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IMMUNITY TO *NEISSERIA MENINGITIDIS* IN UNIVERSITY STUDENTS

by

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ABSTRACT

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In October 1997 a cluster of *N. meningitidis* serogroup C infection occurred amongst first year students at an English university. Six cases were reported, with three fatalities. The measurement of complement-mediated serum bactericidal activity (SBA) is the generally accepted serological correlate of immunity to meningococci. In this thesis, the relationship between SBA and antibody reactivity to individual meningococcal antigens was explored. Individuals who became infected showed low levels of SBA against the outbreak strain; survivors developed SBA that correlated with production of antibodies to group C capsular polysaccharide but not to lipopolysaccharide (LPS) or major outer membrane proteins. Sera obtained one month before the outbreak from asymptomatic classmates of one of the cases, also showed a strong correlation between SBA and the presence of anti-capsular antibodies. No effective vaccine exists for *N. meningitidis* serogroup B, therefore levels of immunity to group B were investigated in the same student population. In contrast to group C, no association was demonstrated between BA directed against group B and the presence of antibodies to the group B capsular polysaccharide but there was a correlation between antibodies reacting with PorA and PorB proteins, and SBA.

A follow-up carrier study was conducted on first year university students in which the dynamics of group B meningococcal carriage and the contribution of carriage to protective immunity against group B was investigated. Acquisition of carriage was always associated with specific SBA, indicating that carriage can induce natural immunity to meningococci. This immunity protected students against both homologous and heterologous strains, and was not associated with antibodies to LPS or group B capsular polysaccharide. A relationship between antibodies reacting with PorA and PorB proteins and bactericidal antibodies was noted.

The results presented in this thesis indicate that antibodies to group B capsular polysaccharide and LPS are not involved in immune protection against group B meningococci. This work validates strategies for prevention of group B infection based on vaccines containing PorA and suggests that PorB may also be an important component of such vaccines.

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PUBLICATIONS

The work presented in this thesis has formed the basis of the following publications:

A) Publications

1. Jones GR, Williams JN, Christodoulides M, Jolley K and Heckels JE. (2000) Lack of immunity in university students before an outbreak of serogroup C meningococcal infection. *Journal of Infectious Diseases*, **181**:1172-5.

2. Jordens JZ, Williams JN, Jones GR, and Heckels JE. (2002) Detection of meningococcal carriage by culture and PCR of throat swabs and mouth gargles. *Journal of Clinical Microbiology,* **40**: 1:75-79.

3. Williams JN, Jones GR, Christodoulides M, and Heckels JE. (2003) Serological Correlates of Protection against Meningococci in a Cohort of University Students Before and During an Outbreak of Serogroup C Infection. *Journal of Infectious Diseases*, **187**:1433-41.

4. Williams JN, Jordens JZ, Jones GR, Christodoulides M, Heckels JE. Carriage and immunity to *Neisseria meningitidis.* Manuscript in preparation.

B) Abstracts

5. Jordens JZ, Williams JN, Jones GR, and Heckels JE. (2000) Detection of nasopharyngeal carriage of *Neisseria meningitidis* by culture and PCR of throat swabs and mouth washings. In: *Proceedings of Public Health Laboratory Service 25th Annual Scientific Conference, University of Warwick.*

6. Williams JN, Jordens JZ, Jones GR, and Heckels JE. (2000) Carriage of a serogroup C meningococcal strain after A-C meningococcal vaccination. In*: International Pathogenic Neisseria Conference 2000: Proceedings of the Twelfth International Pathogenic Neisseria Conference, Texas, America (abstract 143).*

7. Williams JN, Jones GR, Christodoulides M, and Heckels JE. (2000) The immune status of university students before and during an outbreak of serogroup C meningococcal infection. In: *Neisseria2000: Proceedings of the Twelfth International Pathogenic Neisseria Conference, Texas, America (abstract 97*).

8. Jordens JZ, Williams JN, Jones GR, and Heckels JE. (2000) Sensitivity and negative predictive value of culture and PCR of throat swabs and gargles in determining carriage of Neisseria meningitidis. In: *International Pathogenic Neisseria Conference 2000: Proceedings of the Twelfth International Pathogenic Neisseria Conference, Texas, America (abstract 241).*

9. Williams JN, Jordens JZ, Jones GR, Christodoulides M, and Heckels JE. (2001) Dynamics of meningococcal carriage in a student population. In: *Proceedings of Public Health Laboratory Service 26th Annual Scientific Conference University of Warwick Conference, Warwick.*

10. Williams JN, Jones GR, Christodoulides M, Heckels JE. (2002) Serological Correlates of Protection in Non-infected 'at-risk' Students Prior to an Outbreak of Meningococcal Infection at an English University. In: *International Pathogenic Neisseria Conference 2002: Proceedings of the Thirteenth International Pathogenic Neisseria Conference, Oslo, Norway (abstract 226).*

11. Williams JN, Jordens JZ, Jones GR, Christodoulides M, Heckels JE. (2002) Immune Response to Meningococcal Carriage in a Student Population. In: *International Pathogenic Neisseria Conference 2002: Proceedings of the Thirteenth International Pathogenic Neisseria Conference, Oslo, Norway (abstract 225).*

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ABBREVIATIONS

6-FAM	6-carboxyfluorescein
AU	Arbitrary units
A_x	Absorbance nanometers
BA	Bactericidal activity
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
c.f.u.	Colony forming unit
CNS	Central nervous system
CSF	Cerebrospinal fluid
ctrA	Capsular transfer gene
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	2'deoxyribonucleoside
EDTA	Ethylenediamine tetra acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FCS	Foetal calf serum
g	Gravity
HRP	Horseradish peroxidase
HSA	Human serum albumin
Ig	Immunoglobulin
KDa	Kilodaton
LPS	Lipopolysaccharide
mAb	Monoclonal antibody

MenB	Serogroup B meningococcus
MenC	Serogroup C meningococcus
mHSA	Methylated human serum albumin
MRU	Meningococcal Reference Unit
NBT	Nitro blue tetrazolium
NST	Non-subtypeable
NT	Non-typeable
°C	Degrees Celsius
OD	Optical density
OMP	Outer membrane protein
PBS	Dulbecco's phosphate buffered saline
PBSB	Dulbecco's complete phosphate buffered saline
PCR	Polymerase Chain Reaction
PHLS	Public Health Laboratory Service
PMSF	Phenylmethylsulphonylfluoride
PP	Proteose peptone
Rmp	Reduction modifiable protein
SBA	Serum Bactericidal Assay
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
siaD	Sialyltransferase gene
sIgA	Secretory immunoglobulin A
TAMRA	6-carboxy-tetramethyl-rhodamine
TBS	Tris buffered saline
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tm	Melting temperature
TMB	3,3',5,5'-tetramethylbenzidine
Tris	Tris (hydroxymethyl) methylamine
TTBS	Tween-Tris buffered saline

Tween 20	Polyoxyethylenesorbitane monolaurate
UHQ water	Ultra High Quality water
UV	Ultraviolet
v/v	Unit volume per unit volume
VR	Variable Region
w/v	Unit weight per unit volume
WHO	World Health Organisation

CHAPTER 1 INTRODUCTION

1.1 Meningococcal Disease

Clinical descriptions of illness resembling meningococcal disease date back to the 16th century (Cartwright, 1995a), but the etiological agent was not described until 1884 when Marchiafava and Celli found meningococci in cerebrospinal fluid (CSF). Three years later, Anton Weichselbaum isolated the organism from the CSF of patients with meningitis. His observation that the organism was an intracellular diplococcus lead him to name it *Diplokokkus intracellularis meningitidis*. The organism was later renamed *Neisseria meningitidis* after Albert Neisser, who described *Neisseria gonorrhoea* in 1879 (Devoe, 1982).

Infection with *N. meningitidis* remains an important cause of meningitis and septicaemia in the 21st century. Other less common clinical presentations include pneumonia, endophthalmitis, conjunctivitis, sinusitis, tracheobronchitis, pharyngitis, myocarditis, pericarditis, septic arthritis, and urethritis (Leake and Perkins, 2000). The case fatality rate is ca. 7% (Noah and Henderson, 2002) in the UK and approximately one fifth of survivors experience sequelae including: mental retardation, learning disabilities, seizures, hydrocephalus, gangrene of limbs, and hearing or vision loss (Leake and Perkins, 2000;Steven and Wood, 1995).

Meningococcal disease can occur at any time of the year but incidence in the northern hemisphere follows a seasonal pattern with most cases occurring in the winter and spring months (Jones, 1995). In contrast, in the African meningitis belt, epidemic disease occurs at the end of the dry season and is associated with the dry wind coming off the desert (Achtman, 1995). Meningococcal disease can occur at any age, but the highest age specific attack rates are seen in infancy as maternal antibody decreases. Rates decline with age during childhood but a second peak occurs at 15 to 19 years of age (Jones and Mallard, 1993).

1.1.1 Meningococcal Disease and the Military

The occurrence of epidemics of meningococcal disease in the military, making the organism a threat during war provided the impetus for research into the immune response to meningococci and possible control strategies. During the 1960's Goldschneider *et al* (1969a, 1969b) followed an epidemic of serogroup C infection in a military training camp at Fort Dix, New Jersey. These investigators demonstrated an inverse relationship between the presence of antibodies capable of complement-mediated bacteriolysis and the risk of subsequent meningococcal disease. They demonstrated that most military recruits entering the training camp possessed bactericidal antibodies to the epidemic strain and did not subsequently develop infection against this strain. In contrast, invasive disease developed in nearly 40% of recruits who lacked serum bactericidal activity and who acquired the strain.

Data obtained from studies of soldiers have formed the basis for the understanding of meningococcal disease. Military recruits and university students have several common characteristics including: age, crowded living conditions, intensive social mixing and diverse geographical background, therefore it is relevant to compare meningococcal infection in these groups.

1.1.2 Meningococcal Disease and University Students

During the 1990's, the UK experienced a number of outbreaks of meningococcal disease amongst university students. A large outbreak occurred at the University of Wales, Cardiff in November 1996, with another at the University of Southampton in October 1997 (Jones *et al.*, 2000).

Both American (Harrison *et al.*, 2001) and UK (Neal *et al.*, 1999) studies have established that university students are at an increased risk of meningococcal disease compared to non-students of a similar age. The risks of contracting meningococcal disease are increased when many young adults, a group with a high nasopharyngeal meningococcal carriage rate (Stephens, 1999;Jones and Mallard, 1993) are brought into close proximity within the close confines of university, thus facilitating transmission. The extensive opportunities for social mixing are further increased by the provision of catered halls of residence (Neal *et al.*, 1999). Many universities offer catered hall places almost exclusively to first year students (Neal *et al.*, 1999). First year students are most at risk when they first enter the university environment and are exposed to new meningococcal strains not previously

encountered (Ramsay *et al.*, 1997). The student's immunological status at the time of exposure to new strains will likely determine whether the organism is carried or causes disease.

1.2 Meningococcal Carriage

Not everyone exposed to *N. meningitidis* develops invasive meningococcal disease. The human nasopharynx is the only known reservoir of *N. meningitidis* and the usual situation is asymptomatic carriage. Person-to-person transmission occurs via respiratory droplets from a carrier of the organism or, rarely, from a person with invasive disease. The likelihood of contact with a carrier depends partly on the prevalence of carriage in the population.

Around 500 million out of the six billion people in the world are estimated to be carriers of *N. meningitidis* (Stephens, 1999). Carriage rates are lowest in young children, and highest in adolescents and young adults (Jones and Mallard, 1993;Cartwright, 1995b). Meningococcal carriage in open populations in Europe is estimated at around 10% (Cartwright, 1995b) and transmission rates in these circumstances are relatively low (Stephens, 1999). Nasopharyngeal carriage in closed or semi-closed institutions such as universities (Cartwright, 1995b), jails (Tappero *et al.*, 1996) and military establishments (Pether *et al.*, 1988) may rise to > 50%, which results in correspondingly higher transmission rates of meningococci (Stephens, 1999). The carrier state may last for a few days to months and is important because it not only provides a reservoir for meningococcal infection but also may enhance host immunity to meningococci (Jones *et al.*, 1998;Robinson *et al.*, 2002). Between five and twenty percent of normal individuals are carriers at any given time (Greenfield *et al.*, 1971;Fraser *et al.*, 1973), yet few develop meningococcal disease. This low incidence of disseminated disease following colonisation suggests that host rather than bacterial factors play a significant role and that the immunological status of the subject is important in determining disease occurrence and severity.

1.3 Host Defence Against Meningococcal Disease

A thorough understanding of organism - host interaction is essential if the mechanisms of bacterial pathogenicity and immunogenicity are to be elucidated.

1.3.1 Nasopharyngeal mucosa

Initiation of most cases of meningococcal disease begins with host acquisition of a new meningococcal strain by colonisation of epithelial cells lining the mucosal surfaces of the nasopharynx (Epstein *et al.*, 1992), therefore the first and most effective defence against meningococcal disease is an intact mucosal membrane. Chronic irritation of the mucosa due to dust or low humidity, or damage to the mucosa resulting from a concurrent viral or mycoplasmal upper respiratory infection, may be predisposing factors for invasive disease (Tzeng and Stephens, 2000).

1.3.2 Serum Bactericidal Activity

Following the early studies of Heist *et al* (1922), it has become generally accepted that the most important host defense against meningococcal disease is the presence of meningococcal-specific antibody and complement (antibody-mediated, complement-dependent killing). These investigators established that most cases of meningitis occurred amongst individuals that lacked bactericidal activity. Dr. Heist died of meningococcal meningitis and examination of his blood established that he lacked antibodies that were bactericidal to meningococci.

The gold standard for measuring bactericidal activity is the serum bactericidal assay (SBA). The classic studies of military recruits by Goldschneider *et al.,* (1969a,1969b) demonstrated an inverse relationship between the presence of bactericidal antibodies and the risk of subsequent meningococcal disease. These investigators established that antibodies against group C capsular polysaccharide correlated with protection against group C meningococcal disease. Subsequent studies (Rosenqvist *et al.*, 1993;Abdillahi and Poolman, 1988a) have demonstrated that serum bactericidal antibodies may also be directed against noncapsular meningococcal surface antigens. These antibodies are produced in response to meningococcal disease (Jones *et al.*, 2000) and/or colonisation with carrier strains of *N. meningitidis* (Jones *et al.*, 1998;Robinson *et al.*, 2002), *N. lactamica* (Kremastinou *et al.*, 1999), or other nonpathogenic *Neisseria spp.* Protective antibodies may also be stimulated by cross-reacting antigens on other bacterial species (Devoe, 1982). The role of bactericidal antibodies in prevention of invasive disease explains why high attack rates are seen in infants from six to nine months old (Goldschneider *et al.*, 1969b), the age at which maternally acquired antibodies are being lost. In addition to serum bactericidal activity, meningococcal antibodies may confer protection against meningococcal disease through C3b-directed

opsonophagocytosis (Quakyi *et al.*, 1999), however the relative importance of this mechanism remains to be elucidated.

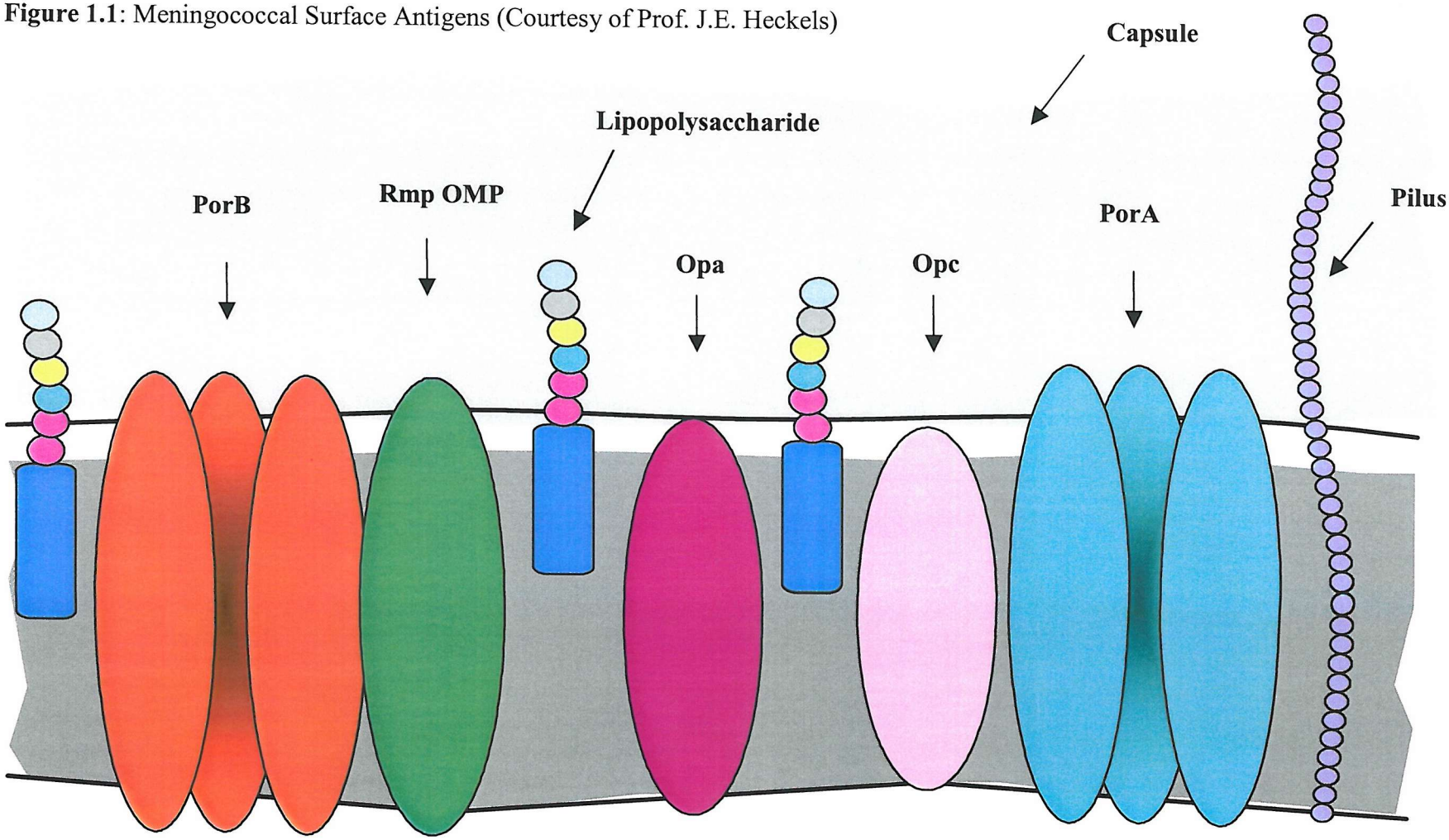
1.4 Molecular Structure of the Meningococcus

A basic understanding of the molecular structure of *N. meningitidis* (Figure 1.1) is required before immunity to meningococci can be studied. *N. meningitidis* is a Gram negative, oxidase positive diplococcus. In common with other Gram-negative organisms, meningococci possess a cytoplasmic membrane, a peptidoglycan layer and an outer membrane containing lipopolysaccharides and proteins. Many meningococci also possess a polysaccharide capsule and surface-exposed pili (Blake and Gotschlich, 1987). Most meningococcal research has focused on the meningococcal capsular polysaccharide and outer membrane, since antigenic components localised on the cell surface are those most likely to interact with the immune system. Microbial membranes are a dynamic environment and membrane components vary rapidly in response to different external stimuli and cellular metabolism. The phase switching (on/off) and antigenic variation of several meningococcal outer membrane proteins (OMPs) are believed to be used by the bacterium to circumvent host immunity and thereby facilitate survival at different sites (Nassif and So, 1995).

1.4.1.1 Outer Membrane Proteins

Meningococcal outer membranes from different strains display distinct protein profiles when subjected to SDS-PAGE analysis (Frasch and Gotschlich, 1974). Using peptide mapping of proteolytic fragments (Tsai *et al.*, 1981) it was discovered that each meningococcal strain produces 3-4 quantitatively dominant OMPs, and several minor proteins, some of which are produced in response to growth conditions. Several of these OMPs have subsequently been shown to have immunological importance. The OMPs were categorised into five classes, designated 1 through 5 in decreasing order of molecular weight range. The nomenclature was subsequently updated to reflect new understanding of meningococcal surface structures (Hitchcock, 1989) and this taxonomy will be used throughout this thesis.

Figure 1.1: Meningococcal Surface Antigens (Courtesy of Prof. J.E. Heckels)



1.4.1.1.1 PorA Protein (Class 1)

The PorA protein is shared by all serogroups and serotypes but shows a quantitative variability in expression; occasionally a strain is found which lacks it completely (Chapter 3 of this thesis) (Jones *et al.*, 2000). This protein functions as a cation selective porin in the outer membrane, allowing the transmembrane diffusion of small solutes into the organism (Blake and Gotschlich, 1987). The PorA protein is surface-exposed and induces bactericidal antibodies (Abdillahi and Poolman, 1988a), which are protective in the experimental rat model (Saukkonen *et al.*, 1987). Researchers have demonstrated the immunogenicity of the PorA protein in humans following either meningococcal infection (Guttormsen *et al.*, 1994), or immunisation with an experimental outer membrane (OMV) complex vaccine (Rosenqvist *et al.*, 1995). Expression of the PorA protein is antigenically stable within a strain, however extensive antigenic diversity is seen between strains and is the basis of serosubtyping. A two-dimensional structural model predicts that the PorA protein consists of eight surface exposed loops (I-VIII) (van der Ley *et al.*, 1991). Epitope mapping studies by McGuinness *et al.* (1990,1993) have shown that PorA proteins from different serosubtypes show considerable structural homology, with variations largely confined to two independent hypervariable regions, designated VR1 and VR2, located in loops I and IV respectively. The epitopes recognised by subtype specific monoclonal antibodies are located at the apices of one or other of these loops, thus generating two potential subtype specificities within one PorA protein. A lesser variable region in loop V of the PorA protein has been denoted VR3 (Maiden *et al.*, 1991).

1.4.1.1.2 PorB Protein (Class 2/3)

The meningococcal porin proteins encoded by the *porB* genes exist as one of two alleles encoding either a PorB2 (Class 2) or a PorB3 (Class 3) OMP that are mutually exclusive, and quantitatively the dominant outer membrane protein. The antigenic diversity of the PorB proteins are utilised in serotyping meningococcal strains. Each strain exhibits only a single serotype unlike the sero-subtype of the PorA protein, where two are present. These proteins exist as trimers and function as anion selective porins in membranes (Frasch *et al.*, 1986). They have the ability to translocate from intact cells and insert into eukaryotic cell membranes to form voltage-dependent channels, a process that may be important in invasion.

Meningococcal disease induces the production of PorB Protein specific antibodies (Guttormsen *et al.*, 1993). Some studies (Frasch *et al.*, 1986;Wright *et al.*, 2002) have shown that such antibodies are bactericidal *in vitro,* however Saukkonen *et al.* (1987) attributed PorB specific antibodies only a minor role in immune protection in the infant rat infection model.

1.4.1.1.3 Rmp (Class 4)

The Reduction-modifiable protein (Rmp) is present in all meningococcal strains in association with the PorB Protein porin (Mcdade and Johnston, 1980). It is highly conserved between strains and antigenically stable (Tinsley and Heckels, 1986). Unlike other OMPs, the Rmp proteins do not induce much Rmp specific antibody in response to meningococcal infection (Mandrell and Zollinger, 1989) or in response to experimental OMV vaccines (Wedege and Froholm, 1986). In fact, the inclusion of this OMP in a vaccine is contraindicated, since this antigen has been shown to produce blocking antibodies which inhibit complement-mediated bactericidal lysis of meningococci (Munkley *et al.*, 1991).

1.4.1.1.4 Opa and Opc Proteins (Class 5)

The Opa (opacity) and Opc proteins are found on both meningococci and gonococci and play an important role in adherence and invasion. Opa and Opc proteins were formerly classified together as part of the Class 5 group of proteins of pathogenic Neisseriae, since they have a similar molecular weight (between 25kDa and 35kDa) (Kawula *et al.*, 1988). These proteins share physico-chemical properties, but are structurally distinct, therefore the Opc protein's classification as an Opa protein was subsequently revised (Olyhoek *et al.*, 1991).

The Opa proteins are believed to be analogous to the gonococcal PII protein since they share a similar amino acid sequence (Achtman *et al.*, 1988) and structure (Tsai *et al.*, 1981). A single meningococcal strain can express one, two or no Opa proteins (Poolman *et al.*, 1980), with each individual protein being encoded by a separate *opa* gene (Achtman *et al.*, 1992), and expression is subject to interstrain and intrastrain variation. Opa proteins are surface exposed and highly immunogenic as observed during meningococcal infection (Poolman *et al.*, 1983) and immunisation with experimental outer membrane vaccines (Wedege and Froholm, 1986). However, monoclonal antibodies raised against the Opa proteins of a meningococcus were found to be bactericidal only against meningococci

expressing the homologous protein (Kawula *et al.*, 1988), therefore a vaccine based solely on Opa proteins is impractical.

The expression of Opa proteins is evenly distributed between nasopharyngeal and systemic isolates. In contrast, a higher proportion of carriage strains express Opc protein as compared to isolates causing disease (Achtman *et al.*, 1991). Meningococcal isolates obtained from the nasopharyngeal mucosa express higher levels of Opc than those obtained from the blood or CSF (Achtman *et al.*, 1991). The Opc protein is expressed by approximately 60% of meningococci belonging to different serogroups (Olyhoek *et al.*, 1991). Within those strains expressing Opc, various levels of expression are seen, with some strains expressing large amounts (Opc^{++}), others smaller amounts (Opc^{+}) and the remaining strains at undetectable levels (Opc^{-}) (Achtman. M., 1988). Switching between these expression levels occurs at a frequency comparable to that of phase variation of Opa proteins (Sarkari *et al.*, 1994).

Although Opc is poorly immunogenic in animals it has been shown to be highly immunogenic in humans, even more so than the PorA outer membrane protein (Wiertz *et al.*, 1996). The antibodies raised however were bactericidal only against Opc^{++} strains and it has been suggested that this is part of a virulence mechanism (Rosenqvist *et al.*, 1993), whereby a small proportion of the bacteria express a highly immunogenic protein that enables invasion of the body, whilst their direct descendants express lower amounts and are therefore protected against the antibodies stimulated by the protein.

1.4.1.2 Capsular Polysaccharide

The presence of a protective polysaccharide capsule is an important virulence determinant in invasive meningococcal infection, since virtually all disease isolates are encapsulated (Band *et al.*, 1983), as compared to around 50% of carriage isolates (Frasch, 1979;Caugant *et al.*, 1994). Capsule serogroups A, B and C have been associated with 90% of invasive disease worldwide; B and C are more common in Europe and America, while A predominates in Africa. The capsule inhibits neutrophil phagocytosis and prevents classical complement pathway bactericidal activity, thus enhancing intravascular bacterial replication and survival (Tunkel and Scheld, 1993;Nassif and So, 1995). Meningococcal invasion into endothelial and epithelial cells can be inhibited by the presence of capsule (Craven *et al.*, 1980;Virji *et al.*, 1992;Stephens *et al.*, 1993). Virji *et al.* (1992) suggest that the organism may be able to vary the level of capsule production depending on its location within the host.

Table 1.1: Chemical Compositions of the Meningococcal Capsular Polysaccharides

Serogroup	Repeating Unit	Linkage
A	O-acetylated-N-acetyl-D-mannosamine-6-phosphate	α(1-6)
B	N-acetylneuramic acid	α(2-8)
C	O-acetylated-N-acetylneuraminic acid	α(2-9)
L	N,N',N''-triacetylglucosamine phosphate	-
W135	D-galactose-α(1-4) - N-acetylneuramic acid	α(2-6)
X	N-acetyl-D-glucosamine-4-phosphate	α(1-4)
Y	D-glucose-α(1-4) - N-acetyl, O-acetylneuramic acid	α(2-6)
Z	N-acetyl-D-galactosamine-α(1-1')-glycerol-3'-phosphate	3'-4
29E	N-acetyl-D-galactosamine-β(1-7)-4,5-O-acetyl KDO	α(2-3)

The chemical compositions of the majority of serogroup capsules have been elucidated, with only serogroups D, H, I and J remaining uncharacterised. Adapted from Apicella (1990) and Devoe (1982).

Abbreviations: Galactosamine; 2-amino-2-deoxy-D-galactopyranose
Glucosamine; 2-amino-2-deoxy-D-glucopyranose
Manosamine; 2-amino-2-deoxy-D-manopyranose
KDO; 2-keto-3-deoxy-octulosonic acid

1.4.1.3 Lipopolysaccharide

N. meningitidis over-synthesises its outer membrane relative to the remainder of the organism during normal log phase growth. The process causes the release of a large number of cell wall blebs (Devoe and Gilchrist, 1973) containing many of the outer membrane proteins, including lipopolysaccharides (LPS) (Frasch and Peppler, 1982). LPS stimulates numerous host responses including complement activation, cytokine release and infiltration of inflammatory cells (Saukkonen *et al.*, 1990). A direct correlation between the level of LPS in the blood and CSF, and the intensity of the inflammatory response has been shown (Vandeuren *et al.*, 1994;Brandtzaeg *et al.*, 1992). LPS sialylation appears to modify adhesion and invasion in unencapsulated meningococcal strains (Nassif and So, 1995). An infection

study performed in an infant mouse infection model showed bacteria switching between a non-sialylated immunotype to a sialylated immunotype during the course of infection (Mackinnon *et al.*, 1993), suggesting that modulation of the LPS may enable the organism to evade the host immune system (van Putten, 1993).

The antigenic properties of the LPS oligosaccharide structure have allowed subdivision of meningococci according to thirteen LPS immunotypes, designated by the letter L (Scholten *et al.*, 1994). LPS immunotypes L2-L4 are predominantly associated with group B and C meningococci (Zollinger and Mandrell, 1977) whilst L9-L11 are found in group A strains (Zollinger and Mandrell, 1980;Scholten *et al.*, 1994). This typing system is not regularly used in epidemiology studies, because although an increasing number of monoclonal antibodies (Mab) have been developed, there is still no Mab for every immunotype. Moreover, the interpretation of immunotyping results is difficult, because meningococci often express several immunotypes, and the expression of immunotypes may be influenced by growth conditions (Poolman *et al.*, 1982;Mandrell *et al.*, 1991).

1.4.1.4 Pili

Meningococci isolated from colonised sites or invasive disease are invariably piliated (Tzeng and Stephens, 2000). Pili are protein-based organelles that aid in attachment of the organism to the host nasopharyngeal mucosa. Two major classes of meningococcal pili termed Class I and Class II can be distinguished serologically and structurally, but appear to have functional similarities in their association with endothelial cells (Virji *et al.*, 1991). Both classes are members of the Type IV family of bacterial pilins, which include pilin from various bacteria including *Moraxella bovis*, *Bacteroides nodus*, *Pseudomonas aeruginosa*, *Vibrio cholera* and enterotoxigenic *Escherichia coli* (Strom and Lory, 1993). In addition to involvement in meningococcal attachment, pili are also responsible for twitching motility.

