuart, J. M., Jones, D. M. and Noah, N. D. (1987). The Stonehouse survey: nasopharyngeal carriage of meningococci and *Neisseria lactamica. Epidemiology and Infection*, **99**, 591-601.

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

Cartwright, K. A. V. (1995). Meningococcal carriage and disease. In *Meningococcal Disease*, 115-146. Edited by K.A.V. Cartwright. Chichester: John Wiley and Sons.

Caugant, D. A., Hoiby, E. A., Magnus, P., Scheel, O., Hoel, T., Bjune, G., Wedge, E., Eng, J. and Froholm, L. O. (1994). Asymptomatic carriage of *Neisseria meningitidis* in a randomly sampled population. *Journal of Clinical Microbiology*, **32**, 323-330.

Caugant, D. A. (1998). Population genetics and molecular epidemiology of *Neisseria meningitidis*. *APMIS*, **106**, 505-525.

Clarke, S. C. Diggle, M. A. and Edwards, G. F. S. (2001a). Semiautomation of Multilocus Sequence Typing for the Characterisation of Clinical Isolates of *Neisseria meningitidis*. *Journal of Clinical Microbiology*, **39**, 3066-3071.

Clarke, S. C., Diggle, M. A. and Edwards, G.F.S. (2001b). Automated non-culture-based sequence typing of meningococci from body fluids. *British Journal of Biomedical Science*, **58**, 230-234.

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

Corless, C. E., Guiver, M., Borrow, R., Edwards-Jones, V., Fox, A. J. and Kaczmarski, E. B. (2001). Simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae* and *Streptococcus pneumoniae* in suspected cases of meningitis and septicemia using real-time PCR. *Journal of Clinical Microbiology,* **39**, 1553-1558.

Darton, M., Guiver, M., Naylor, S., Jack, D. L., Kaczmarski, E. B., Borrow, R, and Read, R. C. (2009). Severity of meningococcal disease associated with genomic bacterial Load. *Clinical Infectious Diseases*, **48**, 587-594.

Davis, D. M., Dulbecco, R., Eisen, H. N. and Ginsberg, H. S. (1980). The Neisseriae. In *Microbiology*, 635-644. Edited by E.C. Gotschlich: Harper.

Davison, K. L., Ramsay, M. E., Crowcroft, N. S., Lieftucht, A., Kaczmarski, E. B., Trotter, C. L., Gungabissoon, U. and Begg, N. T. (2002). Estimating the burden of seogroup C meningococcal disease in England and Wales. *Communicable Disease and Public Health*, **5**, 213-219.

De Filippis, I. and Vicente, A. C. (2005). Multilocus sequence typing and repetitive element-based polymerase chain reaction analysis of *Neisseria meningitidis* isolates in Brazil reveal the emergence of 11 new sequence types genetically related to the ST-32 and ST-41/44 complexes and high prevalence of strains related to hypervirulent lineages. *Diagnostic Microbiology and Infectious Diseases,* **53**, 161-167.

De Greef, S. C., De Melker, H. E., Spanjaard, L., Van Den Hof, S., and Dankert, J. (2003).The first effect of the National vaccination campaign against meningococcal C disease: a rapid and sharp decrease in the number of patients.*Ned Tijdschr Geneeskd*, **147**, 1132-1135.

Diggle, M. A., Bell, C. M. and Clarke, S. C. (2003). Nucleotide sequence-based typing of meningococci directly from clinical samples. *Journal of Medical Microbiology,* **52**, 505-508.

Diggle, M. A., and Clarke, S. C. (2003). Detection and genotyping of meningococci using a nested PCR approach. *Journal of medical Microbiology*, **52**, 51-57.

Dyet, K., Devoy, A., McDowell, R. and Martin, D. (2005). New Zealand's epidemic of meningococcal disease described using molecular analysis: implications for vaccine delivery. *Vaccine*, **23**, 2228-2230.

Eldridge, L., Sutcliffe, E. M., Abbott, J. D. and Jones, D. M. (1978). Serological grouping of meningococci and detection of antigen in cerebrospinal fluid by coagglutination. *Medical Laboratory Science*, **35**, 63-66.

Elias, J., Harmsen, D., Claus, H., Hellenbrand, W., Frosch, M. and Vogel, U. (2006). Spatiotemporal analysis of invasive meningococcal disease, Germany. *Emerging Infectious Diseases*, **12**, 1689-1695.

Ellis, I., Mannion, G. and Warren-Jones, A. (2003). Retained human tissues: a molecular goldmine or modern grave robbing? A legal approach to obtaining and using stored human samples. *Medical Law*, **22**, 357-372.

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

Fox, A. J., Taha, M. K. and Vogel, U. (2007). Standardised nonculture techniques recommended for European reference laboratories. *Federation of European Microbiological Societies reviews*, **31**, 84-88.

Frasch, C. E. (1995). Meningococcal vaccines: past, present, and future. In Meningococcal disease, 246-283. Edited by K. A. V. Cartwright. Chichester, England: John Wiley and Sons.

Goldacre, M. J., Roberts, S. E. and Yeates, D. (2003). Case fatality rates for meningococcal disease in an English population, 1963-98: database study. *British Medical Journal*, **327**, 596-597.

Gorringe, A., Halliwell, D., Matheson, M., Reddin, K., Finney, M. and Hudson, M. (2005). The development of a meningococcal disease vaccine based on *Neisseria lactamica* outer membrane vesicles. *Vaccine,* **23**, 2210-2213.

Gray, S. J., Trotter, C. L., Ramsay, M. E., Guiver, M., Fox, A. J., Borrow, R., Mallard, R. H. and Kaczmarski, E. B. (2006). Epidemiology of meningococcal disease in England and Wales 1993/4 to 2003/4: contribution and experiences of the Meningococcal Reference Unit. *Journal of Medical Microbiology*, **55**, 887-896.

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

Hackett, S. J., Carrol, E. D., Guiver, M., Marsh, J. M., Sills, J. A., Thompson, A. P. J., Kaczmarski, E. B., and Hart, C. A. (2002a). Improved case confirmation in meningococcal disease with whole blood Taqman PCR. *Archive of Disease in Childhood,* **24**, 617-20.

Hackett, S. J., Guiver, M., Marsh, J., Sills, J. A., Thompson, A. P. J., Kaczmarski, E. B., and Hart, C. A. (2002b). Meningococcal bacterial DNA load at presentation correlates with disease severity. *Archives of Disease in Childhood*, **86**, 44-46

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

Harrison, L. H., Trotter, C. L. and Ramsay, M. E. (2009). Global epidemiology of meningococcal disease. *Vaccine*, **27,** supplement 2, B51-63.

Høiby, E. A., Bjune, G., Frøholm, L. O., Eng, J., Halstensen, A., Rosenqvist, E., Rønnild, E., and Wedege, E. (1991). The Norwegian meningococcal serogroup B outer membrane vesicle vaccine protection trials: case tracing, meningococcal antigen detection and serological diagnosis. *National institute of public health annals*, **14**, 107-123.

Holst, J., Martin, D., Arnold, R., Campa Huergo, C., Osper, P., O'Hallahan, J. and Rosenqvist, E. (2009). Properties and clinical performance of vaccines containing outer membrane vesicles from *Neisseria meningitidis*. *Vaccine*, **275**, B3-B12.

Jenson, E. S., Berthelsen, L., Lind, I., Fussing, V., Sorensen, H. T. and Schonheyder, H. C. (2006). Period prevalence and case-fatality rate associated with distinctive clone complexes of *Neisseria meningitidis* serogroups B and C. *European Journal of Clinical Microbiology and Infectious Disease*, **21**, 506-516.

Jolley, K. A., Brehoney, C. and Maiden, M. C. (2007). Molecular typing of meningococci: recommendations for target choice and nomenclature. *Federation of European Microbiological Societies reviews*, **31**, 89-96.

Jones, D. M. and Sutcliffe, E. M. (1990). Group A meningococcal disease in England associated with the Haj. *Journal of Infection*, **21**, 21-25.

Kaczmarski, E. B., Ragunathan, P. L., Marsh, J., Gray, S. J. and Guiver, M. (1998). Creating a national service for the diagnosis of meningococcal disease by polymerase chain reaction. *Communicable Disease and Public Health*, **1**, 54-56.

Kanegaye, J. T., Soliemanzadeh, P. and Bradley, J. S. (2001). Lumbar Puncture in Paediatric Meningitis: Defining the Time Interval for Recovery of Cerebrospinal Fluid Pathogens After Parenteral Antibiotic Pretreatment. *Paediatrics*, **108**, 1169-1174.

Kriz, P., Kalmusova, J. and Felsberg, J. (2002). Multilocus sequence typing of Neisseria meningitidis directly from cerebrospinal fluid. *Epidemiology and Infection* **128**, 157-160.

Kyaw, M. H., Clarke, S. C., Christie, P. and Campbell, H. (2002). Invasive meningococcal disease in Scotland, 1994 to 1999, with emphasis on group B meningococcal disease. *Journal of Clinical Microbiology*, **40**, 1834-1837.

Lancellotti, M., Guiyoule, A., Ruckly, C., Hong, E., Alonso, J. M. and Taha, M. K. (2006). Conserved virulence of C to B capsule switched *Neisseria meningitidis* clinical isolates belonging to ET-37/ST-11 clonal complex. *Microbes and Infection*, **8**, 191-196.

Laval, C. A. B., Pimenta, F. C., De Andrade, J. G., Andrade, S. S. and De Andrade, A. L. S. S. (2003). Progress towards meningitis prevention in the conjugate vaccine era. *The Brazillian Journal of Infectious Diseases*, **7**, 315-324.

Lewis, S., Sadarangani, M., Hoe, J. C. and Pollard, A. J. (2009). Challenges and progress in the development of a serogroup B meningococcal vaccine. *Expert Review Vaccines* , **8**, 729-745.

Maiden, M. C. J., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D. A., Feavers, I. M., Achtman, M. and Spratt, B. G. (1998). Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic micro-organisms. *Proceedings of the National Academy of Sciences USA*, **95**, 3140-3145.