1.4.2 **Serological Classification**

A phenotypic classification system based on meningococcal surface structures (Tsai *et al.*, 1981), has been developed. Strains are characterized by utilizing antibodies that recognize surface-exposed epitopes on the capsule or outer membrane. Meningococci are characterised according to structural differences in the capsular polysaccharide (Table 1.2) giving the following distinct serogroups: A, B, C, etc. Serotyping is based on differences in the conformational epitopes of PorB Protein OMPs (PorB) to give serotypes: 1, 2a, 2b etc.

Serosubtyping utilizes variations in the epitopes of PorA proteins (PorA) to give serosubtypes: P1.1, P1.2, P1.3 etc. Immunotyping is based on the antigenic diversity of meningococcal LPS and gives LPS immunotypes L1, L2, L3 etc. The LPS types are independent of the protein serotypes, although certain combinations frequently occur together.

In summary the current serological classification system utilised is that initially described by Tsai *et al.* (1981), and is based on four groups of antigenic surface components; capsular polysaccharide, PorA Protein, PorB Protein and LPS. Therefore meningococcal isolate designated B:15:P1.7,16:L3,7 would be: Serogroup B, serotype 15, subtypes 7 and 16 and LPS immunotypes L3 and L7.

The information obtained from conventional serological typing methods is limited by antibody availability. Sequencing of the *porA* region which encodes the PorA outer membrane protein has revealed additional serosubtypes based on the variable regions VR1 and VR2, not recognised by the existing antibodies (McGuinness *et al.*, 1993;Brooks *et al.*, 1995). Similarly, sequencing of the *PorB* region (Sacchi *et al.*, 1998) which encodes the PorB OMP has lead to the identification of new meningococcal serotypes. Genotyping assays have the additional advantage that they can be performed on PCR positive patient samples even where no organism has been cultured.

1.5 Disease Prevention

Even with appropriate antibiotic treatment, *N. meningitidis* can kill previously healthy children and adults within hours, therefore meningococcal vaccines are urgently required.

1.5.1 Capsular polysaccharide vaccines and immune response

The first meningococcal vaccines were developed following the observations of the importance of bactericidal antibody in immunity amongst US military recruits, and the particular relevance of capsular polysaccharide to the development of bactericidal antibodies (Goldschneider *et al.*, 1969b;Gotschlich *et al.*, 1969b). The bivalent serogroup A/C capsular polysaccharide vaccine (MACPS) is composed of purified serogroup A and C capsular antigens. As a small but variable amount of disease is associated with groups Y and W135 meningococci, quadrivalent vaccines containing the polysaccharides A, C, Y, and W135 were developed. Until recently, capsular polysaccharide meningococcal vaccines were effective for control of outbreak of meningococcal infections in older children and adults (Swartley *et al.*,

1997), but had limitations for routine use. Polysaccharide antigens are T-independent antigens which are not processed by antigen-presenting cells, but interact directly with B cells, inducing the production of antibodies in the absence of T cells (Goldblatt, 1998). As a result, memory cells are not formed and immunological memory is not induced. In addition, the response to polysaccharide is poorly developed in children under two years of age (Siber, 1994;Goldblatt, 1998). Therefore, vaccines containing purified capsular polysaccharide alone have failed to protect this high-risk group from infection. In older children and adults, antibody responses are induced by polysaccharide antigens, but they are dominated by IgM and are short-lived (Siber, 1994;Goldblatt, 1998). The lack of memory coupled with the rapid decline of antibodies makes revaccination necessary.

1.5.2 Protein conjugated polysaccharide vaccines and immune response

The immunogenicity of polysaccharides can be increased by conjugating them to a carrier protein (Siber, 1994) which converts the polysaccharide into a T-cell-dependent antigen (Siber, 1994;MacLennan *et al.*, 2000;Tunkel and Scheld, 1993) Conjugation of capsular polysaccharide has been used successfully to prepare vaccines against *Haemophilus influenzae* type b (Hib); the incidence of Hib infection has significantly decreased in communities where these conjugate vaccines have been used (Barbour, 1996). The serogroup C meningococcal conjugate vaccine was recently (1999) introduced into the UK immunisation schedule and has proven to be not only more immunogenic in infants, (MacLennan *et al.*, 2000) but also induces immunological memory (MacLennan *et al.*, 2000). Since the introduction of group C conjugate vaccination, cases of serogroup C infection have declined dramatically (Figure 1.2) and group C carriage has also reportedly been reduced (Balmer *et al.*, 2002).

Recent studies have documented the capacity of meningococci to exchange genetic material responsible for capsule production thereby allowing a switch from serogroup C to B and *visa versa* (Swartley *et al.*, 1997). Capsule switching may increase as vaccines that provide serogroup-specific protection become more widely used.

1.5.3 Obstacles to disease prevention

Serogroup B meningococci account for >50% of meningococcal infections in the US and many European countries including the UK (Quakyi *et al.*, 1999). In contrast to the decline in the number of group C infections following introduction of the group C conjugate vaccine, the number of reported cases of confirmed group B infection in England and Wales has increased (Balmer *et al.*, 2002) (Figure 1.2). A vaccine for serogroup B meningococci remains elusive. The development of a serogroup B vaccine is hampered by non-immunogenicity of the B capsule probably as a result of the immunotolerance that results from the similarity between the serogroup B capsular polysaccharide structure and the polysialic acid-containing glycopeptides that are components of human brain tissue (Goldblatt, 1998). Therefore the strategy of polysaccharide-protein conjugation (described in 1.5.2) for vaccination against non-group B meningococci cannot easily be transferred to group B meningococci and various alternative strategies are under development. A novel approach is to use a conjugated chemically modified group B capsule where the N-acetyl group has been replaced by the N-propionyl group (Jennings *et al.*, 1987). The induction of bactericidal antibodies in non-human primates has been shown with PorB as the carrier protein (Fusco *et al.*, 1997). However, some of the antibodies elicited have activity against polysialic acid and therefore have the potential to be autoreactive in humans, raising concerns about safety of this vaccine in humans.

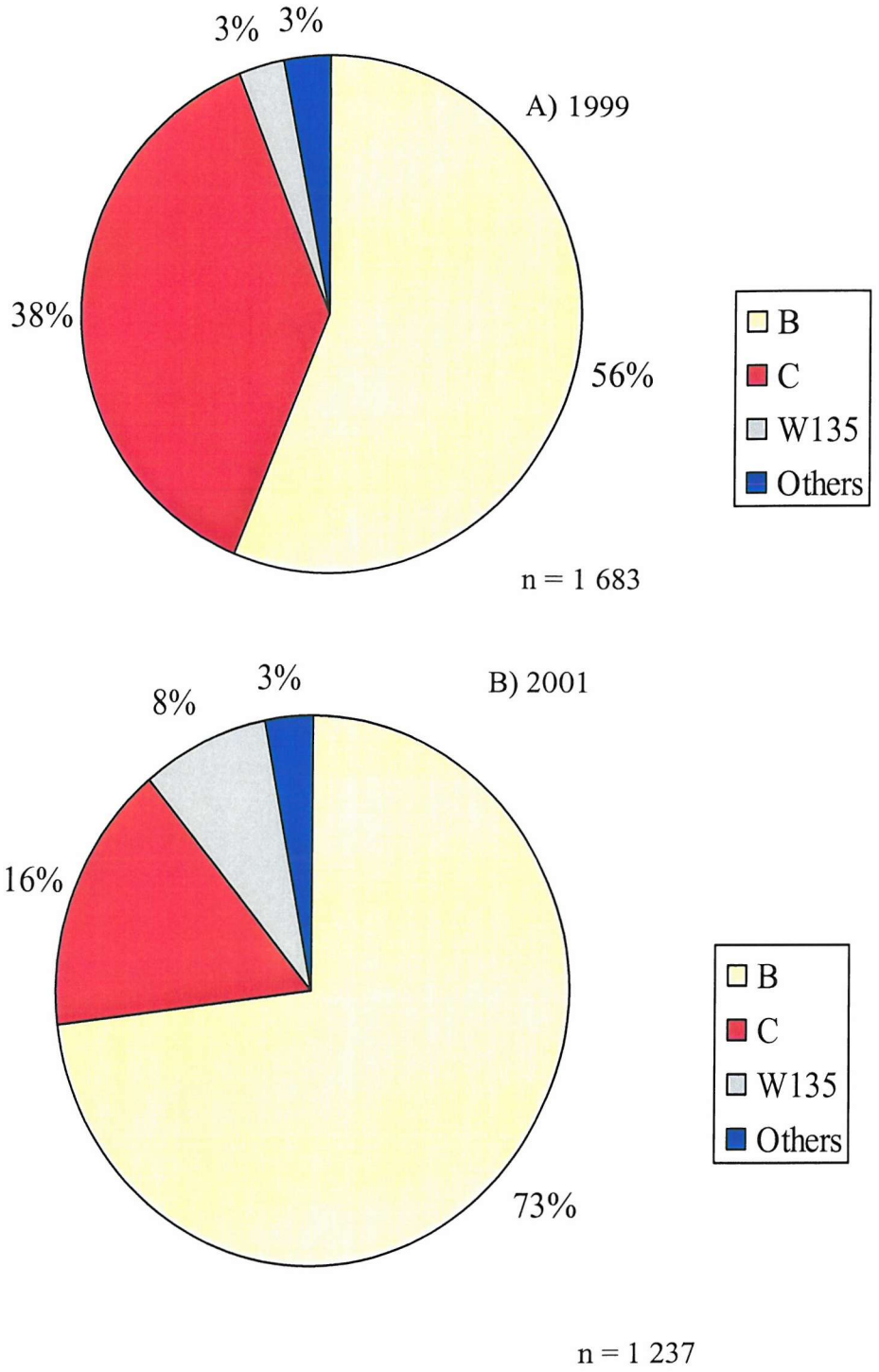
Attempts are therefore underway to identify subcapsular antigens suitable for inclusion in a serogroup B vaccine. Meningococci release cell wall blebs (Devoe and Gilchrist, 1973) containing many outer membrane proteins, including LPS (Frasch and Peppler, 1982) during normal log phase growth. Like the polysaccharide capsule, LPS is highly conserved among invasive isolates and therefore has the potential to offer cross-protective immunity against diverse meningococcal serogroups. Work in animals suggests that vaccines based on detoxified LPS elicit opsonic antibody responses that seem immunotype specific (Verheul *et al.*, 1993) and Plested *et al.* (1999) have demonstrated that the conserved inner core epitopes of LPS can also induce protective immunity. However, clinical trial data are awaited for assessment of these vaccines.

Vaccines based on outer membrane proteins (OMPs) from single strains of group B meningococci have been developed and have been found to be immunogenic (Wedege *et al.*, 1998;Cartwright *et al.*, 1999;Sierra *et al.*, 1991). These monovalent vaccines are potentially useful in management of hyperendemic group B disease involving a single antigenic type (Sierra *et al.*, 1991). The success of such vaccines has however been limited by the relative strain restriction (Tappero *et al.*, 1999), evidence of short duration of immune responses (Wedege *et al.*, 1998;Boslego *et al.*, 1995) and poor immunogenicity in children (Milagres *et al.*, 1994). Since the greatest disease burden is in infants and young children, the poor levels of protection at this age limits the usefulness of these vaccines.

Various strategies have been employed to broaden strain coverage of OMP vaccines, usually involving the use of antigens from multiple antigenic types. The ultimate goal is the discovery of a highly conserved antigen present across all group B strains which is able to induce protective immune responses in both adults and children. The recent sequencing of the complete genome of a serogroup B meningococcal strain (Tettelin *et al.*, 2000) should aid the search for such an antigen.

Research into an effective group B vaccine is being pursued in many directions, and further investigation into vaccines based on the group B capsular polysaccharide, LPS and OMPs are all in progress.

Figure 1.2: Case Isolates of *Neisseria meningitidis* in (A) 1999 and (B) 2001 by Serogroup for England and Wales.



B = serogroup B, C = serogroup C, W135 = serogroup W135, Others = serogroups: 29E, A, Y, Z and non-groupables. Data derived from Balmer *et al.* (2002).

These figures illustrate the serogroup distribution of confirmed cases of meningococcal infection (A) before and (B) after introduction of the group C conjugate vaccine.

1.6 Aims of thesis

Knowledge of the molecular basis of immunity to meningococcal disease is incomplete. It is not clear why or how meningococci cause different disease processes amongst individuals. The aim of the work described in this thesis is to broaden understanding of immunity to *N. meningitidis* in at-risk populations in the context of both outbreak and non-outbreak situations. Meningococcal carriage and disease stimulate antibody to a variety of meningococcal antigens, but which of these antibodies are involved in protection is unknown. The main focus of the studies presented in this thesis was to identify the contribution that individual meningococcal surface antigens make toward natural immunity to meningococci.

CHAPTER 2 MATERIALS AND METHODS

2.1 Sterilisation of Media and Equipment

A) Autoclaving: Pipette tips, growth media and all bulk solutions were routinely sterilised by autoclaving at 120°C (15lb in^{-2}) for 20min.

B) Filtration: Antibiotics and growth supplements were sterilised by passage through a disposable 0.45μm sterile membrane filter (Millipore).

C) Dry heat: Glassware was sterilised by heating to 160°C for 2-3hrs.

2.2 Culture of Meningococci

2.2.1 Media and Growth Conditions for Meningococcal Culture

Meningococci grow optimally on nutritionally enriched media at 37°C in a humid atmosphere in the presence of 5% CO_2 (Cruikshank *et al.*, 1975). The following culture media were used in this study: Proteose Peptone Agar (in-house), Chocolate Agar (Difco) and New York City Medium (Difco) (Le Saux *et al.*, 1992). Chocolate Agar and Proteose Peptone Agar are both non-selective culture media, while New York City Medium has antibiotic additives which are capable of preventing overgrowth of commensal oral flora but will not inhibit growth of *Neisseria spp.*

2.2.1.1 Proteose Peptone Agar

Proteose peptone agar (PP agar) was prepared according to the method of Zak *et al.* (1984) from the components listed in Table 2.1. All ingredients apart from the supplements were sterilised by autoclaving at 15lb in^{-2} for 15min. After cooling the solution to 50°C, 8ml of supplement A (2.1.1) and 2ml of supplement B (2.1.2) were added aseptically. The PP agar was poured into sterile petri dishes (20ml/9cm dish), allowed to set at room temperature, then dried in a sterile microflow cabinet.

Table 2.1: Proteose Peptone Agar

Ingredients	Amount per litre (distilled water)
Bacto-agar (no.1, Oxoid)	10g
$K_2H_2PO_4$	1g
$K_2HPO_4.3H_2O$	5.24g
Proteose peptone (Difco)	10g
Sodium chloride	5g
Starch	1g
Supplement A	8ml
Supplement B	2ml

Supplements A and B were added after autoclaving the other components and allowing the solution to cool to 50°C.

Table 2.1.1: Supplement A

Supplement A:	Amount per 800ml
Co-carboxylase	100mg
Cyanocobalamin	10mg
Ferric nitrate	20mg
Glucose	100g
L-glutamine	10g
Para-amino-benzoic acid	13mg
Thiamine hydrochloride	3mg
β-nicotinamide adenine dinucleotide	250mg

The above components were dissolved in distilled water, filter sterilised and stored at –20°C.

Table 2.1.2: Supplement B

Supplement B:	Amount per 200ml
L-cysteine hydrochloride	26g
Adenine	1g
Guanidine hydrochloride	30mg
Hypoxanthine	320mg
Uracil	800mg

All of the above, with the exception of cysteine, were dissolved in 100ml of boiling 0.1M hydrochloric acid. After cooling to room temperature, the cysteine was added and the final volume made up to 200ml with distilled water. The solution was filter sterilised and stored at –20°C.

2.2.2 Culture of Meningococci from Clinical Specimens

The nasopharyngeal flora of human subjects were sampled with plain cotton swabs (Medical Wire) which were inoculated onto modified New York City (MNYC) selective media (Difco) (Le Saux *et al.*, 1992) and Chocolate Agar (Difco) for the non-selective growth of *Neisseria spp.*

Student volunteers then gargled with 10ml of sterile phosphate-buffered saline, which was transported to the laboratory and processed within 3hrs of collection. An aliquot (1.5ml) of the gargle fluid was transferred to a separate tube and centrifuged at 10 000g for 7.5min. The supernatant fluid was discarded, leaving approximately 100µl of residue which was mixed and used to inoculate MNYC and Chocolate Agar culture plates.

All the plates were incubated at 37°C in an atmosphere of 5% CO_2. The cultures were scrutinised after 48hrs incubation for the greyish, transparent colonies characteristic of meningococci. An oxidase test and a Gram stain (detailed in Section 2.3) were performed on presumptive *Neisseria spp.* Organisms identified as oxidase positive, Gram negative cocci were confirmed as *N. meningitidis* strains on the basis of the API NH (bioMeriex) system of enzymatic and sugar fermentation reactions according to the manufacturers instructions (2.3.3).

2.3 Identification of Neisseria

2.3.1 Oxidase Activity

All members of the Neisseria genus contain cytochrome oxidase in the cell wall, allowing oxidisation of the dye tetramethylparaphenylenediamine hydrochloride (TMPD) from colourless to purple (Cruikshank *et al.*, 1975). Oxidase production was detected by pouring a 1% solution of N,N,N',N'-tetramethyl-*p*-phenylenediamine dihydrochloride (Sigma) over the culture plate on which the colonies were growing. The presence of oxidase activity was signified by a colour change from pale brown to a strong purple within a few minutes. On some occasions a single colony was detected by transferring part of it to a swab that had been impregnated with the indicator so that the colour then developed on the swab.

2.3.2 Gram Staining of Bacteria

Materials:

A) Gram crystal violet (Difco): 0.5% (w/v) crystal violet powder dissolved in water at 100°C

B) Gram iodine (Difco): 1% (w/v) iodine in 12mM potassium iodide

C) Acetone

D) Carbol fuchsin solution (Difco): Basic fuchsin (2g) dissolved in 10g of phenol at 80°C. Absolute alcohol (20ml) was added, followed by 200ml of water. This stock was diluted 1:10 with water before use.

The procedure was carried out as described by Cruikshank *et al.,* (1975). One or two colonies were spread onto a clean glass microscope slide with a drop of distilled water to create an emulsion. The slide was allowed to air dry at room temperature in a laminar flow cabinet. Bacteria were then fixed to the slide by passing it through a Bunsen flame. The slide was flooded with crystal violet for 1min and then rinsed gently with running tap water. Iodine was used as a mordant and was added for 1min and then rinsed off with water. The bacteria were destained with acetone for a few seconds, rinsed with water and the counter stain, carbol fuchsin, was added for 30sec then rinsed off with water. After blotting dry, the sample was

viewed with the oil immersion lens of a Leitz Dialux light microscope under a 100x objective.

2.3.3 API NH Identification System

The API NH (bioMeriex sa, Lyon, France) system consists of microtubes containing dehydrated substances, which enable the performance of enzymatic or sugar fermentation reactions. The organism is inoculated into each well and after incubation a positive result is indicated by a colour change. The system can be used to identify *Neisseria*, *Haemophilus* and *Branhamella catarrhalis* (*Moraxella catarrhalis*), and detect penicillinase. In this study, the API NH was used to confirm the identity of presumptive meningococcal strains obtained from the culture of throat swabs and saline mouth washings for the presence of meningococci. The following materials and reagents were supplied with the kit and used according to the manufacturer's instructions.

Materials:

A) API strip of dehydrated substrates

B) Incubation box for strip

C) NaCl Medium: 0.85% (w/v) in distilled water

D) James Reagent: Compound J2183 (confidential) 0.5g in 100ml HCl 1N.

E) ZYM B Reagent: Fast Blue BB 0.35g, 2-methoxyethanol 100ml

Method:

Throat swabs and saline mouth washings were cultured on MNYC and Chocolate Agar culture media (2.2). The plates were incubated and examined 2 days later for the presence of microbial colonies characteristic of *Neisseria*. Cultures of oxidase positive, Gram negative cocci were subcultured to obtain pure growth onto Chocolate agar plates. The plates were incubated at 37°C in an atmosphere of 5 % CO_2 for 24hrs. The resultant culture was emulsified in NaCl medium (2ml, 0.85%) to create a bacterial suspension with a turbidity equivalent to a 4 McFarland standard and used to inoculate the API strip. Mineral oil was added to the first seven tests and the strip incubated at 37°C in aerobic conditions. After two

hours, the spontaneous reactions were recorded as a '+' or a '-' by referring to the Reading Table in the package insert. ZYM B and James reagents were added to microtubes 8, 9 and 10 and the reaction was noted after 3min.

Interpretation:

On the result sheet, the tests are separated into groups of three and a value 1, 2 or 4 is assigned to each. By adding together the values corresponding to positive reactions within each group, a 4-digit profile was obtained. Identification of the organism was obtained by consulting the numerical profile list in the package insert.

2.4 Storage of Meningococcal Strains

Meningococcal stock cultures were stored in liquid nitrogen as thick suspensions in proteose peptone broth containing 10% (v/v) glycerol. When bacterial strains were required for experiments, the frozen stocks were used to inoculate proteose peptone agar plates that were then incubated at 37°C in a 5% CO_2 atmosphere overnight. A single sub-culture was used to eliminate the potential for strain variation in the expression of cellular components and subsequent effects on experimental assays. Lists of the meningococcal strains used in this work are included in Table 2.2. Meningococcal strains MC161 and MC162 were case isolates from an outbreak of serogroup C meningococcal infection amongst Southampton university students in 1997 (Chapter 3 of this thesis). Strain MenC11 is the reference group C strain recommended by WHO for group C Serum Bactericidal Assays (Maslanka *et al.*, 1997) and was obtained from the Meningococcal Reference Unit based at Manchester. Strain MC58 is a group B case isolate from an outbreak that occurred in Stroud, Gloucester during the 1980's (Tettelin *et al.*, 2000;McGuinness *et al.*, 1990). Meningococcal strains MC168 to MC178 were isolated over the course of a longitudinal study (Chapter 4 of this thesis) of meningococcal colonisation in undergraduate university students.

Table 2.2: Strains of *Neisseria meningitidis* used in this study.

Strain Designation	Characterisation	Source	Reference
MC161	C:2-37:P1.5a,10d:L2	Case 6, Outbreak of meningococcal infection, 1997	(Jones *et al.*, 2000)
MC162	C:2-36:P1.5,2:L2	Case 1, Outbreak of meningococcal infection, 1997	(Jones *et al.*, 2000)
MenC11	C:16:P1.7a,1:L2,L3	Group C Reference Strain, MRU	(Goldblatt *et al.*, 2002)
MC58	B:15:P1.7,16b: L3	Group B Reference Strain	(Tettelin *et al.*, 2000;McGuinness *et al.*, 1990)
MC 168	B:4:P1.5,2:L3	Carriage Strain	
MC 169	B:NT:P1.4,7b:L3	Carriage Strain	
MC170	B:4:P1.15:L3	Carriage Strain	
MC171	B:1:P1.15:L1	Carriage Strain	
MC172	B:1:P1.14:L3	Carriage Strain	
MC173	C:2b:P1.5,2:L3	Carriage Strain	
MC174	29E:4:P1.5	Carriage Strain	
MC175	29E:NT:P1.6	Carriage Strain	
MC176	29E:14:P1.5,2	Carriage Strain	
MC177	NG:NT:NST	Carriage Strain	
MC178	NG:NT:NST	Carriage Strain	

Isolates were characterised by group, type, and subtype antigens by standard methods at the Meningococcal Reference Unit (MRU, Manchester Public Health Laboratory, Manchester, UK). The lipopolysaccharide (LPS) immunotype was determined for the serogroup B and C

strains by the Laboratory for Vaccine Research, RIVM, Bilthoven, The Netherlands (Scholten *et al.*, 1994).

2.5 Extraction of Lipopolysaccharide from Meningococci

The method was essentially that of Lambden and Heckels (1982). Briefly, LPS was extracted from the bacterial pellet using hot phenol-water and then purified from the aqueous phase by ultracentrifugation.

Materials:

A) Phenol: 90% (w/w) in water

B) Na EDTA: 0.1M

C) Tris-acetate: 0.25M, pH 7.2

D) $MgCl_2$: 0.1M

E) RNAse (ICN Biochemicals Inc, Aurora, Ohio)

F) DNAse (ICN Biochemicals)

G) Acetone

Method:

Meningococci were cultured from liquid nitrogen stocks (2.4) on PP Agar (2.2.1.1) plates under standard growth conditions (2.2.2) and then emulsified into proteose peptone broths. To obtain thick growth 30-60 large (14cm) PP plates were each spread with 100µl of the suspension and incubated overnight. The bacteria were harvested into a small volume of water and a 90% (w/w) volume of phenol was added in a 1:1 ratio. The suspension was homogenized for 15min at 70°C, and then centrifuged at 10 000g for 30min to separate the aqueous and phenol phases. The upper aqueous phase was removed and retained, and the phenol plus interface was re-extracted with a further 6.5ml of water at 70°C for 15min. The centrifugation step was repeated and the aqueous phase kept. The aqueous phases were combined and dialysed against running tap water. After 48hrs, a solution of Na EDTA was added to the dialysis bag to give a 5mM final concentration, and the mixture dialysed

overnight against deionised water. Next day, the dialysis water was changed several times and then, a few crystals of $MgCl_2$ followed by 2 volumes of cold acetone were added and left overnight at 4°C. The resulting precipitate was removed and the solution was transferred to glass universals and centrifuged at 10 000g for 10min. The pellet was then placed in a vacuum desiccator for 30min to remove residual acetone. The precipitate was resuspended and dissolved in 0.25M Tris-acetate, pH7.2. A $MgCl_2$ solution was added to give a final concentration of 1mM, then 5mg each of RNAse and DNAse were added. The solution was stirred at room temperature for 3-4hrs and then dialysed against the same Tris/$MgCl_2$ buffer at 37°C overnight. Next day, the mixture was centrifuged at 1 000g for 15min and the pellet discarded, then centrifuged at 100 000g for 2hr and the supernatant solution discarded. The pellet obtained was washed by resuspending it in distilled water and repeating the final centrifugation step. The pellet was then dried under vacuum in a pre-weighed tube and the tube plus dried pellet reweighed to determine the weight of the LPS alone.

2.6 Genetic Analysis

2.6.1 DNA Extraction Methods

2.6.1.1 Method I (Mee *et al.*, 1993)

Meningococcal strains required for DNA amplification were cultured overnight at 37°C in 5% CO_2 on Proteose Peptone Agar (2.2.1.1). A single isolated colony was picked off the plate with a sterile loop and resuspended in 10µl of UHQ water in a sterile microcentrifuge tube. The organisms were lysed by the addition of 10µl potassium hydroxide (0.25M) and then denatured by boiling at 100°C for 5min. Hydrochloric acid (10µl, 0.25M) was added to neutralise the mixture and 10µl of 0.5M Tris-HCl (pH 7.5), was added to ensure the correct pH for PCR. A further 270µl UHQ water was added, the suspension centrifuged at 15 000g and the resultant supernatant solution removed to use directly in PCR assays.

2.6.1.2 Method II: Chelex-100 extraction method (Wilkinson et al., 1998)

One millilitre of CSF was centrifuged, at 15 000g for 30min in a bench microfuge (Heraeus Sepatech, Germany) and the supernatant solution was removed. The resulting CSF pellet was resuspended in 100µl of a 5% (w/v) solution of Chelex-100 (Bio-Rad, Hemel Hempstead, UK) and incubated at 55°C overnight. The reaction mixture was then centrifuged at 15 000g until clear and the supernatant solution used as DNA template in subsequent PCR experiments.

2.6.1.3 Method III: Boiling extraction method

This method for extracting DNA from throat swabs and saline mouth washings prior to TaqMan PCR is described in detail under Section 2.8.

2.6.2 Quantitation of DNA and Purity of DNA

The amount and purity of DNA present in aqueous solutions was determined by measurement of the absorbance at 260nm (A_{260}) and 280nm (A_{280}) using a CE599 automatic scanning spectrophotometer (Cecil Instruments). Samples of the unknown DNA solution were diluted with water to give final volumes of 500µl. Water was used as the reference blank. An A_{260} of 1.0 was taken to be equivalent to a DNA concentration of 50µg ml^{-1}double stranded DNA, 40µg ml^{-1}single stranded DNA or 33µg ml^{-1}oligonucleotide (Sambrook *et al.*, 1989).

An assessment of the purity of the DNA was obtained by measurement of the ratio of A_{260}:A_{280}, which for pure DNA is usually greater than 1.6. (Maniatis *et al.*, 1982).

An alternative method for estimating the concentration of DNA involved analysing a sample of the DNA by agarose gel electrophoresis (2.6.9).

2.6.3 Amplification of Meningococcal DNA by PCR

The polymerase chain reaction (PCR) is an *in vitro* method of producing large amounts of a specific DNA fragment from small amounts of DNA template. Oligonucleotide primers hybridise with the nucleotide sequences on complementary strands at each end of the DNA fragment to be expanded. Cyclical polymerisation by a thermostable DNA polymerase produces millions of copies of the DNA fragment.

The subtype of meningococcal strains was determined by sequencing the meningococcal variable regions VR1 and VR2 by polymerase chain reaction (PCR) using oligonucleotide primers (Table 2.3) corresponding to known conserved areas of the *porA* gene as previously described (Brooks *et al.*, 1995). Initially the whole PorA gene was amplified by utilising primers BIO1 and BIO4. The PCR product obtained from this reaction was used as DNA template to amplify VR1 with primers BIO1 and BIO2, and VR2 with primers BIO3 and BIO4. Next, the PCR product obtained from the amplified VR1 region was

used as template using primers SEQ1 and SEQ2 in individual sequencing reactions, and primers SEQ3 and SEQ4 together with the amplified VR2 region.

Table 2.3: Oligonucleotide Primers used in PCR analysis of the PorA Protein

Region	Forward	Reverse	Sequence
VR1	BIO1		CTTACCGCCCTCGT ATTG
	SEQ1		CCGCACTGCCGCTTG CGG
		SEQ2	GGGCT GAAGGCTGTT TGG
		BIO2	CAAGACGT ATCCGTTGCC
VR2	BIO3		TGGCTTCGCAATTGGGTA
	SEQ3		TTTCAAACGCCACGACGA
		SEQ4	CT ATGCCTTTAA ATATGCG
		BIO4	GA CACGCCAATG TCGGAC

2.6.4 Oligonucleotide Synthesis and Manipulation

Oligonucleotides for PCR were synthesised with phosphoramidites by Dr M. Pickett, in a 3' to 5' direction on a Model 318A automated synthesiser (Applied Biosystems Inc.) employing β-cyanoethyl phosphoramidite (CEP) chemistry and primer sequence information from published data (Brooks *et al.*, 1995). Oligonucleotides were synthesised either onto a column or a filter.

2.6.5 Removal from Solid Support

Oligonucleotides were cleaved from the support matrix by exposure to 0.88 specific gravity ammonia solution overnight at 55°C. After incubation the ammonia solution was allowed to cool to room temperature and transferred to tubes. The oligonucleotide was then precipitated by incubation at -20°C for 2hr with the addition of 0.1 volume of 3M sodium

acetate (pH5.0) and 3 volumes of absolute ethanol. The oligonucleotide was harvested by centrifugation at 10 000g for 10min. The pellets were washed with 1ml of ice cold 80% (v/v) ethanol, recovered by centrifugation at 10 000g for 5min, then dried under vacuum for 15min. One pellet was resuspended in UHQ water (50µl); the concentration was determined by measuring the A_{260} (2.9.2) and the solution was stored at -20°C as working stock. The remaining pellets were all stored at -70°C.