Maiden, M. J. and Stuart, J. M. UK Meningococcal Carriage Group. (2002). Carriage of serogroup C meningococci 1 year after meningococcal C conjugate polysaccharide vaccination. *Lancet*, **359**, 1829-1831.

Marc Laforce, F., Ravenscroft, N., Djingarey, M. and Vivani, S. (2009). Epidemic meningitis due to group A *Neisseria meningitidis* in the African meningitis belt: A persistant problem with an imminent solution. *Vaccine*, **27**, supplement 2, B13-19.

Martin, D. R., Walker, S. J., Baker M. G. and Lennon, D. R. (1998). New Zealand epidemic of meningococcal disease identified by a strain with phenotype B:4:P1.4. *Journal of Infectious Disease*, **177**, 497-500.

McHale, J., Habiba, M., Dixon-Woods, M., Cavers, D., Heney, D. and Pritchard-Jones, K. (2007). Consent for childhood cancer tissue banking in the UK: the effect of the Human Tissue Act 2004. The Lancet Oncology, **8**, 266-272.

Miller, E., Salisbury, D. and Ramsey, M. (2001). Planning, registration, and implementation of an immunisation campaign against meningococcal serogroup C disease in the UK: a success story. *Vaccine*, **20**, 58-67.

Morelli, G., Malorny, B., Müller, K., Seiler, A., Wang, J. F., Del-Valle, J. and Achtman, M. (1997). Clonal descent and microevolution of *Neisseria meningitidis* during 30 years of epidemic spread. *Molecular Microbiology*, **25**, 1047-1064.

Morley, S. L., Cole, M. J., Ison, C. A., Camaraza, M. A., Sotolongo, F., Anwar, N., Cuevas, I., Carbonero, M., Campa, H. C., Sierra, G and Levin, M. (2001). Immunogenicity of a serogroup B meningococcal vaccine against multiple *Neisseria meningitidis* strains in infants. Journal of Paediatric Infectious disease, **20**, 1054-1061.

Nadel, S. (2001). Lumbar puncture should not be performed in meningococcal disease. *Archieves of Diseases in Childhood*, **84**, 375.

Nicolas, P., Norhelm, G., Garnotel, E., Djibo, S. and Caugent, D. A. (2005). Molecular epidemiology of *Neisseria meningitidis* isolated in the African meningitis belt between 1988 and 2003 shows dominance of sequence type 5 (ST-5) and ST11 complexes. *Journal of Clinical Microbiology*, **43**, 5129-5135.

Pace, D., Pollard, A. J. and Messonier, N. E. (2009). Quadrivalent meningococcal conjugate vaccines. *Vaccine*, **27**, supplement 2, B30-41.

Perrett, K. P. and Pollard, A. J. (2005). Towards an improved serogroup B *Neisseria meningitidis* vaccine. *Expert Opinion in Biological Therapy*, **5**, 1611-1625.

Poolman, J. T., van der Ley, P. A. and Tommassen, J. (1995). Surface structures and secreted products of meningococci. In *Meningococcal Disease*, 21-34. Edited by K.A.V. Cartwright. Chichester: John Wiley and Sons.

Ragunathen, I., Ramsay, M., Borrow, R., Guiver, M., Gray, S. and Kaczmarski, E. B. (2000). Clinical features, laboratory findings and management of meningococcal meningitis in England and Wales: report of a 1997 survey. *Journal of Infection*, **40**, 74-79

Read, R. C., Teare, D. M., Pridmore, A. C., Naylor, S. C., Timms, J. M., Kaczmarski, E. B., Borrow, R. and Wilson, A. G. (2009). The tumor necrosis factor polymorphism TNF (-308) is associated with susceptibility to meningococcal sepsis, but not with lethality. *Critical Care Medicine*, **37**, 1237-1243.

Rouaud, P., Perrocheau, A., Taha, M. K., Sesboue, C., Forques, A., Parent du Chatelet, I. and Levy-Bruhi, D. (2006). Prolonged outbreak of B meningococcal disease in the Seine-Martime department, France, January 2003 to June 2005. *Eurosurviellance*, **11**, 178-181.

Russell, J. E., Jolley, K. A., Feavers, I. M., Maiden, M. C. J. and Suker, J. (2004). PorA variable regions of *Neisseria meningitidis. Emerging Infectious Diseases,* **10**, 674-678.

Russell, J. E., Urwin, R., Gray, S., Fox, A. J., Feavers, I. M. and Maiden, M. C. (2008). Molecular epidemiology of meningococcal disease in England and Wales 1975-1995, before the introduction of serogroup C conjugate vaccines. *Microbiology*, **154**, 1170-1177.

Salisbury, D. (2001). Introduction of a conjugate meningococcal type C vaccine programme in the UK. *Journal of Child Health*, **37**, 34-36.

Shigematsu, M., Davison, K. L., Charlett, A. and Crowcroft, N. S. (2002). National enhanced surveillance of meningococcal disease in England, Wales and Northern Ireland, January 1999-June 2001. *Epidemiology and Infection*, **129**, 459-470.