2.6.6 PCR Components

The PCR components per reaction were:

5µl 10x Taq buffer containing 15mM $MgCl_2$ (Promega)

5µl dNTP mix (2mM) (Promega)

5µl forward primer (50ng μl^{-1})

5µl reverse primer (50ng μl^{-1})

25µl UHQ water

5µl template DNA

0.5µl Bio-X-Act polymerase (Bioline)

2.6.7 Thermal cycling conditions

PCR was performed in a GeneAmp PCR System 9600 thermal cycler (Perkin Elmer Cetus) and thermal cycling criteria were selected on the following basis:

A) Denaturation of DNA template at 94°C for 15sec in all reactions.

B) Annealing of primers:

The annealing temperatures were selected on the basis of the Tm of the set of primers, initially 5°C below the lowest Tm of the primer pair. In a series of experiments this was gradually increased towards the Tm to maximise the annealing to the template DNA and therefore the amplification. The reaction mix was held at the optimised annealing temperature for 30sec.

C) Extension of DNA strands:

The temperature at which the Taq polymerase was allowed to polymerise the extension of the new DNA fragment was always 72°C. The time allowed for this extension was 1min since the desired PCR products were between 500bp and 1kb in length.

D) After 30 cycles of PCR, the samples were held at 4°C until removed from the thermal cycler.

2.6.8 GenecleanII™ purification of PCR products

The GenecleanII™ kit (Stratech) was used to purify DNA after PCR amplification in preparation for sequencing. The system utilises the ability of DNA to bind to a silica matrix, termed 'Glassmilk' in the presence of sodium iodide (NaI). All of the solutions were supplied with the kit and used according to the manufacturer's instructions.

To purify DNA from aqueous solution using the GenecleanII™ kit, 3 volumes of 6M NaI were added to the DNA sample and mixed thoroughly. Glassmilk (15µl) was added and the resulting slurry was incubated on ice for 15min, with mixing every 2-3min, to allow the DNA to adsorb to the silica matrix. The Glassmilk/DNA complex was recovered by centrifugation at 8 500g for 30sec and the NaI supernatant solution discarded. Contaminants were removed from the pellet by resuspending it in 500µl of ice-cold NEW wash (NaCl/ethanol/water) by pipetting back and forth. The Glassmilk/DNA complex was recovered by centrifugation at 8 500g for 30sec and the supernatant discarded. The washing procedure was repeated twice more. After the final 30sec centrifugation step, residual NEW wash was removed with an additional centrifugation step of 1min. The washing procedure was repeated once with 80% ice-cold ethanol, the residual ethanol being removed by a final centrifugation step of 1min. The Glassmilk pellet was resuspended in the required volume of UHQ water (typically 2 volumes of 20µl) and the DNA eluted by incubation at 50°C for

5min. The elution process was performed twice to maximise recovery of the DNA and the fractions were analysed by gel electrophoresis to estimate yield.

The GenecleanII™ kit was also used to extract DNA from agarose gels. The desired band was excised from the gel using a sterile scalpel blade and transferred to a 1.5ml centrifuge tube. Its volume was determined by weight using the approximation that 1g equals 1ml. Three volumes of 6M NaI were added and to aid the dissolving of the agarose, incubated at 55°C for 5min. The DNA was then recovered from the solution with the Glassmilk as described.

2.6.9 Agarose Gel Electrophoresis

The method used was that described by Sambrook *et al.,* (1989) and was used to separate and characterise PCR products on a size basis.

Materials:

Electrophoresis was performed using Tris-acetate buffer (TAE), which was made up as a 50x stock solution as follows:

A) 50x Tris-acetate running buffer (TAE): 242g Tris, 200ml 0.5M Na_2EDTA (pH8.0), 57.1ml glacial acetic acid.

The Tris and glacial acetic acid were dissolved in 600ml water, the EDTA was then added and the final volume made up to a litre of water.

B) Agarose (Type II: medium, Sigma)

C) 6x Gel loading buffer (GLB): 18% (w/v) Ficoll 400, 0.18% (w/v) Bromophenol blue, 0.18% (w/v) Xylene cyanol FF, 0.18% (w/v) Orange G, 600mM EDTA (pH8.0)

Depending on the number of DNA samples to be analysed, the agarose gels were cast in either a BRL Horizon 58 (GIBCO BRL), a Wide Mini-Sub™ Cell (BioRad) or a DNA Sub Cell™ (BioRad). A gel matrix consisting of either 1 or 2% agarose (Type II: medium, Sigma) was used to resolve DNA in the 200bp - 10kbp range.

Table 2.4: Casting Gel Dimensions

	Gel dimension (cm)	Bed volume (ml)	Gel thickness (mm)
Horizon 58	5x8	30	7.5
Wide Mini-Sub™ Cell	15x10	80	5.3
DNA Sub Cell™	15x25	120	3.2

When using the DNA Sub Cell™ a 2% agarose gel was used to reduce the frailty of the gel due to its size. A 1% gel was used in the other tanks.

Agarose gels were constructed by dissolving the required amount of agarose in 1x TAE buffer in a microwave oven. The solution was then poured into the desired gel former with the well-forming comb inserted. After approximately 30min the set agarose was submerged in 1x TAE buffer and the well comb removed.

DNA samples were prepared for electrophoresis by the addition of $1/6^{th}$ the volume of 6x gel loading buffer and then loaded into the pre-formed wells. Electrophoresis was carried out at room temperature at 90V until the Orange G dye front reached the end of the gel.

2.6.10 Visualisation of DNA

After electrophoresis, DNA gels were submerged in a 1μg ml^{-1} solution of ethidium bromide (Sigma) in water for 30min in the dark, at room temperature. Excess stain was removed by rinsing with water. The DNA was visualised by ultra-violet illumination using a transilluminator (Ultra-violet Products Inc.) at 302nm and a permanent photographic image was obtained using Polaroid 665 film (8.5 x 10.8cm).

2.6.11 Estimation of DNA Size and Concentration

The sizes of DNA fragments were estimated by comparing their relative mobility through the gel matrix against DNA fragments of a known size. Calibration curves were drawn for the set of standard fragments (see Table 2.5) by plotting the logarithm of their length in bp against their mobility in mm. The standard markers (1kb ladder, GIBCO BRL) used ranged from 75bp to 12kb in size. The ladder was reconstituted in a buffer containg:- 50nm NaCl, 0.1mM Na_2EDTA and 10mM Tris-HCl, pH7.5.

Table 2.5: Fragment Sizes of 1kb Ladder

Size (bp)	Size (bp) cont.	Size (bp) cont.
12 216	5 090	394
11 198	4 072	344
10 180	3 054	298
9 162	2 036	220
8 144	1 635	154
7 126	1 018	142
6 108	516	75

DNA concentration can also be estimated using gel electrophoresis. The intensity of the ethidium bromide staining of the DNA sample was compared against 1µg of a 1kb DNA ladder (BRL Life Technologies) and the sample concentration then estimated.

2.7 DNA Sequencing Reactions

2.7.1.1 Preparation of PCR Product Template

1) Reaction mix:

5µl 10x buffer

5µl dNTPs (2mM)

5µl forward primer (50ng μl^{-1})

5µl reverse primer (50ng μl^{-1})

2µl $MgCl_2$ (50mM)

23µl UHQ water

5µl template DNA

The same precautions against possible contamination sources were taken as before.

2) Thermal cycling conditions:

These were essentially the same as before except that 35 cycles were performed.

2.7.1.2 Sequencing Reactions

DNA sequencing was performed using the PRISM™ ready reaction dyedeoxy terminator cycle sequencing kit (Applied Biosystems Inc.) as described by the manufacturers.

1) PCR Product Sequencing:

The PCR products obtained from amplification using the Bio-X-Act polymerase were cleaned by the GeneCleanII™ procedure (Section 2.6.8).

Sequencing mix:

50ng template DNA

25ng primer

8ml ready reaction mix

Total volume adjusted to 20ml by addition of appropriate volume of UHQ water.

For generation of sequencing products the standard number of cycles used was 30, and was performed in a Perkin Elmer Cetus 9600 Thermal Cycler, using a hot start (60°C).

2.7.2 Removal of excess dyedeoxy terminators from sequencing reactions

High concentrations of unincorporated terminators are present after sequencing, which need to be removed. This was achieved by precipitating the sequencing products with 2M sodium acetate, pH4.5 (2ml) and 95% ethanol (50ml) at -20°C for a minimum of 4hrs. The sequencing products were collected by centrifugation at 15 000g, 30min and washed with 70% ethanol (200ml, room temperature, 10min). The ethanol was removed and the pellet was stored at -20°C.

2.7.3 Preparation of DNA Sequencing Gels

Sequencing gels were poured between two horizontal, low fluorescence glass plates separated by 0.4mm thick spacers (Applied Biosystems Inc.). Before assembly, the plates were cleaned with Alconox detergent powder (Aldrich Chemical Company, Inc., Milwaukee, Wisconsin, USA) and rinsed with hot running water and then distilled water. The plates were then washed with methanol (HPLC grade, BDH), before being left to air dry. Any smears were removed with methanol and lint-free tissue.

10x TBE: 89mM Tris

89mM Boric acid

2mM disodium EDTA

Once dissolved in distilled water, the solution was filtered through a sintered glass filter.

The gel mixture was poured between the horizontal plates, taking care to avoid air bubbles. Upon completion of pouring, the top spacer was inserted and secured using bulldog clips and the gel left to polymerise for a minimum of 4 hours before electrophoresis.

Running buffer: 224ml 10x TBE

1476ml distilled water

2.7.4 Preparation and Loading of Samples

The dried sequencing reaction pellets were resuspended in 4µl of a solution composed of deionised formamide (Applied Biosystems Inc.) and 50mM EDTA (pH8.0) mixed at a 5:1 (v/v) ratio, containing a trace of dextran blue dye. Before loading the gel, the samples were heated at 90°C for two min. Electrophoresis was carried out in an Applied Biosystems Inc. 373A automated DNA Sequencer according to the manufacturers instructions at 30W for 12 hours. The subsequent data were analysed using Applied Biosystems 373A Analysis Software running on a Macintosh computer.

2.7.5 Avoiding Contamination of PCR

Filter tips were used throughout the PCR process to avoid possible sources of contamination. Possible sources of double stranded DNA contamination were eliminated by irradiation of the reaction mixture for 10min with short wave UV (256nm) in an Amplirad

UV illuminator (Genetic Research Instrumentation Ltd., Dunmow, Essex) just prior to addition of *Taq* DNA polymerase and target DNA.

2.8 TaqMan PCR for Detection and Serogroup Determination of Meningococci

TaqMan PCR can be utilised for the detection and serogroup determination of *Neisseria meningitidis* in clinical specimens. The targets of these assays are the meningococcal capsular transfer gene (*ctrA*), and the sialyltransferase gene (*siaD*) for serogroup B and C determination The ABI PRISM™ Sequence Detection Systems use fluorogenic probes to quantitatively detect specific nucleic acid sequences. The TaqMan probe consists of an oligonucleotide labelled at the 5' end with a reporter fluorochrome (usually 6-carboxyfluorescein [6-FAM]) and a quencher fluorochrome (6-carboxy-tetramethyl-rhodamine [TAMRA]) added at any T position or at the 3' end. During PCR, if the target of interest is present, the probe specifically anneals to the target sequence between the forward and reverse primer sites. The nucleolytic activity of the DNA polymerase cleaves the probe between the reporter and quencher only if the region hybridises to the target; the polymerase does not cleave free probe. After cleavage, the shortened probe dissociates from the target and polymerisation of the strand continues. The cleavage of the oligonucleotide between the reporter and quencher dyes results in an increase in fluorescence of the reporter that is proportional to the amount of product accumulated.

2.8.1.1 Sample Preparation for TaqMan PCR.

TaqMan assays were used to detect meningococcal DNA in throat swabs and saline mouth washings of human volunteers. Immediately after inoculating agarose plates for meningococcal culture (Section 2.2), throat swabs were agitated in 200µl of sterile water (Sigma Tissue Culture Grade). The swabs were then broken off, inverted, returned to the tube and centrifuged for 15min at 15 000g to draw out trapped fluid. The swab was then discarded and the washings stored at -80°C. Swab washings (50µl) were thawed, boiled for 10min, cooled on ice and then centrifuged for 10min at 12 500g. The supernatant solution (45µl) was transferred to a fresh tube and used immediately for PCR or stored at -80°C until required.

After culture of saline mouth washings for the detection of meningococci (Section 2.2.2), the gargles were liquefied by treatment with dithiothreitol (33μM) for 15min at room temperature. After centrifugation for 10min at 12 000g the supernatant fluid was discarded and the pellet resuspended in 200μl of sterile water. This was boiled for 10min and centrifuged as above. About 180μl of supernatant fluid was transferred to a fresh tube and used immediately for PCR or stored at -80°C until required.

2.8.1.2 Amplification of Meningococcal ctrA and siaD genes by TaqMan PCR.

The sequences of all primers and probes used are shown in Table 2.4. The primer/probe combination for the capsule transfer *ctrA* gene detects serogroups B, C, Y and W135 (Guiver *et al.*, 2000) (Table 2.4, *ctrA* forward 1, *ctrA* reverse 1 and *ctrA* probe 1). Amplification reactions contained 1μM primers (Cruachem), 0.5μM probe (Scandinavian Gene Synthesis), 1 X buffer A, 3mM $MgCl_2$, 200μM each of dATP, dCTP, dGTP and UTP, 0.025U μl^{-1} AmpliTaq Gold, 0.01U μl^{-1} AmpErase UNG (all provided in the TaqMan Core reagent kit, Applied Biosystems) and 5μl target DNA in a total volume of 25μl. Dilutions of plasmid containing the cloned *ctrA* gene were included in every experiment and used to construct standard curves from which the number of copies of target DNA could be calculated.

A modified *ctrA* primer/probe combination (Table 2.4, *ctrA* forward 2, *ctrA* reverse 2 and *ctrA* probe 2) which detects serogroups A, B, C, X, Y, Z, W135, 29E and some non-groupable strains was also used (Corless *et al.*, 2001). Reactions with this primer/probe combination contained 1 x TaqMan Universal Mix, 0.3μM each primer, 0.2μM probe (all supplied by Applied Biosystems) and 5μl target DNA in a total volume of 25μl. Dilutions (10-fold) of bacterial DNA covering the range 10^3-10^7 copies/ml were included with these *ctrA* assays.

Table 2.6: Oligonucleotide Primers and Probes used in Detection and Serogroup Determination of *Neisseria meningitidis* by TaqMan PCR

Name	5'-3'sequence
ctrA forward 1	TTGTGTGGAAGTTTAATTGTAGGATGC
ctrA reverse 1	TCAGATTGTTGCCCTAAAGAGACA
ctrA TaqMan probe 1	TCCTTCATCAGGCCCCAGCG*
ctrA forward 2	GCTGCGGTAGGTGGTTCAA
ctrA reverse 2	TTGTCGCGGATTTGCAACTA
ctrA TaqMan probe 2	CATTGCCACGTGTCAGCTGCACAT*
group B forward	TGCATGTCCCCTTTCCTGA
group B reverse	AATGGGGTAGCGTTGACTAACAA
group B TaqMan probe	TGCTTATTCCTCCAGCATGCGCAAA*
group C forward	GATAAATTTGATATTTTGCATGTAGCTTTC-3'
group C reverse	TGAGATATGCGGTATTTGTCTTGAAT
group C TaqMan probe	TTGGCTTGTGCTAATCCCGCCTGA*

*labelled with FAM at 5' position and TAMRA at 3' position

Amplification parameters for both primer/probe combinations consisted of 2min at 50°C, 10min at 95°C followed by 50 cycles of 15s at 95°C and 1min at 60°C on a sequence detector system (SDS) 7700 (Applied Biosystems). Positive controls (containing cloned *ctrA* or DNA extracted from group B *N. meningitidis* and group C *N. meningitidis,* as described above) and negative controls (water only) were included in every experiment. The threshold cycle (Ct; the cycle at which sample fluorescence exceeds a threshold value indicating a positive result and which is proportional to the number of genome copies present) was reported for each sample. Samples which were positive with either TaqMan *ctrA* primer/probe combination were characterised by TaqMan *siaD* PCR (Corless *et al.*, 2001;Guiver *et al.*, 2000) with primer/probe combinations which detect group B- and group C- associated DNA (Table 2.6, group B forward, reverse and probe; group C forward, reverse and probe).

2.9 Immunodetection Procedures

2.9.1 Enzyme Linked Immunosorbent Assay (ELISA) Methods

The antibody content of a serum can be assessed by its ability to bind to antigen adsorbed to the wells of a microtitre plate. The following ELISA format was employed to detect various anti-meningococcal antibodies. In each case, the wells were coated with the appropriate meningococcal antigen/s against which detection of the serum antibody response was sought. The wells were then 'blocked' with a nonspecific protein solution to keep background levels low. The samples containing the antibody in solution were added to the wells and then incubated to allow the antibody to bind to the immobilized antigen. A secondary antibody-enzyme conjugate, was added to detect the bound primary antibody. The enzyme conjugated to the secondary antibody catalysed a reaction when the appropriate substrate was added, resulting in coloured reaction products. The intensity of the colour reaction indicates the amount of primary antibody and can be measured in an appropriate ELISA reader.

2.9.1.1 Whole Cell ELISA

This assay of Abdillahi *et al.,* (1988b) was used to detect the total serum antibodies directed against whole meningococcal cells.

Materials:

A) ELISA diluent: 0.85% (w/v) NaCl, 0.06% (w/v) Tris-HCl, 1% (w/v) bovine serum albumin (BSA) (Sigma), 0.05% (v/v) Tween 20, pH 7.4 with acetic acid.

B) Wash buffer: 8.5% (w/v) NaCl, 0.5% (v/v) Tween 20.

C) Substrate buffer components: 0.1M sodium acetate pH 6.0 with 2M citric acid. Enzyme substrate added immediately before use.

D) Enzyme substrate: a 1ml volume of 3,3',5,5'-tetramethylbenzidine (TMB) (3.6mg ml^{-1} dimethyl sulphoxide) and 10µl hydrogen peroxide added to 100ml of substrate buffer.

E) Stop Solution: 1M H_2SO_4

Method:

Meningococcal strains to be tested were grown for 16-18hrs and the growth from one plate was harvested and homogenised into 1ml sterile PBS (pH 7.4) containing 0.05% (w/v) sodium azide and 1mM PMSF. The samples were then inactivated by heating at 56°C for 30min. The absorbance of the bacterial suspensions were measured at 620nm with a Cecil CE559 automatic scanning spectrophotometer. The organism suspensions were adjusted to an optical density (OD) of 0.1 with PBS and 100µl per well added to flat bottomed 96 well polystyrene microtitre plates (Sterilin). The plates were incubated overnight at 37°C in a humid environment. Excess antigen was removed by washing the plates 4 times. ELISA diluent was added to block non-specific binding sites. After 1hr at 37°C, the diluent was removed and the test sera added. The serum samples were assayed in a range of dilutions from 1/30 to 1/10 000 in ELISA diluent and the plates incubated for a further hour at 37°C. Thereafter the sera were removed and the wells washed 4 times. Goat anti-human horseradish peroxidase conjugated antibody (Zymed) was diluted 1/2000 in ELISA diluent and 100µl of this solution added to each well of the microtitre plates. The plates were incubated for 1hr and then washed 4 times. Freshly prepared substrate was added (100µl per well) and the colour was allowed to develop for 10min in the dark at room temperature. The reaction was stopped by the addition of 1M sulphuric acid (50µl per well). The absorbance of each well at 450nm was measured using an Anthos htII microtitre plate reader.

Data Analysis:

The mean optical density was plotted against serum dilution and whole cell ELISA titres were determined graphically as the intercept of the endpoint of an O.D. of 0.5, a value that was selected because it was in the linear range of the assay.

2.9.1.2 Serogroup C Anti-Capsular Polysaccharide ELISA

Antibodies directed against the serogroup C capsular polysaccharide were measured by ELISA as described by Gheesling *et al.* (1994) and compared to a standard reference serum, CDC 1992 (provided by Dr G. Carlone, CDC, Atlanta) (Holder *et al.*, 1995).

Methylation of Human Serum Albumin:

Human serum albumin was methylated as per the method of Gheesling *et al.,* (1994), by suspending 5g of human serum albumin (Sigma) in 500ml of methanol and then adding 2.2ml of 12 M HCl. The mixture was allowed to stand in the dark for three days with occasional agitation. The precipitate was collected by centrifugation and washed twice with methanol and twice with anhydrous ether. Most of the ether was evaporated in a ventilated fume hood, after which the precipitate was dried in a desiccator over KOH pellets. The powder was rehydrated with sterile UHQ water to a concentration of 1mg ml^{-1} and stored at 4°C.

Materials:

A) Methylated Human Serum Albumin (mHSA)

B) Serogroup C polysaccharide (National Institute for Biological Standards and Control, Potters Bar, UK)

C) Antibody buffer: 5% (w/v) bovine serum albumin (BSA) (Sigma), 0.1% (v/v) Tween 20 in PBS (pH 7.4).

D) Wash buffer: 0.1% (v/v) Tween 20 in PBS (pH 7.4)

E) Substrate buffer components: 0.1M sodium acetate pH6.0 with 2M citric acid . Enzyme substrate added immediately before use.

F) Enzyme substrate: A 1ml volume of 3,3',5,5'-tetramethylbenzidine (TMB) (3.6mg ml^{-1} dimethyl sulphoxide) and 10µl H_2O_2 to 100ml of substrate buffer.

G) Stop Solution: 1M H_2SO_4

Method:

A 10µg ml^{-1} solution of group C polysaccharide was added dropwise to a 10µg ml^{-1} solution of mHSA. A multi-channel pipette was used to add 100µl of the resulting mHSA-men C mixture to each well of a flat bottomed 96 well polystyrene microtitre plate (Sterilin). The plate was then incubated overnight at 4°C. Next day excess antigen was removed by washing the plates 4 times. Antibody buffer was added to block non-specific binding sites

and after 1hr at room temperature, the buffer was removed. Serum samples stored at a dilution of 1:1 in glycerol were serially diluted twofold from 1/25 to a final dilution of 1/1600 in antibody buffer and the plates incubated overnight at 4°C. Thereafter the sera were removed and the wells washed 4 times. The secondary antibody, goat anti-human horseradish peroxidase conjugate (Zymed) diluted 1:2000 in diluent (100µl per well) was added and the microtitre plate incubated for 2.5hrs at room temperature. The secondary antibody was removed and the wells washed 4 times. Freshly prepared substrate was added (100µl per well) and the colour allowed to develop for 30min in the dark at room temperature. The reaction was stopped by the addition of 1M sulphuric acid (50µl per well). The absorbance of each well at 450nm was measured using an Anthos htII microtitre plate reader.

Serogroup C Capsule ELISA Data Analysis

Dilutions of sera were tested in duplicate alongside a standard reference serum, CDC1992 (Holder *et al.*, 1995) and the mean absorbance values calculated. A titration curve was obtained for each serum sample by plotting the absorbance values as a function of the logarithm of the reciprocal serum dilution. The antibody concentrations of each test serum were then calculated relative to CDC1992 (provided by Dr G. Carlone, CDC, Atlanta), which has the concentration of 32µg ml^{-1} for group C specific antibody, using the method of Gheesling *et al.* (Gheesling *et al.*, 1994).

$$\left(\frac{\text{Antilog of interpolated dilution from standard curve}}{\text{Antilog of the actual dilution of the serum sample}} \right) \times 32 = X\mu g\ ml^{-1}$$

The lower limit of antibody detection in this assay is approximately 0.1µg ml^{-1} .

2.9.1.3 Serogroup B Anti-Capsular Polysaccharide ELISA

The presence of antibodies reacting with serogroup B capsular polysaccharide was determined by ELISA essentially as described for serogroup C (2.9.1.2), using capsular polysaccharide from an O-acetyl negative mutant *Escherichia coli* K1 (a gift from Dr H. Jennings, Ottawa, Canada), which is structurally and immunologically identical to serogroup B polysaccharide (Devi *et al.*, 1991;Bhattacharjee *et al.*, 1975). As a positive reference control, human serum with antibody activity to group B meningococci was used on each microtitre plate, and the antibody reactivity of each test serum was compared to this control and expressed in arbitrary units (AU). Negative controls contained all reagents except serum.

2.9.1.4 Lipopolysaccharide ELISA

Serum antibody responses to meningococcal lipopolysaccharide were detected by ELISA (Quakyi *et al.*, 1999) with purified LPS (2.5) as antigen.

Materials:

A) Purified Lipopolysaccharide (Section 2.5).

B) $MgCl_2$:(10mM) in PBS (pH 7.4).

C) Antibody buffer: 5% (w/v) bovine serum albumin (BSA) (Sigma), 0.1% (v/v) Tween 20 in PBS (pH 7.4).

D) Wash buffer: PBS (pH 7.4), 0.1% (v/v) Tween 20 Substrate buffer components: 0.1M sodium acetate pH6.0 with 2M citric acid . Enzyme substrate added immediately before use.

E) Enzyme substrate: A 1 ml volume of 3,3',5,5'-tetramethylbenzidine (TMB) (3.6mg ml-1 in dimethyl sulphoxide) and 10µl H_2O_2 to 100ml of substrate buffer.

F) Stop Solution: 1M H_2SO_4

Method:

Flat-bottomed polystyrene microtitre plates (Sterilin) were coated overnight at 4°C with purified LPS (2.5) (10µg ml^{-1}; 100µl per well) in PBS (pH 7.4) and $MgCl_2$ (10mM) and

then blocked with PBS (pH 7.4) containing 5% (w/v) bovine serum albumin and 0.1% (v/v) Tween 20 (antibody buffer) for 1hr at ambient temperature. Sera were serially diluted in antibody buffer and the plates were incubated for three hours at room temperature. Antibody bound to antigen was detected by incubation with goat anti-human horseradish peroxidase conjugated antibody (Zymed) diluted 1:2000 in diluent (100µl per well) for three hours at room temperature and enzyme substrate (100µl per well) containing 3,3',5,5'-tetramethylbenzidine and H_2O_2 (Christodoulides *et al.*, 1993) for 30min at room temperature. The reaction was stopped by the addition of 1M sulphuric acid (50µl per well). The antigen-antibody reactions were then measured at an absorbance of 450nm using an Anthos htII microtitre plate reader. Concentrations of antibody were calculated by reference to a control serum, taken from an individual with significant levels of antibody directed against LPS, which was assigned a concentration of 100 arbitrary units (AU).

2.9.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

The immune response to individual protein antigens can be determined by SDS-PAGE followed by western blotting. After separation of meningococcal antigens according to molecular weight by electrophoresis in a polyacrylamide gel, antigens can be 'blotted' by transverse electrophoresis onto nitrocellulose sheets. Strips cut from the immunoblot can then be incubated with individual sera and immunological reactivity detected by incubation with a secondary antibody-enzyme conjugate. When the appropriate substrate is added, the enzyme component of the conjugate catalyses a reaction resulting in deposition of coloured substrate on the membrane at the reaction site. Control antibodies can be used to indicate the position of specific protein bands on each strip.

2.9.2.1 SDS-PAGE Gels

All SDS-PAGE was performed using a 10 - 25% (w/v) acrylamide gradient as described by Heckels (1981), with the discontinuous buffer system of Laemli (1970).

Materials:

A) Acrylamide monomer solution: 50% (w/v) acrylamide , and 1.3% (w/v) N,N'-methylene bisacrylamide

B) SDS solution: 2% w/v SDS

C) Separating gel buffer: 1.2M Tris-HCl (IBI), pH 8.8.

D) Stacking Buffer: 0.25M Tris-HCl, pH 6.8, 0.08% (v/v) TEMED (IBI).

E) Glycerol .

F) Ammonium persulphate (IBI): 1% (w/v), freshly prepared

G) TEMED

H) Running buffer: 25mM Tris-HCl, 192mM glycine , 0.1% (w/v) SDS, pH 8.3, freshly prepared.

I) Dissociation buffer: 125mM Tris-HCl, pH 6.8. 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol (BDH), 0.002% (w/v) bromophenol blue .

Construction of Gradient Gels

The glass gel casting plates (Life Technologies) were thoroughly cleaned with methanol (100%). The plates were separated by plastic spacers (1mm thick) and were held together with bulldog clips. The edges were sealed with 1% (w/v) molten agarose. Solutions of 10 and 25% acrylamide were prepared according to Table 2.6 and deaerated prior to the addition of ammonium persulphate and TEMED, to avoid inhibition of polymerisation by oxygen. The separating gel was cast using a triple channel peristaltic pump (Pharmacia, P-3) to dimensions of 1 x 110 x 170mm. Water-saturated butanol was carefully layered onto the surface of the gel, to exclude air, and the gel was left to polymerise at room temperature for 1hr. The butanol was removed and the surface of the gel was rinsed with distilled water and blotted dry. The stacking gel was prepared according to Table 2.8 and cast on top of the separating gel. Inserting the appropriate comb into the stacking layer formed the desired number of wells and the gel was left to polymerise at room temperature for 30min. After polymerisation, the comb was removed and the wells were washed with distilled water.

Table 2.7: Composition of Separating Gel

Acrylamide concentration	25%	10%
Acrylamide monomer solution	6.25ml	2.5ml
Separating gel buffer	3.1ml	3.1ml
SDS solution	0.62ml	0.62ml
Glycerol	1.25ml	-
Water	1.1ml	0.25ml
*Ammonium persulphate	0.125ml	0.25ml
*TEMED	6.2ml	6.2ml

*These constituents were added after the solutions were deaerated and immediately before gel casting.

Table 2.8: Composition of Stacking Gel

Acrylamide concentration	4%
Acrylamide monomer solution	1ml
Stacking gel buffer	6.25ml
SDS solution	0.62ml
Water	3.37ml
*Ammonium persulphate	1.25ml

*This was added after the solution was deaerated and immediately before gel casting.

Sample Preparation:

Whole cell lysates were prepared from bacterial strains grown overnight and emulsified in water containing 0.05% (w/v) sodium azide and 1mM PMSF. The protein concentration was measured indirectly using the absorbance of DNA at 260nm. Previous studies have shown that an A_{260} of 12 is equivalent to 1mg ml^{-1} protein. Samples containing ca. 500μg protein were mixed 1:1 with dissociation buffer to a final volume of 1ml and then boiled for 10min. The sample was then loaded into a large single well and protein molecular standards were loaded into small flanking wells. The gel was clamped into an electrophoresis tank (Life Technologies V15.17 gel tanks) and the tank reservoirs filled with running buffer. The gradient gels were run at 200 volts for 18-20hrs at 4°C.

2.9.2.2 *Standards used for Determination of Relative Molecular Masses of Proteins*

A Molecular Weight ladder (Sigma, Aldrich Company, Poole, Dorset, UK) comprising six protein standards ranging from 14 300 kDa to 97 400 kDa was used to estimate the molecular masses of proteins run on the same gel by comparing the distances migrated and plotting a standard curve. In addition a 39 kDa prestained molecular marker (Sigma) was used to visually monitor the migration of proteins while electrophoresis was in progress. The prestained marker also provided a visual check of subsequent electrophoretic transfer efficiency of immunoblotting.

2.9.2.3 *Electrophoretic Transfer of Proteins to Nitrocellulose (Towbin et al., 1979)*

Materials:

A) Semi-dry blotting apparatus (Bio-Rad)

B) Blotting buffer: 20% (v/v) methanol in running buffer

C) Nitrocellulose Protran nitrocellulose, 0.45mm (Schleicher & Schuell)

D) Chromatography paper 17 Chr (Whatman)

The gel was placed in blotting buffer for 30min to equilibrate. Four pieces of chromatography paper and one sheet of nitrocellulose were cut to the same size as the gel and soaked in blotting buffer. The anode surface of the semi-dry transfer blotter was wet with blotting buffer and two sheets of chromatography paper were layered on the anode. The sheet of nitrocellulose was placed on the paper stack and then the gel was arranged on the nitrocellulose. A further two pieces chromatography paper were then layered on top of the previous sheets. Air bubbles were removed as each layer was placed. A constant current of $0.8mA/cm^2$ of gel was applied for 1hr.

2.9.2.4 *Immune Detection of Proteins Bound to Nitrocellulose*

Materials:

A) Tris buffered saline (TBS): 20mM Tris-HCl, 500mM NaCl (Sigma), pH 7.5

B) Tween-Tris buffered saline (TTBS): TBS plus 0.05% (v/v) Tween 20

C) Blocking buffer: 5% (w/v) non-fat milk powder (Marvel^{TM}) in TBS

D) Antibody diluent: 1% (w/v) gelatine in TTBS

E) Substrate components:

E1) Substrate buffer: 100mM Tris-HCl, 100mM NaCl, 2mM $MgCl_2$, pH 9.5

E2) Nitro blue tetrazolium (NBT): 30mg ml^{-1} in 70% dimethyl formamide (DMF)

E3) 5-Bromo-4-chloro-3-indolyl phosphate (BCIP): 15mg ml^{-1} in 100% DMF

Just before use, 1ml each of the NBT and BCIP solutions were added to 100ml of substrate buffer.

Method:

After protein transfer, the nitrocellulose sheet was washed in TTBS (3 x 5min) to remove traces of SDS and then incubated with blocking buffer for 1hr at room temperature to block non-specific antibody binding. Blocking buffer was then removed and the nitrocellulose was washed in TTBS (3 x 5min). The nitrocellulose sheet was then cut into longitudinal strips of ca. 0.3cm width and placed in individual compartments of development trays to facilitate separate incubation with different test antisera and control antibodies directed against meningococcal proteins. Test sera were diluted 1/100 in TTBS containing 1% (w/v) gelatin and incubated with the strips overnight at room temperature.

Unbound antibody was removed by washing in TTBS (3x5min) and the strips were incubated for 1hr at room temperature in the appropriate anti-species alkaline phosphatase conjugated antibody (BioRad) diluted 1:1000 in blocking buffer. The nitrocellulose strips were then washed in TTBS (3 x 5min) followed by TBS (3 x 5min). Antibody bound to the nitrocellulose was detected by addition of the substrate. The colour reaction was monitored carefully and stopped by washing in distilled water once the bands had reached the desired intensity and before background staining had occurred. The blots were then dried and stored in the dark.

Control antibodies were used to indicate the position of specific protein bands on each blot: rabbit polyclonal antibody raised to gonococcal porin, which cross-reacts with

meningococcal PorA and PorB Protein porins (Heckels *et al.*, 1989); monoclonal antibody 4B12/C11 (courtesy of M. Blake, Rockefeller University, New York) for Opa; SM51 (Virji *et al.*, 1989) for Rmp protein; AG123 (courtesy of M. Achtman, Max Plank Institute, Berlin) for Class II pilin and SM1 for Class I pilin (Virji and Heckels, 1983). The molecular weights of bands not coincident with control antibodies were calculated. A standard curve was produced by plotting the distance migrated by the protein standards (log_{10}) against their molecular weights. The apparent molecular masses (m_r) of desired sample proteins were calculated by extrapolating the distances migrated by these proteins on a gel to the standard curve. A MW ladder (Sigma, Aldrich Company, Poole, Dorset, UK) comprising six protein standards ranging from 14 300 kDa to 97 400 kDa was used to plot a standard curve.

2.9.2.4.1 Staining of Polyacrylamide Gels

Gels were stained by gently shaking in a solution containing 10% (v/v) glacial acetic acid, 20% (v/v) isopropanol and 0.5mg ml^{-1} PAGE Blue 83 for 1hr. The gels were then destained in several applications of 10% (v/v) glacial acetic acid and 10% (v/v) isopropanol until the protein bands were visible and background staining was minimal.

2.9.3 Serum Bactericidal Assay

The ability of antibodies to promote complement-mediated bacteriolysis was measured essentially as described by Jones *et al.* (1998).

Materials:

A) PBSB 100 ml PBS + 0.5ml B salts

B) PBSB –FCS (100ml PBSB + 2ml Foetal Calf Serum)

C) External Complement Source

Exogenous Complement Source:

Because the test sera were not processed or stored to preserve endogenous complement, they were heated at 56°C for 30min to inactivate endogenous complement and exogenous complement added. Fresh sera obtained from individual rabbits (3-4 week old) or individual adult human volunteers were screened; those that lacked SBA against the test strain but were able to support killing in the presence of added antibody were used as a

complement source. The optimal final complement concentration for each meningococcal strain was determined to enable standard conditions to be used for all sera tested. Baby rabbit sera were used as the exogenous complement source for serogroup C meningococci (Jones *et al.*, 2000; Maslanka *et al.*, 1997) and human sera for serogroup B meningococci (Jones *et al.*, 1998; Zollinger *et al.*, 1983).

Method:

An overnight growth of meningococci was harvested from proteose peptone agarose plates and resuspended in Dulbecco phosphate buffered saline (Oxoid, PBSB) containing 2% (v/v) heat – inactivated foetal calf serum (Flow) to a concentration of approximately 6 x 10^4 ml^{-1}. A typical bactericidal mixture contained 25µl of the meningococcal suspension, 25µl of heated test serum, 25µl of PBSB and 25µl of freshly thawed exogenous complement. All test sera were assayed three times over a range of final serum dilutions. The amount of test serum added to the bactericidal mixture varied according to the required serum dilution to be tested, however the overall volume of the bactericidal mixture was kept to a constant 100µl by adjusting the volume of PBSB.

Controls included test wells with PBSB in place of serum; heat inactivated complement in place of active complement and a positive control serum with known serum bactericidal activity to the meningococcal strain being tested. The plate was agitated briefly to ensure thorough mixing and then incubated at 37°C for 30min in an atmosphere of 5% (v/v) CO_2. Thereafter, 15µl samples were removed for determination of surviving colony-forming units (c.f.u.). Serum bactericidal titres were expressed as the final serum dilution that resulted in a $\geq$ 50% reduction in surviving c.f.u. compared with an equivalent negative control containing heat-inactivated complement source. All assays were repeated at least once.

CHAPTER 3 THE IMMUNE STATUS OF UNIVERSITY STUDENTS BEFORE, DURING AND AFTER AN OUTBREAK OF SEROGROUP C MENINGOCOCCAL INFECTION

3.1 Introduction

Meningococcal serogroups A, B and C have been associated with 90% of invasive disease worldwide; serogroups B and C are more common in Europe and America, while A predominates in Africa and Asia. Epidemic disease occurs worldwide but is more common in developing countries than in industrialised countries (Hubert and Caugant, 1997a). Most epidemics are caused by group A strains, but small outbreaks have occurred with group B and C strains. Epidemiological data from the late 1990's revealed an increase in meningococcal disease with a greater proportional increase of invasive meningococcal disease due to serogroup C in Europe, (Connolley and Noah, 1999;Hubert and Caugant, 1997b;Ramsay *et al.*, 1997;Neal *et al.*, 1999) Canada, (Mitchell *et al.*, 1996;Whalen *et al.*, 1995) and the USA (Leake and Perkins, 2000;Jackson *et al.*, 1995) This increase in serogroup C disease had been most marked in adolescents and young adults, (Ramsay *et al.*, 1997;Neal *et al.*, 1999) and was associated with a higher death rate (Ramsay *et al.*, 1997) than cases of serogroup B infection.

One of the biggest university outbreaks of meningococcal infection occurred in England in October 1997. This cluster of *N. meningitidis* serogroup C infection amongst first year students at Southampton University provided us with the unique opportunity to investigate levels of immunity to meningococcal infection, both in cases and in uninfected at –risk students, prior to and during the outbreak. Six cases were reported which included five students from the same residential complex. Within this same group, three cases (Cases 3, 4 and 5) were further linked to the same night-club on the same evening, one week prior to presentation. It is national policy to screen all medical students for antibodies to Hepatitis B at the beginning of their training. This enabled us to obtain and study the serum of Case 3 and 76 asymptomatic classmates taken one month prior to the outbreak. Admission sera were available for Cases 1, 3, 4, 5 and 6. In addition, convalescent serum samples were accessible from Cases 4 and 5. Case 2 was admitted to another hospital and it was not possible to obtain serum for immunological analysis. Cases 1, 3 and 6 died, whereas Cases 2, 4 and 5 made a full clinical recovery.

Meningococcal carriage (Table 3.1) at the time of the outbreak was investigated in 587 undergraduate students (Gilmore *et al.*, 1999). Meningococci were isolated from the throats of 25% of the students. Nongroupable (NG) meningococci were most common (11.1%), followed by groups B (6.1%) and Y (2.6%). Group C meningococcal carriage in these asymptomatic at-risk students was low (0.9%) and none of the nongroupable carriage strains possessed the group C *siaD* gene as determined by PCR.

Table 3.1: Carriage Rates by Serogroup in at-risk Students

Serogroup	B	C	29E	W135	X	Y	Z	NG	Total
First Year Carriage Rate (%)*	6.6	0	0.4	2	0	2	0.4	8	19
Second, Third, Fourth and Fifth Year Carriage Rate (%)**	6	1.5	2	2	1.5	3	0	13	30
Total Carriage Rate (%)***	6.1	0.9	1.4	2.0	0.9	2.6	0.2	11.1	25

*Carriage rates of students in their first year of study (n = 258)

** Carriage rates of second, third, fourth and fifth year of study (n = 326)

***Carriage rates of first year students combined with that of second, third, fourth and fifth year students (n = 587). Year of study data missing for three students.

Most of our knowledge of events prior to an outbreak of meningococcal infection is based on the classic studies of Goldschneider *et al.* (1969a). These studies established the importance of bactericidal antibodies in protection against invasive meningococcal disease; however, understanding the contribution that individual meningococcal surface antigens make toward natural immunity to meningococci is incomplete. The advent of modern molecular and biochemical techniques have lead to major advances in understanding of the meningococcal surface structure. Detailed molecular information on the antigenic profile of meningococcal isolates is essential for analysis of the immune response. In this study, modern molecular techniques were used to investigate the association between antibody reactivity to individual meningococcal surface antigens and serum bactericidal activity both in cases and in uninfected at–risk students, prior to and during an outbreakof meningococcal infection.

3.2 Methods and Results

3.2.1 Characterisation of Strains Isolated from Infected Students during a Group C Meningococcal Outbreak

Meningococcal strains isolated from infected students were cultured by standard techniques as described in Section 2.2. Phenotypic characterisation of the strains was performed by the Meningococcal Reference Laboratory, Manchester, UK. The variable regions VR1 and VR2 of the meningococcal *porA* gene were amplified by PCR (Brooks *et al.*, 1995) and sequenced as detailed in Section 2.7. PCR conditions were: 25 cycles of denaturation at 96°C for 40s, annealing at 10°C below the lowest Tm of primers for 40s and extension at 72°C for 45s. Amplified DNA was purified by the GeneCleanII™ (Stratech, UK) procedure as described by the manufacturers (2.6.8). Sequencing was performed using the ThermoSequenase™ ready reaction dyedeoxy terminator cycle sequencing kit (Amersham Life Sciences, Cleveland, Ohio, USA) according to manufacturers instructions. Electrophoresis was carried out in an ABI model 373A automated DNA Sequencer for 12 hours at 30W.

Meningococcal DNA obtained from the blood and cerebrospinal fluid of culture-negative cases were characterised by sequence analysis of the PorA region and by TaqMan sialyltransferase (*siaD*) PCR (2.8) for group B and group C-associated DNA (Guiver *et al.*, 2000).

Table 3.2: Characterisation of Meningococcal Strains from Infected Students during an Outbreak of Meningococcal Disease.

Cases	Day of Presentation	Clinical Outcome	Phenotype	PorA Sequence Subtype	PorB Sequence Type
1	1	died	C:2a:NST[+]	P1.5, 2	2-36
2	17	survived	C:NT:P1.5[#]	P1.5a, 10d	2-37
3	16	died	C *	P1.5a, 10d*	ND^
4	18	survived	-	-	-
5	18	survived	C	-	-
6	20	died	C:NT:P1.5	P1.5a, 10d	2-37

The PorB sequence data in the above table is derived from Gilmore *et al.*, (1999).

*Obtained by PCR analysis of DNA from this patient's CSF.

+ NST Non-Subtypeable

[#] NT Non-Typeable

^ ND Not Determined

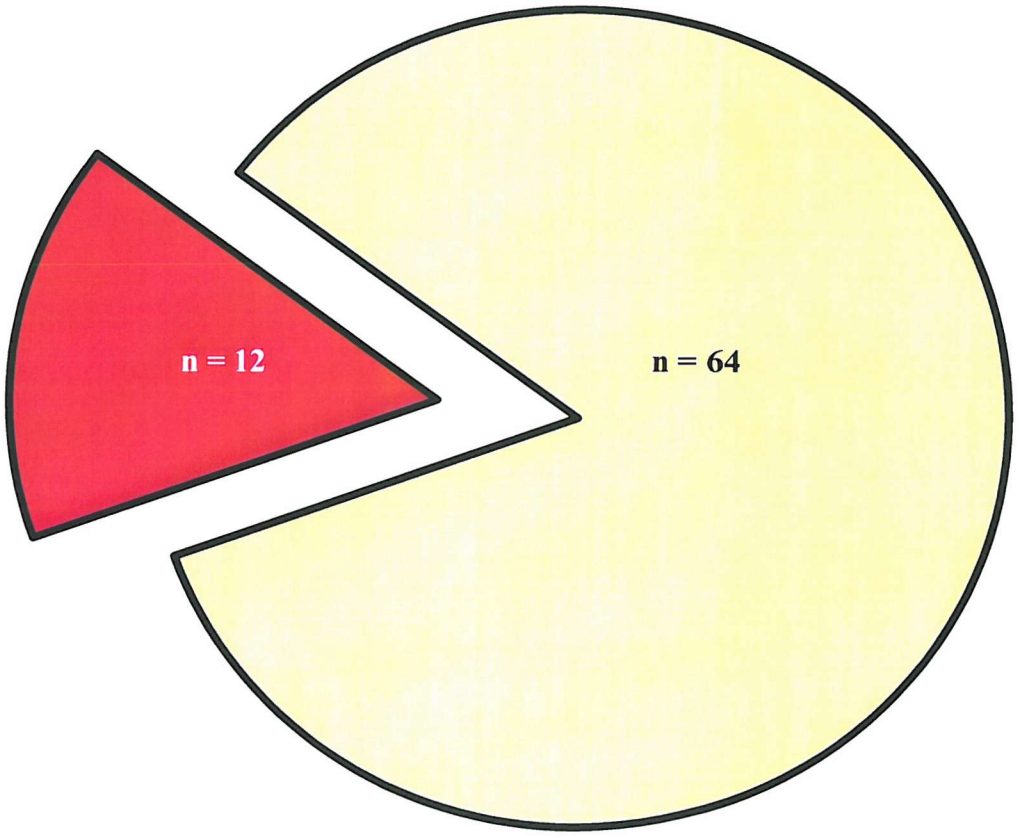
Five of the six cases were confirmed as due to serogroup C meningococcal infection. Cases 1, 2 and 6 were confirmed by culture, Cases 3 and 5 by *siaD* PCR. Case 4 was a probable case as none of the laboratory tests were able to confirm the clinical diagnosis of meningococcal disease. Serology and DNA sequencing of the cultured organisms indicated two distinct serogroup C meningococcal strains associated with the cases. The meningococcal strain obtained from the first case was serologically characterised as serogroup C, serotype 2-36, nonsubtypeable (C:2-36:NST) and designated MC162 (Jones *et al.*, 2000;Feavers *et al.*, 1999). Further investigation indicated that the PorA protein of this strain was not expressed due to the insertion of an IS*1301* element which had inactivated the *porA* gene. Sequencing of the *porA* gene, which encodes the subtype-specific PorA outer membrane protein, demonstrated that this isolate was nonsubtypeable due to nonexpression of a P1.5,2 PorA protein giving the following organism profile: C:2-36:P1.5,2.

In contrast, isolates from Cases 2 and 6 were identified as serogroup C, nontypeable, subtype P1.5 (C:NT:P1.5) by monoclonal antibody typing (courtesy of Meningococcal Reference Laboratory, Manchester, UK). Sequencing of the variable subtype-specific regions of the PorA protein further characterised these strains as subtype P1.5a, 10d. *PorA* sequencing of the cerebrospinal fluid of Case 3 revealed that this patient had been infected with the same strain phenotype as Cases 2 and 6. Sequencing of the *PorB* gene (Gilmore *et al.* 1999, Table 3.2), which encodes the serotype-specific PorB Protein, provided further evidence that the isolate obtained from Case 1 was distinct from the isolates obtained from Cases 2 and 6. It is probable that Case 1 was a sporadic infection with MC162 (C:2-36:P1.5,2), and that given the temporal and geographic proximity of these infections, Cases 2 to 6 comprised the outbreak caused by C:2-37:P1.5a,10d. A representative strain of this phenotype isolated from Case 6 was designated MC161. Neither strain phenotypes C:2-36:P1.5,2 nor C:2-37:P1.5a,10d were detected by Gilmore *et al.*, (1999) in the 587 asymptomatic at-risk students, except in a single case of clearly defined close contact.

3.2.2 Serum Bactericidal Activity Directed Against the Outbreak Strain in Students Prior to Meningococcal Infection

Sera obtained from 76 asymptomatic classmates of Case 3, taken one month prior to the outbreak provided a unique opportunity to investigate the levels of humoral immunity to meningococci in uninfected at–risk students, prior to the outbreak. The sera were tested for bactericidal activity (2.12.3) against the outbreak meningococcal strain MC161 (C:2-37:P1.5a,10d). Only 12 students (14%) had significant bactericidal activity, defined as a ≥ 50% reduction in surviving c.f.u. at a serum dilution of ≥1/8 (Borrow *et al.*, 2001a). Similar results (not shown) were obtained when these sera were tested for bactericidal activity against MC162 (C:2-36:P1.5,2) and the reference group C strain MenC11 (C:16:P1.7a,1). These results suggest that ca. ninety percent of these students were susceptible to invasive disease by the group C strains tested.

Figure 3.1: Serum Bactericidal Activity against the Serogroup C Outbreak Strain in Students before an Outbreak of Serogroup C Meningococcal Infection.



50% Serum Bactericidal Activity

≤ 1/8 titre

> 1/8 titre

n = number of students

This data clearly shows that there was no significant bactericidal activity against the outbreak strain in 64 out of 76 students (84 %).

3.2.3 Specificity of Serum Bactericidal Antibodies directed against Serogroup C Meningococci, in Uninfected Individuals, Prior to an Outbreak of Serogroup C Meningococcal Infection.

An SBA titre of ≥1/8 corresponds with immunity to meningococcal disease, when rabbit serum is used as the external complement source (Borrow *et al.*, 2001a). Applying this definition to the pre-outbreak at-risk students we were able to subdivide the subjects into 'immune' and 'non-immune' groups. In order to determine whether antibody reactivity to specific meningococcal components correlated with serum bactericidal activity, the specificity of reactivity of each serum for the PorA Protein, PorB Protein, Rmp OMP, Opa protein and pili was investigated by western blotting. Whole cell lysates of strain MC161 were separated by SDS-PAGE using a 10-25% (w/v) acrylamide gradient (Laemli, 1970) as described in Section 2.9.2. The presence and strength of antibody reactivity was determined visually and scored with 1 representing a very weak reaction and 5 a very strong reaction, the symbol 0 indicates no reactivity. The identity of reactive bands was determined by the use of control antibodies of defined specificity and by reference to a standard mixture of proteins of known molecular mass.

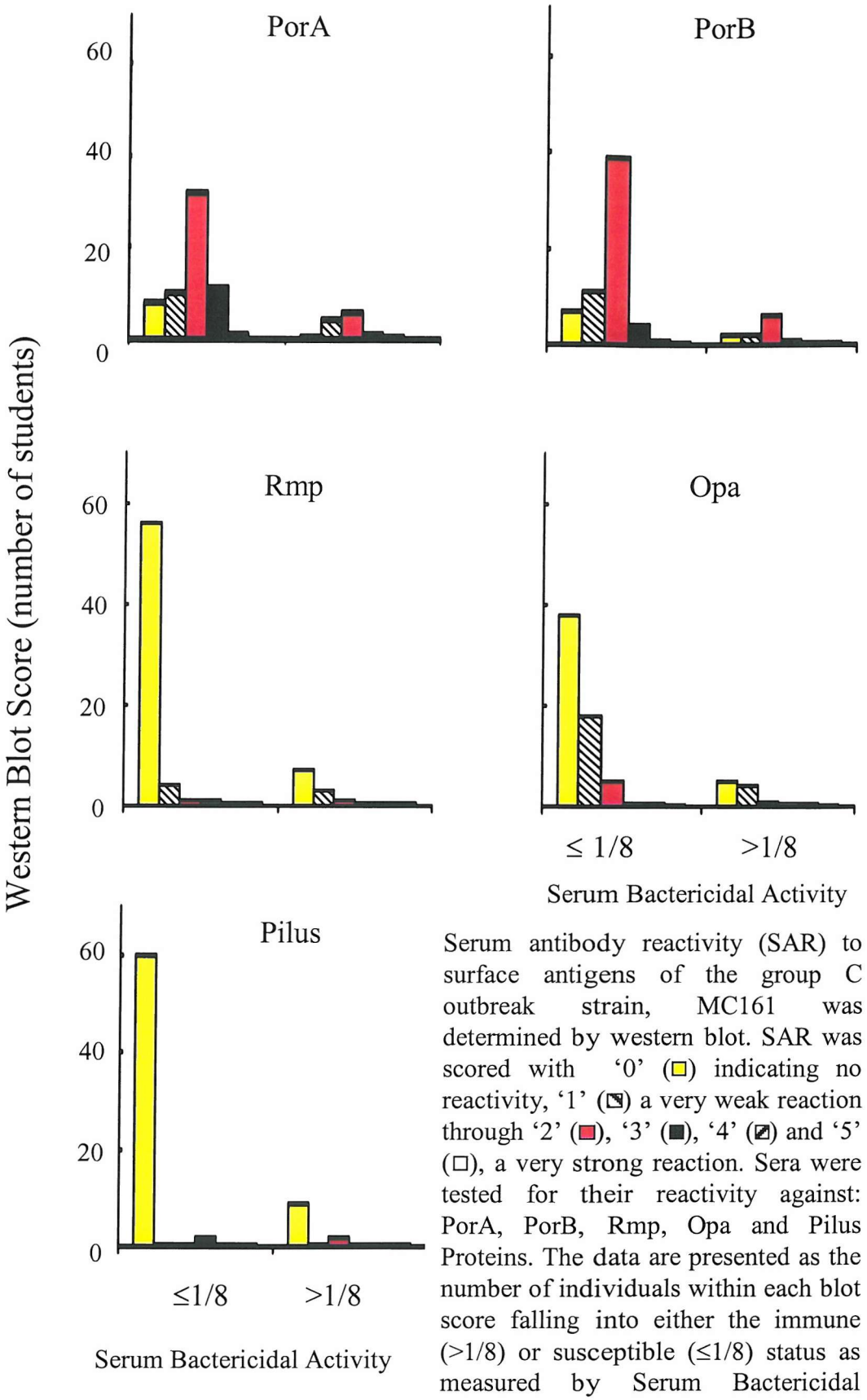
There was no correlation between SBA and antibody to PorA, PorB, Rmp OMP, Opa protein or pili of the homologous strain (Figure 3.2). Similarly, there was no correlation between SBA and a specific immune response to surface antigens of the sporadic (MC162) and reference (MenC11) group C meningococcal strains (results not shown).

Meningococcal group C-specific antibody concentrations of these sera were measured by a standardized ELISA (Gheesling *et al.*, 1994) as described in Section 2.9.1.2. The mean antibody concentration for individuals with SBA titres >1/8 was 55μg ml^{-1}, which was significantly greater ($p<0.05$) than the levels of antibody (2μg ml^{-1}) observed for individuals with SBA titres of <1/8 (Figure 3.3a). There was a significant statistical correlation ($p < 0.05$) between the presence of serum antibodies to group C capsular polysaccharide and SBA against the group C outbreak strain, MC161.

The relatedness of these variables was again apparent when comparing antibody to serogroup C capsule and serum bactericidal activity against the MC162 and MenC11 serogroup C strains (data not shown). The at-risk students were vaccinated against meningococci with the A-C polysaccharide vaccine as part of outbreak control measures initiated at the time of the outbreak. Comparisons were made between levels of antibody to serogroup C capsule and serum bactericidal activity against MC161 in the post vaccination at-risk sera (data not shown). All of the vaccinated students, except one, were protected against the outbreak strain as measured by the serum bactericidal assay. Raised antibody levels to serogroup C capsule were detected in the sera of all the protected students, in contrast the student lacking SBA showed no difference in group C antibody levels after vaccination.

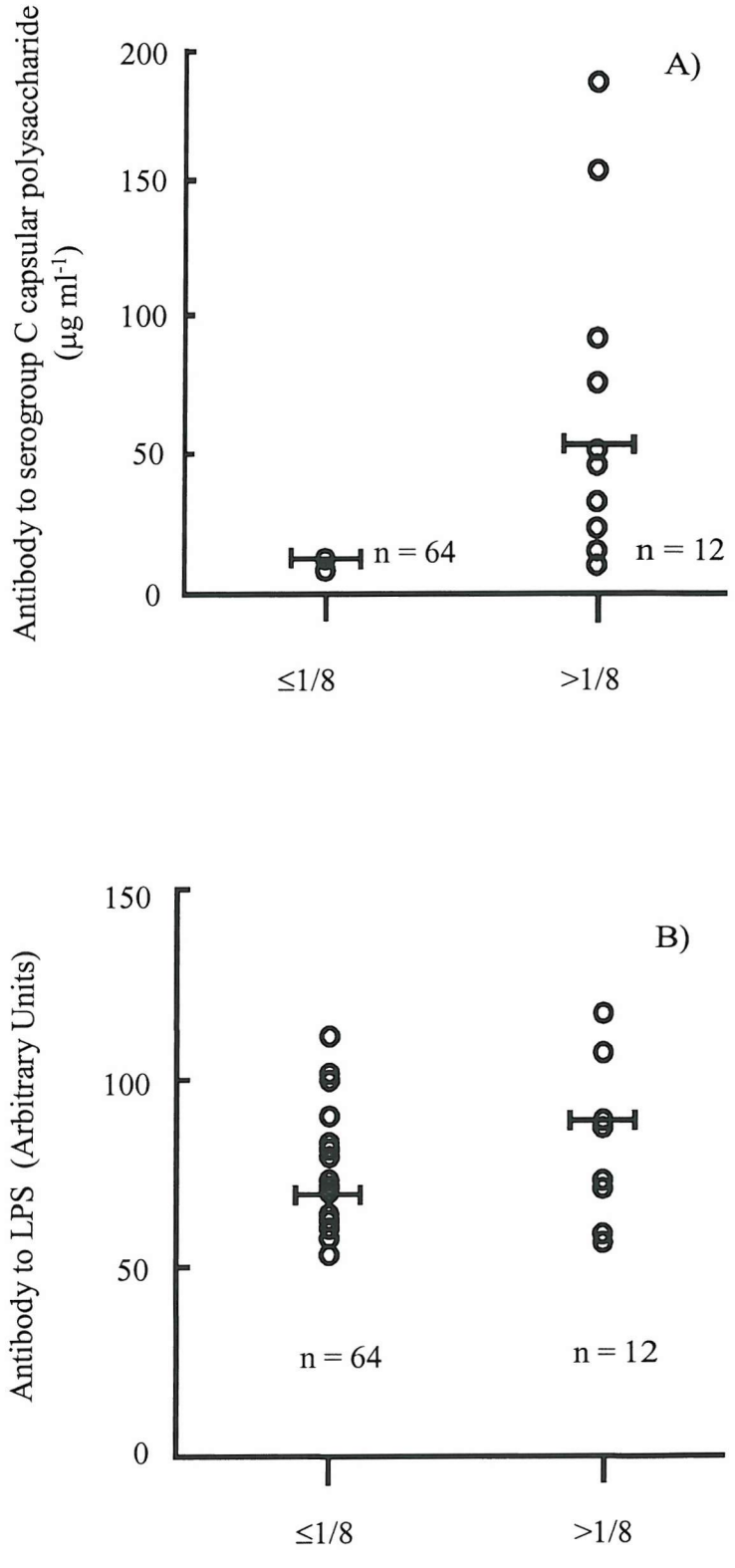
The pre-outbreak sera were tested for serum antibody responses to meningococcal lipopolysaccharide by ELISA (detailed in Section 2.9.1.4.) with purified LPS from the outbreak strain (LPS immunotype L2). The amount of antibody directed against LPS was quantified by comparison to a control serum containing anti-LPS antibodies run on each microtitre plate. Antibody reactivity of each test serum was compared to this control and expressed in arbitrary units (AU). There was no significant association ($p>0.05$) between antibody levels to LPS, measured by ELISA, and SBA (Figure 3.3b). The mean LPS antibody concentrations were 73 and 79 arbitrary units respectively for individuals with SBA titres of $<$ or $>$ 1/8 respectively.

Figure 3.2: Investigation of Humoral Immunity to Serogroup C Meningococci, in Uninfected Individuals, Prior to an Outbreak of Meningococcal Infection.



Serum antibody reactivity (SAR) to surface antigens of the group C outbreak strain, MC161 was determined by western blot. SAR was scored with '0' (□) indicating no reactivity, '1' (▨) a very weak reaction through '2' (■), '3' (■), '4' (▨) and '5' (□), a very strong reaction. Sera were tested for their reactivity against: PorA, PorB, Rmp, Opa and Pilus Proteins. The data are presented as the number of individuals within each blot score falling into either the immune (>1/8) or susceptible (≤1/8) status as measured by Serum Bactericidal Activity against MC161.

Figure 3.3: Antibody Levels to (A) Serogroup C Capsular Polysaccharide and (B) Lipopolysaccharide in Uninfected Students.



3.2.4 Humoral Immune Response to Infection with Group C Meningococci

The humoral immune response to infection was studied for available sera from cases using several *in vitro* assays: determination of antibody to serogroup C capsule and LPS, antibody recognition of surface antigens by western blotting, and the presence of serum bactericidal activity (SBA) against the infecting strain. Paired sera were available from three of the six cases. Serum samples taken one month prior to the outbreak and acute serum samples taken at the time of hospital admission were available for Case 3 and acute and convalescent serum samples were available from Cases 4 and 5. Admission sera were only available for Cases 1 and 6. Case 2 was admitted to another hospital and it was not possible to obtain serum for immunological analysis.