Singleton, R. J., Hennessy, T. W., Bulkow, L. R., Hammitt, L. L., Zulz, T., Hurlburt, D. A., Butler, J. C., Rudolph, K. and Parkinson, A. (2007). Invasive pneumococcal disease caused by nonvaccine serotypes among Akaska native children with high levels of 7-valent pneumococcal conjugate vaccine coverage. *Journal of the American Medical Association*, **297**, 1784-1792.

Smith, I., Caugent, D. A., Hoiby, E. A., Wentzel-Larsen, T. and Halstensen, A. (2006). High case-fatality rates of meningococcal disease in Western Norway caused by serogroup C strains belonging to both sequence type (ST)-32 and ST11 complexes, 1985-2002. *Epidemiologocal Infection*, **134**, 1195-1202.

Stefanelli, P., Fazio, C., Neri, A. T., Sofia, T. and Mastrantonio, P. (2003). First report of capsule replacement among electrophoretic type 37 *Neisseria meningitidis* strains in Italy. *Journal of Clinical Microbiology*, **41**, 5783-5786.

Steven, N. and Wood, M. (1995). The clinical spectrum of meningococcal disease. In Meningococcal Disease, 177-205. Edited by Cartwiright, K. Chichester, England. John Wiley and Sons.

Strange, J. R., and Paugh, E. J. (1992). Reducing case fatality by giving penicillin before admission to hospital. *British Medical Journal,* **305**, 141-143.

Stuart, J., Smyth, B., Ramsay, M., Patterson, W., Monk, P., MacKensie, R., Kroll, S., Kaczmarski, E., Irwin, D., Evans, M. and Duggal, H. (2002). Guidelines for public health management of meningococcal disease in the UK. *Communicable Disease and Public Health,* **5**, 187-204.

Sullivan, C. B., Jefferies, J. M., Diggle, M. A. and Clarke, S. C. (2006). Automation of MLST using third-generation liquid-handling technology. *Molecular Biotechnology,* **32**, 219-226.

Swaminathan, B., Matar, G. M., Reeves, M. W., Graves, L. M., Ajello, G., Bibb, W. F., Helsel, L. O., Morales, M., Dronavalli, H., El-Swify, M., Dewitt, W. and Hunter, S. B. (1996). Molecular subtyping of *Neisseria meningitidis* Serogroup B: Comparison of five methods. *Journal of Clinical Microbiology*, **34**, 1468-1473.

Swartley, J. S., Marfin, A. A., Edupuganti, S., Liu, L. J., Cieslak, P., Perkins, B., Wenger, J. D. and Stephens, D. S. (1997). Capsule switching of *Neisseria meningitidis*. *Proceedings of the National Academy of Sciences USA*, **94**, 271-276.

Taha, M. K. (2002). Molecular detection and characterisation of *Neisseria meningitidis. Expert Review of Molecular Diagnaosis,* **2**, 143-150.

Taha, M. K., Alonso, J. M., Cafferkey, M., Caugant, D. A., Clarke, S. C., Diggle, M. A., Fox, A., Frosch, M., Gray, S. J., Guiver, M., Heuberger. S., Kalmusova, J., Kesanopoulos, K., Klem, A. M., Kriz, P., Marsh, J., Mölling, P., Murphy, K., Olcén, P., Sanou, O., Tzanakaki, G. and Vogel, U. (2005). Interlaboratory comparison of PCR-based identification and genogrouping of *Neisseria meningitidis*. *Journal of Clinical Microbiology*. **43**, 144-149.

Thomson, E. A., Feavers, I. M. and Maiden, M. C. (2003). Antigenic diversity of meningococcal enterobactin receptor FetA, a vaccine component. *Journal of Microbiology*, **149**, 1849-1858.

Trotter, C. L., Ramsay, M. E. and Kaczmarski, E. B. (2002). Meningococcal serogroup C conjugate vaccination in England and Wales: coverage and initial impact of the campaign. *Communicable Disease and Public Health*, **5**, 220-225.

Trotter, C. L., Gay, N. J. and Edmunds, W. J. (2005). Dynamic models of meningococcal carriage, disease, and the impact of serogroup C conjugate vaccination. *American Journal of Epidemiology*, **162**, 89-100.

Trotter, C. L., Chandra, M., Cano, R., Larrauri, A., Ramsay, M. E., Brehony, C., Jolley, K. A., Maiden, M. C., Heuberger, S. and Frosch, M. (2006). A surveillance network for meningococcal disease in Europe. *FEMS Microbiological reviews*, **31**, 27-36.

Trotter, C. L., McVernon, J., Ramsay M. E., Whitney, C. G., Mulholland E. K., Goldblatt, D., Hombach, J., Kieny, M. P. SAGE subgroup. (2008). Optimising the use of conjugate vaccines to prevent disease caused by *Haemophilus influenzae* type b, *Neisseria meningitidis* and *Streptococcus pneumoniae*. *Vaccine,* **26**, 4434-4445.

Tsang, R. S. W., Law, D. K. S., Tyler, S. D., Stephens, G. S., Bigham, M. and Zollinger, W. D. (2005). Potential capsule switching from serogroup Y to B: The characterisation of three such *Neisseria meningitidis* isolates causing invasive meningococcal disease in Canada. *The Canadian Journal of infection diseases in Medical Microbiology*, **16**, 171-174.

Tyler, S., and Tsang, R. (2004). Genetic analysis of Canadian isolates of C:2a:P1.2,5 and B:2a:P1.2,5 *Neisseria meningitidis* strains belonging to the hypervirulent clone of ET-15. *Canadian Journal of Microbiology*, **50**, 433-443.

Urwin, R., Russell, J. E., Thompson, E. A. L., Holmes, E. C., Feavers, I. M. and Maiden, M. C. J. (2004). Distribution of surface protein variants among hyperinvasive meningococci: implications for vaccine design. *Infection and Immunity*, **72**, 5955-5962.

Vogel, U., Claus, H. and Frosch, M. (2000a). Rapid serogroup switching in *Neisseria meningitidis*. *New England Journal of Medicine*, **342**, 219-220.

Vogel, U., Claus, H., Frosch, M. and Caugant, D. A. (2000b). Molecular basis for distinction of the ET-15 clone within the ET-37 complex of *Neisseria meningitidis*. *Journal of Clinical Microbiology,* **38**, 941-942.

WHO. (2001). Meningococcal disease, serogroup W135. *Weekly Epidemiologic Reviews*, **19**, 141-142.

Wilder-Smith, A. and Memish, Z. (2003). Meningococcal disease and travel. *International Journal of Antimicrobial Agents*, 21, 102-106.

Yakubu, D. E., Abadi, F. J. and Pennington, T. H. (1994). Molecular typing methods for *Neisseria meningitidis*. *Epidemiology and Infection*, **113**, 53-65.

Yakubu, D. E. and Pennington, T. H. (1995). Epidemiological evaluation of Neisseria meningitidis serogroup B by pulsed-field gel electrophoresis. *FEMS Immunology and Medical Microbiology*, **10**, 185-189.

Zimmer, S. M. and Stephens, D. S. (2004). Meningococcal conjugate vaccines. *Expert Opinions of Pharmacotherapy*, **5**, 855-863.

Appendices

Appendix A Group B isolates MLST Data Nov 1998-Nov 1999

Appendix A1 Clonal complex

Clonal complex	n=
ST-103 complex	4
ST-11 complex/ET-37	7
ST-1494 complex	1
ST-162 complex	4
ST-167 complex	5
ST-18 complex	15
ST-213 complex	39
ST-22 complex	5
ST-269 complex	237
ST-32 complex/ET-5	95
ST-334 complex	1
ST-35 complex	14
ST-364 complex	5
ST-41/44 complex/Lineage 3	406
ST-461 complex	10
ST-53 complex	1
ST-60 complex	28
ST-8 complex/Cluster A4	11
Unassigned	45

Clonal complex distribution, MLST determination of MRU isolates carried out at Oxford University dept of zoology. Data analysis, this study.

Appendix A2 Breakdown of CC ST41/44 lineage3 into sequence types

Sequence type	Clonal complex	n=
40	ST-41/44 complex/Lineage 3	4
41	ST-41/44 complex/Lineage 3	185
42	ST-41/44 complex/Lineage 3	1
43	ST-41/44 complex/Lineage 3	5
44	ST-41/44 complex/Lineage 3	2
46	ST-41/44 complex/Lineage 3	1
136	ST-41/44 complex/Lineage 3	5
146	ST-41/44 complex/Lineage 3	1
154	ST-41/44 complex/Lineage 3	17
170	ST-41/44 complex/Lineage 3	5
180	ST-41/44 complex/Lineage 3	12
191	ST-41/44 complex/Lineage 3	1
206	ST-41/44 complex/Lineage 3	1
207	ST-41/44 complex/Lineage 3	1
274	ST-41/44 complex/Lineage 3	7
303	ST-41/44 complex/Lineage 3	4
318	ST-41/44 complex/Lineage 3	1
340	ST-41/44 complex/Lineage 3	11
414	ST-41/44 complex/Lineage 3	1
437	ST-41/44 complex/Lineage 3	4
477	ST-41/44 complex/Lineage 3	2
482	ST-41/44 complex/Lineage 3	4
493	ST-41/44 complex/Lineage 3	1
571	ST-41/44 complex/Lineage 3	2
839	ST-41/44 complex/Lineage 3	2
877	ST-41/44 complex/Lineage 3	1
944	ST-41/44 complex/Lineage 3	1
1028	ST-41/44 complex/Lineage 3	1
1091	ST-41/44 complex/Lineage 3	1
1097	ST-41/44 complex/Lineage 3	12
1194	ST-41/44 complex/Lineage 3	1
1200	ST-41/44 complex/Lineage 3	5
1403	ST-41/44 complex/Lineage 3	1
1480	ST-41/44 complex/Lineage 3	4
1489	ST-41/44 complex/Lineage 3	2
1770	ST-41/44 complex/Lineage 3	1
1778	ST-41/44 complex/Lineage 3	3
1823	ST-41/44 complex/Lineage 3	4
1915	ST-41/44 complex/Lineage 3	1

MLST determination carried out at Oxford University dept. of Zoology. Data analysis, this study