Table 3.3: Humoral Immune Response to Infection with Group C Meningococci.

				Western Blot Score against MC161				
Cases	SBA against MC161	Group C antibody ($\mu g\ ml^{-1}$)	MC161 LPS (AU)	PorA Protein	PorB Protein	Rmp OMP	Opa protein	Pilus (Class II)
1 a	<1/4	2	68	0	0	0	0	0
3 p	<1/8	3	76	0	0	1	0	0
3 a	<1/8	2	65	1	1	0	0	0
4 a	1/32	2	68	2	2	1	0	0
4 c	1/2048	29	96	2	2	1	0	0
5 a	1/32	2	71	3	2	0	1	0
5 c	1/2048	30	83	3	2	0	2	0
6 a	<1/8	2	94	1	1	1	4	0

Cases 1 to 6 are numbered in order of notification to the health authority, where 'a' refers to admission sera, 'p' to pre-outbreak serum and 'c' to convalescent sera. The sera were tested for serum bactericidal activity against the outbreak strain MC161 (C:2-37:P1.5a,10d), and serum bactericidal titres were expressed as the final serum dilution that

resulted in a ≥ 50% reduction in surviving c.f.u. compared with an equivalent negative control containing heat-inactivated complement source. Antibody to group C capsule and LPS was quantified by ELISA, and antibody reactivity to surface antigens of strain MC161 was determined by western blot, using a scoring system as described in Figure 3.2.

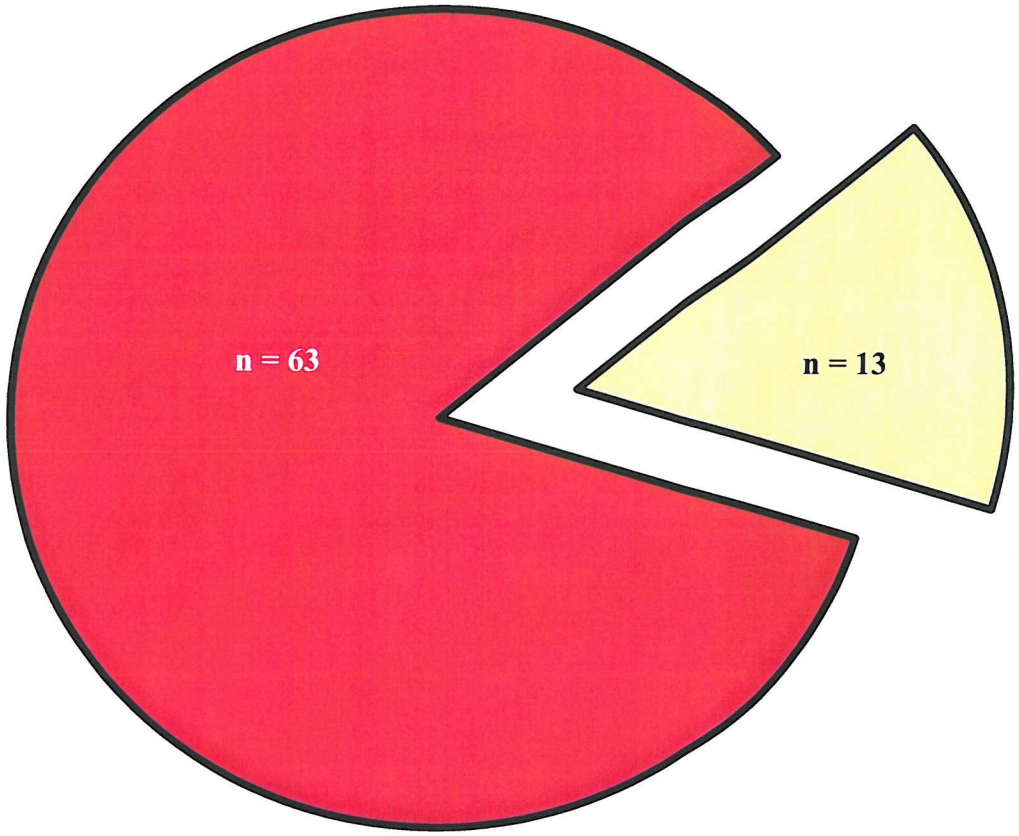
On admission, sera taken from Cases 4 and 5 had significant SBA towards strain MC161 (titres of 1/32), which rose during convalescence (titres of 1/2048) (Table3.3). In contrast, serum taken from Case 3, either prior to the outbreak or on admission, showed no significant serum bactericidal activity. Similarly, serum from both Cases 1 and 6 showed no detectable bactericidal activity at the time of admission (Table 3.3). Identical results were obtained when these sera were tested against the other group C organisms MC162 and MenC11 (data not shown). Serum samples from the infected individuals were also analysed for the presence of antibody to the group C polysaccharide. All of the cases had serum group C antibody levels of around 2μg ml^{-1} on admission to hospital. Serum taken from Cases 4 and 5 showed an increase in anti-capsular antibody from 2μg ml^{-1} to approximately 30μg ml^{-1} during convalescence. In order to study the contribution of specific surface antigens to the immune response, antibody reactivity was investigated with western blotting. No antibodies to pili or any OMPs of strain MC161 were detected in serum from Case 1. Serum from Cases 3 and 6 possessed low levels of antibody to PorA, PorB, and Rmp OMPs on admission. In addition, high levels to the Opa protein was detected in the serum of Case 6 only. No reactivity to pili was detected in serum samples from any of the cases.

No effective vaccine exists for *N. meningitidis* serogroup B, therefore levels of immunity to group B meningococci in the same student population are presented in this thesis.

3.2.5 Serum Bactericidal Activity Directed against Group B Reference Strain in Students Before Meningococcal Outbreak.

The sera of uninfected students taken 28 days prior to the outbreak were tested for SBA against the group B meningococcal strain, MC58 (B:15:P1.7,16) using 5% human serum as the exogenous complement source. Eighty three percent of the students had significant bactericidal activity against MC58, as illustrated in Figure 3.4. These results suggest that most of these students were immune to invasive disease by this group B strain and is in direct contrast to the number of students in this group immune to invasive group C meningococcal disease (ca. 10%).

Figure 3.4: Serum Bactericidal Activity Against the Serogroup B Reference Strain in Students Before an Outbreak of Serogroup C Meningococcal Infection.



50% Serum Bactericidal Activity

≤ 1/8 titre

> 1/8 titre

n = number of students

The sera of most (83%) of the students were able to kill the reference group B strain (MC58) at a titre of 1/8.

3.2.6 Specificity of Serum Bactericidal Antibody Response Directed against Serogroup B Meningococci, in Uninfected Individuals, Prior to an Outbreak of Meningococcal Infection.

The specificity of serum bactericidal reactivity directed against group B meningococci in the uninfected at-risk students was investigated by comparison with antibody reactivity directed against specific meningococcal components. In contrast to serogroup C, there was no association between antibody levels to serogroup B capsule (Figure 3.3B) and SBA of MC58. The relationship between the serum bactericidal activity of each serum and antibody to the PorA Protein, PorB Protein, Rmp OMP, Opa protein and pili of strain MC58 was investigated by western blotting. In contrast to serogroup C, there was a significant association ($p<0.05$) between antibody reactivity to the PorA Protein and SBA (Figure 3.3A). In addition, there was an association between antibody levels to the PorB Protein and SBA ($p=0.058$). There was no correlation between the Rmp OMP, Opa protein, pili (Figure 3.5), or LPS (Figure 3.6) and SBA of the homologous group B strain.

Figure 3.5: Antibody Levels to (A) Serogroup B Capsular Polysaccharide and (B) Lipopolysaccharide in Uninfected Students

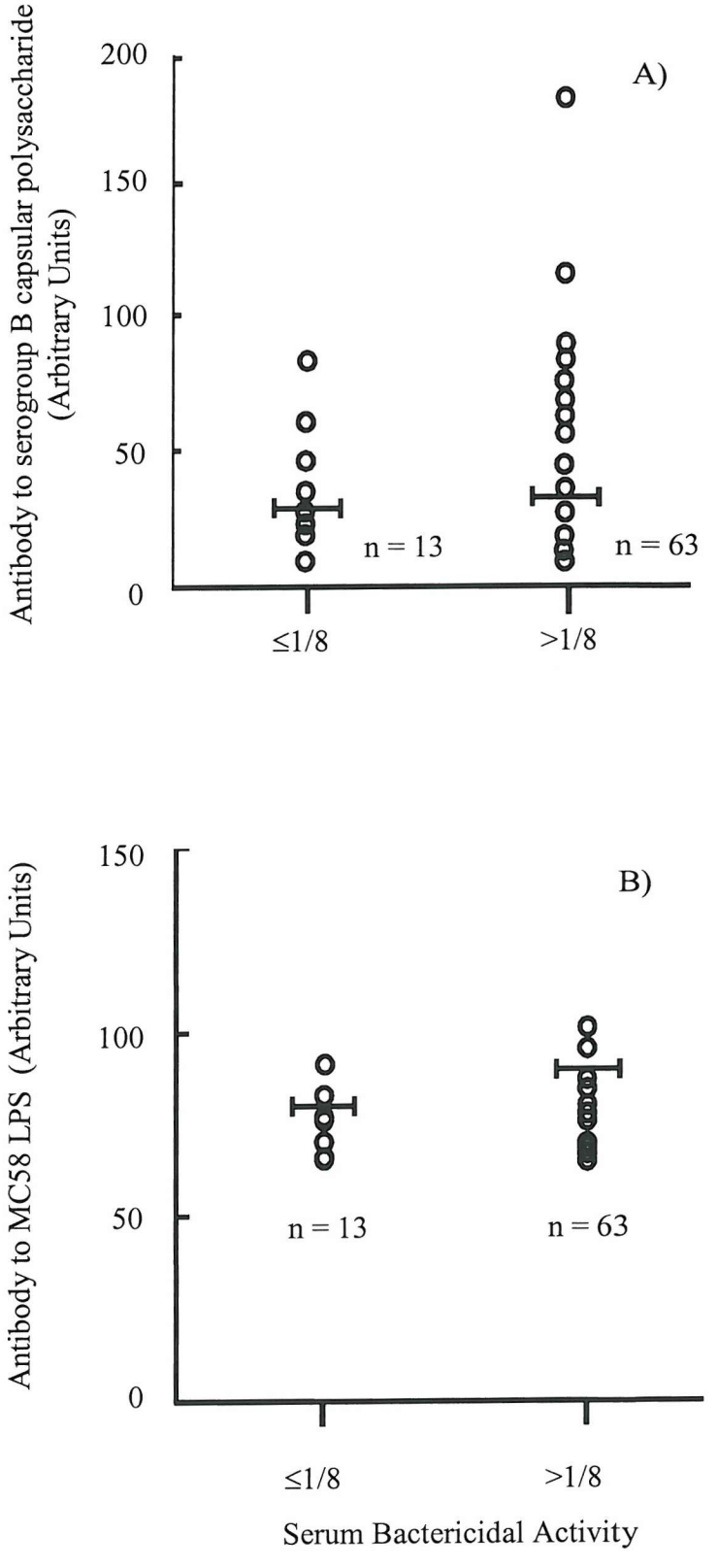
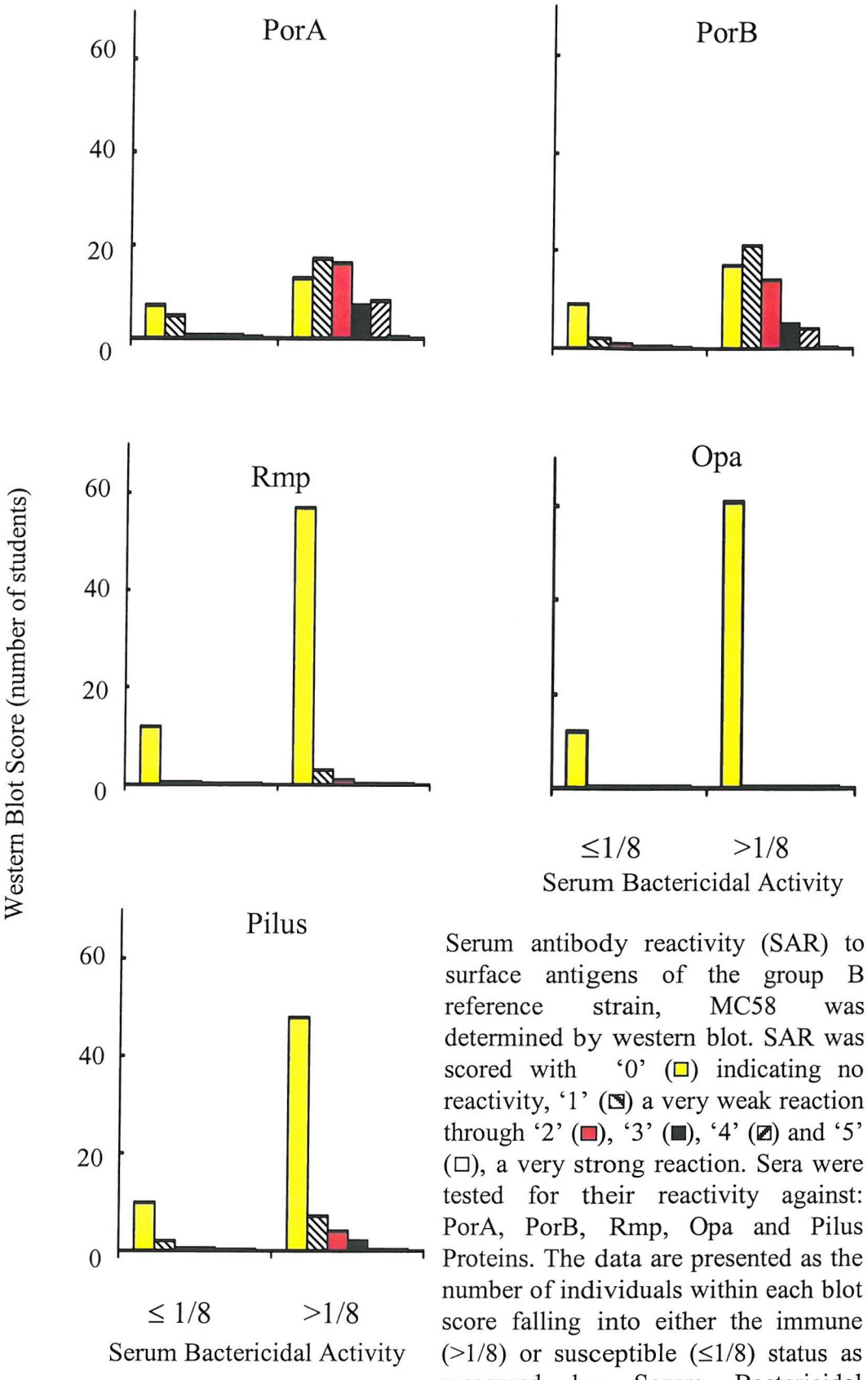


Figure 3.6: Investigation of Humoral Immunity to Serogroup B Meningococci, in Uninfected Individuals, Prior to an Outbreak of Meningococcal Infection.



Serum antibody reactivity (SAR) to surface antigens of the group B reference strain, MC58 was determined by western blot. SAR was scored with '0' (□) indicating no reactivity, '1' (▨) a very weak reaction through '2' (■), '3' (■), '4' (▨) and '5' (□), a very strong reaction. Sera were tested for their reactivity against: PorA, PorB, Rmp, Opa and Pilus Proteins. The data are presented as the number of individuals within each blot score falling into either the immune (>1/8) or susceptible (≤1/8) status as measured by Serum Bactericidal Activity (SBA) against MC58.

3.2.7 Summary of Statistical Relationships Between the Antibody Data

All the antibody data were analysed with SPSS for Windows (version 10.0). The Mann-Whitney *U* test was used to test differences between groups and probability values of <0.05 were considered statistically significant.

Table 3.4: Correlations Between Bactericidal Activity and Antibodies Directed Against Meningococcal Components in Uninfected Students, Prior to an Outbreak of Meningococcal Infection.

	Meningococcal Strains	
Meningococcal Component	MC161 (C:2-37:P1.5a,10d:L2)	MC58 (B:15:P1.7,16b:L3)
Capsule	0.787	0.000
PorA Protein	0.029	0.402
PorB Protein	0.058	0.301
Rmp Protein	0.383	0.069
Opa Protein	*	0.478
Pilus	0.661	0.103
LPS	0.064	0.399

*No statistics computed as MC58 Opa score was a constant figure.

3.3 Discussion

3.3.1 Strain/strains involved in outbreak

An outbreak of serogroup C meningococcal infection occurred at the University of Southampton during October 1997 causing illness in six first year students and three deaths. Although outbreaks of invasive meningococcal infections can be due to more than one circulating strain (Edmond *et al.*, 1995), in this institutional study of serogroup C disease, molecular analysis suggests that only five of the cases (Cases 2 to 6) were of the same strain (MC161, C:2-37:P1.5a,10d:L2), with a single case (Case 1) arising sporadically from a different serogroup C organism (MC162, C:2-36:P1.5,2:L2).

The immune response of infected students to these meningococcal strains was examined in detail. In addition to sera from cases, sera were available for 'at-risk' students prior to the outbreak. This resource provided a unique opportunity to investigate levels of humoral immunity to meningococcal infection in a contemporary 'at-risk' population prior to an outbreak.

3.3.2 Carriage and Immunity to Serogroup C Meningococci in Soldiers in 1967

Data on immunity prior to invasive meningococcal disease is rare, since outbreaks of meningococcal infection are uncommon and unpredictable and therefor access to large numbers of serum samples immediately predating an outbreak is very unusual. Most of our understanding of natural immunity prior to an outbreak of meningococcal infection dates back to the classic studies of Goldschneider *et al.,* (1969b) in which the inverse relationship between bactericidal antibodies and the risk of subsequent disease was demonstrated in a group of military recruits stationed at Fort Dix. During the Fort Dix outbreak >80% of new recruits had an SBA titre of ≥1/4 and none of these developed meningococcal infection. In contrast, infection developed in approximately 40% of recruits who acquired the epidemic strain and who had SBA titres of <1/4. A feature of this institutional serogroup C outbreak was that carriage of serogroup C meningococci was common with 75% of recruits becoming colonized during training (Goldschneider *et al.*, 1969a). These data indicate a high prevalence

of carriage of serogroup C meningococci within the training camp which was associated with a high prevalence of protective immunity to serogroup C strains.

In the Fort Dix study (Goldschneider *et al.*, 1969b) a bactericidal activity titre of ≥1/4 correlated with protection against the homologous meningococcal strain. These investigators used human serum as the exogenous complement source in the SBA against group C meningococci. However, many researchers (Borrow *et al.*, 2001b;Mitchell *et al.*, 1996) have used baby rabbit serum (3 to 4 week old) as the complement source in the group C meningococcal SBA, and it is the source specified in the WHO-recommended procedure (Maslanka *et al.*, 1997). Rabbit serum provides a uniform and standardisable complement source that gives results that can be compared between laboratories. It is generally accepted however, that group C meningococci are more susceptible to group C-specific bactericidal antibodies when using rabbit complement as opposed to human complement, resulting in higher SBA titres (Maslanka *et al.*, 1997;Granoff *et al.*, 1998). Borrow *et al* (2001a) have recently defined correlates of protection to group C meningococcal infection as measured by a SBA incorporating baby rabbit serum (rSBA) as the exogenous complement source. These investigators concluded that a rSBA titre of <1/8 predicted susceptibility to group C infection and this criterion was used to interpret the rSBA results in this thesis.

3.3.3 Carriage and Immunity to Group C Meningococci in Students in 1997

In this thesis, the rSBA was used to screen sera from the 1997 student population prior to the outbreak for bactericidal antibodies to group C meningococci. The results of this study suggest that only ca. 10% of this student population were immune to invasive disease by group C meningococci. The low levels of protective immunity to group C meningococci in this student population were associated with a low (0.9%) carriage rate of group C meningococci prior to the outbreak.

3.3.4 Carriage and Immunity to Meningococcal Disease in 1997 University Students Compared to 1967 Military Recruits.

Military recruits and university students have several common characteristics including: age, crowded living conditions, intensive social mixing and diverse geographical backgrounds. Therefore, data obtained from studies of soldiers have formed the basis for the understanding of meningococcal disease outbreaks in college students. The results presented in this thesis are in contrast to studies undertaken in the 1960's (Goldschneider *et al.*, 1969b), which found a high level of carriage of group C organisms together with a high prevalence of protective immunity to group C meningococci in a cohort of military recruits.

There are significant epidemiological differences between military recruits entering a US training camp in 1967 and students entering an English university in 1997. The conditions of military life such as fatigue and exposure to inclement weather may reduce the normal resistance of new recruits. In addition, the opportunities for dissemination of meningococci are enhanced by overcrowding (Neal *et al.*, 1999) within the close communal existence of barracks. Other predisposing factors such as smoking (Imrey *et al.*, 1996), lower socio-economic status and male gender (Caugant *et al.*, 1994;Harrison *et al.*, 2001) are associated with higher rates of meningococcal carriage. The effects of changes in epidemiological factors over 30 years on the development of natural immunity to meningococcal infection are unknown. However, any tendency toward a reduction in meningococcal carriage may result in a lesser development of natural immunity to infection.

The data presented in this thesis is in accord with more recent research (Richmond *et al.*, 2000) which found a low prevalence of circulating bactericidal antibodies to group C meningococci in healthy adults. In addition, other investigators have reported low levels of serogroup C carriage in association with serogroup C meningococcal disease (Imrey *et al.*, 1996;Conyn-van Spaendonck *et al.*, 1999;Fernandez *et al.*, 1999).

3.3.5 Antibodies Associated with Protective Immunity

The association between individual meningococcal antigens and the development of protective immunity to serogroup C meningococci was studied in sera taken prior to the outbreak. The rSBA was utilised to measure bactericidal antibodies directed against the outbreak, sporadic and reference group C strains. A similar pattern of immunity was observed

regardless of the test strain. These meningococci share a common group (C), but are of different type and subtype, indicating that the serum bactericidal activity was at least partly due to the presence of antibodies directed against the group C polysaccharide. Gotschlich *et al.* (1969a) demonstrated that the group C bactericidal antibody was directed against the group C polysaccharide. Their studies also demonstrated a rise (≥1/4) in SBA after immunisation of adults with group C polysaccharide. The protective level for antibody to the group C polysaccharide is unknown but evidence suggests that it may be close to around 2μg ml^{-1} (Peltola, 1999). However, Granoff and colleagues (1998) have demonstrated that some of the antibody produced against capsular polysaccharide may be of low avidity resulting in low or absent bactericidal function. The levels of anti-group C antibodies were determined in the serum samples from prior to the outbreak. Twenty one (31%) of the 68 students with SBA titres of <1/8 against MC161 had anti-group C antibody levels of >2μg ml^{-1}, in most instances this was in the region of 3-4μg ml^{-1}, with the highest level at 9μg ml^{-1}. Both the mean and median antibody levels to group C capsule for individuals with a serum bactericidal titre of <1/8 were 2μg ml^{-1}. In contrast, the mean antibody concentration for individuals with a serum bactericidal titre of >1/8 was 55μg ml^{-1}, and the median was 29μg ml^{-1}. The correlation between bactericidal activity and anti-group C antibodies was strong ($p < 0.05$) amongst all the group C organisms tested (data not shown).

This relationship was further verified when comparisons were made between antibody to group C capsule and SBA against strain MC161 in the post meningococcal vaccination (A-C polysaccharide vaccine) 'at-risk' students sera (results not shown). All of the vaccinated students, except one, were protected against the outbreak strain as measured by the SBA. Raised antibody levels to group C capsule were detected in the sera of all the protected students, but not the unprotected individual.

In the context of a low prevalence of natural immunity to the outbreak strain, the prompt initiation of chemoprophylaxis and mass vaccination of at-risk students with serogroup C meningococcal polysaccharide during the response to the outbreak at the University of Southampton is likely to have prevented more cases from occurring. Furthermore, the lack of immunity to group C meningococci highlighted in our study, prompted the decision to vaccinate undergraduate students in subsequent academic years. Following the outbreaks of serogroup C infection at UK universities, the MenC polysaccharide conjugate vaccine was introduced into the UK national immunization scheme

and cases of serogroup C infection have since declined dramatically. We have found that students entering university now have high levels of antibodies directed against the serogroup C polysaccharide and correspondingly high SBA titres (Chapter 4 of this thesis).

In addition to a common capsular serotype, the three group C strains share a common LPS immunotype, L3, however the strong correlation between serum bactericidal activity and the presence of antibodies to serogroup C polysaccharide was in contrast with the lack of any correlation with bactericidal antibody levels to LPS. In addition, there was no correlation with the detection of subtype specific antibodies to PorA protein and serum bactericidal activity.

3.3.6 Immunity to Group C Meningococci in Survivors and Non-survivors of Group C Meningococcal Disease

In the current study, serum taken from Case 3 prior to the outbreak had a rSBA titre of <1/8 as did serum taken on admission from the other fatality, Case 6. Although the numbers are not sufficient to draw definitive conclusions, it is interesting to note that the two cases who recovered, Cases 4 and 5, had higher titres on admission (1/32) perhaps indicating the initiation of an immune response. According to the correlates of protection detailed by Borrow *et al.* (2001a), a rSBA titre of 1/32 falls into the equivocal range of results, where some individuals are protected from invasive meningococcal disease. Although the serum bactericidal activity of these individuals was insufficient to prevent meningococcal infection, the presence of some bactericidal antibodies may have prevented a more severe infection and enabled these individuals to survive the disease process. These surviving cases showed a marked rise in serum bactericidal activity (rSBA titre of 1/32 to 1/2048) in conjunction with increased antibodies to group C polysaccharide ($2\mu g\ ml^{-1}$ - $30\mu g\ ml^{-1}$) on convalescence.

In this study, all the cases had anti-group C antibodies of around $2\mu g\ ml^{-1}$ on admission, yet three of these students died. While it is possible that the antibodies to group C polysaccharide in those individuals that did not survive were of insufficient avidity, the main difference between the early sera of Cases 4 and 5, both of which showed some bactericidal activity, and sera from Cases 3 and 6 (non-survivors) were higher levels in the former of antibodies reacting with the PorA and PorB proteins. However, in contrast to group C antibodies the strength of antibody reactivity to these antigens did not increase in strength on convalescence. Case 5 showed a slight increase in reactivity to the Opa protein on convalescence. It was also of interest that admission serum from Case 6, who died, had high

levels of antibody against the Opa protein, demonstrating a lack of protective effect against this antigen. This would be in accord with the occurrence of antigenic shift in Opa expression and observations that during gonococcal infection the antibodies induced by one Opa variant do not recognise other Opa molecules produced by the same strain during the course of the infection (Tinsley and Heckels, 1986).

3.3.7 Carriage and Immunity to Group B Meningococci Prior to an Outbreak of Meningococcal Infection in Students in 1997

This study is an investigation into humoral immunity to group C meningococci in the context of an outbreak of group C meningococcal infection, however the majority of cases of meningococcal infection in the UK, USA and most temperate countries are caused by serogroup B. We therefore investigated the levels of immunity to serogroup B meningococci in the same student population. Compared to serogroup C meningococci, higher levels of carriage (6.1%) of serogroup B were detected during the outbreak and this was accompanied by a high percentage (83%) of students possessing serum bactericidal activity against a serogroup B strain (MC58).

In this study, no significant correlations were drawn between complement-mediated killing of the group B strain and antibody directed against the group B capsule. This observation is consistent with reports that patients recovering from serogroup B infection show elevated levels of anti-polysaccharide antibodies but that the presence of anti-polysaccharide antibodies does not correlate with SBA (Pollard *et al.*, 1999). In this thesis there was also no association observed between group B bactericidal activity and antibodies directed against LPS however, a strong relationship was noted between reactivity to the PorA OMP and SBA of the group B strain. This would be in accord with previous observations showing an association between serum bactericidal activity and the serosubtype specific antibodies directed against PorA in sera of meningococcal carriers (Jones *et al.*, 1998) or volunteers immunised with experimental outer membrane vesicle vaccines (Wedege *et al.*, 1998;Cartwright *et al.*, 1999).

In this study, an association ($p = 0.058$) between group B bactericidal antibodies and PorB was also found. This finding is consistent with the bactericidal activity of some anti-PorB monoclonal antibodies (Saukkonen *et al.*, 1989) and with the induction of a bactericidal

immune response in mice immunised with purified recombinant PorB incorporated into liposomes (Wright *et al.*, 2002).

3.3.8 Conclusions

The SBA measures the presence of serum antibodies that assist in complement-mediated cell death and has been used for decades as an *in vitro* marker for *in vivo* immunity to meningococcal disease. Most understanding of natural immunity prior to an outbreak of meningococcal infection dates back to the classic studies of Goldschneider *et al.* (1969a,1969b) in which the inverse relationship between bactericidal antibodies and the risk of subsequent disease was demonstrated in a group of military recruits stationed at Fort Dix. These investigators demonstrated that the majority of individuals were immune to infection by group C strains and high levels of group C carriage was noted. In contrast, the work presented in this thesis showed that carriage of group C strains was rare, and that 90% of the students were susceptible to infection by group C meningococci.

Antibody to group C polysaccharide was the only individual meningococcal surface component consistently associated with SBA against group C strains in this study. This data is entirely in accord with the classic studies of Goldschneider and his colleagues. In contrast, no correlation was detected between the presence of antibodies to group B capsule and serum bactericidal activity. However, a strong relationship was noted between reactivity to PorA and PorB proteins and bactericidal antibodies directed against the group B strain.

Thus, these studies in a contemporary student population re-enforce the importance of antibodies directed against the capsular polysaccharide for protection against infection with serogroup C meningococci and support the introduction of the conjugate MenC vaccine in the UK and elsewhere. Furthermore, they validate current strategies for prevention of serogroup B infection based on experimental OMV vaccines containing the PorA protein and suggest that the PorB protein may be an important additional component of such vaccines.

CHAPTER 4 DETERMINATION OF THE DYNAMICS OF MENINGOCOCCAL CARRIAGE AND THE IMMUNE RESPONSE TO COLONISATION IN UNIVERSITY STUDENTS

4.1 Introduction

During the late 1990's the incidence of meningococcal disease increased in the UK; a proportionally greater increase was due to serogroup C, which was most marked in adolescents and young adults, (Ramsay *et al.*, 1997;Neal *et al.*, 1999) and was associated with a higher death rate (Ramsay *et al.*, 1997) than cases of serogroup B infection. In the previous chapter it was shown that many students lacked immunity to group C meningococci. Effective vaccines for the control of serogroup C meningococcal infections exist and it is now UK policy to vaccinate first year students against this serogroup, therefore this chapter describes a follow-up investigation into levels of immunity and carriage to group C in a vaccinated student cohort. Samples were taken at four time-points over a 31 week period in order to perform a longitudinal study into the dynamics of meningococcal carriage and the immune response to carriage.

The majority of cases of meningococcal infection in the UK are caused by serogroup B (Balmer *et al.*, 2002) (Figure 1.2) and there is currently no effective vaccine against this serogroup, therefore immunity to group B was investigated in the same group of students. The development of a serogroup B vaccine based on the capsular polysaccharide is hampered by non-immunogenicity of the group B capsule, therefore immunogenic sub-capsular antigens suitable for inclusion in a group B vaccine must be sought. In this study the association between individual meningococcal antigens and immunity to group B meningococcal disease was investigated.

Traditionally, carriage studies are performed on throat swab cultures alone. The outcome of these studies depends heavily on the skill of the operator taking the swab (Cartwright, 1995b). Carriage study results are therefore considered as underestimates of true carriage. In the current study, however, the sensitivity of carriage detection was enhanced by the addition of gargle-culture and PCR of gargles and throat swabs. The ability of these extra techniques in the determination of carriage detection will be assessed.

4.2 Methods and Results

4.2.1 Study population

First year students from a residential university complex were asked to participate in the study. Written informed consent was obtained from all subjects, and ethical permission for the study was obtained from the South West Hampshire local research ethics committee and the Public Health Laboratory Service, Southampton. Mouth washings, throat swabs, and blood samples were collected from 42 vaccinated students at four time-points, over a 31 week period.

4.2.2 Meningococcal Carriage

4.2.2.1 Detection of Meningococcal Colonisation

Nasopharyngeal colonisation of meningococci in the student population was determined by culture and PCR of throat swabs and gargle specimens.

4.2.2.2 Culture and Identification of Meningococcal Strains

Throat swabs and saline mouth washings were inoculated onto modified New York City (MNYC) Agar plates (2.2). The cultures were examined after 48 hours incubation and a Gram stain and an oxidase test (detailed in Sections 2.3.1 and 2.3.2) were performed on presumptive *Neisseria spp*. Negative cultures were incubated for an additional 72hrs and then discarded if still negative for meningococcal growth. Organisms identified as oxidase positive, Gram negative cocci were confirmed as *N. meningitidis* on the basis of the API NH (bioMeriex) system of enzymatic and sugar fermentation reactions according to the manufacturers instructions (2.3.3).

4.2.2.2.1 Characterisation of Meningococcal Isolates

Isolates were serologically characterised according to serogroup, serotype, and serosubtype by standard methods at the Meningococcal Reference Unit (MRU, Manchester Public Health Laboratory, Manchester, UK). The lipopolysaccharide (LPS) immunotype was determined for serogroup B and C strains by the Laboratory for Vaccine Research, RIVM, Bilthoven, The Netherlands (Scholten *et al.*, 1994). Further characterisation of selected

strains was achieved by DNA sequence determination of the PorA variable regions VR1 and VR2 (Brooks *et al.*, 1995) and comparison with the PorA database (http://neisseria.org/nm/typing/pora/) (2.10) by Dr J. Zoe Jordens.

4.2.2.3 TaqMan PCR for Meningococcal Detection and Serogroup Determination

Throat swabs and saline mouth washings were prepared for TaqMan PCR (2.11.1) and screened for all clinically significant serogroups based on the capsule transfer (*ctrA*) and sialyltransferase genes (*siaD*) (2.11.2) by Dr J. Zoe Jordens.

4.2.2.4 Terminology

The following definitions were used in this chapter to describe the meningococcal carriage status of individuals:

A) Carrier: a student detected as positive for meningococcal colonisation by any test (culture or PCR) in any sample (throat swab or gargle) at any time-point.

A) Non-carrier: a student tested as negative for meningococcal colonisation by all tests

B) Acquirer: culture of meningococci or detection of meningococcal DNA from a student from whom all previous tests for the presence of meningococci were negative.

4.2.2.5 Carriage Results

All positive meningococcal carriage results (both culture and PCR) are presented in Table 4.1.

Table 4.1: Detection of Nasopharyngeal Carriage of *Neisseria meningitidis*

Student No.	Session A	Session B	Session C	Session D
5	++++	++++	Did not attend	Did not attend
9	----	Did not attend	+-+-	----
10	---+	----	----	----
11	----	----	---+	----
12	-+--	--+-	----	----
15	----	----	++^{B}+-	++++
17	----	----	+-++	--++
19	----	-+--	Did not attend	Did not attend
20	---+	----	----	----
26	---+^{C}	Did not attend	Did not attend	Did not attend
28	--++^{B}	----	Did not attend	Did not attend
29	----	+-+-	+-+-	+-+-
30	-+^{B}--	+-++^{B}	+-++^{B}	+-++
33	--+-	+-++	--+-	Did not attend
37	----	++^{B}++^{B}	++^{B}++^{B}	++^{B}++^{B}
38	----	++^{B}++^{B}	+-++^{B}	----
39	----	++++	+-++	Did not attend
100	----	----	-+--	----

Nasopharyngeal colonisation of meningococci in the student population was determined by culture and PCR of throat swabs and gargle specimens. Each entry consists of four symbols representing the results (+/-) of throat swab culture, throat swab PCR, gargle culture and gargle PCR respectively. ^{B} = *siaD* PCR detected group B-associated DNA; ^{C} = *siaD* PCR detected group C-associated DNA. Sessions A, B, C, D correspond to sampling weeks 0, 3, 28 and 31.

All students testing positive for meningococcal colonisation at some point during the study (43%) are presented in Table 4.1. Most carriers (12 out of 18) were detected by culture of throat swabs and/or saline gargles and the characteristics of the carried strains are shown in Table 4.2. Some of the cultured meningococcal strains were not detected by *ctrA* TaqMan PCR. For example, at week 3 (Session B), meningococci were cultured from the throat swab and gargle specimens of student 29, but no meningococcal DNA was detected in either of these specimens. The *ctrA* TaqMan PCR procedure sometimes detected the presence of meningococci when culture was negative. At week 0 (Session A), PCR detected meningococcal DNA in the throat swabs of two students (12 and 30), but no meningococci were cultured at this time-point.

The carriage detection rates between specimen types (gargle and throat swab) differed for some individuals. Culture and PCR of the gargle specimen of student 17 at week 30 (Session D) detected meningococcal carriage, but the throat swab at the same time-point was both culture and PCR-negative.

Table 4.2: Characterisation of Carriage Strains Isolated in this Study

Student	Serological Characterisation	PorA VR1	PorA VR2	Specimen & Week	Strain Designation
37	B:4:P1.5,2:L3	5	2	T B	MC168
30[a]	B:NT:P1.4,7b:L3	7-2	4	T B	MC169
9	B:4:P1.15:L3	19	15	T C	MC170
28	B:1:P1.15:L1	19-1	15-11	G A	MC171
15	B:1:P1.14:L3	22	14	T D	MC172
12	C:2b:P1.5,2	5	2	G B	MC173
5	29E:4:P1.5	5-1	10-8	T A	MC174
17	29E:NT:P1.6	18	25	T C	MC175
39[b,c]	29E:14:P1.5,2	5-1	2-2	G B	MC176
33	NG:NT:NST	17	16-24	T B	MC177
29	NG:NT:NST	5-2	10-25	T B	MC178

NG = non-groupable; NT = non-typeable; and NST = non-subtypeable.

T = throat swab; G = saline gargle. A, B, C, D correspond to sampling weeks 0, 3, 28 and 31.

a, 2 isolates were NT; b, 2 isolates were NG; c, 1 isolate was NST

Eleven distinct phenotypic profiles were elucidated. When a strain tested as nongroupable and/or nontypeable, at one or more time-points or sites, but a full antigenic profile was obtained at another time-point from the same carrier, the carried organism was assumed to be the same throughout the history of carriage for that individual. In this way, carrier number 39 was assumed to be carrying strain 29E:14:P1.5,2 throughout the study (see appendix). Meningococci were isolated from this individual in both throat and gargle specimens at two time-points. At week 3 of the study, strain 29E:14:P1.5,2 was isolated from the throat swab and a NG:NT:P1.5,2 strain phenotype from the gargle specimen. At week 28, strain phenotype NG:14:P1.5,2 was cultured from the throat swab and 29E:14:P1.5,2 from

the gargle. Thus, two isolates were Non-groupable and one was Non-typeable, however a full strain profile was available at each time-point.

The carriage rates for the respective meningococcal serogroups were: B (14%), C (4%), 29E (7%) and non-serogroupable (NG, 7%). An additional five carriers were detected by *ctrA* PCR, but were *siaD* PCR negative indicating that these strains were not serogroup B or C meningococci.

4.2.3 Carriage and Immune Response to Group C Meningococci

Students entering the university were shown to have high levels of antibodies directed against the serogroup C polysaccharide. At the beginning of the study the mean antibody levels were measured at 46μg ml^{-1}, and then 37μg ml^{-1} for the next two time-points (sessions B and C) before dropping to 19μg ml^{-1} by the end of the study (session D).

Two (4%) of the students were found to be carrying serogroup C meningococci, despite having recently received the A/C meningococcal vaccine. The serogroup C strain, MC173 (C:2b:P1.2,5:L3), was isolated from volunteer 12 at Sessions A and B within the first few weeks of entry into the study, but by 18 weeks (session C) the organism was no longer being carried. This student's serum samples were analysed in detail to determine the nature and quality of the immune response to serogroup C meningococci. High antibody levels against the homologous serogroup C strain, a reference group C strain (MenC11, C:16:P1.7a,1:L2,L3) (Goldblatt *et al.*, 2002) and against purified group C capsule were detected on entry into the study and these levels declined over time (Table 4.3).

Table 4.3: Immune Response of an Individual Colonised by a Group C Meningococcal Strain After Recent Vaccination against Group C Infection.

Time-point	Antibody to Group C Capsule ($\mu g\ ml^{-1}$)	Serum Bactericidal Activity	
		Carrier Strain, MC173	Reference Strain, MenC11
A	16.4	1/8192	1/8192
B	9.9	1/2048	1/2048
C	9.8	1/2048	1/2048
D	9.9	1/2048	1/2048

A, B, C, D correspond to sampling weeks 0, 3, 28 and 31.

The specificity of the immune response observed by serum bactericidal assays was further determined by SDS-PAGE and Western blotting. Antibody reactivity to the PorA and PorB outer membrane proteins of both the homologous strain and MenC11 were observed in all this student's sera. This technique also revealed the presence of antibodies directed against Opa and Opc OMPs, and antibodies to Class II pili.

Student 26 tested positive for serogroup C-associated DNA at the beginning of the study (Session A), however meningococci were not cultured from this individual, and this student did not attend any further sampling sessions. This student had a level of $27\mu g\ ml^{-1}$ antibodies directed against group C capsular polysaccharide at Time-point A.

4.2.4 Immune Response to Group B Meningococcal Carriage

4.2.4.1 Serum Bactericidal Activity Directed against Neisseria meningitidis.

Bactericidal antibody levels of each student at the beginning (Session A) and end points of the 31-week study (Session D) are shown in Figure 4.1. The five separate graphs (A-E) represent antibody activity specifically directed against each group B strain isolated from carriers (Table 4.2). Serum antibody data for the six group B carriers is represented together with that of the 15 students that attended every session, but had no detectable meningococcal carriage. Student 28 did not attend session D, therefore no data is available for this individual at that time-point.

These assays revealed distinct differences in the ability of individual students' sera to kill different serogroup B carriage strains. Volunteer 32 was the only individual who showed no immunity against any of the serogroup B carriage strains at session A. By session D (31 weeks later), this individual had developed protective immunity against strains MC170 (B:4:P1.15:L3), MC168 (B:4:P1.5,2:L3) and MC169 (B:NT:P1.4,7b:L3); two of these strains (MC168 and MC169) had been detected in more than one student during the study. Similarly, volunteer 36 demonstrated immunity at session A only to strain MC168, by session D (twenty eight weeks later), this student had developed further immunity to strain MC169, but not to the other three group B carriage strains. In contrast, students 29 and 30 were immune to all of the group B carriage strains throughout the study.

The pattern of immunity between the strains also differed. At the beginning of the study 76% of students had immunity to MC168 and MC170 (Figures 4.1.A and C), but only 33% had immunity to MC171 (B:1:P1.15:L1) (Figure 4.1.D).

Figure 4.1: Serum Bactericidal Activity of Students Against Each Serogroup B Meningococcal Carriage Strain

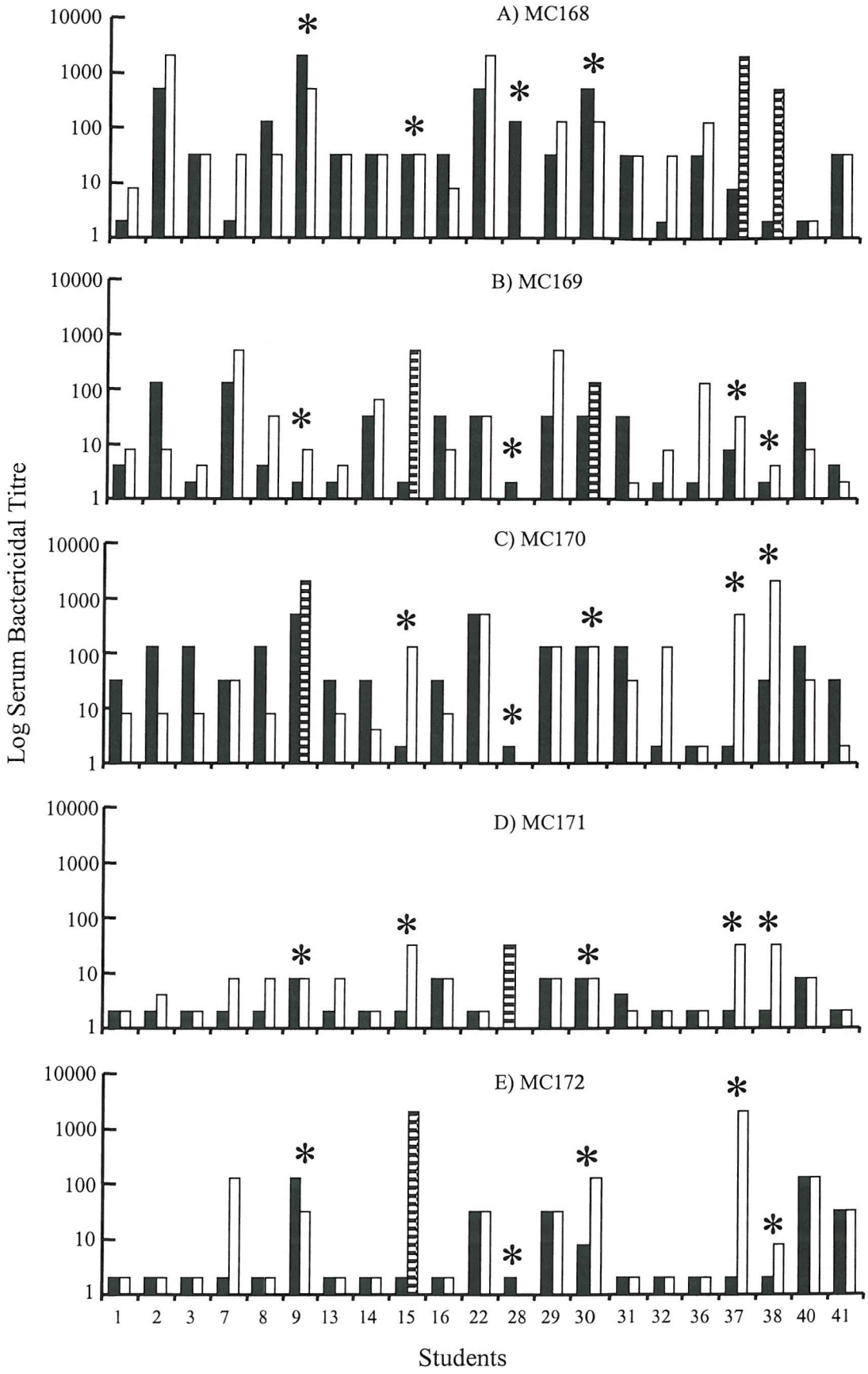
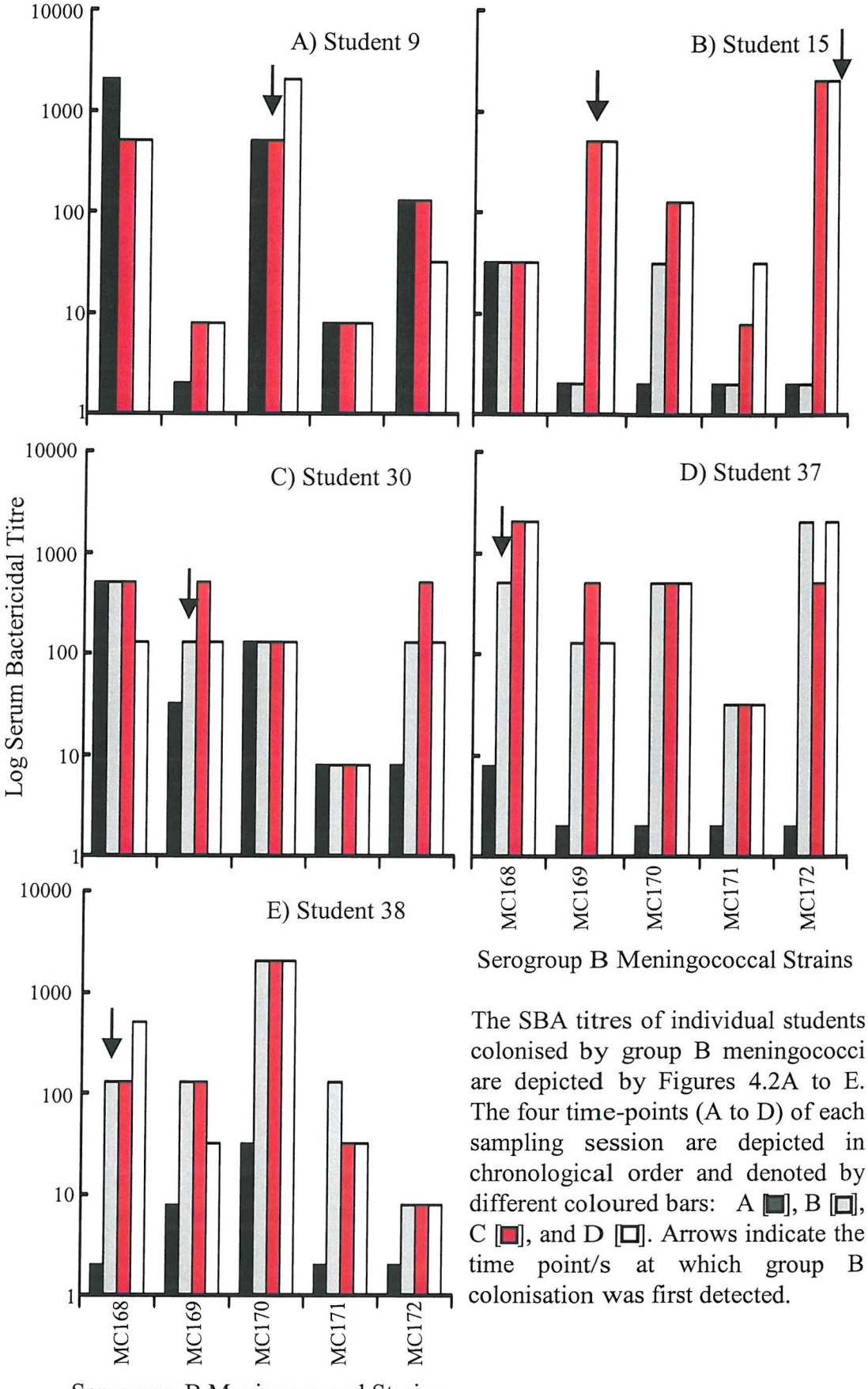


Figure 4.2: Serum Bactericidal Activity of Serogroup B Carriers against all Serogroup B Meningococcal Carriage Strains.



The SBA titres of individual students colonised by group B meningococci are depicted by Figures 4.2A to E. The four time-points (A to D) of each sampling session are depicted in chronological order and denoted by different coloured bars: A [■], B [□], C [■], and D [□]. Arrows indicate the time point/s at which group B colonisation was first detected.

Figure 4.2 shows the effect of acquisition and carriage on the induction of bactericidal activity against homologous and heterologous strains over the 31 weeks of the study. Six students carried group B meningococci at some point during the study. Group B carriage was detected in two students (28 and 30) at week 0. Data for student 28 has been excluded, since samples from only two time-points (A and B) spanning a time-frame of three weeks were available for analysis. Student 30 (Figure 4.2C) was already carrying a group B strain at the start of the study, since *siaD* PCR had detected serogroup B-associated DNA at week 0. Strain MC169 was isolated from this individual at session B and this student was immune to all of the group B strains throughout the study.

Four subjects (9, 15, 37 and 38) became colonised by group B meningococci during the course of the study. Three of these students (subjects 15, 37 and 38) had low bactericidal antibody levels (a titre of <1/4) to their homologous strain/s in sera taken prior to colonisation and a significant rise (fourfold or more) in serum bactericidal titre was detected against the carriage strain/s in sera taken after, or at the time-point that colonisation was detected (Figures 4.2.B, D and E).

Student 15 (Figure 4.2B) carried two group B strains (MC169 and MC172) simultaneously (week 30). This subject had non-protective immunity to these strains prior to the detection of carriage, but subsequently developed a significant increase in bactericidal antibodies to these strains and was immune to all the group B meningococcal strains by 28 weeks into the study (time-point C). Similarly, student 38 lacked immunity to his homologous strain prior to colonisation but protective levels of bactericidal antibodies were detected three weeks later (session B) against the homologous strain as well as the four heterologous strains. In contrast, students 9 (Figure 4.2A) and 37 (Figure 4.2D) were immune to their homologous strains prior to carriage detection. These students lacked immunity to one or more heterologous strains at the start of the study, but had developed protective bactericidal levels to all the strains by the end of the study.

Thus, all students colonised with group B meningococci developed immunity to their homologous strains and subsequently became immune to the other group B strains isolated in the study.

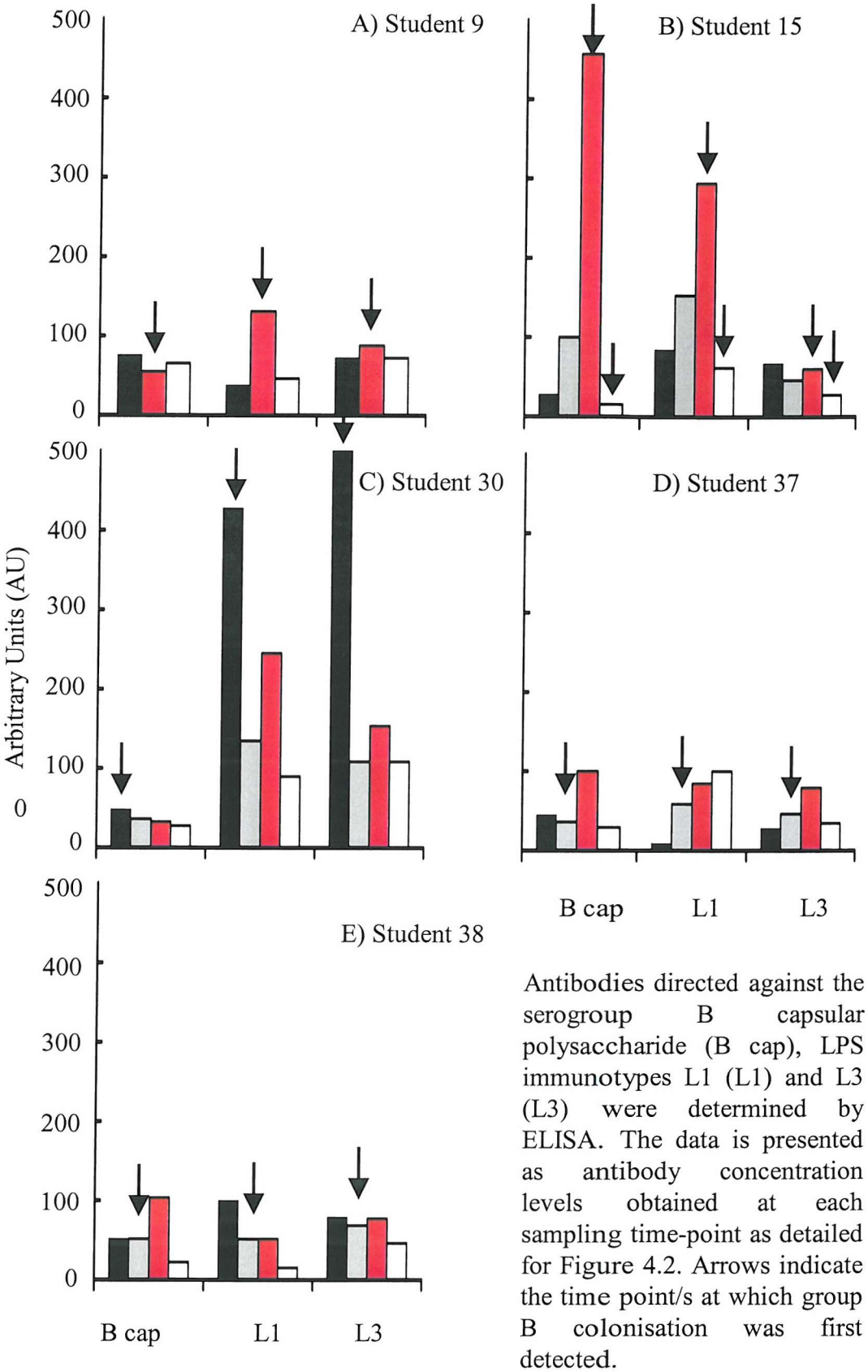
4.2.4.2 Specificity of Immune Response to Group B Meningococci

In order to analyse the immune response to group B antigens, antibodies directed against capsular polysaccharide, lipopolysaccharide and meningococcal surface proteins were determined by ELISA and immunoblotting assays.

4.2.4.2.1 Antibody to Serogroup B Capsular Polysaccharide

Figure 4.3 shows levels of group B capsular antibody concentrations of the group B carriers at each time-point. Among the four (students 9, 15, 37 and 38) group B acquirers the mean antibody concentration increased from 39AU in pre-carriage sera to 144AU after carriage detection. Students 15, 37 and 38 showed a rise in antibody to group B capsule after carriage detection, however this increase was transitory and levels dropped to pre-carriage detection levels by the next sampling session (Figure 4.3B, D and E). There was no significant correlation ($p>0.05$) between serum bactericidal activity against any of the group B strains and antibody levels against the serogroup B capsular polysaccharide (Figure 4.4A).

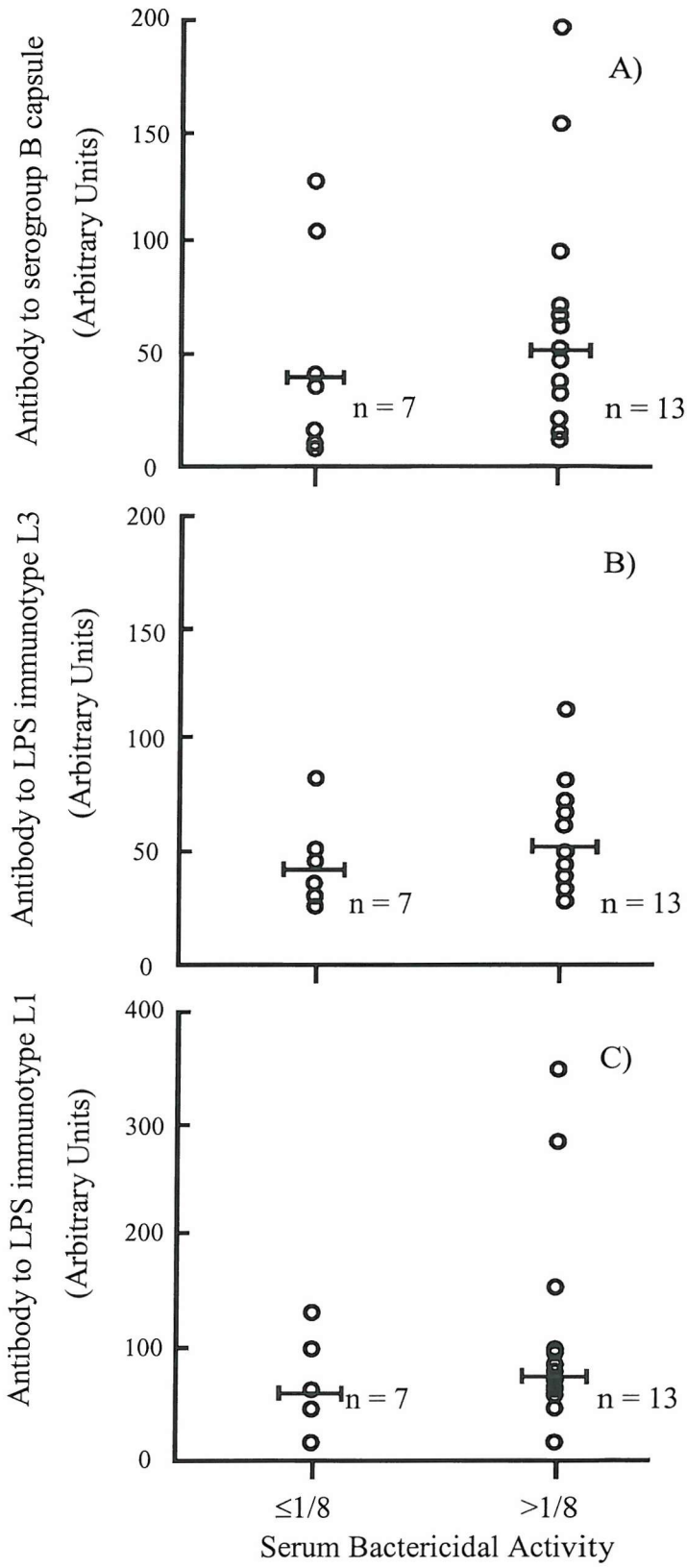
Figure 4.3. Serum Antibodies Directed Against Specific Meningococcal Components in Serogroup B Carriers.



Antibodies directed against the serogroup B capsular polysaccharide (B cap), LPS immunotypes L1 (L1) and L3 (L3) were determined by ELISA. The data is presented as antibody concentration levels obtained at each sampling time-point as detailed for Figure 4.2. Arrows indicate the time point/s at which group B colonisation was first detected.

Antibodies directed against specific meningococcal components

Figure 4.4: Antibody Levels Directed Against (A) Serogroup B Polysaccharide (B) Lipopolysaccharide Immunotype L3 and (C) Lipopolysaccharide Immunotype L1 in Relation to Serum Bactericidal Activity.



4.2.4.2.2 Lipopolysaccharide (LPS) ELISA for detection of antibodies to LPS

Four of the five group B strains in this carriage study possess the LPS immunotype L3, while strain MC171 was characterised as L1. The students' sera were tested for the presence of antibodies directed against both LPS immunotypes in ELISA (Section 2.12.1.4) with LPS from strains MC171 (L1) and MC58 (L3). Concentrations of antibody directed against LPS were calculated by reference to a positive control serum, taken from an individual with significant levels of antibody directed against LPS, which was assigned a concentration of 100 arbitrary units (AU).

Serum antibody data for the six group B carriers is represented together with that of the 15 students that attended every session, but had no detectable meningococcal carriage. All sera demonstrated the presence of antibodies that reacted against both LPS immunotypes. The mean concentration of antibodies directed against LPS immunotype L1 was 142 and 39AU at sessions A and D respectively. Similarly, antibodies directed against LPS immunotype L3 were at levels of 133 and 52AU at the beginning and end of the study. There was no significant correlation ($p>0.05$) between antibodies directed against either LPS immunotypes and bactericidal antibodies directed against any of the group B strains (Figures 4.3B and C).