Appendix A2 continued

2080	ST-41/44 complex/Lineage 3	4
2203	ST-41/44 complex/Lineage 3	1
2211	ST-41/44 complex/Lineage 3	1
2253	ST-41/44 complex/Lineage 3	1
2259	ST-41/44 complex/Lineage 3	1
2279	ST-41/44 complex/Lineage 3	2
2314	ST-41/44 complex/Lineage 3	2
2671	ST-41/44 complex/Lineage 3	1
2691	ST-41/44 complex/Lineage 3	1
2761	ST-41/44 complex/Lineage 3	2
2806	ST-41/44 complex/Lineage 3	1
2821	ST-41/44 complex/Lineage 3	4
2836	ST-41/44 complex/Lineage 3	1
2837	ST-41/44 complex/Lineage 3	1
2916	ST-41/44 complex/Lineage 3	1
3050	ST-41/44 complex/Lineage 3	2
3248	ST-41/44 complex/Lineage 3	2
3457	ST-41/44 complex/Lineage 3	1
3461	ST-41/44 complex/Lineage 3	1
3620	ST-41/44 complex/Lineage 3	1
3754	ST-41/44 complex/Lineage 3	2
3794	ST-41/44 complex/Lineage 3	1
3802	ST-41/44 complex/Lineage 3	1
3866	ST-41/44 complex/Lineage 3	1
4018	ST-41/44 complex/Lineage 3	1
4021	ST-41/44 complex/Lineage 3	2
4026	ST-41/44 complex/Lineage 3	2
4029	ST-41/44 complex/Lineage 3	2
4030	ST-41/44 complex/Lineage 3	1
4031	ST-41/44 complex/Lineage 3	1
4033	ST-41/44 complex/Lineage 3	1
4036	ST-41/44 complex/Lineage 3	1
4039	ST-41/44 complex/Lineage 3	1
4040	ST-41/44 complex/Lineage 3	1
4042	ST-41/44 complex/Lineage 3	1
4048	ST-41/44 complex/Lineage 3	1
4049	ST-41/44 complex/Lineage 3	1
4082	ST-41/44 complex/Lineage 3	1
4084	ST-41/44 complex/Lineage 3	2
4085	ST-41/44 complex/Lineage 3	1

MLST determination carried out at Oxford University dept. of Zoology. Data analysis, this study.

Appendix A2 continued

4086	ST-41/44 complex/Lineage 3	1
4088	ST-41/44 complex/Lineage 3	1
4092	ST-41/44 complex/Lineage 3	1
4097	ST-41/44 complex/Lineage 3	1
4100	ST-41/44 complex/Lineage 3	1
4111	ST-41/44 complex/Lineage 3	1
4112	ST-41/44 complex/Lineage 3	1
4113	ST-41/44 complex/Lineage 3	1
4249	ST-41/44 complex/Lineage 3	1
4359	ST-41/44 complex/Lineage 3	1
4438	ST-41/44 complex/Lineage 3	1
4444	ST-41/44 complex/Lineage 3	1
4448	ST-41/44 complex/Lineage 3	1
4449	ST-41/44 complex/Lineage 3	1
4523	ST-41/44 complex/Lineage 3	1
4525	ST-41/44 complex/Lineage 3	1
4526	ST-41/44 complex/Lineage 3	1
4975	ST-41/44 complex/Lineage 3	1
5281	ST-41/44 complex/Lineage 3	1
5357	ST-41/44 complex/Lineage 3	1
5446	ST-41/44 complex/Lineage 3	1
5503	ST-41/44 complex/Lineage 3	2
5509	ST-41/44 complex/Lineage 3	1
5511	ST-41/44 complex/Lineage 3	1
5513	ST-41/44 complex/Lineage 3	1
5515	ST-41/44 complex/Lineage 3	1
5516	ST-41/44 complex/Lineage 3	1
5534	ST-41/44 complex/Lineage 3	1
5535	ST-41/44 complex/Lineage 3	1
5536	ST-41/44 complex/Lineage 3	1
5537	ST-41/44 complex/Lineage 3	1

MLST determination carried out at Oxford University dept. of Zoology. Data analysis, this study.

Appendix A3 Breakdown of CC ST269 into sequence types

Sequence type	Clonal complex	n=
13	ST-269 complex	2
269	ST-269 complex	108
275	ST-269 complex	24
283	ST-269 complex	26
479	ST-269 complex	2
798	ST-269 complex	1
1049	ST-269 complex	4
1089	ST-269 complex	1
1092	ST-269 complex	4
1095	ST-269 complex	1
1161	ST-269 complex	5
1163	ST-269 complex	5
1195	ST-269 complex	9
1214	ST-269 complex	6
1273	ST-269 complex	1
1284	ST-269 complex	2
1298	ST-269 complex	1
1416	ST-269 complex	4
1774	ST-269 complex	1
1791	ST-269 complex	9
2239	ST-269 complex	1
2803	ST-269 complex	1
2835	ST-269 complex	1
3458	ST-269 complex	1
4020	ST-269 complex	1
4023	ST-269 complex	1
4087	ST-269 complex	1
4116	ST-269 complex	1
4362	ST-269 complex	1
4447	ST-269 complex	1
4451	ST-269 complex	1
5502	ST-269 complex	1
5505	ST-269 complex	1
5514	ST-269 complex	1
5528	ST-269 complex	1
5532	ST-269 complex	1

MLST determination carried out at Oxford University dept. of Zoology. Data analysis, this study.