Results for the serum antibody response of group B carriers directed against LPS are shown in Figure 4.4. There was little difference between LPS antibody levels directed against LPS immunotype L3 before (mean of 65AU) and after (mean of 78AU) carriage detection. In contrast, the immune response to LPS immunotype L1 before (mean of 59AU) and after (mean of 145AU) carriage detection was more marked. Group B carriers 9 and 15, both demonstrated a three-fold increase in antibody to immunotype L1 on acquiring carriage status, despite the fact that their carriage strains were LPS immunotype L3.

4.2.4.2.3 SDS-PAGE and Western blotting for detection of antibodies to meningococcal outer membranes

The specificity of the immune response observed by serum bactericidal assays was further investigated by SDS-PAGE and Western blotting. Western blots incorporating whole cells of isolates of the five group B meningococcal carriage strains were probed with the students' antisera. The immunoblot reactivity results of sequential sera of group B carriers to each of

the five group B strains are presented in Tables E to I (see appendix).

All group B carriers showed antibody reactivity to the PorA protein of their homologous group B strain/s. In addition, all students carrying other group B strains had antibody to the PorA of MC168.

Students 15 and 28 had antibody reactivity to the PorB protein of their homologous strain/s. Although no antibody reactivity to the homologous PorB was seen in the serum samples of student 37, such reactivity was demonstrable on the western blot of a heterologous group B strain (MC170) of the same PorB serotype ('4') as the carriage strain. The sera of student 37 also reacted with the PorB serotype '1' on the western blot of strain MC172.

Reactivity to the Rmp, Opa and pilus proteins seemed to be randomly spread amongst the students and showed no association with the particular carriage strain. In general serum antibody reactivity to the Rmp and Pilus proteins was low or absent in these students, while reactivity to Opa proteins was sometimes high.

In summary, all carriers had antibody reactivity to the PorA protein of the homologous strains/s. Antibody to PorB was less common but also associated with carriage. In contrast, there was no link between reactivity to the other outer membrane antigens and meningococcal colonisation.

Figures A to E (see appendix) show the relationship between SBA and Western blot reactivity of meningococcal surface proteins of both carriers and non-carriers at the last time-point of the study. A summary of the statistical relationships between SBA and meningococcal surface structures of the homologous strains is presented in Table 4.4.

4.2.5 Summary of Statistical Relationships Between the Antibody Data

Differences between antibody levels to meningococcal surface structures of immune and non-immune students were compared with the Mann-Whitney *U* test and probability values of <0.05 were considered statistically significant.

Table 4.4A: Correlations Between Bactericidal Activity and Antibodies Directed Against Meningococcal Components in Students' Sera

	Meningococcal Strains				
Meningococcal Component	MC168 [a]	MC169 [b]	MC170 [c]	MC171 [d]	MC172 [e]
Capsule	0.380	0.184	1.000	0.620	0.360
LPS	0.066	0.242	0.430	0.869	0.147

[a] (B:4:P1.5,2:L3), [b] (B:NT:P1.4,7b:L3), [c] (B:4:P1.15:L3), [d] (B:1:P1.15:L1), [e] (B:1:P1.14:L3).
There was no association between antibody reactivity to capsule or lipopolysaccharide (p>0.05) and serum bactericidal activity directed against any of the strains.

Table 4.4B: Relationship between Bactericidal Antibody Activity and Antibodies Directed Against Meningococcal Components in Students' Sera

	Meningococcal Strains				
Meningococcal Component	MC168 [a]	MC169 [b]	MC170 [c]	MC171 [d]	MC172 [e]
PorA	0.006	0.188	0.797	0.725	0.006
PorB	0.849	0.859	N/A	0.000	0.000
Rmp	0.612	0.859	N/A	N/A	N/A
Opa	0.636	0.666	0.684	0.000	0.631
Opc	ND	ND	N/A	ND	ND
Pilus	N/A	0.600	N/A	ND	0.277

ND Not Detected; [a] (B:4:P1.5,2:L3), [b] (B:NT:P1.4,7b:L3), [c] (B:4:P1.15:L3), [d] (B:1:P1.15:L1), [e] (B:1:P1.14:L3). N/A Probability value could not be calculated as the western blot reactivity score was a constant value.

Bactericidal activity was compared to antibody reactivity to meningococcal surface antigens of the homologous strain (Figures A to E, see appendix) of carriers and non-carriers. The data was compared with regression analysis and a summary of the probability values is tabulated above. Probability values of <0.05 were considered statistically significant.

Antibody reactivity to the PorA protein was associated (p <0.05) with immune protection for strains MC168 and MC172. Antibody directed against the PorB protein was associated with immunity to MC171 and MC172. In addition, there was an association between antibody levels to the Opa Protein and bactericidal antibodies against MC171. No association between antibody directed against any of the meningococcal surface proteins tested and bactericidal activity against MC169 and MC170 was found.

4.3 Discussion

Temporary nasopharyngeal carriage is characteristic of meningococci (Peltola, 1999) and may be an important immunising event leading to the development of bactericidal antibodies (Caugant *et al.*, 1994). This chapter describes the role of carriage in inducing natural humoral immunity to meningococci in first year residential students attending an English university in 1999. Estimates of meningococcal carriage may be higher if more sensitive identification techniques are used for meningococcal detection. This data shows that the detection of carriage can be improved by the addition of gargle culture and PCR of throat swabs and gargle specimens. Together these methods provide more accurate information for the assessment of carriage. In this study, gargle culture performed better than throat swab culture indicating that gargle culture is a reasonable alternative to throat swab culture in carriage detection studies. Since the taking of mouth washings for gargle culture does not require great expertise, this method could be used as an alternative to throat swab culture in the event of large-scale outbreaks or carriage studies.

Meningococcal carriage was detected in 18 students (43%) over the study period and the carriage rate increased progressively from 19% at the beginning of the study (session A) to a peak of 35% after 18 weeks (session C). Increased carriage rates were also a feature of other longitudinal carriage studies of students (Neal *et al.*, 2000;Robinson *et al.*, 2002). In this study, the predominant colonising serogroup was B (14%) followed in order of magnitude by 29E (7%), non-serogroupable (NG, 7%) and C (4%) strains respectively.

The expression of serotype and serosubtype by the same strain can be intermittent (Gilmore *et al.*, 1999) and serological identification of isolates may be influenced by the amount of polysaccharide covering the proteins used in the typing system (Pether *et al.*, 1988), rendering some strains non-typeable or non-subtypeable. Some isolates in this study were not typeable by serological methods so DNA sequencing of the variable regions of *por*A was used to determine their relationship to strains isolated during the study. Non-typeable and non-serosubtypeable isolates of a group B strain were isolated from both gargle and throat swab specimens of student 15 at time-point C (see appendix). Sequencing of the variable regions of *por*A showed that these strains were both serosubtype P1.4,7b and the PorA region

of this organism was serologically typeable as such when next isolated from this student (session D).

Following the outbreaks of serogroup C infection at some UK universities (Jones *et al.*, 2000) the previous year, students were vaccinated with the combined serogroup A and C plain polysaccharide (MACP) vaccine prior to arrival at university. In this study, students entering the university were shown to have high levels of antibodies directed against the serogroup C polysaccharide (mean of 46μg ml^{-1}). Two (4%) of the vaccinated students in this carriage study were found to be carrying serogroup C meningococci. The students' serum samples were analysed in detail to determine the nature and quality of the immune response to group C meningococci. High levels of bactericidal antibody directed against serogroup C meningococci and purified group C capsule were detected (Table 4.7). This data is consistent with the strong association between group C bactericidal antibodies and antibody to group C polysaccharide observed by many investigators (Maslanka *et al.*, 1997;Goldschneider *et al.*, 1969b). The group C carriers in this study had both bactericidal antibodies and antibody levels to group C polysaccharide above levels thought to be protective, yet these individuals were colonised. Ranta *et al.* (1999) suggest that a relatively low concentration of serum antibodies is required to prevent disease, whereas a higher concentration is required to prevent nasopharyngeal carriage. Similarly, for *Haemophilus influenzae* type b, a 10-100 fold higher concentration of serum antibodies is required to prevent carriage than to prevent disease in infant rats (Kauppi *et al.*, 1993). The persistence of group C carriage in vaccinated individuals is of concern as it provides a potential reservoir of infection for non-immune individuals.

Since the introduction of immunisation with conjugate group C vaccines into national immunisation programmes, cases of serogroup C infection have declined dramatically and group C carriage has also reportedly been reduced (Balmer *et al.*, 2002). However, even before the widespread introduction of the MenC conjugate vaccine, the majority of cases of meningococcal infection in the UK, USA and most temperate countries were caused by serogroup B (Quakyi *et al.*, 1999). The low immunogenicity of the group B capsule has prevented the development of an effective vaccine against this serogroup, therefore it is essential to identify and characterise the contribution that sub-capsular antigens of carried strains have on host immunity for possible inclusion in a serogroup B vaccine.

The sera of both carriers and non-carriers of group B meningococci were screened for serum bactericidal activity. Four out of the six carriers of group B developed protective levels of bactericidal antibodies against the carriage strains by time point B (three weeks into the study). Edwards *et al.* (1977) note an average of nine days between bacterial colonisation and a significant bactericidal antibody response, with peak titres attained at 15-21 days following first isolation and significant SBA titre rises by day ten. In this study, the detection of meningococcal colonisation correlated with significant bactericidal activity against the homologous strain and peak bactericidal titres had been attained by the next sampling session (median of 15 weeks) after colonisation detection. Subject 15 carried two group B strains simultaneously (MC169 and MC172) and had attained peak SBA levels at the time-point of carriage detection for both homologous strains. In fact, SBA titres to MC172 had reached a peak at the time-point prior to detection of this strain, indicating that it may have been present but not detected.

In this study, the serum bactericidal assay revealed distinct differences in the ability of individual students' sera to kill different serogroup B carriage strains. This suggests that the serum bactericidal activity is directed against antigens other than the capsular polysaccharide common to all serogroup B meningococcal strains. The lack of correlation between antibody directed against the group B capsule and complement-mediated killing of any group B strain is consistent with the observation that patients recovering from serogroup B meningococcal infection show elevated levels of anti-polysaccharide antibodies but their presence does not correlate with serum bactericidal activity (Granoff *et al.*, 1995). In contrast to Wedege *et al.* (1998), no association ($p > 0.05$) was observed in this study between group B bactericidal activity and antibodies directed against LPS. The lack of correlation between SBA titre and the presence of antibodies directed against serogroup B capsular polysaccharide, or antibodies to LPS, shown in this study indicates a role for other components in the development of natural immunity.

Previous studies of the immune response to an experimental outer-membrane vesicle vaccine showed an association between the presence of antibodies reacting with PorA and protection against invasive disease (Wedege *et al.*, 1998). In this study two of the group B carriage strains appear to have the same PorA protein (subtype P1.15) but the pattern of serum bactericidal activity of individuals to these strains was markedly different. Many more students (67%) were susceptible to MC171 than to MC170 (24%) (Figure 4.2). Sequencing of the *por*A region revealed these strains to be different variants of subtype epitope 15 (Table

4.2). This emphasises the limitations of serological typing methods and indicates the highly specific nature of the immune response to PorA.

The combination of techniques used to detect carriage in this study contributed significantly to understanding the role of carriage in protective immunity. Carriage stimulated bactericidal antibodies directed against homologous group B strains and these antibodies were cross-reactive to some of the other group B strains isolated in the study. Bactericidal antibody activity in this study was both strain and individual dependent. Ten percent of the students in this study were immune to all the circulating group B strains and one individual was susceptible to infection by all the group B strains isolated during this study. It is important to note, however that a lack of bactericidal antibody activity to a particular strain does not necessarily lead to infection when coming into contact with the same strain. Two individuals who lacked bactericidal antibodies at the beginning of the study were subsequently colonised by group B strains and developed protective immunity.

There is still no vaccine for group B meningococci, and since many students are not protected against all circulating group B strains, it is important to raise awareness of the signs and symptoms of meningococcal disease amongst students at the beginning of each academic year.

CHAPTER 5 GENERAL DISCUSSION

5.1 Introduction

Transient asymptomatic nasopharyngeal carriage is the most common manifestation of meningococcal infection. It is likely that the occurrence of invasive meningococcal disease in a small percentage of carriers is determined not only by the introduction of new virulent strains, but also by factors that facilitate meningococcal transmission and the susceptibility of the host population. Pre-existing humoral immunity (antibody directed against *N. meningitidis*) is probably the most important host factor in determining whether or not a person will succumb to the organism and develop meningococcal infection. Humoral immunity in populations may be important in determining whether a population is at risk of a meningococcal disease outbreak. Outbreaks of meningococcal infection may not occur until humoral immunity to a particular strain in a population has declined. Most of our knowledge of immunity prior to an outbreak of meningococcal infection is based on the classic studies of Goldschneider *et al.* (1969a) which established the importance of bactericidal antibodies in protection against invasive meningococcal disease. However, understanding the contribution that individual meningococcal surface antigens make toward natural immunity to meningococci is incomplete.

5.2 Immunity to Serogroup C meningococci

Chapter 3 of this thesis describes the immunological investigation into an outbreak of group C meningococcal disease which occurred amongst first year students attending Southampton University in 1997. Six cases were reported, five students from the same university residential complex. Modern molecular techniques were applied to the investigation of immunity to meningococcal infection both in cases and in uninfected at-risk students before and during the outbreak. The most important defence against meningococcal infection is the presence of serum bactericidal activity against the prevalent strains. This student population had low levels of bactericidal antibody activity directed against serogroup C meningococci prior to the outbreak of serogroup C meningococcal infection. This lack of immunity was associated with a low carriage rate of group C meningococci. This data is in contrast to studies undertaken in the 1960's (Goldschneider *et al.*, 1969a;Goldschneider *et*

al., 1969b), which found a high level of carriage of group C organisms together with a high prevalence of protective immunity in a cohort of military recruits. A correlation between levels of nasopharyngeal carriage and immunity to meningococcal infection is consistent with the concept of meningococcal colonisation stimulating the production of bactericidal antibodies (Caugant *et al.*, 1994).

In this thesis, the nature of the antigens associated with protective immunity was investigated. Meningococci express a number of variable components on the cell surface which facilitate survival of the bacterial population in changing environments. Due to the correlation between bactericidal antibodies and protection against meningococcal disease, the surface components which elicit bactericidal antibodies are considered to be the best candidates for inclusion in meningococcal vaccines. Antibody reactivity to capsule and LPS were quantified by ELISA, and antibody levels to meningococcal outer membrane proteins PorA, PorB, Rmp, Opa, and pili of the group C outbreak strain were determined by western blot. Antibody directed against group C polysaccharide was the only individual meningococcal surface component consistently associated with serum bactericidal antibody activity directed against serogroup C strains. This association has been noted before (Gotschlich *et al.*, 1969a), and re-enforces the importance of antibodies directed against the capsular polysaccharide for protection against infection with serogroup C meningococci. Therefore, the conclusion from this study of a modern outbreak, that the presence of antibodies against capsular polysaccharide is the best correlate of protection against serogroup C infection, is entirely in accord with the classic studies of Goldschneider and colleagues. Effective vaccines for the control of serogroup C meningococcal infections exist and it is now policy to vaccinate first year students against this serogroup. Chapter 4 of this thesis details a longitudinal carrier study which was conducted on the 1999/00 intake of first year residential students attending Southampton University. Two (4%) of the students were found to be carrying serogroup C meningococci, despite having recently received the A/C meningococcal vaccine. The group C carriers had both bactericidal antibodies and antibody levels to group C polysaccharide above levels thought to protect against meningococcal infection, yet these individuals were colonised. It has been suggested that a relatively low concentration of serum antibodies is required to prevent disease, whereas a higher concentration may be required to prevent nasopharyngeal carriage (Ranta *et al.*, 1999). The persistence of group C carriage in vaccinated individuals is of concern as it provides a

potential reservoir of infection for non-immune individuals. It has been reported that the new conjugate group C vaccines recently introduced into the UK immunisation schedule have not only caused a dramatic decline in cases of serogroup C infection, but group C carriage has also been reduced (Balmer *et al.*, 2002).

5.3 Immunity to Serogroup B meningococci

The majority of cases of meningococcal infection in the UK are caused by serogroup B (Balmer *et al.*, 2002) (Figure 1.2) and there is currently no effective vaccine against this serogroup, therefore data on the levels of immunity to serogroup B meningococci in both student cohorts are presented in this thesis. Since bactericidal antibodies are the generally accepted correlate of protection against meningococci, initial studies were directed at establishing levels of antibodies with the ability to promote complement-mediated bacteriolysis of group B strains. Compared with serogroup C, a considerably greater proportion (83%) of the 1997 student cohort had protective immunity against a serogroup B strain and this was associated with higher levels of serogroup B carriage (6.1%). The follow-up carrier study conducted on the 1999 cohort of students showed that meningococcal carriage stimulated bactericidal antibody activity against homologous group B strains. The importance of bactericidal activity in protection against meningococcal infection is well established, however a lack of bactericidal antibody activity to a particular strain does not necessarily lead to infection when coming into contact with the same strain. Two students who lacked bactericidal antibodies at the beginning of the carriage study were subsequently colonised by group B strains and developed protective immunity.

Since there is no effective vaccine against serogroup B meningococci, this study focused in particular on the association between individual meningococcal antigens and immunity to group B meningococcal disease. Serum bactericidal assays in the 1999 student cohort revealed distinct differences in the ability of individual students' sera to kill the five serogroup B carriage strains. This suggests that the serum bactericidal activity is directed against antigens other than the capsular polysaccharide common to all serogroup B meningococcal strains. This finding is consistent with the 1997 student cohort study (Chapter 3) in which no statistical association was found between the presence of antibodies to group B capsule and serum bactericidal activity against a group B strain and with the poor immunogenicity of the group B capsule noted by other investigators (Pollard *et al.*, 1999).

The low immunogenicity of the group B capsule has been attributed to immunological tolerance induced by similarity of the capsule to structures in the developing foetal brain. Jennings *et al.* (1987) attempted to overcome the immunological tolerance by chemically modifying the group B capsule and conjugating it to a carrier protein. The induction of bactericidal antibodies in non-human primates has been shown with PorB as the carrier protein (Fusco *et al.*, 1997). However, some of the antibodies elicited have the potential to be autoreactive in humans, raising concerns about safety of this vaccine in humans.

Problems associated with serogroup B vaccines based on the capsular polysaccharide have focused attention on subcapsular antigens suitable for inclusion in a serogroup B vaccine. In this thesis natural immunity to group B meningococci was strongly associated with antibodies directed against the PorA outer membrane protein of the homologous strain. This data is in accord with the immunogenicity of the PorA protein for humans observed following immunisation with an experimental outer membrane (OMV) complex vaccine (Rosenqvist *et al.*, 1995). The protection afforded by these vaccines was found to specific for strains possessing the homologous PorA protein. Two of the group B strains in the carriage study described in this thesis appear to have the same PorA protein (subtype P1.15), but the pattern of serum bactericidal activity of individuals to these strains was markedly different. Sequencing of the porA region revealed these strains to be different variants of subtype epitope 15, perhaps indicating the highly specific nature of the immune response to PorA.

The PorA protein is a good candidate for inclusion in meningococcal vaccines since it is shared by all serogroups and serotypes and is antigenically stable within a strain, however extensive antigenic diversity is seen between strains and is the basis of serosubtyping In addition, the PorA protein shows a quantitative variability in expression and occasionally a strain is found which lacks it completely.

It has been suggested that the reduced size of the meningococcal PorB protein in relation to the PorA protein make it poorly accessible to antibodies and therefore less capable of inducing an immune response (Michaelsen *et al.* 2001). However, data presented in this thesis indicate that the PorB porin protein is another outer membrane protein which induces antibodies associated with immunity to some group B meningococci. However, this research suggests that some PorB types e.g. '15' may be more immunogenic than others e.g. types '1' and '4'. The association of SBA titer with the presence of antibodies to PorB is consistent

with the induction of a bactericidal immune response in mice immunized with purified recombinant porB incorporated into liposomes (Wright *et al.*, 2002) and the induction of PorB Protein specific antibodies after immunisation with experimental OMV vaccines (Guttormsen *et al.*, 1993). The PorB protein has some advantages over PorA in that clinical isolates show less variation in PorB serotype than they do in PorA subtype and no natural isolates lacking PorB have been reported.

No association was found in this thesis between antibodies reactive against the Rmp OMP (formerly Class 4) and immunity to group B meningococci. This protein is highly conserved between strains and antigenically stable (Tinsley and Heckels, 1986), however inclusion of this OMP in a vaccine is contraindicated, since this antigen has been shown to block the bactericidal activity of anti-PorA or anti-PorB antibodies *in vitro* (Munkley *et al.*, 1991).

Other studies have shown the Opc protein to be highly immunogenic after immunisation of Norwegian adolescents with a group B OMV vaccine but immunity was of short duration (Rosenqvist *et al.*, 1993). These investigators found an association between bactericidal activity and anti-Opc antibodies detected by ELISA but not by immunoblotting. In the carriage study described in this thesis, only one of the five group B carriage strains expressed this protein, and no correlation between antibodies directed against this protein measured by immunoblotting and immunity to group B was found. It has been suggested that human antibodies to Opc might recognise conformational epitopes which are denatured during immunoblotting. Jolley *et al.* (2001) noted that the protective effect in strains expressing the homologous Opc protein was only apparent in a minority of isolates expressing high levels of the protein. It is possible that the carriage strain described in this thesis was expressing Opc protein at too low a level to induce a protective immunogenic response.

Many individuals from both student cohorts had antibody reactive against the Opa protein, however these antibody levels did not correlate with serum bactericidal activity against group C or B meningococci. The admission serum of one of cases who died of group C invasive disease had high levels of antibody against the Opa protein, demonstrating a lack of protective effect against this antigen.

LPS is a major component of the meningococcal outer membrane which is highly conserved with ca. 80% of invasive group B and C strains possessing the LPS L3 immunotype (Jones *et al*, 1992). LPS in conjunction with the polysaccharide capsule enable the meningococcus to evade the immune system. Vogel *et al.* (1997) reported that meningococcal capsulation and sialylation of LPS were both indispensable for group B resistance to complement-mediated bactericidal killing. However, the studies of Kahler *et al.* (1998) indicate that it is the LPS structure regardless of sialylation which contributes to the organism's ability to resist bacteriolysis. In particular, the alpha-chain structure of the L2 and L3 immunotypes were found to facilitate meningococcal resistance to bactericidal activity.

In this thesis, four out of the five group B carriage strains of the carrier study possess the LPS immunotype L3, only one strain was characterised as L1. The group B reference strain, used to assess immunity to group B meningococci in the group C outbreak investigation was immunotype L3, while the group C outbreak strain was immunotype L2. Consistent with the observations of Kahler *et al.* (1998) no association was demonstrated between bactericidal activity and antibodies directed against LPS immunotypes L2 or L3. However, in accord with Wedege *et al.* (1998) there was also no correlation shown in this thesis between the LPS immunotype L1 and bacteriolysis. In contrast to the results presented in this thesis, Wedege *et al.* (1998) did find a correlation between the LPS immunotype L3 and bactericidal activity.

The lack of correlation between SBA titre and the presence of antibodies directed against serogroup B capsular polysaccharide, LPS, PorA, PorB or Opa protein in some of the group B carrier strains in this study indicates a role for other components in the development of natural immunity. Many previously unrecognised membrane proteins have been discovered during the meningococcal group B genome sequencing project (Tettelin et al., 2000). Some of these proteins are conserved and elicit serum bactericidal antibody responses in mice, therefore they represent promising new vaccine candidates.

The ultimate goal is the discovery of a highly conserved antigen present across all meningococcal strains which is able to induce protective immune responses in both adults and children. It is unclear whether antibody directed at an individual antigen, or several antigens are needed to confer natural immunity against group B meningococci. Meningococci are successful pathogens because of their ability to vary their immunogenic surface structures and evade immunological attack, therefore vaccines directed at a single variable surface

structure are unlikely to be successful. The problem might however be overcome by the use of multicomponent vaccines.

5.4 Further Work

If greater resources and time had been available, this study into immunity of students to meningococci could have been expanded further. The immune response to serogroup C and B meningococci was measured, however some of the students were colonised by other serogroups. The SBA may measure antibodies to outer membranes which are not serogroup specific. Testing of the same sera for bactericidal activity against the other serogroups may indicate the degree of cross-protection between strains induced by carriage. Important information might have been gained about the nature of the antigen/s responsible for such immune protection.

5.5 Conclusions

Work presented in this thesis has shown that a lack of protection against meningococci was linked to low carriage of the particular serogroup. Acquisition of carriage was always associated with specific bactericidal activity, indicating that carriage can induce natural immunity to meningococci and this immunity protected students against both homologous and heterologous strains. Protection against group C infection correlated with production of antibodies to group C capsular polysaccharide but not to lipopolysaccharide (LPS) or major outer membrane proteins. In contrast to group C, no association was demonstrated between bactericidal activity against group B strains and the presence of antibodies to group B capsular polysaccharide but there was a correlation with antibodies reacting with PorA and PorB proteins, and bactericidal activity. These results are in accord with the protection afforded against group C meningococcal disease by anti-C capsular vaccines and demonstrate that both the PorA and PorB proteins are potential candidates for inclusion in a group B vaccine.

The extensive opportunities for social mixing within universities facilitate meningococcal transmission and many universities have persistently raised rates of meningococcal disease amongst student populations which are mostly attributed to group B. Of concern in this study was the fact that most (90%) of the students of the 1999 cohort were not immune to all the circulating group B strains, therefore a vaccine conferring immunity to

a wide range of types and subtypes of group B meningococci is urgently required for the pre-university age group.

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APPENDICES

Characterisation of Meningococcal Isolates detected during a Longitudinal Carriage Study

First year students from a residential university complex were asked to participate in a longitudinal meningococcal carriage study. Mouth washings, throat swabs, and blood samples were collected from 42 vaccinated students at four time-points, over a 31 week period. Each of Tables A to D show meningococcal carriage at a single time-point and the data is further summarised according to the specimen and student from which the meningococcal strain was isolated. The full list of students that were colonised at some point during the study is presented, so that the carriage history of each student can be traced. Isolates were serologically characterised according to group, type, and subtype antigens by standard methods at the Meningococcal Reference Unit (MRU, Manchester Public Health Laboratory, Manchester, UK). Further characterisation of selected strains was achieved by DNA sequence determination of the PorA variable regions VR1 and VR2 (Brooks *et al.*, 1995) and comparison with the porA database (http://neisseria.org/nm/typing/pora/) (2.10) by Dr J. Zoe Jordens.

Table A: Characterisation of Meningococcal Carriage Isolates detected at Time-point A

Student.	Throat swabs		Saline Gargles	
	Characterisation of Isolates		Characterisation of Isolates	
	Serological	Molecular*	Serological	Molecular*
5	29E:4:P1.5	5-1, 10-8	29E:4:P1.5	ND
9	NEG	NEG	NEG	NEG
10	NEG	NEG	NEG	NEG
11	NEG	NEG	NEG	NEG
12	NEG	NEG	NEG	NEG
15	NEG	NEG	NEG	NEG
17	NEG	NEG	NEG	NEG
19	NEG	NEG	NEG	NEG
20	NEG	NEG	NEG	NEG
26	NEG	NEG	NEG	NEG
28	NEG	NEG	B:1:P1.15	19-1, 15-11
29	NEG	NEG	NEG	NEG
30	NEG	NEG	NEG	NEG
33	NEG	NEG	NG:NT:NST	ND
37	NEG	NEG	NEG	NEG
38	NEG	NEG	NEG	NEG
39	NEG	NEG	NEG	NEG
100	NEG	NEG	NEG	NEG

NG = non-groupable; NT = non-typeable; NST = non-subtypeable; NEG = meningococci not detected; ND = Not Determined; * PorA sequence serosubtype.

Table A shows meningococcal carriage data at Time-point A, week 0 of the study.

Table B: Characterisation of Meningococcal Carriage Isolates detected at Time-point B

Student.	Throat swabs		Saline Gargles	
	Characterisation of Isolates		Characterisation of Isolates	
	Serological	Molecular*	Serological	Molecular*
5	29E:4:P1.5	ND	29E:4:P1.5	ND
9	NEG	NEG	NEG	NEG
10	NEG	NEG	NEG	NEG
11	NEG	NEG	NEG	NEG
12	NEG	NEG	C:2b:P1.5,2	5, 2
15	NEG	NEG	NEG	NEG
17	NEG	NEG	NEG	NEG
19	NEG	NEG	NEG	NEG
20	NEG	NEG	NEG	NEG
26	NEG	NEG	NEG	NEG
28	NEG	NEG	NEG	NEG
29	NG:NT:NST	5-2, 10-25	NG:NT:NST	ND
30	B:NT:P1.4	7-2, 4	B:NT:P1.4	ND
33	NG:NT:NST	17, 16-24	NG:NT:NST	ND
37	B:4:P1.5,2	5, 2	B:4:P1.5,2	ND
38	B:4:P1.5,2	ND	B:4:P1.5,2	ND
39	29E:14:P1.5,2	ND	NG:NT:P1.5,2	5-1, 2-2
100	NEG	NEG	NEG	NEG

NG = non-groupable; NT = non-typeable; NST = non-serosubtypeable; NEG = meningococci not detected; ND = Not Determined; * PorA sequence serosubtype.

Table B shows meningococcal carriage data at Time-point B, 3 weeks into the study.

Table C: Characterisation of Meningococcal Carriage Isolates detected at Time-point C

Student.	Throat swabs		Saline Gargles	
	Characterisation of Isolates		Characterisation of Isolates	
	Serological	Molecular*	Serological	Molecular*
5	Did not attend	Did not attend	Did not attend	Did not attend
9	B:4:P1.15	19, 15	B:4:P1.15	ND
10	NEG	NEG	NEG	NEG
11	NEG	NEG	NEG	NEG
12	NEG	NEG	NEG	NEG
15	B:NT:NT	7-2, 4	B:NT:NT	7-2, 4
17	NG:NT:P1.6	18, 25	29E:NT:P1.6	18, 25
19	Did not attend	Did not attend	Did not attend	Did not attend
20	NEG	NEG	NEG	NEG
26	NEG	NEG	NEG	NEG
28	Did not attend	Did not attend	Did not attend	Did not attend
29	NG:NT:NST	ND	NG:NT:NST	ND
30	B:NT:P1.4	ND	B:NT:P1.4	ND
33	NEG	NEG	NG:NT:NST	ND
37	B:4:P1.5,2	ND	B:4:P1.5,2	ND
38	B:4:P1.5,2	ND	B:4:P1.5,2	5, 2
39	NG:14:P1.5,2	ND	29E:14:P1.5,2	ND
100	NEG	NEG	NEG	NEG

NG = non-groupable; NT = non-typeable; NST = non-subtypeable; NEG = meningococci not detected; ND = Not Determined; * PorA sequence serosubtype.

Table C shows meningococcal carriage data at Time-point C, 28 weeks into the study.

Table D: Characterisation of Meningococcal Carriage Isolates detected at Time-point D

Student.	Throat swabs		Saline Gargles	
	Characterisation of Isolates		Characterisation of Isolates	
	Serological	Molecular*	Serological	Molecular*
5	Did not attend	Did not attend	Did not attend	Did not attend
9	NEG	NEG	NEG	NEG
10	NEG	NEG	NEG	NEG
11	NEG	NEG	NEG	NEG
12	NEG	NEG	NEG	NEG
15	B:1:P1.14	22, 14	B:NT:P1.4	7-2, 4
17	NEG	NEG	NG:NT:P1.6	18, 25
19	Did not attend	Did not attend	Did not attend	Did not attend
20	NEG	NEG	NEG	NEG
26	NEG	NEG	NEG	NEG
28	Did not attend	Did not attend	Did not attend	Did not attend
29	NG:NT:NST	ND	NG:NT:NST	ND
30	B:NT:P1.4	ND	B:NT:P1.4	ND
33	Did not attend	Did not attend	Did not attend	Did not attend
37	B:4:P1.5,2	ND	B:4:P1.5,2	ND
38	NEG	NEG	NEG	NEG
39	Did not attend	Did not attend	Did not attend	Did not attend
100	NEG	NEG	NEG	NEG

NG = non-groupable; NT = non-typeable; NST = non-subtypeable; NEG = meningococci not detected; ND = Not Determined; * PorA sequence serosubtype.

Table D shows meningococcal carriage data at Time-point D, 31 weeks into the study.

Table E: Immune Response of Group B Carriers to Strain MC168 (B:4:P1.5,2:L3).

				Western Blot Score against MC168				
Carriers	SBA against MC168	Group B antibody (AU)	L3 LPS (AU)	PorA Protein	PorB Protein	Rmp OMP	Opa protein	Pilus
9A	1/2048	76	74	1	0	0	0	0
9C	1/512	56	89	1	0	0	0	0
9D	1/512	66	74	1	0	0	0	0
15A	1/32	29	68	1	0	0	0	0
15B	1/32	100	48	1	0	0	0	0
15C	1/32	457	62	1	0	0	0	0
15D	1/32	16	28	1	0	0	0	0
28A	1/128	162	62	1	0	0	1	0
28B	1/32	229	28	0	0	0	1	0
30A	1/512	50	500	1	0	0	0	0
30B	1/512	37	110	0	0	0	0	0
30C	1/512	33	155	1	0	0	0	0
30D	1/128	29	110	1	0	0	0	0
*37A	1/8	46	28	1	0	0	1	0
*37B	1/512	37	48	2	0	2	3	0
*37C	1/2048	100	81	2	0	0	2	0
*37D	1/2048	30	35	3	0	0	0	0
*38A	<1/4	52	81	0	0	0	0	0
*38B	1/128	52	69	1	0	0	0	0
*38C	1/128	105	78	1	0	0	0	0
*38D	1/512	23	48	1	0	0	0	0

Carriers matched with their homologous strains are marked with an asterix (*). Sera were tested for their reactivity against: PorA, PorB, Rmp, Opa, Opc and Pilus Proteins by western blot. Antibody reactivity was scored with '0' indicating no reactivity, '1' a weak reaction through '2' and '3', with progressively stronger reactions. In addition, bactericidal antibody activity against the homologous strain, antibody directed against the group B capsule and antibody directed against homologous LPS are presented. A, B, C, D correspond to sampling weeks 0, 3, 28 and 31.

Table F: Immune Response of Group B Carriers to Strain MC169 (B:NT:P1.4,7b:L3).

				Western Blot Score against MC169				
Carriers	SBA against MC169	Group B antibody (AU)	L3 LPS (AU)	PorA Protein	PorB Protein	Rmp OMP	Opa protein	Pilus
9A	<1/4	76	74	0	0	0	0	0
9C	1/8	56	89	0	0	0	0	0
9D	1/8	66	74	0	0	0	0	0
*15A	<1/4	29	68	0	0	0	0	0
*15B	<1/4	100	48	0	0	0	0	0
*15C	1/512	457	62	2	1	0	3	0
*15D	1/512	16	28	2	0	0	2	0
28A	<1/4	162	62	0	0	0	3	0
28B	1/4	229	28	0	0	0	3	0
*30A	1/32	50	500	1	0	0	0	0
*30B	1/128	37	110	0	0	0	0	0
*30C	1/512	33	155	0	0	0	0	0
*30D	1/128	29	110	0	0	0	0	0
37A	<1/4	46	28	0	0	0	0	0
37B	1/128	37	48	3	1	1	0	0
37C	1/512	100	81	3	1	1	0	0
37D	1/128	30	35	3	1	1	0	0
38A	1/8	52	81	0	0	0	0	0
38B	1/128	52	69	0	0	0	0	0
38C	1/128	105	78	0	0	0	0	0
38D	1/32	23	48	0	0	0	0	0

Carriers matched with their homologous strains are marked with an asterix (*). Sera were tested for their reactivity against: PorA, PorB, Rmp, Opa, Opc and Pilus Proteins by western blot. Antibody reactivity was scored with '0' indicating no reactivity, '1' a weak reaction through '2' and '3', with progressively stronger reactions. In addition, bactericidal antibody activity against the homologous strain, antibody directed against the group B capsule and antibody directed against homologous LPS are presented. A, B, C, D correspond to sampling weeks 0, 3, 28 and 31.

Table G: Immune Response of Group B Carriers to Strain MC170 (B:4:P1.15:L3).

Carriers	SBA against MC170	Group B antibody (AU)	L3 LPS (AU)	Western Blot Score against MC170					
				PorA Protein	PorB Protein	Rmp OMP	Opa protein	Opc protein	Pilus
*9A	1/512	76	74	0	0	0	0	0	0
*9C	1/512	56	89	2	0	0	0	0	0
*9D	1/2048	66	74	0	0	0	0	0	0
15A	<1/4	29	68	0	0	0	0	0	0
15B	1/32	100	48	0	0	0	0	0	0
15C	1/128	457	62	2	0	0	0	0	0
15D	1/128	16	28	2	0	0	0	0	0
28A	<1/4	162	62	1	0	0	0	0	0
28B	1/128	229	28	2	0	0	0	0	0
30A	1/128	50	500	0	0	0	0	0	0
30B	1/128	37	110	1	0	0	0	0	0
30C	1/128	33	155	0	0	0	0	0	0
30D	1/128	29	110	0	0	0	0	0	0
37A	<1/4	46	28	1	0	0	0	0	0
37B	1/512	37	48	3	1	1	0	1	0
37C	1/512	100	81	3	0	0	0	0	0
37D	1/512	30	35	3	0	0	0	0	0
38A	1/32	52	81	0	0	2	0	0	0
38B	1/2048	52	69	0	0	0	0	0	0
38C	1/2048	105	78	0	0	0	0	0	0
38D	1/2048	23	48	0	0	0	0	0	0

Carriers matched with their homologous strains are marked with an asterix (*). Sera were tested for their reactivity against: PorA, PorB, Rmp, Opa, Opc and Pilus Proteins by western blot. Antibody reactivity was scored with '0' indicating no reactivity, '1' a weak reaction through '2' and '3', with progressively stronger reactions. In addition, bactericidal antibody activity against the homologous strain, antibody directed against the group B capsule and antibody directed against homologous LPS are presented. A, B, C, D correspond to sampling weeks 0, 3, 28 and 31

Table H: Immune Response of Group B Carriers to Strain MC171 (B:1:P1.15:L1).

				Western Blot Score against MC171			
Carriers	SBA against MC171	Group B antibody (AU)	L1 LPS (AU)	PorA Protein	PorB Protein	Rmp OMP	Opa protein
9A	1/8	76	38	0	0	0	0
9C	1/8	56	132	0	0	0	0
9D	1/8	66	47	0	0	0	0
15A	<1/4	29	85	0	0	0	0
15B	<1/4	100	155	0	0	0	0
15C	1/8	457	295	0	0	0	0
15D	1/32	16	63	0	0	0	0
*28A	1/32	162	1585	3	2	0	3
*28B	1/128	229	1738	3	2	0	3
30A	1/8	50	427	0	0	0	1
30B	1/8	37	135	0	0	0	1
30C	1/8	33	245	0	0	0	1
30D	1/8	29	91	0	0	0	1
37A	<1/4	46	10	0	0	0	0
37B	1/32	37	59	1	0	0	3
37C	1/32	100	85	0	0	0	0
37D	1/32	30	100	0	0	0	0
38A	<1/4	52	100	0	0	0	0
38B	1/128	52	52	0	0	0	0
38C	1/32	105	52	0	0	0	0
38D	1/32	23	16	0	0	0	0

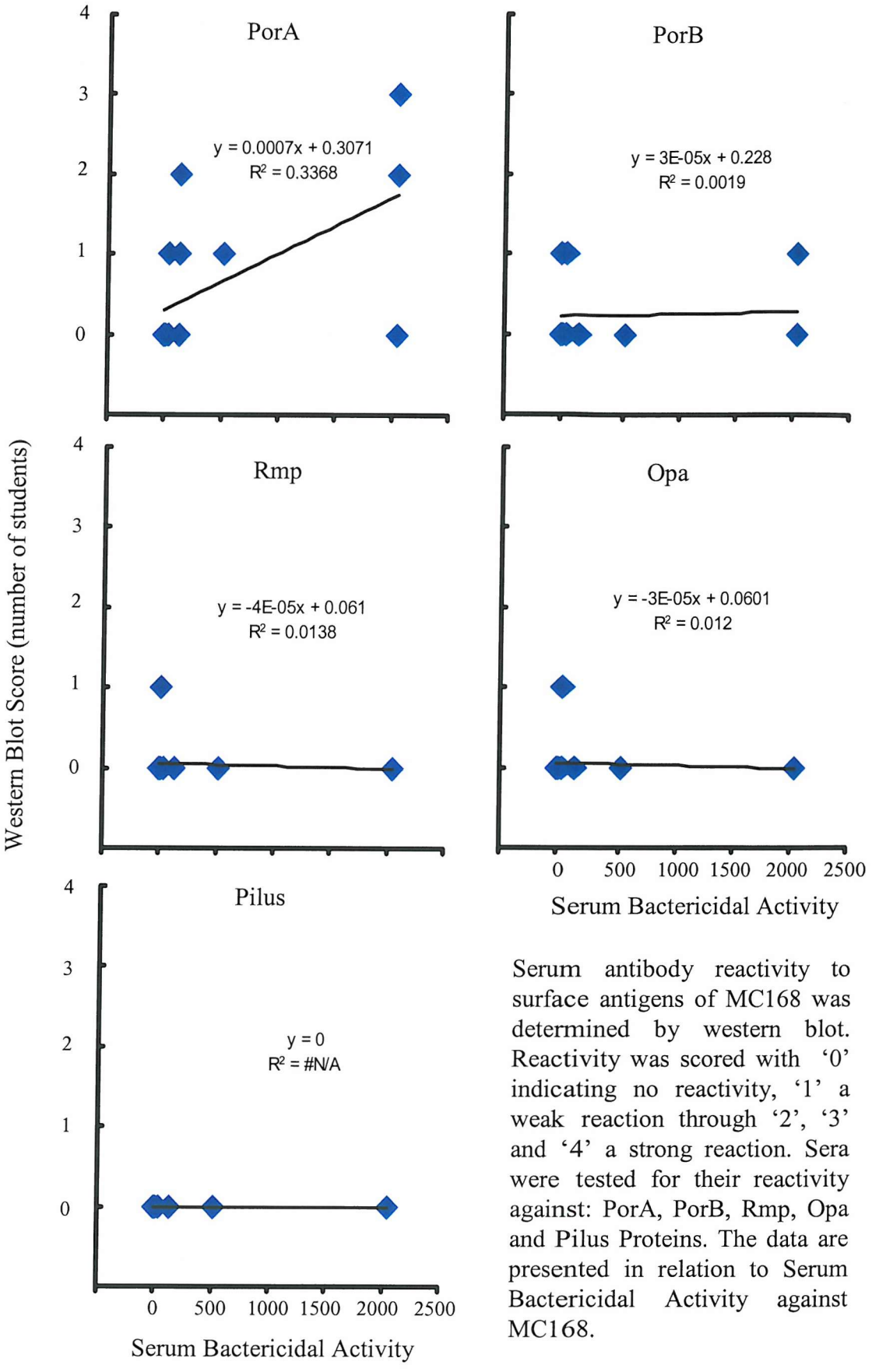
Carriers matched with their homologous strains are marked with an asterix (*). Sera were tested for their reactivity against: PorA, PorB, Rmp, Opa, Opc and Pilus Proteins by western blot. Antibody reactivity was scored with '0' indicating no reactivity, '1' a weak reaction through '2' and '3', with progressively stronger reactions. In addition, bactericidal antibody activity against the homologous strain, antibody directed against the group B capsule and antibody directed against homologous LPS are presented. A, B, C, D correspond to sampling weeks 0, 3, 28 and 31

Table I: Immune Response of Group B Carriers to Strain MC172 (B:1:P1.14:L3).

				Western Blot Score against MC172				
Carriers	SBA against MC172	Group B antibody (AU)	L3 LPS (AU)	PorA Protein	PorB Protein	Rmp OMP	Opa protein	Pilus
9A	1/128	76	74	0	0	0	0	0
9C	1/128	56	89	0	0	0	0	0
9D	1/32	66	74	0	0	0	0	0
*15A	<1/4	29	68	0	0	1	1	1
*15B	<1/4	100	48	0	0	1	1	1
*15C	1/2048	457	62	1	1	0	0	1
*15D	1/2048	16	28	1	1	0	0	1
28A	<1/4	162	62	0	0	0	0	2
28B	<1/4	229	28	0	0	0	0	2
30A	1/8	50	500	0	0	0	0	0
30B	1/128	37	110	0	0	0	0	0
30C	1/512	33	155	0	0	0	0	0
30D	1/128	29	110	0	0	0	0	0
37A	<1/4	46	28	0	0	0	0	0
37B	1/2048	37	48	1	1	1	1	1
37C	1/512	100	81	1	1	0	0	0
37D	1/2048	30	35	1	1	0	0	1
38A	<1/4	52	81	0	0	0	0	0
38B	1/8	52	69	1	1	0	0	2
38C	1/8	105	78	1	1	0	0	1
38D	1/8	23	48	1	1	0	0	1

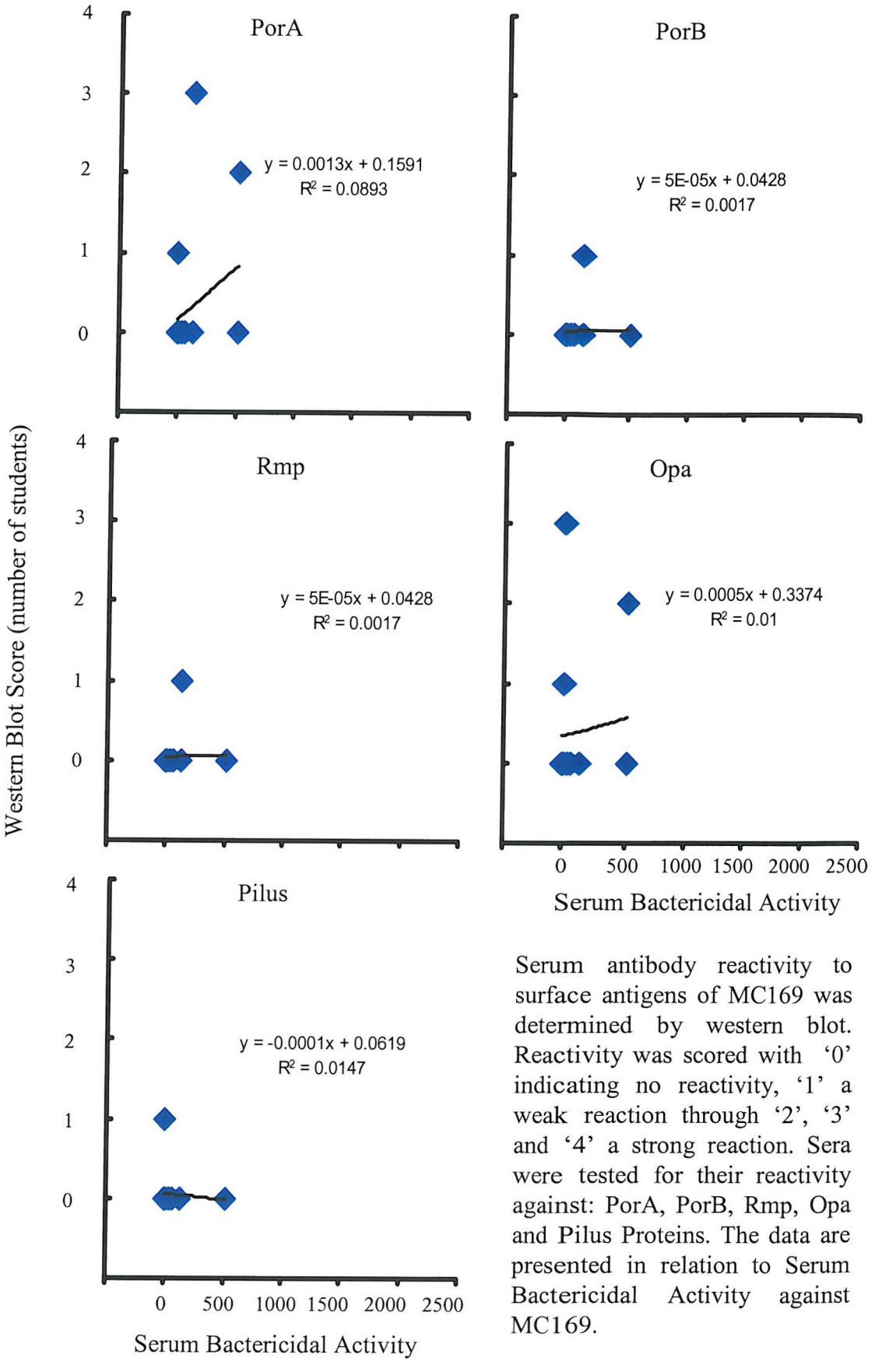
Carriers matched with their homologous strains are marked with an asterix (*). Sera were tested for their reactivity against: PorA, PorB, Rmp, Opa, Opc and Pilus Proteins by western blot. Antibody reactivity was scored with '0' indicating no reactivity, '1' a weak reaction through '2' and '3', with progressively stronger reactions. In addition, bactericidal antibody activity against the homologous strain, antibody directed against the group B capsule and antibody directed against homologous LPS are presented. A, B, C, D correspond to sampling weeks 0, 3, 28 and 31

Figure A: Investigation of Humoral Immunity to Serogroup B Strain MC168 in Carriers and Non-carriers



Serum antibody reactivity to surface antigens of MC168 was determined by western blot. Reactivity was scored with '0' indicating no reactivity, '1' a weak reaction through '2', '3' and '4' a strong reaction. Sera were tested for their reactivity against: PorA, PorB, Rmp, Opa and Pilus Proteins. The data are presented in relation to Serum Bactericidal Activity against MC168.

Figure B: Investigation of Humoral Immunity to Serogroup B Strain MC169 in Carriers and Non-carriers



Serum antibody reactivity to surface antigens of MC169 was determined by western blot. Reactivity was scored with '0' indicating no reactivity, '1' a weak reaction through '2', '3' and '4' a strong reaction. Sera were tested for their reactivity against: PorA, PorB, Rmp, Opa and Pilus Proteins. The data are presented in relation to Serum Bactericidal Activity against MC169.

Figure C: Investigation of Humoral Immunity to Serogroup B Strain MC170 in Carriers and Non-carriers

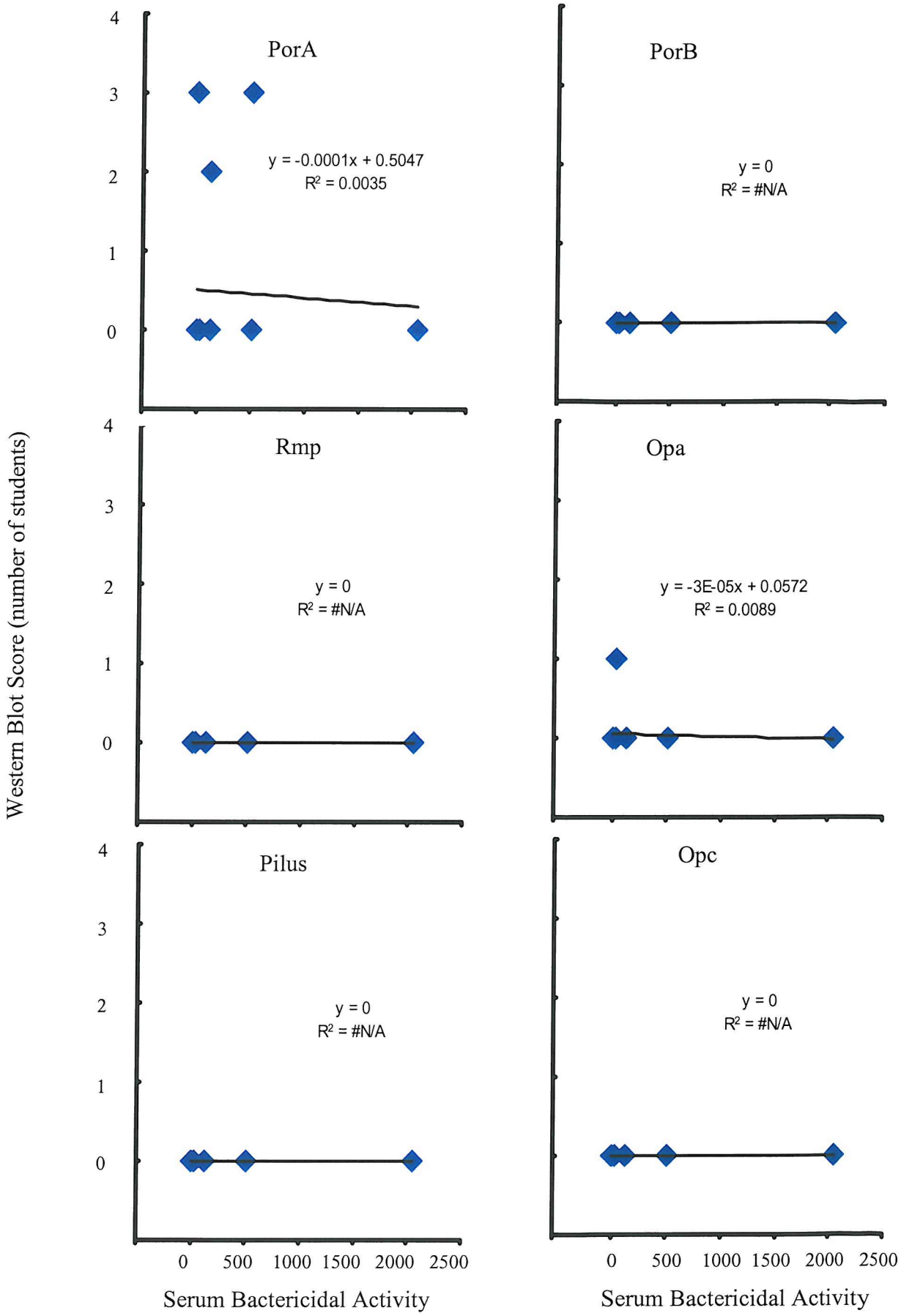
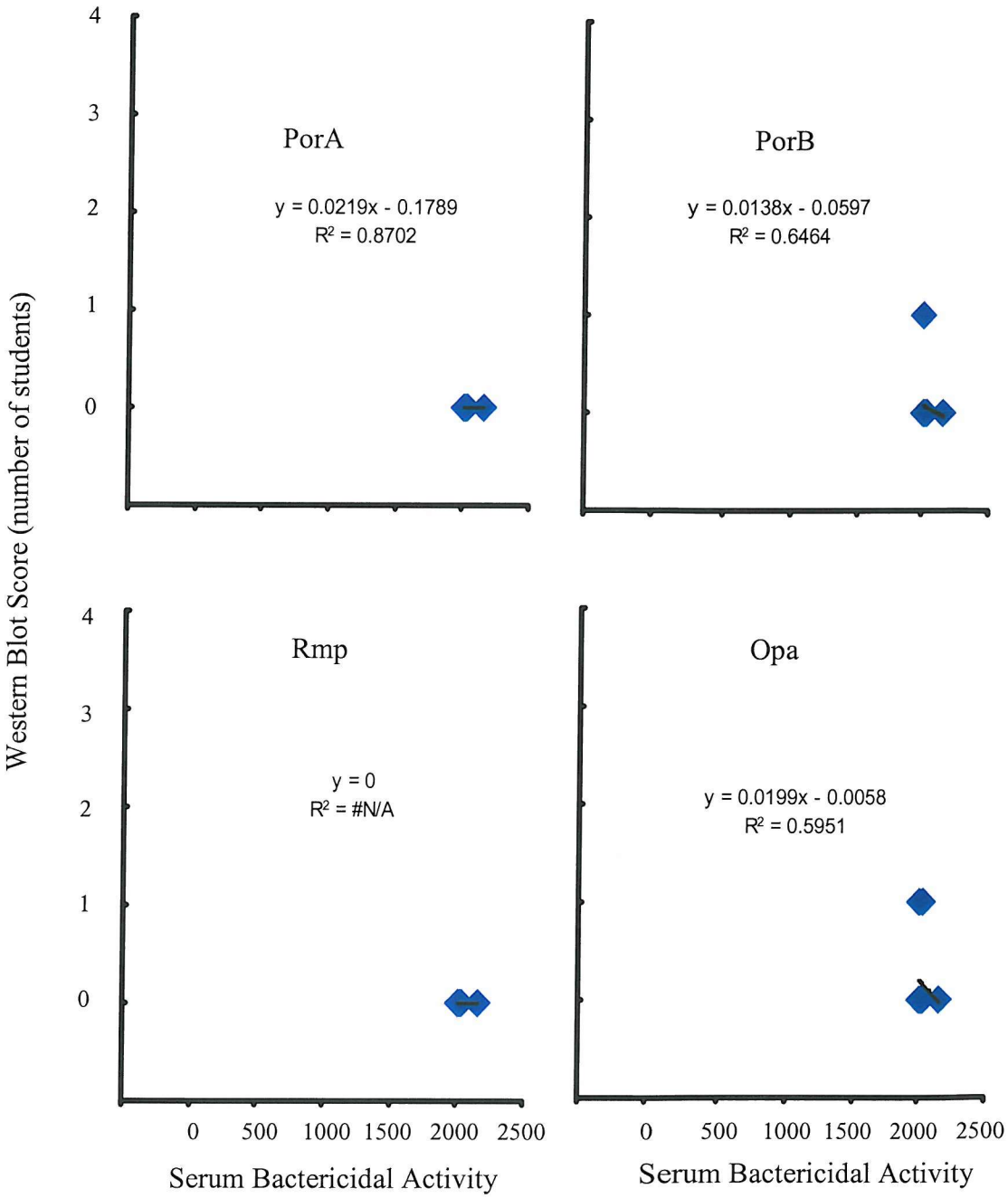
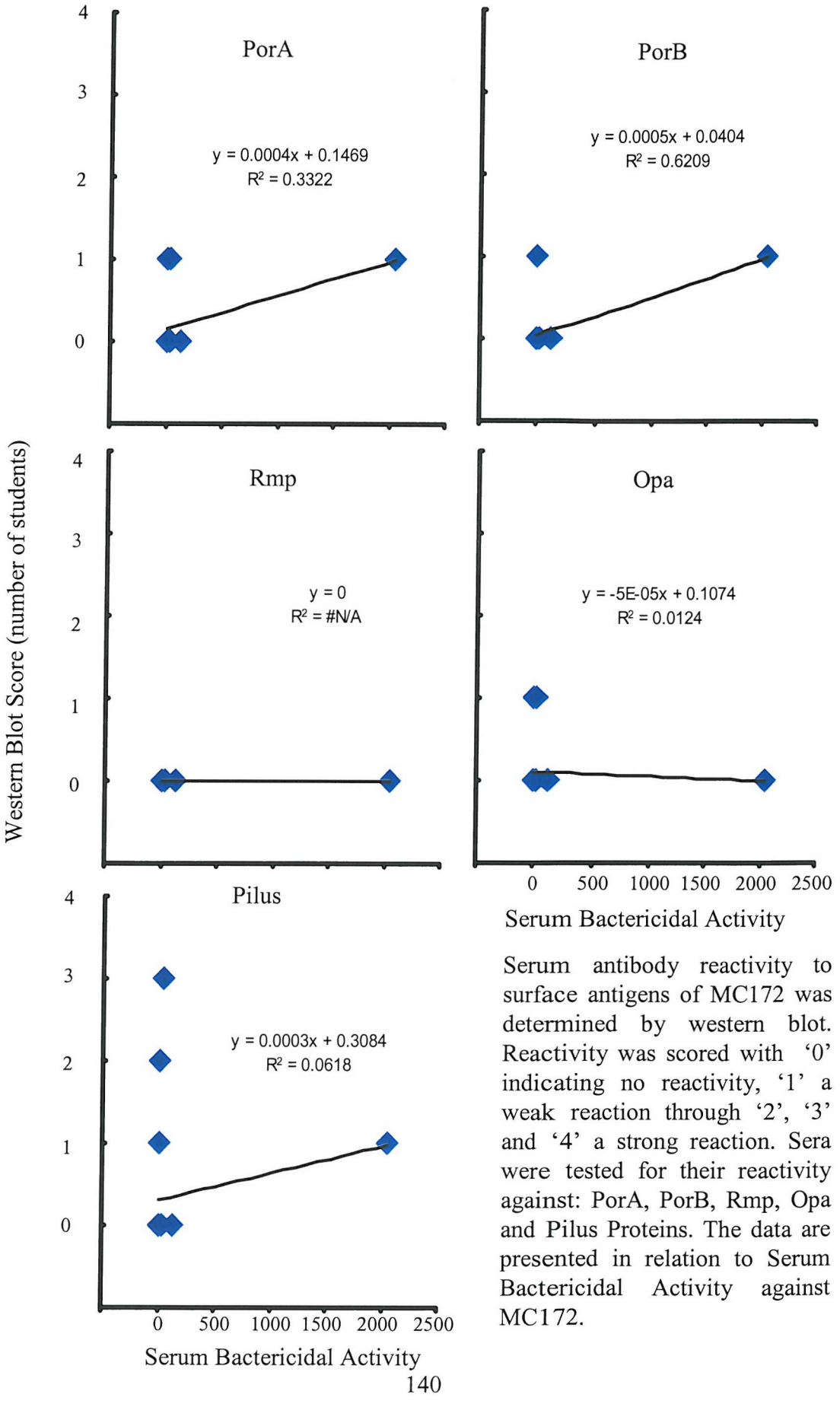


Figure D: Investigation of Humoral Immunity to Serogroup B Strain MC171 in Carriers and Non-carriers



Serum antibody reactivity to surface antigens of MC171 was determined by western blot. Reactivity was scored with '0' indicating no reactivity, '1' a weak reaction through '2' '3' and '4' a strong reaction. Sera were tested for their reactivity against: PorA, PorB, Rmp, Opa and Pilus Proteins. The data are presented in relation to Serum Bactericidal Activity against MC171.

Figure E: Investigation of Humoral Immunity to Serogroup B Strain MC172 in Carriers and Non-carriers



Serum antibody reactivity to surface antigens of MC172 was determined by western blot. Reactivity was scored with '0' indicating no reactivity, '1' a weak reaction through '2', '3' and '4' a strong reaction. Sera were tested for their reactivity against: PorA, PorB, Rmp, Opa and Pilus Proteins. The data are presented in relation to Serum Bactericidal Activity against MC172.