Appendix B Group C isolates MLST Data Nov 1998-Nov 1999

Appendix B1 Clonal complex

Clonal complex	n=
ST-103 complex	1
ST-11 complex/ET-37 complex	544
ST-18 complex	2
ST-213 complex	1
ST-23 complex/Cluster A3	1
ST-254 complex	1
ST-269 complex	8
ST-32 complex/ET-5 complex	2
ST-334 complex	1
ST-35 complex	1
ST-41/44 complex/Lineage 3	10
ST-60 complex	3
ST-8 complex/Cluster A4	73
Unassigned	7

Clonal complex distribution, MLST determination carried out at Oxford University dept. of Zoology. Data analysis, this study.

Appendix B2 Breakdown of CC ST11 into sequence types

Sequence type	Clonal complex	n=
1025	ST-11 complex/ET-37 complex	4
11	ST-11 complex/ET-37 complex	495
1287	ST-11 complex/ET-37 complex	1
1410	ST-11 complex/ET-37 complex	1
1988	ST-11 complex/ET-37 complex	4
214	ST-11 complex/ET-37 complex	1
2709	ST-11 complex/ET-37 complex	1
2942	ST-11 complex/ET-37 complex	1
3035	ST-11 complex/ET-37 complex	1
3455	ST-11 complex/ET-37 complex	4
3456	ST-11 complex/ET-37 complex	1
3462	ST-11 complex/ET-37 complex	1
3751	ST-11 complex/ET-37 complex	1
4025	ST-11 complex/ET-37 complex	1
4044	ST-11 complex/ET-37 complex	1
4091	ST-11 complex/ET-37 complex	2
4093	ST-11 complex/ET-37 complex	1
4098	ST-11 complex/ET-37 complex	1
4115	ST-11 complex/ET-37 complex	1
4357	ST-11 complex/ET-37 complex	2
4445	ST-11 complex/ET-37 complex	1
491	ST-11 complex/ET-37 complex	2
4976	ST-11 complex/ET-37 complex	1
4977	ST-11 complex/ET-37 complex	1
50	ST-11 complex/ET-37 complex	1
52	ST-11 complex/ET-37 complex	1
5517	ST-11 complex/ET-37 complex	1
67	ST-11 complex/ET-37 complex	11

MLST determination carried out at Oxford University dept. of Zoology. Data analysis, this study.

Appendix B3 Breakdown of CC ST8 into sequence types

Sequence type	Clonal complex	n=
3459	ST-8 complex/Cluster A4	1
4035	ST-8 complex/Cluster A4	1
4047	ST-8 complex/Cluster A4	5
4089	ST-8 complex/Cluster A4	1
66	ST-8 complex/Cluster A4	5
760	ST-8 complex/Cluster A4	2
8	ST-8 complex/Cluster A4	58

MLST determination carried out at Oxford University dept. of Zoology. Data analysis, this study.

Appendix C Group B isolates MLST Data Nov 2000-Nov 2001

Clonal complex

Clonal complex	n=
ST-103 complex	5
ST-11 complex/ET-37 complex	14
ST-1157 complex	4
ST-162 complex	1
ST-167 complex	2
ST-18 complex	14
ST-213 complex	50
ST-22 complex	1
ST-254 complex	2
ST-269 complex	266
ST-32 complex/ET-5 complex	78
ST-35 complex	13
ST-364 complex	3
ST-41/44 complex/Lineage 3	367
ST-461 complex	10
ST-60 complex	31
ST-750 complex	1
ST-8 complex/Cluster A4	8
Unassigned	63

MLST determination carried out at Oxford University dept. of Zoology. Data analysis, this study.

Appendix D Laboratory confirmed Group B cases 1984-2006. MRU data.

Serogroup B Cases	Culture only	Culture and PCR	PCR only	Total		Culture only	Culture and PCR	PCR only
1984	300			300		100%	0%	0%
1985	377			377		100%	0%	0%
1986	573			573		100%	0%	0%
1987	679			679		100%	0%	0%
1988	852			852		100%	0%	0%
1989	885			885		100%	0%	0%
1990	996			996		100%	0%	0%
1991	964			964		100%	0%	0%
1992	923			923		100%	0%	0%
1993	928			928		100%	0%	0%
1994	789			789		100%	0%	0%
1995	904			907		100%	0%	0%
1996	761	68	26	855		89%	8%	3%
1997	621	261	291	1173		53%	22%	25%
1998	552	324	336	1212		46%	27%	28%
1999	556	423	496	1475		38%	29%	34%
2000	555	401	703	1659		33%	24%	42%
2001	522	418	770	1710		31%	24%	45%
2002	424	323	638	1385		31%	23%	46%
2003	403	353	619	1375		29%	26%	45%
2004	325	294	525	1144		28%	26%	46%
2005	327	293	659	1279		26%	23%	52%
2006	297	248	511	1056		28%	23%	48%

Note: PCR analysis introduced in October 1996.

Appendix E. Laboratory confirmed Group C cases 1984-2006. MRU data.

Serogroup C Cases	Culture only	Culture and PCR	PCR only	Total		Culture only	Culture and PCR	PCR only
1984	125			125		100%	0%	0%
1985	180			180		100%	0%	0%
1986	392			392		100%	0%	0%
1987	442			442		100%	0%	0%
1988	464			464		100%	0%	0%
1989	436			436		100%	0%	0%
1990	466			466		100%	0%	0%
1991	393			393		100%	0%	0%
1992	326			326		100%	0%	0%
1993	319			319		100%	0%	0%
1994	291			291		100%	0%	0%
1995	465			465		100%	0%	0%
1996	509	72	30	611		83%	12%	5%
1997	406	204	209	819		50%	25%	26%
1998	353	237	236	826		43%	29%	29%
1999	349	310	341	1000		35%	31%	34%
2000	260	194	261	715		36%	27%	37%
2001	126	78	117	321		39%	24%	36%
2002	74	54	40	168		44%	32%	24%
2003	45	19	34	98		46%	19%	35%
2004	19	15	25	59		32%	25%	42%
2005	13	4	11	28		46%	14%	39%
2006	12	6	11	29		41%	21%	38%
2007	18	4	17	39		46%	10%	44%

Note: PCR analysis introduced in October 1996.

Appendix F Fatal group B case isolates from 1999 with phenotype (MRU) and MLST data (Oxford University).

Lab No.	site	grp	type	sub-type	ST	CC
M99.240178	TS	B	NT	P1.15	269	ST-269 Complex
240148	BC	B	4	P1.4	340	ST-41/44 Lineage 3
240278	BC	B	4	P1.4	340	ST-41/44 Lineage 3
240364	TS	B	1	P1.10	4110	Unassigned
240531	BC	B	4	P1.2.P1.5	4028	ST-35 complex
240689	BC	B	4	P1.4	340	ST-41/44 Lineage 3
240960	BC	B	15	P1.7,P1.16	259	ST-32/ET-5 complex
240930	BC	B	4	P1.4	4036	ST-41/44 Lineage 3
240928	BC	B	4	P1.10	4002	ST-32/ET-5 complex
241116	TRACH	B	15	P1.6	41	ST-41/44 Lineage 3
241417	BC	B	4	P1.4	41	ST-41/44 Lineage 3
241418	BC	B	NT	P1.9	4050	Unassigned
241547	BC	B	4	P1.4	41	ST-41/44 Lineage 3
241619	BC	B	1	P1.15	284	Unassigned
241735	BC	B	2B	NT	4437	ST-8 Cluster A4
242004	BC	B	22	P1.14	18	ST-18 complex
241999	BC	B	NT	P1.4	41	ST-41/44 Lineage 3
242146	BC	B	14	P1.4	154	ST-41/44 Lineage 3
242269	BC	B	15	P1.7,P1.16	32	ST-32 ET-5 complex
242546	BC	B	4	P1.15	34	ST-32 ET-5 complex
243163	BC	B	NT	P1.3,P1.6	1617	ST-22 complex
243265	BC	B	4	P1.6	5537	ST-41/44 Lineage 3
243289	BC	B	4	P1.15	33	ST-32 ET-5 complex
243340	CSF	B	4	P1.15	5539	ST-32 ET-5 complex
243657	BC	B	4	P1.5	NA	NA
243755	BC	B	4	P1.4	NA	NA
240096	BC	B	4	P1.4	340	ST-41/44 Lineage 3
240871	CSF	B	4	P1.4	5513	ST-41/44 Lineage 3
242067	BC	B	NT	NT	5527	ST-18 complex
242178	BC	B	1	P1.4	2837	ST-41/44 Lineage 3
242391	BC	B	4	NT	NA	NA
243610	BRAIN PUS	B	22	NT	NA	NA
243951	TS	B	4	P1.4	NA	NA
240076	BC	B	NT	P1.6	479	ST-269 complex
240087	BC	B	NT	P1.9	1195	ST-269 complex
240265	BC	B	NT	P1.6	479	ST-269 complex
240178	TS	B	NT	P1.15	269	ST-269 complex
240514	BC	B	4	P1.14	4432	Unassigned
241186	BC	B	NT	NT	4041	ST-60 complex
241198	BC	B	22	P1.14	18	ST-18 complex
241477	BC	B	NT	P1.15	269	ST-269 complex
241781	BC	B	15	P1.7,P1.16	259	ST-32 ET-5 complex
241931	BC	B	4	P1.16	944	ST-41/44 Lineage 3
242053	BC	B	15	P1.1	32	ST-32 ET-5 complex
242196	BC	B	NT	P1.9	275	ST-269 complex

Appendix F continued

242331	BC	B	4	P1.4	274	ST-41/44 Lineage 3
242476	BC	B	NT	NT	269	ST-269 complex
243316	CSF	B	4	P1.4	41	ST-41/44 Lineage 3

NA= not available (for 1999 only isolates up to the end of October had MLST analysis carried out). MLST determination of MRU isolates carried out at Oxford University dept. of Zoology.

TS = throat swab, BC = Blood culture, CSF = cerebro-spinal fluid.